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Relationship between the results of *in vitro* receptor binding assay to human estrogen receptor α and *in vivo* uterotrophic assay: Comparative study with 65 selected chemicals

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Abstract

For screening chemicals possessing endocrine disrupting potencies, the uterotrophic assay has been placed in a higher level in the OECD testing framework than the ER binding assay to detect ER-mediated activities. However, there are no studies that can demonstrate a clear relationship between these assays. In order to clarify the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays and to determine meaningful binding potency from the ER binding assay, we compared the results from these assays for 65 chemicals spanning a variety of chemicals classes. Under the quantitative comparison between logRBAs (relative binding affinities) and logLEDs (lowest effective doses), the log RBA was well correlated with both logLEDs of estrogenic and anti-estrogenic compounds at $r^2 = 0.67$ ($n = 28$) and 0.79 ($n = 23$), respectively. The RBA of 0.00233% was found to be the lowest ER binding potency to elicit estrogenic or anti-estrogenic activities in the uterotrophic assay, accordingly this value is considered as the detection limit of estrogenic or anti-estrogenic activities in the uterotrophic assay. The usage of this value as cutoff provided the best concordance rate (82%). These findings are useful in a tiered approach for identifying chemicals that have potential to induce ER-mediated effects *in vivo*.

Keywords: Binding assay; Uterotrophic assay; Concordance

1. Introduction

After a variety of studies showed that certain chemicals might disrupt the sex hormonal systems of wildlife and humans, the Organization for Economic Cooperation and Development (OECD), European countries, the United States and Japan initiated efforts to develop and validate assays and screen chemicals for their potential to disrupt the endocrine system of human and wildlife (CEC, 2004; EPA, 1998; Gelbke et al., 2004). Under the OECD activity, "Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals" was developed. This framework has five components or levels each corresponding to a different level of biological complexity (OECD, 2002). The *in vitro* assays such as receptor binding assay are placed in level 2 in this framework to provide mechanistic information and serve for screening purposes. As a tool to detect hormonal effects mediated

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thorough specific hormone receptors, especially for estrogen receptors (ERs), the application of the ER binding assay has been investigated as a quick and cost-effective method. Furthermore, the rodent uterotrophic assay, which has been placed in level 3 to provide data about single endocrine mechanism and effects, has also been recognized as an *in vivo* assay to detect estrogen receptor mediated effects (Kanno et al., 2001; Owens and Koeter, 2003).

Since the uterotrophic assay and ER binding assay both detect ER-mediated activities, a clear relationship between the results from both two assays is expected. However, there are no studies that can demonstrate a clear relationship between two assays on a variety of chemical structures, although studies comparing at limited number of chemical structures has been available (Takemura et al., 2005; Hong et al., 2005; Legler et al., 2002; Yamasaki et al., 2004). Since the receptor binding assay is considered as one of the screening assays to determine if a chemical has potential to trigger receptor-mediated endocrine disruption such as ER and androgen receptor (AR). The results of receptor binding studies need to be related to biological relevance. The purpose of this study is to clarify the relationships between the results obtained in the *in vitro* ER binding assay and *in vivo* uterotrophic assay, and to determine the meaningful cutoff value from the ER binding assay. For this purpose, we compared the results of receptor binding assay using human ERα (hERα) with immature rat uterotrophic assay for 65 selected chemicals.

2. Materials and methods

2.1. Chemicals

The chemical names, CAS numbers and sources of 65 test chemicals subjected in this study are listed in Table 1. Parabenes, benzophenones, biphenyls, diphenylmethanes, diphenylethylenes, phthalates, phenols and other chemical structure classes were included. All test chemicals used in this study had more than 95% purity. Tris(hydroxymethyl)aminomethane (Tris), phenylmethylsulfonyl fluoride (PMSF) and 17β-Estradiol were purchased from Sigma-Aldrich. Dimethylsulfoxide (DMSO), leupeptin hemisulfate monohydrate, sodium metavanadate, ($NaVO_3$) dithiothreitol (DTT), glycerol and bovine serum albumin (BSA) were obtained from Wako Pure Chemical Industries, Ltd. and disodium salt dihydrate, ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were from Dojindo Laboratories Inc.

2.2. In vitro ER binding assay

The receptor binding assay using recombinant human estrogen receptor α (hERα) was conducted by the method previously reported (Nakai et al., 1999; Yamasaki et al., 2004). Briefly, a recombinant hERα ligand binding domain (hERα-LBD) fused with glutathione-*S*-transferase (GST) was expressed in *E. coli* and purified using affinity chromatography technique. After the addition of sample solution (10 μL) of varied concentration (1×10^{-11} to 1×10^{-4} M as final concentrations) and [2,4,6,7,16,17-^{3}H] 17β-estradiol ([^{3}H]-E2, 10 μL; final conc. 0.5 nM, 155 Ci/mmol, Amarsham Biosciences Corp.) in Tris-HCl (pH 7.4, 50 μL) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 1 mM DTT, 10% glycerol, 10 mg/mL BSA, 0.5 mM PMSF, and 0.2 mM leupeptin, a solution of recombinant hERα-LBD (30 μL; final conc. 0.2 nM) was gently mixed. This mixture solution was incubated for 1 h at 25 °C. Free radiolabelled ligands were removed by incubation with 0.4% dextran-coated charcoal (Sigma) (100 μL) for 10 min at 4 °C followed by filtration. The radioactivity of residual radiolabelled ligands bound to receptors in filtrate were measured by liquid scintillation counting. The assay was repeated more than three times for each test chemical.

2.3. Immature rat uterotrophic assay

The chemicals listed in Table 1 were tested in the immature rat uterotrophic assay according to the OECD draft test guideline as previously reported (Yamasaki et al., 2004) in compliance with good laboratory practice (GLP).

Crj:CD (SD) IGS female rat pups (10-day old) purchased from Charles River Japan Inc. (Shiga, Japan) were weaned with their dams and individually housed until 19-days old. These immature female rats were weighted and weight-ranked to assign to each of the treated and control groups (6 rats/group). Three doses were used for each test chemical and the highest dose was set at the maximum tolerance dose based on the results of dose-range finding test. The limit dose was set at 1000 mg/kg/day. Each group of six immature, 20-days old female rats received subcutaneous injections of a test chemical into their back for three consecutive days (4 mL/kg/day) for evaluation of estrogenic activity. The vehicle control group treated with olive oil (s.c.) and the positive control group for estrogenic activity (s.c. 0.6 μg/kg/day of 17α-ethynylestradiol (EE, Sigma)) were concurrently run for each test chemical. To evaluate anti-estrogenic activity in another set of treatment groups, EE (0.6 μg/kg/day) was co-administered with the test chemical. The positive control group for anti-estrogenic activity received subcutaneous co-administration of 1 mg/kg/day of tamoxifen (TAM, Sigma) and 0.6 μg/kg/day of EE. The animals were sacrificed by bleeding from the abdominal vein under deep ether anesthesia 24 h after the final administration, and body weight and uterine weight of each animal were recorded.

2.4. Data analysis

The resulting data from the ER binding assay were analyzed using GraphPad Prism®, Version 4 (GraphPad Software, Inc.), and the IC_{50} value for each test chemical was obtained by logistic equation. The relative binding affinity

Table 1
List of test chemicals and summary results of ER binding and uterotrophic assays

Chemical Name	CAS No.	Source[a]	logRBA	logLED (μmol/kg/day)	
				Estrogenic	Anti-estrogenic
17β-Estradiol	50-28-2	NA	2.00	<−2.43[b]	N.A.
4-*n*-Amylphenol	14938-35-3	TCI	−2.49	3.69	–
p-Dodecyl-phenol	104-43-8	Kanto Chem.	−0.62	2.18	–
p-*tert*-Butylphenol	98-54-4	Wako	−2.63	2.82	3.30
p-(*tert*-Pentyl) phenol = *p*-(*tert*-Amyl) phenol	80-46-6	Wako	−1.76	3.09	3.09
4-Cyclohexylphenol	1131-60-8	TCI	−1.40	3.05	–
4-(1-Adamantyl)phenol	29799-07-3	Aldrich	0.23	1.54	–
2,4-Di-*tert*-butylphenol	96-76-4	TCI	−2.81	–	–
Pentachlorophenol	87-86-5	Wako	N.B.	–	–
2-Naphthol	135-19-3	Wako	−2.98	–	–
p-Hydroxybenzoic acid	99-96-7	Wako	N.B.	–	–
Ethyl-*p*-hydroxybenzoate	120-47-8	Wako	N.D.	–	–
2-Ethylhexyl-4-hydroxybenzoate	5153-25-3	Wako	−1.28	2.90	2.90
4,4′-Dimethoxybenzophenone	90-96-0	TCI	N.B.	–	–
4-Hydroxybenzophenone	1137-42-4	Sigma	−1.97	3.00	3.00
4,4′-Dihydroxybenzophenone	611-99-4	Wako	−1.77	2.97	2.97
2,4-Dihydroxybenzophenone	131-56-6	TCI	−1.86	2.67	3.15
2,4,4′-Trihydroxybenzophenone	1470-79-7	Aldrich	−1.13	2.24	2.24
2,2′,4,4′-Tetrahydroxybenzophenone	131-55-5	Wako	−1.03	2.91	2.21
4-Fluoro-4′-hydroxybenzophenone	25913-05-7	TCI	−2.50	2.67	–
2,3,4-Trihydroxybenzophenone	1143-72-2	Wako	−2.05	3.11	3.64
2,2-Bis(4-hydroxyphenyl)-4-methyl-n-pentane	6807-17-6	Wako	0.45	0.87	0.87
4,4′-Cyclohexylidenebisphenol	843-55-0	TCI	−0.67	2.05	2.05
4,4′-(Octahydro-4,7-methano-5H-inden-5-ylidene) bisphenol	1943-97-1	Acros	0.34	0.80	0.80
4,4′-(Hexafluoroisopropylidene)diphenol	1478-61-1	Aldrich	−0.11	1.08	1.08
4-(Phenylmethyl)-phenol	101-53-1	TCI	−1.65	3.04	3.04
4,4′-Dihydroxydiphenylmethane	620-92-8	TCI	−1.14	2.70	2.70
4,4′-Sulfonyldiphenol	80-09-1	TCI	−2.26	1.90	3.30
4,4′-Thiobis-phenol	2664-63-3	TCI	−0.61	0.96	1.66
Clomiphene citrate (*cis* and *trans* mixture)	50-41-9	ICN	1.57	0.52	0.52
4,4′-Dimethoxytriphenylmethane	7500-76-7	KKC	N.D.	–	–
3,3′-Dichlorobenzidine dihydrochloride	612-83-9	SIGMA	−3.36	–	–
4,4′-Biphenol	92-88-6	TCI	−1.05	2.51	2.51
4′-Hydroxy-4-biphenylcarbonitrile	19812-93-2	Wako	−2.84	–	–
3,3′,5,5′-Tetramethyl-(1,1′-bisphenyl)-4,4′-diol	2417-04-1	Aldrich	−2.39	–	–
Diethylphthalate	84-66-2	Kanto Chem.	N.B.	–	–
Di-*n*-propyl phthalate	131-16-8	TCI	N.D.	–	–
Di-*n*-pentyl phthalate	131-18-0	TCI	−2.78	–	–
Di-*n*-hexyl phthalate	84-75-3	TCI	−3.04	–	–
Diheptyl phthalate	3648-21-3	Aldrich	−2.95	–	–
Diisononyl phthalate	28553-12-0	Wako	−3.49	–	–
Diisodecyl phthalate	26761-40-0	TCI	−3.46	–	–
Di(2-ethylhexyl) phthalate	117-81-7	Wako	−1.15	–	–
Diallyl tetephthlate	1026-92-2	TCI	N.B.	–	–
Testosterone enanthate	315-37-7	Wako	N.B.	1.40	–
Methyltestosterone = 17α-Methyltestosterone	58-18-4	Wako	N.D.	1.52	–
N-Cyclohexyl-2-benzothiazolesulfenamide	95-33-0	TCI	−2.33	–	–
2,2′-Dibenzothiazolyl disulfide = 2,2′-Dithiobis[benzothiazole]	120-78-5	Wako	−1.89	–	–
2-Benzothiazolethiol = 2-Mercaptobenzothiazole	149-30-4	Wako	−2.78	–	–
4-*tert*-Butylpyrocatechol = 4-*tert*-Butylcatechol	98-29-3	Wako	−1.72	3.78	3.26
p-Dichlorobenzene	106-46-7	TCI	N.B.	–	–
Benzanthrone	82-05-3	Wako	N.B.	–	3.64
Flutamide	13311-84-7	SIGMA	N.B.	–	–
3-Amino-1,2,4-triazole	61-82-5	TCI	N.B.	–	–
Benomyl	17804-35-2	SIGMA	N.B.	–	–
Hexachlorocyclopentadiene	77-47-4	Wako	−1.97	–	–
Captafol; 1,2,3,6-Tetrahydro-*N*-(1,1,2,2-tetrachloroethylthio)phthalimide	2425-06-1	Wako	−1.34	–	–
Di (2-ethylhexyl) adipate= Bis(2-ethylhexyl)adipate	103-23-1	Wako	N.B.	–	–
Disulfiram	97-77-8	Wako	−1.34	–	2.53
4,4′-(1,3-Phenylendiisopropylidene)bisphenol	13595-25-0	Aldrich	−0.76	2.16	0.76
1,1,3-Tris(2-methyl-4-hydroxy-5-*tert*-butylphenyl)butane	1843-03-4	Wako	−1.67	–	–

(*continued on next page*)

Table 1 (*continued*)

Chemical Name	CAS No.	Source[a]	logRBA	logLED (μmol/kg/day)	
				Estrogenic	Anti-estrogenic
3,3,3′,3′-Tetramethyl-1,1′-spirobisindane-5, 5′,6,6′-tetrol	77-08-7	TCI	−1.00	–	3.37
Diphenyl-*p*-phenylenediamine	74-31-7	Wako	−1.87	2.58	–
Atrazine	1912-24-9	TCI	N.B.	–	2.97
4-Hydroxyazobenzene	1689-82-3	Wako	−1.13	2.30	–
4-Diethylaminobenzaldehyde	120-21-8	Wako	N.B.	3.05	–

N.A.: not available, N.B. (not bound): the maximum displacement of radiolabelled ligand was below 20%, N.D. (not determined): IC_{50} was not calculated and the maximum displacement of radiolabelled ligand was between 20–50%.

–: Significant increase or decrease of uterine weight was not observed in estrogenic or anti-estrogenic assay systems, respectively.

[a] TCI; Tokyo Chemical Industry Co., Ltd., Kanto Chem.; Kanto Chemical Co., Inc., Wako; Wako Pure Chemical Industries, Ltd., Acros; Acros Organics, ICN; KKC; Kankyo Kagaku Center Inc.

[b] The agonistic logLED of E2 was determined by the subcutaneous injection for three consecutive days to immature rat (from 23-days old, SD rat) conducted by Padilla-Banks et al., 2001). This value was not used for the quantitative and qualitative analyses in the study.

(RBA) of each test chemical was calculated using the following equation:

$$\mathrm{RBA} = (\mathrm{IC_{50}}\text{ for }\mathrm{E_2})/(\mathrm{IC_{50}}\text{ for test chemical}) \times 100$$

When IC_{50} was not calculated and the maximum displacement of radiolabelled ligand was between 20% and 50%, the binding potency of test chemical was shown as "N.D. (not determined)". When the maximum displacement of radiolabelled ligand was below 20%, the binding potency of test chemical was shown as "N.B. (not bound)".

The Dunnet test was used to analyze the data from the uterotrophic assay. When the significant increase of uterine weights in agonism assay or significant decrease in antagonism assay were observed, the test chemical was evaluated as estrogenic or anti-estrogenic, respectively.

The lowest effective dose (LED, μmol/kg/day), the lowest dose showing a statistically significant effect in this assay, was employed as a quantitative parameter in this comparison study with logRBAs from the *in vitro* ER binding assay, and the correlation coefficients (r^2) and its *P* values were calculated by GraphPad Prism® version 4 (GraphPad Software, Inc.). Also, contingency analyses were performed to calculate concordance (the rate agreement of the results among assays), false-negative (the rate of negatives in the ER binding assay identified as positive in the uterotrophic assay) and false-negative rates (the rate of positives in the ER binding assay identified as negatives in the uterotrophic assay).

3. Results

3.1. In vitro ER binding assay

The results of the *in vitro* binding assay to hERα for 65 chemicals are shown in Table 1.

RBA values were obtained for 47 of 65 chemicals. The highest and lowest logRBAs were 1.57 of clomiphene citrate and −3.49 of diisononyl phthalate, respectively.

The remaining 18 chemicals were regarded as negatives (non-binders) in the concentration tested. Among them, although 4 chemicals, i.e., ethyl *p*-hydroxybenzoate, di(*n*-propyl)phthalate, 17α-methyltestosterone and 4,4'-dimethoxytriphenylmethane showed 20–50% displacement and they were regarded as negatives in this study.

3.2. Immature rat uterotrophic assay

Sixty-five chemicals were tested by immature rat uterotrophic assay in both estrogenic and anti-estrogenic assay systems. The test chemical was evaluated as estrogenic or anti-estrogenic if the uterine weights were significantly increased in the estrogenic assay or decreased in the anti-estrogenic assay. In such cases, the log lowest effective doses (logLED, μmol/kg/day) were shown in Table 1.

Based on this evaluation, 31 and 25 chemicals were identified as estrogenic and anti-estrogenic in immature rat uterotrophic assay, respectively. Twenty-one, including all diphenylmethanes tested, exhibited both estrogenic and anti-estrogenic responses. On the other hand, none of the phthalates tested in this study have either estrogenic nor anti-estrogenic.

3.3. In vitro ER binding assay vs. in vivo uterotrophic assay

3.3.1. Comparison between logRBA and logLED values

The logRBAs obtained from the *in vitro* ER binding assay were compared with logLEDs from the uterotrophic assay. As shown in Fig. 1, the logRBAs were well correlated with both logLEDs in estrogenic and anti-estrogenic assay systems at $r^2 = 0.67$ ($n = 28$, $P < 0.0001$) and 0.79 ($n = 23$, $P < 0.0001$), respectively.

The lowest logRBA that can detect estrogenic or anti-estrogenic response in the uterotrophic assay was −2.63 (RBA = 0.00233%) of *p-tert*-butylphenol, respectively.

3.3.2. Consistency between ER binding and uterotrophic assays for detecting estrogenicity/anti-estrogenicity

The results of ER binding and uterotrophic assays based on the evaluation of the ER related response as detectable (positive) or not (negative) are compared in Table 2-1. The rates of concordance, false negative and false positive for all chemicals tested was 66%, 14% and 57%, respectively.

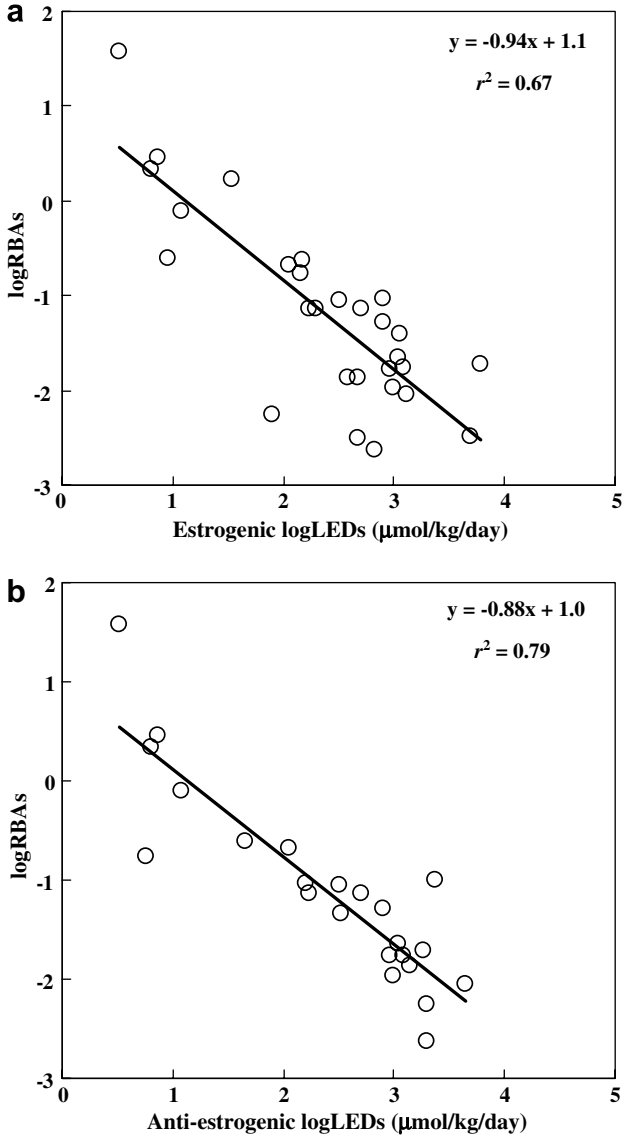

Fig. 1. Relationships between logLED and logRBA values. a, Relationship between estrogenic logLEDs and logRBAs b, Relationship between anti-estrogenic logLEDs and logRBAs.

Seventeen chemicals found to bind to the ER were neither estrogenic nor anti-estrogenic in the uterotrophic assay. Among these chemicals, 3 benzothiazoles and 6 phthalates were included, and the logRBAs of the remaining 8 chemicals were relatively weak ranging from –3.36 to –1.34. Five chemicals were classified as non-binders that showed estrogenic or anti-estrogenic responses in the uterotrophic assay. All 3 non-ER binder chemicals that showed estrogenicity in the uterotrophic assay were androgens (testosterone enanthate and 17α-methyltestosterone) and *p*-diethylaminobenzaldehyde. The other 2 non-ER binders that showed anti-estrogenic effects were atrazine and benzanthrone.

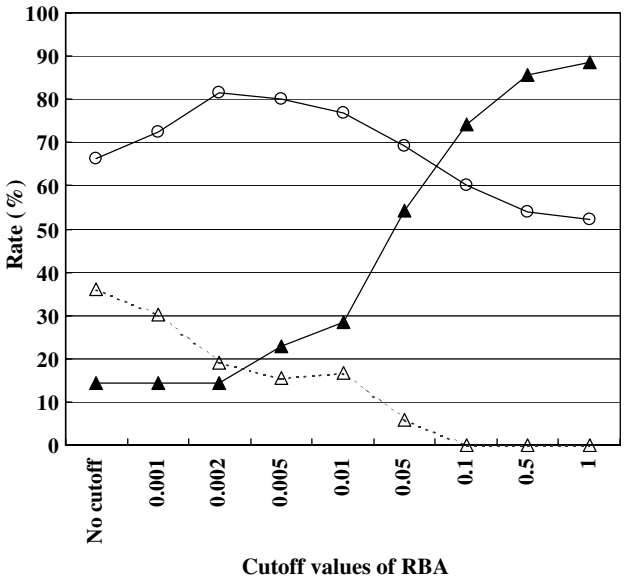

Fig. 2. Changes of indexes by contingency analysis depending on cutoff of RBA. The rates of concordance, false negative and false positive are shown as open circle with solid line, closed triangle with solid line and open triangle with dash line, respectively.

Based on the comparison above, the ER binding assay seemed to have higher sensitivity than immature rat uterotrophic assay. In order to determine the lowest biologically effective binding potency in the ER binding assay, the relationship between RBA cutoff value and the rates of concordance, false negative and false positive from contingency analysis were investigated (Fig. 2). As mentioned above, the lowest RBA that showed estrogenic or anti-estrogenic responses in the uterotrophic assay was −2.63 (RBA = 0.00233) of *p-tert*-butylphenol in this study. When this value is used as a cutoff value, the rates of concordance and false positive rate ratios were refined at 82% and 23%, respectively without increasing the false-negative rate

Table 2-1
Contingency table between ER binding and uterotrophic assays without applying cutoff

		ER-binding assay		Total	Index	Rate(%)
		P	N			
Uterotrophic assay	P/P	*21*	0	21	Concordance	66
Estrogenic	P/N	*7*	3	10		
/Anti-estrogenic	N/P	*2*	2	4	False negative	14
activities	N/N	17	*13*	30		
Total		47	18	65	False positive	57

P: positives, N: negatives, P/N: positives in estrogenic and negatives in anti-estrogenic activities, P/P: positives in both estrogenic and anti-estrogenic activities, N/P: negatives in estrogenic and positives in anti estrogenic activities and N/N: negatives in both estrogenic and anti-estrogenic activities.

Table 2-2
Contingency table based on the RBA giving a maximum concordance

		ER-binding assay		Total	Index	Rate(%)
		P	N			
Uterotrophic assay	P/P	*21*	0	21	Concordance	82
Estrogenic	P/N	*7*	3	10		
/Anti-estrogenic	N/P	*2*	2	4	False negative	14
activities	N/N	7	*23*	30		
Total		37	28	65	False positive	23

P: positives, N: negatives, P/N: positives in estrogenic and negatives in anti-estrogenic activities, P/P: positives in both estrogenic and anti-estrogenic activities, N/P: negatives in estrogenic and positives in anti estrogenic activities and N/N: negatives in both estrogenic and anti-estrogenic activities.

(Table 2-2). This cutoff achieved the best concordance and lowest false-negative ratios as shown in Fig. 2.

4. Discussion

After the potential of chemicals to disrupt the endocrine became apparent, numerous efforts have been made to test and assess chemicals for their endocrine disrupting potential. To detect ER mediated effects, the application of the *in vitro* ER binding assay and *in vivo* rodent uterotrophic assay have long been investigated since ER mediation has been considered as a major mechanism of endocrine disruption of exogenous chemicals.

In order to understand the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays and to investigate the biologically meaningful binding potency from an *in vitro* assay, we compared the results obtained from a receptor binding assay using hERα and the immature rat uterotrophic assay for 65 chemicals spanning a variety of chemicals classes.

For a quantitative comparison of logRBAs and logLEDs, the log RBA was found to be well correlated with both log LEDs of estrogenic and anti-estrogenic assay results at $r^2 = 0.67$ and 0.79, respectively (Fig. 1). These results strongly suggest that there was a positive relationship between the two assays and that both assays detect same biological mechanism, i.e., ER mediated biological responses. It also suggests that the result from the uterotrophic assay can be predicted, in some instances, from the results of the ER binding assay. However, care must be taken to extrapolate *in vitro* data because some important factors, such as the interaction of the ER with other endocrine related systems and metabolism of the test chemical in*in vivo* situation cannot be negligible.

The contingency table analysis of the results from the *in vitro* ER binding and the *in vivo* uterotrophic assays for all 65 chemicals revealed a relatively good concordance ratio (66%). In this comparison, androgens, phthalates and other classes of chemicals were identified as presenting conflicting results in the two assays under the test conditions. Two androgens, testosterone enanthate and 17α-methyltestosterone, were identified as non-ER binders that were estrogenic in the uterotrophic assay. The potential of androgens to stimulate uterine growth in immature female rat is known (Armstrongm et al., 1976). Armstrongm et al. (1976) investigated the effect of testosterone on uterine weight of immature female rat by subcutaneous administration, and clearly demonstrated the increase of uterine weight and the potential of aromatization to convert testosterone to E2. And the enzymatic activity of aromatase in immature female rat has been also observed (el-Maasarany et al., 1991). Testosterone enanthate could be converted to testosterone, i.e. the precursors of estrogens, by hydrolysis in the body. The aromatization of 17α-methyltestosterone to 17α-methylestradiol has been confirmed in *in vitro* assay using human aromatase (de Gooyer et al., 2003). Thus, both testosterone enanthate and 17α-methyltestosterone can be precursors of estrogens and can elevate the estrogen levels caused by aromatization of these administrated androgens, and this would be expected to result in an increase of the uterine weight. At this moment, the metabolic fate of test chemicals in the immature rat uterotrophic assay cannot be estimated precisely and therefore the impact of metabolic system on the inconsistency between these assays cannot be fully explained. Accordingly, the metabolic issue on their assay systems should be extensively explored in the future. *p*-Diethylaminobenzaldehyde that showed the same discrepancy as androgens has been reported as androgen receptor antagonist in the transcriptional activation assay (Araki et al., 2005). But its anti-androgenic effect on the uterotrophic assay is not known and the further investigation may be necessary. There were seventeen chemicals that had ER binding potency but neither estrogenic nor anti-estrogenic activities in the uterotrophic assay. Three benzothiazoles and six phthalates were included among these chemicals. Benzothiazoles seems to be readily metabolized and at least two benzothiazoles that had more than 0.002% of RBA would be metabolized to 2-mercaptobenzothiazole having 0.00165% of RBA (el Dareer et al., 1989; Elfarra and Hwang, 1990; Fukuoka and Tanaka, 1987). In this study, 9 phthalates were tested and 6 of them had ER binding affinity ranging from −3.49 to −1.15 as logRBA. However, none of phthalates elicited estrogenic or anti-estrogenic responses in the uterotrophic assay in this study. Some phthalates showed ER-mediated activities in *in vitro* assays but no estrogenic response in *in vivo* model as shown in this study (Hong et al., 2005; Zacharewski et al., 1998). These discrepancies

between *in vitro* and *in vivo* assays in phthalates are probably caused by the deactivation of phthalates to mono alkyl phthalates (Harris et al., 1997; Picard et al., 2001; Zacharewski et al., 1998). The other chemicals with inconsistent response outliers between the *in vitro* and *in vivo* assay comparison had relatively weak ER binding potencies.

The quantitative comparison found that the 0.00233% of RBA of *p-tert*-butylphenol was the lowest ER binding potency detected in the ER binding assay that elicited estrogenic or anti-estrogenic activities in the immature rat uterotrophic assay and this RBA is considered as the detection limit of estrogenic or anti-estrogenic activities observed in the uterotrophic assay. The use of this cutoff value considerably improved the concordance between the two assays without increasing the false negative rate by excluding the weak ER binders for which estrogenic or anti-estrogenic activities cannot be detected in the *in vivo* assay.

Our studies revealed that the quantitative relationship between the ER receptor binding assay and uterotrophic assay, and the application of cutoff based on meaningful ER binding affinity can provide the best concordance between two assays. These findings are useful in a tiered approach for identifying chemicals that have potential to induce ER-mediated effects in *in vivo*, though it is necessary to consider the metabolic capacity in *in vivo* situation.

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