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Application of a yeast estrogen screen in non-biomarker species *Varicorhinus barbatulus* fish with two estrogen receptor subtypes to assess xenoestrogens

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Abstract

Xenoestrogens can interfere with normal estrogen signaling by competitively binding to the estrogen receptor (ER) and activating transcription of target genes. In this study, we cloned the estrogen receptor alpha (*vb*ERα) and beta 2 (*vb*ERβ2) genes from liver of the indigenous Taiwanese cyprinid fish *Varicorhinus barbatulus* and tested the direct impact of several xenoestrogens on these ERs. Transcriptional activity of xenoestrogens was measured by the enzymatic activity of estrogen responsive element (ERE)-containing β-galactosidase in a yeast reporter system. The xenoestrogens tested were phenol derivatives, DDT-related substances, phthalic acid esters, and polychlorinated biphenyls, with 17β-estradiol (E2) as a subjective standard. The phenol derivatives [4-nonylphenol (4-NP), 4-*t*-octylphenol (4-*t*-OP) and bisphenol A (BPA)] exhibited significant dose-dependent responses in both ligand potency and ligand efficiency. Consistent with yeast assays using human or rainbow trout ERs, we observed a general subtype preference in that *vb*ERα displayed higher relative potencies and efficiencies than *vb*ERβ2, although our assays induced a stronger response for xenoestrogens than did human or trout ERs. Whereas 4-NP and 4-*t*-OP have similar EC50 values relative to E2 for both ER subtypes, the strong estrogenic response of BPA markedly differentiates *vb*ERα from *vb*ERβ2, suggesting possible species-specific BPA sensitivity. We report that the ameliorative yeast tool is readily applicable for indigenous wildlife studies of the bio-toxic influence of xenoestrogens with wildlife-specific estrogen receptors.

Keywords: Estrogen receptor; Ligand efficiency; Ligand potency; Transcriptional activation; Xenoestrogen

1. Introduction

Xenoestrogens, one type of endocrine disrupting chemical (EDC), can mimic the action of physiological estrogens through interaction with estrogen receptors, possibly resulting in health problems in humans and wildlife (Daston et al., 1997). Xenoestrogens thus have generated sufficient public concern to be included in the Food Quality Protection Act (Public Law 104–170) by the Environment Protection Agency. This trend has stimulated the development of various assays for the assessment of xenoestrogens, which are widely present in pesticides, plasticizers, synthetic hormones, and food supplements. Many assays have

Abbreviations: EDCs, endocrine disrupting chemicals; *vb*ERα, estrogen receptor alpha of *Varicorhinus barbatulus*; *vb*ERβ2, estrogen receptor beta 2 of *Varicorhinus barbatulus*; PCR, polymerase chain reaction; 4-NP, 4-nonylphenol; 4-*t*-OP, 4-*t*-octylphenol; BPA, bisphenol A; BBP, benzyl butyl phthalate; di-*n*-BP, di-*n*-butyl phthalate; DDT, 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene; PCBs, polychlorinated biphenyls; E1, estrone; E2, 17β-estradiol; ERE, estrogen response element; YES, yeast estrogen screen; SD, synthetic dropout; 5′-RACE, 5′-rapid amplification of cDNA ends; 3′-RACE, 3′-rapid amplification of cDNA ends; NCBI, the National Center for Biotechnology Information; DMSO, dimethyl sulfoxide; ONPG, *o*-nitrophenyl-D-galactopyranoside; OD, optical density; *vb*ERα-YES, *vb*ERα transformed yeast estrogen screen; *vb*ERβ2-YES, *vb*ERβ2 transformed yeast estrogen screen.

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been developed for a primary screening of EDCs. Significant progress in human estrogen receptor-mediated assays has allowed identification of the mechanism and genome-wide signatures of xenoestrogen-specific target genes (Terasaka et al., 2004; Moggs, 2005). Therefore, the desperate need to assess xenoestrogen impact on wildlife can be met by taking advantage of the well-established, nearly standardized human ER-mediated assays for a rapid, low-cost and wildlife-specific assessment strategy.

Estrogen exerts its function through interaction with membrane-associated receptors or intracellular ERs (Hall et al., 2001). A general mechanism of ER activation involves diffusion of estrogen into the cell nucleus, binding of estrogen to the ER, and dimerization of ERs to form a transcription factor activating transcription of estrogen response element (ERE)-containing target genes to control many cellular responses. Estrogen action is generally mediated by two ER subtypes, alpha (ERα) and beta (ERβ) (Dechering et al., 2000). Although ERα and ERβ share more than 60% sequence homology and cooperate at the molecular level in estrogen-responsive pathways, they differ in ligand affinity and transcriptional activity (Kuiper et al., 1997). For example, the physiological estrogen binds with higher affinity to murine ERα than to murine ERβ (Tremblay et al., 1997). Moreover, different functional effects are observed in rat uterine studies showing that ERβ can antagonize the proliferative functions of ERα (Weihua et al., 2000). Binding of E2 to the ERα receptor activates gene transcription, while binding of E2 to the ERβ receptor shows an inhibitory effect on transcription through the promoter regulatory AP1 site. Binding of the ER antagonist tamoxifen to ERβ at the AP1 site activates, while binding to ERα inhibits (Paech et al., 1997). In addition, the two subtypes differ in expression patterns and transcriptional actions on certain response elements, leading to species-, cell-, and promoter-specific actions of estrogens and antiestrogens (Kuiper et al., 1998). Significant differences in response occur through the two estrogen pathways depending on environmental chemicals. Two types of ERβ, namely β1 and β2, have been identified in teleost (Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2002), but the physiological roles of these ERβ subtypes are largely unknown. Therefore, the necessity of delineating the differential effects of xenoestrogens on different ER subtypes in wildlife, and the assessment of both subtypes as EDCs, could provide full-scope understanding of the potential risk.

Different in vitro assays, some even applied to samples from the fields, were developed to pre-screen for EDCs (Rehmann et al., 1999; Fang et al., 2000; Garcia-Reyero et al., 2001) based on the idea that the ER signaling pathway is evolutionarily conserved from yeast to human (Metzger et al., 1995). Recombinant yeast cells were designed to contain the exogenous ER receptor of interest as well as the responsive reporter stably integrated into the yeast genome. The measurement of reporter activity is a function of how xenoestrogens activate the ER, which in turn transcribes the ERE controlled reporter that brings about a color reaction (Balmelli-Gallacchi et al., 1999), bioluminescent activity (Leskinen et al., 2005) or green fluorescent protein activity (Bovee et al., 2004a). The human ER-mediated yeast estrogen screen (YES) is a reliable and robust assessment method and BLYES (bacterial luminescence yeast estrogen screen) is a modified method to shorten the reporter detection time (Sanseverino et al., 2005). Since first developed by Routledge and Sumpter (1996), the YES assay has been widely used for in vitro assays to detect xenoestrogenic compounds. This paper aims to modify this well-known tool by switching the central concerns from human to wildlife via the usage of fish ERs in this assay. Moreover, most of these methods were focused only on ERα, which may underestimate the ligand influence on other ER subtypes as was found for phytoestrogens (Harris et al., 2002).

2. Materials and methods

2.1. Fish treatment

The Taiwan cyprinid fishes, *Varicorhinus barbatulus (V. barbatulus)*, were purchased wild-caught from the fishery in Nanjuang (Miaoli, Taiwan). Adult female fishes (about 7–8 inches of body length) in a semi-recirculating tank at room temperature were fed a commercial fish diet and treated with 40 μg/l 17β-estradiol for two weeks before sacrifice to induce over-expression of ER genes. Fish livers were removed and were immediately frozen in liquid nitrogen, and total RNA was extracted using Trizol reagent (Gibco-BRL, Gaithersburg, MD) following the manufacturer's instructions.

2.2. Cloning the full length ER genes

To isolate the estrogen receptor cDNA of *V. barbatulus*, we utilized a strategy that was based upon the common identities of ER homologs from six different fish species including gilthead seabream (Accession No. AJ006039), medaka (Accession No. D28954), rainbow trout (Accession No. AJ242740), channel catfish (Accession No. AF061275), Atlantic croaker (Accession No. AF298181), and goldfish (Accession No. AF061269). ClustalW software (Thompson et al., 1994) was used for multiple sequence alignment of these gene sequences to identify conserved nucleotide sequences. Two degenerate primers (primer 1 and 2; see Table 1) based on the region overlapping the DNA and hormone binding domains were designed. Total RNA from fish liver was reverse-transcribed using the Superscript II (Invitrogen) one-step reverse transcriptase PCR kit and random primers. Polymerase chain reaction (PCR) amplification was carried out with the initial denaturation at 94 °C for 3 min, and each sample was subjected to 30 cycles of denaturation for 30 s at 94 °C, annealing at 55 °C for 30 s, and extension for 1 min at 72 °C. The generated PCR fragment was purified and cloned into the

Table 1
Primers used to clone the full length *vb*ERα and *vb*ERβ2 cDNAs from the cyprinid fish (*Varicorhinus barbatulus*)

Primer	Primer sequence	Nucleotide position
1	5′-CA(G/A)GG(T/A)CACAATGA(T/C)TA(C/T)AT-3′	*vbER*α(830–849); *vb*ERβ2(633–652)
2	5′-TG(C/G)TCCATGCCTTTGTT(A/G)CT-3′	*vb*ERα(1745–1764); *vb*ERβ2(1560–1579)
3	5′-CATCATGCCCACTTCATAGCAC-3′	*vb*ERα(922–943); *vb*ERβ2(725–746)
4	5′-CCAACACCTGCCTGCTGAGA-3′	*vb*ERα(626–645)
5	5′-TGTACTCTGGATCAAGAGCCG-3′	*vb*ERα(687–707)
6	5′-GTCAGTGCTTTATGTATGCCTC-3′	*vb*ERα(1106–1127)
7	5′-TCATTCTGCTCCAGTCCAGT-3′	*vb*ERα(1571–1590)
8	5′-ACCATCACCATCCAGTTGCTG-3′	*vb*ERβ2(477–497)
9	5′-GAAACTCATGTTCTCACCTGACC-3′	*vb*ERβ2(1208–1230)
10	5′-CAGCAACAGTCCATCCGGCT-3′	*vb*ERβ2(1500–1519)
11	5′-CATCGAGTGGACATGGACACAG-3′	*vb*ERβ2(1833–1854)

pGEM-T Easy vector (Promega) for sequencing. The sequence was confirmed as part of *vb*ERα or *vb*ERβ2 using BLAST (Altschul et al., 1990) on NCBI (National Center for Biotechnology Information, National Institutes of Health). To obtain the full-length cDNA, the 5′ end of *vb*ERα or *vb*ERβ2 was extended using gene specific primers (primer 4 for *vb*ERα and primer 8 for *vb*ERβ2, see Table 1) for 5′-RACE with the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacture's protocol. A 3′-RACE procedure using gene specific primers (primer 5–7 for *vb*ERα; primer 9–11 for *vb*ERβ2, see Table 1) was also performed to obtain the full 3′ extensions of both genes. Various length 5′-RACE and 3′-RACE products were cloned and sequenced. The full-length cDNAs of *vb*ERα and *vb*ERβ2 were assembled by overlapping sequences. Analyses of amino acid composition, molecular weight of the proteins, and signature domains derived from Krust et al. (1986) were assessed using the package of DNAMAN software. The nucleotide sequences reported in the present study have been submitted to the DDBJ/EMBL/GenBank data bank under the Accession Numbers AJ547632 (*vb*ERα) and AJ314603 (*vb*ERβ2).

2.3. Constructing the recombinant yeast assays

The yeast Match-maker One-Hybrid System (Clontech, Palo Alto, CA, USA) was used to monitor the ER-dependent transcriptional effect of xenoestrogens. The full length cDNA of *vb*ERα was reconstructed by PCR using primer-αf containing an *Eco*R1 cleavage site (5′-AGGAATTCATGTACCCTAAGGAGGAGCA-3′) and primer-αr containing a *Bam*H1 cleavage site (5′-CAGGATCCTCAGGGGTCTGGACTCTGGT-3′). The full length cDNA of *vb*ERβ2 was reconstructed using primer-βf (5′-CAGGAATTCATGAGCTCTTCCCTTGGTCC-3′) and primer-βr (5′-CAGGATCCTCAATCTGTGTCCATGTCC-3′) with *Eco*R1 and *Bam*H1 sites, respectively. The PCR products were cloned into the Topo TA cloning vectors for sequence confirmation. The full length cDNA of *vb*ERα or *vb*ERβ2 was excised with *Eco*R1 and *Bam*H1 and directionally cloned into the multiple cloning sites of the pGAD424 expression vector (named as pGAD424-*vb*ERα and pGAD424-*vb*ERβ2, respectively) to produce ER protein subtypes in yeast. The β-galactosidase reporter construct was prepared by cloning three tandem repeats of the double-stranded *cis*-element (ER responsive element, 5′-GGTCACAGTGACC-3′) (Petit et al., 1995) for transcription activity of *vb*ERα and *vb*ERβ2 into the minimal target promoter (P_{CYC1}) to drive production of the β-galactosidase reporter (named ERE-p*LacZi*). The individual subtype, pGAD424-*vb*ERα or pGAD424-*vb*ERβ2, was transformed together with the reporter ERE-p*LacZi* and integrated into genomic DNA of the host yeast (YM4271) via double crossover. After 17–24 h of mating, the yeast cells were harvested, washed with 1 × TE buffer, pH 8.0, containing 25 μg/ml kanamycin, resuspended in 5 ml of the same buffer and spread on plates with selective media (–Leu, –His) containing 15 mM 3-amino-1,2,4-triazole (3-AT) to reduce possible leaky expression of the HIS3 gene. Yeast containing the individual pGAD424-*vb*ERα or pGAD424-*vb*ERβ2 subtype together with the reporter ERE-p*LacZi* were used to investigate the estrogenic activity of chemicals simply by observing the color reaction produced by the β-galactosidase reporter activated through *vb*ERα or *vb*ERβ2.

2.4. Screening for xenoestrogens

The 11 xenoestrogens in this study consist of four groups: the first group, three phenol derivatives including 4-nonylphenol (4-NP), 4-*t*-octylphenol (4-*t*-OP) and bisphenol A (BPA); the second group, DDT-related substances including 4,4′-DDT, 4,4′-DDD and 4,4′-DDE; the third group, phthalate esters including benzyl butyl phthalate (BBP) and di-*n*-butyl phthalate (DBP); and the fourth group, polychlorinated biphenyls (PCBs) including Aroclor® 1248 and Aroclor® 1260. 4-nonylphenol (4-NP) is used to make nonionic surfactants, or stabilizing agents in industrial and agricultural products. 4-*t*-OP is used in industrial neutral detergents. BPA as a cross-linking chemical is widely used industrially to produce plastic polymers, making it one of the highest-volume chemicals produced in the world, such that exposure to BPA is detected in human

urine (Ikezuki et al., 2002). The insecticide 4,4′-DDT, is metabolized by aerobic degradation into 4,4′-DDD and stabilized as 4,4′-DDE in mammals (Suggs et al., 1970). Phthalate esters are used as plasticizers in polyvinylchloride plastics.

All chemicals bought from manufacturers were reagent-grade without further purification. 17β-estradiol (E2) was purchased from Sigma (St Louis, MO, USA). Chemicals were obtained from the following sources: BPA and 4-NP from Aldrich Chemical Co. (Milwaukee, WI, USA); 4-*t*-OP, di-*n*-BP, BBP, 4,4′-DDT, 4,4′-DDD, and 4,4′-DDE from Chem Service (West Chester, PA); and two PCBs (Aroclor® 1248 and Aroclor® 1260) from Supelco (Supelco Park, Bellefonte, USA). Chemicals were dissolved using dimethyl sulfoxide (DMSO) into stock solutions and stored at −20 °C before further analysis.

2.5. Transcriptional activity between vbERα and vbERβ2-mediated assay

The liquid culture assay for ß-galactosidase activity, expressed in Miller units, was measured as described in the Yeast Protocols Handbook (Clontech) using *o*-nitrophenyl-D-galactopyranoside (ONPG) as a substrate. First, the recombinant yeast was incubated overnight on SD medium containing 3-amino-1,2,4 triazole without uracil and leucine at 30 °C, 180 rpm. After the culture was diluted to $OD_{600} = 0.5$ with SD medium, individual xenoestrogens or DMSO solvent alone were added and the culture incubated for an additional 4 h. The concentration of DMSO was controlled to not exceed 2% in the final screening test. After incubation, the culture (V1) was collected by centrifugation and resuspended in 0.1 ml Z buffer (V2) (0.1 M sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, pH = 7). The V1/V2 ratio was defined as a concentration factor. The cell extracts were then obtained by three freeze/thaw cycles. After adding 0.7 ml Z buffer containing β-mercaptoethanol, the activity of β-galactosidase was measured immediately by mixing with its substrate, 160 µl ONPG (4 mg/ml). When the yellow color developed, 0.4 ml 1 M Na_2CO_3 was added to stop the reaction. The cell debris was removed by centrifugation at 14,000 rpm for 5 min and optical density (OD) was recorded at 420 nm. The β-galactosidase activity was calculated using the following equation: $1000 \times OD_{420}/(OD_{600} \times \text{incubation time (in minutes)} \times 0.1 \times \text{concentration factor})$.

2.6. Curve fitting

After the β-galactosidase assay, the concentration-response curve was determined for each chemical to adopt a best-fit using Eq. (1) (Rehmann et al., 1999; Graumann et al., 1999):

$$y = \frac{A - D}{\{1 + (C/\chi)^B\}} + D \quad (1)$$

where

- x the concentration of the individual xenoestrogen examined,
- y the β-galactosidase activity at a specific concentration,
- A maximum β-galactosidase activity,
- B relative slope of the mid-region of the curve as estimated from a linear/log regression of the linear part in the concentration response curve,
- C chemical concentration that produced half-maximal β-galactosidase activity,
- D minimum β-galactosidase activity.

We also obtained two parameters to measure the sensitivities of the compounds tested and their relative maximal transcriptional activity, designated "ligand potency" (EC_{50}) and "ligand efficiency", respectively. The relative ligand potency and relative ligand efficiency were then calculated and normalized against E2 values.

3. Results

3.1. Sequence analysis of both *vb*ER subtypes

In this study, we isolated and cloned two ER subtypes (*vb*ERα and *vb*ERβ2) encompassing open reading frames of 568 amino acids (~63 kDa) and 612 amino acids (~68 kDa), respectively, from livers of the cyprinid fish (*V. barbatulus*). As expected, *vb*ERα and *vb*ERβ2 share strong similarity of signature domains with other ERs. For example, *vb*ERα shares 91% similarity with goldfish ERα and *vb*ERβ2 shares 90% similarity with goldfish ERβ2 (Ma et al., 2000; Choi and Habibi, 2003). The newly-cloned *vb*ERα and *vb*ERβ2 cDNAs code for proteins of six theoretical domains (A/B, C, D, E/F) as expected in the typical ER proposed by Krust et al. (1986). Consistent with subtype similarity in humans, a similar percentage of inter-subtype conservation (73–82%) was also observed between *vb*ERα and *vb*ERβ2.

3.2. β-galactosidase assay

The recombinant yeast sets were constructed so the yeast genome was hardwired to respond to either *vb*ERα or *vb*ERβ2 as a receptor/transcription factor and to β-galactosidase as a reporter construct. Therefore, the induction of β-galactosidase activity for the reporter gene was strictly dependent on the presence of recombinant *vb*ERα or *vb*ERβ2 and the action of xenoestrogens. By testing 11 suspected xenoestrogens in comparison with the natural estrogen E2, the three phenol derivatives 4-NP, 4-*t*-OP, and BPA, produced significant dose-dependent responses as shown in Figs. 1(a) and 2(a). However, the activity of β-galactosidase often decreased after reaching the recorded maximal response for 4-NP, 4-*t*-OP, and BPA, possibly due to saturation of chemical solubility or to toxicity to the yeast. In Table 2, no subtype difference

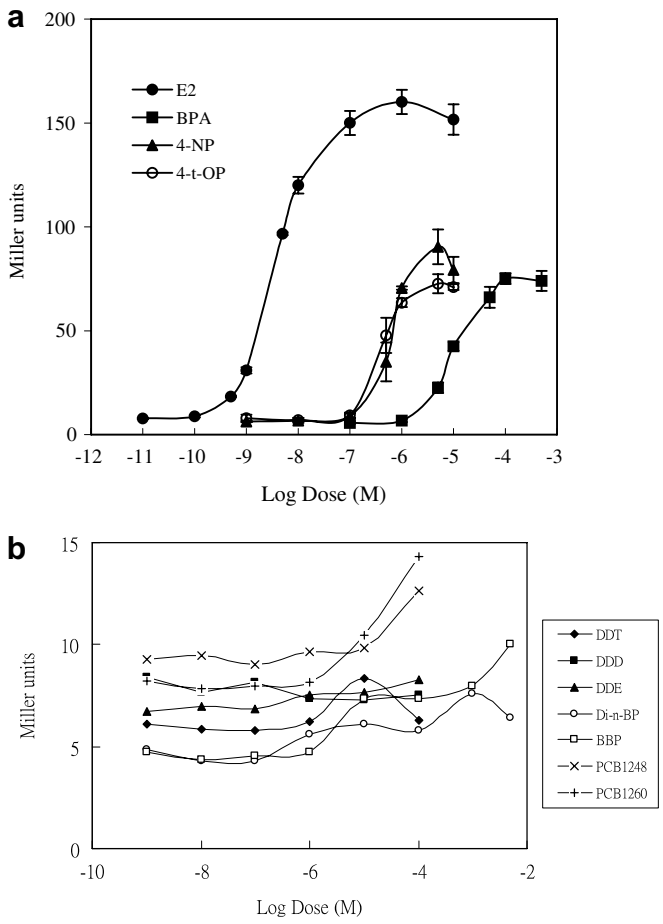

Fig. 1. Xenoestrogens potentiate ligand-dependent transactivation of fish ERα subtype to target β-galactosidase reporter in yeast cells. Concentration-response curves for E2, 4-NP, 4-*t*-OP, BPA (a) and DDT, DDD, DDE, Di-n-BP, BBP, PCB1248, PCB1260 (b) were observed in the *vb*ERα mediated bioassays. Each point and vertical bar represents the mean ± SD. All data are obtained in triplicate; $p < 0.05$ compared with DMSO vehicle control.

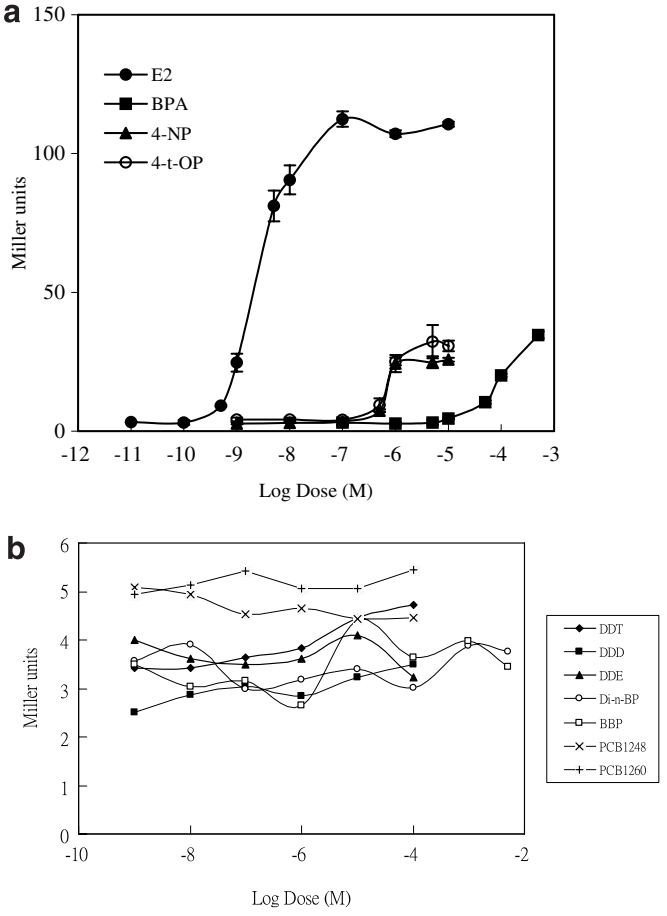

Fig. 2. Xenoestrogens potentiate ligand-dependent transactivation of fish ERβ2 subtype to target β-galactosidase reporter in yeast cells. Concentration-response curves for E2, 4-NP, 4-*t*-OP, BPA (a) and DDT, DDD, DDE, Di-n-BP, BBP, PCB1248, PCB1260 (b) were observed in the *vb*ERβ2 mediated bioassays. Each point and vertical bar represents the mean ± SD. All data are obtained in triplicate; $p < 0.05$ compared with DMSO vehicle control.

was observed in the initial response concentrations for 4-NP and 4-*t*-OP. Only BPA had an initial response concentration of 0.5 nM in ERα-transfected yeast and 50 μM in ERβ2-transfected yeast. However, the DDT-related substances, phthalic acid esters and PCB revealed little or no significant transcriptional activities. The corresponding parameters such as ligand potency (EC_{50}) and ligand efficiency calculated by Eq. (1) are listed in Table 3. The order of sensitivities (designated ligand potencies or EC50) of the compounds tested, either *vb*ERα or *vb*ERβ2, were as following: E2 > 4-NP, 4-*t*-OP > BPA. The relative ligand potency was then calculated and normalized against E2 potency. The values of relative ligand potency, in the ERα-mediated assay, were 6.4×10^{-3} for 4-NP, 1×10^{-2} for 4-*t*-OP, and 4.1×10^{-4} for BPA, whereas in the *vb*ERβ2-mediated assay, the relative ligand potencies were 4.6×10^{-3} for 4-NP, 3.9×10^{-3} for 4-*t*-OP, and 3.2×10^{-5} for BPA. *vb*ERα generally had higher maximal β-galactosidase activities (1.4 to 12.8-fold) than *vb*ERβ2 for all three compounds.

Table 2
*vb*ERα and *vb*ERβ2-mediated yeast assays showing the lowest concentration exhibiting a clear transcriptional response for 11 compounds

Type of compound	Substance	LCR	
		*vb*ERα-YES	*vb*ERβ2-YES
Mammalian steroid hormones	17β-Estradiol	0.5 nM	0.5 nM
Phenol derivatives	4-Nonylphenol	0.5 μM	0.5 μM
	4-*tert*-Octylphenol	0.5 μM	0.5 μM
	Bisphenol A	5 μM	50 μM
DDT-related substances	4,4′-DDT	–	–
	4,4′-DDD	–	–
	4,4′-DDE	–	–
Phthalic acid esters	Di-*n*-butyl phthalate	–	–
	Benzyl butyl phthalate	–	–
Polychlorinated biphenyls	Aroclor® 1248	–	–
	Aroclor® 1260	–	–

LCR: Lowest concentration exhibiting a clear response; –: no obvious response in all concentrations tested.

Table 3
The ligand potencies and efficiencies of phenol-derived xenoestrogens in the *vb*ERα and *vb*ERβ2-mediated yeast bioassays

Chemical	ERα-transformed yeast				ERβ2-transformed yeast			
	Potency (M)	Relative potency	Efficiency (Miller)	Relative efficiency	Potency (M)	Relative potency	Efficiency (Miller)	Relative efficiency
17β-Estradiol	4.1×10^{-9}	1	160	1	2.9×10^{-9}	1	112	1
4-Nonylphenol	6.4×10^{-7}	6.4×10^{-3}	90	0.56	6.3×10^{-7}	4.6×10^{-3}	26	0.23
4-*t*-Octylphenol	4.1×10^{-7}	1×10^{-2}	73	0.46	7.5×10^{-7}	3.9×10^{-3}	32	0.29
Bisphenol A	1.0×10^{-5}	4.1×10^{-4}	75	0.47	9.1×10^{-5}	3.2×10^{-5}	35	0.31

For xenoestrogens, relative ligand potencies and efficiencies are calculated by normalizing against 17β-estradiol values to obtain their E2 equivalents.

E2 displayed the highest maximal transcriptional activity (designated ligand efficiency) of all chemicals tested. The phenol derivatives 4-NP, 4-*t*-OP, and BPA showed only 0.56, 0.46, and 0.47-fold ligand efficiency relative to E2, respectively, in ERα-transformed yeast. Meanwhile in ERβ2-transformed yeast, 4-NP, 4-*t*-OP, and BPA showed only 0.23, 0.29, 0.31-fold of E2 equivalent in ligand efficiency, respectively. Therefore, the relative ligand efficiency was also higher in the *vb*ERα-mediated assay than in the *vb*ERβ2-mediated assay for all chemicals tested.

4. Discussion

Here we demonstrate successful isolation and cloning of two estrogen receptor genes from the cyprinid fish, *V. barbatulus*, in Taiwan. This implies that direct assay of wildlife estrogen receptors is possible even without the availability of genomic DNA or mRNA sequences. We used full-length cDNAs of both alpha and beta estrogen receptor subtypes (*vb*ERα and *vb*ERβ2) from fish liver to measure the transcriptional activities triggered by estrogenic agents. Estrogenic environmental contaminants most readily accumulate in liver, making liver-expressed ERs the most likely active tissue source. *vb*ERα and *vb*ERβ2 have been successfully used in the yeast reporter system to assess the potential activity of xenoestrogens.

In this study, we used a reliable and robust assay, the yeast estrogen screen (YES), in combination with two ER genes from the cyprinid fish (*V. barbatulus*) to facilitate the assessment and compare ligand-dependent transcriptional potencies of EDCs. To exogenously hard-wire ER signaling into yeast as a tool, a pair of plasmids was homologously integrated into the yeast genome with *vb*ERα or *vb*ERβ2 receptor/transcription factor as the detector and β-galactosidase under the control of the hormone responsive ERE promoter as the reporter. To ensure the integrity of ER signaling, we inserted the entire coding region of each ER and three tandem copies of the consensus ERE (Petit et al., 1995), reflecting the transcriptional potency of EDCs through β-galactosidase activity. Under physiological conditions, the presence of ER subtype-specific target genes enables distinct physiological outcomes (Kian Tee et al., 2004; Stossi et al., 2004); in our assay this outcome difference is prevented by converging the responses to a single β-galactosidase reporter.

The field concentrations of various xenoestrogens in surface waters of Taiwan have been previously investigated (Iwata et al., 1994; Wang et al., 2001; Ding and Wu, 2000; Yuan et al., 2002; Chen and Yang, 2004). The following ranges of test compound concentrations were detected: E2 (negligible ~ 0.62 nM), 4NP (4.04–226.91 nM), 4-*t*-OP (<0.24 nM), BPA (<0.22–13.14 nM), BBP (<3.20 nM), DBP (<3.59–48.50 nM), total DDT (0.01–0.19 ng/l), total PCB (0.09–2.10 ng/l). There was no significant difference in surface water concentration between Taiwan and other countries for most compounds except 4-NP (<0.52–2.90 nM detected in USA) (Thiele et al., 1997) and DBP (0.25–0.83 nM detected in USA) (Ray et al., 1983).

The instream concentrations for 4-NP and DBP were considerably higher in Taiwan than in other countries, which could be related to the absence of sewage collection systems in the area where the studies were conducted. Compared to the results from our ER-mediated assays, as depicted in Fig. 1(a) or Fig. 2(a), the actual aquatic concentrations of E2 and 4NP are already high enough to induce substantial estrogenic responses. For example, the background concentration of E2 (0.62 nM) detected in Taiwan's rivers could induce 14% (in the ERα assay) and 12% (in the ERβ2 assay) of the maximal responses. Similarly, the field concentration of 4NP (0.23 μM) could produce 25% and 21% of the maximal responses in our ERα and ERβ2 mediated assays, respectively. In addition, since previous reports indicated that reproductive abnormalities could occur in wild fish populations at very low levels of estrogenic chemicals (Jobling et al., 1998; van Aerle et al., 2001), the combined effects of xenoestrogen mixtures is another important consideration in risk assessment of estrogenic chemicals that should be carefully evaluated in the future. For example, Brian et al. (2005) observed a synergistic effect in a combination of five estrogenic chemicals (estradiol, ethynylestradiol, nonylphenol, octylphenol, and bisphenol A), although each chemical individually produced no response.

Using the natural estrogen 17β-estradiol as an authentic standard, the EC_{50} values for our recombinant *vb*ERα in yeast compared similarly to values for recombinant human ERα (Bovee et al., 2004a), indicating similar sensitivities to estrogen of both ERs in yeast . The 11 xenoestrogens used in this study consist of four groups: phenol derivatives, DDT-related substances, phthalate esters, and polychlori-

nated biphenyls. Our results show that the three phenol derivatives 4-NP, 4-*t*-OP and BPA in either *vb*ERα or *vb*ERβ2 yeast assays exhibited an apparent dose-dependent response, while little to no response was observed in the eight compounds from three other EDC groups, including 4,4′-DDT, 4,4′-DDD, 4,4′-DDE, benzyl butyl phthalate, di-n-butyl phthalate, Aroclor® 1248, and Aroclor® 1260. Consistent with our results, a previous yeast estrogen screen found little response of the ER towards phthalic acid esters (Rehmann et al., 1999). For the responsive chemicals, compared to prior studies of the human ER, our ERα-mediated system showed relative ligand potencies about 32-fold higher for 4-NP (Gaido et al., 1997), about 45-fold higher for 4-*t*-OP (Rehmann et al., 1999) and about 9.1-fold (Gaido et al., 1997) to 29-fold (Rehmann et al., 1999) higher for BPA, suggesting either an order of magnitude greater sensitivity in our systems or differences in response by species-specific ERs. When results of fish-related tests were compared to relative ligand potencies as measured against rainbow trout ERα (Ackermann et al., 2002), our ERα-mediated system showed potencies about 2.4-fold higher for 4-NP (Gaido et al., 1997) and 4-fold higher for 4-*t*-OP (Rehmann et al., 1999), suggesting comparable results between fish homologues in yeast models. Interestingly, an inverse response was seen with BPA in that the relative ligand potency of BPA against rainbow trout ERα measured by others was 13.2-fold more sensitive than BPA measured through our *vb*ERα system. Thus we propose that species-specific differences in the EC50 of ER-mediated xenoestrogen effects may be significant and suggest that future studies examine EDCs using wildlife-specific ERs. In the study of *o*,*p*′-DDT-related chemical ecotoxicity, Fent (2001) found that irreplaceable differences created significant differences between mammalian and fish systems, and suggested that a risk evaluation for fish could only be meaningfully assessed in fish-specific systems (Fent, 2001).

In addition, subtype-specific differences in relative ligand potencies were also observed in the three responsive chemicals. In the *vb*ERα-mediated compared with the *vb*ERβ2-mediated yeast system, EC50s were moderately higher for 4-NP (1.4-fold) and 4-*t*-OP (2.6-fold), and much higher for BPA (12.8-fold). This indicates that, compared with E2, an equivalent EC50 was observed for 4-NP and 4-*t*-OP through either fish ER subtype, while a stronger estrogenic influence was observed for BPA through *vb*ERα.

On the other hand, the relative maximal transcriptional activity, or "ligand efficiency", for the three responsive chemicals (4-NP, 4-*t*-OP, and BPA) detected by *vb*ERα were moderately higher (1.3-fold to 2.4-fold) compared with the *vb*ERβ2-mediated response. Maximal transcriptional activity equivalent to E2 was consistently observed in 4-NP, 4-*t*-OP and BPA with either human ER subtype (Bovee et al., 2004b). When concentrations higher than the recorded maximal responses of 4-NP, 4-*t*-OP and BPA were tested, a drastic decrease in β-galactosidase activity was observed. The recorded maximal responses to 4-NP, 4-*t*-OP and BPA were less than the maximal equivalent estrogenic response to E2. Thus, the potency and efficiency of response to each xenoestrogen might be dependent on both the system and duration. For instance, Harris et al. (2002) found BBP to be a partial agonist that showed only 50% of E2 maximum response with overnight incubation. Only when the assay incubation was extended to 6 days was the full response observed. Our system may therefore assay short term effects of these chemicals aimed at immediate and robust properties. Thus, species-specific differences in the maximal transcriptional activity of ER-mediated effects of xenoestrogens may be less significant.

A potential limitation of our method is underestimating the effect of EDCs on fish due to other routes of ER activation, such as ERE element-independent and ligand-independent gene activation. Ligand-independent gene activation is mediated by serine-phosphorylation of the AF-2 domain of both ERs through growth factor-activated kinase signaling (Metivier et al., 2001). It is suggested that co-activators or co-repressors might display critical roles in differential functions of ER subtypes in fish physiology (Horwitz et al., 1996; Darimont et al., 1998; Lewandowski et al., 2005). In vitro experiments using animal cell lines provide supplemental data. Nevertheless, this simplified and standardized ER signaling in recombinant ER yeast systems allows cross-species comparisons of the impacts of EDCs. Simplified ER systems also provide an easily interpretable assay for future investigations of ER subtype interaction. ERα and ERβ with opposite functional effects have been reported (Weihua et al., 2000), and human ERβ transcriptionally modulates ERα (Hall and McDonnell, 1999). Therefore, it would be intriguing to see whether this interaction occurs in yeast systems. This question can be addressed by using yeast containing both genes with a dual-receptor/single reporter. Yeast assays provide a reliable, rapid and sensitive method to evaluate the estrogenic impact and direct use of wildlife-specific ER not constrained to the study of the Taiwan fish, *V. barbatulus*. We anticipate that yeast assays will be widely applicable to ER-containing wildlife studies for EDCs.

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