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:

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

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.

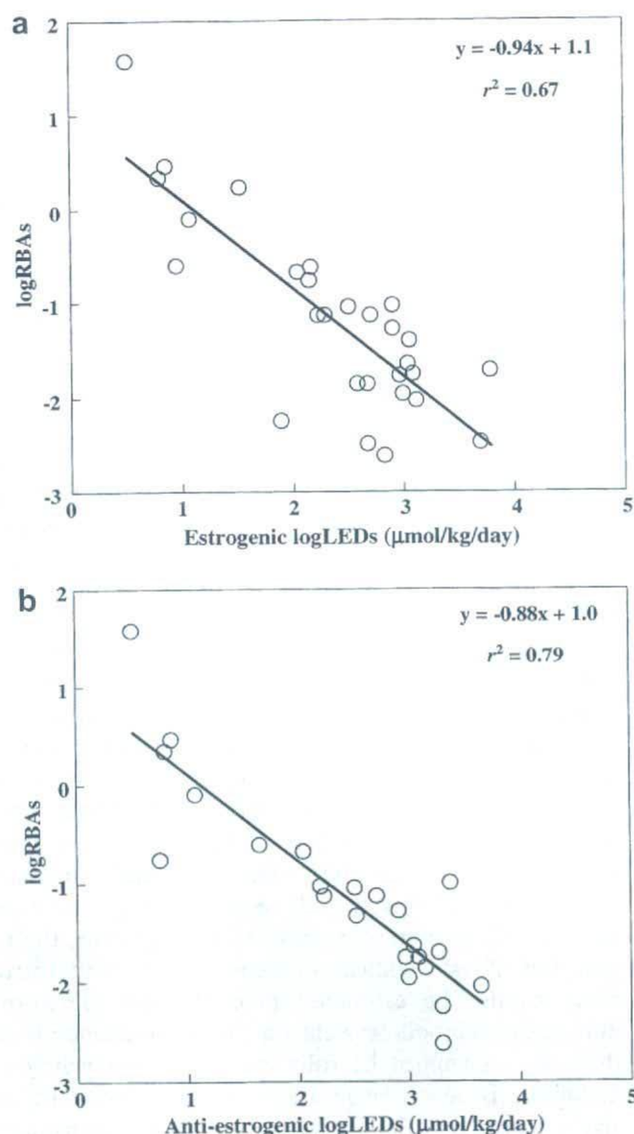

Fig. 1. Relationships between logLED and logRBA values. a, Relationship between estrogenic logLEDs and logRBAs b, Relationship between anti-estrogenic logLEDs and logRBAs.

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.

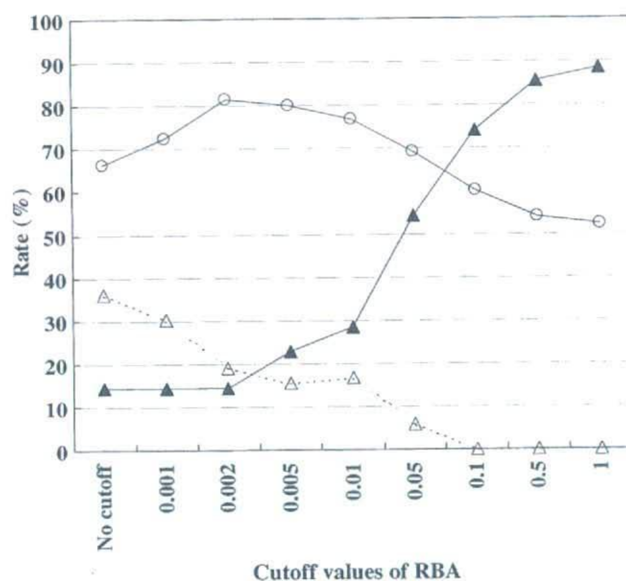

Fig. 2. Changes of indexes by contingency analysis depending on cutoff of RBA. The rates of concordance, false negative and false positive are shown as open circle with solid line, closed triangle with solid line and open triangle with dash line, respectively.

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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The Conformation Change-Sensing Antibodies for Retinoid-Related Orphan Receptor Family

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Rapid and efficient procedures for evaluation of endocrine disrupting chemicals are required extensively in the fields. We have established a novel assay procedure to evaluate simultaneously the binding ability and the biological activity of the particular compound for the target nuclear receptors (NRs). We designated this method as the conformation change-sensing assay. In order to expand this method to all NRs, we initiated the preparation of conformation sensing polyclonal antibodies for retinoid-related orphan receptors.

Keywords: conformation change-sensing antibody, endocrine disruptors, nuclear receptor, retinoid-related orphan receptor (ROR)

Introduction

Endocrine disruptors are thought to mediate some effects or influences through the transcriptional nuclear receptors (NRs). In order to evaluate the binding ability and biological activity of the particular compound for NRs, we have established a novel assay procedure, in which the conformation change along with the ligand binding is measured quantitationaly by antibodies. Antibodies allow for NRs to evaluate simultaneously the receptor binding and hormonal activity of chemicals. Such conformation sensing polyclonal antibodies can recognize the ligand-induced conformation change of helix-12 (H12) of NRs.

Recently, endocrine disruptors have acknowledged to influence on not only the estrogen receptor (ER) but also all other NRs. Thus, it is keen to develop the assay methodology applicable to all of 48 human NRs. In the present study, we prepared polyclonal antibodies specific for H12 of retinoid-related orphan receptor (ROR) family. This family consists of three members of subtypes, RORα, RORβ, and RORγ, which have a wide range of functions including the born metabolism, maintenance of circadian rhythm, and the gain of immunologic function [1, 2]. Therefore, it is important to establish an efficient assay method for RORs' disturbances. We describe here the design and preparation of antigen peptides and the specificity of antibodies prepared.

RORα: --PDI**VRLHFPPLYKELFTSEF**EPAM---
RORβ: --PEI**VNTLFPPLYKELFNPD**STGCK---
RORγ: --PIVVQ**AAFPPLYKELFSTETE**ESPV---

Fig. 1. Amino acid sequences of C-terminal region of ligand binding domain of RORs. The sequences underlined indicate the helix 12, and the bold letters indicate the fragment used as antigen peptide raising specific antibodies anti-ROR-helix 12.

Results and Discussion

The C-terminal region of RORs is highly conserved. In particular, the amino acid sequences of H12 are completely identical. To obtain either specific or nonspecific antibodies for each ROR, H12-containing antigen peptides were designed as shown in Fig. 1. Peptides synthesized by the solid phase method were conjugated to a carrier protein, keyhole (KLH) for immunization. Obtained rabbit polyclonal antibodies were purified by KLH immunoprecipitation followed by affinity chromatography using H12 peptide-linked agarose gel.

To examine the ability of antibodies obtained to bind to the target peptides, competitive ELISA was performed (Fig. 2). RORα-antibody recognized RORβ and RORγ antigen peptides as well as the antigen peptide prepared for RORα itself, suggesting that it recognizes the sequence common to all these RORs. That is definitely H12. RORγ-antibody similarly recognized antigens for RORα and RORβ. On the other hand, RORβ-antibody recognized their own antigen very strongly. Thus, RORβ-antibody recognized the unique sequence of the RORβ-antigen peptide. A combination of these antibodies may afford the structural information to evaluate the receptor activities of chemicals.

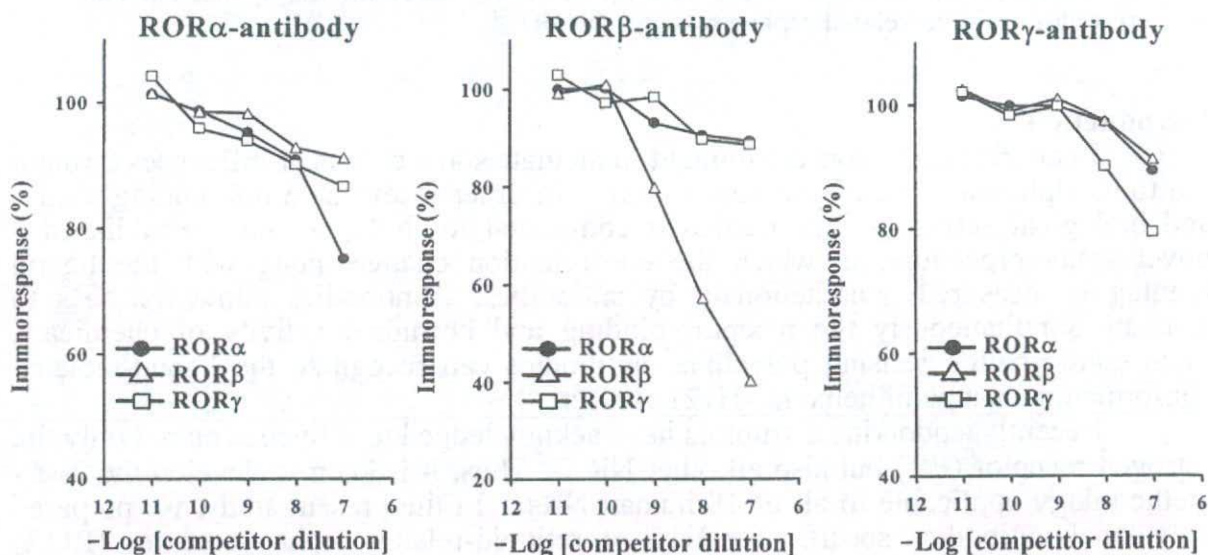

Fig. 2. The results of competitive ELISA. BThG-linked antigen peptides were coated at the varying concentration.

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A Docking Modelling Rationally Predicts Strong Binding of Bisphenol A to Estrogen-Related Receptor γ

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Abstract: A computer-aided docking study was carried out to quickly clarify the binding structure of the ligand-receptor complex between bisphenol A (BPA), a well-known endocrine disruptor, and estrogen-related receptor γ (ERRγ). The resulting complex indicated that BPA binds to the ligand-binding pocket of ERRγ without any disruptions of the activation conformation.

Keywords: Estrogen-related receptor γ, bisphenol A, endocrine disruptors, docking calculation.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are chemicals that mimic the effects of hormones and thereby disrupt endocrine systems. Numerous common industrial chemicals are suspected of being EDCs. Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, is a strong EDC candidate. BPA is now important as a raw material for epoxy resins and polycarbonate plastics. In 1993, Krishnan *et al.* reported that BPA leaked from a flask made of polycarbonates and caused abnormal growth of MCF-7 human breast cancer cells by mimicking the activity of the native estrogen 17β-estradiol (E2) [1]. Also, Gaido *et al.* described that BPA as well as E2 exhibited transactivation activity in a yeast-based estrogen receptor gene transcription assay [2]. Although the activities of these BPAs were much weaker than that of E2 (1/5,000 to 1/15,000 of the activity of E2), BPA was acknowledged as one of the EDCs that act upon estrogen receptor (ER).

Nuclear receptors are a family of 48 or more intracellular receptors in humans. Estrogen-related receptor (ERR) is a subfamily of human nuclear receptors closely related to ER [3–5]. In spite of their high homology to ER, ERR members do not respond to E2, and constitutively activate the transcription in eukaryotic cells. Meanwhile, vom Saal *et al.* have extensively documented numerous low-dose effects of BPA [6]. The low-dose effects of BPA have also been reported by many other groups (for review vom Saal *et al.* [7]); for example, Belcher *et al.* reported that BPA disrupts neural development in the rat fetus [8]. For these low-dose effects of BPA, it has been thought that ER is a target receptor. However, Takayanagi *et al.* reported recently that BPA strongly binds to ERRγ [9]. These results raise the possibility that BPA may be an EDC of ERRγ possessing unidentified activity. Thus, this unpredictable strong binding potency of BPA has underscored the need for development of a new rapid procedure to assess the risk posed to all nuclear receptors.

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As a strategy to screen a large number of chemicals with or without endocrine disruption potentials, studies on the quantitative structure-activity relationship (QSAR) have been carried out, especially for ER [10–12]. Recently, computational docking operation becomes a useful vehicle for investigating the molecular binding interactions [13-27]. Advances in three-dimensional (3D) modeling and docking strategies allow the application of *in silico* structure-based drug design studies (SBDD) to such assessments. These were originally designed to predict how small molecules such as ligands or drug candidates bind to a receptor whose 3D structure has been clarified. Indeed, if an *in silico* EDC screening system based on SBDD was available, such a system could perform a high-speed screening of chemicals against nuclear receptor-LBDs, thereby providing an effective risk assessment without the need for costly and time-consuming wet experiments.

The present study aims to examine the question of how BPA docks with the LBD, based on the fact that BPA shows strong binding activity to ERRγ. Heretofore, the attention has been paid to the sex steroid hormone receptors ER and AR as targets of EDCs. However, the binding of BPA to ERRγ invokes to involve all the nuclear receptors to investigate. In this report, we performed computer-aided docking studies on the BPA and ERRγ-LBD complexes to clarify the structural essentials by which they bind to each other. The complex structure of BPA/ERRγ-LBD, which was calculated in this study, successfully described its high constitutive activity. BPA bound to ERRγ has been found as a quite unique space-filler in the ligand-binding domain.

MATERIALS AND METHOD

3D structures of BPA and other ligands were constructed by the program Sketch, one of the modules of Insight II (Accerlys, San Diego, CA). In order to prepare the receptor molecule appropriately in the docking calculation, hydrogens were added onto heavy atoms identified by X-ray crystallography (1TFC: PDB code), and the charges were assigned by Biopolymer module in the neutral condition. CFF91 force field (Accerlys) was used in all molecular mechanics calcu-

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lations. For calculation of the volume of the ligand-pocket, the 3D structure of apo-form ERRγ-LBD (1TFC) was used [28]. The volume size of vacant ligand-pocket was estimated and determined by means of an active site finding tool called Binding Site Analysis (Accelrys). Using volume keyword, molecular volumes were computed by Gaussian 03 equipped with 6-31G basis set, following energy minimization step [29]. Structural formulas of all the ligands used in this study are shown in Fig. 1.

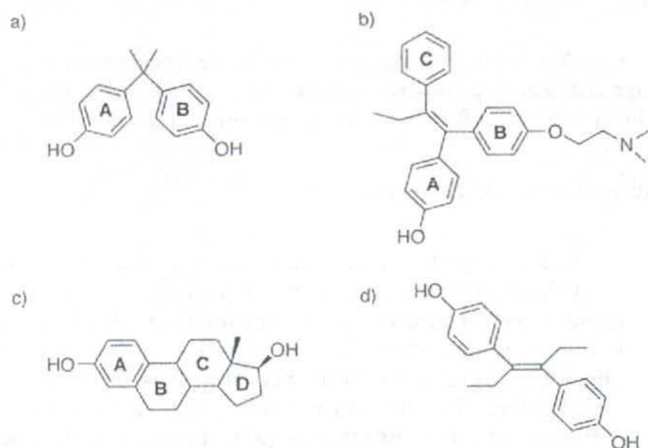


Figure 1. The structural formulas of bisphenol A (BPA), 4-hydroxytamoxifen (4-OHT), 17β-estradiol (E2), and diethylstilbestrol (DES). a: BPA, BPA possesses the two phenol groups A and B together with the two methyl groups. b: 4-OHT, 4-OHT possesses three benzene rings on the trans-ethylene double bond: i.e., A, phenol; B, *p*-β -dimethylethoxyphenyl; and C, phenyl rings. c: E2, and d: DES.

Docking calculations between BPA and ERRγ-LBD (1TFC) were carried out by using Affinity program (Accelrys) in grid docking methodology with CFF91 force field on SGI O2 workstation [30, 31]. The flexible region of the docking calculation includes the BPA molecule initially placed and all the residues in an 8 Å-surrounding distance in the ligand pocket of LBD. BPA was placed at three different positions by referring to the structure of 4-OHT in the 4-OHT/ERRγ-LBD complex (1S9Q) [28].

With the aim of binding energy calculation of BPA in each complex, 6-31G level *ab initio* FMO-MP2 calculations were performed by ABINIT-MP (Advanced Soft, Tokyo, Japan) with BPA and amino acid residues of ERRγ-LBD being within 6 Å from BPA [32-35]. FMO calculations were carried out on a parallel UNIX server, IBM eServer p5 model 595, at the computing and communications center of Kyushu university. The binding energies (ΔE) between BPA and ERRγ-LBD were calculated from the computed results of the FMO calculations by the method described by Fukuzawa *et al.* [36]. Binding energy (ΔE) between BPA and ERRγ-LBD can be expressed in the equation **1** as the difference in each energy value of the receptor ($E_{receptor}$), ligand (E_{ligand}), and complex ($E_{complex}$) [36].

$$\Delta E = E_{complex} - (E_{receptor} + E_{ligand}) \quad (1)$$

RESULTS AND DISCUSSION

ERRγ is a constitutively active and orphan receptor. Although no natural ligand is known, ERRγ is deactivated by DES and 4-OHT [37-39]. To date, the data on five 3D structures of ERRγ-LBD have been deposited in the RCSB-protein data bank. Two of the five structures explain the non-liganded apo-form [28, 40], and the other three structures show the holo-structures of ERRγ-LBD bound with either 4-OHT or DES [28]. In order to discuss the probability of BPA binding with ERRγ-LBD, we first calculated the volume of the ligand-pocket of ERRγ-LBD and the molecular volume of BPA. Since the binding sites of 4-OHT and DES in ERRγ-LBD have been determined to be the same by the X-ray crystal analysis, we first selected the one as a putative ligand binding pocket for BPA. The volume size of vacant ligand space in the apo-form ERRγ-LBD was calculated to be 293.6 $Å^3$. The molecular volume of BPA was computed precisely by using the volume keyword in Gaussian 03 and the calculated volume was 295.2 $Å^3$. Although the program Binding Site Analysis provided several other pockets, their volume sizes were much smaller than BPA's molecular size.

As a result, we could obtain compatible values for volumes of the ligand and the receptor. Based on this finding, BPA was judged to have a sufficient volume to bind to the vacant space of the ERRγ-LBD apo-form.

To examine how BPA binds to ERRγ-LBD, flexible docking calculations were carried out using the program Affinity (Accelrys) with the apo-form ERRγ-LBD (1TFC) as a template [30, 31]. In this study, BPA was manually placed at three different positions by referring to the structure of 4-OHT in the 4-OHT/ERRγ-LBD complex (1S9Q) before the docking calculation [28]. As shown in Fig. **1** and **2**, 4-OHT possesses 3 different aromatic rings, namely, the phenol (A-ring), the *p*-β -dimethylethoxyphenyl (B-ring) and the phenyl (C-ring) on the trans-ethylene double bond. BPA has two phenol groups (A and B) on the sp^3-carbon atom. Placing the A-ring of BPA at the point where the A-ring of 4-OHT is located, we attempted to place the B-ring of BPA at the points corresponding to the point where the B- or C-ring of 4-OHT is located. In addition to these two arrangements, we further attempted to place BPA to take the initial positioning with the B- and C-rings of 4-OHT. From each docking calculation, 5-7 different structures of the BPA/ERRγ-LBD complex were obtained, and their affinity scores are listed in Table **1**.

Complexes **1-7**, **2-3**, and **3-6** gave the best affinity score in each calculation, and we selected these as the representative complexes. When BPA was placed at random in the LBD, the Affinity docking calculations resulted in the structures similar to **1-7** and **2-3**. Since these structures never gave the Affinity scores greater than **1-7** and **2-3**, we just selected the complexes **1-7**, **2-3**, and **3-6** as the structures for further examinations.

Fig. **3** illustrates these selected docked structures of BPA. It should be noted that BPA has almost the same position in each of these docked structures, even though the calculations were initiated from completely different placements of BPA. The binding structure of **2-3** is almost completely compatible to that of **3-6**, although BPA in **1-7** is in a different orientation. In particular, one of the methyl groups of **1-7**, the left

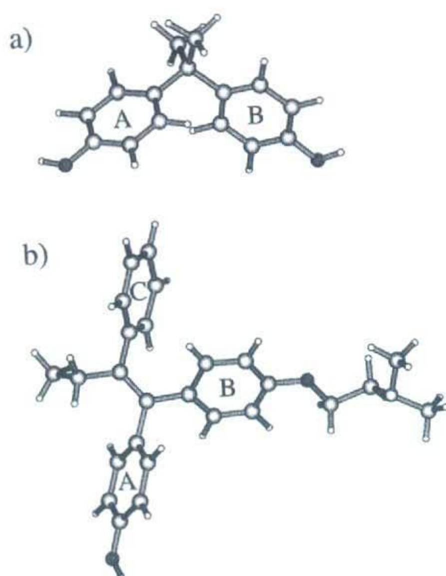


Figure 2. The three dimensional structures of bisphenol A (BPA) and 4-hydroxytamoxifen (4-OHT). a: BPA is shown by its minimum energy conformation. b: 4-OHT is shown by the structure pulled out from the 4-OHT/ERR γ-LBD complex (1S9Q). Characters (A and B in 'a' and A, B and C in 'b') indicated the same ring structures as shown in Fig. **1**.

side methyl group (α in Fig. **3a**), was found to be apart from that of **2-3** (Fig. **3**). As a result, the methyl group α of **1-7** is located in the hydrophobic pocket constructed by Met306, Leu309, and Ile310. On the other hand, the methyl group α' of **2-3** (Fig. **3b**) is in close proximity to the benzene ring of Phe450 (< 3.1 Å), which may be responsible for the CH/π interaction. These results clearly indicate that the pocket or vacant space available for BPA is uniform and there are only a limited number of attachment positions by which BPA can occupy it.

We preliminary carried out the Affinity docking calculations to complex BPA into other templates derived from ERRγ-LBD/4-OHT or DES (1S9Q or 1S9P). However, the resulting BPA-binding structures were found to leave a considerably large empty space, with the activation function (AF)-containing H12 being in deactivation conformation. Apparently, this is inappropriate to explain BPA's high binding affinity and high basal constitutive activity.

For detailed comparison of the binding energies of BPA in these three BPA/ERRγ-LBD complexes, we carried out *ab initio* (HF and MP2 level) calculations by the fragment molecular orbital (FMO) method [32-36]. As shown in Table **2**, HF and MP2 calculations afforded the results of negative ΔE values for BPAs, indicating a structural stabilization due to the ligand binding. Such negative ΔE values reveal that BPA is a favorable binder of ERRγ, as 4-OHT and DES are.

Table 1. Results of the Flexible Docking Calculations of Bisphenol A (BPA) to the ERRγ-LBD apo-form by the Computer Program Affinity

Complex No.[a]	Number of Appearances[b]	Energy (kcal/mol)	Ranking
1-1	9	–864.683	7
1-2	16	–887.407	6
1-3	15	–887.565	5
1-4	13	–890.651	3
1-5	33	–890.461	4
1-6	15	–898.950	2
1-7	13	–898.996	1
2-1	7	–921.715	5
2-2	20	–928.930	4
2-3	18	–930.596	1
2-4	14	–930.595	2
2-5	30	–928.931	3
3-1	6	–842.793	5
3-2	4	–848.302	4
3-3	1	–840.223	6
3-4	1	–880.767	3
3-5	26	–882.895	2
3-6	33	–882.896	1

[a]Complex number **1**, **2**, and **3** represent the calculations started from different initial positionings, respectively. In complex **1**, the phenol rings of BPA are placed at the positions of the A- and B-rings of 4-OHT (see Fig. **2**). Complex **2** is placed in the positions of the A- and C-rings, and complex **3** is placed in the positions of the B- and C-rings.
[b]It means the times appeared as the result in each affinity calculation.

To compare the binding energy of BPA with that of a weak binder, we selected E2, an endogenous ligand of ER. Flexible docking calculations between E2 and ERRγ-LBD (1TFC) followed by FMO calculations were carried out. It was found that E2 exhibits ΔE value of +19.8 kcal/mol, which is much larger than those of binders 4-OHT, DES and BPA in the HF calculation (Table 2). This is a demonstration that E2 is indeed a weak binder of ERRγ. This notably large ΔE value obtained by the HF calculation indicates that there was an unfavorable spatial contact and conformation change along with a complex formation between E2 and ERRγ-LBD. In the calculated E2/ERRγ-LBD complex, the steroid structure of E2 in a planer configuration warped almost 45 degree at the B-ring. The ligand binding pocket of ERRγ-LBD (1TFC) was too small to bind E2.

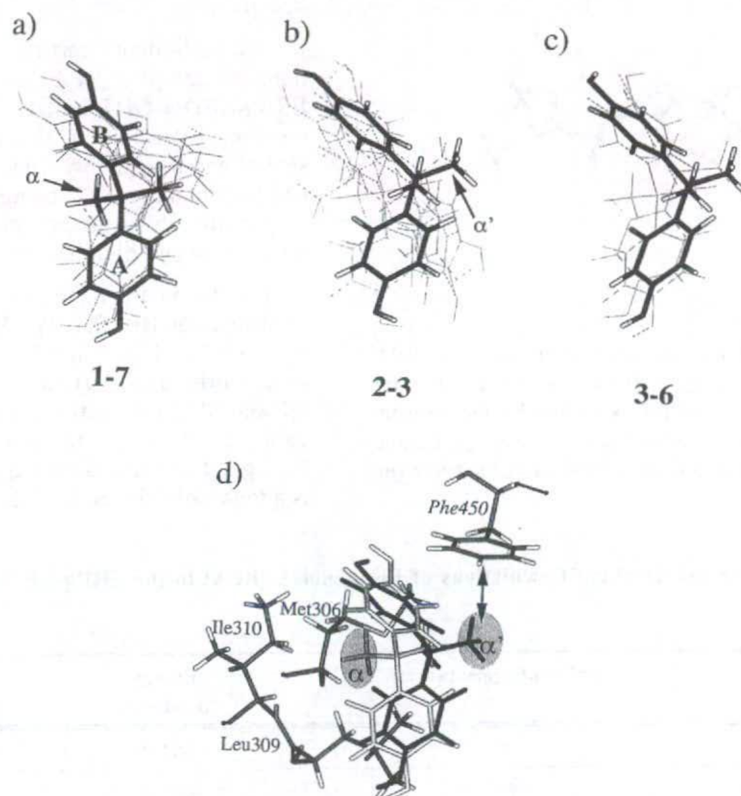


Figure 3. The three-dimensional structures of bisphenol A (BPA) docked in the apo-form ERRγ-LBD. Each structure (a)-(c) was obtained by calculations starting from different dockings with initial placements. The calculations were carried out by the computer program Affinity. **1-7** (a), **2-3** (b), and **3-6** (c) show the structure of BPA (bold sticks) obtained with the best Affinity score (see Table **1**). In each calculation, the other structures of BPA are shown by thin stick lines. A and B are labeled on the two phenol rings of BPA. α: The methyl group on the left side of **1-7**; and α': Another methyl group on the right side of **2-3**. (d) Structural comparison of **1-7** (white molecule) and **2-3** (black molecule). All amino acid residues were from the results of **1-7**, with the only exception being *Phe450* from the results of **2-3**. α and α' are described above.

Table 2. Calculated Binding Energies (ΔE) of the ERRγ Complexes with BPA, 4-OHT, DES and E2 by *ab initio* Calculations

Complex (No.)	ΔE (HF)	ΔE (MP2)
1TFC+BPA (1-7)	−7.90	−57.6
1TFC+BPA (2-3)	−14.4	−68.8
1TFC+BPA (3-6)	−0.14	−48.1
1S9Q (4-OHT)[a,b]	−10.6	−82.4
1S9P (DES)[a]	−4.03	−64.5
1TFC+E2	19.8	−49.3

Energies are in kcal/mol.
[a]Crystal Structure, in which water molecule(s) are ignored. [b]A cholic acid, closely existed with 4-OHT in 1S9Q, regarded as a part of the receptor molecule in the calculations.

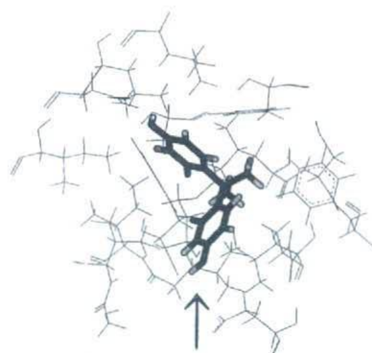
The differences between the HF and MP2 calculations may be due to the exclusion and inclusion of the weak hydrogen bonding, especially the π interaction in calculating the BPA-binding to ERRγ-LBD [41]. The MP2 method practically corrects the electron correlation energy, which was ignored by the HF method. It is clear that, among the three different BPA complexes, **2-3** is judged to be the most stable with the smallest energy values. As shown in Fig. **3**, **2-3** and **3-6** are in almost the same binding conformation, although their apparent binding energies calculated are considerably different (Table **2**). This difference in binding energies is probably due to the difference in the energies of intermolecular interactions involving the phenol-hydroxyl groups. The hydroxyl groups in **2-3** and **3-6** direct towards different receptor sites, and as a result **2-3** won the largest energy stabilization.

These results indicate that, when performing the energy calculations, it is important to compute the electron correlation by involving the π interaction. Since even the E2-ERRγ complex afforded a considerably lower energy value (–49.8 kcal/mol) in the MP2 calculation, the usage of both HF and MP2 methods appears necessary for a correct prediction.

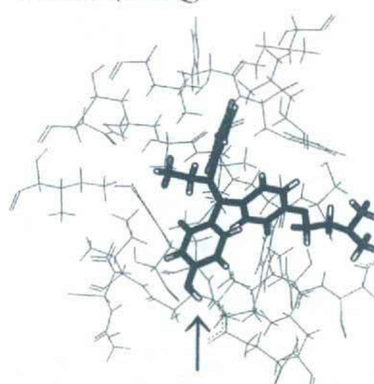
Fig. **4** illustrates the ligand binding site of ERRγ complexed with ligands BPA, 4-OHT and DES. When the most stable complex **2-3** was compared with the 4-OHT and DES complexes, one of the two phenol-benzene rings of BPA, namely, the A-ring, was found to be placed at the same position where the phenol-benzene group (A-ring) of 4-OHT and DES are placed (Fig. **4**). However, another phenol-benzene ring of BPA, the B-ring, is not sitting on the position where the second benzene group of 4-OHT or DES is placed. As seen in (Fig. **5a**), the calculated structure of the BPA/ERRγ-LBD complex, namely **2-3**, well defined to discuss the binding manner of BPA with the ligand binding pocket of ERRγ-LBD. It should be noted that the BPA locates in the activation conformation of ERRγ-LBD. Phe450 present on the inside surface of helix 12 is placed to direct towards the ligand-binding pocket, with the result that the helix is held in a position in which the cofactors can bind correctly. Although Greschik *et al.* reported that antagonism induced by DES and 4-OHT is ascribed to the rotation of the side chain of Phe-435 [39], such a rotation of Phe435, defined as antagonist binding, was not observed in this BPA/ERRγ-LBD complex (Fig. **5b**). Consequently, the characteristics of the binding mode of BPA became prominent, since BPA binds to the ERRγ-LBD apo-form without any disruption to its activation conformation.

In a calculated complex **2-3**, three hydrogen-bondings are present between BPA and LBD. One of the phenol-hydroxyl groups (B-ring) of BPA forms a hydrogen-bond with the side-chain amide group of Asn346 (2.0 Å) and also with the side-chain hydroxy group of Tyr326 (2.5 Å). On the other side, another phenol group (A-ring) forms a hydrogen bond with the side-chain carboxyl group of Glu275 (2.9 Å) and with the side-chain guanidino group of Arg316 (3.1 Å) (Fig. **5b**). It is also identified that another hydrogen bond between the hydroxy group of A-ring and α-carbonyl group of Tyr326 exists (3.5 Å). In the 4-OHT or DES/ERRγ-LBD complex, a water molecule is present near the space of Glu275, and this H_2O participates in the hydrogen bonding with the 4-OHT- and DES-phenol-hydroxyl group. In this study, the docking program utilized ignores or excludes the water molecule while docking calculations occur. If we could simulate water molecules in the calculations, it would

a) BPA (This Study)



b) 4-OHT (1S9Q)



c) DES (1S9P)

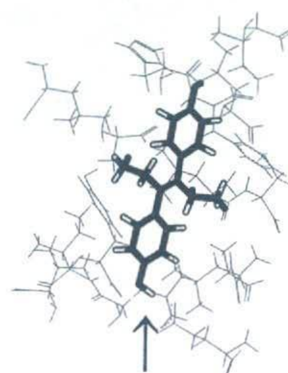


Figure 4. Complex structure of bisphenol A (BPA), 4-hydroxytamoxifen (4-OHT), and diethylstilbestrol (DES) bound to ERRγ-LBD. a) Calculated complex **2-3** in this study, b) 1S9Q, and c) 1S9P. Bold sticks display the ligand molecules BPA, 4-OHT, and DES, respectively. Arrows indicate the position of the phenol-benzene A ring in each ligand.

be possible to predict the binding energies and structures of complexes more precisely.

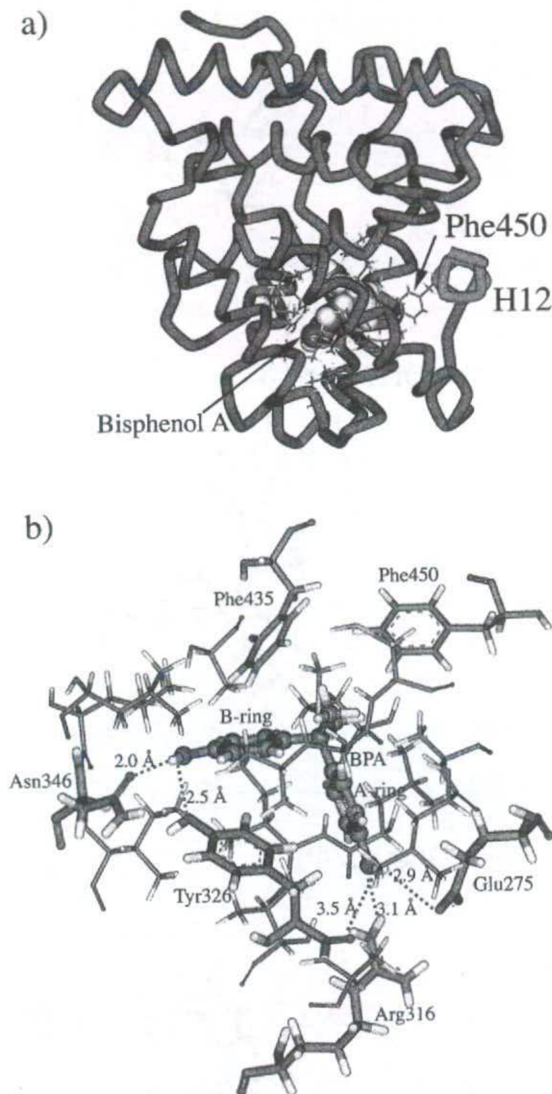


Figure 5. Calculated complex structure of bisphenol A (BPA)/ERRγ-LBD. a) Tube-model of the complex. H12 indicates the number 12 α-helix of ERRγ-LBD. b) The structure of BPA in the ligand-binding pocket. Broken lines (orange) indicate the hydrogen bonds in a reasonable distance. BPA represents a structure **2-3** calculated in Table **1** and Fig. **3**.

In the present study, we described the flexible docking calculation of BPA with ERRγ-LBD, and the results revealed that BPA is a strong binder of ERRγ with high spontaneous constitutive activity. This agrees well with the results reported [6]. Furthermore, we provided evidence that BPA in the complex is indeed a space-filler of ERRγ-LBD. This conformation is characterized by one of the BPA-phenol-benzene rings, which is placed at the vacant space, but not by the placement of the benzene rings of 4-OHT and DES. Consequently, BPA has a unique binding site in ERRγ-LBD. In the future, in order to better understand ERRγ it will be necessary to clarify the roles of BPA binding or of the BPA/ERRγ complex, which sustains a high constitutive activity.

ACKNOWLEDGEMENT

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ABBREVIATIONS

BPA = Bisphenol A
DES = Diethylstilbestrol
EDC = Endocrine disrupting chemical
E2 = 17β-estradiol
ER = Estrogen receptor
ERR = Estrogen-related receptor
HF = Hartree-Fock
LBD = Ligand binding domain
MP2 = Second order Møller-Plesset perturbation theory
4-OHT = 4-Hydroxytamoxifen
QSAR = Quantitative structure-activity relationship
SBDD = Structure-based drug design studies

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Direct Measure of Fluorescence Intensity for Efficient Receptor-binding Assay: Conjugates of Ethinylcarboxyestradiol and 5(and 6)-Carboxyfluorescein via α,ω-Diaminoalkanes as a Tracer for Estrogen Receptor

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Steroidal nuclear receptors (NRs) have been acknowledged as a target binding protein of so-called endocrine disruptors. It is therefore necessary to develop an efficient assay system for screening these endocrine-disrupting chemicals. We here describe the first exemplification of a direct measure of fluorescence intensity for a binding assay of NRs. We designed and synthesized a series of conjugates of 17α-ethinylcarboxyestradiol with carboxyfluorescein, both carboxyl groups of which were cross-linked with α,ω-diaminoalkanes. The resulting fluorescein-linked estradiol derivatives E2(n)cF (n = 2, 4, 6, 8, 10 and 12) were evaluated for their fluorescence and receptor-binding characteristics. E2(4)cF and E2(8)cF exhibited the sufficient binding affinity to the recombinant estrogen receptor (ER) in the radiolabel binding assay using [^{3}H]17β-estradiol, and showed excellent fluorescent characteristics in the fluorescence measurements with and without ER. They exhibited sufficiently large specific binding characteristics with adequate K_d- and B_{max}-values. When these fluorescent ligands were used as a tracer for the binding assay against the ER, assay data of various compounds were shown to be compatible with those obtained from the ordinary binding assay using [^{3}H]17β-estradiol. The present study clearly shows that measurement of fluorescence intensity, instead of fluorescence polarization, affords an adequate receptor-binding assay system.

Key words: endocrine disruptors, estrogen receptor, fluorescence intensity, fluorescent tracer, receptor-binding assay.

Abbreviations: Cbz, carbobenzoxy; DMF, *N,N*-dimethylformamide; DMSO, *N,N*-dimethyl sulfoxide; E2(n)cF, the conjugates between 17α-ethinylcarboxyestradiol (E2) and caryboxyfluorescein (cF) via α,ω-diaminoalkanes -NH-$(CH_2)_n$-NH-; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ER, estrogen receptor; ERR, estrogen-related receptor; ESI, electrospray ionization; EtOAc, ethyl acetate; FAB, fast atom bombardment; GST, glutathione-*S*-transferase; HOBt, 1-hydroxybenzotriazole; HP-TLC, high-performance thin-layer chromatography; LBD, ligand binding domain; MS, mass spectrometry; PBS, phosphate buffer saline; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid and THF, tetrahydrofuran.

INTRODUCTION

With the accomplishment of the human genome project in 2001, it became evident that the nuclear receptors (NRs) form a superfamily of proteins that includes 48 different receptor proteins (*1, 2*). The NRs were first discovered as a binding protein for steroids, thyroid hormones and retinoic acids. The NR elicits a transcriptional activity that is modulated by binding of the agonist or antagonist ligand. This activity affects cell growth and cell differentiation. The estrogen receptors (ERα and ERβ) are a member of the steroid hormone receptor protein family (*3–6*), which includes such receptors as estrogen-related receptors (ERRα, ERRβ and ERRγ), glucocorticoid receptor, mineral corticoid receptor, progesterone receptor and androgen receptor.

ER has been acknowledged as a target binding protein of a number of environmental chemicals called endocrine disruptors. Endocrine disruptors are suspected to cause interference or disorder in the endocrine system, producing undesirable effects on the reproductive system related to fetal development in animals and humans (*7, 8*). These chemicals have such a damaging influence

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upon not only ERs, but also other NRs. Most recently, we have reported that bisphenol A, one of the endocrine disruptor candidates, binds strongly to ERRγ (*9–11*). This finding was confirmed only after many difficulties in establishing a specific receptor-binding assay. It is imperative that an efficient binding assay system be developed to screen for these endocrine disruptors.

Assays using the high specificity of NRs make it possible to quantify many chemicals present in only minute traces in environmental substances or complex biological materials. Efforts have continued toward increasing the sensitivity, specificity and convenience of such assays. The methods depend upon labelling of the ligand being quantified. The general types of label that afford the requisite sensitivity include primarily radiolabels and fluorescence labels. The readout of an assay with radiolabels then finally depends upon a determination of the amount of label present, usually ^{3}H or ^{125}I, by counting the radioactivity. This line of radiolabel receptor-binding assays for ER has been extensively developed during the last 15 years (*12–15*).

In the assays with fluorescence labels, determination of the amount can be given by the fluorescence polarization. The method for measuring fluorescence polarization has been applied to a binding assay for ER (*16–18*). However, this method often faces central problems. For example, the measurement should be dictated by special instrumentation, in which any fluorometer must be equipped with polarization capability to determine binding by steady-state fluorescence polarization measurements. This instrumentation is usually quite expensive, and parts replacement to improve sensitivity or to optimize the emission signals relative to background is often necessary.

Fluorescence polarization is determined principally based on fluorescence intensities polarized either parallel or perpendicular to the direction on the electric vector of the exciting wave (*16*, *17*). It should be noted that the results of an assay with fluorescence labels are to be obtained also by measurement of fluorescence intensity, instead of fluorescence polarization (*19*). To date, no systemic and complete investigations have been carried out to establish the receptor-binding assay based on fluorescence intensity *per se*. This is presumably due to the lack of proper fluorescence labels that can afford sufficient specific binding.

Fluorescent probes or tracers should retain high-receptor specificity in addition to essential fluorescent characteristics. Two different types of fluorescent estrogens have been reported: one is a group of estrogen derivatives in which the fluorophore is connected chemically to the estrogen (*20–27*). However, most of these ligands generally show low-specific binding affinity for the receptor, but high non-specific binding. A group of inherent fluorescent ligands possesses fluorochrome built within the structure of the ligand (*28–34*). This type of ligand usually suffers from suboptimal fluorescence or binding characteristics, and the molecular design is extremely difficult. In the present study, we attempted to identify the best fluorescence label from very common fluorescent estrogens.

Recent X-ray structural analyses have revealed the important structural essentials for the interaction between estrogen ligand (17β-estradiol) and receptor (*35*, *36*). For the design of fluorescence labels, all these structural requirements are to be satisfactorily retained, and we therefore selected 17β-estradiol (E2) as a pharmacophore. Choosing carboxyfluorescein (cF) as a fluorophore, we decided to prepare the E2-cF conjugates by cross-linking with a series of α,ω-diaminoalkanes with varying methylene chain lengths (Fig. 1). We here describe the synthesis and characterization of these fluorescence ligands and their usage in a binding assay for the ER.

MATERIALS AND METHODS

Materials—17α-Ethinylestradiol and *m*-cresol were purchased from Wako (Osaka) and methyllithium in diethyl ether was from Kanto Chemicals (Tokyo). The 5(and 6)-Carboxyfluorescein *N*-succinimidyl ester was obtained from Molecular Probes (Leiden, The Netherlands) and thioanisole and α,ω-diaminoalkanes ($n = 2$, 4, 6, 8, 10 and 12) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Carbobenzoxy chloride (Cbz-Cl) and *N*,*N*-dimethyl sulfoxide (DMSO) were purchased also from Tokyo Kasei. All other chemicals of the best grade available were obtained from several different sources. Tetrahydrofuran (THF) was purified by distillation from sodium benzophenone, and *N*,*N*-dimethylformamide (DMF) was of the grade suitable for peptide synthesis (Kanto Chemicals).

Recombinant Human Estrogen Receptor α Ligand-binding Domain—The recombinant human estrogen receptor α ligand-binding domain (ERα-LBD) was expressed as a glutathione-*S*-transferase (GST)-fused protein. The LBD was ligated into a prokaryotic expression vector pGEX-4T1 (Amersham Pharmacia Biotech, Amersham, Bucks, UK) at the *Bam*HI and *Not*I sites. *Escherichia coli* BL21α transformed with the expression plasmid was cultured in 1 l of L-broth containing 0.16 mg/ml of ampicillin, and protein expression was induced by isopropyl 1-thio-β-D-galactoside. The cells were harvested by centrifugation (3,000*g*, 10 min, 4°C) and resuspended in 4 ml of 50 mM Tris–HCl (pH 8.0) containing 50 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol. After sonication and centrifugation (17,800*g*, 30 min, 4°C), a soluble fraction was loaded to the affinity column of Glutathione-Sepharose 4B (Amersham Pharmacia Biotech). After incubation for 60 min at 4°C, the column was washed three times with phosphate buffered saline containing 0.2% (v/v) Triton X-100 (PBST), and the fusion protein was eluted with PBST containing 20 mM reduced glutathione.

Chemical Synthesis and Characterization—Methods—High-performance thin-layer chromatography (HP-TLC) was carried out on silica gel 60 (Merck, Frankfurt, Germany) with the following solvent systems (v/v): R_f, $CHCl_3$-MeOH-AcOH (50:10:2). For structural verification, ^{1}H-NMR spectra, ESI mass spectrometry (MS) (Micro Mass Quatro-2 spectrometer) and/or FAB MS spectra (JEOL SX/SX 102A tandem mass spectrometer) were measured.

Figure 1 shows the synthetic scheme of fluorescein-labelled estrogens. The synthesis includes four different reaction steps, as follows: i.e. step 1, the carboxylation of 17α-ethinylestradiol by CO_2 under the catalytic MeLi; step 2, the coupling of 17α-ethinylcarboxyestradiol with *N*-Cbz-α,ω-diaminoalkanes; step 3, deprotection of

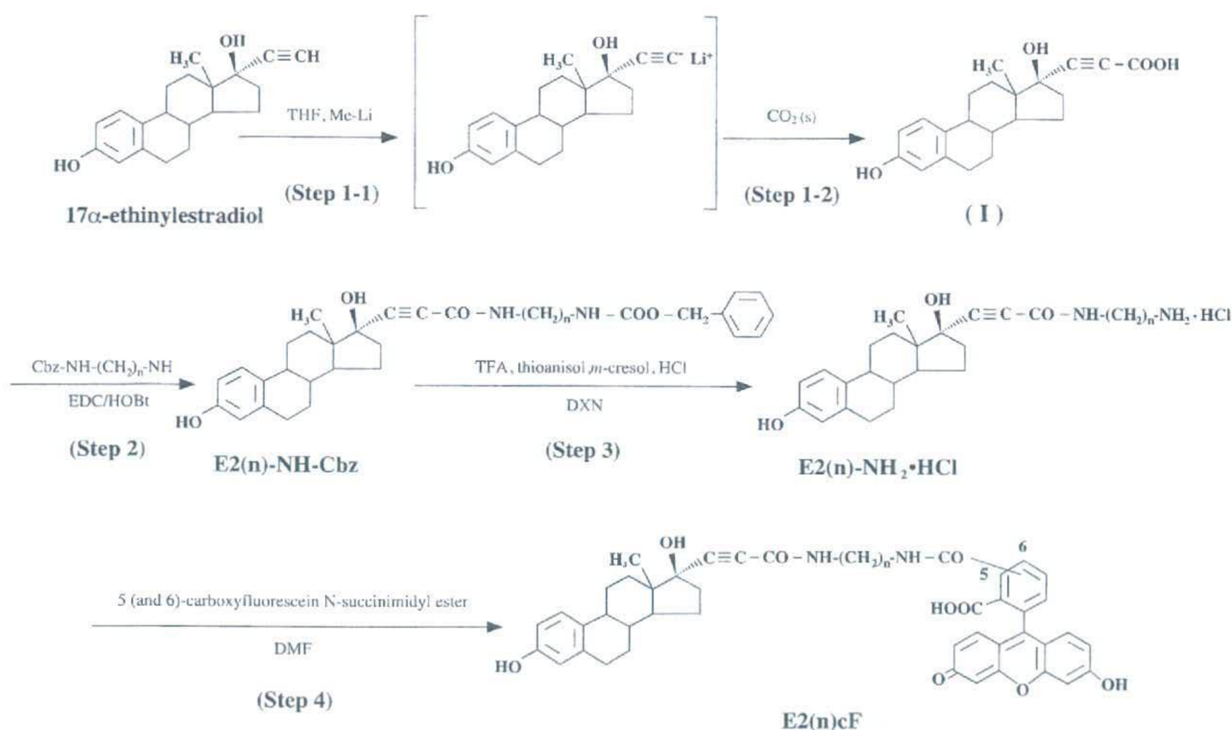

Fig. 1. **Synthetic scheme of the conjugates of 17α-ethinylestradiol and carboxyfluorescein via α,ω-diaminoalkanes H_2N-$(CH_2)_n$-NH_2 (*n* = 2, 4, 6, 8, 10 and 12).**

the Cbz group by trifluoroacetic acid (TFA) in the presence of scavengers and step 4, the coupling with carboxyfluorescein.

17α-Ethinylcarboxyestradiol (I)—Under the condition of dry N_2 pressure, a solution of 17α-ethinylestradiol (4.60 g, 15.5 mmol) in THF (155 ml) was added to a solution of 1.14 M MeLi (46.5 mmol) in ether (40.8 ml) at approximately −70°C, and the solution was stirred for 30 min at the same temperature. Small pieces of dry ice (~300 g) were added to the reaction mixture in 30 min. During this addition, the solution was vigorously mixed with a magnetic stirrer, and the temperature was kept under −10°C. After removing the cooling bath, the reaction was continued for an additional 2 h at room temperature. The resulting white suspension was poured into 5.5 M ammonium chloride (20 ml), and the product, namely 17α-ethinylestradiol carboxylic acid denoted as 17α-ethinylcarboxyestradiol, was extracted with aq. NaOH. The alkaline solution was washed with ether to remove unreacted 17α-ethinylestradiol, and acidified with conc. HCl. The resulting solid was extracted with ethylacetate (EtOAc), and the solution was washed with saturated NaCl and dried over Na_2SO_4. After evaporation, the product 17α-ethinylcarboxyestradiol **I** was recrystallized from MeOH. Yield, 5.10 g (96%); HP-TLC, R_f 0.32; ^{1}H NMR (DMSO-d_6) δ 0.78 (s, 3H, CH_3), 5.76 (s, 1H, 17β-O*H*), 6.44–7.07 (m, 3H, Ar*H*), 8.98 (br, 1H, Ar O*H*); MS (FAB) m/z M^+ 340.24 (Calcd. for $C_{21}H_{24}O_4$, 340.4).

N-Cbz-α,ω-diaminoalkanes—A series of *N*-Cbz-α,ω-diaminoalkanes was prepared by the single carbobenzoxylation of α,ω-diaminoalkanes essentially as reported (*37*), but with some modifications. In this study, target compounds were obtained by careful purification of the reaction products. As a representative example, the synthesis of *N*-Cbz-1,6-diaminohexane (Cbz-NH-$(CH_2)_6$-NH_2) is described. To a solution of 1,6-diaminohexane (11.6 g, 100 mmol) in MeOH (100 ml) was added dropwise Cbz-Cl (14.3 ml, 100 mmol) at 0°C. The resulting precipitate (mostly *N,N*-diCbz-diaminohexane) was filtered off, and the filtrate was acidified with 3.6% HCl. The precipitate, mainly *N*-Cbz-1,6-diaminohexane, was collected by filtration. This precipitate was washed with ether and 4% $NaHCO_3$ and eventually treated with 4 M HCl/dioxane to afford the hydrochloride. *N*-Cbz-1,6-diaminohexane was also recovered from the filtrate. After extraction with ether to remove the dicarbobenzoxylated derivative, the filtrate was neutralized with 4% $NaHCO_3$ and extracted with ether. The organic solution was washed with saturated NaCl and dried over Na_2SO_4. After evaporation, 4 M HCl/dioxane was added dropwise to afford the product of Cbz-NH-$(CH_2)_6$-NH_2·HCl, namely, *N*-Cbz-1,6-diaminohexane hydrochloride. The combined yield was 7.00 g (24%). All other products (*n* = 2, 4, 8, 10 and 12) were prepared in a similar way.

17α-Ethinylcarboxyestradiol-N-Cbz-α,ω-diaminoalkanes (E2(n)-NH-Cbz)—17α-Ethinylcarboxyestradiol I (340 mg, 1.0 mmol), 1-hydroxybenzotriazole (HOBt: 168 mg, 1.1 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl: 211 mg, 1.1 mmol) were added to a solution of *N*-Cbz-1,6-diaminohexane hydrochloride (287 mg, 1.0 mmol) and Et_3N (140 μl, 1.0 mmol) in DMF (100 ml) at 0°C. The reaction mixture was stirred for 2 h at 0°C and overnight at room temperature, and then evaporated *in vacuo*. The residue was crushed into fine

Table 1. **Physicochemical properties of E2(n)-NH-Cbz, 17α-ethinylcarboxyestradiol-*N*-Cbz-α,ω-diaminoalkanes.**

Compounds E2(n)-NH-Cbz	Yield (%)	Mp (°C)	R_f[a]	m/z (M+H$^+$)	
				Found	Calcd.
E2(2)-NH-Cbz	82	106–108	0.23	517.3	517.6
E2(4)-NH-Cbz	86	93–94	0.19	545.2	545.7
E2(6)-NH-Cbz	81	84–85	0.26	573.3	573.7
E2(8)-NH-Cbz	98	70–71	0.37	601.3	601.8
E2(10)-NH-Cbz	96	91–92	0.44	629.4	629.8
E2(12)-NH-Cbz	98	79–80	0.46	657.4	657.8

[a]$CHCl_3$-MeOH-AcOH (50:10:2, v/v).

Table 2. **Physicochemical properties of E2(n)-NH_2·HCl, 17α-ethinylcarboxyestradiol-α,ω-diaminoalkanes hydrochloride.**

Compounds E2(n)-NH_2·HCl	Yield (%)	Mp (°C)	R_f[a]	m/z (M+H$^+$)	
				Found	Calcd.
E2(2)-NH_2·HCl	61	108–109	0.24	382.1	382.5
E2(4)-NH_2·HCl	89	93–94	0.23	411.2	411.6
E2(6)-NH_2·HCl	92	83–84	0.20	439.3	439.6
E2(8)-NH_2·HCl	83	69–70	0.25	467.3	467.7
E2(10)-NH_2·HCl	59	89–90	0.24	495.4	495.7
E2(12)-NH_2·HCl	63	80–81	0.28	523.4	2523.8

[a]$CHCl_3$-MeOH-AcOH (50:10:2, v/v).

powder in water. This rather viscous solid was washed successively with 4% $NaHCO_3$, 5% $KHSO_4$ and water, and then dissolved in EtOAc. The solution was dried over Na_2SO_4. After evaporation, the residue was recrystallized from MeOH–water to obtain the final compound E2(6)-NH-Cbz. All other E2(n)-NH-Cbz (n = 2, 4, 8, 10 and 12) products were prepared in a similar way. The yields and mass numbers of the compounds are shown in Table 1.

17α-Ethinylcarboxyestradiol-α,ω-diaminoalkanes hydrochloride (E2(n)-NH_2·HCl)—17α-Ethinylcarboxyestradiol-*N*-Cbz-1,6-diaminohexane E2(6)-NH-Cbz (1.15 g, 2.0 mmol) was dissolved in a mixture of TFA (80 ml), thioanisole (9.34 ml, 80 mmol), and *m*-cresol (8.37 ml, 80 mmol) and the solution was stirred for 4 h at room temperature. After evaporation, the residue was dissolved in ether, and the solution was treated with 4 M HCl/dioxane. The resulting precipitate was washed with pet. ether by decantation, and recrystallized from ether to obtain the final compound E2(6)-NH_2·HCl: yield, 0.89 g (92%). All other E2(n)-NH_2·HCl (n = 2, 4, 8, 10 and 12) products were prepared in a similar way. The yield, melting point and mass number of the compounds are shown in Table 2.

17α-ethinylcarboxyestradiol-α,ω-diaminoalkane-carboxyfluorescein (E2(n)cF)—The solution of 17α-ethinylcarboxyestradiol-α,ω-diaminohexane hydrochloride E2(6)-NH_2·HCl (48 mg, 0.1 mmol) in DMF (1 ml) was neutralized with Et_3N (14 µl, 0.1 mmol), and 5(and 6)-carboxyfluorescein *N*-succinimidyl ester (57 mg, 0.12 mmol) was added. The reaction mixture was stirred overnight at room temperature. After water (100 µl) was added to consume the unreacted *N*-succinimidyl ester, the solution was purified directly by gel filtration using a column (2 × 140 cm) of LH-20 eluted with DMF. Fractions

Table 3. **Physicochemical properties of E2(n)cF, 17α-ethinylcarboxyestradiol-α,ω-diaminoalkane-carboxyfluorescein.**

Compounds E2(n)cF	Yield (%)	Mp (°C)	R_f[a]	m/z (M+H$^+$)	
				Found	Calcd.
E2(2)cF	42	248–250	0.67	741.3	740.8
E2(4)cF	42	208–210	0.67	769.2	768.8
E2(6)cF	34	198–200	0.64	797.2	796.9
E2(8)cF	29	189–191	0.74	825.5	824.9
E2(10)cF	61	154–156	0.86	852.7	853.0
E2(12)cF	52	142–144	0.87	881.1	881.0

[a]$CHCl_3$-MeOH-AcOH (50:10:2, v/v).

containing the product were collected, and the solution was evaporated in vacuo. The residue was solidified with H_2O, and the obtained precipitate was finally purified by reverse-phase high-performance liquid chromatograph (RP-HPLC). The final product of 17α-ethinylcarboxyestradiol-α,ω-diaminoalkanes-5(and 6)-carboxyfluorescein are designated as E2(n)cF hereafter, in which the number 'n' denotes the chain length of the linker polymethylene. E2(6)cF was obtained as a white powder: yield, 12 mg (34%). All other E2(n)cF (n = 2, 4, 8, 10 and 12) products were prepared in a similar way. The purity was verified by HP-TLC, analytical RP-HPLC and ESI-MS (Table 3).

Radio-ligand Receptor-binding Assay—In order to assess the binding activity for the ER, a series of the conjugates E2(n)cF (n = 2, 4, 6, 8, 10 and 12) were examined for their ability to inhibit the receptor binding of tritium-labelled estrogen. ER preparations used in this binding study were a full-length protein expressed in Sf9 cells (a kind gift from Sumitomo Chemical Co., Ltd) and a ligand-binding domain expressed in *E. coli* as a GST-fused protein. A solution (10 µl) of the recombinant estrogen receptor ERα-LBD (~1 nM) was added to Tris–HCl (pH 7.4, 70 µl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 10 mg/ml BSA, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. After a sample solution (10 µl) of E2(n)cF and 10 nM [^{3}H]17β-estradiol (10 µl; 4.4–6.6 TBq/mmol, Amersham, Buckinghamshire, UK) was added, the reaction mixture (100 µl in total) was incubated for 1 h at room temperature. The compounds were dissolved in DMSO, the final concentration of which was adjusted to not exceed 3%.

Free radioligand was removed by centrifugation (10 min, 17,500g) after incubation with 0.4% dextran-coated charcoal (Sigma) in 0.2 M PBS (pH 7.4) for 10 min at 4°C. Scintillation counting of the supernatant was performed to measure [^{3}H]17β-estradiol bound to the receptor. To estimate the binding affinity, the IC_{50} values, the concentrations for the half maximal inhibition, were calculated from the dose-response curves evaluated by the non-linear analysis program ALLFIT (*38*).

Optimization of Receptor Concentration for Change in Fluorescence Intensity—The fluorescence intensity of E2(n)cF (n = 2, 4, 6, 8, 10 and 12) would be different under conditions with and without the ER, if the fluorescein moiety interacts non-specifically or specifically with the receptor. A solution (100 nM, 20 µl) of recombinant human ERα, which was expressed as

a ligand-binding domain protein (ERα-LBD) at a concentration of ~10 μM, was dissolved in Tris–HCl (pH 7.4, 160 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. After a sample solution dissolved in DMSO (20 μl) was added, the reaction mixture (200 μl in total) was incubated for 1 h at room temperature. Free estradiol derivatives were removed by incubating with 0.04% or 0.4% dextran-coated charcoal (Sigma) in 0.2 M PBS (pH 7.4, 200 μl) for 10 min at 4°C. All these experiments were performed on a 96-well polypropylene plate (Nunc A/S; Roskilde, Denmark), and the solution contained 0.5 mg/ml bovine γ-globulin to prevent adsorption of compounds to the plate wall. Plate centrifugation was carried out at 4°C for 10 min over 3,000*g* on a KUBOTA 6200 Centrifuge with a PF21 plate rotor (Kubota Co., Tokyo).

For measurement of fluorescence intensity, 200 μl of solution in each well was transferred into another 96-well FluoroNunc polystyrene plate (Nunc), a plate suitable for fluorometry measurements, by using an 8-channel dispenser (Nichiryo Co., Tokyo). The plate was placed on a microplate reader, the Wallac 1420 ARVOsx (Perkin Elmer, Turku, Finland), to measure the fluorescence intensity. Estimation of the emissions at 535 nm with excitation at 485 nm was carried out with the excitation energy around 10,000–40,000 for 1–3 s (see RESULTS section). Non-specific binding of fluorescein-linked estradiol derivatives was assessed by adding 10 μM of 17β-estradiol.

Fluorescence Measurement of E2(4)cF and E2(8)cF in the Presence of Estrogen Receptor—For E2(4)cF or E2(8)cF (500 nM), estrogen receptor ERα-LBD at 100, 250 and 500 nM concentrations was added at room temperature. Emission spectra were observed by the excitation at either 485 or 500 nm on a Spectrofluorometer FP-550A (Jasco, Tokyo).

Saturation Binding Assay of Fluorescein-ligand—The binding of fluorescent ligands to recombinant estrogen receptor ERα-LBD in the presence or absence of unlabelled estradiol was examined. A solution (100 nM, 20 μl) of ERα-LBD was dissolved in Tris–HCl (pH 7.4, 140 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 0.5 mg/ml bovine γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. After a fluorescent ligand (0.1–1 mM; 20 μl each) was added, the reaction mixture (200 μl) was incubated for 1 h at room temperature. Free estradiol derivatives of E2(4)cF or E2(8)cF were removed by incubating with 0.4% dextran-coated charcoal in 0.2 M PBS (pH 7.4, 200 μl) for 10 min at 4°C. Plate centrifugation was carried out at 4°C for 10 min as described above, and the fluorometry measurements were carried out with an excitation energy of 40,000 for 3 s. Non-specific binding was assessed by adding 10 μM 17β-estradiol.

Competitive Receptor-binding Assay by Using a Fluorescence Probe—To assess the ability of a compound to bind to the ER, the competitive binding assay was constructed by using the fluorescent ligand developed in the present study. A solution (100 nM, 20 μl) of the recombinant ERα-LBD was dissolved in Tris–HCl (pH 7.4, 140 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 0.5 mg/ml bovine γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. After a sample solution (20 μl) and fluorescent ligand E2(4)cF or E2(8)cF (50 nM, 20 μl) were added, the reaction mixture (200 μl) was incubated for 1 h at room temperature. After B/F separation with 0.04% dextran-coated charcoal followed by plate centrifugation at 4°C, the supernatant was used for fluorometry measurements carried out with an excitation energy of 40,000 for 3 s. Dose–response curves were assessed by the computer program ALLFIT. The binding affinity of the compounds was estimated as IC_{50} values, which exhibit the concentration for the half-maximal inhibition.

RESULTS

Chemical Synthesis of Fluorescein-linked 17α-Ethinylcarboxyestradiols—Figure 1 shows the scheme for a four-step synthesis of conjugates of 17α-ethinylcarboxyestradiol and CF, starting from 17α-ethinylestradiol. Cross-linking was performed by α,ω-diaminoalkanes ($n = 2$, 4, 6, 8, 10 and 12) in order to obtain a conjugate with an optimized methylene chain length for enhanced specific binding to the ER. Thus, '*n*' indicates eventually the number of methylene chains between estradiol moiety and fluorescein in the conjugates. The use of α,ω-diaminoalkanes with varying methylene chain lengths was eventually judged to be an important and critical issue to obtain the best fluorescent tracer for the receptor-binding assay.

The most difficult synthesis was carboxylation of 17α-ethinylestradiol, namely, the step 1. According to the method described by Carlson *et al.* (*34*), carbon dioxide (CO_2) gas was used first. However, almost no reaction occurred, and we therefore used dry ice in small pieces instead of CO_2 gas. This made the reaction in THF proceed very smoothly, but only under strict control of the reaction temperature. We kept the reaction temperature strictly under −10°C during the initial 30 min, which resulted in a considerably high-reaction yield (81–98%).

17α-Ethinylcarboxyestradiol-*N*-Cbz-α,ω-diaminohexane E2(*n*)-NH-Cbz ($n = 2$, 4, 6, 8, 10 and 12) was prepared from 17α-ethinylcarboxyestradiol **I** and *N*-Cbz-α,ω-diaminoalkanes ($n = 2$, 4, 6, 8, 10 and 12). Although removal of the Cbz group was carried out by TFA containing thioanisole and *m*-cresol, the TFA salt could not be crystallized. Instead, the HCl salt was obtained successfully by treatment with 4 M HCl/dioxane. For purification of the final compounds, namely, carboxyfluorescein-linked 17α-ethinylcarboxyestradiol E2(*n*)cF, there were several problems to be solved. Because of their extremely high hydrophobic nature and thus their serious adsorption nature to the gel, the compounds could not be recovered very well from the column. For RP-HPLC, the sampling solution could be prepared only by using 75% acetic acid. Although E2(*n*)cF exists as a mixture of isomers, the cross-linker of which is attached to either position 5 or 6 of fluorescein, we could not separate these isomers, even on HPLC.

Receptor-binding Affinity of Fluorescein-linked 17α-Ethinylcarboxyestradiols—The binding affinity of synthesized conjugates E2(*n*)cF for the ER was evaluated

Table 4. **Receptor-binding affinity of the conjugates of 17α-ethinylcarboxyestradiol with carboxyfluorescein cross-linked by α,ω-diaminoalkanes and their intermediate precursors.**

Chain length (n) of polymethylene	Receptor binding affinity, IC_{50} (μM)		
	Cbz-protected derivatives E2(n)-NH-Cbz	Amino-free derivative $E2(n)-NH_2 \cdot HCl$	Fluorescent ligands E2(n)cF
2	0.29 ± 0.12	1.60 ± 1.2	12.0 ± 8.0
4	0.16 ± 0.15	0.72 ± 0.30	2.0 ± 2.0
6	0.27 ± 0.20	0.89 ± 0.10	1.8 ± 1.2
8	0.71 ± 0.11	0.44 ± 0.11	0.19 ± 0.15
10	0.75 ± 0.10	0.48 ± 0.13	0.90 ± 0.62
12	0.74 ± 0.42	0.10 ± 0.12	0.53 ± 0.23

The IC_{50} values for derivatives are the averages ± SEM of at least three separated experiments. In this assay, using 10 nM [^{3}H]17β-estradiol (4.4–6.6 TBq/mmol) and GST-fused ER-LBD, B/F separation was performed with 0.4% dextran-coated charcoal. To estimate the binding affinity, the IC_{50} values, the concentrations for the half maximal inhibition, were calculated from the dose–response curves evaluated by the non-linear analysis program ALLFIT.

first by the conventional binding assay using [^{3}H]17β-estradiol as a tracer. It was found that 17α-ethinylestradiol (IC_{50} = 0.87 nM) was able to bind to the ER as strongly as 17β-estradiol (IC_{50} = 1.20 nM). However, its carboxylic acid derivative drastically reduced the affinity (IC_{50} = 25 μM, approximately four orders of magnitudes weaker than 17β-estradiol). This extremely diminished activity may have been due to the steric hindrance or the electrostatic effects of the attached carboxyl group, apparently being unfavourable in binding to the receptor. It was reported that the receptor-binding affinity of 17α-substituted estradiol derivatives is reasonably low (*39–41*). Indeed, it was found that the receptor-binding affinities of 17α-ethinylestradiol cross-linked with α,ω-diaminoalkanes and its *N*-Cbz derivatives are only moderately high, although they are apparently more potent than their parent carboxylic acid derivatives (Table 4). These results suggest that the hydrophobic interaction between the methylene chain and the receptor is superior to that of free carboxylic acid. The diaminoalkane derivatives lacking a fluorophore did not show a binding affinity dependent upon methylene chain lengths (Table 4).

When the final compounds of carboxyfluorescein-linked 17α-ethinylcarboxyestradiol derivatives E2(*n*)cF were assayed, it was found that they bind quite strongly to the ER in a dose-dependent manner. The receptor-binding affinity of a series of compounds was found to be dependent upon the lengths of the polymethylene chains–$(CH_2)_n$– of the linkers. Since carboxyfluorescein itself was absolutely inactive in this binding assay, it is unlikely that carboxyfluorescein binds to the binding pocket of 17β-estradiol. In a series of E2(*n*)cF, E2(8)cF with 1,8-diaminooctane (*n* = 8) exhibited the highest affinity to the receptor (IC_{50} = 186 nM, Table 4). This relatively enhanced binding activity is probably due to the moderately enhanced receptor interaction of carboxyfluorescein at a site other than the E2 binding pocket. Octamethylene–$(CH_2)_8$–is to occupy an optimum distance between E2 and cF, which must be positioned somewhere

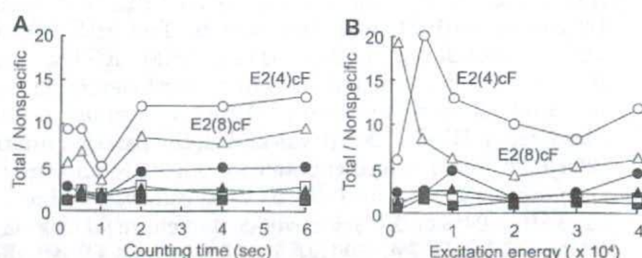

Fig. 2. **Effects of the fluorescence excitation energy and counting time on the specific and non-specific bindings of fluorescent ligands E2(*n*)cF.** Each 100 nM fluorescent ligand E2(*n*)cF, namely, E2(2)cF (filled circle), E2(4)cF (open circle), E2(6)cF (filled triangle), E2(8)cF (open triangle), E2(10)cF (filled square), E2(12)cF (open square), was incubated with ER (10 nM) at room temperature for 1 h either with (non-specific binding) or without (total binding) 17β-estradiol (E2: 10 μM). Specific binding to estrogen receptor was estimated by the subtraction of non-specific binding from total binding. Fluorescence intensity was measured after removal of unbound free fluorescent ligand by using 0.4% (w/w) dextran-coated charcoal. Fluorescent ligand was individually excited at 485 nm, and the fluorescence intensity was monitored at 535 nm. (A) The intensity ratio of total binding to non-specific binding was analysed under different counting times (seconds). (B) The intensity ratio of total binding to non-specific binding was analysed under different excitation energies.

near the binding pocket of E2. Pseudo-specific binding of cF to the receptor would be substantiated with hydrophobic and electrostatic interactions, since the cF moiety has a few functional groups. Nonetheless, it should be noted that E2(*n*)cF is considerably potent to bind to the ER.

Determination of Fluorescent Probe for Receptor-binding Assay—In order to use the conjugates of E2(*n*)cF as a fluorescent probe of the receptor-binding assay for ER, each fluorescent ligand was carefully evaluated for their fluorescence characteristics and receptor-binding characteristics. First of all, in order to optimize the measurement conditions of the fluorometry, the excitation energy and counting time were examined for all E2(*n*)cF (*n* = 2, 4, 6, 8, 10 and 12) (Fig. 2). When the intensity ratio of the total binding versus non-specific binding was plotted against either the counting time or the excitation energy, it was found that E2(4)cF and E2(8)cF provide specific-receptor binding that is clearly higher than others. For stable fluorometry measurements, we selected three different instrumentation conditions; i.e. a combination of excitation energy and counting time, 10,000/1 s, 20,000/2 s and 40,000/3 s, respectively.

In the fluorometry measurements, the fluorescence intensity decreased gradually to reach the equilibrium constant. This was prominent when the time-course of change in the specific binding of E2(4)cF to the ER was plotted in the presence or absence of 17β-estradiol (Fig. 3). During the initial several minutes after centrifugation for B/F separation, the solutions exhibited a rapid decline in fluorescence intensity. It takes ~10 min to reach a steady state. Consequently, the sample solutions were measured for their fluorescence intensity at more than ~10 min after the B/F separation. In this B/F separation, we found that dextran-coated charcoal (dry preparation) should be