Several Environmental Pollutants Have Binding Affinities for Both Androgen Receptor and Estrogen Receptor α

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(Received May 8, 2001; Accepted July 4, 2001)

We determined the binding affinities of some chemicals suspected of having endocrine-disrupting effects for androgen and/or estrogen receptors (ADR and ERα) by a non-radioisotope (RI) receptor binding assay. Tributyltin had the highest binding affinity for ADR with an IC_{50} of 7.6×10^{-6} M, but no affinity for ERα. Bisphenol A and 4-nonylphenol strongly bound to both ADR (IC_{50} values of 7.9×10^{-6} and 1.3×10^{-5} M, respectively) and ERα (IC_{50} values of 7.8×10^{-6} and 7.2×10^{-7} M, respectively). Octachlorostyrene had affinity for both receptors (IC_{50} for ADR, 2.7×10^{-5} M; and for ERα, 7.0×10^{-5} M). Although 4-octylphenol had a low affinity for ADR, it had a high affinity for ERα (IC_{50} of 9.8×10^{-6} M). Di-*n*-butyl phthalate, dicyclohexyl phthalate, and di(2-ethylhexyl) phthalate had low affinities for both ADR and ERα. The affinity of benzophenone was low for both receptors and *n*-butylbenzene had no affinity for either. Styrene trimers such as 1a-phenyl-4a-(1′-phenylethyl)tetralin (ST-2), 1a-phenyl-4e-(1′-phenylethyl)tetralin (ST-3), 1e-phenyl-4a-(1′-phenylethyl)tetralin (ST-4), and 1e-phenyl-4e-(1′-phenylethyl)tetralin (ST-5) had relatively high affinities, with IC_{50} values of $1.2–3.1 \times 10^{-5}$ M. Styrene dimers showed lower affinities for ADR than the trimers. Some styrene oligomers have been previously reported to have binding affinities for ERα. These findings suggest that some chemicals possess binding affinities for ADR and ERα. It is necessary to examine the effects of substances on various hormone receptors to elucidate their endocrine-disrupting activities.

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Key words —— endocrine disrupter, androgen receptor, estrogen receptor, tributyltin, styrene oligomer

INTRODUCTION

A variety of synthetic chemicals has been released into the environment, some in large quantities, during the past several decades. There is increasing scientific evidence that many substances with different chemical structures can interfere with the normal hormonally regulated biological processes to adversely affect development and/or reproductive function in wildlife, experimental animals, and humans.[1–3] These environmental contaminants are able to alter the normal functioning of the endocrine and reproductive systems by mimicking or inhibiting endogenous hormone actions, or modulating the synthesis of hormones.[4] These types of chemicals have been given the term "environmental endocrine disrupters" or "hormonally active agents." Endocrine disrupters are composed of numerous types of chemicals used in a wide variety of herbicides, fungicides, insecticides, detergents, materials of plastics, and many others. To clarify the endocrine-disrupting effect in chemical substances, versatile international collaborations have been performed. The Ministry of the Environment, Government of Japan, released a document titled "Strategic Programs on Environmental Endocrine Disrupters SPEED '98" in 1998 and a second edition in 2000, and 67 substances were listed as chemicals suspected of causing endocrine disruption in the document. Furthermore, tributyltin, 4-octylphenol, nonylphenol, di-*n*-butyl phthalate, dicyclohexyl phthalate, di(2-etylhexyl) phthalate, octachlorostyrene, benzophenone, and *n*-butylbenzene have been selected as substances on which priority risk assessments will be conducted as part of a three year program starting in 2000 under the government's Millennium Project.

A series of *in vitro* assays have been developed for the detection of disruptive activity as a first screening. One is an estrogen receptor (ER) competitive binding assay that measures the binding affinity of chemicals for receptors. Recently, it has become possible to perform the assays without using radioisotope (RI)-labeled compounds. We have applied this assay to determine whether the chemicals had estrogenic activity, and confirmed the binding affinities of some parabens for human ERs α and

β (ERα and ERβ).[5] Furthermore, a non-RI competitive binding assay for androgen receptor (ADR) has been established as a screening for hormonally active chemicals. We evaluated the binding affinities for both ADR and ER to determine whether the nine substances listed above have androgenic and estrogenic effects. It was determined that the styrene oligomers (1,3-diphenyl propane, SD-1; 2,4-diphenyl-1-butene, SD-2; *cis*-1,2-diphenylcyclobutane, SD-3; *trans*-1,2-diphenylcyclobutane, SD-4; 2,4,6-triphenyl-1-hexene, ST-1; 1a-phenyl-4a-(1′-phenylethyl)tetralin, ST-2; 1a-phenyl-4e-(1′-phenylethyl)tetralin, ST-3; 1e-phenyl-4a-(1′-phenylethyl)tetralin, ST-4; 1e-phenyl-4e-(1′-phenylethyl)tetralin, ST-5; and 1,3,5-triphenylcyclohexane (ST-6) had estrogenic effects.[6] In this study, the androgenic binding affinities of the styrene oligomers were examined.

MATERIALS AND METHODS

Reagent —— Chemicals were of the highest grade commercially available or for the environmental analysis. The styrene dimers SD-1 (> 99.5% pure), SD-2 (> 99.0% pure), SD-3 (100% pure), and SD-4 (> 98.5% pure), the styrene trimers ST-1 (> 97.8% pure), ST-2 (> 99.7% pure), ST-3 (> 99.0% pure), ST-4 (> 99.6% pure), ST-5 (> 99.8% pure), and ST-6 (> 99.9% pure), tri-*n*-butyltin (IV) acetate (tri-*n*-butyltin, > 95% pure), 4-nonylphenol (> 98% pure), octachlorostyrene (> 99% pure), *n*-butylbenzene (> 99% pure), and benzophenone (> 99% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4-Octylphenol (> 98% pure), di-*n*-butyl phthalate (> 98% pure), dicyclohexyl phthalate (> 99.0% pure), di(2-ethylhexyl) phthalate (> 98.0% pure), bisphenol A (> 99.0% pure), and diethylstilbestrol (DES, > 98.0% pure) were purchased from Tokyo Chemical Industries Co., Ltd. (Tokyo, Japan). These chemicals were prepared as solutions in dimethyl sulfoxide.

ADR and ER Competitive Binding Assay —— The ADR competitive binding assay was determined using a Ligand Screening System-Androgen Receptor kit (Toyobo Co., Ltd, Osaka, Japan). Solutions of human ADR, unlabeled testosterone, and chemicals were reacted at 4°C for 1 hr. The liberated testosterone was allowed to compete with anti-testosterone antibody and peroxidase-labeled testosterone at 4°C for 1 hr. Plates were washed using a wash solution, and then the substrate solution was added. The developed color was read at 450 nm on a microplate-spectrophotometer.

The ERα competitive binding assay was determined using an Estrogen-R (α) Competitor Screening kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), essentially according to the method reported previously.[5] Solutions of fluorescence-labeled 17β-estradiol (E_2) and chemicals were added to the wells of a plate coated with human ERα, and they were allowed to compete at room temperature for 2 hr. The plates were washed using the wash solution, and then the assay solution was added to each well. The fluorescence intensity was detected with a microplate-fluoresencephotometer (excitation at 485 nm, emission at 535 nm).

RESULTS

Binding of Chemicals to ADR

Various concentrations of each chemical were reacted with human ADR with testosterone, and the competition curves are shown in Fig. 1a–1d. The IC_{50} values (concentration of chemical required to reduce the specific testosterone binding by 50%) were obtained from the curves, and the relative binding affinities for ADR (RBA-A) were indicated as the ratio of IC_{50} of mibolerone, a synthetic anobolic testosterone, to that of each chemical (Tables 1 and 2)

Tributyltin: Tri-*n*-butyltin bound the most tightly to ADR among tested chemicals. A slight inhibition of the specific binding appeared at 1.9×10^{-6} M. The inhibition was concentration dependent, and complete inhibition appeared at 1.9×10^{-5} M. The IC_{50} and RBA-A for ADR were 7.6×10^{-6} M, and 0.224, respectively.

Phenolic compounds: Bisphenol A and 4-nonylphenol had high binding affinity for ADR. The IC_{50} of the former was 7.9×10^{-6} M and that of the latter was 1.3×10^{-5} M. The RBA-As were 0.215 and 0.131, respectively. 4-Octylphenol did not completely inhibit the specific binding, and only 34% inhibition was obtained at 1.9×10^{-4} M.

Phthalates: Di-*n*-butyl phthalate, dicyclohexyl phthalate or di(2-ethylhexyl) phthalate had the partial inhibitory effects on specific binding to ADR. About 45% inhibition of specific binding occurred with each chemical at 1.9×10^{-4} M.

Styrene oligomers: Among the styrene dimers, SD-2 completely inhibited specific binding to ADR. The IC_{50} and RBA-A were 4.3×10^{-5} M and 0.040, respectively. SD-3 and SD-4 did not completely in-

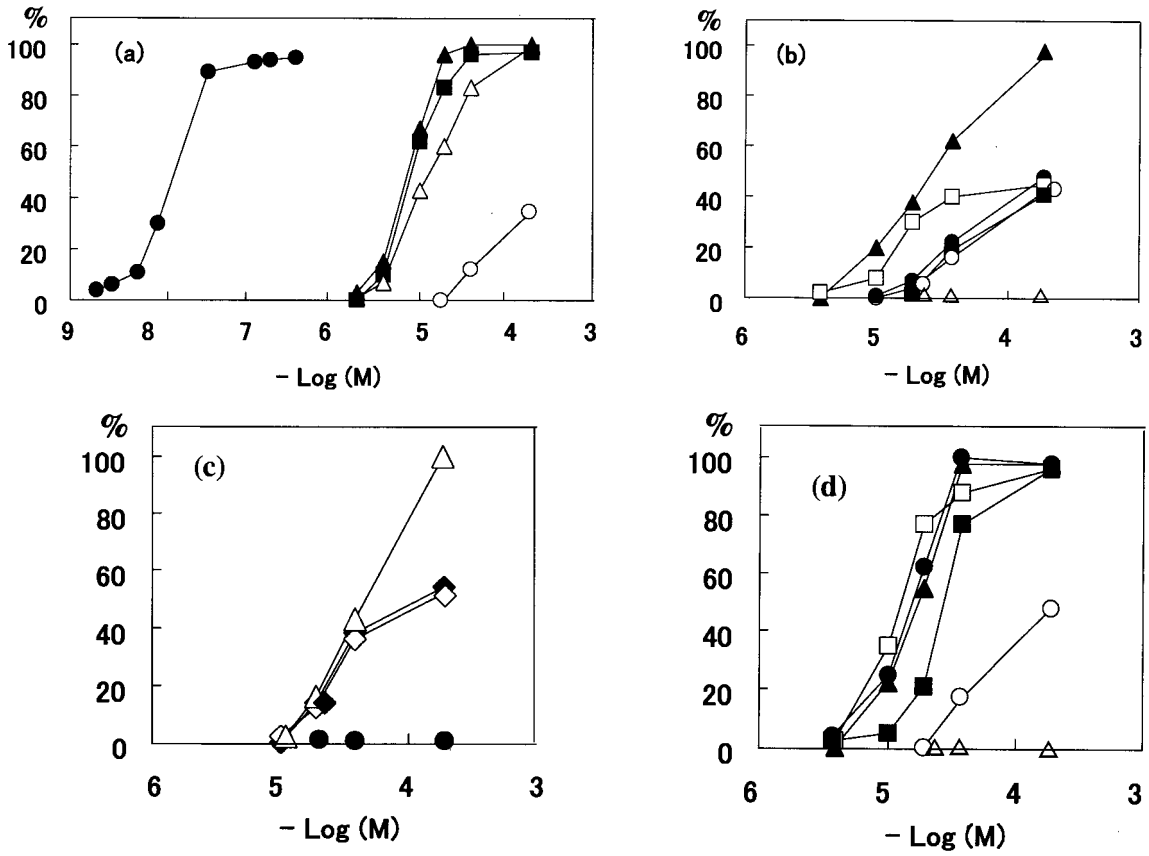

Fig. 1. Competition by Chemicals for Testosterone Binding to Androgen Receptor

Ligand binding experiments were carried out using a Ligand Screening System-Androgen Receptor kit (Toyobo, Japan). The SD was less than 2.6% ($n = 5$). Ordinates: % of inhibition = A – B/A (A and B were the fluorescence intensities in the absence and presence of competitor, respectively. (a) ●, mibolerone; ■, bisphenol A; △, 4-nonylphenol; ○, 4-octylphenol; ▲, tri-*n*-butyltin(IV) acetate. (b) ○, benzophenone; ▲, octachlorostyrene; ●, di-*n*-butyl phthalate; □, dicyclohexyl phthalate; ■, di(2-ethylhexyl) phthalate; △, *n*-butylbenzene. (c) ●, SD-1; △, SD-2; ◆, SD-3; ◇, SD-4. (d) ○, ST-1; □, ST-2; ●, ST-3; ■, ST-4; ▲, ST-5; △, ST-6.

Table 1. Binding Affinities of Chemicals for ADR and ERα

	ADR		ERα	
Compound	IC_{50} (M)	RBA-A[a)]	IC_{50} (M)	RBA-E[b)]
Mibolerone	1.7×10^{-8}	100	—	—
DES	—	—	1.6×10^{-8}	100
Tri-*n*-butyltin	7.6×10^{-6}	0.224	NE[c)]	—
4-Octylphenol	$> 1.9 \times 10^{-4}$	< 0.009	9.8×10^{-6}	0.163
4-Nonylphenol	1.3×10^{-5}	0.131	7.2×10^{-7}	2.222
Bisphenol A	7.9×10^{-6}	0.215	7.8×10^{-6}	0.205
Di-*n*-butyl phthalate	$> 1.9 \times 10^{-4}$	< 0.009	1.9×10^{-4}	0.008
Dicyclohexyl phthalate	$> 1.9 \times 10^{-4}$	< 0.009	5.8×10^{-5}	0.028
Di(2-ethylhexyl) phthalate	$> 1.9 \times 10^{-4}$	< 0.009	1.5×10^{-4}	0.011
Octachlorostyrene	2.7×10^{-5}	0.063	7.0×10^{-5}	0.023
Benzophenone	$> 1.9 \times 10^{-4}$	< 0.009	$> 1.9 \times 10^{-4}$	< 0.008
n-Butylbenzene	NE[c)]	—	NE[c)]	—

a) RBA-A and *b*) RBA-E were calculated as a ratio of the IC_{50} value of mibolerone (synthesis of anabolic testosterone) to that of the chemical, and a ratio of the IC_{50} value of diethylstilbestrol (DES) to that of the chemical, respectively. *c*) NE; no effect at 1.9×10^{-4} M.

Table 2. Binding Affinities of Styrene Oligomers for ADR

Compound	IC_{50} (M)	RBA-A[a)]
Mibolerone	1.7×10^{-8}	100
1,3-diphenyl propane (SD-1)	NE[b)]	—
2,4-diphenyl-1-betene (SD-2)	4.3×10^{-5}	0.040
cis-1,2-diphenylcyclobutane (SD-3)	1.4×10^{-4}	0.012
trans-1,2-diphenylcyclobutane (SD-4)	1.5×10^{-4}	0.013
2,4,6-triphenyl-1-hexene (ST-1)	$> 1.9 \times 10^{-4}$	< 0.009
1a-phenyl-4a-(1′-phenylethyl)tetralin (ST-2)	1.2×10^{-5}	0.142
1a-phenyl-4e-(1′-phenylethyl)tetralin (ST-3)	1.6×10^{-5}	0.106
1e-phenyl-4a-(1′-phenylethyl)tetralin (ST-4)	3.1×10^{-5}	0.055
1e-phenyl-4e-(1′-phenylethyl)tetralin (ST-5)	1.8×10^{-5}	0.094
1,3,5-triphenylcyclohexane (ST-6)	NE[b)]	—

a) RBA-A was calculated as a ratio of the IC_{50} value of mibolerone to that of the styrene oligomer. *b*) NE; no effect at 1.9×10^{-4} M.

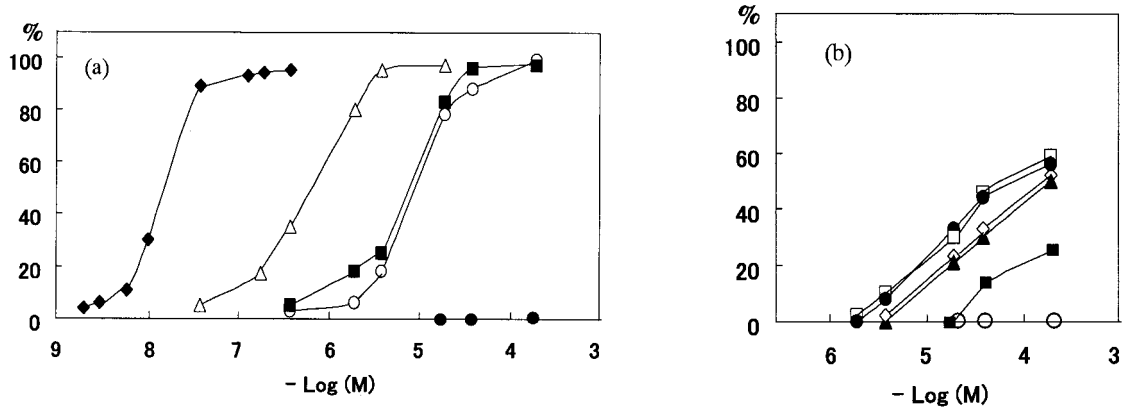

Fig. 2. Competition by Chemicals for Estradiol Binding to Estrogen Receptor α

Ligand binding experiments were carried out using an Estrogen-R (α) Competitor Screening kit (Wako, Japan). The SD was less than 2.5% ($n = 5$). Ordinates: % of inhibition = A – B/A (A and B were optical density in the absence and presence of competior, respectively). (a) ◆, diethylstilbestrol; △, 4-nonylphenol; ■, bisphenol A; ○, 4-octylphenol; ●, tri-*n*-butyltin (IV) acetate. (b) □, dicyclohexyl phthalate; ●, octachlorostyrene; ◇, di(2-ethylhexyl) phthalate; ▲, di-*n*-butyl phthalate; ■, benzophenone; ○, *n*-butylbenzene.

hibit binding, and about 53% inhibition was obtained with each chemical at 1.9×10^{-4} M. SD-1 did not bind to ADR.

The binding affinities of the styrene trimers for ADR were relatively higher than those of the dimers. The IC_{50} values of ST-2, ST-3, ST-4, and ST-5 were 1.2×10^{-5}, 1.6×10^{-5}, 3.1×10^{-5}, and 1.8×10^{-5} M, respectively. The RBA-As were 0.142, 0.106, 0.055, and 0.094, respectively. The binding affinity of ST-1 was lower than that of the other trimers, and 48% inhibition appeared at 1.9×10^{-4} M. ST-6 did not bind to ADR.

Other chemicals: The inhibition of specific binding to ADR by octachlorostyrene was concentration dependent. The IC_{50} and RBA-A were 2.7×10^{-5} M and 0.063, respectively. Benzophenone at 1.9×10^{-4} M inhibited the binding to ADR by 48%. *n*-Butylbenzene did not bind to ADR.

Binding of chemicals to ERα

Different concentrations of each chemical were reacted with human ERα with E_2, and the competition curves were obtained (Fig. 2a and 2b). The IC_{50} values were obtained from the curves, and the relative binding affinities for ERα (RBA-E) were indicated as the ratio of the DES IC_{50} to that of each chemical (Table 1).

Phenolic compounds: These chemicals had relatively high affinities for ERα. The affinity of 4-nonylphenol was the highest among the tested chemicals, and the IC_{50} value and RBA-E were 7.2×10^{-7} M and 2.222, respectively. The IC_{50} and

RBA-E of bisphenol A were 7.8×10^{-6} M and 0.205, respectively. The two values for 4-octylphenol were 9.8×10^{-6} M and 0.163, respectively.

Phthalates: The phthalates tested in this experiment did not completely inhibit specific binding to ERα. The IC_{50} value and RBA-E of di-*n*-butyl phthalate were 1.9×10^{-4} M and 0.008, respectively. The values of dicyclohexyl phthalate were 5.8×10^{-5} M and 0.028, respectively, and those of di(2-ethylhexyl) phthalate were 1.5×10^{-4} M and 0.011, respectively.

Other chemicals: Octachlorostyrene inhibited the specific binding to ERα in a concentration-dependent manner. The IC_{50} value and RBA-E were 7.0×10^{-5} M and 0.023, respectively. The inhibition by benzophenone at 1.9×10^{-4} M was only 25%. The binding of tri-*n*-butyltin and *n*-butylbenzene to ERα was not detected.

DISCUSSION

Organotin compounds had been used worldwide for antifouling paint of ship hulls and for fishing nets since 1969, but they have not been approved for use from about 12 years ago in Japan. However, these substances still remain in marine organisms and sediments on the seabed.[7] These organotins have been reported to cause imposex in marine female gastropods.[8,9] It has been suggested that tributyltin brought about imposex in female marine gastropods mediated by an increasing androgen level caused by the inhibition of aromatase activity by tributyltin.[10–12] On the other hand, Shiraishi *et al*.[13] suggested that organotin compounds were estrogen antagonists. In this study, tri-*n*-butyltin bound to ADR although it had no affinity for ERα. The binding of tributyltin to ADR is thought to be a possible mechanism for the occurrence of the imposex. Tributyltin may be considered to work as an androgen agonist based on our results.

4-Octylphenol, 4-nonylphenol, and bisphenol A are industrial raw materials used in large quantities in a wide variety of surfactants and plastics and have been responsible for contaminating food and the environment worldwide.[14,15] There is accumulating evidence that these compounds influcence the actions of estrogen.[1–3,16–19] Moreover, bisphenol A has been reported to have anti-androgenic activity, and 4-nonylphenol was a weak androgen agonist in *in vitro* yeast-based assays.[4] We found that bisphenol A and 4-nonylphenol had relatively high binding affinities for both ADR and ERα. These compounds have estrogenic activity, and furthermore they might block or mimic the action of androgen through binding to an ADR.

Phthalate esters are produced in extremely large volumes and used as plasticizers in polymeric materials such as polyvinylchloride.[20] These substances such as di(2-ethylhexyl) phthalate, di-*n*-butyl phthalate, and dicyclohexyl phthalate are released into the environment and food samples (cheese, cream, butters, *etc*.) via volatilization and leaching from plasticware and other sources.[21] There is some evidence of the estrogenic behavior of certain phthalates from *in vitro*[22–25] and *in vivo*[26] studies. Our experiment shows that the phthalates have binding affinities for both ADR and ERα.

Octachlorostyrene, a by-product of organic chlorine compounds, is an environmental contaminant in the Great Lake regions of North America and the Norwegian coast in Europe,[27,28] and was found to be concentrated in the human plasma of aluminum foundry workers.[29,30] *In vivo* experiments have recognized histological and functional changes in the thyroid.[28] We found that octachlorostyrene had binding affinities for both ADR and ERα. The results suggest that they have the mimicking or blocking effects on the actions of androgen and estrogen.

Benzophenone is widely used as a synthetic raw material for medical products, perfumes, cosmetics *etc*., and is a constituent of fruits such as the muscat grape and mango. Vaz *et al*. have indicated antagonist impediment by benzophenone to aromatase cytochrome P450 *in vitro*.[31] Benzophenone has binding affinities for both ADR and ERα. Furthermore, *p*-hydroxybenzophenone, a metabolite of benzophenone, had proliferative activity in MCF7 cells.[32] We consider it necessary to examine the binding affinity of the metabolites for hormone receptors.

n-Butylbenzene, a synthesis intermediate in the chemical reaction, induced cytochrome P450 isozymes in rat liver, and it inhibited testosterone hydroxylation activity.[33] As the substance did not bind to ADR and ERα at high concentration (1.9×10^{-4} M), it was considered to have no estrogenic or androgenic activities.

Styrene oligomers, especially styrene trimers, are contaminats in polystyrene ware used widely for food containers.[34–36] These styrene oligomers migrate from the polystyrene containers into the contents, such as instant food, when heated in a microwave oven or incubated at 20°C for 24 hr.[34,35,37] The styrene trimers (ST-1, ST-2, ST-3, ST-4, and ST-5)

were shown to be estrogenic according to the assay of MCF-7 proliferative activity and the competitive binding assay to ERα.[6] Presently, styrene trimers (ST-2, ST-3, ST-5) also have relatively high binding affinities for ADR. We must investigate further the hormone-like activities of the styrene trimers.

This study demonstrates that hormonally active agents in the environment have multifarious activities, which may make it difficult to interpret their mechanisms of action *in vivo*. The kits determining the binding assay for ADR and ERα are useful for the prescreening of many chemicals.

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