**Table 1.** The Relative Binding Affinity of 4-Nonylphenol and *p*-Octylphenol for Various ERα and ERβ Determined by Competitive Enzyme Immunoassay

(A) ERα

| | DES | |
|---|---|---|
| | $IC_{50}$ (M) | RBA (%) |
| human | $3.2 \times 10^{-8} \pm 3.0 \times 10^{-9}$ | 100 |
| quail | $3.8 \times 10^{-9} \pm 1.0 \times 10^{-9}$ | 100 |
| *Xenopus* | $7.8 \times 10^{-9} \pm 9.2 \times 10^{-10}$ | 100 |
| medaka | $6.5 \times 10^{-8} \pm 1.5 \times 10^{-9}$ | 100 |
| | NP | |
| | $IC_{50}$ (M) | RBA (%) |
| human | $9.2 \times 10^{-6} \pm 1.1 \times 10^{-6}$ | 0.35 ± 0.047 |
| quail | $6.0 \times 10^{-8} \pm 9.7 \times 10^{-9}$ | 6.4 ± 1.9 |
| *Xenopus* | $2.9 \times 10^{-7} \pm 3.6 \times 10^{-8}$ | 2.8 ± 0.43 |
| medaka | $3.1 \times 10^{-7} \pm 1.2 \times 10^{-8}$ | 21 ± 0.98 |
| | OP | |
| | $IC_{50}$ (M) | RBA (%) |
| human | $1.4 \times 10^{-5} \pm 1.0 \times 10^{-6}$ | 0.23 ± 0.030 |
| quail | $1.3 \times 10^{-7} \pm 2.3 \times 10^{-8}$ | 3.3 ± 1.2 |
| *Xenopus* | $1.3 \times 10^{-6} \pm 1.5 \times 10^{-7}$ | 0.63 ± 0.091 |
| medaka | $1.9 \times 10^{-7} \pm 6.3 \times 10^{-8}$ | 34 ± 2.4 |

(B) ERβ

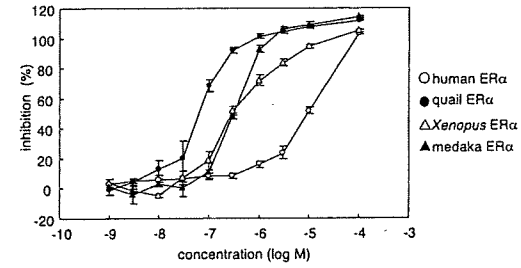
| | DES | |
|---|---|---|
| | $IC_{50}$ (M) | RBA (%) |
| human | $5.3 \times 10^{-9} \pm 1.8 \times 10^{-9}$ | 100 |
| quail | $1.3 \times 10^{-8} \pm 4.2 \times 10^{-10}$ | 100 |
| medaka | $3.2 \times 10^{-8} \pm 2.3 \times 10^{-9}$ | 100 |
| | NP | |
| | $IC_{50}$ (M) | RBA (%) |
| human | $5.7 \times 10^{-7} \pm 1.3 \times 10^{-8}$ | 0.83 ± 0.33 |
| quail | $5.1 \times 10^{-7} \pm 3.5 \times 10^{-8}$ | 2.5 ± 0.19 |
| medaka | $7.3 \times 10^{-7} \pm 7.2 \times 10^{-8}$ | 4.5 ± 0.79 |
| | OP | |
| | $IC_{50}$ (M) | RBA (%) |
| human | $4.9 \times 10^{-7} \pm 2.7 \times 10^{-8}$ | 0.98 ± 0.41 |
| quail | $9.9 \times 10^{-7} \pm 2.3 \times 10^{-7}$ | 1.3 ± 0.32 |
| medaka | $1.4 \times 10^{-6} \pm 1.3 \times 10^{-7}$ | 2.3 ± 0.093 |

RBA (%) = (DES $IC_{50}$)/(test sample $IC_{50}$) × 100.

6. Both 4-nonylphenol and *p*-octylphenol bound to ERβ of human, quail and medaka with similar affinity. On the other hand, in the case of ERα, different binding patterns were obtained. In Fig. 5A, 4-nonylphenol bound to quail ERα with higher affinity, whereas it bound to human ERα with lower affinity. Almost the same pattern was obtained in the binding of *p*-octylphenol to ERα (Fig. 6A).

The results are summarized by showing the $IC_{50}$ and RBA (relative binding affinity) (%) in Table 1. The RBA (%) values were lower than 7% in all cases,

(A) 4-Nonylphenol-ERα




(B) 4-Nonylphenol-ERβ

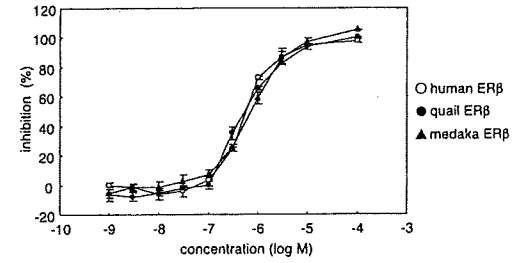
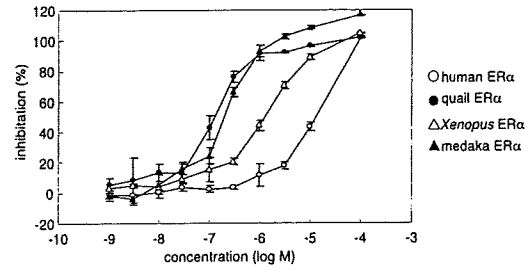



**Fig. 5.** Inhibition Curves of 4-Nonylphenol for Various ERα and ERβ Obtained by Competitive Enzyme Immunoassay
Values are the mean and standard deviation ($n$ = 3–4).

(A) *p*-Octylphenol-ERα




(B) *p*-Octylphenol-ERβ

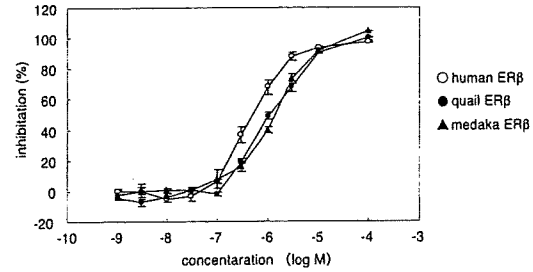



**Fig. 6.** Inhibition Curves of *p*-Octylphenol for Various ERα and ERβ Obtained by Competitive Enzyme Immunoassay
Values are the mean and standard deviation ($n$ = 3–4).

except for medaka ERα which showed 21 and 34% for 4-nonylphenol and *p*-octylphenol, respectively. Thus, 4-nonylphenol and *p*-octylphenol seem to have a strong ability to bind medaka ERα. Fig. 7 shows the inhibition curves of DES and *p*-octylphenol for ERα in human and medaka. These data were from Figs. 4 and 6. Although the affinity of DES for ERα in human and medaka was almost the same, that of *p*-octylphenol was not, indicating that *p*-octylphenol bound to medaka ERα with higher affinity than human ERα. On the other hand, in the case of quail ERα, the inhibition curve of *p*-octylphenol revealed a similar pattern to medaka ERα. However, the patterns of DES were different between quail and medaka ERα, resulting in the marked difference in RBA (%) (Fig. 8, Table 1). These results indicate that there is species specificity in the ability of not only 4-nonylphenol and *p*-octylphenol but also DES to bind ERs.

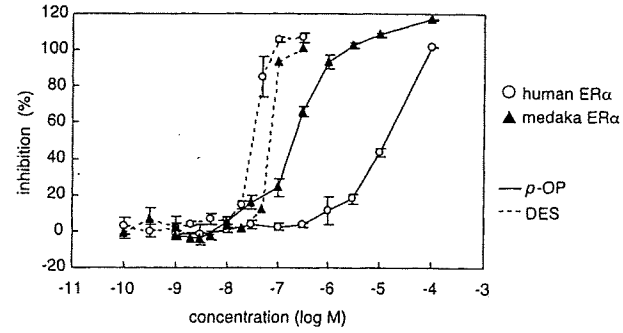



**Fig. 7.** Inhibition Curves of *p*-Octylphenol for Human and Medaka ERα Obtained by Competitive Enzyme Immunoassay

The solid and dotted lines show inhibition curves of *p*-octylphenol and DES, respectively. Values are the mean and standard deviation ($n$ = 3–4).

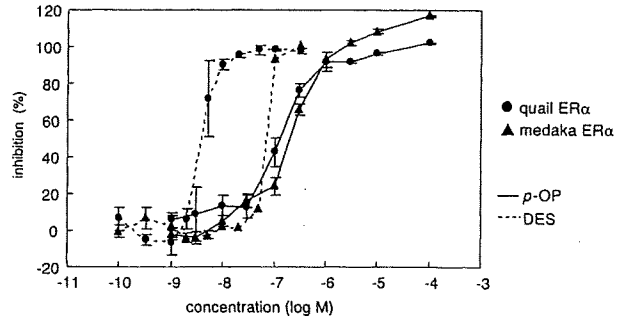



**Fig. 8.** Inhibition Curves of *p*-Octylphenol for Quail and Medaka ERα Obtained by Competitive Enzyme Immunoassay

The solid and dotted lines show inhibition curves of *p*-octylphenol and DES, respectively. Values are the mean and standard deviation ($n$ = 3–4).

## DISCUSSION

As a first screening method for the evaluation of EDCs, *in vitro* binding analyses are very useful, and many methods have been developed. These include the yeast two-hybrid assay, fluorescence polarization method, radio-competitive assay and competitive EIA. Although the capacity to bind human ERα and ERβ is well characterized, it is pointed out that the effects of EDCs on not only humans but also wildlife should be clalified.

We have previously established a competitive EIA for the detection of EDCs bound to quail ERα and ERβ. Using this system, we evaluated 20 test chemicals selected by MoE.[5] The competitive EIA has several advantages described above. Since no *in vitro* binding assay has been established for the frog, we first developed a competitive EIA for *Xenopus* ERα. Then, we established similar systems for human and medaka ERα and ERβ. The only difference among these systems is GST-ER-LBD. Therefore, once the GST-ER-LBD of other species is available, the development of a species specific EIA for ERs would seem to be very easy.

Next, we characterized the species specificities of the binding of 4-nonylphenol and *p*-octylphenol to various ERs, since there is no report of a comparison of the binding affinity of 4-nonylphenol and *p*-octylphenol for various ERs using the same protocol. No significant differences in the ability of 4-nonylphenol and *p*-octylphenol to bind ERβ in human, quail and medaka were observed. However, in the case of medaka ERα, the RBA (%) was a large value compared with that of ERα in human, quail and *Xenopus*. These results are partly due to the sequence difference with medaka ERα. However, it should be noted that RBA (%) is a relative binding affinity compared with the $IC_{50}$ for DES. $IC_{50}$s obtained from inhibition curves for 4-nonylphenol and *p*-octylphenol revealed higher values for human ERα in comparison with those for quail, *Xenopus* and medaka (Table 1 and Figs. 5 and 6).

It is also pointed out that $IC_{50}$s obtained from standard curves for DES to ERα and ERβ in various species showed slight differences in binding abilities. These data strongly suggest that the binding affinities of E2, DES, 4-nonylphenol and *p*-octylphenol for ER, especially ERα, might differ in a species-specific manner. The reports presented

previously are all based on the relative binding affinity for E2 or DES. Therefore, for further analyses of the binding of chemicals to ERs, a comparison of the affinity constant of the chemical itself as well as E2 and DES for the various ERs is required.

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# Development of a Competitive Enzyme Immunoassay for Detection of Capacity of Chemicals to Bind Quail Estrogen Receptor $\alpha$ and $\beta$

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*In vitro* binding assays are useful in the initial screening of endocrine disrupting chemicals. Such assays should be applied to the estrogen receptors (ER) of not only humans but also wildlife. As a system for birds is yet to established, we expressed the ligand binding domain (LBD) of quail ER$\alpha$ and ER$\beta$ as a fusion protein with glutathione S-transferase and using these proteins, developed two systems (a competitive enzyme immunoassay and a fluorescence polarization assay) for assaying the capacity to bind ERs *in vitro*. Moreover, 20 test chemicals selected by Ministry of the Environment of Japan were evaluated in terms of binding ability. Both systems worked well, the competitive enzyme immunoassay proving especially powerful, since it needs no special equipment. This system is applicable to other species including fish, amphibians and reptiles when information on the LBD of ER is available.

**Key words** —— endocrine disrupting chemicals, estrogen receptor, enzyme immunoassay, quail, endocrine disruptor, *in vitro* binding assay

## INTRODUCTION

Endocrine disrupting chemicals (EDCs) also called endocrine disruptors (EDs) seem to affect reproductive functions through the estrogen-estrogen receptor (ER) signaling pathway.[1,2] Although the mechanisms behind the disruption are not fully understood, the development of screening methods is definitely needed. Indeed, one-generation study and uterotrophic assay for use *in vivo*, and the receptor binding assay and reporter gene assay for use *in vitro* have been developed.[1–4] The Ministry of the Environment of Japan (MoE) released a document entitled "Strategic Programs on Environmental Endocrine Disrupters '98 (SPEED'98)" in 1998, and selected 65 substances as high-priority chemicals to be tested. The tests for some chemicals have been completed.[5]

For the initial screening *in vitro*, the receptor binding assay is often utilized. The yeast two-hybrid assay is particularly useful, since it is easy and relatively cheap to perform, and also no special equipment is needed.[6,7] This method is based on the interaction between the ligand binding domain (LBD) in the hormone receptor and the coactivator in the ligand-dependent manner.[6] Recently, it was proposed that the effect of EDCs should be considered not only in humans but also in wildlife, and indeed adverse effects on humans and wildlife were reported. However, information on coactivators is restricted to specific species, such as humans, rats and mice.[1,2] Therefore, the yeast two-hybrid assay does not seem to be a suitable method for use *in vitro* among various species.

The Organization for Economic Cooperation and Development (OECD) and MoE have started to develop various test methods for humans, fish, reptiles, amphibians, and birds.[5,8,9] As *in vitro* binding assay, several methods have been established, and

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some for human ERs are commercially available. These include the fluorescence polalization method[10] (TAKARA BIO Inc., Otsu, Shiga, Japan), a competitive enzyme immunoassay (EIA) (TOYOBO Co. Ltd., Osaka, Japan) and a radio-competitive assay. For the testing birds, Japanese quail was used in a vitellogenin assay, and a sex-reversal assay.[5] We have previously reported the cloning of quail ER$\alpha$.[11] The cloning of ER$\beta$ was also reported.[12] However, an *in vitro* binding assay for birds is yet to be established.

We report here the establishment of a competitive EIA for analysis of the capacity to bind quail ER$\alpha$ and ER$\beta$, and the subsequent testing of chemicals selected by MoE. We also established a fluorescence polalization method for quail ER$\alpha$ and ER$\beta$, and compared the usefulness of these two approaches.

## MATERIALS AND METHODS

**Chemicals** —— The test chemicals (12 and 8 chemicals selected by MoE in 2000 and 2001, respectively)[5] were supplied by Dr. Kawashima at JAPAN NUS Co. Ltd. (Japan), where large amounts of chemicals of reagent grade have been stocked for the various tests at the request of MoE. All test chemicals were dissolved in dimethyl sulfoxide (DMSO). All other chemicals are of reagent grade.

**Plasmid Construction** —— A DNA fragment containing LBD of quail ER$\alpha$ or ER$\beta$ was amplified by polymerase chain reaction (PCR) using KOD-Plus (TOYOBO Co. Ltd.), and the products were recovered. The 5′-end of these fragments was phosphorylated by T4 polynucleotide kinase and subcloned into the *Eco*RV site of pBluescript KS+. After confirmation of the nucleotide sequences using an automated DNA sequencer DSQ1000 (Shimadzu Corp., Kyoto, Japan), the *Eco*RI-*Sal*I fragment of ER$\alpha$-LBD or ER$\beta$-LBD was cloned into the same restriction sites in pGEX-4T-1, which codes glutathione S-transferase (GST) (Amersham Pharmacia Biotech). Finally, the resultant recombinant plasmid was transformed into *Escherichia coli* (*E. coli*), BL21 (DE3) (Novagen, EMD Biosciences Inc., Germany).

**Expression and Purification of GST-ER Fusion Protein** —— BL21 (DE3) harboring pGEX-4T-1-quail ER$\alpha$ or ER$\beta$ was cultured in Luria-Bertani (LB) medium at 30°C. At an $OD_{600}$ of 0.4, isopropyl $\beta$-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM. After incubation for 2 hr, the cells were harvested, suspended in 14.4 ml of B-0.1 [20 mM Tris (pH 7.5), 10% glycerol, 0.1 M KCl, 5 mM $MgCl_2$, and 1 mM DTT]. Then, 1.6 ml of 1 mg/ml lysozyme in B-1 [20 mM Tris (pH 7.5), 10% glycerol, 1 M KCl, 5 mM $MgCl_2$, and 1 mM dithiothreital (DTT)] was added and the cells were disrupted by sonication. This suspension was mixed with 16 ml of B-1, and further stirred gently for 0.5 hr at 4°C. By centrifugation at 12000 rpm for 15 min at 4°C, the soluble fraction including GST-ERs was obtained.

For the purification of GST-ERs, 0.42 ml of glutathione (GSH)-Sepharose 4B (Amersham Pharmacia Biotech) was added to 32 ml of the soluble fraction, and stirred gently for 0.5 hr at 4°C, and then the mixture was packed into the column. After washing thoroughly, the bound GST-ERs were eluted with 1 ml of 20 mM GSH in B-1.

**Competitive Enzyme Immunoassay** —— The competitive enzyme immunoassay was performed using the kit, Ligand Screening System-ER$\alpha$ or ER$\beta$ (TOYOBO Co. Ltd.), according to the manufacturer's instructions. However, the human ER$\alpha$ or ER$\beta$ in the kit was replaced with quail GST-ER$\alpha$ or GST-ER$\beta$. 17$\beta$-Estradiol (E2) and diethylstilbestrol (DES) were diluted with DMSO at a concentration of $8/3 \times 10^{-4}$ M–$8/3 \times 10^{-8}$ M, and further diluted with the dilution buffer in the kit at a final concentration of $8/3 \times 10^{-6}$ M–$8/3 \times 10^{-10}$ M (The final DMSO concentration is 1%). The test chemicals were diluted in the same way at a final concentrations of $8/3 \times 10^{-4}$ M–$8/3 \times 10^{-8}$ M.

Next, 20 $\mu$l of quail GST-ER, 30 $\mu$l of test chemical or standard DES, and 30 $\mu$l of E2 were mixed and incubated for 1 hr on ice. After the incubation, 50 $\mu$l of the mixture was transferred to an anti-E2 antibody-coated plate, and 50 $\mu$l of E2-horse radish peroxidase (HRP) solution was added. After incubation for 1 hr on ice, the plate was washed and the enzyme reaction was run. The absorbance at 450 nm was measured with a 1420 ARVO Multilabel Counter (Wallac, Gaithersburg, U.S.A.).

The percent inhibition of binding was calculated as follows: $(A_{\text{DMSO control}} - A_{\text{test sample}})/(A_{\text{DMSO control}} - A_{\text{DES at 112 nM}}) \times 100$. The $IC_{50}$ was obtained from the concentration giving 50% inhibition when the inhibition by 112 nM DES is 100%. Relative binding affinity (RBA) was obtained by dividing the $IC_{50}$ of DES by that of the $IC_{50}$ at test sample. When percent inhibition was less than 50%, 20% relative effective concentration (REC20) was also calculated.

**Fluorescence Polarization Method** —— For the

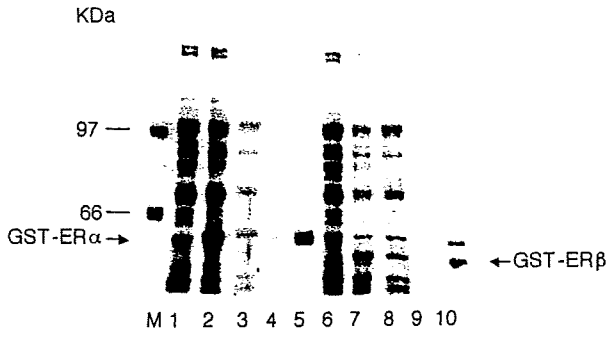



**Fig. 1.** Expression and Purification of Quail GST-ER$\alpha$ and ER$\beta$

The quail GST-ER$\alpha$ and GST-ER$\beta$ fusion proteins were expressed in *E. coli* BL21 (DE3), and then purified using GSH-Sepharose. The samples were loaded on SDS-polyacrylamide gel, and stained with coomassie brilliant blue. Lane M: size marker; Lanes 1–5: GST-ER$\alpha$; Lanes 6–10: GST-ER$\beta$; Lanes 1&6: whole protein solubilized from untreated cells; Lanes 2&7: whole protein solubilized from IPTG-treated cells; Lanes 3&8: soluble fraction from IPTG-treated cells; Lanes 4&9: purified GST-ERs from soluble fraction; Lanes 5&10: purified GST-ERs from soluble fraction (40-fold more than in Lanes 4 &9).

fluorescence polarization method of assaying binding to ERs,[10] an FP Screen-for-Competitors Kit, ER-$\alpha$ or ER$\beta$, high sensitivity (TAKARA BIO Inc.), was used. The human ER$\alpha$ or ER$\beta$ in the kit was replaced with quail GST-ER$\alpha$ or GST-ER$\beta$. The assay was performed according to the manufacturer's instructions. In brief, the test chemicals were diluted with DMSO at a concentration of $10^{-2}$ M–$10^{-6}$ M, and 1 $\mu$l of each chemical was added to 99 $\mu$l of Fluomone ES1 and quail GST-ER complex. After incubation for 1 hr at room temperature, the fluorescence polarization was measured with a Full-Range Beacon 2000 (TAKARA BIO Inc.).

## RESULTS

### Expression and Purification of GST-ER Fusion Protein

For the development of *in vitro* binding assay systems, we first expressed the LBD of quail ER$\alpha$ and ER$\beta$ as a GST fusion protein in *E. coli*. With the addition of IPTG, GST-ERs were induced and recovered mainly in the soluble fraction (Fig. 1). This soluble fraction was applied to a GSH-Sepharose column and eluted with GSH. The purity and amount of the resultant purified GST-ER$\alpha$ (MW = 65 kDa) and GST-ER$\beta$ (MW = 60 kDa) were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1).

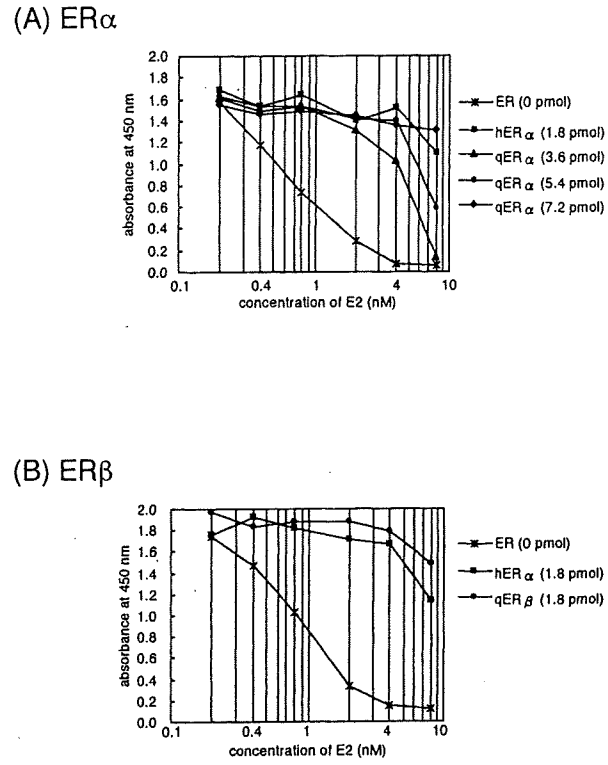



**Fig. 2.** Measurement of the Amount of Free Ligand in the Presence of Quail ERs

For determination of the most suitable amount of ERs to use, the free ligand test was performed using various amounts of quail ERs. As a control, human ER$\alpha$ was used. (A): quail ER$\alpha$; (B): quail ER$\beta$.

### Development of a Competitive Enzyme Immunoassay for the Capacity to Bind Quail ERs

For the development of a competitive EIA for quail ERs, the optimum amount of ERs used should be determined. Using various amounts of E2 and quail ERs, the amount of free ligand which does not make a complex with ERs was determined (Fig. 2). Since the concentration of E2 used in the kit is 4 nM, it is required that up to 4 nM be trapped by ER. At above 4 nM, E2 exists freely. In this regard, the optimum amount of ER used was determined as 5.4 pmol/well and 1.8 pmol/well for quail ER$\alpha$ and ER$\beta$, respectively.

Next, we developed a competitive EIA using these amounts of recombinant ERs. The standard curve was made taking percent inhibition with 112 nM DES as 100% (Fig. 3). The $IC_{50}$ of DES for quail ER$\alpha$ and ER$\beta$ was $6.0 \times 10^{-9}$ M and $1.3 \times 10^{-8}$ M, respectively. When using the human ERs in the kit, those values were $1.0 \times 10^{-8}$ M and $5.0 \times 10^{-9}$ M, respectively. Thus, $IC_{50}$ values were similar among the two species and two types of ER.

The competitive EIA established for quail ERs

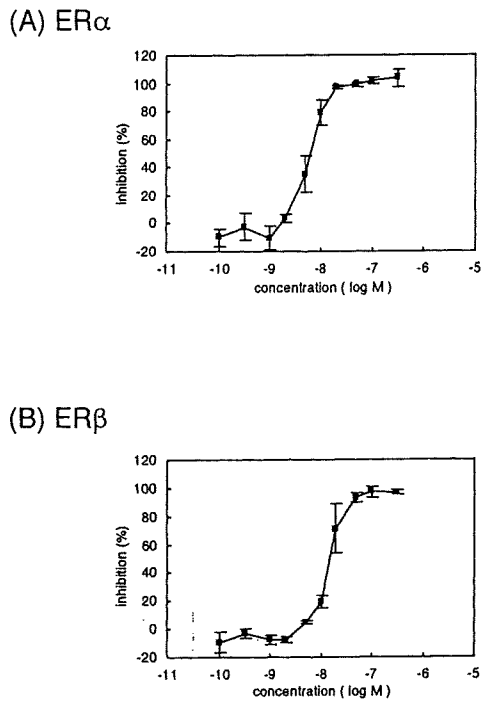



**Fig. 3.** Standard Curve of DES Obtained by Competitive Enzyme Immunoassay

The inhibition curves against DES for quail ERα (A) and ERβ (B) are shown. Values are the mean and standard deviation ($n = 4$).

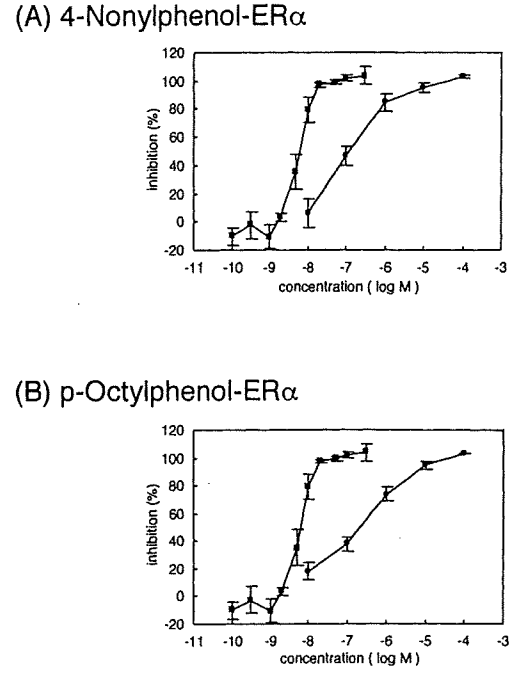



**Fig. 4.** Inhibition Curves of 4-Nonylphenol and *p*-Octylphenol for Quail ERα Determined by Competitive Enzyme Immunoassay

The squares show the inhibition curves of DES. The circles show the inhibition curves of 4-nonylphenol (A) and *p*-octylphenol (B). Values are the mean and standard deviation ($n = 4$).

was next applied to the test chemicals. MoE listed 65 chemicals suspected of having endocrine-disrupting effects in SPEED'98. Of these, 12 and 8 chemicals were selected in 2000 and 2001, respectively, as high-priority chemicals to be tested. Therefore, we next tested the capacity of these 20 chemicals to bind quail ERs using the competitive EIA developed above. Figures 4 and 5 show the inhibition curves of 4-nonylphenol and *p*-octylphenol for quail ERα and ERβ, respectively. The results for 20 chemicals were summarized by showing the $IC_{50}$ or REC20 in Table 1. Among those chemicals tested, 4-nonylphenol, p-octylphenol and bisphenol A exhibited relatively strong binding to both quail ERα and ERβ, although in each case the $IC_{50}$ was $10^{-7}$ M and the RBA was less than 10%.

The competitive EIA is based on the antigen-antibody reaction. If the test chemical inhibits this reaction, a false positive result might be obtained. Therefore, it is necessary to check the inhibitory effect of the test chemical on the antigen-antibody interaction. Figures 6–8 show the inhibitory effects on the immunoreaction as well as the results for the competitive EIA for ERα and ERβ. Bisphenol A (Fig. 6) had the capacity to bind quail ERα and ERβ, although an inhibitory effect on the immunoreaction

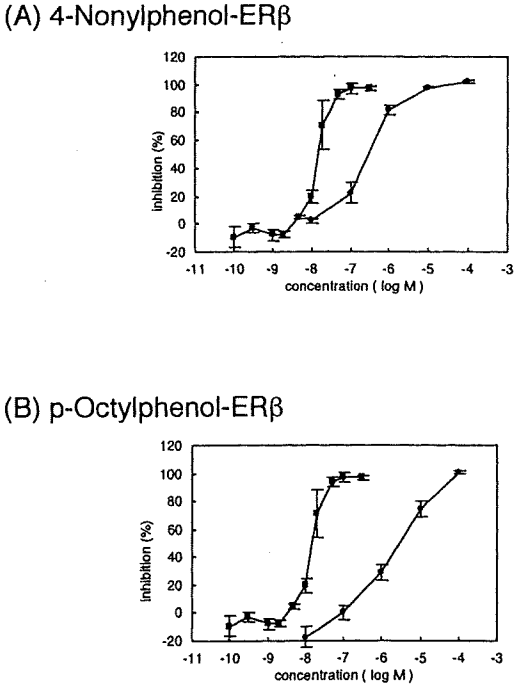



**Fig. 5.** Inhibition Curves of 4-Nonylphenol and *p*-Octylphenol for Quail ERβ Determined by Competitive Enzyme Immunoassay

The squares show the standard curves of DES. The circles show the inhibition curves of 4-nonylphenol (A) and *p*-octylphenol (B). Values are the mean and standard deviation ($n = 4$).

**Table 1.** The Relative Binding Affinity of Test Chemicals to Quail ERα and ERβ Evaluated by Competitive Enzyme Immunoassay

| | | qERα | | | qERβ | | |
|---|---|---|---|---|---|---|---|
| | Chemicals | $IC_{50}$ (M) | REC20 (M) | RBA (%) | $IC_{50}$ (M) | REC20 (M) | RBA (%) |
| | DES | $6.0 \times 10^{-9}$ | | 100 | $1.3 \times 10^{-8}$ | | 100 |
| 1 | Benzophenone | | $> 10^{-4}$ | | | $> 10^{-4}$ | |
| 2 | Octachlorostyrene | | $> 10^{-4}$ | | | $4.0 \times 10^{-5}$ | |
| 3 | Diethyl phthalate | | $> 10^{-4}$ | | | $> 10^{-4}$ | |
| 4 | Benzyl-n-butyl phthalate | $2.0 \times 10^{-5}$ | | 0.030 | $9.0 \times 10^{-5}$ | | 0.014 |
| 5 | Bis(2-ethylhexyl) adipate | | $> 10^{-4}$ | | | $> 10^{-4}$ | |
| 6 | Triphenyltin chloride | $1.0 \times 10^{-5}$ | | 0.060 | $1.0 \times 10^{-5}$ | | 0.130 |
| 7 | Bis(2-ethylhexyl) phthalate | $3.0 \times 10^{-6}$ | | 0.200 | | $2.0 \times 10^{-5}$ | |
| 8 | Dicyclohexyl phthalate | $1.0 \times 10^{-4}$ | | 0.006 | | $9.0 \times 10^{-6}$ | |
| 9 | Di-n-butyl phthalate | | $> 10^{-4}$ | | | $> 10^{-4}$ | |
| 10 | Tributyltin(IV) chloride | $2.0 \times 10^{-5}$ | | 0.030 | | $2.0 \times 10^{-5}$ | |
| 11 | 4-Nonylphenol | $1.0 \times 10^{-7}$ | | 6.00 | $3.0 \times 10^{-7}$ | | 4.30 |
| 12 | p-Octylphenol | $1.0 \times 10^{-7}$ | | 6.00 | $3.0 \times 10^{-6}$ | | 0.430 |
| | DES | $4.7 \times 10^{-9}$ | | 100 | $1.2 \times 10^{-8}$ | | 100 |
| 13 | Bisphenol A | $3.0 \times 10^{-7}$ | | 1.57 | $1.8 \times 10^{-7}$ | | 6.67 |
| 14 | 2,4-Dichlorophenol | $1.0 \times 10^{-5}$ | | 0.047 | | $2.8 \times 10^{-5}$ | |
| 15 | 4-Nitrotoluene | | $> 10^{-4}$ | | | $> 10^{-4}$ | |
| 16 | Di-n-pentyl phthalate | | $> 10^{-4}$ | | | $> 10^{-4}$ | |
| 17 | Di-n-propyl phthalate | | $> 10^{-4}$ | | | $> 10^{-4}$ | |
| 18 | Pentachlorophenol | | $5.0 \times 10^{-5}$ | | | $1.9 \times 10^{-5}$ | |
| 19 | Amitrole | | $> 10^{-4}$ | | | $> 10^{-4}$ | |
| 20 | Di-n-hexyl phthalate | | $> 10^{-4}$ | | | $> 10^{-4}$ | |

$IC_{50}$ (M): the concentration giving 50% inhibition when the inhibition by 112 nM DES is 100%. REC20 (M): 20% relative effective concentration when percent inhibition was less than 50%. RBA (%): relative binding affinity was obtained by dividing the $IC_{50}$ of DES by that of the $IC_{50}$ at test sample. Since the experiments of Nos. 1–12 and Nos. 13–20 were performed separately, $IC_{50}$ for DES were shown in each experiment.

was also obtained at a higher concentration.

On the other hand, the percent inhibition by pentachlorophenol was similar to the inhibitory effect on the immunoreaction, indicating that this chemical had no capacity to bind ERs at the concentration tested (Fig. 7). In the case of 2, 4-dichlorophenol, a slight binding capacity was obtained only for ERα (Fig. 8). Both triphenyltin and tributyltin exhibited the weak binding, but had the same inhibitory effect on the immunoreaction (data not shown). Thus, it is necessary to check whether test chemicals having the capacity to bind ERα or ERβ exhibit an inhibitory effect on the immunoreaction.

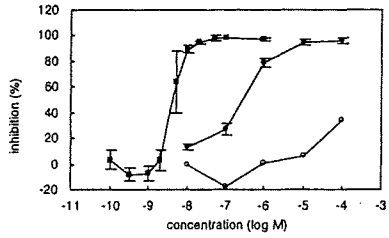
### Development of the Fluorescence Polarization Method for Assaying the Capacity to Bind Quail ERs

A kit for the fluorescence polarization method for human ERα and ERβ is available. Therefore, by replacing human ERs with quail ERs, we established a fluorescence polarization method for assaying the capacity to bind quail ERs. First, we determined the optimum (saturating) concentration of quail ERs to be 100 nM ERα and 50 nM ERβ with Fluomone ES1. Under these conditions, we established the fluorescence polarization method, and obtained a standard curve and the inhibition curve of 4-nonylphenol and *p*-octylphenol (Figs. 9 and 10, respectively). Moreover, 12 chemicals selected in 2000 by MoE were tested. The results on binding capacity were basically the same as those obtained by competitive EIA (data not shown).

## DISCUSSION

As a method for screening EDCs, *in vitro* binding analysis is a quite useful. So far, the capacity of EDCs to bind human ERα and ERβ has been well characterized. Recently, it was pointed out that the effects of EDCs on not only humans but also wildlife should be clarified. For this purpose, a cheap

(A) Bisphenol A-ERα




(B) Bisphenol A-ERβ

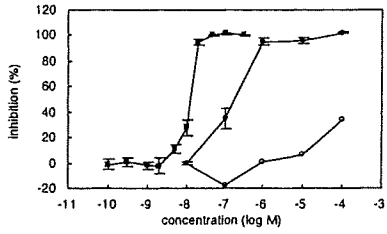
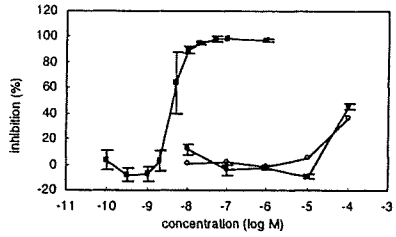



**Fig. 6.** Inhibition Curves of Bisphenol A for Quail ERα and ERβ Determined by Competitive Enzyme Immunoassay and the Inhibitory Effect on the Antigen-Antibody Reaction

The squares show the standard curves of DES. The closed circles show the inhibition curves of bisphenol A for ERα (A) and ERβ (B). The open circles show the inhibitory effect of bisphenol A on the antigen-antibody reaction. Values are the mean and standard deviation ($n$ = 4).

(A) Pentachlorophenol-ERα




(B) Pentachlorophenol-ERβ

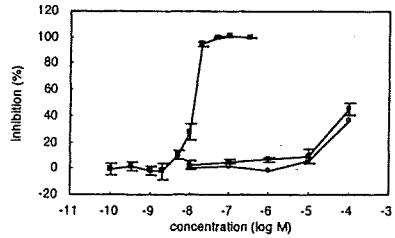
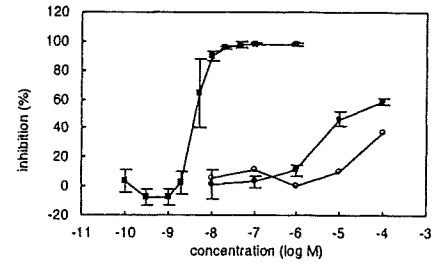



**Fig. 7.** Inhibition Curves of Pentachlorophenol for Quail ERα and ERβ Determined by Competitive Enzyme Immunoassay and the Inhibitory Effect on the Antigen-Antibody Reaction

The squares show the standard curves of DES. The closed circles show the inhibition curves of pentachlorophenol for ERα (A) and ERβ (B). The open circles show the inhibitory effect of pentachlorophenol on antigen-antibody reaction. Values showed the mean and standard deviation ($n$ = 4).

(A) 2, 4-Dichlorophenol-ERα




(B) 2, 4-Dichlorophenol-ERβ

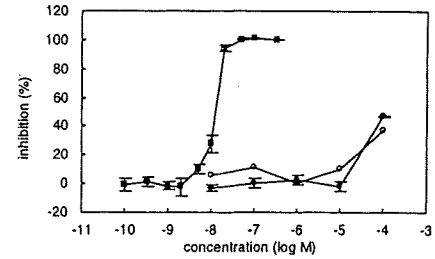



**Fig. 8.** Inhibition Curves of 2, 4-Dichlorophenol for Quail ERα and ERβ Determined by Competitive Enzyme Immunoassay and the Inhibitory Effect on the Antigen-Antibody Reaction

The squares show the standard curves of DES. The closed circles show the inhibition curves of 2, 4-dichlorophenol for ERα (A) and ERβ (B). The open circles show the inhibitory effect of 2, 4-dichlorophenol on the antigen-antibody reaction. Values are the mean and standard deviation ($n$ = 4).

and easy screening method was required. However, competitive assays using radiolabeled estrogen need special equipment and handling. Although a kit for fluorescence polarization is available for human ERα and ERβ, this system also needs special equipment. The yeast two-hybrid system is superior in terms of cost and handling. However, since the cofactor of each species is necessary, it has disadvantages for the evaluation of the binding capacity for many species of ERs.

Competitive EIAs for detecting the ability of EDCs to bind ERs have several advantages. First, it requires neither special equipment nor special techniques. Second, it costs less. Therefore, once the recombinant ERs are ready, the assay system can be established quickly for ERs in all species. Although quail is used as a test bird for the evaluation of EDCs, an *in vitro* binding assay system for birds is yet to be established. In this report, we have established a competitive EIA for the detection of EDCs bound to quail ERα and ERβ. Using this system, we evaluated the 20 test chemicals selected by MoE.

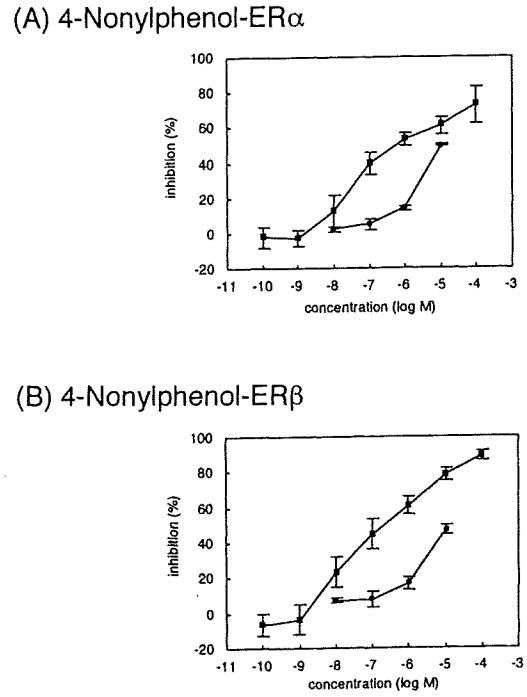



**Fig. 9.** Standard Curve of E2 and Inhibition Curve of 4-Nonylphenol Obtained by the Fluorescence Polalization Method

The squares show the standard curve of E2. The circles show the inhibition curves of 4-nonylphenol for ERα (A) and ERβ (B). Values are the mean and standard deviation ($n$ = 3).

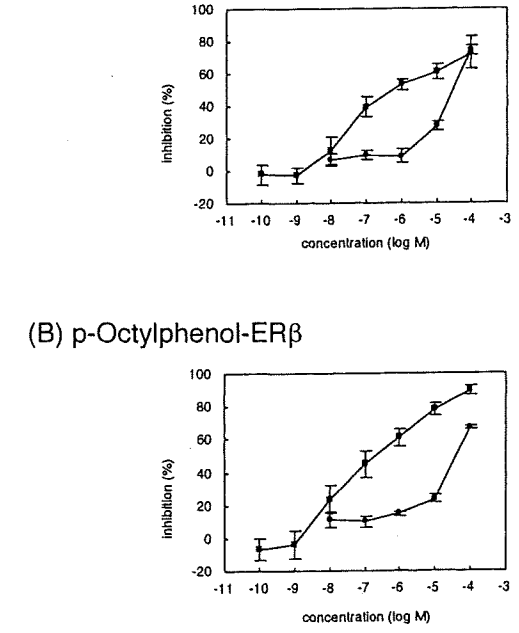



**Fig.10.** Standard Curve of E2 and Inhibition Curve of p-Octylphenol Obtained by the Fluorescence Polalization Method

The squares show the standard curve of E2. The circles show the inhibition curves of $p$-octylphenol for ERα (A) and ERβ (B). Values are the mean and standard deviation ($n$ = 3).

The *in vitro* binding method for birds presented in this paper seems to be well developed, since the relative affinity concentrations of 20 test chemicals in this assay system are well interrelated with those obtained by the fluorescence polarization method for human ER,[5] the yeast two-hybrid assay using rat ER and rat cofactor,[6,7] and the radio-competitive assay for fish.[5] Moreover, the fold differences of the binding concentration between E2 and test chemicals including 4-nonylphenol, $p$-octylphenol and bisphenol A are basically the same when compared with those obtained by *in vivo* studies, which is 3-day uterotrophic assay in rats.[13]

However, the species specificity was also observed. Unfortunately, it is hard to discuss with this specificity in detail, because all *in vitro* binding assays estabilished previously have shown the relative binding activity to E2 or DES, and the binding affinity of E2 and DES itself to ER are not characterized yet. Therefore, for characterization of the species specificity, the affinity constant itself of E2, DES and other test chemicals including 4-nonylphenol, $p$-octylphenol and bisphenol A are definitely needed. We are now establishing an assay system for use in frogs as well as humans and medaka, and also the affinity constant of several chemicals having estrogenic activity for evaluating species-specific effects of EDCs.

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# 1-Chloro-2,4-Dinitrobenzene Stimulates the Estrogenic Activity in MCF-7 Cells

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The estrogenic effect of chemicals, including 1-chloro-2,4-dinitrobenzene (CDNB), in the combination with 17$\beta$-estradiol ($E_2$) was screened by a reporter gene assay using breast cancer cells (MCF-7). It was found that CDNB stimulated $E_2$-induced transcriptional activity of estrogen receptor (ER) and down-regulated both ER protein and mRNA. However, CDNB alone and CDNB metabolite(s) showed no estrogenic activity and no binding activity to ER, suggesting an indirect pathway other than ER. CDNB gave no transcriptional activity for an aryl hydrocarbon receptor (AhR), suggesting no possibility of a pathway through cross-talk between AhR and ER. On the other hand, CDNB enhanced the mitogen activated protein kinase (MAPK) pathway, suggesting estrogenic action *via* MAPK. These results indicated that CDNB possessed estrogen-like activity in the transcription and regulation of ER though a different pathway from $E_2$.

**Key words** —— 1-chloro-2,4-dinitrobenzene, metabolite, aryl hydrocarbon receptor, mitogen activated protein kinase, MCF-7 cell

## INTRODUCTION

Estrogen plays important roles in the function, growth and differentiation of mammary glands, the uterus, and ovaries. It also affects other tissues, including bone, liver, the cardiovascular system, and brain. Estrogen acts primarily through the estrogen receptor (ER), which is a member of the nuclear hormone receptor superfamily and a ligand-dependent transcription factor.[1] ER reacts specifically with estrogen, and is transported as a dimer into the site of the estrogen response element (ERE) in the nucleus. The estrogen-ER complex recruits coactivators and general transcription factors, and then regulates the expression of target genes.[2,3]

However, certain chemicals, so-called endocrine disruptors (EDs), affect the endocrine system containing estrogen. EDs are defined as xenobiotics that interfere with the function of natural hormones in terms of production, release, transport, metabolism, receptor binding, or excretion in the body, resulting in disturbing the maintenance of homeostasis and the regulation of developmental processes.[4] It is important and urgent task in the risk assessment of ED to screen and list the positive chemicals from a huge number of chemicals using *in vivo* and *in vitro* tests and many have been reported. For instance, bisphenol A and nonylphenol mimic estrogens *via* binding to ER.[5,6] However, most of these tests have been performed on single pure chemicals; nevertheless, the exposure occurs actually as mixtures of chemicals and not a single chemical. At least, the chemicals should express an effect in the presence of natural hormones such as 17$\beta$-estradiol ($E_2$).

Therefore, we studied the effect of chemicals on the estrogenic activity in the coexistence of $E_2$, expecting to find new chemicals with inhibitory or stimulative activity for $E_2$. In our previous paper, the yeast two-hybrid assay revealed inhibitory chemicals including hexachlorophene, pentachlorophenol, and vitamin K3 (menadione; K3).[7] In this paper, we examined the estrogenic activity in the presence of $E_2$ on chemicals negative in the yeast two-hybrid assay using an MCF-7 reporter gene assay, and found that 1-chloro-2,4-dinitrobenzene (CDNB) showed an enhancing effect by activation of mitogen activated protein kinase (MAPK), not ER.

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## MATERIALS AND METHODS

**Chemicals** —— CDNB was purchased from Nacalai Tesque Co. (Kyoto, Japan), $E_2$ and $\beta$-napthoflavone were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and MG-132 was obtained from Calbiochem (Germany). 4-Hydroxytamoxifen (4-OHT) was a gift from Dr. Hayakawa, K. (Kanazawa University). The other chemicals were of the highest grade commercially available, and were used without further purification. These chemicals were prepared as solutions in dimethylsulfoxide (DMSO).

**Cells** —— MCF-7 cells were grown for routine maintenance in Eagle's Minimal Essential Medium (EMEM) with phenol red (Nissui Pharmaceuticals Co. Ltd., Tokyo, Japan), supplemented with 10 mM non-essential amino acids (Nacalai Tesque Co., Tokyo, Japan) and 10% heat-inactivated fetal bovine serum (FBS). Cells were maintained in a humidified environment at 37°C with 5% $CO_2$ in air.

**Reporter Gene Assay** —— For the reporter gene assay, $1.5 \times 10^5$ cells were seeded into 35 mm dishes in phenol red-free EMEM containing 10 mM non-essential amino acids and 10% charcoal-dextran treated FBS. The following day, cells were transently transfected with 1 $\mu$g of plasmid (400 ng of reporter plasmid with 4xERE-TATA-luciferase and 100 ng of pSV40-$\beta$-gal to measure the transfection efficiency, and 500 ng of pBluescript) using the Fugene6 transfection reagent (Roche Diagnostics Co., Indianapolis, U.S.A.) according to the manufacturer's protocol. The cells were incubated for 24 hr after transfection, and exposed to chemicals with and without $E_2$ in fresh medum. The test chemicals were dissolved in DMSO (final concentration in the culture medium did not exceed 0.1%). After incubation for 24 hr, cells were washed two times with PBS, lyzed and assayed for luciferase activity using a luminometer (Lumat LB9501, Berthold GmbH & Co., Germany). Luciferase activity was presented after normalization to $\beta$-galactosidase activity.

**Northern Blots** —— MCF-7 cells were cultivated in 100 mm tissue culture plates for 48 hr, and then treated for 24 hr with DMSO, 1 nM $E_2$, 10 $\mu$M CDNB or CDNB with $E_2$. Twenty micrograms of total RNA per treatment group was separated by electrophoresis on 1% agarose gels, transferred onto nylon membrane (HybondN+, Amersham Pharmacia Biotech, England), bound to the membrane by UV crosslinking, and dried at 80°C for 2 hr. The membrane was then prehybridized in a solution containing 1% bovine serum albumin (BSA) (Sigma, U.S.A.), 7% sodium dodecyl sulfate (SDS) and 0.5 M sodium phosphate buffer (pH 7.2) for 18 hr to 24 hr at 60°C and hybridizied in the same buffer for 24 hr with the [$\alpha$-$^{32}$P]dCTP cDNA probes. Levels of specific mRNA transcripts were standardized as a value relative to G3PDH mRNA in the same sample and band intensities were determined on BAS images (Fujifilm, Tokyo, Japan).

**Protein Isolation and Western Blots** —— MCF-7 cells, after cultivation in 35 mm dishes for 48 hr, were treated with chemicals for the indicated times. After treatment, the cells were washed twice with PBS and then lysed in 70 $\mu$l of lysis buffer containing 8 M urea, 1% NP-40, and 2% 2-mercaptoethanol. After removing the cell debris, the supernatants were used for protein concentration assays. The proteins were boiled for 2 min, applied to a 10% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore immobilon transfer membrane, U.S.A.). The membrane was blocked using 3% skim-milk (Yukijirushi, Japan) or 2.5% BSA overnight at 4°C and probed with primary antibodies; ER$\alpha$ (1 : 200 in 1% skim-milk, Santa Cruz Biotechnology Inc., U.S.A.), extracellular signal-regulated kinases 1 (ERK1) (1 : 1000 in 1.25% BSA, BD Biosceinces, U.S.A.), or phospho-p44/p42 MAP kinase (1 : 1000, Cell signaling technology). Following incubation with a peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (Amersham Pharmacia Biotech, U.S.A.).

**ER Competitive Binding Assay** —— The binding of the chemicals to ER$\alpha$ was determined using a fluorescence polarization assay, an FP Screen-for-Competitors Kit (ER$\alpha$, high sensitivity; PanVera, Madison, U.S.A.). Briefly, 1 $\mu$l of each chemical solution was added to 49 $\mu$l of screening buffer in tubes and mixed well by shaking. Then, 50 $\mu$l of ER$\alpha$-fluorescence estrogen (ES1) complex solution was added to the tube, incubated at room temperature for 1 hr and the fluorescence was determined using BEACON2000 (PanVera). DMSO instead of the chemical solution was used as a negative control (0% inhibition), and 10 $\mu$l of ES1 (50 nM) instead of ER$\alpha$-ES1 complex as a positive control (100% inhibition). Curve fitting was performed by GraphPad Prism 2.01 software to obtain $IC_{50}$.

**CDNB Metabolism** —— CDNB was treated with rat liver S-9 mixture (Oriental Yeast Co. Ltd., Japan) for 1 hr at 37°C, according to the manufacturer's protocol. The rat liver S-9 mixture contained 20 $\mu$l/ml

S-9, 0.8 mM NADPH, 0.8 mM NADH, 1.0 mM glucose-6-phosphate (G-6-P), 0.4 u/ml G-6-P dehydrogenase, 20 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 6.6 mM KCl, and 1.6 mM $MgCl_2$.

**Yeast Assay for Aryl Hydrocarbon Receptor (AhR) Ligand Activity** —— The yeast transformed with the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator (AhR/ARNT) plasmid and XRE plasmid was used as described by Miller.[8] The AhR ligand activity was determined essentially according to the method of Adachi *et al.*[9] The yeast strain YCM3 was grown for 5 hr at 30°C in synthetic dropout (SD) medium lacking tryptophan. Test chemicals (2 $\mu$l in DMSO), 5 $\mu$l of culture, and 200 $\mu$l of SD medium containing 2% galactosidase were mixed and incubated overnight at 30°C. The cell densities were determined by reading the OD at 595 nm. After 10 $\mu$l of the reaction mixture was added to 140 $\mu$l of Z-buffer, $\beta$-galactosidase activity was determined by incubation with o-nitrophenol-$\beta$-D-galactopyranoside for 60 min at 37°C. The absorbance of the $\beta$-galactosidase assay was read at 415 nm.

## RESULTS

### Enhancement of $E_2$-Induced Transcription by CDNB in MCF-7 Cells

The effect of CDNB on $E_2$-induced transcriptional activity was determined by a reporter gene assay, using MCF-7 cells transfected with the ERE-TATA-luciferase plasmid. When CDNB was exposed to the cells for 24 hr with and without $E_2$, CDNB enhanced dose–dependently the transcriptional activity under the condition of 1 nM $E_2$, while CDNB alone did not show any estrogenic activity (Fig. 1).

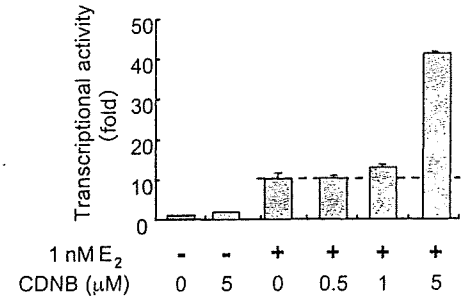


**Fig. 1.** Enhancement of $E_2$-Induced Transcriptional Activity by CDNB

MCF-7 cells were transiently transfected with ERE-TATA-luciferase plasmids as described in MATERIALS AND METHODS. The transcriptional activity was determined as luciferase activity using a reporter gene assay. Luciferase activity was normalized to $\beta$-galactosidase activity in each sample. Transcriptinal activity was expressed when the luciferase activity of DMSO was 1. Values represent the mean ± S.D. ($n$ = 3).

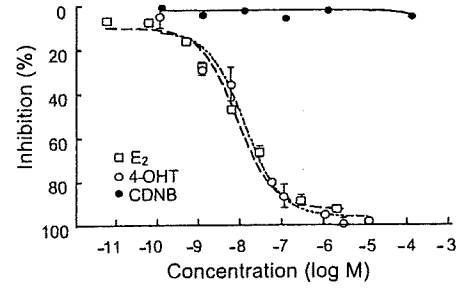


**Fig. 2.** Competitive Binding Assay of CDNB to ER

Binding of fluorescein-labeled estrogen to ER was inhibited competitively by increasing concentrations of $E_2$ and 4-OHT, but not CDNB. The ratio of displacement was measured using a luminescence meter. Values represent the mean ± S.D. ($n$ = 3). gene assay. Luciferase activity was normalized to $\beta$-galactosidase activity in each sample. Transcriptinal activity was expressed when the luciferase activity of DMSO was 1. Values represent the mean ± S.D. ($n$ = 3).

### No Binding Activity of CDNB to ER$\alpha$

CDNB showed no binding activity to ER$\alpha$ in the system where non-labeled $E_2$ and 4-OHT, a known antagonist, inhibited dose–dependently the binding of fluorescein-labeled estrogen to ER$\alpha$ (Fig. 2). This indicated that CDNB did not enhance the $E_2$ activity through ER$\alpha$.

### Decrease in ER mRNA and Protein Levels by CDNB

The expression of ER mRNA was repressed by CDNB, as well as $E_2$, but not 4-OHT. Furthermore, CDNB with $E_2$ decreased more than $E_2$ alone (Fig. 3A). ER protein levels were also down-regulated by CDNB with $E_2$ more than CDNB or $E_2$ alone (Fig. 3B).

### No Effect of CDNB Metabolite on Transcriptional Activity of ER$\alpha$

It is known that certain chemicals acquire estrogenic activity after being metabolized. Here, it was expected to be the same in the case of CDNB, so the transcriptional activity of ER$\alpha$ was examined on metabolite(s) of CDNB, obtained after incubation of CDNB with a rat liver S-9 mixture at 37°C for 1 hr. Then, the activities of CDNB metabolite(s) were compared with and without $E_2$. CDNB metabolite(s) with $E_2$ did not enhance the transcriptional activity, unlike the CDNB case (Fig. 4). Furthermore, the CDNB metabolite(s) itself did not have the estro-

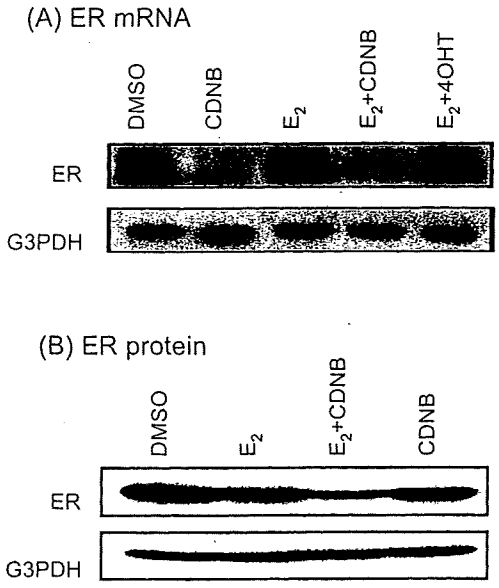


**Fig. 3.** Decrease in ER mRNA and Protein by CDNB

MCF-7 cells were exposed to DMSO, 1 nM $E_2$, 10 $\mu$M CDNB, or CDNB with $E_2$, or 4-OHT with $E_2$. ER mRNA (A) and ER protein (B) were detected by Northern blotting after 24 hr exposure and by Western blotting after 12 hr exposure, respectively. G3PDH was shown as a control of constant protein loading.

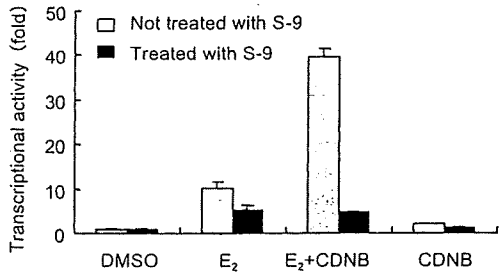


**Fig. 4.** Effect of CDNB Metabolite(s) on Transcriptional Activity

The transcriptional activity was determined by the reporter gene assay as described in MATERIALS AND METHODS. As CDNB metabolite(s), 5 mM CDNB treated with an S-9 mixture at 37°C for 1 hr was used, and MCF-7 cells were exposed to DMSO, 1 nM $E_2$M CDNB, 5 $\mu$M CDNB metabolite(s), CDNB with $E_2$, and CDNB metabolite(s) with $E_2$. Values represent the mean ± S.D. ($n$ = 3).

genic activity, the same as CDNB. This result suggested that the enhancive effect of CDNB was not affected by the CDNB metabolite(s).

### No Effect of CDNB on AhR

AhR was reported to cross-talk with ER,[10] so the yeast AhR reporter assay was used to determine if the effect of CDNB was through AhR or not. However, CDNB did not have agonistic activity for AhR in a range from $10^{-9}$ to $10^{-6}$ M, while CDNB did not affect the activity of 10 $\mu$M $\beta$-napthoflavone (NF), a known agonist for AhR (Fig. 5).

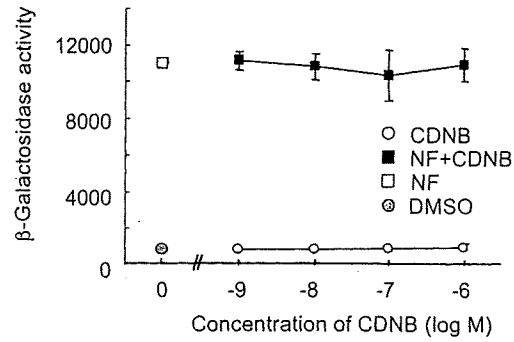


**Fig. 5.** Effect of CDNB on AhR Activity

$\beta$-Galactosidase activity was determined by the yeast assay as described in MATERIALS AND METHODS. Yeasts were exposed to DMSO, the given concentrations of CDNB, 100 nM NF, and concentrations of CDNB with 100 nM NF. Values represent the mean ± S.D. ($n$ = 3).

### Induction of Phosphorylation of ERK by CDNB

Activated MAPK induces the phosphorylation of ER, enhances the transcriptional activity of $E_2$ and decreases the ER protein level.[11] It was investigated whether CDNB activated MAP kinase or not by detecting the phosphorylated ERK using Western blotting analysis. CDNB and CDNB with $E_2$, or $E_2$ alone induced the phosphorylation of ERK, whereas DMSO did not (Fig. 6A). The phosphorylation of ERK in cells treated with $E_2$ alone was weakly detected at 2 hr. In contrast, CDNB retained the ability to phosphorylate ERK from 1 hr to 8 hr. Furthermore, CDNB with $E_2$ strongly phosphorylated ERK from 1 hr and showed a peak at 2 hr. On the other hand, CDNB, CDNB with $E_2$, $E_2$ alone or DMSO did not change the expression of ERK (Fig. 6B).

These results suggested that CDNB activated MAPK to enhance $E_2$-induced transcriptional activity and to decrease the ER protein level.

## DISCUSSION

Estrogen produces physiological actions within a variety of target sites by activating ER.[12] For example, the growth of breast cancer cells is dependent on estrogen through ER.[13] In this study, ER$\alpha$-positive MCF-7 breast cancer cells were used to investigate estrogen-dependent responses and the cross-talk between ER and AhR, and the stimulative effect was found to be due to CDNB. In the

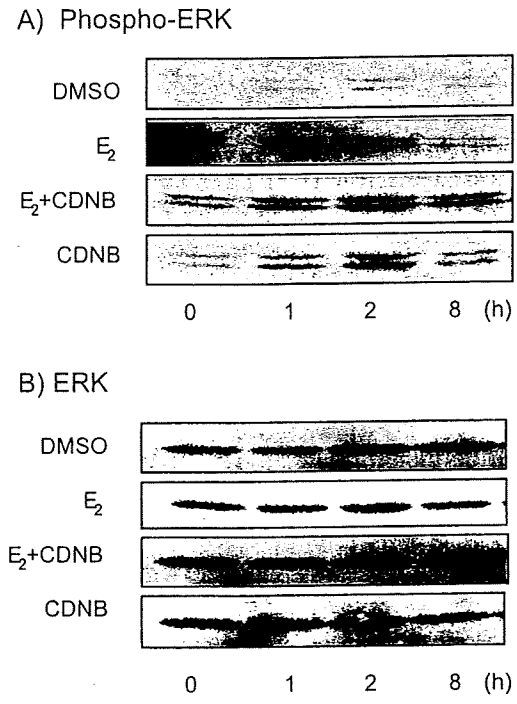


**Fig. 6.** Kinetics of ERK Phosphorylation

MCF-7 cells, cultivated for 4 days, were exposed for various periods of time to DMSO, 1 nM $E_2$, 10 $\mu$M CDNB, and $E_2$ with CDNB. Phospho-ERK (A) and ERK (B) were detected by Western blotting.

previous paper, we did screening using a yeast two-hybrid assay and found inhibitory compounds, but no enhancing compounds.[7] Here, we found why this occurred, that is, the yeast two-hybrid assay is based on the ligand-dependent interaction between ER and a coactivator.[14]

Certain chemicals existing in the environment have a harmful effect on the hormonal system of the human body. Many investigators have reported the risk analysis associated with these chemicals. However, the effect of the chemicals actually appears as mixture with estrogen in the body. In spite of this fact, the combined action has not been well studied *in vitro*. In this study, we showed the effect of CDNB on estrogenic activity with coexistence of $E_2$.

CDNB was evaluated as the stimulator of $E_2$ activity, although CDNB alone did not have any agonistic activity for ER in the MCF-7 cell reporter gene assay (Fig. 1). The enhancing effect of CDNB on $E_2$ activity was not induced through the binding of ER$\alpha$ (Fig. 2). However, Fig. 3 demonstrates that CDNB decreased the ER level as like $E_2$.[15]

To explain the mechanism for this action, we first investigated the metabolite of CDNB. Xenobiotics were metabolized in the body, and then several of their metabolites showed estrogenic action. For example, *trans*-stilbene and methoxychlor showed agonistic ER activity through metabolism.[16–20] However, the results showed that the metabolite of CDNB did not affect the estrogenic action (Fig. 4). Furthermore, 2,4-dinitrophenol, a possible CDNB metabolite, did not have the estrogenic activity in the reporter gene assay (data not shown). These results strongly suggested that the CDNB mechanism of action did not depend on ER.

ER-independent activation can also occurr in breast cancer cells. One example is the cross-talk between ER and AhR as reported recently.[21] Dioxins mimicked the effect of estrogen through a mechanism that involved the activation of ER by the transcriptionally active AhR-ARNT complex.[22] Also, a 90-kDa heat shock protein (HSP90) mediated cross-talk between the ER and AhR signal transduction pathway,[23] and the cross-talk between intracellular signaling pathways influenced ER transcriptional activity in a tissue and cell-specific manner.[24] Agonists for AhR inhibited ER signaling and the expression of target genes.[25,26] Furthermore, resveratrol, known as an agonist of ER,[27] inhibited the AhR action.[28–30] If CDNB is an antagonist for AhR, it would stimulate estrogenic action. However, CDNB did not show any agonistic or antagonistic activity for AhR (Fig. 5). The results suggested that the stimulation of $E_2$ activity by CDNB did not operate *via* AhR.

Another mechanism for activation of ER is *via* ER phosphorylation. It has been reported that MAPK phosphorylates the N-terminal domain of ER,[31] to enhance estrogen-induced transcriptional activity of ER[32] by affecting the recruitment of coactivator.[33] In addition, ER phosphorylation by MAPK is required for full activity of ER activation function 1 and enhanced transcriptional activity.[32] The major MAPK cascades involve ERK including Raf-1, c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), or p38 MAPK. Because the ERK cascade was relevant to breast cancer,[34] we focused on the action of CDNB on ERK. The result showed the activation of ERK by CDNB (Fig. 6). Our findings clarified that CDNB stimulated estrogenic action *via* MAPK. The activation of ERK, one of the MAPK pathways, by CDNB enhanced ER-dependent transcription (Fig. 1). Furthermore, the decrease in ER protein level by CDNB (Fig. 3B) supported the report that activation of MAPK reduced the ER expression.[35] Recently, it was reported that CDNB induced the activation of dendritic cells through MAPK.[36]

It was revealed from our results that CDNB pos-

sessed estrogen-like activity in the transcription and regulation of ERα in a different manner from $E_2$, *via* MAPK. This implies that various mechanisms should be taken into consideration, particularly when risks of chemicals with hormonal effects are evaluated by *in vitro* assays.

According to the chemical hazard data book,[37] CDNB is produced near 200 t in Japan as materials for dyes, pigments, UV absorbents, and others. There has no data of detection from water and sediment samples although it is hard-of-biodegradation and low-of-bioaccumulation. It shows high acute ecotoxcity, whereas comparatively weak acute toxicity to human. But it shows some effects on the motility of sperm and the development of embryo in animals. Although we cannot say about relationship of *in vitro* effect of CDNB with *in vivo* toxicity, it is concluded that *in vitro* studies provides important information for risk assessment of chemicals.

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# Endocrine disruptors induce cytochrome P450 by affecting transcriptional regulation via pregnane X receptor

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**Abstract**

Pregnane X receptor (PXR) is a nuclear receptor that regulates the expression of genes for cytochrome P450 3A (CYP3A), multidrug resistance 1 (MDR1), and organic anion-transporting peptide 2 (OATP2). These genes control the metabolism (CYP3A subfamily) and aspects of the pharmacokinetics (MDR1 and OATP2) of both endogenous and xenobiotic compounds. Since PXR is important in understanding the actions of endocrine disruptors (EDs), we determined the ability of suspected EDs to interact with PXR. In our study, 7 of 54 xenobiotics compounds interacted with PXR, including methoxychlor and benzophenone. All of the chemicals activated PXR in vitro and induced CYP3A mRNA in the male rat liver. In addition, CYP2C11 was also induced by some PXR agonists and converted methoxychlor into xenoestrogen. These findings suggest that some EDs affect sex hormone receptor indirectly by induction of metabolic enzyme via PXR, to produce rapidly higher concentrations of effective metabolites, leading to disturbance of the endocrine system.


*Keywords:* Pregnane X receptor; Endocrine disruptors; CYP2C11; Estrogenic metabolite; Methoxychlor

## Introduction

The concept of endocrine disruption was introduced by Colborn and Clement at the Work Session on "Chemically Induced Alterations in Sexual Development: The Wildlife/Human Connection" in 1991. A variety of environmental chemicals can disrupt the normal endocrine functions. They include phytochemicals, pesticides, pollutants, and industrial chemicals that mimic the action of hormones and disrupt the endocrine system. Studies in natural and laboratory settings show that endocrine disruptors (EDs) affect the hormonal systems in many species and produce adverse effect on reproduction, growth, and the immune system. While EDs act through a variety of pathways, their mechanism of action is not completely understood (Colborn et al., 1996; Gaido et al., 2000; Laws et al., 2000; Gray et al., 2001).

The importance of nuclear hormone receptors in endocrine function is well established from many studies. These receptors (homodimer or heterodimer) bind to specific DNA elements (hormone response elements) in the promoters of target genes and regulate the transcription of genes (Evans, 1988; O'Malley et al., 1991; Mangelsdorf and Evans, 1995; Blumberg and Evans, 1998; McKenna et al., 1999). Some chemicals or their metabolites bind to sex steroid hormone receptors (androgen receptor (AR) and estrogen receptor (ER)) directly and influence the expression of target genes (Nishihara et al., 2000; Blair et al., 2000; Yoshihara et al., 2001; Sultan et al., 2001). In this study, we focused on the pregnane X receptor (PXR) and explored how suspected EDs affect PXR.

PXR is activated by pregnenolone 16α-carbonitrile (PCN), which is a cytochrome P450 (CYP) 3A gene inducer in rodents (Kliewer et al., 1998). Activated PXR binds to the response element as a heterodimer with retinoid X receptor and induces the expression of target genes, such as CYP3A,

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multiple drug resistance 1 (MDR1), and organic anion-transporting polypeptide 2 (OATP2) (Guo et al., 2002a, 2002b; Kauffmann et al., 2002). The CYP3A subfamily are metabolic enzymes of many endogenous steroid hormones (such as pregnenolone and progesterone) and xenobiotic compounds (Lee et al., 2001; Yamada et al., 2001). Additionally, both MDR1 and OATP2 control the distribution and elimination of a wide range of chemicals. During the last few years, it has been reported that PXR also plays a key role in lipid homeostasis together with liver X receptor and farnesoid X receptor (Kliewer and Willson, 2002). Since PXR is concerned with biosynthesis, distribution, metabolism, and elimination of endogenous and xenobiotic compounds, it must play an important role in endocrine disruption. Recently, it was demonstrated that nonylphenol and phthalic acid, suspected xenoestrogens, activate PXR to induce CYP3A in rat (Masuyama et al., 2000). However, there are few reports about a relationship between PXR and EDs.

In the current study, we investigated the ability of EDs to activate PXR using in vitro assay systems in comparison with AR and ERα and assessed the effect of these EDs on the expression of target gene of PXR in the rat liver.

## Methods

*Materials.* Alachlor (>98%), benzophenone (>99%), benzene hexachloride (γ-BHC) (>99%), 17β-estradiol (>97%), PCN (>99%), testosterone (>97%), and vinclozolin (>99%) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 5α-dihydrotestosterone (>99%) (Sigma Chemical Co., St. Louis, MO), methoxychlor (99%) (Dr. Ehrenstorfer), and trifluralin (>99%) (Kanto Chemical, Japan) were used. These chemicals were of the highest purity available. Molecular biological reagents, unless otherwise cited, were from Toyobo (Osaka, Japan).

*Yeast two-hybrid assay.* The MATHMACHER two-hybrid system (Clontech, Palo Alto, CA) was used to determine whether the ligand-binding domain (LBD) of nuclear receptor interacts with steroid receptor coactivator 1 (SRC-1) in the presence of test compounds. The two different expression vectors, pGBT9 (Clontech) and pACT (Clontech), were used to generate hybrid protein in yeast. The LBD of rPXR (from 108 to stop codon) was amplified by RT–PCR using total RNA from rat liver as a template. The amplified fragments were subcloned in-frame fusion to the GAL4 DNA-minding domain in the yeast expression vector pGBT9 with *SmaI–SalI* sites. The other yeast expression plasmids were previously described (Nishikawa et al., 1998; Onate et al., 1995). Each construct of pGBT9-AR, -ERα, or -PXR was cotransformed with pACT–SRC1 into the yeast strain Y190 (Clontech), which was made competent by lithium acetate method. Yeast transformants were selected by growth on Sabourand Dextrose (SD) medium lacking leucine and tryptophan. Triplicates of independent colonies were grown and used for the assay according to the method described in our previous paper (Nishikawa et al., 1998). In this study, we represented the 10% relative effective concentrations (REC10) against to the maximum activity by known ligand in each receptor (AR, 1 μM DHT; ER, 1 μM 17β-estradiol; PXR, 1 μM PCN).

*Animal experiment.* Male Sprague–Dawley rats (Crj:CD) were purchased at 3–4 weeks of age from Charles River Laboratories Japan, Inc. (Shiga, Japan) and used after acclimation for a week. They were maintained in a room with controlled temperature (24 ± 2°C), relative humidity (55 ± 10%), and a 12-h light/dark cycle and given free access to food and water. Rats were randomly assigned to groups based on body weight and treated with benzophenone (50, 100, or 250 mg/kg/day ip) and methoxychlor (50, 100, or 250 mg/kg/day po) in corn oil or vehicle alone for 3 days. In the second study, rats were divided into groups and given a single intraperitoneal injection of alachlor (100 mg/kg/day), γ-BHC (20 mg/kg/day), PCN (20 mg/kg/day), trifluralin (100 mg/kg/day), vinclozolin (250 mg/kg/day), or corn oil vehicle. The dosages of chemicals were based on previous studies (Wilson et al., 1996; Laws et al., 1994; Linder et al., 1992; Morrell et al., 2000; Byrd et al., 1995).

*Isolation of RNA and RT–PCR analysis.* Rats were killed 24 h after treatment by decapitation and their livers were removed, examined, and weighed. A piece of liver was immediately frozen in liquid nitrogen and stored at −80°C until use. Approximately 0.1 g frozen liver tissue was homogenated and RNA was isolated using the guanidine idiothiocyanate method (Trizol; GibcoBRL). For the quantitative RT–PCR assay, we performed competitive RT–PCR using TAKARA RNA LA PCR (AMV) Kit Version 1.1 and Rat Cytochrome P450 Competitive RT–PCR Set (TaKaRa) according to the manufacturer's instructions. The RT reactions were carried out using 500 ng RNA under coexistence with four stepwise-diluted concentrations of RNA competitor per one sample. CYP2B1/2, 2C11, 3A1, and cyclophilin then were amplified. The aliquots of PCR products were electrophoresed in 3% Nu-Sieve agarose gel and the amount of each separated band was quantified densitometrically using FAS-II (TOYOBO) and the public domain NIH Image program version 1.60.

*In vitro microsomal metabolism.* Incubation mixtures contained 10 pmol of each recombinant CYP protein (GENETEST Co., Woburn, MA), 100 μM substrate (methoxychlor or benzophenone), 1.3 mM NADP+, 3.3 mM glucose 6-phosphate, and 3.3 mM $MgCl_2$ in 100 mM potassium phosphate buffer (pH 7.4) to give a final volume of 0.5 ml. Following preincubation at 37°C for 5 min in a water bath, the reaction was initiated by the addition of 0.4 U/ml glucose 6-phosphate dehydrogenase. After the 0-, 30-, or 60-min incubation period, the reaction was terminated by