Evaluation of Ability of Chemicals to Bind Frog (*Xenopus laevis*) Estrogen Receptor by *in Vitro* Binding Assay

Eiko Suzuki, Masaaki Kunimoto, Makoto Nishizuka, and Masayoshi Imagawa*

Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3–1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467–8603, Japan

(Received July 27, 2004; Accepted August 5, 2004; Published online August 23, 2004)

The *in vitro* binding assay seems to be a useful first screening method for endocrine disrupting chemicals. Various methods have been developed and applied to the testing of chemicals. Since these assays should be applied to estrogen receptors (ER) of not only humans but also wildlife, we previously developed a standardized *in vitro* binding assay system for human, quail, Japanese medaka, and *Xenopus laevis* ERs using a competitive enzyme immunoassay. Since that was a first report on an *in vitro* binding assay system for *Xenopus* ERα, and the capacities of chemicals to bind *Xenopus* ERα were not tested yet, we here evaluated the ability of 20 test chemicals, which were selected by the Ministry of the Environment of Japan, to bind *Xenopus* ERα. Of these, 4-nonylphenol, *p*-octylphenol and bisphenol A had relatively high binding capacity, and these results are similar to those obtained previously in quail ERα.

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

*To whom correspondence should be addressed: Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3–1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467–8603, Japan. Tel. & Fax: +81-52-836-3455; E-mail: imagawa@phar.nagoya-cu.ac.jp

INTRODUCTION

Endocrine disrupting chemicals (EDCs) seem to be substances affecting reproductive functions through the estrogen-estrogen receptor (ER) signaling pathway.[1,2] Although the mechanisms of disruption are not fully understood, screening methods are definitely needed. Indeed, *in vivo* approaches including a one-generation study and uterotrophic assay, and *in vitro* techniques including a receptor binding assay and reporter gene assay 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. For initial screening *in vitro*, the receptor binding assay is often utilized. Using the fluorescence polalization method, the capacity of chemicals to bind human ERα and ERβ was evaluated. The Japanese medaka (*Oryzias latipes*) ERα and ERβ were tested using a radio-competitive assay (Ministry of Environment, Japan; http://www.env.go.jp/en/topic/edcs.html). We previously established a competitive enzyme immunoassay (EIA) for analysis of the capacity to bind quail ERα and ERβ, and then tested the chemicals selected by MOE.[5]

Although it has recently been proposed that the effects of EDCs are considered not only in humans but also in wildlife,[3,6,7] such as birds, frogs and fish, and indeed adverse effects on human and wildlife were reported (Ministry of Environment, Japan; http://www.env.go.jp/en/topic/edcs.html),[7] a standardized method is not yet established. Moreover, a method for use with amphibian ERs such as frog ER had not been developed. The Organization for Economic Cooperation and Development (OECD) and MOE have started the development of various test methods for humans, fish, reptiles, amphibians, and birds (Ministry of Environment, Japan; http://www.env.go.jp/en/topic/edcs.html, OECD; http://www.oecd.org/home/).[8] For this purpose, we developed a standardized assay system for ERs in various species.[9] This method is based on the competitive EIA. It needs no special equipment and no special technique, and is less expensive than other methods, such as a radio-competitive assay and a fluorescence polalization method described above.

Although we established a competitive EIA system for *Xenopus laevis* ERα, the binding ability of chemicals except 4-nonylphenol and *p*-octylphenol was not evaluated. As described above, MOE se-

lected 65 substances to be tested. Of these, 12 and 8 chemicals were selected in 2000 and 2001, respectively, as higher-priority chemicals. Therefore, we evaluated these 20 chemicals in this paper.

MATERIALS AND METHODS

Chemicals —— The test chemicals (12 and 8 chemicals selected by MOE in 2000 and 2001, respectively) (Ministry of Environment, Japan; http://www.env.go.jp/en/topic/edcs.html) were supplied by Dr. Kawashima at JAPAN NUS Co., Ltd. (Tokyo, Japan), where large amounts of chemicals of reagent grade have been stocked for various tests at the request of MOE. All test chemicals were dissolved in dimethyl sulfoxide (DMSO). All other chemicals are of reagent grade.

Competitive Enzyme Immunoassay —— The principle of the *in vitro* binding assay for *Xenopus* ERα is based on the enzyme-linked competitive immunoassay, and was described previously.[9] This assay was performed using the kit, Ligand Screening System-ERα (TOYOBO Co., Ltd., Osaka, Japan), according to the manufacturer's instructions. The only change was the replacement of the human ERα in the kit with *Xenopus* glutathioneS-transferase-ERα-ligand binding domain (GST-ERα-LBD). The expression and purification of *Xenopus* GST-ERα-LBD were described previously.[9] 17β-Estradiol (E2) and diethylstilbestrol (DES) were diluted with DMSO at a concentration of $8/3 \times 10^{-4}$–$8/3 \times 10^{-8}$ M, and further diluted with the dilution buffer in the kit at a final concentration of $8/3 \times 10^{-6}$–$8/3 \times 10^{-10}$ M (final DMSO concentration, 1%). The test chemicals were diluted in the same way at a final concentration of $8/3 \times 10^{-4}$–$8/3 \times 10^{-8}$ M.

The assay was done according to the manufacturer's instructions, and the optimum conditions were determined in the previous paper.[9] The protocol consists of three steps as follows. Step 1: receptor-ligand binding; 20 μl (0.9 pmol) of *Xenopus* GST-ERα-LBD in B-1 buffer [20 mM Tris (pH 7.5), 10% glycerol, 1 M KCl, 5 mM $MgCl_2$, and 1 mM DTT] including 20 mM GSH, 30 μl of test chemical or standard DES in the dilution buffer in the kit, and 30 μl of E2 ($8/3 \times 4$ nM) in the dilution buffer in the kit were mixed and incubated for 1 hr on ice. Step 2:

Table 1. The Relative Binding Affinity of Test Chemicals for *Xenopus* ERα Evaluated by Competitive Enzyme Immunoassay

	Chemicals	IC_{50} (M)	REC20 (M)	RBA (%)
	DES	4.5×10^{-9}		100
1	Benzophenone		5.0×10^{-5}	
2	Octachlorostyrene		2.8×10^{-5}	
3	Diethyl phthalate		$> 10^{-4}$	
4	Benzyl-n-butyl phthalate	1.9×10^{-5}		0.024
5	Bis(2-ethylhexyl) adipate		$> 10^{-4}$	
6	Triphenyltin chloride	3.0×10^{-6}		0.150
7	Bis(2-ethylhexyl) phthalate		6.8×10^{-5}	
8	Dicyclohexyl phthalate	8.1×10^{-5}		0.006
9	Di-n-butyl phthalate		2.8×10^{-5}	
10	Tributyltin(IV) chloride	6.3×10^{-5}		0.007
11	4-Nonylphenol	2.5×10^{-7}		1.800
12	*p*-Octylphenol	7.2×10^{-7}		0.625
13	Bisphenol A	4.7×10^{-7}		0.957
14	2, 4-Dichlorophenol	5.0×10^{-5}		0.009
15	4-Nitrotoluene		$> 10^{-4}$	
16	Di-n-pentyl phthalate		2.4×10^{-5}	
17	Di-n-propyl phthalate		7.9×10^{-6}	
18	Pentachlorophenol	2.5×10^{-5}		0.018
19	Amitrol (3-Amino-1,2,4-triazole)		$> 10^{-4}$	
20	Di-n-hexyl phthalate		7.4×10^{-6}	

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 the IC_{50} of the test sample.

antigen-antibody reaction; after the incubation, 50 μl out of 80 μl of the mixture was transferred to an E2-coated plate, and 50 μl of E2-horse radish peroxidase (HRP) solution was added. The mixture was incubated for 1 hr on ice. Step 3: enzyme reaction; the plate was washed and the enzyme reaction was conducted at 37°C for 20 min. 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: $(A_{DMSO\ control}-A_{test\ sample})/(A_{DMSO\ control}-A_{DES\ at\ 112\ nM}) \times 100$. 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 the IC_{50} of the test sample. When percent inhibition was less than 50%, a 20% relative effective concentration (REC20) was also calculated.

RESULTS AND DISCUSSION

We evaluated the capacity of 20 chemicals to bind *Xenopus* ERα using the competitive EIA. The results for the 20 chemicals are summarized by showing the IC_{50} and RBA (%) or REC20 in Table 1. Among those chemicals tested, 4-nonylphenol, *p*-octylphenol and bisphenol A exhibited relatively strong binding to *Xenopus* ERα as observed for quail ERα,[5] although in each case the IC_{50} was 10^{-7} M and the RBA was less than 2%. Nine chemicals including 4-nonylphenol, *p*-octylphenol and bisphenol A gave an IC_{50}, and 7 chemicals showed very weak binding and gave a REC20.

Although both triphenyltin and tributyltin exhibited the weak binding activity, it was reported that both tin compounds denatured the GST-ER protein (Ministry of Environment, Japan; http://www.env.go.jp/en/topic/edcs.html). Therefore, this is not a real binding activity and this system does not work on tin chemicals.

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. We next determined the inhibitory effect of 16 chemicals, which revealed the ability to bind *Xenopus* ERα. No inhibitory effect was observed for 9 chemicals, while 7 chemicals showed 30% or more inhibition at 10^{-4} M (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. In consideration of the inhibitory effect on the immunoreaction, we concluded that 4-nonylphenol, *p*-octylphenol and bisphenol A have some activity

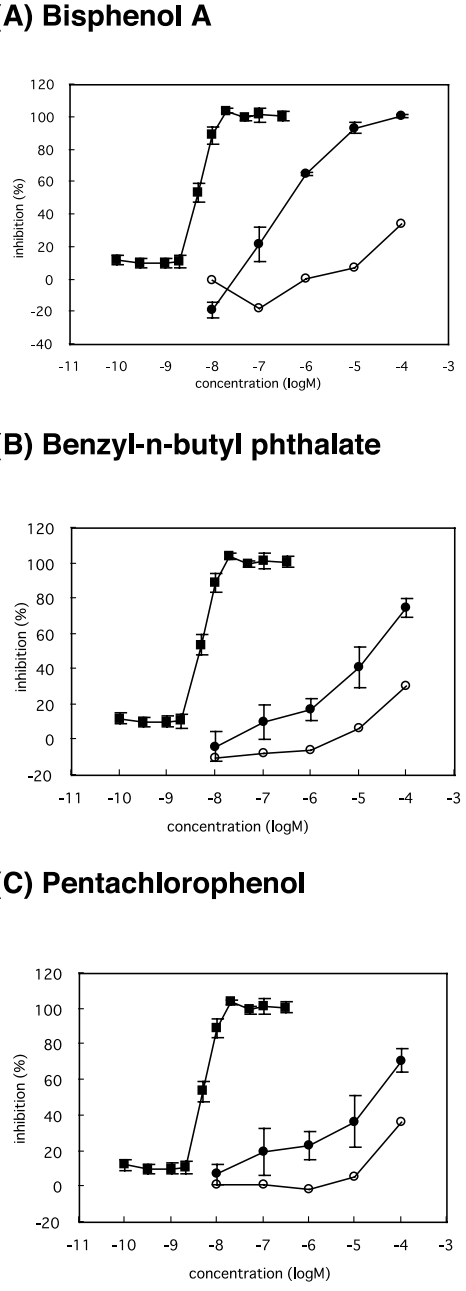

Fig. 1. Inhibition Curves of Bisphenol A, Benzyl-n-butyl Phthalate and Pentachlorophenol for *Xenopus* ERα Determined by Competitive Enzyme Immunoassay, and the Inhibitory Effect on the Antigen-Antibody Reaction

The squares show the standard curves of DES. The closed circles show the inhibition curves of bisphenol A (A), benzyl-n-butyl phthalate (B) and pentachlorophenol (C) for *Xenopus* ERα. The open circles show the inhibitory effect of each chemical on the antigen-antibody reaction. Values are the mean and standard deviation (n = 4).

to bind *Xenopus* ERα, and benzyl-n-butyl phthalate and pentachlorophenol might have very weak binding activity. Figure 1 shows the data for bisphenol A, benzyl-n-butyl phthalate and pentachlorophenol. The other chemicals with weak binding seem to have no capacity to bind *Xenopus* ERα at the concentration tested. The results obtained here are basically very similar to those for quail ERα.[5)]

Acknowledgements We thank Dr. Y. Kawashima (JAPAN NUS Co., Ltd.) for generously donating the test chemicals. This study was supported by the fund for endocrine disruptors from the Ministry of the Environment, Japan.

REFERENCES

1) Crews, D., Willingham, E. and Skipper, J. K. (2000) Endocrine disruptors: present issues, future directions. *Q. Rev. Biol.*, **75**, 243–260.
2) Hutchinson, T. H., Brown, R., Brugger, K. E., Campbell, P. M., Holt, M., Lange, R., McCahon, P., Tattersfield, L. J. and van Egmond, R. (2000) Ecological risk assessment of endocrine disruptors. *Environ. Health Perspect.*, **108**, 1007–1014.
3) Kavlock, R. J. (1999) Overview of endocrine disruptor research activity in the United States. *Chemosphere*, **39**, 1227–1236.
4) Iguchi, T., Sumi, M. and Tanabe, S. (2002) Endocrine disruptor issues in Japan. *Congenit. Anom.* (Kyoto), **42**, 106–119.
5) Maekawa, S., Nishizuka, M., Heitaku, S., Kunimoto, M., Nishikawa, J., Ichikawa, K., Shimada, K. and Imagawa, M. (2004) Development of a competitive enzyme immunoassay for detection of capacity of chemicals to bind quail estrogen receptor α and β. *J. Health Sci.*, **50**, 25–32.
6) Lathers, C. M. (2002) Endocrine disruptors: a new scientific role for clinical pharmacologists? Impact on human health, wildlife, and the environment. *J. Clin. Pharmacol.*, **42**, 7–23.
7) Iguchi, T., Sumi, M. and Tanabe, S. (2002) Endocrine disruptor issues in Japan. *Congenit. Anom.* (Kyoto), **42**, 106–119.
8) Baker, V. A. (2001) Endocrine disrupters-testing strategies to assess human hazard. *Toxicol. In Vitro*, **15**, 413–419.
9) Nishizuka, M., Heitaku, S., Maekawa, S., Nishikawa, J. and Imagawa, M. (2004) Development of standardized *in vitro* assay system for estrogen receptors and species specificity of binding ability of 4-nonylphenol and *p*-octylphenol. *J. Health Sci.*, **50**, 511–517.