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Modeling binding equilibrium in a competitive estrogen receptor binding assay

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Abstract

Although the free concentration is more significant in the environmental chemistry and toxicology of receptor-mediated toxicants, few studies have been conducted to use it as a dose-metric. The relative binding affinity of three model endocrine disrupting compounds, diethylstilbestrol (DES), ethynylestradiol (EE2), and bisphenol A (BPA), were evaluated using a competitive ELISA with human estrogen receptor α. After measuring the available receptors and the dissociation constant for 17β-estradiol, binding inhibition curves using the free concentration as the dose-metric were obtained by assuming species equilibrium in the ELISA system and compared with apparent inhibition curves generated using the nominal concentration as the dose-metric. Because ligand binding to estrogen receptors may reduce its free concentration in the assay system, the differences between the two curves for free and nominal concentrations are more significant for more strongly binding ligands. The ratio of a compound's nominal concentration causing 50% inhibition (IC50) to the IC50 of DES, the positive control, was strongly affected by specific assay conditions, while that estimated by modeling free concentration is independent of receptor concentration, indicating that the free concentration is a better dose-metric for a competitive binding assay.

Keywords: Free concentration; Endocrine disrupting chemicals; ELISA; Inhibition curve

1. Introduction

The presence of estrogenic endocrine disrupting chemicals (EDCs) has been of significant concern for decades (Vethaak et al., 2005; Campbell et al., 2006). The estrogenic potential of suspected EDCs and extracts prepared from environmental samples has been evaluated by various in vitro (e.g., Soto et al., 1995; Routledge and Sumpter, 1996; Kuiper et al., 1997, 1998; Nishikawa et al., 1999; Koda et al., 2002; Ohno et al., 2002; Beck et al., 2006; Xiao et al., 2006) and in vivo (e.g., Vom Saal et al., 1997; Allen et al., 1999; Hemmer et al., 2002; Labadie and Budzinski, 2006) methods. Competitive binding to estrogen receptors is one of the most popular endpoints during the initial screening stage, because ligand binding to the receptors is the initial step for most hormonal actions. Recently, competitive binding assays applying fluorescence polarization (Ohno et al., 2002), enzyme-linked immunosorbant assays (ELISA) (Koda et al., 2002; Morohoshi et al., 2005; Kuruto-Niwa et al., 2007), and using bacterial magnetic particles (Itak et al., 1992; Yoshino et al., 2005) have been developed to replace conventional assays using radio labeled compounds.

It is generally accepted that the free concentration is the driving force for toxic effects and the environmental fate of aquatic pollutants (Heringa et al., 2004; Reichenberg and Mayer, 2006). However, the free concentration is rarely used as a dose-metric for conventional in vitro screening methods, including competitive receptor binding assays. For example, Heringa et al. (2004) have shown that significant underestimation of the estrogenic potential of highly hydrophobic EDCs, such as alkylphenols, may be caused by their binding to serum proteins and surfaces of plastic

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labware. Although serum proteins are not typically added in competitive receptor binding assays, binding of EDCs to estrogen receptors may significantly reduce the free concentrations in the assay solution, especially for strong ligands. This variability in the proportionality between free and nominal concentrations may cause inconsistency in results obtained from different assay conditions.

In this study, we evaluated the binding affinity of three model EDCs, diethylstilbestrol, 17α-ethynylestradiol, and bisphenol A, using a competitive hER-α binding assay detecting released estrogen by ELISA. The assay was chosen because it is high-throughput and does not require expensive experimental devices. In order to predict the bias of the results in different assay conditions, the competitive binding assay was performed at two different hER-α concentrations. Free concentrations of EDCs in a given assay condition was calculated using an equilibrium binding model assuming that equilibrium is achieved rapidly. The robustness of the assay results using free concentration as a dose-metric was compared with those using nominal concentration.

2. Materials and methods

2.1. Materials

Three model endocrine disrupting chemicals were selected to evaluate the effects of binding affinity on the inhibition curves. Diethylstilbestrol (DES) and 17α-ethynylestradiol (EE2) were chosen as examples of the strong ligands and bisphenol A (BPA) was chosen as a model weak ligand. All chemicals were of high purity. DES and EE2 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 17β-estradiol (E2) and BPA were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Human estrogen receptor α (hER-α) was purchased from PanVera (Madison, WI, USA). Aqueous estradiol not bound to hER-α was detected using an ELISA kit for the detection of E2 (Neogen Corp., Lexington, KY, USA). Measured E2 concentration is not expected to be affected by co-existing EDCs concentration because the cross-reactivity to the coated antibody is typically much less than 1% for other steroids according to the manufacturer.

2.2. Competitive estrogen receptor binding assay

Relative binding affinity and inhibition curves of three selected estrogenic chemicals were obtained using a competitive estrogen receptor binding assay in the absence of radio-labeled compounds. The assay was performed using a procedure described in literature (summarized as depicted in Fig. 1; for details, see Koda et al., 2002; Morohoshi et al., 2005) with slight modifications. Briefly, 25 μl of chemical solutions containing endocrine disrupting chemicals (EDCs) were incubated for 1 h with receptor solution (15 μl) and a 17β-estradiol (E2) solution (7.2 nM, 25 μl). Two receptor concentrations were used in order to evaluate the dependency of the assay results for different ligands. A custom-made Teflon® well plate was used as the reaction plate to minimize any possible interactions with surface material. To detect E2 concentrations not bound to hER-α, 50 μl of the reaction solution was transferred to an antibody plate coated with estradiol antibody and incubated with an equal amount of estradiol-horseradish peroxidase (E2-HRP) obtained from Neogen for 1 h. After the plate was cleaned three times using a washing solution (50 mM disodiumphosphate, 150 mM NaCl, 0.05% Tween 20, 200 μl), 150 μl of a substrate solution (3,3′,5,5′-tetramethylbenzidine) was added and the antibody plate was further incubated for 30 min at 25 °C. The absorbance of each well was measured at 650 nm using a Synergy™ HT multi-detection microplate reader (BIO-TEK Instruments, Inc., Winooski, VT, USA). The measured absorbance was normalized by the absorbance from the blank sample (aqueous buffer + E2-HRP). The reaction solution incubated with excess diethylstilbestrol (500 nM) was used as a positive control. Inhibition (I) of E2 binding to hER-α in the reation plate, in relation to EDC application was calculated from the absorbance of the antibody plate as: $I = \frac{[B]-[S]}{[B]-[P]}$, where [B], [S], and [P] are normalized absorbance $\left(\frac{A}{A0}\right)$ for the negative control (buffer and 7.2 nM E2) in the absence of EDC, in the presence of EDC and in the presence of 500 nM DES (positive control) in the reaction plate, respectively.

2.3. Equilibrium binding model

Because ligand–receptor binding and antigen–antibody binding are thought to be very fast reactions, it is reasonable to assume that all chemical species are in equilibrium for both the reaction and the antibody plates. Thus, competitive binding in the reaction plate can be expressed by two equilibrium binding reactions

$$\mathrm{E2} + \mathrm{ER} \Longleftrightarrow \mathrm{ER-E2} \quad K_{\mathrm{D,E2}} = \frac{\mathrm{free[ER]free[E2]}}{\mathrm{[ER-E2]}} \tag{1}$$

$$\mathrm{EDC} + \mathrm{ER} \Longleftrightarrow \mathrm{ER-EDC} \quad K_{\mathrm{D,EDC}} = \frac{\mathrm{free[ER]free[EDC]}}{\mathrm{[ER-EDC]}} \tag{2}$$

Mass balance equations for E2, EDC, and ER in the antibody plate are

$$\mathrm{total[E2]} = \mathrm{unbound[E2]} + \mathrm{[ER-E2]} = \mathrm{free[E2]} + \mathrm{[Ab-E2]} + \mathrm{[ER-E2]} \tag{3}$$

$$\mathrm{total[EDC]} = \mathrm{free[EDC]} + \mathrm{[ER-EDC]} \tag{4}$$

$$\mathrm{total[ER]} = \mathrm{free[ER]} + \mathrm{[ER-E2]} + \mathrm{[ER-EDC]} \tag{5}$$

where [Ab-E2] represents 17β-estradiol bound to the antibody and unbound[E2] represents E2 not bound to the hER-α (i.e., free[E2] and [Ab-E2]). In the absence of any other competing ligands, unbound[E2] can be estimated from measured $\frac{A}{A0}$ by interpolation using the two nearest

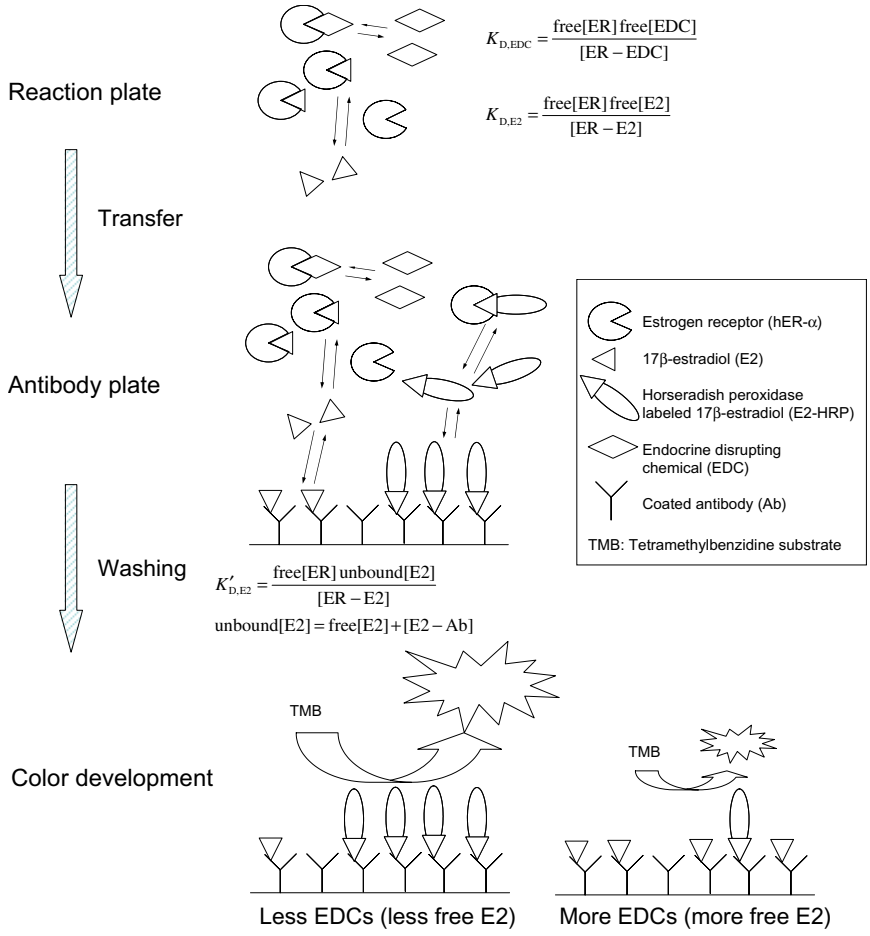

Fig. 1. Principle of the competitive receptor binding assay using an enzyme-linked immunosorbant assay for the detection of free estrogens. (Copyright© (2002) Society of Environmental Toxicology and Chemistry from *Environmental Toxicology and Chemistry*, by Koda et al., 2002. Reprinted with slight modifications by permission of Alliance Communications Group, a division of Allen Press, Inc.).

$\frac{A}{A0}$ values from the standard curve. Under the assumption that free[E2] is proportional to unbound[E2], Eq. (1) can be written using unbound[E2] instead of free[E2] with the modified dissociation constant, $K'_{D,E2}$:

$$K'_{D,E2} = \frac{free[ER]unbound[E2]}{[ER-E2]} \quad (6)$$

Plugging Eq. (3) into Eq. (6) and rearranging, one finds

$$\frac{[ER-E2]}{unbound[E2]} = \frac{1}{K'_{D,E2}}(total[ER] - [ER-E2]) \quad (7)$$

Thus, the modified dissociation constant and total amount of active hER-α can be obtained by plotting $\frac{[ER-E2]}{unbound[E2]}$ vs. [ER − E2].

In the presence of competing ligands, unbound[E2] can be calculated from EDC dose, either total[EDC] or free[EDC] with the estimated $K_{D,EDC}$ by solving Eqs. (2)–(6) (see Appendix for a more detailed derivation). Corresponding $\frac{A}{A0}$ and inhibition in color development (I) were calculated from unbound[E2]. Therefore, measured $\frac{A}{A0}$ values were used to obtain $K_{D,EDC}$ as a best-fit parameter. A non-linear least square regression analysis was performed using SPSS for Windows (Ver. 12.0; SPSS, Chicago, IL, USA). Relative binding affinity (RBA) is calculated as the ratio $\frac{K'_{D,E2}}{K_{D,EDC}}$.

3. Results and discussion

3.1. Determination of the dissociation constant (K_D) and active ER concentration

Fig. 2 shows a typical standard curve without estrogen receptors or EDCs and normalized absorbance values $\left(\frac{A}{A0}\right)$ measured after incubation in the reaction plate with hER-α. Horizontal differences between the two series represent 17β-estradiol (E2) bound to the receptor, [ER − E2]. The dissociation constant for E2 from hER-α and the active concentration of hER-α (total[ER]) were determined using a Scatchard plot ($\frac{[ER-E2]}{unbound[E2]}$ vs. [ER-E2] as shown in Fig. 3). From the slope and the intercept of the regression described in Eq. (7), $K'_{D,E2}$ was determined to be 1.09 ± 0.17 nM and the active receptor concentration was 3.84 ± 0.38 nM. The measured $K'_{D,E2}$ in this study is in good agreement with literature K_D values, typically reported to be

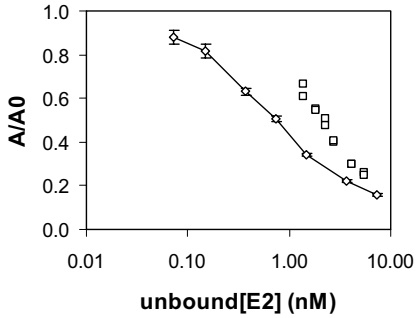

Fig. 2. A typical standard curves of the competitive receptor binding assay, relating unbound E2 to the normalized absorbance $A/A0$, in the absence (◊) and the presence (□) of hER-α. Error bars denote standard deviations.

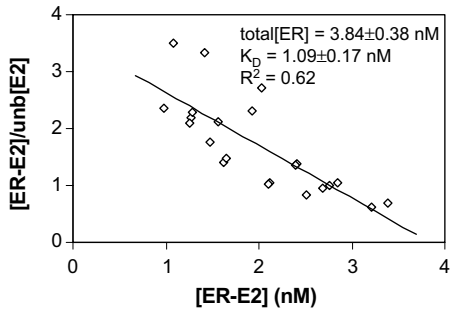

Fig. 3. Scatchard plot ([ER-E2]/unb[E2] vs. [ER-E2]) for 17β-estradiol (E2) binding to human estrogen receptor α (hER-α).

between 0.1 and 2.0 nM, obtained using various techniques (Kuiper et al., 1997, 1998; Matthews et al., 2000; Ohno et al., 2002; Rich et al., 2002; Usami et al., 2002; Yoshino et al., 2005), although $K'_{D,E2}$ may overestimate the dissociation constant because unbound[E2] used in Eq. (6) is greater than free[E2]. The measured active receptor concentration is slightly less than the value of 4.6 nM calculated by the manufacturer using a dilution factor. This could be due to denaturation of the receptor during the preparation steps.

3.2. Competitive estrogen receptor binding assays

Fig. 4 shows inhibition curves for DES, EE2, and BPA obtained from the receptor and E2 concentrations described above. Solid lines are theoretical inhibition curves obtained using the least-square method. The estimated relative absorbance $\left(\frac{A}{A0}\right)$ was calculated from the unbound[E2] concentration which is determined by dissociation constants for the EDCs (Eqs. (2)–(6)). Dissociation constants ($K_{D,EDC}$) for DES, EE2, and BPA, obtained as the best-fit parameters, are 0.91 ± 0.14, 1.65 ± 0.37, and 945 ± 132 nM, respectively. Inhibition curves calculated using the free concentration of the EDC as a dose-metric are also presented (as dashed lines) in Fig. 4. Because Fig. 4 has a log scale on the abscissa axis, spanning 8 orders of magnitude, in order to compare directly strong and

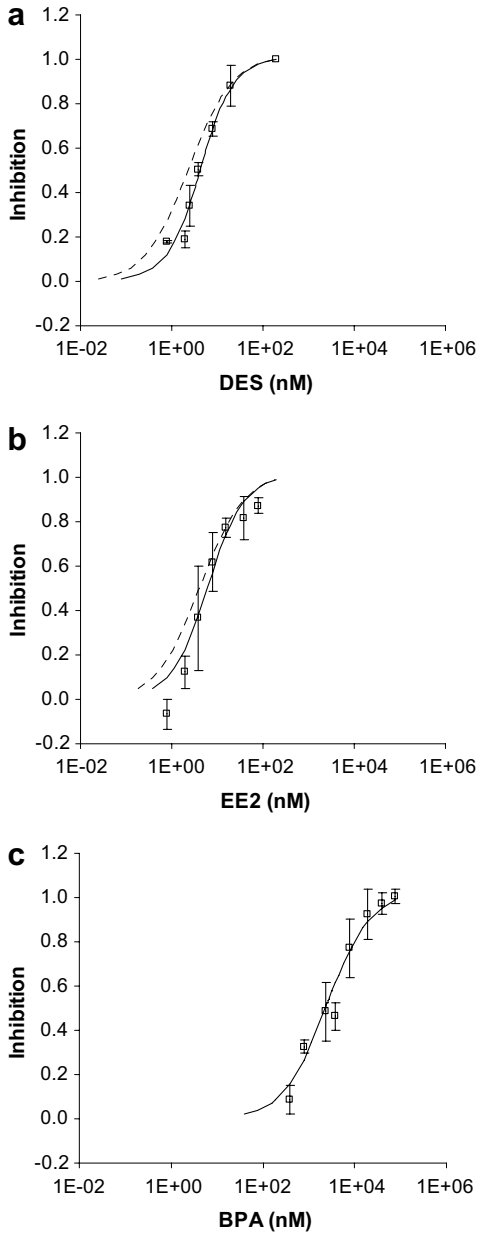

Fig. 4. Inhibition curves for (a) diethylstilbestrol (DES), (b) ethynylestradiol (EE2), and (c) bisphenol A (BPA). Theoretical inhibition curves are shown in solid lines using nominal concentration as a dose-metric and in dashed lines using free concentration as a dose-metric. Error bars denote the standard deviation in inhibition from at least three independent measurements.

weak ligands, this compressed scale makes the effects appear minor. However, the horizontal difference between two curves at IC50 is significant for strong ligands. This difference is 0.46 log unit for DES. As can be seen from Fig. 4, the discrepancy between the two inhibition curves strongly depends on the binding affinity of an EDC to hER-α, because binding of a strong ligand to the receptor significantly lowers the free concentration of the ligand in the assay system. In other words, the apparent inhibition curve of a strong ligand shifts to the right from the free concentration inhibition curve in relation to the concentra-

tion of E2 and hER-α. Although dissociation constants are unchanged at a given experimental temperature, apparent IC50 values obtained from the experimental inhibition curve depend on specific assay conditions. More receptors or more E2 in the reaction plate should increase measured IC50 values determined from the experimental inhibition curve. Significant overestimation of the relative binding affinity $\left(\frac{IC50_{DES}}{IC50_{EDC}}\right)$ may result when the relative binding affinity is normalized by the apparent IC50 of the positive control (DES), as reported in literature (Koda et al., 2002; Morohoshi et al., 2005). The horizontal difference of the two inhibition curves in Fig. 5 illustrates the relative binding affinity of BPA normalized by that of DES. The relative binding affinity of BPA could be overestimated by a factor of 3 because $IC50_{DES}$ using nominal concentration is almost three times that using free concentration as mentioned earlier.

3.3. Prediction of inhibition curves using an equilibrium binding model

In order to validate the equilibrium binding model, the competitive estrogen binding assay was conducted using a 7.7 nM hER-α concentration. Fig. 6 shows the shift of the inhibition curves from the previous assay condition (3.8 nM hER-α). Inhibition curves were generated using $K_{D,EDC}$ values obtained in the previous experiment. Dashed lines and solid lines represent the estimated inhibition curves at hER-α = 3.8 nM and hER-α = 7.7 nM, respectively. As it can be seen from the figures, the experimental values agree well with the predicted inhibition, indicating that the chemistry of competitive binding in the ELISA system can be described by the equilibrium binding model. In addition, the effects of receptor concentration on IC50 are greater for stronger ligands.

The increased sensitivity of detecting inhibition with decreasing receptor and estradiol concentration in Koda et al. (2002) can be explained quantitatively as presented

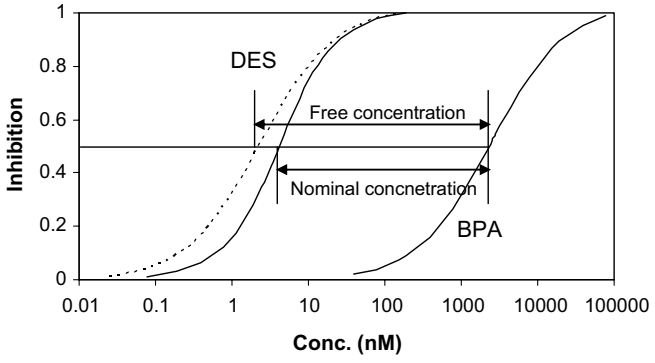

Fig. 5. An illustration of the overestimation of binding affinity when calculated using the apparent inhibition curves. Whereas the free concentration inhibition curve (dashed line) is unaffected by assay condition, the apparent concentration inhibition curve (solid line) is affected by changing receptor and estrogen concentrations.

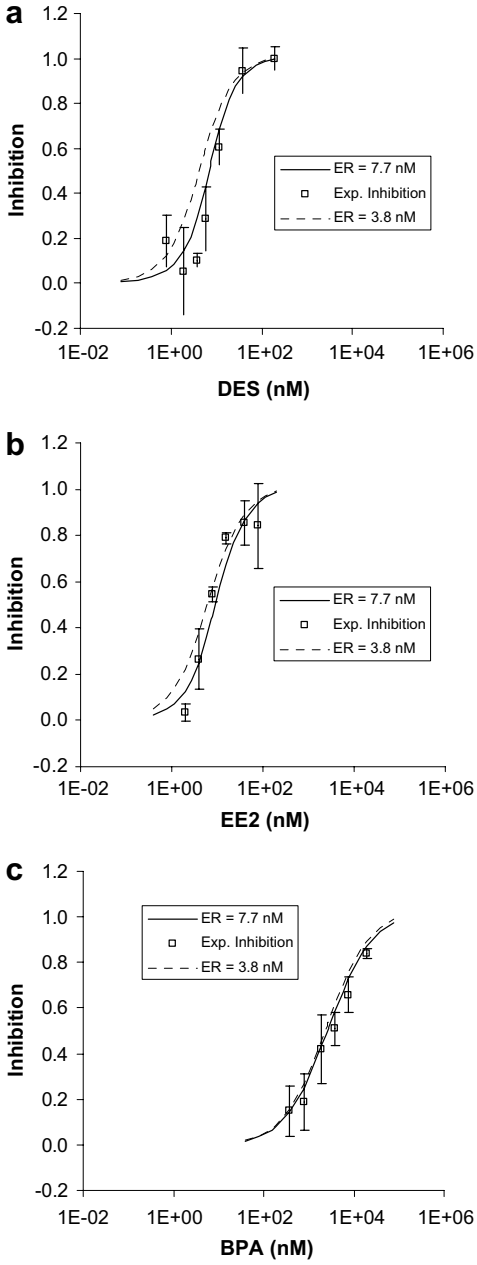

Fig. 6. Estimated inhibition curves for (a) diethylstilbestrol (DES), (b) ethynylestradiol (EE2), and (c) bisphenol A (BPA) with experimental inhibition results. Estimated inhibition curves with receptor concentration of 7.7 nM are represented in solid lines. Dashed lines indicate the inhibition curves from Fig. 4 (hER-α = 3.8 nM).

in this study. However, $\frac{IC50_{DES}}{IC50}$ may depend on the assay condition, unless free concentration IC50 or dissociation constants obtained as a best-fit parameter are used. Fig. 7 shows predicted changes in apparent $\frac{IC50_{DES}}{IC50}$ using BPA as an example of a weak ligand. Two squares in Fig. 7 are the experimental ratios obtained at 3.8 nM and 7.7 nM of hER-α. Apparent $\frac{IC50_{DES}}{IC50_{BPA}}$ increases with increasing receptor concentration because the inhibition curve for a stronger ligand is shifted more to the right than that for a weak ligand. This supports the hypothesis that more

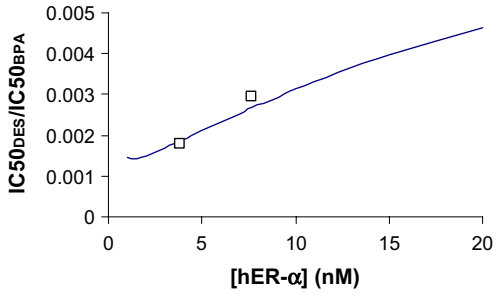

Fig. 7. The effects of receptor concentration on the apparent IC50DES/IC50 using bisphenol A as an example ligand. Open squares denote experimental values.

reliable results for relative binding affinity can be obtained from estimated dissociation constants rather than apparent IC50 values.

3.4. Implication to endocrine disruption in aquatic animals

The competitive receptor binding assay has been typically used to evaluate relative binding affinity (RBA) of a chemical or an environmental sample to receptors (Kuiper et al., 1997, 1998; Koda et al., 2002; Ohno et al., 2002). However, an RBA value obtained from a specific assay condition may not be robust, unless dissociation constants (or free concentration) are used. Because free concentration is the driving force for the fate of aquatic pollutants, better prediction of RBA in terms of aquatic toxicology can be obtained from models incorporating measured dissociation constants.

Although endocrine disruption in wildlife is the result of several complicated processes in series, ligand binding to the hormone receptor is the initial step for the hormone action. Although many potential estrogenic EDCs have been evaluated by their binding affinity to different estrogen receptors isolated from different species (e.g., Lutz and Kloas, 1999; Matthews et al., 2000; Menuet et al., 2002), little is known about the relationship between the amount of the activated receptors from foreign ligands and the various endpoints for the detection of estrogenicity. Because the toxicity of receptor mediated toxicant at the species level may not be evaluated by simply combining bioconcentration rates and in vitro toxicity endpoints, further investigation is need to fill the gaps among different in vitro and in vivo assays. Free concentration as evaluated in this study would be critical to explain the differences in responses.

Appendix A

Symbols

$\frac{A}{A0}$	normalized absorbance
Ab	estradiol antibody
BPA	bisphenol A
DES	diethylstilbestrol
E2	17β-estradiol
EDCs	endocrine disrupting chemicals
EE2	17α-ethynylestradiol
hER-α	human estrogen receptor α
HRP	horseradish peroxidase
K_D	dissociation constant
K'_D	modified dissociation constant
RBA	relative binding affinity

Mathematical solution for the systems of linear equations

At the equilibrium binding condition, there are five equations (Eqs. (2)–(6)) with five unknowns. If $K'_{D,E2}$ and $K_{D,EDC}$ are not identical, a cubic equation for unbound[E2] is obtained by substituting relationships for free[ER], free[EDC], and [ER-EDC] based on unbound[E2] and known total concentrations as follows:

$$\text{unbound[E2]}^3 + p\ \text{unbound[E2]}^2 + q\text{unbound[E2]} + r = 0 \tag{A.1}$$

where $p = -\frac{\left[K'_{D,E2}\left(\text{tot[EDC]}-\text{tot[ER]}+2\text{tot[E2]}-K'_{D,E2}\right)+K_{D,EDC}\left(\text{tot[ER]}-\text{tot[E2]}+K'_{D,E2}\right)\right]}{K'_{D,E2}-K_{D,EDC}}$,

$q = -\frac{K'_{D,E2}\text{tot[E2]}\left(\text{tot[EDC]}-\text{tot[ER]}+\text{tot[E2]}-2K'_{D,E2}+K_{D,EDC}\right)}{K'_{D,E2}-K_{D,EDC}}$, and $r = -\frac{K'^{2}_{D,E2}\text{tot[E2]}^2}{K'_{D,E2}-K_{D,EDC}}$.

This cubic equation may be reduced to the form,

$$x^3 + ax + b = 0 \tag{A.2}$$

by substitution of the variable $x - \frac{p}{3}$ for the unbound[E2]. Here

$$a = \frac{1}{3}(3q - p^2) \text{ and } \quad b = \frac{1}{27}(2p^3 - 9pq + 27r)$$

Eq. (A.2) can be solved by transforming it to the trigometric identity (Selby, 1969)

$$4\cos^3\theta - 3\cos\theta - \cos(3\theta) = 0 \tag{A.3}$$

Let $x = m\cos\theta$, then

$$x^3 + ax + b = 4\cos^3\theta - 3\cos\theta - \cos(3\theta) = 0 \tag{A.4}$$

where $m = 2\sqrt{-\frac{a}{3}}$ and $\cos(3\theta) = \frac{3b}{am}$.

Any solution θ_1 which satisfies will also have the solutions $\theta_1 + \frac{2\pi}{3}$ and $\theta_1 + \frac{4\pi}{3}$.

Therefore, the roots of the cubic equations, $x^3 + ax + b = 0$ are $2\sqrt{-\frac{a}{3}}\cos\theta_1$, $2\sqrt{-\frac{a}{3}}\cos\left(\theta_1+\frac{2\pi}{3}\right)$, $2\sqrt{-\frac{a}{3}}\cos\left(\theta_1+\frac{4\pi}{3}\right)$.

In the case that $K_{D,EDC}$ equals to $K'_{D,E2}$, unbound[E2] can be solved by a quadratic equation

$$\text{unbound[E2]}^2 + s\ \text{unbound[E2]} + t = 0 \tag{A.5}$$

where $s = -\frac{\text{tot[E2]}\left(\text{tot[EDC]}-\text{tot[ER]}+\text{tot[E2]}-K'_{D,E2}\right)}{\text{tot[EDC]}+\text{tot[E2]}}$ and $t = -\frac{K'_{D,E2}\text{tot[E2]}^2}{\text{tot[EDC]}+\text{tot[E2]}}$.

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