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

Sayaka Hari,[a] Jun-ichi Nishikawa,[a] Kikumi Horiguchi,[a] Mitsuru Iida,[b] and Tsutomu Nishihara*[, a, 1]

[a]*Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan and* [b]*EDC Analysis Center, Otsuka Pharmaceutical Company LTD., Kawauchi, Tokushima 771–0132, Japan*

(Received February 16, 2006; Accepted June 12, 2006; Published online July 3, 2006)

When 56 selected environmental chemicals were tested for the androgenic activity to Yeast Two-hybrid and reporter gene assay in the presence of 5α-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 5α-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

[1]Present address: Osaka University, Center for Advanced Science and Innovation, A-407, 2–1 Yamada-oka, Suita, Osaka 565–0871, Japan.

*To whom correspondence should be addressed: Osaka University, Center for Advanced Science and Innovation, A-407, 2–1 Yamada-oka, Suita, Osaka 565–0871, Japan. Tel.: +81-6-6879-7423; Fax: +81-6-6879-7426; E-mail: r-nishihara@dol.hi-ho.ne.jp

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 μg/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 μg/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 μl), pre-incubated overnight at 30°C in synthetic defined (SD) medium free from tryptophan and leucine, were incubated with NOCs (2.5 μl) and DHT (40 nM final concentration) in SD medium lacking tryptophan and leucine (150 μl) 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 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml in Z-buffer) and incubated at 37°C for 1 hr. Finally, added 100 μl 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 β-galactosidase activity was calculated from these 3 absorbances. The anti-androgenic activity was expressed as the percentage against β-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 1×10^5 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
γ-Hexachlorocyclohexane (γ-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 μl of NOC/DMSO solution was added to 49 μl of AL green assay buffer in the small test tube. Additionally, added 50 μl 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 μl 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 2.0×10^5 cells/ml in RPMI 1640 medium supplemented with 10% char-

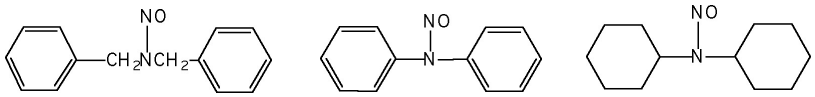
Table 2. Anti-Androgenic Activity of 17 Environmental Chemicals by Yeast Two-Hybrid Assay and Reporter Gene Assay

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 1.5×10^5 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 [α-^{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 μm. 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 μm in the Yeast Two-hybrid assay and 5, 17 and 12 μm in the AR-EcoScreen cell reporter gene assay, whereas IC_{50} of F was 5 and 0.2 μm, 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 β-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 μm 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.

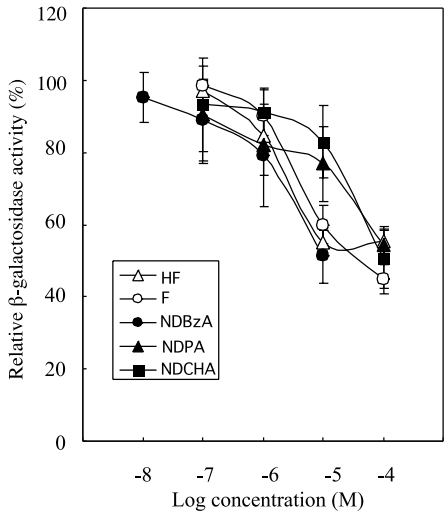


Fig. 2. Dose-Dependent Curves of NOCs in Yeast Two-Hybrid Assay System

Relative β-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. ($n = 3$).

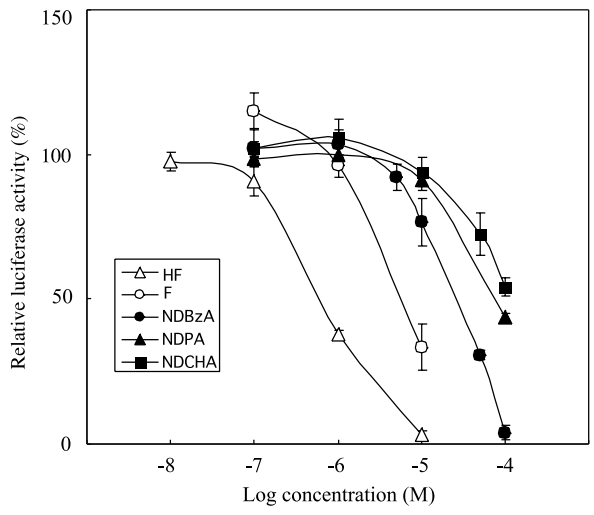


Fig. 3. Dose-Dependent Curves of NOCs in AR-EcoScreen Cell Reporter Gene Assay

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 $IC_{50}^{a)}$ (μM)	Yeast Two-hybrid assay $IC_{50}^{a)}$ (μM)	AR binding assay $IC_{50}^{a)}$ (μM)
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.

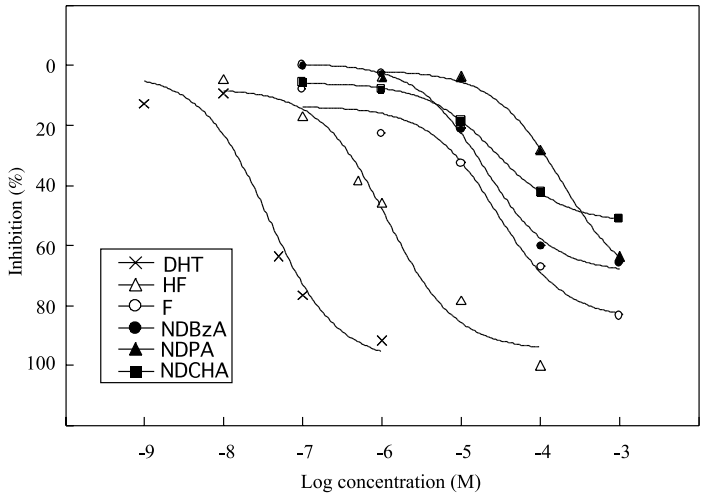


Fig. 4. Competitive Binding of NDBzA, NDPA and NDCHA against AR/AR-Ligand Complex to AR

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).

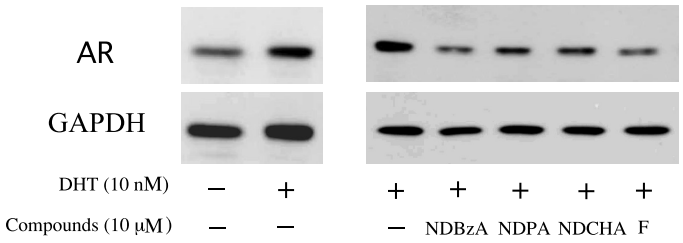


Fig. 5. Effect of NDBzA, NDPA and NDCHA on the Protein Level of AR

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

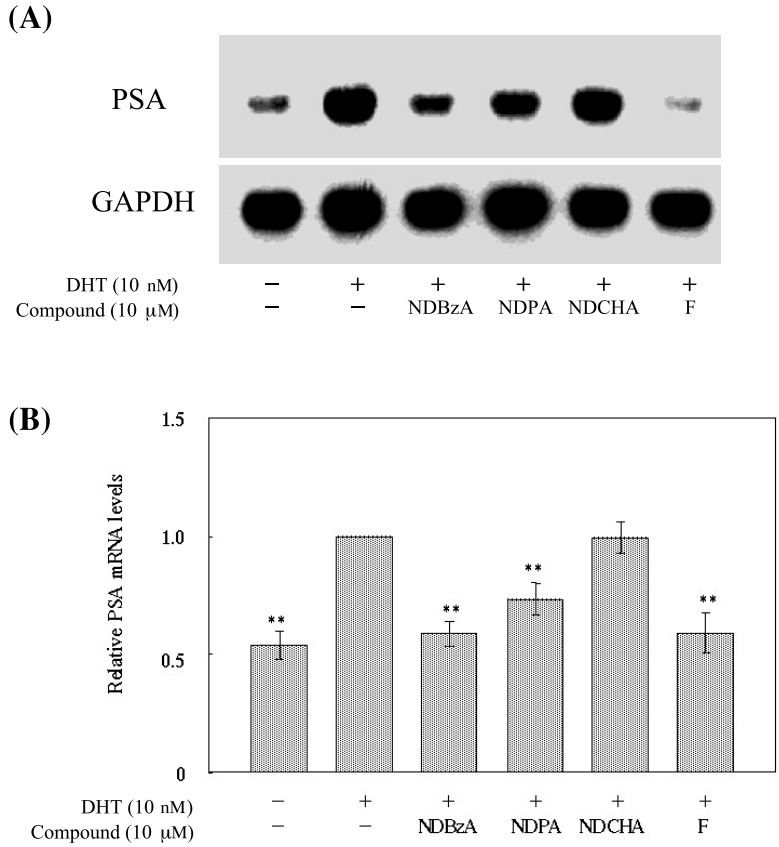


Fig. 6. Effect of NDBzA, NDPA and NDCHA on the Expression of AR Target Gene, PSA

(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.

Acknowledgements We thank Drs. M. Kawase at Osaka University and S. Osada at Nagoya City University for their valuable discussion and advice during this study, and also Dr. Yong K. Oh in U.S.A. for checking the English of this manuscript.

REFERENCES

1) Mangelsdorf, H., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M. and Chambon, P. (1995) The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835–839.
2) Beato, M., Herrlich, P. and Schutz, G. (1995) Steroid hormone receptors: many actors in search of plot. *Cell*, **83**, 851–857.
3) Cleutjens, K. B., van Eekelen C. C., van der Korput H. A., Brinkmann A. O. and Trapman, J. (1995) Two androgen respose regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promorter. *J. Biol. Chem.*, **271**, 6379–6388.
4) Gobinet, J., Ppujol, N. and Sultan, C. (2002) Molecular action of androgens. *Mol. Cell. Endocrinol.*, **198**, 15–24.
5) Oesterling, J. E. (1991) Prostate specific antigen: a critical assessment of the most useful tumor marker for adenocarcinoma of the prostate. *J. Urol.*, **145**, 907–923.
6) Colborn, T., Dumanoski, D. and Myers, J. P. (1996) *Our stolen future*, Dutton, New York.
7) Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S. and Utsumi, H. (2000) Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *J. Health Sci.*, **46**, 282–298.
8) Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S. L., Moland, C. L., Tong, W., Shi, L., Perkins, R. and Sheehan, D. M. (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol. Sci.*, **54**, 138–153.
9) Sultan, C., Balaguer, P., Terouanne, B., Georget, V., Paris, F., Jeandel, C., Lumbroso, S. and Nicolas, J. (2001) Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation. *Mol. Cell. Endocrinol.,* **178**, 99–105.
10) Kojima, H., Katsura. E., Takeichi, S., Niiyama, K. and Kobayashi, K. (2004) Screening for estrogen and androgen receptor activities in 200 pepsticides by *in vitro* receptor gene assays using chinese hamster ovary cells. *Environ. Health Perspect.*, **112**, 524–531.
11) Kelce, W. R., Monosson, E., Gamcsik, M. P., Laws, S. C. and Gray, L. E. (1994) Environmental hormone disruptors: evidence that vinclozolin development toxicity is mediated by antiandrogenic metabolites. *Toxicol. Appl. Pharmcol.*, **126**, 276–285.
12) Kelce, W. R., Stone, C. R., Laws, S. C., Gray, L. E., Kemppainen, J. A. and Wilson, E. M. (1995) Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature* (London), **375**, 581–585.
13) Gray, L. E., Ostby, J. S. and Kelce, W. R. (1994) Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol. Appl. Pharmacol.*, **129**, 46–52.
14) Gray, L. E., Wolf, C., Lambright, C., Mann, P., Cooper, R. L. and Ostby, J. (1999) Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinete, p,p'-DDE, and ketoconazol) and toxic substances (dibutyl-and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol. Ind. Health*, **15**, 94–118.
15) Monosson, E., Kelce, W. R., Lambright, C., Ostby, J. and Gray, L. E. (1999) Peripubertal exporsure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. *Toxicol. Ind. Health*, **15**, 65–79.
16) Lambright, C., Ostby, J., Bobseine, K., Wilson, V., Hotchkiss, A. K., Mann, P. C. and Gray, E. (2000) Cellular and molecular mechanisms of action of linuron: an antiandrogenic herbicide that produces reproductive malformations in male rats. *Toxicol. Sci.*, **56**, 389–399.
17) Akaza, H., Usami, M., Kotake, T., Koiso, K. and Aso, Y. (1993) A randomized phase II traial of flutamide vs chloromadinone acetate in previously untreated advanced cancer. The Japan Flutamide Study Group. *Jpn. J. Clin. Oncol.*, **23**, 178–185.
18) Lijinsky, W. (1987) Carcinogenecity and mutagenecity of *N*-nitroso compounds. *Mol. Toxicol.*, **1**, 107–119.
19) Mirvish, S. S. (1995) Role of *N*-nitroso compounds (NOC) and *N*-nitrosation in ethiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exporsures to NOC. *Cancer Lett.*, **93**, 17–48.
20) Tricker, A. R., Spiegelhalder, B. and Preussmann,

R. (1989) Environmental exporsure to performed nitroso compounds. *Cancer Surv.*, **8**, 251–272.
21) Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M. and Nishihara, T. (1999) New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmcol.*, **154**, 76–83.
22) Satoh, K., Ohyama, K., Aoki, N., Iida, M. and Nagai, F. (2004) Study on anti-androgenic effects of bisphenol a diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives using cells stably transfected with human androgen receptor, AR-EcoScreen. *Food Chem. Toxicol.*, **42**, 983–993.
23) Torchiba, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K. and Rosenfeld, M. G. (1997) The transcriptional coactivater p/CIP binds CBP and mediates nuclear-receptor function. *Nature* (London), **387**, 677–684.
24) Henttu, P. and Vihko, P. (1993) Growth factor regulation of gene expression in the human prostatic carcinoma cell line LNCaP. *Cancer Res.*, **53**, 1051–1058.
25) Lee, C., Sutkowski, D. M., Sensibar, J. A., Zelner, D., Kim, I., Amsel, I., Shaw, N., Prins, G. S. and Kozlowski, J. M. (1995) Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by Dihydrotestosterone. *Endocrinology*, **136**, 796–803.
26) Dioxin, S. C., Knopf, K. B. and Figg, W. D. (2001) The control pf prostate-specific antigen expression and gene regulation by pharmacological agents. *Pharmacol. Rev.*, **53**, 73–91.