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Combination of receptor-binding assays and designed mutant receptors for discerning agonists and antagonists

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Abstract

Competitive receptor-binding assays are convenient for analyzing interactions between receptors and their ligands and for screening pharmaceutical drugs and potential endocrine-disrupting chemicals. Although these assays can be used for high-throughput screening, they cannot discern antagonists and agonists. Based on three-dimensional structures of complexes between ligand-binding domain of human estrogen receptor-α and its ligands, we designed mutant receptors with modified mode of ligand-binding. In the current studies we examined the binding of endogenous ligands, artificial ligands, and potent endocrine-disrupting chemicals to wild-type and Asp351 mutants of the human estrogen receptor-α ligand-binding domain.

The new combination assay showed the decrease of relative biding affinity (RBA) values for antagonists. For example, RBA for tamoxifen was changed from 4.8 (using the Asp351 receptor) to less than 1.5 (using the Glu351 receptor). On the other hand, the agonists showed increase of RBA values. For example, RBA for bisphenol A was changed from 0.011 (using the Asp351 receptor) to less than 0.030 (using the Glu351 receptor). The variation of RBA was dependant on the type of mutant receptors. The change of RBA from wild-type to mutant-type can be an index for discerning agonists and antagonists. Comparison of RBA values obtained by assays using wild-type and mutant receptors is a simple way of discerning agonists and antagonists, and this approach could be extended to other types of receptors, if information of the receptors was enough to construct a designed mutant receptor.

Keywords: Receptor binding assay; Pharmaceutical drug; Endocrine disrupting chemicals; High-throughput screening; Antagonist; Agonist; Ligand binding domain; Human estrogen receptor; Mutant receptors; Relative binding affinity

1. Introduction

Antagonists for nuclear receptors have proven to be effective therapeutics, especially for the treatment of cancer. For example, tamoxifen and raloxifene are selective estrogen receptor modulators that work, at least in part, by antagonizing estrogen binding [1,2]. In addition to their use in the treatment of cancer, such estrogen receptor antagonists are beginning to be used for the treatment of cardiovascular diseases and osteoporosis [3,4].

Precisely some compounds have antagonist and agonist activity. For example, tamoxifen acts not only as an antagonist but also as an agonist of the estrogen receptor, although generally it is classified as antagonist. This agonist activity results in undesirable side-effects such as a promotion of uterine cancer. Raloxifene has much weaker agonist activity than tamoxifen and is thought to be a better choice for the treatment of cancer. In addition, new more selective antagonists are still desired [5].

The molecular effects of inhibiting and activating nuclear receptors have recently been the subject of intense investigation. For such investigations, many kinds of nuclear receptors have been produced using recombinant techniques [6–10], and some of these have been crystallized [11–16]. Receptor antagonism depends not only on the structure of the ligand-binding site but also on the transactivation sites where cofactor proteins bind. Information on the structure of the receptor–ligand complex is, however, essential for the design of receptor antagonists. For example, the crystal structures of the human estrogen receptor-α (hERα) ligand-binding domain (LBD) with raloxifene, diethylstilbestrol, 17β-estradiol, and tamoxifen reveal that Glu353, Arg394, and His524 play important roles in agonist effects, whereas Asp351 is important for antagonist effects [11,13,17].

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Although it is possible to determine which amino acids are important for agonist or antagonist effects, it is difficult to differentiate a compound as a whole is agonist or antagonist with a receptor-binding assay, and *in vivo* tests or *in vitro* assays using living cells or animals may be necessary [18–20]. Also, yeast two-hybrid assays can be used to distinguish antagonists and agonists, but these experiments are laborious and time-consuming compared to receptor-binding assays. Furthermore, *in vivo* tests and *in vitro* tests using living cells have other difficulties; for example, the effects of test compounds can depend on the animal species, the environmental conditions, the cell line, and complications of growing cells in culture. Also, the induction of metabolic enzymes such as cytochrome P450s can affect *in vivo* tests.

In the current studies, we adopted maltose binding protein (MBP) as a fusion partner [21] because of its superior characteristics including stability and solubility. Using MBP fusion receptors which can be easily designed for our purposes, we tried to investigate the possibilities to differentiate a compound as a whole with a combination of two receptor-binding assays and also tried to find an index for distinction.

2. Materials and methods

2.1. Chemicals and culture media

[^{3}H]-17β-estradiol (E2) was purchased from NEN Life Science. Bacterial culture media, yeast extract, and bacto-tryptone were from Difco. All other chemicals were purchased from Nacalai tesque and Sigma–Aldrich.

2.2. Modified pMalc2e vector

To prevent cleavage of the linker peptide in the pMalc2e vector (New England Biolabs) by bacterial proteases, the Lys in the sequence Asp-Asp-Asp-Asp-Lys-Val-Pro-Glu-Phe was converted to Glu using the primers shown in Table 1 and the QuickChange^{TM} Site-directed Mutagenesis Kit (Stratagene).

2.3. Construction of expression vectors for wild-type and mutant receptors

The hERα LBD cDNA fragment (Fig. 1) was generated by PCR using *Pfu* turbo DNA polymerase (Stratagene), primers HERABAMH1F and HERSAL1R (nucleotides 759–1785; amino acids 253–595; Table 1), and PCR-Ready Human cDNAs (Maxim Biotec), which were derived from human placenta. The PCR fragment was digested with *Bam*HI and *Sal*I (Stratagene) and ligated into the modified vector using T4 ligase (Stratagene), generating pHERAKM2. After that, wild-type pHERAKM2 was modified by site-directed mutagenesis using the following primer sets: ERAE351F1/ERAE351R1 to generate the Asp351Glu mutant, ERAG351F/ERAG351R to generate Asp351Gly, ERAS351F/ERAS351R to generate Asp351Ser, and ERAN351F/ERAN351F to generate Asp351Asn.

2.4. PCR

PCR for human ERα LBD cDNA fragment was carried out with 35 cycles of 30 s of denaturing (95 °C), 30 s of annealing (57 °C), and 85 s of extension (72 °C) and using the *Pfu* turbo DNA polymerase and the supplied buffer (Stratagene).

2.5. Sequencing

DNA sequencing was performed using an ABI PRISM Big Dye Terminator Cycle Sequencing Kit, an ABI PRISM Model 310, and sequencing primers SEQM1, SEQM2, SEQM3, and SEQER1 (Table 1). The sequences were confirmed at least twice. The constructed vectors did not have any unexpected or artificial mutations [23].

2.6. Transformant

Escherichia coli (JM109) was transformed with the expression vectors by using calcium chloride competent cells. Transformants were purified, isolated, and frozen as glycerol stocks at −80 °C [22].

Table 1
Oligonucleotide primers used for sequencing, vector modifications, PCRs

Primer name	Sequence
PMUT1F	5′-GGGGATGACGATGACGAGGTACCGGAATTCGG-3′
PMUT1R	5′-CCGAATTCCGGTACCTCGTCATCGTCATCCCC-3′
HERABAM1F	5′-CGCGCGGGATCCATGGATCCAGGTGGGATACGAAAAGACCGAAGA-3′
HERASAL1R	5′-ATGCCCGTCGACTCAGACTGTGGCAGGGAAACCCTCTGCCTCCC-3′
SEQM1	5′-CTGGGTGCCGTAGCGCTGAAGTCT-3′
SEQM2	5′-GGTCGTCAGACTGTCGATGAAGCC-3′
SEQM3	5′-CGCCAGGGTTTTCCCAGTCACGAC-3′
SEQER1	5′-GATGGGCTTACTGACCAACCTGGC-3′
ERAE351F1	5′-CTGACCAACCTGGCAGAGAGGGAGCTGGTTCAC-3′
ERAE351R1	5′-GTGAACCAGCTCCCTCTCTGCCAGGTTGGTCAG-3′
ERAG351F1	5′-CTGACCAACCTGGCAGGAAGGGAGCTGGTTCAC-3′
ERAG351R1	5′-GTGAACCAGCTCCCTTCCTGCCAGGTTGGTCAG-3′
ERAS351F1	5′-GGCTTACTGACCAACCTGGCATCTAGGGAGCTGGTTCAC-3′
ERAS351R1	5′-GTGAACCAGCTCCCTTCTTGCCAGGTTGGTCAGTAAGCC-3′
ERAN351F1	5′-CTGACCAACCTGGCAAACAGGGAGCTGGTTCAC-3′
ERAN351R1	5′-GTGAACCAGCTCCCTGTTTGCCAGGTTGGTCAG-3′

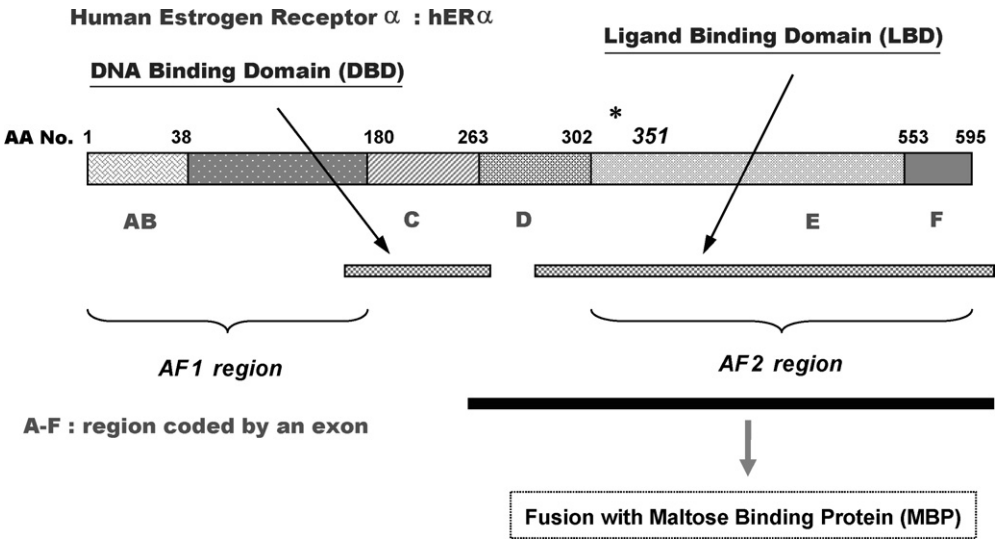

Fig. 1. An illustlation of sequences for human estrogen receptor α. The exons of the receptor are indicated as A–F. The amino acid numbers are shown above each receptor sequence bar. The regions of DNA binding domain (DBD) and ligand-binding domain (LBD) of each receptor are shown as thin gray bars. Regions fused with maltose binding protein (MBP) also are shown as thin black bars. The 351st amino acid of hERα LBD is changed for mutant receptors.

2.7. Production of fusion proteins

Transformed cells expressing MBP-receptor fusions were grown overnight at 37 °C in SB media (10 g/L sodium chloride, 30 g/L bacto-tryptone, and 20 g/L yeast extract) containing 100 μg/mL ampicillin. Next, 1 mL of the overnight culture was added into a 2-L flask containing 500 mL of SB medium supplemented with 100 μg/mL ampicillin and 20 mM glucose and grown at 37 °C with rotation at 150 rpm until the OD_{600} reached 0.8–1.2. Protein expression was induced by adjusting the culture to 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 30 °C with shaking at 150 rpm.

2.8. Isolation of fusion proteins

Cells expressing fusion proteins were harvested by centrifugation for at 5000 × *g* for 10 min at 9 °C. The bacterial pellets were resuspended in ice–PEGV buffer (50 mM PIPES, pH 7.4, 1 mM sodium ethylenediamine acetic acid, 10% glycerol, 1 mM sodium vanadate, 150 mM sodium chloride, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mM dithiothreitol: DTT) supplemented with 0.1% (w/v) lysozyme. The solution was mixed with polyethyleneimine solution (0.004%, w/v), and insoluble components were removed by filtration through a glass-fiber filter (Whatman's GF/D). The nucleotides were then precipitated from the filtrate by centrifugation at 5000 × *g* for 20 min. This crude extract was used in some receptor-binding assays. For other experiments, the supernatant was precipitated with 40% ammonium sulfate, and the precipitate was resuspended in a one-fifth volume of the supernatant before precipitation. Finally, the resuspended solution was centrifuged at 5000 × *g* for 30 min at 4 °C to remove insoluble components. This partially purified receptor solution was adequately diluted with PEGV and used for the following receptor-binding assays.

2.9. Receptor-binding assay

The partially purified receptor solution including was diluted in PEGV buffer containing 0.2% (w/v) porcine gelatin along with 25 μL of 30 nM [^{3}H]-labeled E2 and 25 μL of test chemical solution (E2, tamoxifen, hydroxyl-tamoxifen, daidzein, genistein, 17-epi-estrone, diethylstilbestrol, bisphenol A, or nonylphenol; Fig. 2). After a 3 h incubation at 4 °C, the reactions were terminated by adding 50 μL of dextran–charcoal matrix solution (5% (w/v) activated charcoal (Nacalai Tesque) and 0.5% (w/v) dextran T70 (Amersham Pharmacia Biotech) in PEGV without sodium vanadate). After 3 min at 4 °C, the mixture was centrifuged at 1000 × *g*, and 200 μL of the supernatant was immediately transferred to a scintillation vial containing 2 mL of Clear-sol I (Nacalai tesque) and counted with a Packard 1900TR scintillation counter. All experiments were repeated two to four times. The coefficient of variation values in the experiments were within 15%.

3. Results and discussion

All designed mutants retained ligand-binding activity and mutations at Asp351 had little effect on the binding of the ERα LBD to E2 (Fig. 3a). Inhibition curves for each of the tested chemicals are shown in Fig. 3b–d as examples. The inhibition profiles for the antagonists, tamoxifen and hydroxyl-tamoxifen were greatly affected by the mutations on Asp351 (Fig. 3c and d). Table 2 shows the concentrations giving 50% inhibition values (IC_{50}) for wild-type (Asp351) and mutant receptors (Table 2).

We next calculated the relative binding affinity (RBA) for each of the compounds and the various fusion receptors using mean values of IC_{50} as follows: RBA (%) = 100 × {IC_{50} (E2)/IC_{50} (tested chemicals)} [24] (Table 3). The RBA values of Glu351 become greater than those of Asp351 (wild-type)

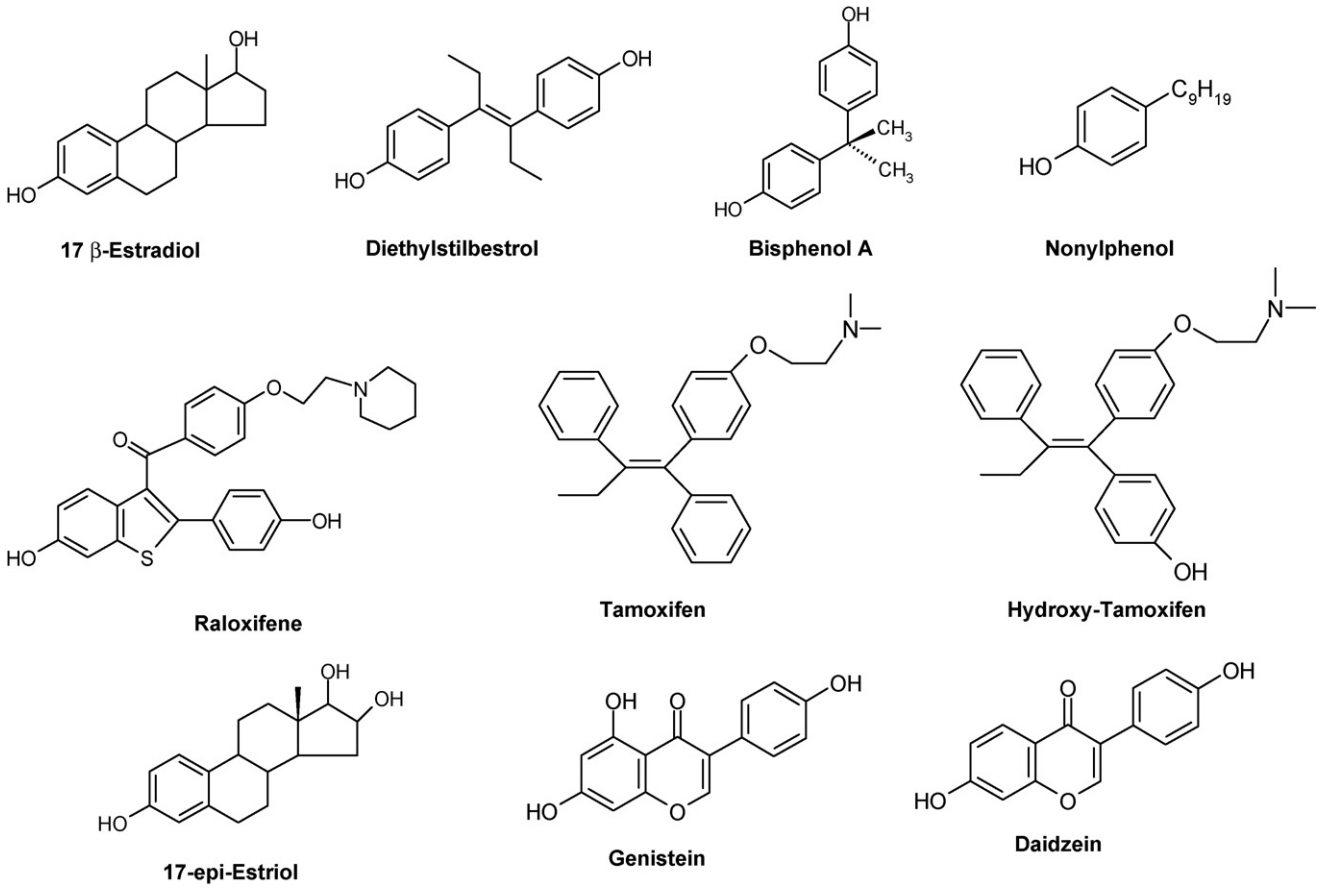

Fig. 2. The chemical structures of tested compounds.

for diethylstilbestrol, bisphenol A, nonylphenol classified as endocrine disruptors and agonists. The RBA values of Glu351 smaller than those of wild-type for OH-tamoxifen and tamoxifen classified as strong antagonists as a whole molecule, and for genistein and daizein classified plant phytoestrogens and antagonists, and for 17-epi-estriol that is a metabolite of β-estradiol. As for OH-tamoxifen, tamoxifen and genistein, we confirmed the decrease of RBA against Glu351, Asn351, Ser351, and Gly351 designed receptors. Bispenol A and nonylphenol showed the increase of RBA against Glu351, Asn351, Ser351, and Gly351 receptors. The results of RBA for daizein were dependent on the designed receptors (increase by Ser351, Gly351 mutants and decrease by Glu351, Asn351 mutants). The deference may reflect an electric charge of amino acid side chain on the position 351 of ERα.

Glu351 or Asn351 mutant showed the good correspondence to the general classification of agonists and antagonists for the ERα receptor. Those designed mutant receptors have a possibility to discriminate a compound as a whole.

To discern agonists and antagonists by our combination assays using wild-type and designed receptors, the change of RBA value is important. For our purpose the Glu351 receptor is

Table 2
IC_{50} values using wild type or designed type receptors

Chemical	Receptor				
	Asp351	Glu351	Asn351	Ser351	Gly351
Estradiol	2.2 ± 0.2	3.0 ± 0.1	2.5 ± 0.2	2.2 ± 0.1	2.3 ± 0.2
OH-tamoxifen	5.0 ± 0.2	10.8 ± 1.8	8.4 ± 0.8	9.4 ± 1.1	13.3 ± 3.7
Tamoxifen	46.3 ± 1.0	>200	>200	>200	>200
17-epi-Estriol	2.5 ± 0.1	>5	2.6 ± 0.3	2.7 ± 0.6	3.3 ± 0.1
Diethylstibestrol	3.1 ± 0.3	3.9 ± 0.4	3.8 ± 0.2	3.3 ± 0.2	3.5 ± 0.2
Genistein	110 ± 10	350 ± 10	160 ± 10	120 ± 20	90 ± 10
Daidzein	2070 ± 280	4000 ± 90	2770 ± 520	1790 ± 40	1610 ± 340
Bisphenol A	19300 ± 500	10000 ± 1650	6770 ± 330	6000 ± 2000	5800 ± 1100
Nonylphenol	3790 ± 120	1900 ± 110	2580 ± 260	1900 ± 170	1940 ± 70

Asp351 = wild-type form, Glu351, Asn351, Ser351, Gly351 = mutant-type form.

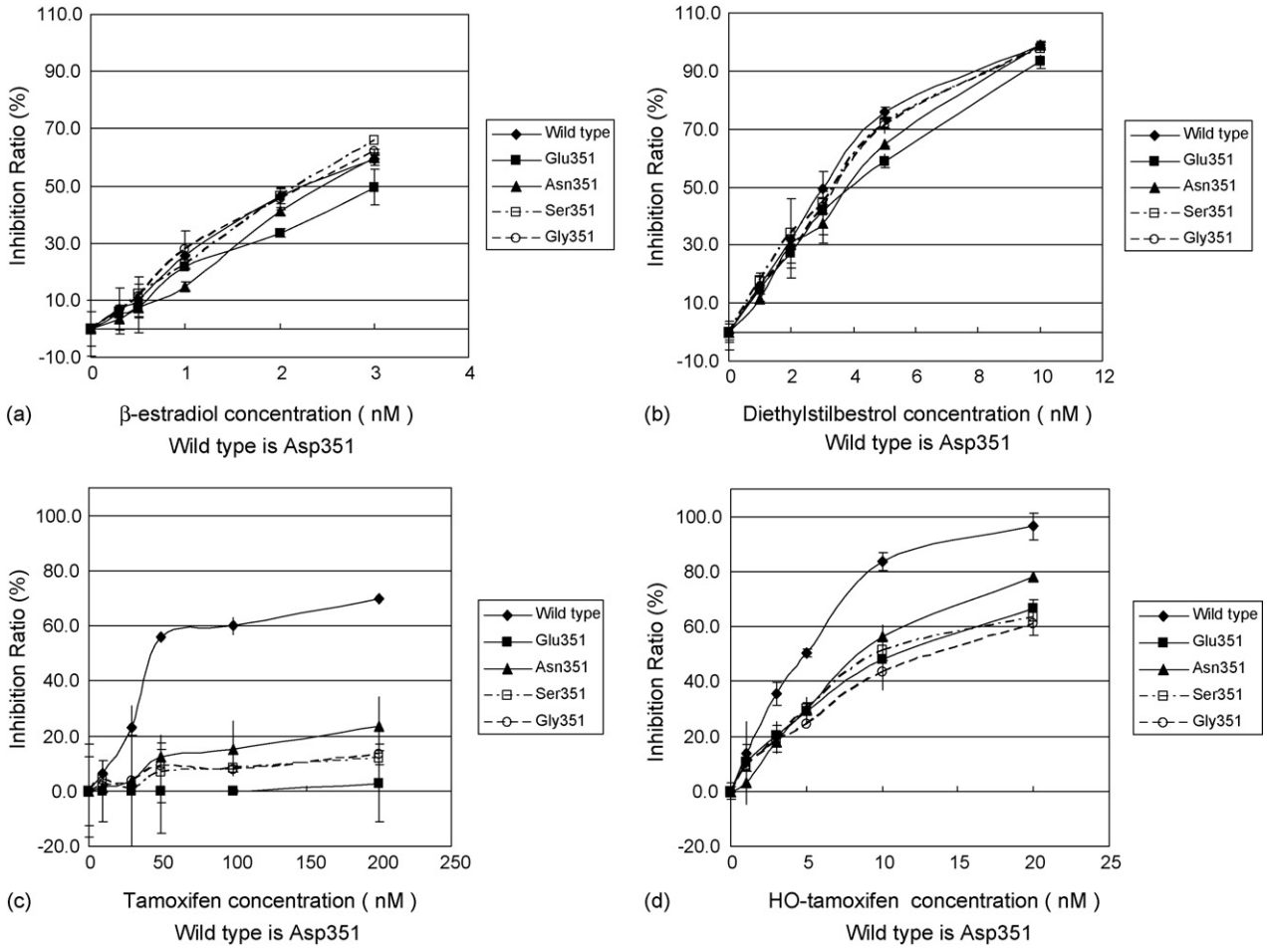

Fig. 3. Inhibition curves of each cold ligand against MBP-hERα LBDs. Each cold ligand has been competed with ^{3}H estradiol 3 nM. (a) β-estradiol, (b) diethylstibestrol, (c) tamoxifen, (d) hydroxyl-tamoxifen (OH-tamoxifen). Wild-type is Asp351 and mutant receptors are Glu351, Asn351, Ser351, Gly351.

most adequate because it shows the change of RBA values most clearly for tested chemicals. We propose the index of {RBA value (Glu351 mutant)/RBA value (Asp351 wild-type)} for discerning. If the index of chemical A is over 1.3, chemical A is antagonist as a whole molecule. If the index of chemical B is under 1.3, it is agonist as a whole. The value of 1.3 proposed from limited data, must be adjusted by additional enough amount of data.

By the way, antagonism of receptor may occur by mechanisms related to binding of compounds to other sites. For example, some of antagonists could affect dimerization of hERα, binding to AF regions of hERα, or affect the binding between hERα DNA binding domain and the estrogen response element (ERE) regions of genes. For complete comprehension of antagonism, transactivation in AF regions and dimerization of receptors and other interactions must be considered. The

Table 3
RBA values using wild type or designed type receptors

Chemical	Receptor				
	Asp351	Glu351	Asn351	Ser351	Gly351
Estradiol	100	100	100	100	100
OH-tamoxifen	44.0	<27.8	<29.8	<23.4	<17.3
Tamoxifen	4.75	<1.50	1.25	1.10	1.15
17-epi-Estriol	88.0	60.0	96.2	81.5	69.7
Diethylstibestrol	71.0	84.6	65.8	66.7	65.7
Genistein	2.00	0.86	1.56	1.83	2.56
Daidzein	0.106	0.075	0.063	0.123	0.143
Bisphenol A	0.011	0.030	0.037	0.037	0.040
Nonylphenol	0.058	0.158	0.097	0.116	0.119

Asp351 = wild-type form, Glu351, Asn351, Ser351, Gly351 = mutant-type form.

mode of binding between the LBD and ligands is, however, a still and more important issue because specific ligands on LBD site are better drugs that can control functions of ERα specifically. Compounds that effect on other sites are difficult to be controlled well and they are not so specific to ERα. The new superior drug without any side-effects will be created by sufficient knowledge about the LBD site and its known ligands.

Our results should be tested by enough compounds, however, our purpose is not to define a compound strictly as a final conclusion, but to propose a tool which can screen enormous amount of compounds rapidly. That is requested for the first drug screening and endocrine disruptor screening that must test thousands of compounds. Our tool can provide much more information compared to usual competitive assays. It includes the strength of binding to the target and rough information about agonist or antagonist activity as a whole molecule. We admit our approach may not be able to define a compound completely and find exceptions if we tested a large amount of chemicals, however, situation is same as the quantitative structure–activity relationship approach. After we recognize exceptions and problems, for example, we will be able to modify or reconstruct or adjust our method to cover much more chemicals.

To design adequate mutant receptors like Glu351 ERα is also possible in case of other types of receptors. Much information of three-dimensional (3-D) structures of target proteins and various approaches have been reported. The information includes the mode of binding between receptors and their ligands. To find key amino acids of receptor functions and design new mutant receptors, we can utilize such 3-D information. Instead of the information in 3-D structures of complexes, obtained by crystallization and X-ray analysis, such knowledge can be provided from docking simulations [25] for a wild-type of receptor and its ligands or predicted structures built from sequences of a receptor using computational methods like structure-based drug design approaches improved recently and spread widely [26].

4. Conclusion

The RBA of designed mutant receptors may be useful for identifying antagonists; specifically, if the RBA of a compound for the mutant receptor is smaller than that for the wild-type receptor, the compound classified as an antagonist, whereas if the values are greater, it is estimated as an agonist. We are currently examining the effects of a variety of other chemicals by our system to attempt to validate these preliminary findings.

It may be possible to extend these methods to the β-type estrogen receptor or other receptors, although the following must be known: (1) the three-dimensional structure; (2) the mechanism of interaction between the native target protein and its ligands; and (3) modes of binding between the receptor and a typical antagonist or agonist. (1)–(3) are required to know the key amino acids to be changed. If enough structural information is available, specific mutants can be designed that can allow discernment of agonists and antagonists. This should facilitate the characterization of newly synthesized compounds as a first screening test without the need for complicated cell culture or *in vivo* assays.

Further experiments using a larger number of compounds must be performed to confirm that the RBA values can be used to discern agonists and antagonists of hERα. Also we noticed the Asp351 may be better choice for finding antagonists but not the best amino acid for investigating agonists. Other residues also have a possibility.

Finally, in the current studies, we used [^{3}H]-labeled compounds for the binding assays, but fluorescent methods are now available and can be useful for developing high-throughput assays [27] as well as other non-radio isotope methods. We therefore plan to explore the use of such fluorescent methods after we confirm the initial results reported here.

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