Effects of a Diphenyl Ether-type Herbicide, Chlornitrofen, and Its Amino Derivative on Androgen and Estrogen Receptor Activities

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Chlornitrofen (CNP) was widely used in large quantities as a herbicide in rice paddy fields in Japan during 1965–1994. Recently, there has been concern that chemicals in the environment may disrupt the endocrine function of wildlife and humans, but little is known about the effect of CNP on endocrine function. We have developed reporter gene assays for human androgen receptor (hAR) and human estrogen receptor-α (hERα) using Chinese hamster ovary cells. Using this assay method, we measured CNP and its amino derivative (CNP-amino) for hAR and hERα agonist/antagonist activities, comparing them with several well-known AR antagonists or ER agonists. We found that CNP and CNP-amino have potent antiandrogenic activities as well as estrogenic activities. The order of their antiandrogenic activity was CNP > vinclozolin > *o,p′*-DDT = *p,p′*-DDE > CNP-amino, and the order of their estrogenic activity was *o,p′*-DDT > CNP-amino > *p,p′*-DDT > CNP. We investigated the binding ability of CNP and CNP-amino to hAR and hERα using a receptor competitive-binding assay. The order of their binding potencies to hAR was CNP > *o,p′*-DDT = *p,p′*-DDE = CNP-amino > vinclozolin, and that of their binding potencies to hERα was *o,p′*-DDT > CNP-amino > *p,p′*-DDT = CNP. These results suggest that both CNP and CNP-amino may act as endocrine disruptors via AR and ERα in humans and other animals. Our reporter gene assays are highly sensitive and specific and are suitable for screening AR and ERα agonist/antagonists among numerous environmental chemicals. *Key words:* antiandrogenic activity, Chinese hamster ovary cells, chlornitrofen, chlornitrofen-amino, estrogenic activity, human androgen receptor, human estrogen receptor α, reporter gene assay. *Environ Health Perspect* 111:497–502 (2003). doi:10.1289/ehp.5724 available via *http://dx.doi.org/* [Online 1 November 2002]

Chlornitrofen [2,4,6-trichlorophenyl-4′-nitrophenyl ether (CNP); Figure 1] was widely used in large quantities as a herbicide to control various weeds in rice fields in Japan during the period 1965–1994. This herbicide was produced and used mostly in Japan. The amount of the active ingredient of CNP used in Japan was estimated to be 82,359 tons (Masunaga et al. 1998). Several studies reported unusually high levels of CNP residue in freshwater fish and shellfish during the application period (Ohyama et al. 1986; Watanabe et al. 1981, 1983; Yamagishi and Akiyama 1981). CNP is also known to convert to its corresponding amino derivative [2,4,6-trichlorophenyl-4′-aminophenyl ether (CNP-amino); Figure 1] by reduction of the CNP nitro group in the soil of paddy fields (Kuwatsuka 1977; Shimotori and Kuwatsuka 1978). There have also been reports of the isolation of CNP and CNP-amino from tap water and shellfish (Adachi 1994; Suzuki et al. 1983). Yamamoto et al. (1987) reported that the standardized mortality ratios of biliary tract cancer were high in Niigata prefecture, especially in the Niigata plain, and that this phenomenon could be related to the use of CNP. Thus, the use of CNP is thought to cause water pollution in rice-growing areas in Japan and lead to a high accumulation of CNP and CNP-amino in fish and shellfish in lakes and seas surrounding areas of rice cultivation.

Recently, it has been well documented that several chemicals from agricultural, industrial, and household sources possess endocrine-disrupting properties that are a potential threat to human and wildlife reproduction (Colborn 1995; Colborn et al. 1993; Jensen et al. 1995). The mechanism of action of these effects is considered to consist mainly of agonistic or antagonistic effects on hormone receptors. For example, it has already been reported that several pesticides or their metabolites such as vinclozolin, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p′*-DDE), fenitrothion, and procymidone are androgen receptor (AR) antagonists (Kelce et al. 1995; Ostby et al. 1999; Tamura et al. 2001; Wong et al. 1995) and that pesticides such as 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p′*-DDT) and methoxychlor are estrogen receptor (ER) agonists (Chen et al. 1997; Shelby et al. 1996). Moreover, it has been reported that some of the environmental estrogens such as 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane (*o,p′*-DDT), bisphenol A, and butyl benzyl phthalate also have antiandrogenic activitiy (Sohoni and Sumpter 1998). Although CNP and CNP-amino are thought to form methemoglobin, induce hepatic drug-metabolizing enzymes, and display mutagenicity (Hanioka et al. 1995; Miyauchi et al. 1981, 1983; Oguri et al. 1995), the endocrine-disrupting effects of CNP or CNP-amino have not yet been described.

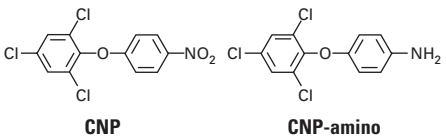

Figure 1. Chemical structures of CNP and CNP-amino.

The reporter gene assay has been widely used as an *in vitro* method for clarifying the ligand–receptor interaction by receptor agonists and antagonists. To detect the (anti)hormonal activities of environmental chemicals, some investigators have performed reporter gene assays using yeast cells, HepG2 cells, Hela cells, and so forth (Gaido et al. 1997; Maness et al. 1998; Nishikawa et al. 1999; Saito et al. 2000). However, these assays all encounter problems in the membrane transport of chemicals, sensitivity, or complicated procedures. In this study, we established two transient reporter gene assays for detecting transcriptional activities via AR and ER activities using transfection reagent FuGene6 and Chinese hamster ovary (CHO) cells based on the method of Vinggaard et al. (1999). The method is rapid, sensitive, and reproducible. Using this assay, we investigated the effects of CNP and CNP-amino on androgenic and estrogenic activities. In addition, the binding affinities of CNP and CNP-amino to human androgen receptor (hAR) and human estrogen receptor-α (hERα) were also investigated using a receptor competitive-binding assay (Satoh et al. 2000, 2001). Here we provide the first evidence that CNP and CNP-amino might be endocrine-disrupting chemicals with both antiandrogenic and estrogenic activities that act via hormone receptors.

Materials and Methods

Chemicals and cell culture materials. 5α-Dihydrotestosterone (DHT, 95% pure), testosterone (> 97% pure), 17β-estradiol (E_2, > 97% pure), estrone (98% pure), progesterone (98% pure), cortisol (> 97% pure), and dimethylsulfoxide (DMSO), used for confirming the specificity of the assay system,

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were purchased from Wako Pure Chemical Industries (Osaka, Japan). CNP (99% pure), CNP-amino (> 98.5% pure), vinclozolin (> 99% pure), *p,p′*-DDT (> 99% pure), *o,p′*-DDT (> 99% pure), and tamoxifen citrate (98% pure) were also obtained from Wako Pure Chemical Industries. *p,p′*-DDE (99% pure) was obtained from GL Sciences (Tokyo, Japan). Test compounds were solubilized in DMSO. The luciferase reporter vectors pGL3-Basic (containing the firefly luciferase gene) and pRL-SV40 (containing the *Renilla* luciferase gene, transfections and toxicity control), and the dual-luciferase reporter assay system were purchased from Promega (Madison, WI, USA). The transfection reagent FuGene6 was obtained from Roche Diagnostics Corp. (Indianapolis, IN, USA). Dulbecco's modified Eagle's minimum essential medium (DMEM/F-12) and penicillin–streptomycin solution (antibiotics) were obtained from GIBCO-BRL (Rockville, MD, USA). Fetal bovine serum (FBS) and charcoal–dextran-treated FBS (CD-FBS) were obtained from Hyclone (Logan, UT, USA). CHO-K1 cells obtained from the American Type Culture Collection were grown at 37°C in DMEM/F-12 supplemented with 10% FBS and antibiotics.

Construction of plasmids. AR cDNA and ERα cDNA were cloned by reverse transcriptase–polymerase chain reaction from human prostate and human placenta RNA (Clontech, Palo Alto, CA, USA), respectively. The sequences of the cloned hAR and hERα cDNA were verified and were inserted into mammalian expression vector pZeoSV2(-) and pcDNA3.1Zeo(-) (Invitrogen, San Diego, CA, USA), creating pZeoSV2AR and pcDNAERα, respectively.

We constructed a reporter plasmid for the AR-mediated transcriptional assay (AR assay) based on the mammalian inducible expression vector pIND/Hygro (Invitrogen), which originally contains ecdysone/glucocorticoid-responsive element (ecdysone/GRE). Briefly, the luciferase gene (digested *Hind*III and *Xba*I) from pGL-3 basic was cloned into pIND/Hygro, creating pIND-LUC. To remove the ecdysone/GRE and create a new multicloning site (MCS), pIND/Hygro was digested with *sma*I. The digested fragment (about 1,500 bp of *sma*I-*sma*I) contains a minimal heat shock (hs) promoter without ecdysone/GRE. Then, the oligonucleotides 5′-GATCTATCGATTCTAGAGGATCCTCGAGATATCCC-3′ and 5′-GGGATATCTCGAGGATCCTCTAGAATCGATGA-3′ (containing *Bgl*II, *Cla*I, *Xba*I, *Bam*HI, *Xho*I, *Eco*RV) were ligated to this *sma*I-*sma*I fragment from pIND/Hygro and then digested with *Bgl*II and *Hind*III (about 300 bp, contains MCS and hs). And this small fragment was inserted into the pIND-LUC (digested with *Bgl*II and *Hind*III), creating pIND-MCS-LUC. To introduce the androgen responsive element (ARE) into the newly created MCS, kinated oligonucleotides 5′-gatccatcatAGTACGtgaTGTTCTcaagaa-3′and 5′-gatcttcttgAGAACAtcaCGTACTatgatg-3′(flanking the *Bgl*II site) containing ARE of the C3 gene of prostatic binding protein (Karvonen et al. 1997) were ligated and then inserted into the *Bgl*II site of the pIND-MCS-LUC, creating pINDARE (Figure 2A).

For the ERα-mediated transcriptional assay (ERα assay), we constructed a reporter plasmid pGL3-tkERE based on the pGL3 basic vector. A plasmid pRL-TK (Promega) was digested with *Ava*II, followed by blunt-ended treatment with Klenow fragment, and then digested with *Hind*III. The digested small fragment (about 70 bp) from pRL-TK, containing a deletion tk promoter, was cloned into the blunt-ended *Bgl*II/*Hind*III site of the pGL3 basic vector, creating pGL3-tk. This vector has the minimal tk (–40 to +31) promoter and carries only the TATA box of the regulatory element. Then the kinated strands of the oligonucleotides containing a perfectly palindromic estrogen-responsive element (ERE, AGGTCA cag TGACCT) from the *Xenopus* vitellogenin gene (Klein-Hitpass et al. 1986) were cloned into the kpnI site of pGL3-tk, creating pGL3-tkERE (Figure 2B). Sequencing verified that the pIND-ARE and the pGL3-tkERE carried four tandem repeats of ARE or ERE upstream of their promoter.

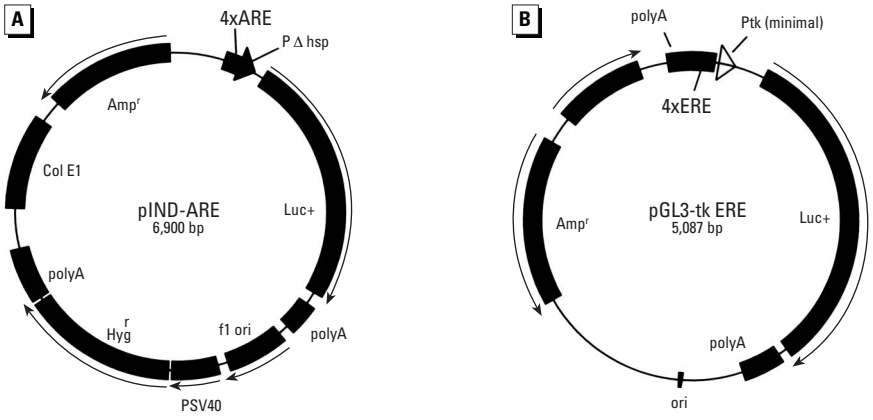

Figure 2. Structures of luciferase reporter plasmids.

Reporter gene assays for hAR and hERα. The host CHO-K1 cells were plated in 96-well microtiter plates (Nalge, Nunc, Denmark) at a density of 8,400 cells per well in phenol red-free DMEM/F-12 containing 5% CD-FBS (complete medium) 1 day before transfections. For detection of hAR activity, we transfected cells with 2.5 ng pZeoSV2AR, 62.5 ng pIND-ARE, and 5.0 ng pRL-SV40 per well using the transfection reagent FuGene6. For detection of hERα activity, we transfected cells with 6.25 ng pcDNAERα, 62.5 ng pGL3-tkERE, and 5.0 ng pRL-SV40 per well. After a 3-hr transfection period, cells were dosed with various concentrations of test compounds or with 0.1% DMSO (vehicle control) in complete medium. For measurement of hAR and hERα antagonist activity, we added 10^{-10}M of DHT and 10^{-11} M of E_2 to the cell cultures, respectively. After an incubation period of 24 hr, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50 µL/well) provided with the dual-luciferase reporter assay kit. The firefly luciferase activity was measured before measuring *Renilla* luciferase activity in one reaction tube with 5-µL aliquots of cell lysates using the dual-luciferase reporter assay system following the manufacturer's instructions with a MiniLumat LB 9506 luminometer (Berthold, Germany). We normalized the firefly luciferase activity to the *Renilla* luciferase activity.

Competitive binding assay for AR and ERα. We determined competitive binding of CNP and CNP-amino against the binding of the index hormone to AR by non-radioisotopic receptor binding assay using a ligand screening system-androgen receptor kit (Toyobo Co., Ltd., Osaka, Japan) as reported by Satoh et al. (2001). The solutions of human AR, unlabeled testosterone, and test chemical (competitor) dissolved in DMSO were reacted at 4°C for 1 hr. The liberated testosterone was allowed to compete with the antitestosterone antibody and peroxidase-labeled testosterone at 4°C for 1 hr. After plates were washed with a wash solution, the substrate solution was added. We measured the developed color at 450 nm on a microplate-spectrophotometer (MPRA4i; TOSOH Co., Ltd., Tokyo, Japan).

Competitive binding assay of CNP and CNP-amino to ERα was performed using a ligand screening system-estrogen receptor α kit (Toyobo Co., Ltd., Osaka, Japan) as reported by Satoh et al. (2000). Briefly, the solutions of human ERα, unlabeled E_2, and test chemical dissolved in DMSO were reacted at 4°C for 1 hr. The liberated E_2 was

allowed to compete with the anti-E_2 antibody and peroxidase-labeled E_2 at 4°C for 1 hr. We then assayed the developed color as described above for AR binding assay.

We calculated the binding levels of the chemicals to the respective receptors from the decreases in absorbance rate. We used mibolerone and diethylstilbestrol (DES) as positive controls from the AR and ERα-binding kits, respectively.

Data analysis. We evaluated the statistical significance of differences using the Student's *t*-test (two-tailed, equal variance) calculated by software (Excel; Microsoft, Redmond, WA, USA). The level of significance was $p < 0.05$. Data are presented as the mean and, where shown, the SD of at least three separate experiments with duplicate wells.

Results

Sensitivity and specificity of the reporter assays for hAR and hERα. We established two transient reporter gene assays for detecting transcriptional activities via hAR and hERα using transfection reagent FuGene6 and CHO cells. To confirm the sensitivity and specificity of our reporter gene assays, we tested various endogenous steroids for hAR- and hERα-mediated transcriptional activities, respectively. Figure 3A shows the results for androgenic activity. The androgenic activity of DHT was observed in a dose-dependent manner at concentrations above 10^{-11}M, and the maximum induction was 16-fold that of the solvent control. The androgenic activity of testosterone was observed at concentrations above 10^{-10} M, and its transcriptional activity was approximately 1/10 that of DHT. Cortisol was also able to stimulate luciferase synthesis, but the activity was about 1/1,000 that of DHT. E_2, estrone, and progesterone showed only low activity at the concentrations tested.

The results for estrogenic activity are shown in Figure 3B. E_2 activity became detectable at the concentrations of more than 10^{-12} M, and the maximum induction was 9-fold that of the solvent control. The E_2 metabolite, estrone, was as effective as E_2 at inducing luciferase activity. DHT showed approximately 1/2,000 the intensity of E_2 and estrone. Testosterone and cortisol were also tested and found positive by this assay at high concentrations. Progesterone was inactive at the concentration tested.

Effects of CNP and CNP-amino on the hAR- and hERa-mediated reporter gene assays. To investigate whether CNP and CNP-amino have endocrine-disrupting effects, we determined their androgenic and estrogenic activities using the two assay systems described above. Figure 4A shows the hAR-mediated transcriptional activities of CNP, CNP-amino, vinclozolin, *p,p′*-DDE and *o,p′*-DDT at a concentration of 10^{-5} M in the absence or presence of 10^{-10} M DHT. CNP was weakly androgenic, but CNP-amino, as well as vinclozolin, *p,p′*-DDE, and *o,p′*-DDT displayed no androgenicity (Figure 4A). CNP and CNP-amino, however, inhibited the hAR-mediated transcriptional activity by DHT, as did the AR antagonists vinclozolin, *p,p′*-DDE, and *o,p′*-DDT (Figure 4A).

The dose-responsive inhibitory effects of CNP and CNP-amino on DHT-induced androgenic response are depicted in Figure 4B. CNP showed an antiandrogenic effect in a dose-dependent manner at concentrations from 10^{-8} to 10^{-6} M, but the inhibition curve of CNP turned upward at the concentrations > 10^{-6} M. CNP-amino showed an antiandrogenic effect at concentrations of $10^{-7} – 10^{-5}$M. These effects of CNP and CNP amino were detectable without changes of *Renilla* luciferase activity in cells (data not shown), indicating that the present experimental condition did not affect the cellular viability. When the antiandrogenic potencies of each compound were expressed as the concentration exhibiting 50% inhibition of the androgenic activity of 10^{-10} M DHT (IC_{50}), the antiandrogenic potencies of CNP ($IC_{50} = 1.7 \times 10^{-7}$ M) were approximately 3.5-, 14-, and 10-fold higher than those of vinclozolin ($IC_{50} = 5.8 \times 10^{-7}$ M), *p,p′*-DDE ($IC_{50} = 2.4 \times 10^{-6}$ M), *o,p′*-DDT ($IC_{50} = 1.6 \times 10^{-6}$ M), respectively. The antiandrogen potency of CNP-amino ($IC_{50} = 2.5 \times 10^{-6}$ M) was similar to those of *p,p′*-DDE and *o,p′*-DDT, and more than 1/10 the intensity of CNP.

Figure 5A shows the hERα-mediated transcriptional activities induced by CNP, CNP-amino, *p,p′*-DDT, and *o,p′*-DDT at a concentration of 10^{-5} M and by therapeutic antiestrogen, tamoxifen, at concentrations of 10^{-8} and 10^{-7} M in the absence or presence of 10^{-11} M E_2. CNP and CNP-amino exhibited potent estrogenic activity, as did *p,p′*-DDT

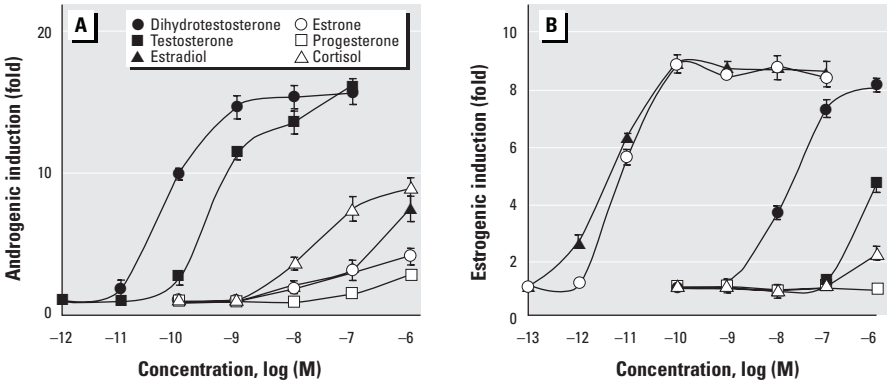

Figure 3. Dose response of various steroids on the human AR and human ERα reporter gene assays. CHO cells were transiently transfected with expression plasmids for human AR (*A*) or ERα (*B*) plus relative receptor responsive firefly luciferase reporter plasmids and a constitutively active *Renilla* luciferase expression plasmid (transfection and toxicity control). Cells were treated with increasing concentrations of various steroids to detect agonist activity. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Values represent the means ± SD of three independent experiments and are presented as mean fold-induction over the vehicle control.

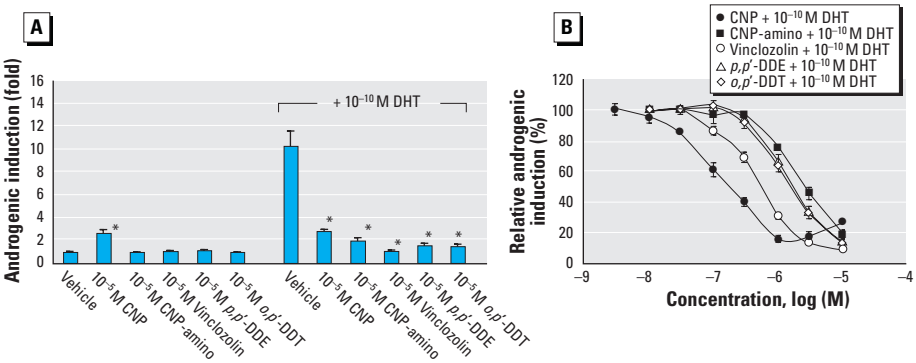

Figure 4. Effects of CNP and CNP-amino on human AR activity. (*A*) CHO cells, transiently cotransfected with pZeoSV2AR, pIND-ARE and pRL-SV40, were incubated with the vehicle control (0.1% DMSO) or 10^{-5} M of CNP, CNP-amino, vinclozolin, *p,p′*-DDE, or *o,p′*-DDT in the absence or presence of 10^{-10} M DHT. Values represent the means ± SD of three independent experiments and are presented as mean fold-induction over the vehicle control. (*B*) Cells were incubated with various concentrations of CNP, CNP-amino, vinclozolin, *p,p′*-DDE, or *o,p′*-DDT in the presence of 10^{-10} M DHT. Values represent the means ± SD of three independent experiments and are presented as percentage induction, with 100% activity defined as the activity achieved with 10^{-10} M DHT.
*Significant difference at $p < 0.05$ by Student's *t*-test.

and *o,p′*-DDT, the ER agonists, at the concentration of 10^{-5} M, but tamoxifen was inactive at 10^{-8} and 10^{-7} M (Figure 5A). In the presence of 10^{-11} M E_2, CNP, CNP-amino, *p,p′*-DDT, and *o,p′*-DDT did not show any significant differences from the vehicles, whereas tamoxifen inhibited the estrogenic activity of E_2 in a dose-dependent manner at 10^{-8} and 10^{-7} M (Figure 5A). The dose–response curves for the estrogenic activities of CNP and CNP-amino are shown together with those of *p,p′*-DDT, *o,p′*-DDT, and tamoxifen in Figure 5B. When the estrogenic potencies of each compound were expressed as the concentration showing 50% the estrogenic activity of 10^{-10} M E_2 (EC_{50}), the EC_{50} values of CNP, CNP-amino, *p,p′*-DDT, and *o,p′*-DDT were 1.0×10^{-5} M, 9.3×10^{-7} M, 3.8×10^{-6} M, and 4.6×10^{-7} M, respectively. The order of the estrogenic potencies of the five compounds was as follows: *o,p′*-DDT > CNP-amino > *p,p′*-DDT > CNP.

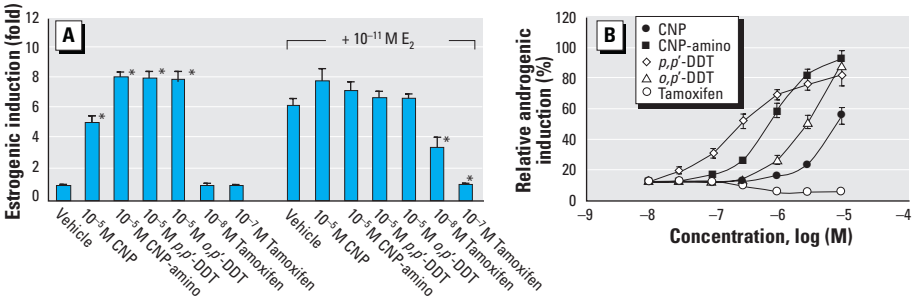

Figure 5. Effects of CNP and CNP-amino on human ERα activity. (*A*) CHO cells, transiently cotransfected with pcDNAERα, pGL3-tkERE and pRL-SV40, were incubated with the vehicle control (0.1% DMSO) or 10^{-5} M of CNP, CNP-amino, *p,p′*-DDT, *o,p′*-DDT, or 10^{-7} M of tamoxifen in the absence or presence of 10^{-11} M E_2. Values represent the means ± SD of three independent experiments and are presented as mean fold-induction over the vehicle control. (*B*) Cells were incubated with various concentrations of CNP, CNP-amino, *p,p′*-DDT, or *o,p′*-DDT. Values represent the means ± SD of three independent experiments and are presented as percentage induction, with 100% activity defined as the activity achieved with 10^{-10} M E_2.
*Significant difference at $p < 0.05$ by Student's *t*-test.

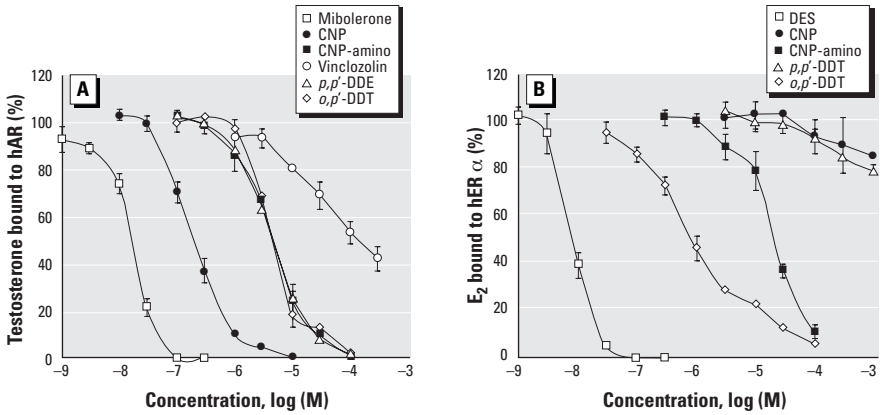

Figure 6. Competitive binding of CNP and CNP-amino to human AR and human ERα. Competitive binding assay was carried out using a ligand screening system-androgen receptor kit (*A*) and an ERα kit (*B*). Percentage of testosterone bound to hAR was calculated as ([optical density in the presence of competitor – optical density in the presence of 3×10^{-7} M mibolerone]/[optical density in the absence of competitor – optical density in the presence of 3×10^{-7} M mibolerone]) × 100. Percentage of E_2 bound to hERα was calculated as ([optical density in the presence of competitor – optical density in the presence of 3×10^{-7} M DES]/[optical density in the absence of competitor – optical density in the presence of 3×10^{-7} M DES]) × 100. Each point is the mean ± SD of three independent experiments with duplicate wells.

Table 1. Competitive binding abilities of CNP and CNP-amino for hAR and hERα.

	hAR		hERα	
Compound	IC_{50} (M)[a]	RBA-A[b]	IC_{50} (M)[c]	RBA-E[d]
Mibolerone	1.9×10^{-8}	100	ND	ND
DES	ND	ND	8.6×10^{-9}	100
CNP	2.2×10^{-7}	8.64	$> 10^{-3}$	< 0.0009
CNP-amino	5.7×10^{-6}	0.33	2.4×10^{-5}	0.036
Vinclozolin	1.5×10^{-4}	0.013	ND	ND
p,p′-DDT	ND	ND	$> 10^{-3}$	< 0.0009
p,p′-DDE	5.4×10^{-6}	0.35	ND	ND
o,p′-DDT	5.6×10^{-6}	0.34	9.1×10^{-7}	0.95

ND, no data.
[a]IC_{50}, the concentration of test compound exhibiting 50% inhibition against the binding of teststerone to hAR. [b]RBA-A (relative binding affinity for hAR) was expressed as a ratio of IC_{50} of mibolerone to that of test compounds. [c]IC_{50}, the concentration of test compound exhibiting 50% inhibition against the binding of E_2 to hERα. [d]RBA-E (relative binding affinity for hERα) was expressed as a ratio of IC_{50} of DES to that of test compounds. RBA values for mibolerone and DES were arbitrarily set at 100.

Competitive inhibition of the binding of testosterone and estradiol to hAR and hERα by CNP and CNP-amino. Figure 6A shows the competition curves depicting the effects of various doses of CNP, CNP-amino, vinclozolin, *p,p′*-DDE, *o,p′*-DDT, and mibolerone, a synthetic anabolic testosterone, on the binding of testosterone to hAR. CNP and CNP-amino inhibited the binding of testosterone to hAR in a dose-dependent manner, as did *p,p′*-DDE, *o,p′*-DDT, and mibolerone, and complete inhibition was achieved by CNP and CNP-amino at concentrations > 10^{-5} M and 10^{-4} M, respectively. Vinclozolin, in contrast, showed very low binding affinity for hAR. The IC_{50} (concentration of test compound exhibiting 50% inhibition against the binding of testosterone to hAR) values were obtained from the curves, and the relative binding affinities for hAR (RBA-A) were expressed as the ratio of the IC_{50} of mibolerone to that of each compound (Table 1). The IC_{50} values of CNP and CNP-amino were 2.2×10^{-7} M and 5.7×10^{-6} M, respectively. The RBA-A of CNP and CNP-amino were 8.64 and 0.33 compared to 100 for mibolerone.

Figure 6B shows the competition curves showing the effects of CNP, CNP-amino, *p,p′*-DDT, *o,p′*-DDT, and DES, a synthetic estrogenic drug, on the binding of E_2 to hERα. The effect of CNP on the binding of E_2 to hERα was very low, similar to that of *p,p′*-DDT. However, CNP-amino and *o,p′*-DDT showed inhibition ranging from 3×10^{-6} to 10^{-4} M and 3×10^{-7} to 10^{-4} M, respectively. The IC_{50} (concentration of test compound exhibiting 50% inhibition against the binding of E_2 to ERα) values were obtained from the curves, and the relative binding affinities for ERα (RBA-E) were expressed as the ratio of the IC_{50} of E_2 to that of each compound (Table 1). The IC_{50} of CNP and CNP-amino were > 1×10^{-3} M and 2.4×10^{-5} M, respectively. The RBA-E of CNP and CNP-amino were < 0.0009 and 0.036 compared to 100 for DES.

Discussion

In this study, we first developed the hAR- and hERα-mediated reporter gene assays using CHO-K1 cells to examine the effects of CNP and CNP-amino on sex hormone receptors. Our AR and ER assay systems showed high sensitivity to androgenic and estrogenic compounds, respectively, when compared with other assay systems using yeast cells and

HepG2 cells (Gaido et al. 1997; Maness et al. 1998; Nishikawa et al. 1999). This is thought to be the result of the high transfection efficiency of the FuGene transfection reagent for CHO cells, as reported by Vinggaard et al. (1999). In addition, our assay systems were highly specific to androgenic and estrogenic compounds, similar to the results obtained by Gaido et al. (1997) with yeast cells. These results suggest that both the hAR and hERα assays described in the present study are superior to other reporter gene assays in terms of rapidity, sensitivity, and reproducibility and are useful in identifying endocrine disruptors via AR and ERα from a large number of chemicals.

Using our assay systems, we examined the effects of CNP and CNP-amino on hAR and hERα activities. In the hAR assay, both CNP and CNP-amino were found to have a dose-dependent, suppressive effect on the DHT-induced transcriptional activity. It is noteworthy that the antiandrogenic potency of CNP was higher than that of well-known AR antagonists such as vinclozolin, *p,p′*-DDE, and *o,p′*-DDT. Moreover, CNP-amino also showed an antiandrogenic activity almost equal to those of the AR antagonists, indicating not only that the parent compound CNP but also its principal metabolite possess serious antiandrogenic activities. What is intriguing was finding that CNP also displayed weak AR agonistic activity at high concentrations, while CNP-amino did not (Figure 4B). These diverse effects of CNP are similar to those of the M2 (3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide), one of the two primary metabolites of vinclozolin, and hydroxyflutamide (an active metabolite of flutamide), both of which partially inhibit DHT activity and display some agonistic activity at high concentrations (Kelce and Wilson 1997; Wong et al. 1995). Thus, CNP is defined as a partial AR agonist/antagonist.

In the hERα assay, we found that CNP and CNP-amino also possess estrogenic activity but not antiestrogenic activity. The estrogenic activity of CNP was lower than the activities of *p,p′*-DDT and *o,p′*-DDT, well-known ER agonists; however, the estrogenic activity of CNP-amino was about 4-fold higher than that of *p,p′*-DDT. Nevertheless, the estrogenic potencies of CNP and CNP-amino were about 10^7- and 10^5-fold less potent than that of the endogenous estrogen E_2. Nishikawa et al. (2000) examined the estrogenic activities of 517 different kinds of chemicals by a yeast two-hybrid assay and judged CNP to be negative for estrogenic activity at 10^{-5} M. This again suggests that our hERα assay is more sensitive than the yeast two-hybrid assay.

Using the competitive receptor-binding assays, we identified that the antiandrogenic and estrogenic activities of CNP and CNP-amino measured by our AR and ER assay systems were mediated by way of the binding of CNP and CNP amino to AR and ERα. The binding abilities of CNP and CNP-amino, as well as *p,p′*-DDE and *o,p′*-DDT, for hAR were consistent with the antiandrogen activity defined in the reporter gene assay. In contrast, vinclozolin, which showed potent antiandrogenic activity in the hAR assay, showed poor binding ability to hAR. Kelce et al. (1994) reported that metabolites of vinclozolin exhibited a potent binding ability to AR, whereas the parent compound showed little activity. In this context, it is likely that CHO-K1 cells possess at least some biotransformation capacity, producing metabolites of vinclozolin, as Vinggaard et al. (1999) pointed out. The binding ability of CNP-amino and *o,p′*-DDT for hERα well reflected the hERα-transcriptional activation, whereas that of CNP and *p,p′*-DDT for hERα was somewhat low and did not correlate well with the hERα-transcriptional activation. The latter discrepancy may represent a difference in sensitivity between the reporter gene assay and receptor-binding assay.

In this study, we demonstrated for the first time that CNP and CNP-amino possess both antiandrogenic and estrogenic activities similar to *o,p′*-DDT. This in turn indicates that, in terms of the environment, CNP and CNP-amino should be considered serious endocrine-disrupting agents similar to other well-known AR antagonists or ER agonists. The present study also demonstrates the effectiveness of our reporter gene assays for detecting chemical interactions with hAR and hERα and for discerning receptor agonists from antagonists. It has been reported that many chemicals have more than one type of activity, and, in particular, a single chemical can have pleiotropic effects, being able to bind to both the androgen and estrogen receptors (Gaido et al. 2000; Satoh et al. 2001; Sohoni and Sumpter 1998). At present, we are searching for similar effects in yet undefined chemicals using our reporter gene assays.

There are a number of points of interest in the chemical structures of CNP and CNP-amino. The difference in chemical structure between CNP and CNP-amino is that a nitro group connected to the benzene ring in CNP is replaced by an amino group. This indicates that the difference between the nitro and amino group in their structure regulates their binding affinities to hAR and hERα and that the nitro group of CNP and the amino group of CNP-amino may play important roles in the transcriptional activity through the binding to the ligand-binding domain of hAR and hERα, respectively. Such a phenomenon may occur in other diphenylether herbicides such as nitrofen, chlomethoxynil, and bifenox, which have molecular structures similar to that of CNP and are converted to corresponding amino derivatives in the environment (Kuwatsuka 1977). Furthermore, Tamura et al. (2001) demonstrated that the organophosphate insecticide fenitrothion, which has a nitro group connected to the benzene ring similar to pharmaceutical antiandrogen flutamide, possesses potent antiandrogenic activity *in vitro* and *in vivo*. The existence of nitro benzene in the molecular structure may be an important key in identifying antiandrogenic compounds.

Because vinclozolin and *p,p′*-DDE, which were used as positive control chemicals in this study, are known to have *in vivo* antiandrogenic activity (Gray et al. 1994; Kelce et al. 1995, 1997), further studies are required to confirm the AR antagonist and ER agonist effects of CNP and CNP-amino by *in vivo* assays such as the Hershberger antiandrogen assay (Kelce et al. 1997; Lambright et al. 2000) and the uterotrophic assay (Odum et al. 1997).

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