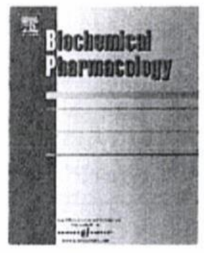
ELSEVIER

available at www.sciencedirect.com

SCIENCE DIRECT

journal homepage: www.elsevier.com/locate/biochempharm



Organotin compounds enhance 17β-hydroxysteroid dehydrogenase type I activity in human choriocarcinoma JAr cells: Potential promotion of 17β-estradiol biosynthesis in human placenta

Tsuyoshi Nakanishi [a,*], Youhei Hiromori [a], Hideaki Yokoyama [a], Mihoko Koyanagi [a], Norio Itoh [a], Jun-Ichi Nishikawa [b], Keiichi Tanaka [a]

[a] Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan
[b] Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan

ARTICLE INFO

Article history:
Received 4 November 2005
Accepted 24 January 2006

Keywords:
Organotin
Tributyltin
Triphenyltin
17β-Hydroxysteroid dehydrogenase type I (17β-HSD I)
Placenta
Estrogen
Retinoid X receptor (RXR)

Abbreviations:
TBT, tributyltin
TPT, triphenyltin
17β-HSD I, 17β-hydroxysteroid dehydrogenase type I
17β-HSDs, 17β-hydroxysteroid dehydrogenases
E1, estrone
E2, 17β-estradiol
9cRA, 9-*cis* retinoic acid
atRA, all-*trans* retinoic acid

ABSTRACT

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), are typical environmental contaminants and suspected endocrine-disrupting chemicals because they cause masculinization in female mollusks. However, it remains unclear whether organotin compounds also cause crucial toxicities in human sexual development and reproductive functions. We investigated the effects of 17 tin compounds on the catalytic activity and mRNA expression of 17β-hydroxysteroid dehydrogenase type I (17β-HSD I) in human choriocarcinoma JAr cells. At nontoxic concentrations, both trialkyltins with propyl, butyl or cyclohexyl substituents on the tin atom and triphenyltin (TPT) enhanced 17β-HSD I mRNA transcription and enzyme activity in a dose-dependent fashion. Although tetraalkyltin compounds such as tetrabutyltin and tributylvinyltin also increased the mRNA expression and enzyme activity of 17β-HSD I, the concentrations necessary for activation were >30–100 times greater than those for trialkyltins. Inorganic tin had no effect on the catalytic activity and mRNA expression of 17β-HSD I. Interestingly, diphenyltin and monophenyltin, which are metabolites of TPT, enhanced 17β-HSD I activity with a concomitant increase in mRNA expression, whereas dibutyltin and monobutyltin, which are metabolites of tributyltin, enhanced 17β-HSD I activity without a concomitant increase in mRNA expression. These results suggest that organotin compounds are potent stimulators of 17β-estradiol biosynthesis to enhance 17β-HSD I activity in the human placenta in vitro; the placenta represents a potential target organ for these compounds, whose endocrine-disrupting effects might be the result of local changes in 17β-estradiol concentrations in pregnant women.

© 2006 Elsevier Inc. All rights reserved.

* *Corresponding author at*: Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6, Yamadaoka Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 8232; fax: +81 6 6879 8234.
E-mail address: nakanishi@phs.osaka-u.ac.jp (T. Nakanishi).

0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.01.014

FCS, fetal calf serum
MEM, minimal essential medium
RXR, retinoid X receptor
LG, LG100268
RAR, retinoic acid receptor

1. Introduction

The placenta plays a vital role in maintaining pregnancy by delivering oxygen and nutrients from the maternal circulation to the fetus and by returning fetal metabolites to the mother. In addition, the placenta performs many crucial endocrine functions. For example, the human placenta is the main source of estrogenic steroids during human pregnancy [1].

17β-Hydroxysteroid dehydrogenases (17β-HSDs) catalyze the interconversion of 17-ketosteroids and 17β-hydroxysteroids, such as estrone (E1) to 17β-estradiol (E2), and androstenedione and testosterone. Thus, these members of the short-chain alcohol dehydrogenase protein family catalyze the conversion of low-activity steroids to high activity forms and vice versa. So far, multiple different types of 17β-HSDs have been cloned [2], and these isoenzymes have been found to differ from each other in substrate specificity as well as in tissue distribution and subcellular localization. In the human placenta and ovarian granulosa cells, the type I enzyme (17β-HSD I) is highly expressed [3–5] and catalyzes primarily the reaction from low-activity E1 to the biologically more active form E2. In addition to being found in steroidogenic tissues, 17β-HSD I is present in some estrogen target cells, such as breast [6] and endometrial epithelial cells [7], which suggest its involvement in the regulation of intracellular E2 supplies for estrogen receptors. Given the pivotal functional roles of 17β-HSD I, the developmental and reproductive toxicity of environmental contaminants known to have endocrine-disrupting effects plausibly might involve 17β-HSD I.

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), have been widely used as biocides, agriculture fungicides, wood preservatives, and disinfecting agents in circulating industrial cooling waters, as well as antifouling paints for marine vessels [8,9]. There are many reports of the biological effects of organotin compounds, which vary in their toxic effects on eukaryotes. One of the most notable toxicities in sexual development and reproduction is that of TBT- and TPT-mediated endocrine disruption in some species of gastropods [10,11]. This phenomenon is known as "imposex"—the superimposition of male genitalia on female animals. Therefore, these organotin compounds are suspected to cause endocrine-disrupting effects in mammals, including humans. Human exposure to organotin compounds may result from consumption of organotin-contaminated meat and fish products or occupational exposure during the manufacture and formulation of organotin compounds or the application and removal of organotin-containing paints [12,13]. The possible exposure of humans to organotins therefore has prompted great concern about potential toxicities.

To facilitate the application of current knowledge of the toxicity of organotin compounds to sexual development and reproduction in humans, we assessed the possible effects of 17 tin compounds on E2 production and mRNA expression of 17β-HSD I in human placental cells by using human choriocarcinoma JAr cells. We discuss the potential toxicity of organotin compounds as endocrine disruptors in humans.

2. Materials and methods

2.1. *Chemicals and cell culture*

Tin compounds tested in this study are listed in Table 1. 9-*cis* retinoic acid (9cRA) and all-*trans* retinoic acid (atRA) were from Wako Pure Chemicals (Osaka, Japan). LG100268 (LG, >95% pure) was obtained from Astellas Pharma (Tokyo, Japan). All chemicals were dissolved in DMSO (Wako Pure Chemicals). The human choriocarcinoma cell line JAr was obtained from American Type Culture Collection (ATCC; Rockville, MD). JAr cells (ATCC No. HTB-144) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM pyruvate, 4.5 g/l glucose, and 10% fetal calf serum (FCS). JEG-3 cells (ATCC No. HTB-36) were cultured in minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 0.1 mM MEM nonessential amino acid solution (Invitrogen, Carlsbad, CA), and 10% FCS. To determine the effect of tin compounds on [^{3}H]thymidine incorporation and mRNA expression of JAr cells, cells were seeded, precultured for 24 h, and then treated with either various concentrations of tin compounds in 0.1% DMSO or vehicle alone (0.1% DMSO) for another 24 or 48 h. In control experiments, 0.1% DMSO did not affect the [^{3}H]thymidine incorporation, catalytic activity, and mRNA expression of 17β-HSD I.

2.2. *[^{3}H]thymidine uptake assay*

To determine the cytotoxicity of tin compounds, JAr cells (10^3 cells/well) were seeded in 96-well plates. After 24 h, cells were treated with various concentrations of these compounds for another 48 h. Each culture was pulsed with 20 kBq of [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) for 2 h before harvesting, and the radioactivity incorporated into cells was determined by liquid scintillation. A nontoxic concentration of a tin compound was defined as a concentration at which the uptake of [^{3}H]thymidine was ≥80% that seen with the vehicle alone.

2.3. *17β-HSD I activity measurements*

JAr cells (3×10^4 cells/well) were plated in 24-well plates. After 24 h of culture, JAr cells were treated with various concentrations of tin compounds for a further 48 h. At the end point of

Table 1 – Tin compounds tested in this study

Tin compounds	Abbreviation	Structure	Purify (%)	CAS No.	Maximum nontoxic concentration[a]	Source
Trimethyltin chloride	TMTCl		>98	1066-45-1	1 μM	Aldrich Chemicals
Triethyltin bromide	TETBr		>97	2767-54-6	100 nM	Aldrich Chemicals
Tripropyltin chloride	TPrTCl		>98	2279-76-7	30 nM	Merck
Tributyltin chloride	TBTCl		>95	1416-22-0	100 nM	Tokyo Kasei Kogyo
Triphenyltin chloride	TPTCl		>95	639-58-7	100 nM	Aldrich Chemicals
Tricyclohexyltin hydroxide	TChTOH		>99	13121-70-5	30 nM	Aldrich Chemicals
Trioctyltin hydride	TOTH		>95	869-59-0	>10 μM	Tokyo Kasei Kogyo
Butyltin trichloride	$MBTCl_3$		>95	1118-46-3	>10 μM	Aldrich Chemicals
Dibutyltin dichloride	$DBTCl_2$		>97	683-18-1	30 nM	Tokyo Kasei Kogyo
Tetrabutyltin	TeBT		>93	1461-25-2	3 μM	Aldrich Chemicals
Phenyltin trichloride	$MPTCl_3$		>98	1124-19-2	3 μM	Aldrich Chemicals
Diphenyltin dichloride	$DPTCl_2$		>96	1135-99-5	300 nM	Aldrich Chemicals
Tributyltin hydride	TBTH		>98	688-73-3	100 nM	Aldrich Chemicals
Tributyltin bromide	TBTBr		>90	1461-23-0	100 nM	Aldrich Chemicals
Triphenyltin hydroxide	TPTOH		>95	76-87-9	100 nM	Aldrich Chemicals
Tributylvinyltin	TBVT		>97	7486-35-3	>10 μM	Tokyo Kasei Kogyo
Tin chloride	$SnCl_4$		>98	7646-78-8	>10 μM	Wako Pure Chemicals

[a] Maximum nontoxic concentration of each tin compound was defined as the maximum concentration at which the uptake of [^{3}H]thymidine was ≥80% of that of the vehicle alone.

each treatment, cells were rinsed with fresh serum-free culture medium and then added 0.5 ml of fresh serum-free culture medium supplemented with 1 μM E1 (Sigma, St. Louis, MO). After incubation for 4 h at 37 °C in an atmosphere of 5% CO_2, culture media were collected, and the total E2 content was determined by assay with a Correlate-EIA 17β-estradiol Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI).

2.4. *Quantitative reverse transcription-PCR (RT-PCR)*

JAr cells were treated with various tin compounds in regular culture medium supplemented with 5% charcoal-stripped FCS instead of 10% normal FCS, and then total RNA was extracted from the cells by using TRIzol reagent (Invitrogen). mRNA expression of 17β-HSD I in JAr cells was determined by quantitative RT-PCR. We reverse-transcribed 5 μg total RNA extracted from JAr cells in a total volume of 20 μl by using SuperScript III reagent (Invitrogen) and oligo-(dT) as primer and incubating for 1 h at 42 °C. After termination of cDNA synthesis, each reaction mixture was diluted with the addition of 80 μl TE buffer. Aliquots (2 μl) of diluted reverse-transcription products were amplified in a reaction mixture containing QuantiTect SYBR Green PCR reagent (Qiagen, Valencia, CA) and 0.5 μM of each primer using LightCycler (Roche Diagnostics, Mannheim, Germany). After preincubation of reaction mixtures at 95 °C for 15 min, PCR amplification was performed with 35–40 cycles of denaturation at 95 °C for 15 s, annealing at 65 °C for 30 s, and elongation at 72 °C for 10 s. Primers used were for human 17β-HSD I (HSD17B1), 5′-GGGCTGCCTTTCAATGACGTTT-3′ and 5′-ATCAGGCTCAAGTGGACCCCAA-3′, and human β-actin, 5′-CTACGAGCTGCCTGACGGC-3′ and 5′-GCCACAGGACTCCATGCCC-3′.

2.5. Statistics

Data were analyzed with Tukey's multiple comparisons test by using SPSS software (Chicago, IL). Control and treatment group data always were obtained from equal numbers of replicate experiments, and experiments were performed independently at least twice. Values at which P was <0.05 were considered statistically significant.

3. Results

3.1. *Effects of organotin compounds on DNA synthesis in JAr cells*

To confirm the nontoxic concentration ranges of 17 tin compounds (Table 1) and to determine whether treatment with organotin compounds was associated with cytotoxic effects, we performed DNA synthesis assays. JAr cells were treated for 48 h with tin compounds at various concentrations, and DNA synthesis was evaluated by [^{3}H]thymidine incorporation. To help interpret the results, we classified these experiments into three groups as follows: Group I, comparison of different structures of alkyl and aryl chains in trialkylated and triarylated tin compounds; Group II, comparison of different numbers of alkyl or aryl chains in butyltin and phenyltin compounds; and Group III, comparison of different fourth functional groups on the tin of TBT and TPT (Fig. 1).

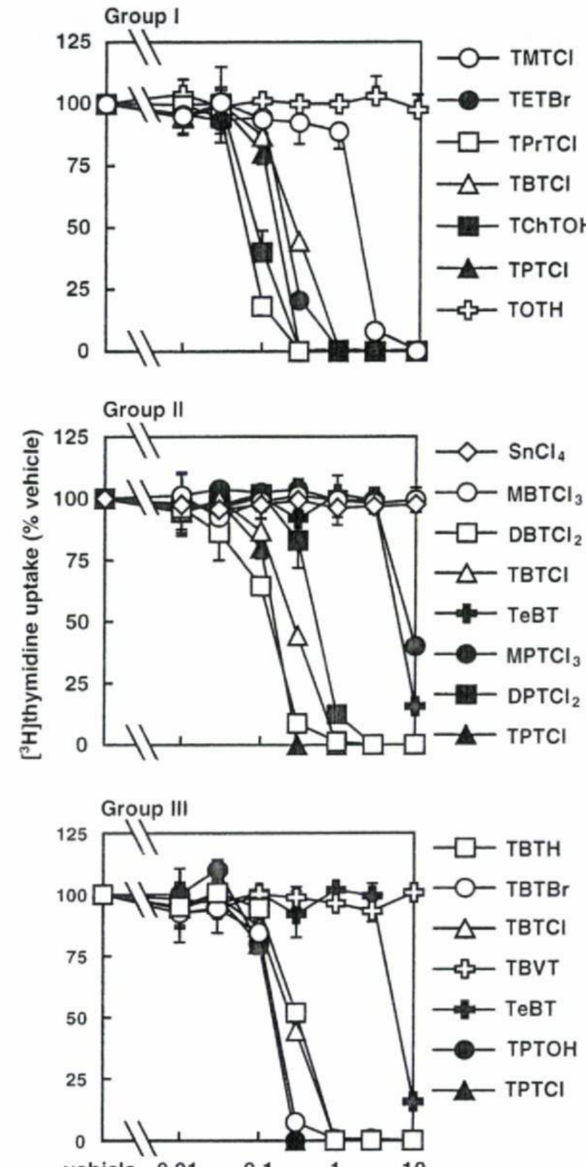


Fig. 1 – Effect of organotin compounds on DNA synthesis in JAr cells. Cells were treated with various concentrations of tin compounds for 48 h. Results are expressed as mean ± 1 S.D. of triplicate cultures. The radioactivity in vehicle-only cells, calculated from all experiments, was 111,709 ± 6182 cpm (*n* = 15). Group I, comparison of different structures of alkyl and aryl chains in trialkylated and triarylated tin compounds; Group II, comparison of different numbers of alkyl or aryl chains in butyltin and phenyltin compounds; and Group III, comparison of different fourth functional groups on the tin of TBT and TPT. The abbreviation for each compound used are indicated in Table 1.

Many of the Group I trialkylated and triarylated tin compounds, which have ethyl, propyl, butyl, cyclohexyl, or phenyl groups on the tin atom, were highly toxic, and

exposure to >100–300 nM significantly inhibited [^{3}H]thymidine incorporation in JAr cells. Although TMTCl was one of the less toxic chemicals of Group I, a striking reduction of [^{3}H]thymidine incorporation to 5% of the control value occurred after treatment with >1 μM. TOTH had no significant effect on [^{3}H]thymidine incorporation at a concentration range of 10 nM to 10 μM. Among the Group II chemicals, the cytotoxicity of $DBTCl_2$ was nearly as high as that of the most highly toxic trialkyltins. $DPTCl_2$ was also toxic but less so than $DBTCl_2$. Although TeBT and $MPTCl_3$ were less toxic than other Group II compounds, they induced marked reduction of [^{3}H]thymidine incorporation at 10 μM. $SnCl_4$ and $MBTCl_3$ showed no effect, even at concentrations of 10 μM. Among the Group III chemicals, the TBT and TPT derivatives were similar in toxicity, and exposure to doses of 300 nM decreased [^{3}H]thymidine incorporation to <50% of control levels. TBVT showed no significant effect at the concentration range of 10 nM to 10 μM. Using these results as a guide, we established the maximal nontoxic concentration of each compound for use in investigating possible effects on the 17β-HSD I activity of JAr cells (Table 1).

3.2. *Effect of organotin compounds on 17β-HSD I activity in JAr cells*

At lethal concentrations, at which uptake of [^{3}H]thymidine was <10% of control levels, all organotin compounds abolished E2 production because of extinction of the cells. All tested TBT and TPT derivatives (Group III) were active and induced 17β-HSD I activity (Fig. 2): exposure to 100 nM of each of these organotin compounds caused statistically significant increases in 17β-HSD I activity in JAr cells. There were no significant differences in 17β-HSD I activity among the TBT and TPT derivatives (Group III), suggesting that the ligand on the trialkylated and triarylated tin compounds (as long as it is not an alkyl or aryl group) is relatively unimportant to stimulation of endocrine functions. However, the presence of a fourth alkyl group on the tin atom decreased the potency of the organotin compounds in inducing 17β-HSD I activity, because both TeBT and TBVT failed to stimulate this placental function at doses of <100 nM (Fig. 2, Group III). Among the other trialkyltin compounds (Group I), TETBr, TPrTCl, and TChTOH were significantly active. Metabolites of both TBTCl and TPTCl ($MBTCl_3$, $DBTCl_2$, $MPTCl_3$ and $DPTCl_2$; Group II) also altered 17β-HSD I activity, but the level of activation decreased in proportion to the dealkylation or dearylation of these organotin compounds (mono- < di- < tri-). These results suggest that the potency of the effects induced by organotin compounds is related to both the number and structure of the alkyl and aryl groups.

3.3. *Effect of organotin compounds on mRNA expression of 17β-HSD I (HSD17B1) in JAr cells*

We investigated the tin compound-induced mRNA expression of 17β-HSD I (*HSD17B1* gene) in JAr cells at either the concentration that elicited the greatest response in catalytic activity or the maximal nontoxic concentration. Except for

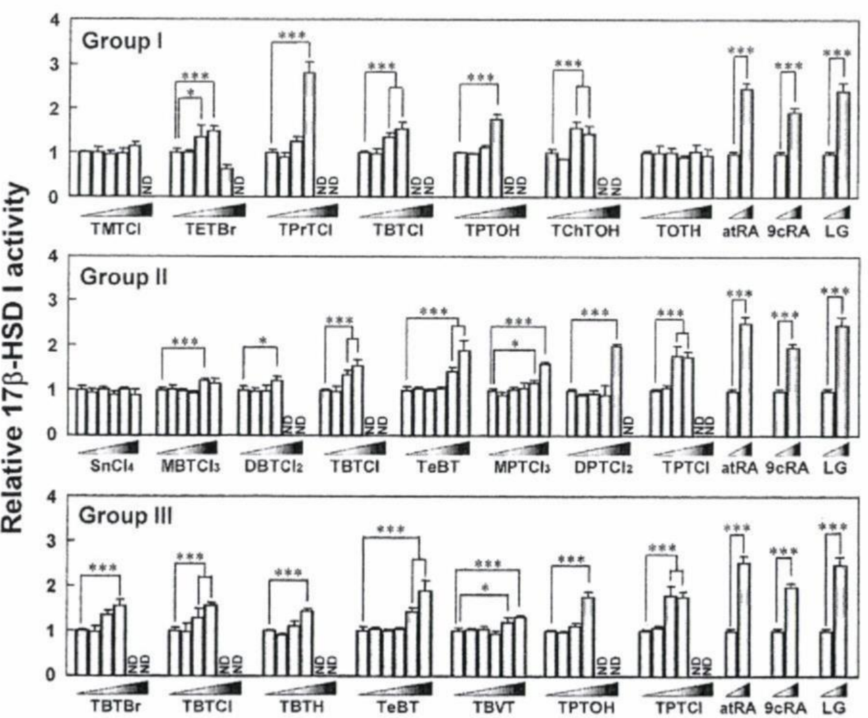


Fig. 2 – Effects of tin compounds on 17β-HSD I activity in JAr cells. Cells were treated for 48 h with tin compounds at various concentrations (0 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) or with 0 or 1 μM of atRA, 9cRA, or LG. Results are expressed as mean ± 1 S.D. of triplicate cultures. The 17β-HSD I activity (amount of converted E2) in vehicle-only cells, calculated from all experiments, was 4.44 ± 1.01 ng/well/4 h (*n* = 18). Groups I, II, and III correspond to the groups described in the legend for Fig. 1. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.005$ indicate values significantly different from vehicle-control values. ND, not detectable.

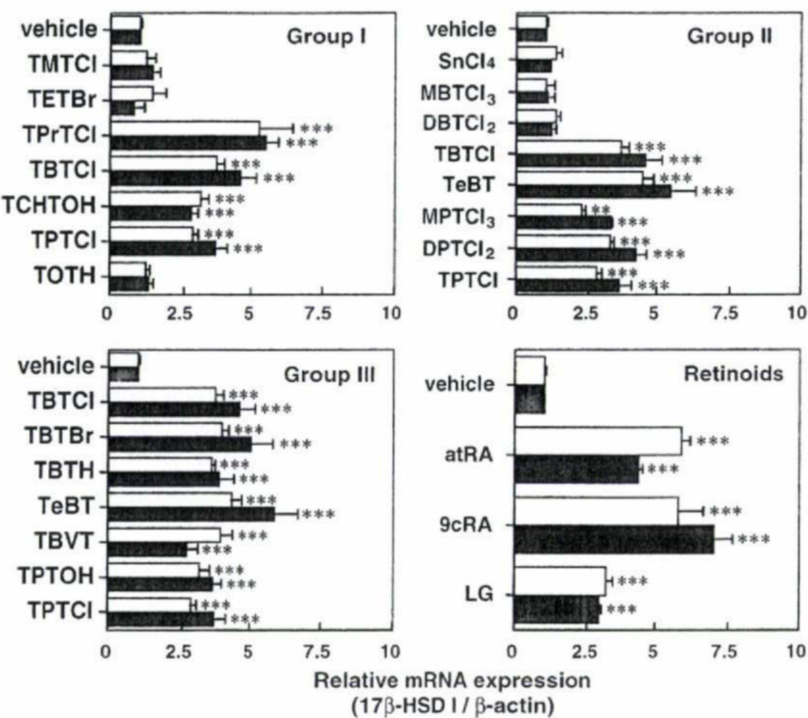


Fig. 3 – Effects of tin compounds on the mRNA expression of 17β-HSD I (HSD17B1) in JAr cells. Total RNA was isolated from JAr cells treated with tin compounds for 24 h (open bars) and 48 h (solid bars). The doses of each compound were: 100 nM of TETBr, TPrTCl, TBTCl, TChTOH, TPTOH, TPTCl, TBTH, TBTBr and $DBTCl_2$; 1 μM of TMTCl, $MBTCl_3$, $DPTCl_2$, 9cRA, atRA and LG; and 10 μM of TOTH, $SnCl_4$, $MPTCl_3$, TeBT and TBVT. The relative mRNA levels for each condition were determined by quantitative RT-PCR assays for each of the 3 independent cultures (see Section 2). Results are expressed as means ± 1 S.D. of three independent cultures. Groups I, II, and III correspond to the groups described in the legend for Fig. 1. **$P < 0.01$; and ***$P < 0.005$ represents values significantly different from vehicle-control values.

TETBr, $MBTCl_3$, and $DBTCl_2$, the organotin compounds that significantly enhanced the catalytic activity of 17β-HSD I also significantly increased its mRNA expression. However, the mRNA effects were much more pronounced than the changes in catalytic activity (Figs. 2 and 3). Furthermore, atRA and 9cRA, which are known to enhance 17β-HSD I activity with mRNA expression, also showed their induction of mRNA expression more obviously than that of catalytic activity (Figs. 2 and 3).

In a recent study, we demonstrated that some organotin compounds function as agonists for retinoid X receptor (RXR), a nuclear receptor for retinoids, to stimulate the mRNA expression of human placental aromatase and human chorionic gonadotropin in human choriocarcinoma cells [14]. As shown in Figs. 2 and 3, organotin compounds that induced the transactivation function of RXR in our previous study (TBT and TPT derivatives, TPrTCl, TChTOH, TeBT, and TBVT) also enhanced 17β-HSD I mRNA transcription and enzyme activity. In addition, a synthetic RXR-specific ligand LG also enhanced 17β-HSD I enzyme activity and mRNA transcription (Figs. 2 and 3). These results suggest that these organotin compounds induce the expression of 17β-HSD I mRNA via RXR transactivation.

However, organotin compounds that did not induce the transactivation function of RXR in our previous study (TETBr, $MBTCl_3$, $DBTCl_2$, $MPTCl_3$ and $DPTCl_2$) also significantly enhanced 17β-HSD I enzyme activity. Interestingly, $MPTCl_3$ and $DPTCl_2$ significantly enhanced mRNA expression of 17β-HSD I, whereas TETBr, $MBTCl_3$, and $DBTCl_2$ had little effect on mRNA expression (Fig. 3). These results indicate that the observed organotin-induced alterations in 17β-HSD I activity are due not only to regulation at the mRNA level but also another mechanism.

4. Discussion

Recently, organotin compounds have become recognized as endocrine-disrupting chemicals, because numerous marine organisms have been shown to exhibit sexual abnormalities after exposure to TBT or TPT. In gastropod mollusks, which are among the species most sensitive to organotin compounds, these chemicals have been demonstrated to induce the superimposition of male sex organs, such as a penis and/or a vas deferens, over female sex organs, a phenomenon known as imposex [10,11]. It has been theorized that TBT increases androgen levels through inhibition of aromatase activity or suppression of androgen excretion [15–17]; nevertheless this theory is not well founded. Although these organotin compounds also are reported to inhibit the catalytic activity of human steroidgenic enzymes, including aromatase [18,19], 3β-hydroxysteroid dehydrogenase type II, 5α-reductase type II, and 17β-HSD I and III [20,21], the concentrations effective for the inhibition of these enzymes were relatively high (>1 μM)

and generally toxic to mammalian cells [22–24], including human choriocarcinoma cells (Fig. 1). Therefore, in regard to effects on humans, we have to distinguish between the nonspecific cellular toxicity of organotins and their inhibition of steroidgenic enzymes. We previously demonstrated that nanomolar concentrations (i.e., 3–100 nM) of some organotin compounds, including TBT and TPT, enhance aromatase activity, which catalyzes the conversion androgen to estrogen, and that this increase in enzymatic activity occurs concurrently with increases in mRNA expression and E2 biosynthesis from androstenedione in human choriocarcinoma cells [14,22]. In addition, our current study showed that many of the same organotin compounds also enhance 17β-HSD I activity, which predominantly catalyzes the conversion of the weakly estrogenic chemical E1 into the strongly estrogenic compound E2 in JAr cells (Figs. 2 and 3). Our findings suggest that the endocrine-disrupting action of these organotin compounds in pregnant women may be to promote the biosynthesis of E2 in the placenta, an effect opposite to that theorized in gastropods.

Our current study has demonstrated that organotin compounds alter E2 biosynthesis in human placental cells in vitro. Although several reports have established the in vivo reproductive toxicity of organotin compounds in rodents [25–28], there are no reports on whether organotin-induced production of placental E2 is associated with teratogenetic effects. Accordingly, it remains unclear which endocrine-disrupting effects or malformations result, at least in part, from organtoin-induced local changes in E2 concentrations of the placenta in vivo. Further, the in vivo endocrine effects of environmental contaminants on the human placenta are difficult to estimate from animal studies, particularly those involving rodents, because the endocrine functions of the placenta vary considerably among different species. In particular, estrogen biosynthesis during pregnancy in humans is much different from that in rodents. In humans, ovarian function gradually declines after fertilization, as the placenta becomes the primary site of estrogen biosynthesis during pregnancy [29]. In contrast to the process in humans, the ovary (not the placenta) is the main source of estrogen during pregnancy in rodents, because the placenta of rodents expresses neither aromatase nor 17β-HSD I [30–32]. It has been suggested that rodents are therefore unsuitable for evaluating the effects of environmental contaminants on estrogen biosynthesis in the human placenta. The regulation of estrogen biosynthesis in placenta is very important for human embryo because altering placental function can cause permanent effects in the embryo. For example, the lack of placental aromatase causes female pseudohermaphroditism, as is seen in patients with aromatase deficiency [33]. Consequently, there is an urgent need to establish effective tools to evaluate the endocrine-disrupting effects and teratogenicity of environmental contaminants that induce changes in local estrogen concentrations of the placenta in vivo.

Several stimuli, such as cAMP analogues [34], the natural retinoic acid receptor (RAR) ligand atRA [35], and the natural RAR and RXR ligand 9cRA [36], induce the mRNA expression of 17β-HSD I, thereby increasing its activity. Organotin compounds, such as TBT and TPT, have no effect on intracellular cAMP production [22]. In a recent study, we demonstrated that some organotin compounds function as RXR agonists to stimulate the mRNA expression of human placental aromatase and human chorionic gonadotropin in human choriocarcinoma cells [14]. These chemicals bind directly to the ligand-binding domain of RXR and function as transcriptional activators. The RXR-agonistic organotin compounds also enhanced the expression of 17β-HSD I mRNA in the present study. The level of activation was nearly equal to the level of RXR activation induced by these compounds [14]. The RAR response element is located at −512 to −479 bp in the promoter region of 17β-HSD I [35], but the RXR response element has not yet been identified. However, Ro41, a specific RAR antagonist, fails to abrogate 9cRA-induced expression of 17β-HSD I mRNA [36]. In addition, we found that the RXR-specific ligand LG consistently enhanced 17β-HSD I enzyme activity and mRNA transcription (Figs. 2 and 3). Unlike 9cRA, these organotin compounds are RXR-specific and do not activate the RAR pathway [14]. In light of these findings, although transcriptional regulation in the 17β-HSD I promoter is not yet fully understood, we suggest that the cis-elements which have a response to the RXR-dependent signaling pathway may be located in the promoter region and that RXR-agonistic organotin compounds induce the expression of 17β-HSD I mRNA via RXR transactivation.

We assayed 17 tin compounds for their ability to induce both the catalytic activity and mRNA expression of 17β-HSD I in JAr cells. The concentrations needed to induce these two features of 17β-HSD I did not differ significantly among the TBT and TPT derivatives. Compared with those for the TBT and TPT derivatives, approximately 30- to 100-fold higher concentrations of tetraalkyltin compounds (e.g., TeBT and TBVT) were needed to affect 17β-HSD I activity or transcript levels. This observation may indicate that tetraalkyltin compounds are metabolically converted to trialkyltins, which are the active form, in the cells. This hypothesis is supported, in a broad sense, by previous results that show that organotin compounds undergo dealkylation by the microsomal monooxygenase system, which is dependent on cytochrome P450 in the liver and other organs [37–39]. In addition, our previous study suggested that it may be necessary for activation of RXR by these tetraalkyltin compounds to metabolically convert them into the active dealkylated form (e.g., TBT) in cells, because the presence of a fourth alkyl group on the tin atom interferes with the binding of alkyltin compounds to the receptor [14]. These findings support our speculation that these organotin compounds induce the expression of 17β-HSD I mRNA via RXR transactivation.

Tin compounds that failed to act as RXR agonists ($MBTCl_3$, $DBTCl_2$, $MPTCl_3$, $DPTCl_2$ and TETBr) [14] also significantly increased the catalytic activity of 17β-HSD I. Strangely enough, metabolites of TPT ($MPTCl_3$ and $DPTCl_2$) significantly enhanced mRNA expression of 17β-HSD I, whereas metabolites of TBT ($MBTCl_3$ and $DBTCl_2$) and TETBr had little effect on mRNA expression (Figs. 2 and 3). It remains unclear why these organotin compounds enhanced the activity of 17β-HSD I with or without altering its mRNA expression. At least, the induction appears due to a mechanism other than activation of RXRs.

To our knowledge, our study is the first to show that organotin compounds potentially promote estrogenic action to enhance 17β-HSD I activity in human placenta. However, the mRNA changes that the compounds induced were not comparable to the changes in catalytic activity. Consequently, we conclude that the observed organotin-induced alterations in JAr cells are due to other mechanisms in addition to regulation of 17β-HSD I mRNA levels. The toxic mechanisms of organotin compounds appear very intricate. For instance, organotin compounds function as inhibitors of steroidogenic enzymes [18–21] and RXR ligands [14] but also have been shown to enhance histone acetyltransferase activity [40]. Future studies need to clarify the precise mechanism of action of organotin compounds in human endocrine disruption in vitro and in vivo.

Acknowledgments

This research was supported in part by Grants in Aid for Scientific Research (No. 15201012) from the Ministry of Education, Science, Sports, and Culture of Japan; The Industrial Technology Research Grant Program in 2001 from NEDO (New Energy and Industrial Technology Development Organization of Japan); Health and Labor Sciences Research Grants (Research on Advanced Medical Technology) from the Ministry of Health, Labor, and Welfare of Japan; the fund for endocrine disruption research from the Ministry of the Environment of Japan; and the Long-range Research Initiative (LRI) by Japan Chemical Industry Association (JCIA). We thank Astellas Pharma for providing LG100268.

REFERENCES

[1] Albrecht ED, Pepe GJ. Placental steroid hormone biosynthesis in primate pregnancy. Endocr Rev 1990;11:124–50.

[2] Luu-The V, Dufort I, Pelletier G, Labrie F. Type 5 17β-hydroxysteroid dehydrogenase: its role in the formation of androgens in women. Mol Cell Endocrinol 2001;171:77–82.

[3] Bonenfant M, Provost PR, Drolet R, Tremblay Y. Localization of type 1 17β-hydroxysteroid dehydrogenase mRNA and protein in syncytiotrophoblasts and invasive cytotrophoblasts in the human term villi. J Endocrinol 2000;165:217–22.

[4] Ghersevich SA, Poutanen MH, Rajaniemi HJ, Vihko RK. Expression of 17β-hydroxysteroid dehydrogenase in the rat ovary during follicular development and luteinization induced with pregnant mare serum gonadotrophin and human chorionic gonadotrophin. J Endocrinol 1994;140:409–17.

[5] Sawetawan C, Milewich L, Word RA, Carr BR, Rainey WE. Compartmentalization of type I 17β-hydroxysteroid oxidoreductase in the human ovary. Mol Cell Endocrinol 1994;99:161–8.

[6] Poutanen M, Moncharmont B, Vihko R. 17β-hydroxysteroid dehydrogenase gene expression in human breast cancer cells: regulation of expression by a progestin. Cancer Res 1992;52:290–4.

[7] Maentausta O, Sormunen R, Isomaa V, Lehto VP, Jouppila P, Vihko R. Immunohistochemical localization of 17β-hydroxysteroid dehydrogenase in the human endometrium during the menstrual cycle. Lab Invest 1991;65:582–7.

[8] Boyer IJ. Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. Toxicology 1989;55:253–98.

[9] Fent K. Ecotoxicology of organotin compounds. Crit Rev Toxicol 1996;26:1–117.

[10] Horiguchi T, Shiraishi H, Shimizu M, Morita M. Effects of triphenyltin chloride and five other organotin compounds on the development of imposex in the rock shell, Thais clavigera. Environ Pollut 1997;95:85–91.

[11] Matthiessen P, Gibbs PE. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ Toxicol Chem 1998;17:37–43.

[12] Kannan K, Tanabe S, Tatsukawa R. Occurrence of butyltin residues in certain foodstuffs. Bull Environ Contam Toxicol 1995;55:510–6.

[13] Kannan K, Tanabe S, Iwata H, Tatsukawa R. Butyltins in muscle and liver of fish collected from certain Asian and Oceanian countries. Environ Pollut 1995;90:279–90.

[14] Nakanishi T, Nishikawa J, Hiromori Y, Yokoyama H, Koyanagi M, Takasuga S, et al. Trialkyltin compounds bind retinoid X receptor to alter human placental endocrine functions. Mol Endocrinol 2005;19:2502–16.

[15] Bettin C, Oehlmann J, Stroben E. TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. Helgol Meeresunters 1996;50:299–317.

[16] Ronis MJJ, Mason AZ. The metabolism of testosterone by the periwinkle (Littorina littorea) in vitro and in vivo: effects of tributyltin. Mar Environ Res 1996;42:161–6.

[17] Spooner N, Gibbs PE, Bryan GW. The effects of tributyltin upon steroid titers in the female dogwhelk, Nucella lapillus, and the development of imposex. Mar Environ Res 1991;32:37–49.

[18] Cooke GM. Effect of organotins on human aromatase activity in vitro. Toxicol Lett 2002;126:121–30.

[19] Heidrich DD, Steckelbroeck S, Klingmuller D. Inhibition of human cytochrome P450 aromatase activity by butyltins. Steroids 2001;66:763–9.

[20] Doering DD, Steckelbroeck S, Doering T, Klingmuller D. Effects of butyltins on human 5α-reductase type 1 and type 2 activity. Steroids 2002;67:859–67.

[21] Lo S, Allera A, Albers P, Heimbrecht J, Jantzen E, Klingmuller D, et al. Dithioerythritol (DTE) prevents inhibitory effects of triphenyltin (TPT) on the key enzymes of the human sex steroid hormone metabolism. J Steroid Biochem Mol Biol 2003;84:569–76.

[22] Nakanishi T, Kohroki J, Suzuki S, Ishizaki J, Hiromori Y, Takasuga S, et al. Trialkyltin compounds enhance human CG secretion and aromatase activity in human placental choriocarcinoma cells. J Clin Endocrinol Metab 2002;87:2830–7.

[23] Saitoh M, Yanase T, Morinaga H, Tanabe M, Mu YM, Nishi Y, et al. Tributyltin or triphenyltin inhibits aromatase activity in the human granulosa-like tumor cell line KGN. Biochem Biophys Res Commun 2001;289:198–204.

[24] Watanabe H, Adachi R, Hirayama A, Kasahara T, Suzuki K. Triphenyltin enhances the neutrophilic differentiation of promyelocytic HL-60 cells. Biochem Biophys Res Commun 2003;306:26–31.

[25] Crofton KM, Dean KF, Boncek VM, Rosen MB, Sheets LP, Chernoff N, et al. Prenatal or postnatal exposure to bis(tri-n-butyltin)oxide in the rat: postnatal evaluation of teratology and behavior. Toxicol Appl Pharmacol 1989;97:113–23.

[26] Ema M, Kurosaka R, Amano H, Ogawa Y. Further evaluation of the developmental toxicity of tributyltin chloride in rats. Toxicology 1995;96:195–201.

[27] Noda T, Morita S, Yamano T, Shimizu M, Yamada A. Effects of triphenyltin acetate on pregnancy in rats by oral administration. Toxicol Lett 1991;56:207–12.
[28] Noda T, Morita S, Yamano T, Shimizu M, Nakamura T, Saitoh M, et al. Teratogenicity study of tri-n-butyltin acetate in rats by oral administration. Toxicol Lett 1991;55:109–15.
[29] Simpson ER, MacDonald PC. Endocrine physiology of the placenta. Annu Rev Physiol 1981;43:163–88.
[30] Akinola LA, Poutanen M, Vihko R, Vihko P. Expression of 17β-hydroxysteroid dehydrogenase type 1 and type 2, P450 aromatase, and 20α-hydroxysteroid dehydrogenase enzymes in immature, mature, and pregnant rats. Endocrinology 1997;138:2886–92.
[31] Durkee TJ, McLean MP, Hales DB, Payne AH, Waterman MR, Khan I, et al. P450(17α) and P450SCC gene expression and regulation in the rat placenta. Endocrinology 1992;130:1309–17.
[32] Jackson JA, Albrecht ED. The development of placental androstenedione and testosterone production and their utilization by the ovary for aromatization to estrogen during rat pregnancy. Biol Reprod 1985;33:451–7.
[33] Shozu M, Akasofu K, Harada T, Kubota Y. A new cause of female pseudohermaphroditism: placental aromatase deficiency. J Clin Endocrinol Metab 1991;72:560–6.
[34] Tremblay Y, Beaudoin C. Regulation of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase messenger ribonucleic acid levels by cyclic adenosine 3′,5′-monophosphate and phorbol myristate acetate in human choriocarcinoma cells. Mol Endocrinol 1993;7:355–64.
[35] Piao YS, Peltoketo H, Oikarinen J, Vihko R. Coordination of transcription of the human 17β-hydroxysteroid dehydrogenase type 1 gene (EDH17B2) by a cell-specific enhancer and a silencer: identification of a retinoic acid response element. Mol Endocrinol 1995;9:1633–44.
[36] Zhu SJ, Li Y, Li H, Wang YL, Xiao ZJ, Vihko P, et al. Retinoic acids promote the action of aromatase and 17β-hydroxysteroid dehydrogenase type 1 on the biosynthesis of 17β-estradiol in placental cells. J Endocrinol 2002;172:31–43.
[37] Kimmel EC, Fish RH, Casida JE. Bioorganotin chemistry. Metabolism of organotin compounds in microsomal monooxygenase systems and in mammals. J Agric Food Chem 1976;25:1–9.
[38] Ohhira S, Matsui H. Metabolism of a tetraphenyltin compound in rats after a single oral dose. J Appl Toxicol 2003;23:31–5.
[39] Ohhira S, Watanabe M, Matsui H. Metabolism of tributyltin and triphenyltin by rat, hamster and human hepatic microsomes. Arch Toxicol 2003;77:138–44.
[40] Osada S, Nishikawa J, Nakanishi T, Tanaka K, Nishihara T. Some organotin compounds enhance histone acetyltransferase activity. Toxicol Lett 2005;155:329–35.

Environmental Sciences, 13, 2 (2006) 089–100
MYU Tokyo

ES612

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

Tsuyoshi Nakanishi*, Jun-ichi Nishikawa[1] and Keiichi Tanaka

Department of Toxicology, Graduate School of Pharmaceutical Sciences,
Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan
[1]Department of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences,
Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan

(Received May 12, 2005; accepted May 28, 2005)

Key words: organotin, aromatase, endocrine disruptor, RXR, PPARγ

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.

---

*E-mail: nakanishi@phs.osaka-u.ac.jp

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

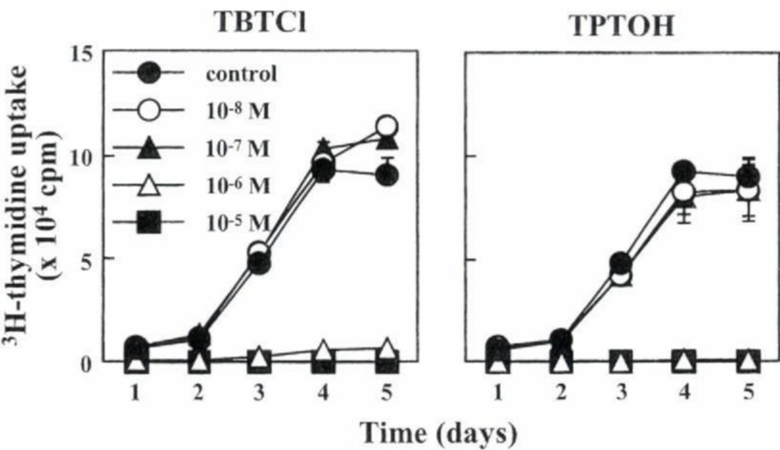


Fig. 1. Effect of organotin compounds on DNA synthesis in JAr cells. Cells (10^3 cells/well) were cultured in 96-well microtiter plates. After 24 h of culture, cells were treated with various concentrations of TBT chloride (TBTCl) or TPT hydroxide (TPTOH) for 1 to 5 days. Each culture was pulsed with 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.

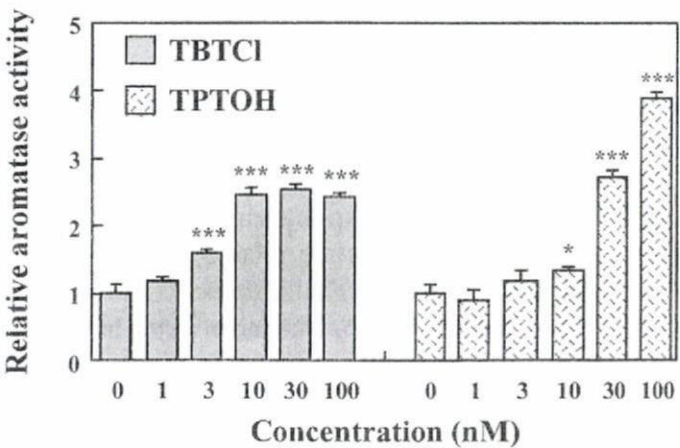


Fig. 2. Effect of organotin compounds on aromatase activity in JAr cells. Cells (1.5×10^5 cells/well) were seeded in 12-well plates. After 24 h of culture, cells were treated with various concentrations of TBTCl or TPTOH for 48 h. At the end point of each treatment, cells were washed and aromatase activity 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

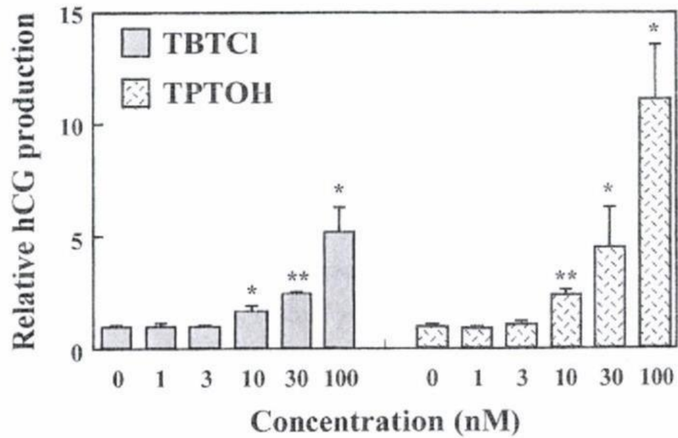


Fig. 3. Effect of organotin compounds on hCG secretion in JAr cells. Cells (4×10^4 - 25 cells/well) were seeded in 48-well plates. After 24 h of culture, cells were treated with various concentrations of TBTCl or TPTOH for 48 h. At the end point of each treatment, cells were washed three times and cultured 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.

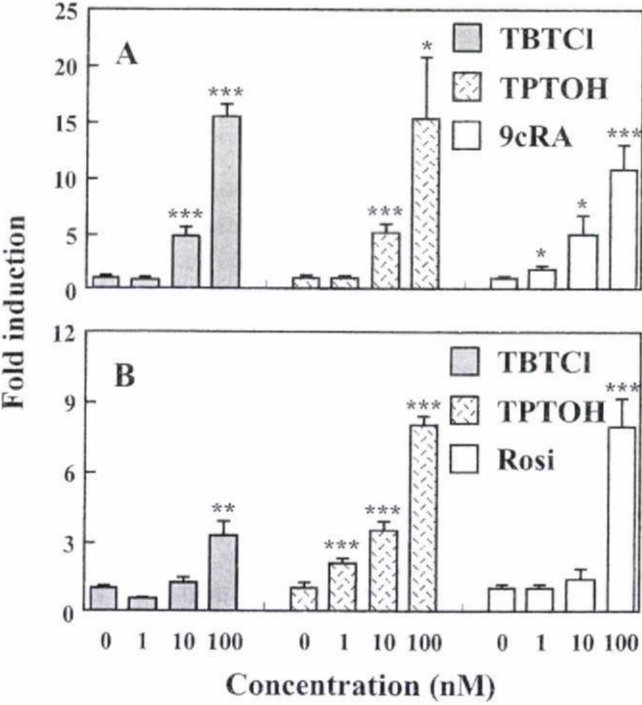


Fig. 4. Organotin compounds induce transcriptional activity through RXR α and PPAR γ. Ligand-dependent transactivation of RXR α and PPAR γ was detected as luciferase activity. (A) JEG-3 cells were cotransfected with a GAL4-DBD-RXR α expression plasmid and a GAL4-responsive reporter plasmid. (B) JEG-3 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 γ 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 γ 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 γ 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.

Acknowledgements

We thank the staff of our laboratory for their helpful discussions. We also thank Teikoku Hormone MFG for providing standardized hCG for ELISA. The work of our laboratory has

been supported in part by: Grants-in-Aid for Scientific Research (Nos. 11877400, 13470499, and 15201012) from the Ministry of Education, Science, Sports and Culture of Japan; the Industrial Technology Research Grant Program in 2001 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan; Health and Labor Sciences Research Grants (Research on Advanced Medical Technology) from the Ministry of Health, Labor and Welfare of Japan; the Long-Range Research Initiative, Japan (LRI); the Senri Life Science Foundation; and the Showa Shell Sekiyu Foundation for the Promotion of Environmental Research.

References

1 Boyer, I.J. (1989): Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. *Toxicology* **55**: 253–298.

2 Fent, K. (1996): Ecotoxicology of organotin compounds. *Crit. Rev. Toxicol.* **26**: 1–117.

3 Kannan, K., Tanabe, S., Iwata, H. and Tatsukawa, R. (1995): Butyltins in muscle and liver of fish collected from certain Asian and Oceanian countries. *Environ. Pollut.* **90**: 279–290.

4 Kannan, K., Tanabe, S. and Tatsukawa, R. (1995): Occurrence of butyltin residues in certain foodstuffs. *Bull. Environ. Contam. Toxicol.* **55**: 510–516.

5 Horiguchi, T., Shiraishi, H., Shimizu, M. and Morita, M. (1997): Effects of triphenyltin chloride and five other organotin compounds on the development of imposex in the rock shell, *Thais clavigera*. *Environ. Pollut.* **95**: 85–91.

6 Matthiessen, P. and Gibbs, P.E. (1998): Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. *Environ. Toxicol. Chem.* **17**: 37–43.

7 Spooner, N., Gibbs, P.E., Bryan, G.W. and Goad, L.J. (1991): The effects of tributyltin upon steroid titers in the female dogwhelk, *Nucella lapillus*, and the development of imposex. *Mar. Environ. Res.* **32**: 37–49.

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

9 Ronis, M.J.J. and Mason, A.Z. (1996): The metabolism of testosterone by the periwinkle (*Littorina littorea*) *in vitro* and *in vivo*: Effects of tributyltin. *Mar.Environ. Res.* **42**: 161–166.

10 Escriva, H., Delaunay, F. and Laudet, V. (2000): Ligand binding and nuclear receptor evolution. *BioEssays* **22**: 717–727.

11 Escriva, H., Safi, R., Hanni, C., Langlois, M.C., Saumitou-Laprade, P., Stehelin, D., Capron, A., Pierce, R. and Laudet, V. (1997): Ligand binding was acquired during evolution of nuclear receptors. *Proc. Natl. Acad. Sci. U. S. A.* **94**: 6803–6808.

12 Laudet, V. (1997): Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J. Mol. Endocrinol.* **19**: 207–226.

13 Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D., Mendelson, C.R. and Bulun, S.E. (1994): Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr. Rev.* **15**: 342–355.

14 Nakajin, S., Shinoda, M. and Hall, P.F.(1986): Purification to homogeneity of aromatase from human placenta. *Biochem. Biophys. Res. Commun.* **134**: 704–710.

15 Kellis, J.T., Jr. and Vickery, L.E. (1987): Purification and characterization of human placental aromatase cytochrome P-450. *J. Biol. Chem.* **262**: 4413–4420.

16 Simpson, E.R., Zhao, Y., Agarwal, V.R., Michael, M.D., Bulun, S.E., Hinshelwood, M.M., Graham-Lorence, S., Sun, T., Fisher, C.R., Qin, K. and Mendelson, C.R. (1997): Aromatase expression in health and disease. *Recent. Prog. Horm. Res.* **52**: 185–213; discussion 213–214.

17 Bulun, S.E., Economos, K., Miller, D. and Simpson, E.R. (1994): *CYP19* (aromatase cytochrome P450) gene expression in human malignant endometrial tumors. *J. Clin. Endocrinol. Metab.* **79**: 1831–1834.

18 Bulun, S.E., Zeitoun, K.M., Takayama, K., Simpson, E. and Sasano, H. (2000): Aromatase as a therapeutic target in endometriosis. *Trends. Endocrinol. Metab.* **11**: 22–27.

19 Heidrich, D.D., Steckelbroeck, S. and Klingmuller, D. (2001): Inhibition of human cytochrome P450 aromatase activity by butyltins. *Steroids* **66**: 763–769.

20 Cooke, G.M. (2002): Effect of organotins on human aromatase activity *in vitro*. *Toxicol. Lett.* **126**: 121–130.

21 Nakanishi, T., Kohroki, J., Suzuki, S., Ishizaki, J., Hiromori, Y., Takasuga, S., Itoh, N., Watanabe, Y., Utoguchi, N. and Tanaka, K. (2002): Trialkyltin compounds enhance human CG secretion and aromatase activity in human placental choriocarcinoma cells. *J. Clin. Endocrinol. Metab.* **87**: 2830–2837.

22 Saitoh, M., Yanase, T., Morinaga, H., Tanabe, M., Mu, Y.M., Nishi, Y., Nomura, M., Okabe, T., Goto, K., Takayanagi, R. and Nawata, H. (2001): Tributyltin or triphenyltin inhibits aromatase activity in the human granulosa-like tumor cell line KGN. *Biochem. Biophys. Res. Commun.* **289**: 198–204.

23 Watanabe, H., Adachi, R., Hirayama, A., Kasahara, T. and Suzuki, K. (2003): Triphenyltin enhances the neutrophilic differentiation of promyelocytic HL-60 cells. *Biochem. Biophys. Res. Commun.* **306**: 26–31.

24 Sebastian, S. and Bulun, S.E. (2001): A highly complex organization of the regulatory region of the human *CYP19* (aromatase) gene revealed by the Human Genome Project. *J. Clin. Endocrinol. Metab.* **86**: 4600–4602.

25 Bulun, S.E., Sebastian, S., Takayama, K., Suzuki, T., Sasano, H. and Shozu, M. (2003): The human *CYP19* (aromatase P450) gene: update on physiologic roles and genomic organization of promoters. *J. Steroid Biochem. Mol. Biol.* **86**: 219–224.

26 Bamberger, A.M., Ezzat, S., Cao, B., Wong, M., Parker, K.L., Schulte, H.M. and Asa, S.L. (1996): Expression of steroidogenic factor-1 (SF-1) mRNA and protein in the human placenta. *Mol. Hum. Reprod.* **2**: 457–461.

27 Simpson, E.R., Michael, M.D., Agarwal, V.R., Hinshelwood, M.M., Bulun, S.E. and Zhao, Y. (1997): Cytochromes P450 11: expression of the *CYP19* (aromatase) gene: an unusual case of alternative promoter usage. *FASEB J.* **11**: 29–36.

28 Michael, M.D., Kilgore, M.W., Morohashi, K. and Simpson, E.R. (1995): Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (*CYP19*) gene in the ovary. *J. Biol.Chem.* **270**: 13561–13566.

29 Kanayama, T., Mamiya, S., Nishihara, T. and Nishikawa, J. (2003): Basis of a high-throughput method for nuclear receptor ligands. *J. Biochem.* **133**: 791–797.

30 Kanayama, T., Kobayashi, N., Mamiya, S., Nakanishi, T. and Nishikawa, J. (2005): Organotin compounds promote adipocyte differentiation as agonists of the peroxisome proliferator-activated receptor γ/retinoid X receptor pathway. *Mol. Pharmacol.* **67**: 766–774.

31 Chambon, P. (1996): A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10**: 940–954.

32 Aranda, A. and Pascual, A. (2001): Nuclear hormone receptors and gene expression. *Physiol. Rev.* **81**: 1269–1304.

33 Giguere, V. (1994): Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. *Endocr. Rev.* **15**: 61–79.

34 Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A. and Evans, R.M.(1992): Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* **358**: 771–774.

35 Issemann, I., Prince, R.A., Tugwood, J.D. and Green, S. (1993): The peroxisome proliferator-activated receptor:retinoid X receptor heterodimer is activated by fatty acids and fibrate hypolipidaemic drugs. *J. Mol. Endocrinol.* **11**: 37–47.

36 Bardot, O., Aldridge, T.C., Latruffe, N. and Green, S. (1993): PPAR/RXR heterodimer activates a peroxisome proliferator response element upstream of the bifunctional enzyme gene. *Biochem. Biophys. Res. Commun.* **192**: 37–45.

37 Westin, S., Kurokawa, R., Nolte, R.T., Wisely, G.B., McInerney, E.M., Rose, D.W., Milburn, M.V., Rosenfeld, M.G. and Glass, C.K. (1998): Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature* **395**: 199–202.

38 Schulman, I.G., Shao, G. and Heyman, R.A. (1998): Transactivation by retinoid X receptor-peroxisome proliferator-activated receptor γ(PPAR γ) heterodimers: intermolecular synergy requires only the PPAR γhormone-dependent activation function. *Mol. Cell. Biol.* **18**: 3483–3494.

39 Laffitte, B.A., Kast, H.R., Nguyen, C.M., Zavacki, A.M., Moore, D.D. and Edwards, P.A. (2000): Identification of the DNA binding specificity and potential target genes for the farnesoid X-activated receptor. *J. Biol. Chem.* **275**: 10638–10647.

40 Mukherjee, R., Davies, P.J., Crombie, D.L., Bischoff, E.D., Cesario, R.M., Jow, L., Hamann, L.G., Boehm, M.F., Mondon, C.E., Nadzan, A.M., Paterniti, J.R., Jr. and Heyman, R.A. (1997): Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature* **386**: 407–410.

41 Germain, P., Iyer, J., Zechel, C. and Gronemeyer, H. (2002): Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. *Nature* **415**: 187–192.

42 Thompson, P.D., Jurutka, P.W., Haussler, C.A., Whitfield, G.K. and Haussler, M.R. (1998): Heterodimeric DNA binding by the vitamin D receptor and retinoid X receptors is enhanced by 1,25-dihydroxy vitamin D3 and inhibited by 9-cis-retinoic acid. Evidence for allosteric receptor interactions. *J. Biol. Chem.* **273**: 8483–8491.

43 Forman, B.M., Umesono, K., Chen, J. and Evans, R.M. (1995): Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell* **81**: 541–550.

44 Nakanishi, T., Nishikawa, J., Hiromori, Y., Yokoyama, H., Koyanagi, M., Takasuga, S., Ishizaki, J.I., Watanabe, M., Isa, S.I., Utoguchi, N., Itoh, N., Kohno, Y., Nishihara, T. and Tanaka, K. (2005): Trialkyltin compounds bind retinoid X receptor to alter human placental endocrine functions. *Mol. Endocrinol.* **19**: 2502–2516.

45 Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M. and Kliewer, S.A. (1995): An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ(PPAR γ). *J. Biol. Chem.* **270**: 12953–12956.

46 Tontonoz, P., Hu, E. and Spiegelman, B.M. (1994): Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell* **79**: 1147–1156.

47 Sun, T., Zhao, Y., Mangelsdorf, D.J. and Simpson, E.R. (1998): Characterization of a region upstream of exon I.1 of the human *CYP19* (aromatase) gene that mediates regulation by retinoids in human choriocarcinoma cells. *Endocrinology* **139**: 1684–1691.

48 Mu, Y.M., Yanase, T., Nishi, Y., Takayanagi, R., Goto, K. and Nawata, H. (2001): Combined treatment with specific ligands for PPAR γ/RXR nuclear receptor system markedly inhibits the expression of cytochrome P450arom in human granulosa cancer cells. *Mol. Cell. Endocrinol.* **181**: 239–248.

49 Mu, Y.M., Yanase, T., Nishi, Y., Waseda, N., Oda, T., Tanaka, A., Takayanagi, R. and Nawata, H. (2000): Insulin sensitizer, troglitazone, directly inhibits aromatase activity in human ovarian granulosa cells. *Biochem. Biophys. Res. Commun.* **271**: 710–713.

50 Fan, W., Yanase, T., Morinaga, H., Mu, Y.M., Nomura, M., Okabe, T., Goto, K., Harada, N. and Nawata, H. (2005): Activation of peroxisome proliferator-activated receptor-$\gamma$and retinoid X receptor inhibits aromatase transcription via nuclear factor-κB. *Endocrinology* **146**: 85–92.