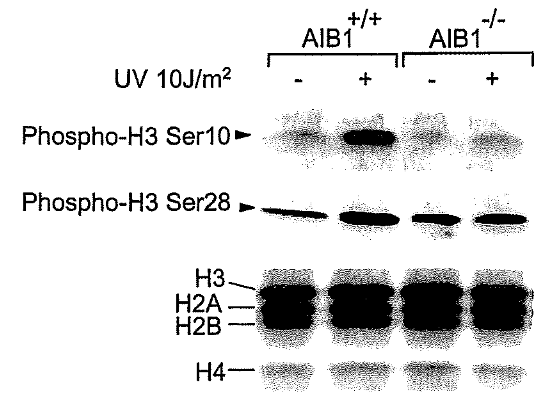


Fig. 6. **Knockout of AIB1 leads to inhibition of UV-induced phosphorylation of histone H3 at serine 10.** Western blot analyses are shown with the specific antibodies indicated on the left, in wild-type or AIB1-knockout cells either treated or untreated by UV irradiation (10 J/m$^2$). Coomassie Blue–stained 15% polyacrylamide SDS–containing gels indicated equal loading of proteins.

following UV irradiation (data not shown). These results suggest that AIB1 plays a critical role in the signaling cascade for both Akt activation and modulation of the phosphorylation of histone H3 at serine 10 in response to cellular stress.

## DISCUSSION

Recent studies have demonstrated that AIB1 plays a pivotal role in activation of the intrinsic IGF-1–driven cell survival pathway, which is mediated through the PI3K/Akt pathway (*15*). Although the signal transduction pathways that lead to the positive control of AIB1 have been studied extensively, the critical targets of this kinase that mediate the stress response remain to be determined. We showed here that AIB1-deficient DT40 cells are extremely sensitive to be killed by cellular stresses such as serum deprivation and UV irradiation. This susceptibility was correlated with a reduction in the ability to restore DNA-synthesis levels under stress conditions. Moreover, we showed that, with serum deprivation or UV irradiation treatment, the induction of phosphorylation of both JNK and c-Jun in AIB1-knockout DT40 cells was much greater than in wild-type DT40 cells. These results are consistent with those of previous studies, which demonstrated that activation of the JNK/c-Jun pathway mediates the induction of cell death by DNA damage agents (*33*, *34*). Our results therefore indicate that the presence of AIB1 is required to suppress activation of the JNK/c-Jun signaling pathway in DNA replication under cellular stress conditions. On the other hand, in ER-negative cancer cells subjected to cellular stress, high levels of AIB1 production have been shown to promote activation of the Akt/p65 survival pathway (*15*). It has been shown that activation of c-Jun by the JNK apoptosis pathway is required to suppress NF-κB transcription (*35*, *36*). Our study is consistent with the result of a recent report, which demonstrated that Akt inhibits stress-activated JNK through activation of NF-κB (*36*). Collectively, these data indicate that AIB1 plays a key role as a mediator between the Akt/NF-κB and JNK/c-Jun pathways in controlling cell fate in response to cellular stress.

Importantly, various signal transduction pathways can modulate the interactions of specific coregulators with nuclear receptors or mediate their activities (*37*). Recent studies suggest that the transcriptional corepressors NCoR and SMRT interact with, and exert repressive effects on AP-1 or NF-κB (*38–41*). It has been proposed that the transactivation potential of c-Jun is repressed by histone deacetylase (HDAC) complexes and these repressor complexes are dissociated by JNK-mediated phosphorylation (*42*). As can be seen from our stress assays in DT40 cells, presence of AIB1 is an important key to modulate the switch from transcriptional repression to activation in association with the diverse protein kinase-dependent signalling pathways in response to cellular stress.

We have shown that, in addition to its role in signal ransduction pathways, AIB1 production is correlated with UV-induced phosphorylation of histone H3 at serine 10, but not with acetylation of histone H3 at lysine 9 or 14. These results suggest that AIB1 is essential in mediation of the phosphorylation of histone H3 in chromatin remodeling. Although it has been shown that the phosphorylation of histone H3 is mediated by the Aurora B kinases (*43*), IKK-*α* (*45*), MSK1 and MSK2 (*45*), and RSK-2 (*46*), we need to reveal by future studies a kinase that can phosphorylate histone H3 in association with AIB1 directly. Previous studies have reported that IκB kinase, a positive regulator of NF-κB activation, is activated by Akt (*13*, *14*). However, production of endogenous IKKα or IKKβ proteins did not change detectably in response to activated Akt in DT40 cells (data not shown). NF-κB activation by stress stimuli has also been shown to be independent of phosphorylation of IκBα at Ser 32/36, and to be IKK-independent (*47*). Moreover, NF-κB, which is usually maintained in an inactive state by protein–protein interaction with inhibitor IκBs, is constitutively active in ER-negative breast cancer cell lines (*48*). Thus, in DT40 cells AIB1 might be produced as one of the downstream targets interacting directly with Akt, independently of IKK.

Previous studies have found that, under conditions of stress, Akt interacts with JIP1 in primary neurons and thus inhibits JNK activation. Therefore, ectopic expression of Akt attenuated stress-induced apoptosis while Akt1 gene deletion rendered neurons more sensitive to stress stimulus than wild-type neurons (*49*). Moreover, recent studies have shown that activation of NF-κB is required for inhibition of JNK in response to TNF-α or UV stimulation (*21–24*). These previous studies and our findings suggest that the level of production of AIB1 is a key determinant of cell susceptibility to cellular stress, in association with phosphorylation cascades. Furthermore, we have elucidated the molecular mechanisms by which, in response to cellular stress, AIB1 plays a critical role in DNA replication or phosphorylation of histone H3 at serine 10, in association with active Akt/NF-κB pathway.

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# Anti-Androgenic Activity of *N*-Nitrosodibenzylamine, *N*-Nitrosodiphenylamine and *N*-Nitrosodicyclohexylamine

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When 56 selected environmental chemicals were tested for the androgenic activity to Yeast Two-hybrid and reporter gene assay in the presence of 5$\alpha$-dihydrotestosterone (DHT), the activity was inhibited by some of the chemicals including *N*-nitrosodiphenylamine (NDPA), a novel anti-androgenic compound, and one of suspected carcinogenic *N*-nitrosocompounds (NOCs) commonly used as material of rubber and plastic goods. We further examined 15 NOCs for anti-androgenic activity, and found that *N*-nitrosodibenzylamine (NDBzA) and *N*-nitrosodicyclohexylamine (NDCHA) as well as NDPA inhibited the activity of DHT in a dose-dependent manner. These compounds showed the competitive binding to androgen receptor (AR) against DHT and decreased the level of AR protein. Furthermore, 3 NOCs down-regulated the prostate specific antigen (PSA) at the transcriptional level in LNCaP cells. These results suggest that some NOCs antagonized the androgenic effect of DHT in the same manner as the synthetic anti-androgen, flutamide (F).

**Key words** —— anti-androgenic activity, androgen receptor, *N*-nitrosocompound, *N*-nitrosodibenzylamine, *N*-nitrosodiphenylamine, *N*-nitrosodicyclohexylamine

## INTRODUCTION

The androgens, testosterone (T) and its metabolite 5$\alpha$-dihydrotestosterone (DHT), play an important role in the development and function of male reproductive organs such as prostate and testis, as well as non-reproductive organs including muscle, hair follicles and brain. Their biological effects are mediated by one of the nuclear receptor superfamily of ligand-regulated transcription factors, androgen receptor (AR).[1,2] T is synthesized mainly in the Leydig cells of testes and converted in the prostate to DHT, a more potent androgen than T. Upon DHT binding to AR in the cytosol, the complex translocates to the nucleus, where AR-DHT complex binds to androgen response element (ARE) in the promoter region of target genes and regulate the transcription of them.[3,4] The androgen target gene, a member of the human kallikrein gene family, produces prostate specific antigen (PSA), which is well known as a marker protein of prostate cancer.[5]

It has been noticed that some environmental and industrial chemicals interfere with endogenous androgen function in humans and wildlife. These compounds are referred to as endocrine disruptors (EDs). Interference with androgenic action can occur in a various developmental and reproductive abnormalities of the male sex functions.[6] Although there have been many reports on EDs, most of them are estrogenic action via estrogen receptor (ER). We therefore have been focused on anti-androgenic compounds, showing female phenotype via AR.

There have been many studies of screening for EDs by *in vitro* assays, such as Yeast Two-hybrid, reporter gene, and receptor binding assay.[7–10] Environmental anti-androgens, such as *p,p'*-dichlorodiphenyldichloroethylene (DDE), vinclozolin and linuron, compete with endogenous androgens for AR, to alter androgen-dependent transcriptions by inhibition of binding to AR.[11–16] Now more than

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50000 chemicals are distributed in the world, among which novel and potent anti-androgenic chemicals may exist. We should therefore assess androgenic action of these chemicals before they affect to humans and wildlife.

In this study, we first tested anti-androgenic activity of a total of 56 environmental chemicals by Yeast Two-hybrid and AR-EcoScreen cell reporter gene assay. These were performed flutamide (F) and hydroxyflutamide (HF) as a positive compound. F is well known as a synthetic anti-androgen and used for drug therapy of prostate cancer.[17] HF is an active metabolite of F.

We found a novel anti-androgenic compound, *N*-nitrosodiphenylamine (NDPA). *N*-Nitroso compounds (NOCs) including NDPA are well known to have carcinogenic and mutagenic properties, such that gastric, esophageal, nasopharyngeal, bladder and colon cancers.[18,19] However there have been no reports that NOCs affect endocrine systems. Exposure to environmental NOCs is through various pathways, for example, life-style (tobacco, food, cosmetic products and household commodities), occupational (rubber, leather, and material industry) and uptake of precursors (nitrite, nitrate and amine).[20] Thus it is thought that humans and wildlife have chance affected by NOCs. Then we tested anti-androgenic activity of 15 NOCs by using Yeast Two-hybrid and reporter gene assay. Finally we investigated the mechanism of anti-androgenic action of positive compounds.

## MATERIALS AND METHODS

**Chemicals and Cells** —— All chemicals of the highest grade commercially available were used without further purification. Most of 56 test chemicals listed in Table 1 are the same used in the previous paper.[7] NOCs listed in Table 2 were purchased from Wako Pure chemicals (Osaka, Japan) and dissolved in dimethyl sulfoxide (DMSO) for use. AR EcoScreen cells were grown in 10 cm dishes using DMEM/F-12 (GIBCO, BRL, Inc., U.K.) supplemented with 5% heat-inactivated fetal bovine serum (FBS, ICN Biomedical, Inc., Aurora, Ohio), penicillin (100 U/ml), streptomycin (100 $\mu$g/ml) (Nakarai Tesque Co., Kyoto, Japan) in a humidified 5% $CO_2$ incubator. LNCaP cells, the androgen-sensitive human prostate cancer cell line, were cultured in 10 cm dishes using RPMI 1640 (Nacalai Tesque Co., Kyoto, Japan) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 $\mu$g/ml) (Nacalai Tesque Co., Kyoto, Japan) in a humidified 5% $CO_2$ incubator.

**Yeast Two-Hybrid Assay (AR:SRC-1)** —— The Yeast Two-hybrid assay system with the rat AR and the coactivator, steroid receptor coactivator-1 (SRC-1), was prepared by modifying the method described in previous reports.[7,21] Briefly, two expression plasmids, pGBT9-AR-LBD and pACT2-SRC-1, were transformed into yeast cells (Saccharomyces cerevisiae Y190). The yeast cells (100 $\mu$l), pre-incubated overnight at 30°C in synthetic defined (SD) medium free from tryptophan and leucine, were incubated with NOCs (2.5 $\mu$l) and DHT (40 nM final concentration) in SD medium lacking tryptophan and leucine (150 $\mu$l) at 30°C for 4 hr. After the absorbance at 595 nm was measured, the cultured cells were digested enzymatically with zymolyase 20T (Seikagaku Co., Tokyo, Japan) at 37°C for 15 min. Then the lysate was mixed with 40 $\mu$l of *o*-nitrophenyl-$\beta$-D-galactopyranoside (4 mg/ml in Z-buffer) and incubated at 37°C for 1 hr. Finally, added 100 $\mu$l of 1 M $Na_2CO_3$ to stop the reaction and then absorbance at 420 and 570 nm were measured by using a 96-well microplate reader (Model 550 MICROPLATE READER, BIO RAD) and $\beta$-galactosidase activity was calculated from these 3 absorbances. The anti-androgenic activity was expressed as the percentage against $\beta$-galactosidase activity of 40 nM DHT without chemicals (100%). It was judged to be positive when the inhibition was more than 20% and the cytotoxicity was not observed at these concentrations. Cytotoxicity of the compound was confirmed by using control yeast cells which transformed pGBT9-p53 and pGAD3F-SV40 into yeast cells. $IC_{50}$ values were calculated using GraphPad Prism 2.01 software.

**Reporter Gene Assay for AR (AR-EcoScreen)** —— The reporter gene assay using AR-EcoScreen cells was performed as previously described.[22] AR EcoScreen can evaluate androgenic activity and toxicity of compound. Briefly, in 24 well plates, AR-EcoScreen cells were seeded $1 \times 10^5$ cells/ml in phenol red free DMEM/F12 containing 5% charcoal-dextran treated fetal bovine serum (FBS). After 24 hr of culturing, medium was changed and added NOCs with 0.5 nM DHT. Following 16–24 hr of culturing, cells were washed twice with phosphate buffered saline (PBS), lysed with Passive Lysis Buffer (Promega Co., WI, U.S.A.) and assayed using Dual luciferase assay system (Promega Co., WI, U.S.A.) with luminometer (Lumat LB9501, Berthold

**Table 1.** Names of 56 Test Chemicals and Anti-Androgenic Activity in the Yeast Two-Hybrid Assay (AR:SRC-1)

| Group, compounds[a)] | |
|---|---|
| Pesticides and related (21) | Benzens and heterocyclics (9) |
| 1,2-Dibromo-3-chloropropane | 2,4-Dinitroaniline |
| 2,4,5-Trichlorophenol | 2,5-Dinitroaniline |
| 2,4-Dichlorophenoxyacetic acid | 2-Phenylendiamine |
| 2,4,5-Trichlorophenoxyacetic acid | 4-Chloroaniline |
| Alachlor[b)] | Benzophenone[b)] |
| Aldicarb | Biphenyl[b)] |
| Captan | *N*-Ethylaniline |
| Carbaryl (NAC)[b)] | 4-Nitrotoluene |
| $\gamma$-Hexachlorocyclohexane ($\gamma$-HCH)[b)] | *N*-Nitrosodiphenylamine (NDPA)[b)] |
| Hexachlorophene[b)] | Phthalates and adipate (9) |
| Maneb | Di-*n*-ethyl phthalate[b)] |
| Manzeb | Di-*n*-propyl phthalate[b)] |
| Methomyl | Di-*n*-butyl phthalate[b)] |
| Methoxychlor (MXC) | Di-*n*-pentyl phthalate |
| Molinate[b)] | Di-*n*-hexyl phthalate |
| Pentachlorophenol | Butylbenzyl phthalate[b)] |
| Thiobencarb[b)] | 2-Ethylhexyl phthalate |
| Thiuram | 2-Cyclohexyl phthalate |
| Vinclozolin[b)] | 2-Ethylhexyl adipate |
| Simazine | Aliphatics (4) |
| Ziram | Cyclohexyl amine |
| Phenols (9) | *N,N*-Dimethylformamide |
| 2,4-Dichlorophenol | Nitrilotriacetic acid |
| 2,4-Dinitrophenol | *N*-Nitrosodimethylamine |
| 2,4,6-Tribromophenol | Flavonoids (4) |
| 2,5-Dichlorophenol | Coumestrol |
| 4-Cresol | Daizein |
| 4-Nonylphenol[b)] | Genistein |
| Bisphenol A[b)] | Naringenin |
| Diethylstilbesterol (DES) | |
| *N*-Phenyl-1-naphthylamine[b)] | |

*a*) Compounds marked by *b*) were positive in Yeast Two-hybrid assay (AR:SRC-1).

GmbH & Co.). The anti-androgenic activity was expressed as the percentage against 0.5 nM DHT without chemicals (100%). We judged as anti-androgen when 20% or more inhibition was calculated without cytotoxicities. $IC_{50}$ values were calculated using GraphPad Prism 2.01 software.

**Competitive Binding Assay for AR** —— The binding affinity of NOCs to AR was determined by a fluorescene polarization assay using ANDROGEN RECEPTOR COMPETITOR, GREEN Kit (Pan Vera, Madison, U.S.A.). Briefly, 1 $\mu$l of NOC/DMSO solution was added to 49 $\mu$l of AL green assay buffer in the small test tube. Additionally, added 50 $\mu$l of AR-ligand binding domain (LBD) (25 nM) / Fluormone AL green (1 nM) complex to the same tube and mixed. The assay tube covered to protect the reagents from light was incubated at 22°C for 5 hr. Finally, sample fluorescence was measured on BEACON 2000 (Pan Vera, Madison, U.S.A.). DMSO (0% inhibition) instead of the compound solution was used as a negative control, and 0.5 $\mu$l of Fluormone AL green (1 nM) instead of AR-LBD/ Fluormone AL green complex as a positive control (100% inhibition). $IC_{50}$ values were calculated using GraphPad Prism 2.01 software.

**Protein Preparation and Western Blotting** —— LNCaP cells were seeded at $2.0 \times 10^5$ cells/ml in RPMI 1640 medium supplemented with 10% char-

**Table 2.** Anti-Androgenic Activity of 17 Environmental Chemicals by Yeast Two-Hybrid Assay and Reporter Gene Assay

| Group, compounds | Yeast Two-hybrid assay $IC_{20}$ [a] | Reporter gene assay $IC_{20}$ [b] |
|---|---|---|
| Pesticides and related | | |
| Alachlor | + | ++ |
| Carbaryl | ++ | – |
| Hexachlorophene | ++ | – |
| γ-Hexachlorocyclohexane | + | – |
| Molinate | + | – |
| Thiobencarb | ++ | ++ |
| Vinclozolin | ++ | +++ |
| Phenols | | |
| 4-Nonylphenol | +++ | ++ |
| Bisphenol A | + | ++ |
| *N*-Phenyl-1-naphtylamine | + | – |
| Benzens and heterocyclics | | |
| Benzophenone | + | – |
| Biphenyl | ++ | – |
| *N*-Nitrosodiphenylamine (NDPA) | ++ | ++ |
| Phthalates | | |
| Di-*n*-ethyl phthalate | + | – |
| Di-*n*-propyl phthalate | ++ | +++ |
| Di-*n*- butyl phthalate | ++ | ++ |
| Butylbenzyl phthalate | + | +++ |

*a*) Concentration of the test compounds showing 20% inhibition of the androgenic activity induced by 40 nM DHT. *b*) Concentration of the test compounds showing 20% inhibition of the androgenic activity induced by 0.5 nM DHT. Symbols: +++, anti-androgenic activity ($IC_{20} < 1$ μM); ++, anti-androgenic activity (1 μM $\leq IC_{20} <$ 10 μM); +, anti-androgenic activity (10 μM $\leq IC_{20}$); –, no effect.

coal-stripped FBS. After 24 hr of incubating, the cells in fresh medium were incubated for 10 hr with NOCs in the presence of 10 nM DHT. After the treatment, the cells were collected Passive Lysis Buffer (Promega Co., WI, U.S.A.) and centrifuged for 5 min. The supernatant was collected as a sample of Western blotting. 15 μg aliquots were separated by SDS-PAGE (7.5% acrylamide gel) and transferred to poly(vinylidene fluoride) (PVDF) membrane. The membrane was probed with rabbit anti-androgen receptor antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.), followed by peroxidase-conjugated anti-rabbit IgG antibody (Amersham Biosciences, Piscataway, NJ, U.S.A.). The membrane was then visualized using an electrochemical luminescence (ECL) detection system.

**RNA Preparation and Northern Blotting** —— LNCaP cells were seeded at $1.5 \times 10^5$ cells/ml in RPMI 1640 medium supplemented with 10% charcoal-stripped FBS. After 24 hr incubation, the medium was changed and NOC was added with 10 nM DHT. After 18 hr treated, total RNA was isolated using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, U.S.A.). Total RNA (12 μg) was denatured in 50% formamide and 17.5% formaldehyde at 65°C and fractionated by electrophoresis on a 1% agarose gel containing 18% formaldehyde. Samples were transferred to nylon membrane (Hybond $N^+$, Amersham Life Sciences, Little Chalfont, Buckinghamshire, U.K.) in 20 × SSC (1 × SSC is 0.15 M NaCl and 0.0015 M sodium citrate). The DNA probes for PSA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were generated from PCR products. PCR primers used for PSA (418-939 bp) were: forward; 5′-GGCAGGTGCTTGTAGCCTCTC-3′, reverse; 5′-CACCCGAGCAGGTGCTTTTGC-3′, and for GAPDH: forward; 5′-ACCACAGTCCATGCCATCA-3′, reverse; 5′-TCCACCACCCTGTTGCTGTA-3′. These products were labeled with [$\alpha$-$^{32}$P]dCTP using the BcaBEST™ Labeling Kit (TaKaRa Bio. Inc., Ohtsu, Japan). Hybridization was performed overnight at 65°C in 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA), 137 mM $Na_2HPO_4$

NDBzA NDPA NDCHA

**Fig. 1.** Chemical Structures of 3 NOCs Showing Anti-Androgenic Activity

and 63.2 mM $NaH_2PO_4$.

**Scanning Bio-Imaging Analysis** —— Scanning bio-imaging analysis was performed with a BAS-2500 (FUJI PHTO FILM Co. LTD., Kanagawa, Japan). The area of PSA was integrated by GAPDH.

**Statistics** —— All results are expressed as means ± standard deviations (S.D.). Statistical analysis was performed by Dunnett's method.

## RESULTS

### Screening for Anti-Androgenic activity of the 56 Environmental Chemicals by Yeast Two-Hybrid Assay (AR:SRC-1) and AR-EcoScreen Cell Reporter Gene Assay

For anti-androgenic activity, the test chemicals were examined in the presence of 40 and 0.5 nM DHT in Yeast Two-hybrid assay (AR:SRC-1) and AR-EcoScreen cell assay, respectively. The 2 concentrations of DHT in the assay were corresponding to 50% and 70% of the maximum activity in each assay, and it was judged to be positive for the chemicals having $IC_{20}$ values of lower than 10 $\mu$m. Names of the 56 chemicals tested are listed in Table 1, and positive in the Yeast Two-hybrid assay were marked by *.

As seen in Table 1, about one third, 17 of the 56 chemicals, were positive in the Yeast Two-hybrid assay. When these positive compounds were applied to the cell assay, 9 of them were positive in the reporter gene assay (Table 2). The result shows that 6 (thiobencarb, vinclozolin, 4-nonylphenol, NDPA, di-n-proyl phthalate, and di-n-butyl phthalate) were agreed in both assays, but the remaining were disagreed. Except for NDPA and di-n-proyl phthalate, their anti-androgenic activities had already been reported.

### Screening for Anti-Androgenic Activity on 15 NOCs

In the last section, we found that NDPA and di-n-propyl phthalate were only newly found anti-androgenic compounds. Because anti-androgenic activities of the other phthalates had been tested, we focused on the anti-androgenic activity of NDPA, and NOC with testing 15 chemicals by two *in vitro* assays. Although no androgenic activity was observed on the 15 NOCs (data not shown), 3 NOCs [*N*-nitrosodibenzylamine (NDBzA), NDPA and *N*-nitrosodicyclohexylamine (NDCHA)] shown in Fig. 1 indicated significant and dose-dependent anti-androgenic activities by two *in vitro* assays (Table 3, Fig. 2, 3). $IC_{50}$ values of them were 3, 28, and 55 $\mu$m in the Yeast Two-hybrid assay and 5, 17 and 12 $\mu$m in the AR-EcoScreen cell reporter gene assay, whereas $IC_{50}$ of F was 5 and 0.2 $\mu$m, respectively (Table 4). Thus anti-androgenic activity of NDBzA was thought to be near F, whereas that of NDPA and NDCHA was about 10 times lower than these compounds.

### Mode of Action of NDBzA, NDPA and NDCHA

Many anti-androgens inhibited androgenic action by competition on binding to AR. As shown in Fig. 4, NDBzA, NDPA and NDCHA showed the binding affinity to AR by the competitive binding assay using fluorescent labeling AR ligand. $IC_{50}$ values of NDBzA, NDPA, NDCHA, F and DHT were 20, 183, 27, 29 and 0.04 *m*m, respectively (Table 4). And the binding affinity of NDBzA and NDCHA was estimated to be similar to F, and NDPA was about 10 times lower, although these affinities were 1000 to 10000 times lower than DHT.

The effect of these NOCs on the level of AR protein expression was examined in androgen dependent LNCaP cells. When the cells were treated with DHT for 10 hr, the level of AR increased. Then when LNCaP cells were treated with NDBzA, NDPA, NDCHA and F in the presence of DHT, they decreased the level of AR induced with DHT (Fig. 5). These results suggest that NDBzA, NDPA and NDCHA prevented the DHT induced AR level to inhibit the androgenic action of DHT and another pathway.

Northern blot analysis was applied to determine effect of NOCs on the expression of an endogenous androgen responsive gene in LNCaP cells. The level

**Table 3.** Effect of NOCs on Luciferase Activity

| Compound | Relative $\beta$-galactosidase activity (%) | Relative luciferase activity[a] (%) | Source |
|---|---|---|---|
| *N*-Nitrosodimethylamine | 95.7 ± 4.2 | 105.1 ± 13.9 | Wako |
| *N*-Nitrosodiethylamine | 103.6 ± 3.1 | 105.8 ± 9.5 | Wako |
| *N*-Nitrosodipropylamine | 108.3 ± 2.4 | 94.4 ± 9.1 | SUPELCO |
| *N*-Nitrosodibutylamine | 103.6 ± 2.9 | 95.8 ± 10.5 | SIGMA |
| *N*-Nitrosodiisobutylamine | 106.4 ± 1.7 | 95.3 ± 6.0 | Wako |
| *N*-Nitrosomethylbutylamine | 102.1 ± 4.5 | 102.8 ± 5.7 | SIGMA |
| *N*-Nitrosoethylbutylamine | 101.0 ± 2.9 | 99.5 ± 8.1 | SIGMA |
| *N*-Nitrosodiethanolamine | 113.4 ± 5.6 | 98.0 ± 8.4 | SIGMA |
| *N*-Nitrosodiisopropanolamine | 109.5 ± 2.8 | 91.7 ± 5.1 | SIGMA |
| *N*-Nitrosodicyclohexylamine | 50.7 ± 8.9** | 54.2 ± 3.3** | SIGMA-ALDRICH |
| *N*-Nitrosodiphenylamine | 54.4 ± 5.1** | 43.7 ± 1.6** | Wako |
| *N*-Nitrosodibenzylamine | 51.6 ± 7.7** | 3.8 ± 2.5** | SIGMA-ALDRICH |
| *N*-Nitrosopiperidine | 110.5 ± 2.8 | 97.0 ± 3.8 | SIGMA |
| *N*-Nitrosopyrrolidine | 114.4 ± 2.2 | 103.4 ± 8.7 | SIGMA-ALDRICH |
| *N*-Nitrosomorpholine | 109.7 ± 4.2 | 96.7 ± 8.8 | SIGMA |

*N*-Nitroso compound at 10 $\mu$m was tested and the relative activity in the presence of 40 nM DHT in the Yeast Two-hybrid assay and 0.5 nM DHT in the AR-EcoScreen cell reporter gene assay were calculated as the percentage against DHT without chemicals (%). Values represent the mean ± S.D. ($n = 3$). **$p < 0.01$ compared to DHT without chemicals.

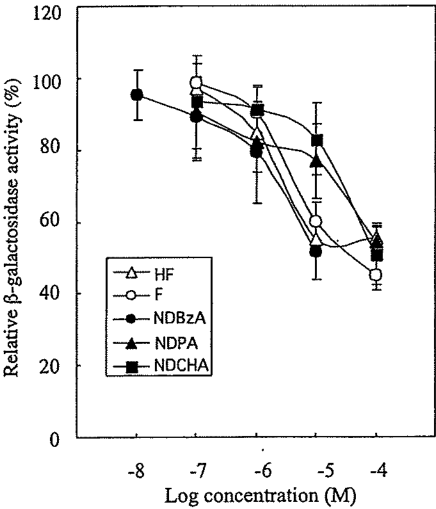



**Fig. 2.** Dose-Dependent Curves of NOCs in Yeast Two-Hybrid Assay System

Relative $\beta$-galactosidase activity of NDBzA, NDPA and NDCHA in the presence of 40 nM DHT was calculated as the percentage against DHT without chemicals (100%). Values represent the mean ± S.D. ($n = 3$).

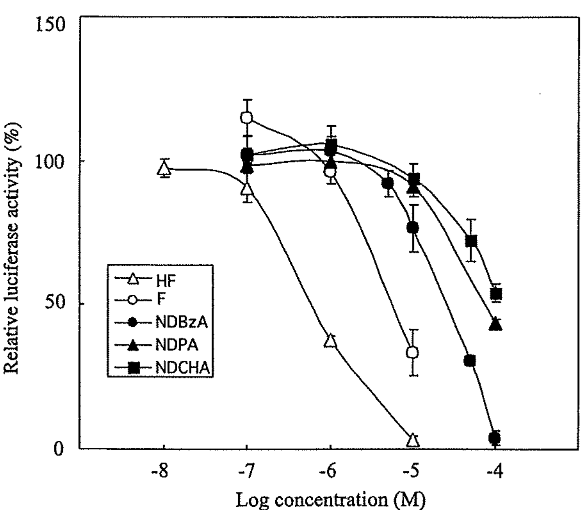
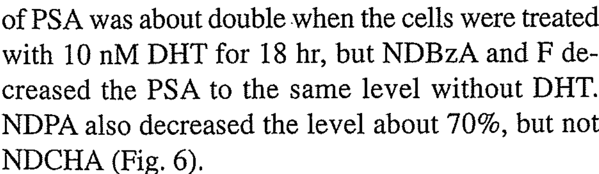



**Fig. 3.** Dose-Dependent Curves of NOCs in AR-EcoScreen Cell Reporter Gene Assay

Relative luciferase activity of NDBzA, NDPA and NDCHA in the presence of 0.5 nM DHT was calculated as the percentage against DHT without chemicals (100%). Values represent the mean ± S.D. ($n = 3$).



of PSA was about double when the cells were treated with 10 nM DHT for 18 hr, but NDBzA and F decreased the PSA to the same level without DHT. NDPA also decreased the level about 70%, but not NDCHA (Fig. 6).

## DISCUSSION

In this study, we assessed the anti-androgenic activity on 56 chemicals using Yeast Two-hybrid assay (AR:SRC-1) and AR-EcoScreen cell reporter gene assay, and found NDPA as a novel anti-androgen. Then we tested on 15 NOCs and found that NDBzA and NDCHA as well as NDPA inhibited the

**Table 4.** Effects of Compounds on the Inhibition of DHT Activity by the Various Assays

| Compounds | Reporter gene assay $IC_{50}$[a] ($\mu$M) | Yeast Two-hybrid assay $IC_{50}$[a] ($\mu$M) | AR binding assay $IC_{50}$[a] ($\mu$M) |
|---|---|---|---|
| F | 0.223 | 4.56 | 28.6 |
| NDBzA | 4.95 | 2.60 | 19.9 |
| NDPA | 16.9 | 27.6 | 183 |
| NDCHA | 11.7 | 54.8 | 27.4 |

*a*) $IC_{50}$ denotes the concentration that chemicals inhibited 50% of DHT without chemicals as described under method.

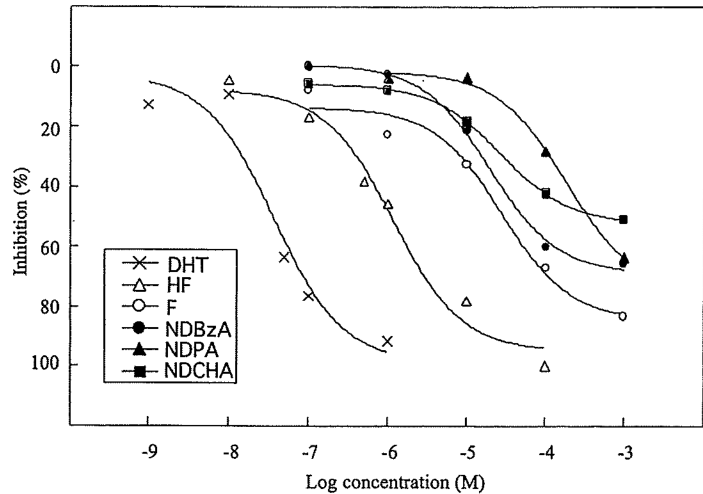



**Fig. 4.** Competitive Binding of NDBzA, NDPA and NDCHA against AR/AR-Ligand Complex to AR

NDBzA, NDPA and NDCHA competed against Fluormone AL green (fluorescent labeling AR lignads) on binding to human AR. Values represent the mean ± S.D. ($n = 3$).

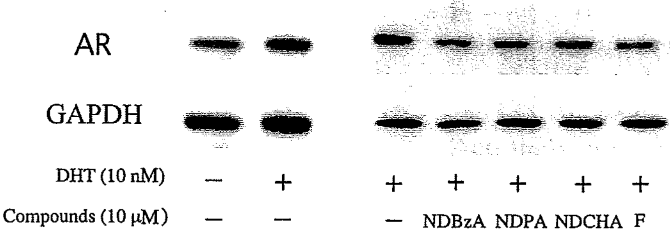



**Fig. 5.** Effect of NDBzA, NDPA and NDCHA on the Protein Level of AR

LNCaP cells were treated with NDBzA, NDPA and NDCHA in the presence of 10 nM DHT for 10 hr. The level of AR was detected by Western blot analysis.

androgenic activity of DHT.

To estimate the anti-androgenic activity in the Yeast Two-hybrid assay required simpler technique and shorter time, however the sensitivity was lower than the reporter gene assay. Therefore we tested all of the 56 chemicals for anti-androgenic activity by the two *in vitro* assays. Then 9 chemicals found as positive compounds by both. On the other hand, 3 chemicals were positive only by Yeast Two-hybrid assay. The different judgment may be due to difference of used cofactors. Androgen directly interacts with AR, and the complex stimulates transactivation of target genes through interaction with cofactors such as SRC-1, transcription intermediary factor (TIF2), and amplified in breast cancer (AIB1).[23] The AR-EcoScreen cells contain all cofactors, but yeast cells do SRC-1 alone. Another cause may be difference in cell membrane permeability between two

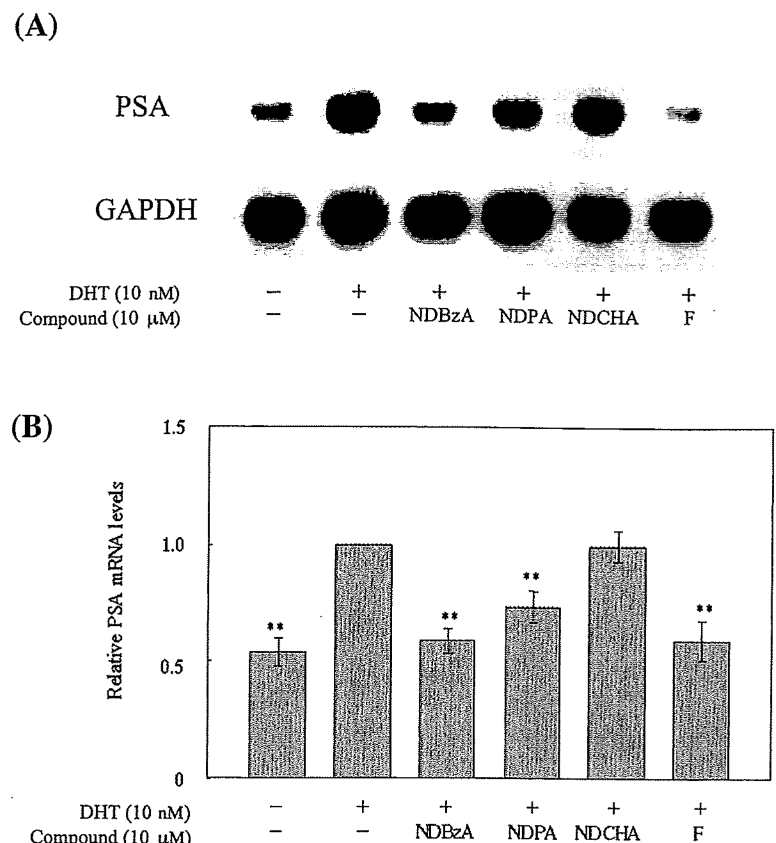



**Fig. 6.** Effect of NDBzA, NDPA and NDCHA on the Expression of AR Target Gene, PSA

(A) The PSA mRNA levels were determined by Northern blot analysis. LNCaP cells were treated with NOCs in the presence of 10 nM DHT for 18 hr. Total RNA fractions (12 μg each) were subjected to Northern blots. (B) The value of each PSA mRNA level was rectified with the GAPDH. Relative PSA mRNA levels were compared to 10 nM DHT without chemicals (relative PSA mRNA level = 1). Values represent the mean ± S.D. ($n = 3$). $^{**}p < 0.01$ compared to 10 nM DHT without chemicals.

cells and in assay condition such as the treating time of compounds.

Among positive compounds we found one of the NOCs, NDPA, as a novel anti-androgenic compound. Therefore we measured anti-androgenic activity of 15 NOCs to determine whether or not *N*-nitroso group correlated with anti-androgenic activity. As a result NDBzA and NDCHA were positive as well as NDPA, but the others were not. These positive compounds had ring structure other than *N*-nitroso groups. Anti-androgenic activity of NDBzA was estimated the highest, as much as F. NDPA was considered to be higher than NDCHA. These 3 NOCs were competitive in the binding to AR, although binding affinities were low. If the anti-androgenic activity and binding affinity were compared, there is likely to be no correlativity. Because anti-androgenic activity of NDPA was higher than NDCHA, whereas binding affinity of NDPA was less than NDCHA. These results suggest that NDBzA, NDPA and NDCHA antagonized with DHT on the process of competitive binding to AR.

Androgens increase the level of AR protein and AR plays an important role in the nucleus. These NOCs decreased the level of AR, suggesting that NDBzA, NDPA and NDCHA inhibited the androgenic action. The expression of PSA, AR target gene, is regulated by the AR and is thought to function as a growth factor in LNCaP cells.[24–26] Northern blot showed that NDBzA and NDPA inhibited transcriptional level of PSA in LNCaP cells. The same results were obtained by RT-PCR (data not shown). NDBzA inhibited the level of PSA as much as F and NDPA also decreased. These results suggest that 3 NOCs down-regulate the AR target genes mRNA level by antagonizing against DHT in binding process to AR.

This study shows first the anti-androgenic activity of NDBzA, NDPA and NDCHA, that some NOCs may affect endocrine system of humans and wildlife. Thus there are still anti-androgenic compounds that nobody knows. From now on, when we

perform risk assessment of chemicals, it is need to test the androgenic and anti-androgenic effect on endocrine system as well as carcinogenicity and others.

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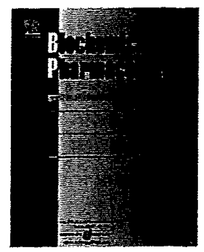

# Organotin compounds enhance 17β-hydroxysteroid dehydrogenase type I activity in human choriocarcinoma JAr cells: Potential promotion of 17β-estradiol biosynthesis in human placenta

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*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.

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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 ($3 \times 10^4$ 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 | $(CH_3)_3Sn-Cl$ | >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 | $SnCl_4$ | $Cl-SnCl_2-Cl$ | >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′-GCCACAGGA-CTCCATGCCC-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.

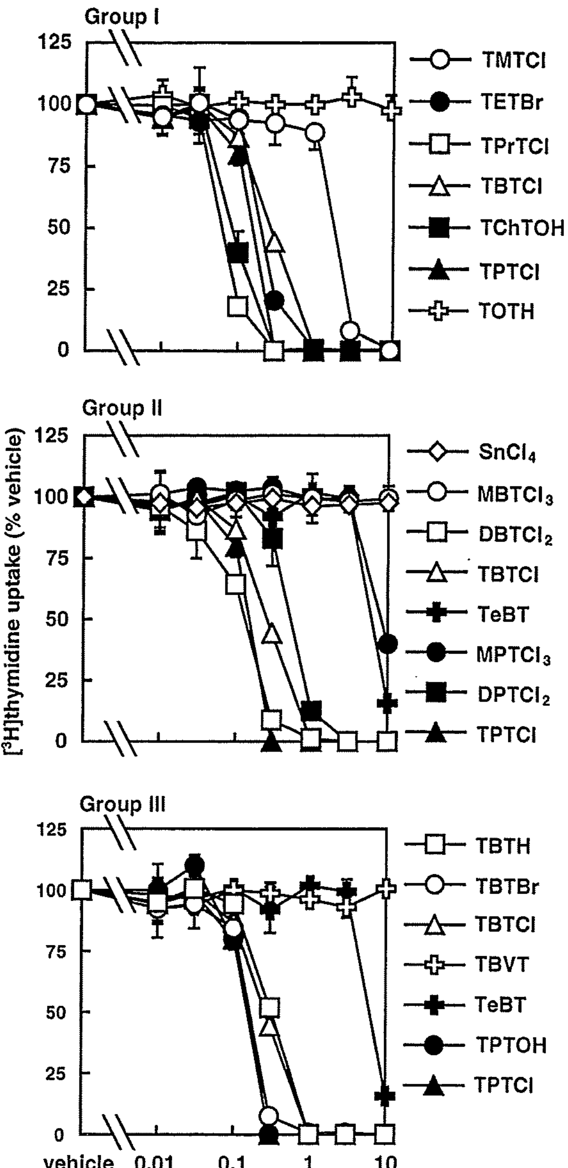


**Fig. 1 – Effect of organotin compounds on DNA synthesis in JAr cells. Cells were treated with various concentrations of tin compounds for 48 h. Results are expressed as mean ± 1 S.D. of triplicate cultures. The radioactivity in vehicle-only cells, calculated from all experiments, was 111,709 ± 6182 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.**

## 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).

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

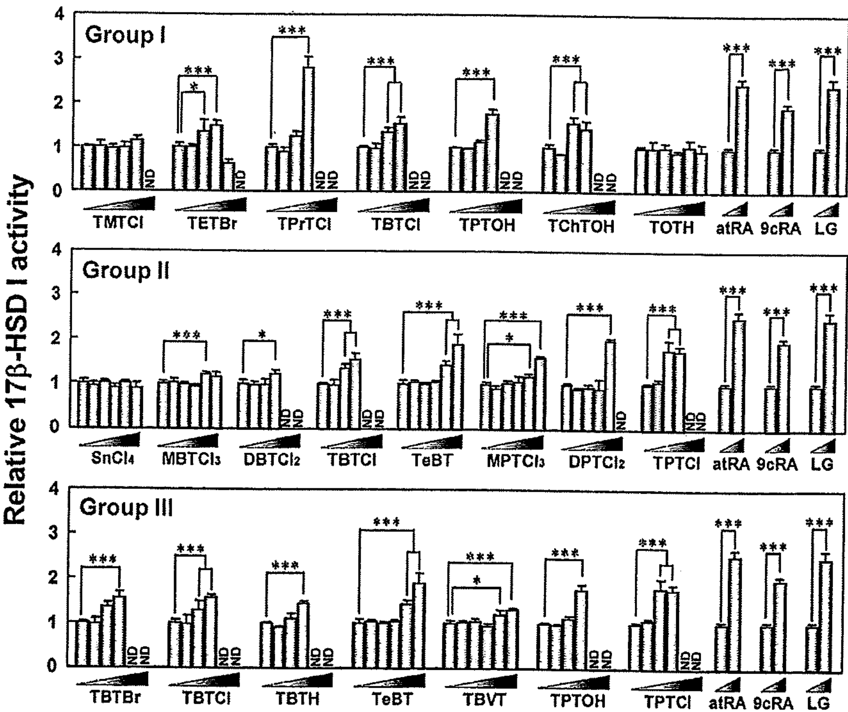


**Fig. 2 – Effects of tin compounds on 17β-HSD I activity in JAr cells. Cells were treated for 48 h with tin compounds at various concentrations (0 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) or with 0 or 1 μM of atRA, 9cRA, or LG. Results are expressed as mean ± 1 S.D. of triplicate cultures. The 17β-HSD 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.**

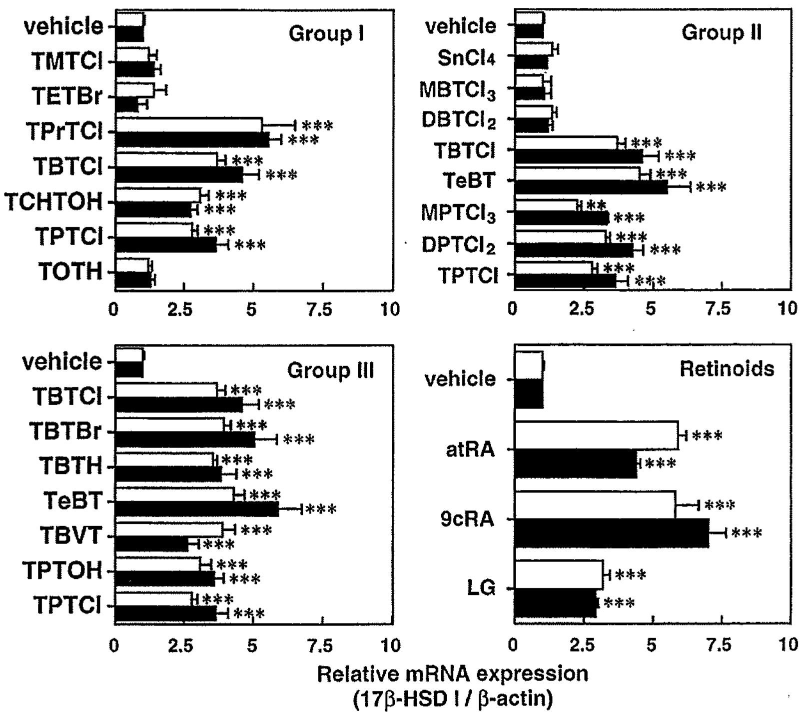


**Fig. 3 – Effects of tin compounds on the mRNA expression of 17β-HSD I (HSD17B1) in JAr cells. Total RNA was isolated from JAr cells treated with tin compounds for 24 h (open bars) and 48 h (solid bars). The doses of each compound were: 100 nM of TETBr, TPrTCl, TBTCl, TChTOH, TPTOH, TPTCl, TBTH, TBTBr 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.