

Research Paper

The estrogen receptor: a structure-based approach to the design of new specific hormone–receptor combinations

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Abstract

Background: The specificity of hormone action arises from complementary steric and electronic interactions between a hormonal ligand and its cognate receptor. An analysis of such key ligand–receptor contact sites, often delineated by mutational mapping and X-ray crystallographic studies, can suggest ways in which hormone–receptor specificity might be altered.

Results: We have altered the hormonal specificity of the estrogen receptor α (ER) by making ‘coordinated’ changes in the A-ring of the ligand estradiol and in the A-ring binding subpocket of ER. These changes were designed to maintain a favorable interaction when both E and ER are changed, but to

disfavor interaction when only E or ER is changed. We have evaluated several of these altered ligand and receptor pairs in quantitative ligand binding and reporter gene assays.

Conclusions: In best cases, the new interaction is sufficiently favorable and orthogonal so as to represent the creation of a new hormone specificity, which might be useful in the regulation of transgene activity. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Estrogen receptor; Hormone specificity; Receptor specificity reengineering

1. Introduction

The specificity that is inherent in biological regulatory systems can usually be traced to the specific recognition of a hormone by its cognate cellular receptor, and is thought to arise, at least in part, from the complementary shape and functional interactions in contact regions between the hormone and receptor. In the nuclear hormone receptors, a large superfamily of ligand-activated transcription factors, hormone–receptor specificity is illustrated by the highly specific binding and activating characteristics shown by the receptors from different subfamilies. For example, while estrogens and androgens activate their cognate receptors at subnanomolar hormone concentrations, cross-receptor activation typically requires 10 000–100 000

higher concentrations of the heterologous hormone. Curiously, certain receptor mutations can alter hormone–receptor specificity. For example, compared to wild type androgen receptor (AR), certain AR mutants have expanded binding preference for androgens [1]. In the progesterone receptor (PR), a single residue is responsible for pronounced species differences in antagonist binding [2], and a C-terminal truncation gives a mutant that binds antagonists but not agonists [3]. Similarly, two C-terminal mutations in the estrogen receptor (ER) have a similar effect on antagonist vs. agonist binding [4–6], and other mutations invert the activity of estrogens and anti-estrogens [7]. Although these mutants were characterized some time ago, the molecular basis for their altered specificity can now be analyzed within the context of recently described antiparallel α -helical triple sandwich structures of the ligand binding domains (LBD) of these receptors [8]. Also, the utility of such specificity-shifted nuclear hormone receptors in regulating the activity of transgenes has not been overlooked (see Section 3) [9–11].

Prior to the availability of X-ray crystal structures of

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steroid receptor LBDs, we investigated in some detail the nature of the hormone specificity of ER and AR by mutagenesis. Through alanine scanning mutagenesis studies on ER, we had concluded that the A-ring phenol of the ligand was positioned near portions of helices 3 and 5 [12,13]. It seemed most likely that the phenol was interacting with a glutamate residue (E353) in ER, because in AR this residue was replaced by a glutamine (Q711); this change was well matched to the need of the cognate ligands for hydrogen bonding at the C(3) position (i.e., the hydrogen bond donor of the estradiol (E) phenol was matched with the acceptor glutamate in ER, and the hydrogen bond acceptor of the androgen 3-keto group was matched with the donor glutamine in AR). We supported this hypothesis by finding that the ER E353Q mutant was activated by estrogens with lower potency, but by androgens with higher potency than wild type ER [14], a gain of function–loss of function outcome that was expected from a direct interaction between the E phenol and the E353 carboxylate. This interaction was subsequently demonstrated directly by X-ray crystallography [8].

Our experience in shifting the binding specificity of ER by making a defined, structure-based change in both the receptor and the ligand, suggested that this ‘reciprocal alteration of ligand–receptor functionalities’ might be a general paradigm for designing hormone–receptor pairs with unique specificities. In this report, we describe the design and evaluation of a series of ER mutants and complementary ligands in which we have made different reciprocal changes of functionality, based on demonstrated or presumed contact sites between ligand and receptor. Conceptually, our approach is related to the ‘bump–hole’ approach used by Schreiber and Shokat to alter the specific-

ity small molecule–protein interactions in other systems [15]. Some of these new receptor–ligand pairs that we have produced have a specificity that is distinctly different from that of the wild type ER and E, and they might prove useful as transgene regulators in vivo.

2. Results

2.1. General scheme for altering hormone–receptor specificity by reciprocal changes in ligand receptor contact sites

A general scheme for achieving the desired shift in specificity by making reciprocal changes at the ligand–protein interface is illustrated in Fig. 1. Starting from a particular interaction at the E–ER interface that is an important determinant of the specificity of hormone–receptor recognition (ER–E box ‘*MATCH*’), one makes two changes: the E molecule is altered to produce a modified ligand (E’, left bottom), and a coordinated or reciprocal change is made in the receptor (ER’, top right). The ultimate goal of these changes is to achieve a new ligand–receptor specificity that is ‘orthogonal’ to the original one (ER’–E’ box, ‘*REMATCH*’). Attaining the desired orthogonality requires, as well, that the modified ligand E’ interact poorly with the wild type ER (ER–E’ box, ‘*Ligand Mismatch*’), and that the mutant receptor ER’ interact poorly with the natural ligand E (ER’–E box, ‘*Receptor Mismatch*’). We have found the new terms ‘ligand mismatch’ and ‘receptor mismatch’ useful in describing the two different ways in which the new specificity needs to be sufficiently orthogonal to the original one.

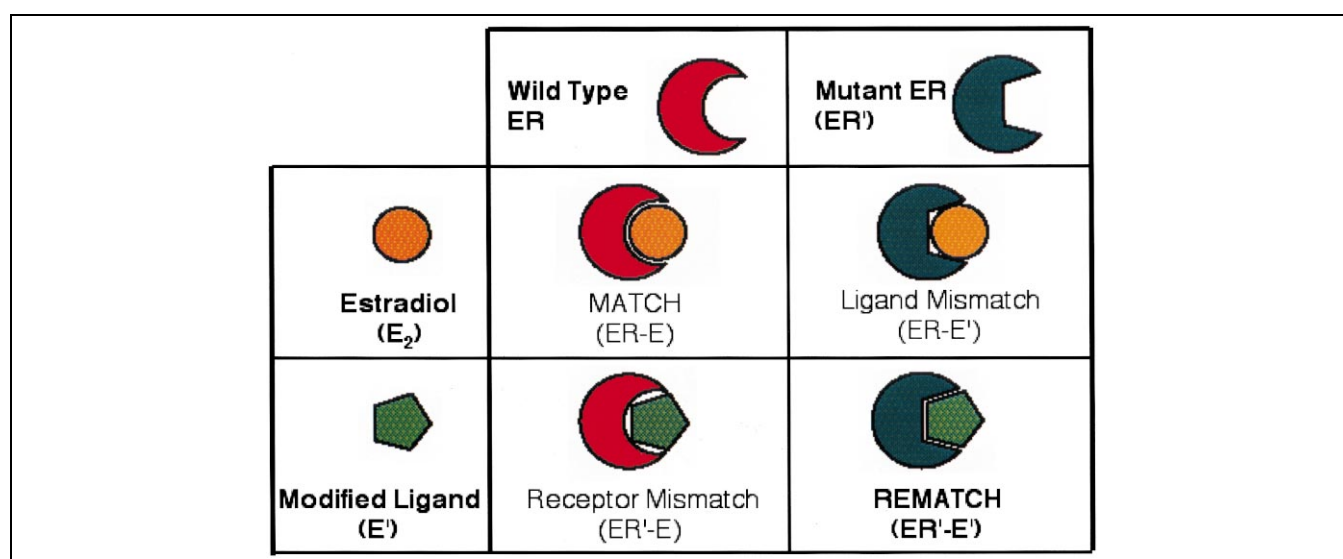


Fig. 1. Scheme illustrating the interaction between ER and its ligand E. The natural match, between wild type ER and E (ER–E box) is redesigned by altering the ligand (E to E') and the receptor (ER to ER') in a reciprocal fashion, so that they rematch (ER'–E' box) in an energetically favorable fashion. The mismatch between altered ligand and receptor (ER–E' box) and normal ligand and mutant receptor (ER'–E box) are to be energetically unfavorable.

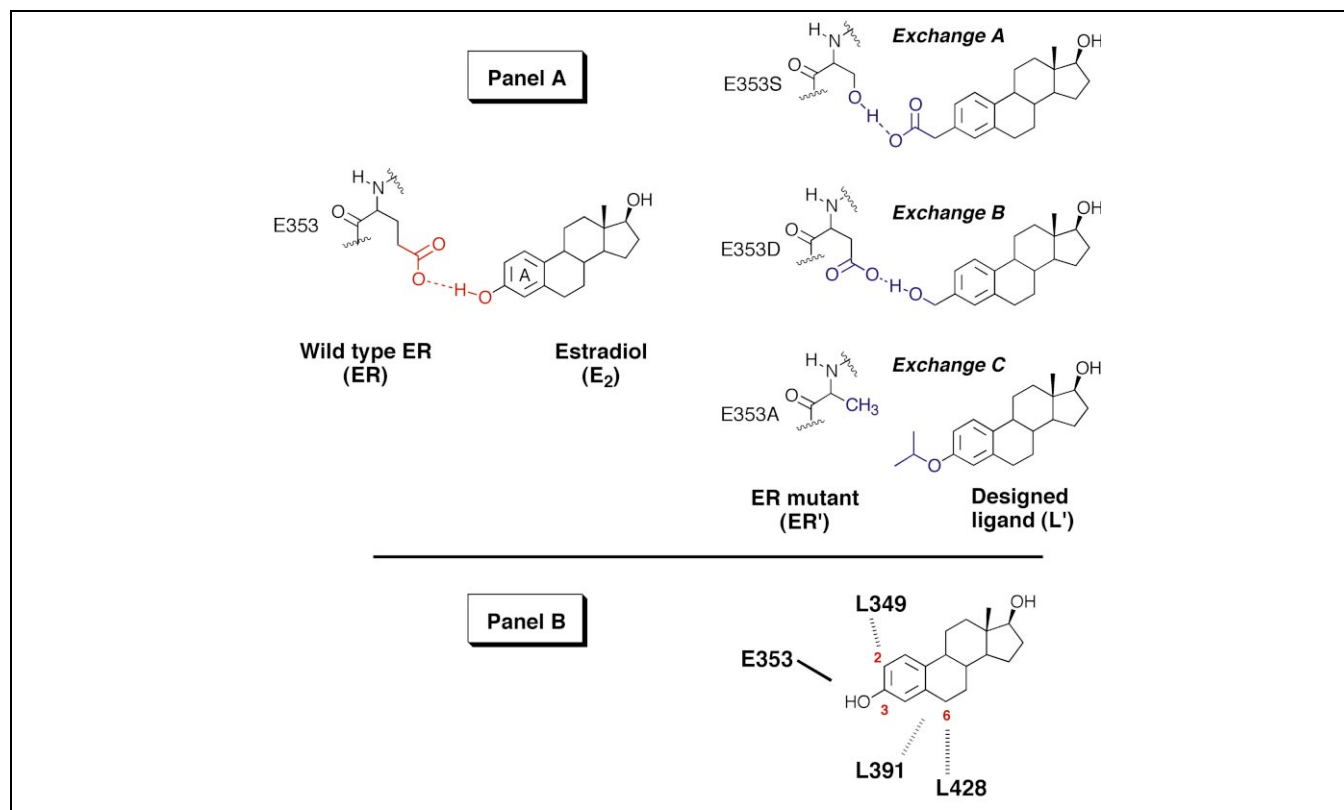


Fig. 2. Map of interactions between ER α residues and various positions in E. A: Interactions at E353 in ER α and C(3) in E. The natural interaction (left) is to be replaced by reciprocal changes in the receptor and ligand that are designed to maintain a favorable interaction between receptor and ligand. B: Interactions between various residues in ER α and C(2) and C(6) of E.

2.2. Reciprocal exchange of substituents and functionalities at contact sites between E and the ER α : the C(3)–E353 contact, and contacts at adjacent sites

Initially, we sought to expand on the presumed contact between ER E353 and C(3) position of the ligand that we had defined by our mutagenesis experiments (Fig. 2A, left) [14] by modifying the E molecule at this site in different ways and attempting to compensate for these modifications with a reciprocal change in the ER (Fig. 2A, right).

Thus, in exchange A, we reversed the roles of ligand hydrogen bond donor and receptor hydrogen bond acceptor at this site, by changing the 353 residue from an acceptor (glutamate) to a donor (serine) and the ligand correspondingly from a donor (phenol) to an acceptor (carboxylate). In exchange B, we shifted the position of the natural interaction by one carbon atom, changing the receptor site from a glutamate to an aspartate, and the ligand from a phenol to a benzylic alcohol. In principle, exchanges A and B both maintain the polar hydrogen bonding interaction between ligand and receptor, although in a manner that differs from the glutamate–phenol interaction in the wild type ER–E complex. By contrast, exchange C is designed to replace this polar interaction with a non-polar one, by substituting the glutamate on ER with an alanine and the phenol on the ligand with an ether.

Once the coordinates of the ER–E complex became

available [8], we confirmed these initial choices by molecular modeling, and we expanded the exchanges to encompass positions adjacent to C-3 on the ligand, namely C(2), C(4), and C(6), and the residues in primary contact with these sites (Fig. 2B). We restricted our investigation to the region near the A-ring subpocket in ER α because here the receptor is comparatively tightly packed around the ligand, meaning that there are a number of close receptor–ligand contacts in and near the A-ring. By contrast, elsewhere the fit is loose and the receptor is rather flexible [8,16]. Because the ER residues that make closest contact with E positions C(2), C(4), and C(6) are all non-polar, in each case we mutated the contact residue to an alanine and replaced the lost bulk with a suitable non-polar substituent on E.

2.3. Activation of ER mutants with E: the receptor mismatch

The effect of mutations in ER α on receptor interaction with E was evaluated by comparing the potency of E on wild type ER α and ER α mutants in cotransfection assays. These assays were performed in human breast cancer cells (MDA-MB-231 cells), using an expression plasmid for ER α (or various ER α mutants) and an estrogen-responsive reporter plasmid containing estrogen response elements (2ERE-pS2-CAT). Dose-response curves for E

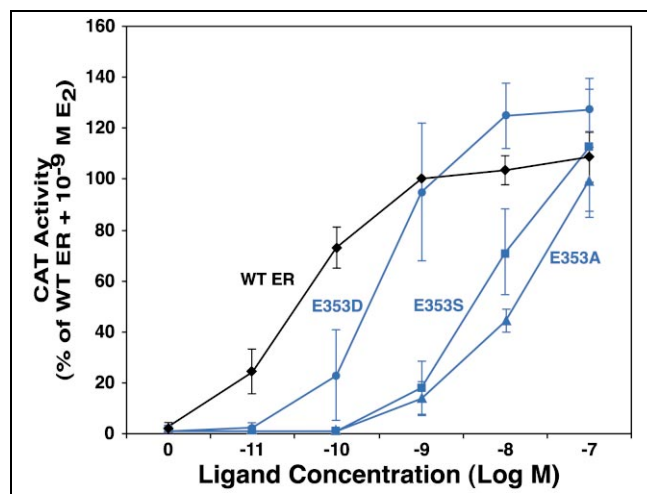


Fig. 3. ER α mutant mismatches with E. Dose-response curves for activation of transcription by E through the wild type ER α (black curve) or three ER α mutants at amino acid 353 (E353D, E353S, and E353A) (blue curves) were determined in a cell transfection assay in MDA-MB-231 breast cancer cells using an estrogen-responsive responder gene (2ERE-pS2-CAT). For a description of the color scheme, see the legend of Fig. 4.

with wild type ER α and various mutant ERs are shown in Fig. 3. In such assays, E activates reporter gene transcription through wild type ER α with an EC₅₀ of ca. 0.03 nM; its potency on the mutant receptors is distinctly lower.

It is convenient to express the transcriptional potencies

Table 1

Activation of ER α mutants by E (receptor mismatch)

ER α	Relative transcriptional potency (RTP) of E ^a
Wild type	100
E353S	0.66
E353A	0.25
E353D	22
L349A	0.42
L391A	19
L428A	14

^aRTP is defined as [EC₅₀(for E on wild type ER α)/EC₅₀(for the mismatched or rematched ligand-receptor pairs)] \times 100. On this scale, the potency of E on wild type ER is 100.

of E on the ER α mutants relative to its potency on wild type ER α . Similarly, as described below, the potency of various modified estrogens on both wild type ER α and ER α mutants can also be expressed relative to the potency of E on wild type ER α . Therefore, the potency shown by various receptors and ligands in transcription assays is expressed as relative transcriptional potency (RTP) values, RTP = [EC₅₀(for E on wild type ER α)/EC₅₀(for the mismatched or rematched ligand-receptor pairs)] \times 100 (see Table 1, and later Table 3). These RTP values are directly comparable to the relative binding affinity (RBA) values (Table 2), which are determined in competitive binding assays, as defined below.

All of the ER mutations at 353 reduced the potency of E (Fig. 3 and Table 1). The E353S and E353A mutants

Table 2

RBA values of E derivatives for wild type ER^a

Entry	R [C(3)]	RBA (%)	Entry	R ² [C(2)]	RBA (%)	Entry	R ³ [C(6 α)]	RBA (%)
1	OH (E ₂)	(100)	12	CH ₂ COOH	0.04	17	α -C ₆ H ₅	0.30
2	COOH	3.0	13	CH ₂ CH=CH ₂	12	18	α -CH ₂ C ₆ H ₅	0.32
3	CH ₂ OH	8.3	14	CH ₂ CH=C(CH ₃) ₂	0.18			
4	CH ₂ CN	1.8	15	CH ₂ CH ₂ CH ₂ CH ₃	0.28			
5	CH ₂ CONH ₂	3.6	16	CH(CH ₃) ₂	0.02			
6	CH ₂ COOH	0.04						
7	OCH ₂ COOH	< 0.03						
8	OCH ₂ CH ₃	0.54						
9	OCH(CH ₃) ₂	0.30						
10	OCH ₂ CF ₃	0.22						
11	CH ₂ CH ₂ CH ₃	0.16						

Entry	R ⁴ [C(2)-C(3)]	RBA (%)
19	-CH ₂ -C(O)-	0.07
20	-CH=CH-	3.0
21	-CH=C(CH ₃)-	1.6
22	-CH=C(CH ₂ CH ₃)-	0.25

^aRBA values are determined in a competitive radiometric binding assay, using [³H]E as tracer, radioinert compound as competitor, and charcoal-dextran to adsorb free ligand. On the RBA scale, the affinity of E is set at 100%.

Table 3
Summary of RTP of the most significant ligand-ER α combinations investigated^a

Ligand no.	Ligand structure	RBA	RTP ^b			
			wt ER	E353S ER	E353A ER	E353D ER
1	E ₂	100	100	0.66	0.25	22
2	-CO ₂ H	3.0	3.1	2.7	–	9.4
3	-CH ₂ OH	8.3	4.4	–	–	1.4
6	-CH ₂ CO ₂ H	0.04	0.0056	0.078	–	–
7	-OCH ₂ CO ₂ H	< 0.03	0.14	1.0	–	–
8	-OCH ₂ CH ₃	0.54	0.044	–	1.5	–
9	-OCH(CH ₃) ₂	0.3	0.025	–	0.25	–
10	-OCH ₂ CF ₃	0.22	0.075	–	0.075	–
11	-CH ₂ CH ₂ CH ₃	0.16	0.01	–	0.52	–
19	lactone	0.07	0.001	< 0.001	–	–
20	H-furan	3.0	0.005	–	0.003	–
21	Me-furan	1.59	0.10	–	0.92	–
22	Et-furan	0.25	0.008	–	0.25	–

^aRTP is defined as $[\text{EC}_{50}(\text{for E on wild type ER}\alpha)/\text{EC}_{50}(\text{for the mismatched or rematched ligand-receptor pairs})] \times 100$. On this scale, the potency of E on wild type ER is 100.

^bBold numbers indicate the RTP values for E and the three best new hormone-receptor specificity pairs.

show a dose-response to E that is shifted to the right by 160 and 400 fold, respectively, indicating that the very significant functional mismatch between these residues and the phenol of E gives an effective 'receptor mismatch'. The E353D mutant, however, showed only a 5 fold right shift. This mutation is the least perturbing of ER ligand interaction and presumably requires only a minor shift in ligand and/or residue positions to restore a productive polar interaction between the mutant receptor and the natural ligand E. Mutations at receptor sites close to E positions C(2) (i.e., L349A) and C(6) (i.e., L391A and L428) had variable effects on the potency of E (Table 1). The L349A mutation reduced the potency of E more than 200 fold, but the L428A and the L391A mutants are still very well activated by E. Some of these RTP values are also given in the summary Table 3.

2.4. Affinity of E derivatives with wild type ER: the ligand mismatch

The degree to which a change in ligand structure affects interaction with wild type ER can be assessed in two ways, by determining ligand binding affinity with wild type ER and by measuring potency in cell cotransfection assays using wild type ER. The binding assays can be done very conveniently in a competitive manner, whereby even low affinities are readily measurable. The results are given in Table 2, as RBA values, $\text{RBA} = [\text{IC}_{50}(\text{for E on wild type ER}\alpha)/\text{IC}_{50}(\text{for the ligand on wild type ER}\alpha)] \times 100$. These RBA values are directly comparable to the RTP values (defined above), and some of these values are presented in Table 3.

The degree to which a structural change at the C-3 position of E results in a ligand binding mismatch with wild type ER α is generally understandable from the nature of the structural and functional alteration. Simply extend-

ing the hydroxyl group by one carbon atom (from phenol to benzylic alcohol, ligand 3) has the least effect on binding affinity, a feature which also compromises the effectiveness of exchange B (cf., Fig. 2, and see below). The C-3 carboxylate (ligand 2), which is a synthetic precursor for ligand 3, still has significant affinity for wild type ER α , even though it has the same charge as the glutamate residue at 353 with which it is presumed to be in contact. The carboxymethyl analog (ligand 6, for exchange A, Fig. 2) is very well mismatched with ER α , having an affinity that is

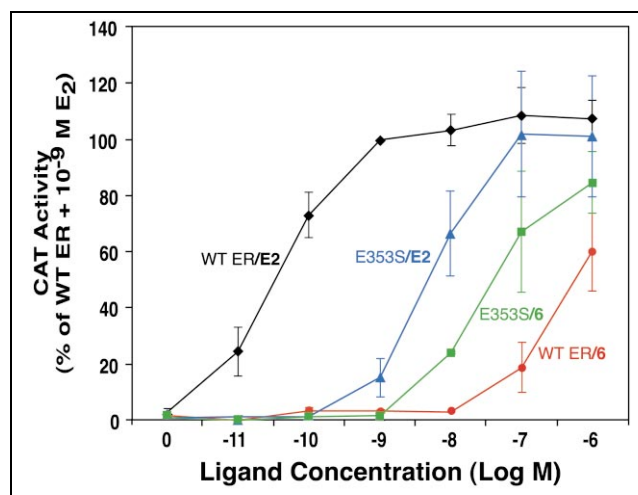


Fig. 4. Analysis of the transcription activation by wild type ER α with E (black) or compound 6 (red), and by the E353S ER α mutant with E (blue) or compound 6 (green). MDA-MB-231 cells were transfected with the mutant or wild type ER α expression plasmids and with the 2ERE-pS2-CAT reporter and the pCMV5b internal control plasmid, and were treated with the indicated concentrations of E or compound 6 for 24 h. CAT activities were normalized to β -galactosidase activity and are expressed relative to maximal wild type ER α activity with 10^{-9} M E, which is set at 100%. A consistent color scheme is used in Figs. 3–6: Match – black; receptor mismatch – blue; ligand mismatch – red; re-match – green. See Fig. 1 for a description of these terms.

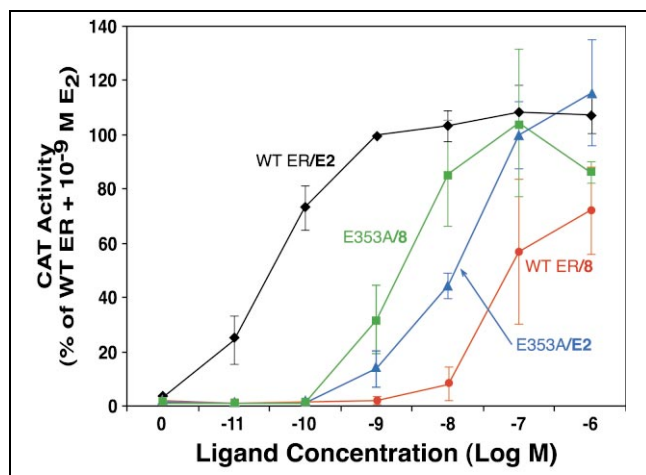


Fig. 5. Analysis of the transcription activation by wild type ER α with E or compound **8**, and by the E353S ER α mutant with E or compound **8**. Assays protocol and color scheme follow that described in the legend to Fig. 4.

2500 fold less than E. Ligands **4** and **5**, which are synthetic precursors for ligand **6**, have significantly higher affinities for ER α than does ligand **6**, probably because they have other hydrogen bonding possibilities. Both ethers (ligands **8** and **9**; for exchange C) also have greatly reduced affinities for ER α , as does the propyl derivative **11**. In the furan series (**20–22**), the affinity for ER α decreases with the increased size of the substituent. The binding affinities of the E derivatives bearing substituents at C(2) and C(6) (**12–18**), designed to be matched with the mutations shown in Fig. 2B, are very low, except for the C(2) allyl analog (**13**).

2.5. Potency of E derivatives on ER mutants: the ligand–receptor rematch

From dose–response curves for E and the designed ligands with wild type ER α and the various ER mutants in cell transfection assays, we can obtain a quantitative indication of the extent to which the designed ligands are mismatched with wild type ER α and are rematched with the mutant ERs. Representative examples of such dose–response curves are shown in Figs. 4–6 (note color scheme described in the legend to Fig. 4), with the mismatches and rematches schematized in Fig. 7. The RTP values (defined above) of the designed ligands for ER α and the various mutants are summarized in Table 3.

2.6. Ligand rematches with E353S ER α

With the E353S ER and the carboxymethyl–E pair (exchange A, Fig. 2A), the mutant receptor mismatch with E is 160 fold, as was noted from Fig. 3 and Table 1. As shown in Fig. 4, the mismatch of ligand **6** with wild type ER α in terms of potency in activating transcription is extremely high, ca. 10 000 fold. This is consistent with

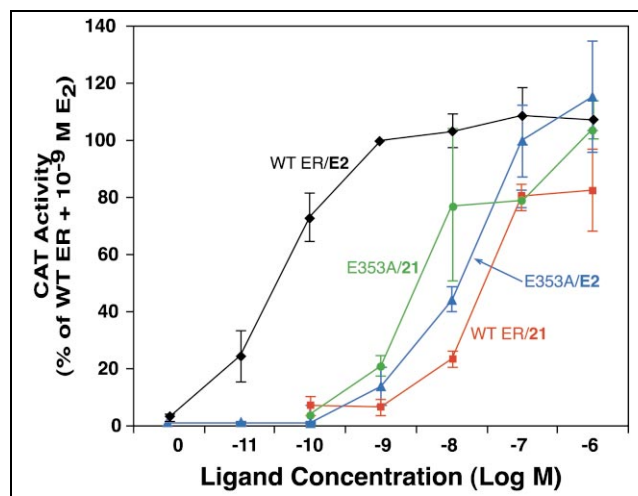


Fig. 6. Analysis of the transcription activation by wild type ER α with E or compound **21**, and by the E353S ER α mutant with E or compound **21**. Assays protocol and color scheme follow that described in the legend to Fig. 4.

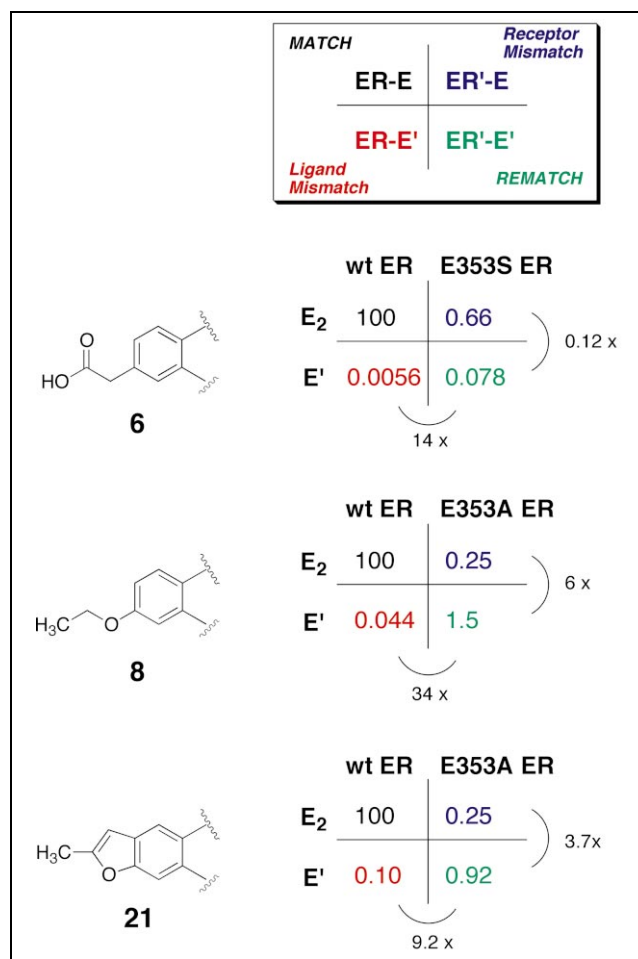


Fig. 7. Summary of the three best new hormone–receptor specificity pairs. The RTP values are summarized in an array that corresponds to that in Fig. 1. The fold change in RTP values is shown next to each pair of numbers.

the very low binding affinity measured for this ligand with wild type ER α (Table 2). The rematch of ligand **6** with E353S ER α is considerable, so that this ligand is ca. 15 fold more potent on the E353S mutant ER than on wild type ER α . However, because the receptor mismatch of E353S ER α with E is not very high, the potency of E on the mutant receptor is still 5 fold greater than that of the designed ligand.

We also investigated several other ligands, related to ligand **6** that might have rematched with the E353S ER α mutant (Table 3). The carboxylate ligand **2** is not effective; it is still relatively potent on wild type ER α , and it is not well rematched with E353S ER α . The C(3)-carboxymethyl ether, ligand **7**, has the carboxyl functionality for interaction with S353 and an ether oxygen to accept hydrogen bonds from R394. It is well mismatched with ER α , and it does rematch with the E353S ER mutant to some extent, but not as well as does ligand **6**, probably because it has an extra atom linking the carboxyl group to the aromatic A-ring. Lactone ligand **19** is smaller and has similar functionality for accepting hydrogen bonds from E353S and R394; again, it was very effectively mismatched with wild type ER α , but its rematch with the mutant E353S ER α was surprisingly poor.

2.7. Ligand rematches with E353A ER α

As was noted in Fig. 3, the receptor mismatch of E with E353A ER α is ca. 400 fold, which is better than its mismatch with E353S ER α . The transcriptional potency mismatch of the designed ligand **8** with wild type ER α is very large, ca. 2000 fold (Fig. 5 and Table 3), which is even greater than its affinity mismatch. Gratifyingly, the rematch of ligand **8** with E353A ER α is very good. As a result, the designed ligand shows a ca. 35 fold greater potency on this mutant ER than it does on wild type ER α ; its potency is even 6 fold greater than that of E on E353A ER α . Thus, as we had sought, the ligand **8**–E353A pair rematch displays a potency that is greater than either of the two mismatched pairs, although the potency of this reengineered hormone–receptor pair is still ca. 65 fold below that of E on wild type ER α . Our original plan for exchange C was to match E isopropyl ether (ligand **9**) with E353A ER α ; however, the rematch of this ligand was less good than was that of the ethyl ether **8** (Table 3).

In vivo, ethers are often rapidly dealkylated by cytochrome P450-mediated metabolism. Therefore, one might anticipate problems in extending the ligand **8**–E353A ER α pair to in vivo situations, because the dealkylation product would be the potent ligand E. To address this issue, we prepared an analog of this ether having three fluorine substituents positioned β to the ether function (ligand **10**), because such fluorine substitution is known to retard metabolic dealkylation reactions of ethers [17]. Although the trifluoroether analog had very low affinity for wild type ER α and a very good ligand potency mismatch, it

did not show an effective rematch with the E353A mutant ER (Table 3). Molecular modeling suggests that the larger fluorine substituents are difficult to accommodate in the constrained E353A subpocket. The large group dipole of the trifluoromethyl substituent might also be contributing to these results. As another analog of ethyl ether **8**, we prepared the 3-propyl derivative **11**. It is isosteric with the ethyl ether, but lacking the ether oxygen, it loses the capacity to accept a hydrogen bond from R394. As expected, this ligand has low affinity and low potency on ER α (good ligand mismatch), but it had only a modest (2–5 fold) rematch (Table 3).

In a different approach to metabolically stable analogs of ligand **8**, we prepared the fused furans, ligands **20–22**. The alkyl-substituted furans **21** and **22** have low affinity for wild type ER α (i.e., good ligand mismatch), but the unsubstituted furan **20** has substantial affinity (Table 2). In transfection assays (Fig. 6), the methyl furan (**21**) worked the best; it shows both a good ligand potency mismatch with wild type ER α and an effective rematch with E353A ER α . The ethyl furan (**22**) also gave a rematch, but less well than did **21** (not shown).

Thus, for the E353A ER α mutant, we have designed a ligand that works very well (ligand **8**) but might be metabolically unstable in vivo, and another ligand (methyl furan **21**) that works nearly as well and is likely to be more metabolically stable.

2.8. Other ligand rematches

As we noted in the proposed exchange B (Fig. 2A), both the receptor mismatch of the E353D ER α mutant with E (5 fold) and the corresponding ligand mismatch of the E benzylic alcohol ligand **3** were relatively minimal (12 fold in terms of binding affinity and 20 fold in terms of RTP). Thus, the opportunity for achieving a distinctive rematch with the ligand **3**–E353D ER α pair was relatively limited, as proved to be the case (Table 3).

In the exchanges shown in Fig. 2B, we planned to remove non-polar residues at certain sites on ER α that were in contact with atoms in E near C(3). According to this strategy, we prepared the L349A, L391A, and L428A ER α mutants. In each case, these mutants were to be paired with the corresponding E derivatives substituted at C(2) and C(6). While the specificity shift with all of these new exchanges turned out to be less complete than with the E353S and E353A ER α mutants discussed above, an analysis of their behavior is instructive.

Residue L349 in ER α is in close contact with the C(2) position on E, and the L349A mutant is very effectively mismatched with E (200 fold; Table 1). Of the four C(2) alkyl-substituted ligands we prepared, **13–16**, three of them, the 2-(3-methyl-2-butenyl) derivative (**14**), the 2-butyl derivative (**15**), and the 2-isopropyl derivative (**16**), showed good ligand affinity mismatch with ER α (Table 2). Surprisingly, the C(2)-allyl derivative **13** had quite

high affinity for ER α , too high for it to be useful. The rematch for ligands **14–16** was very minimal, and the L349A mutant was only partially activated by the modified compounds at high concentration (10^{-6} M) (not shown).

The two ER α mutants designed to interact with C(6)-substituted estrogens, L391A and L428A, were both still activated rather effectively by E (Table 1). Because of this rather feeble receptor mismatch, these receptors were of little practical use in rematching studies. The two C(6)-substituted estrogen we prepared, ligands **17** and **18**, while considerably affinity and potency mismatched with wild type ER α , were not very effectively rematched (not shown).

3. Discussion

3.1. Coordinated changes at contact sites in estrogens and the ER can create novel hormone–receptor specificities

We have developed a simple structure-based design protocol to create new hormone–receptor specificities by making coordinated changes to the ligand and to the receptor at contact sites, changes that together maintain the ener-

getically favorable interaction between ligand and receptor, but separately disrupt this interaction. We focused our work on the subpocket of ER α that binds the A-ring of E, because interactions here are tight and energetically important [8,16].

The three best new hormone–receptor specificity pairs we have created are displayed in Fig. 7, which presents a summary of their transcriptional potency data in an outline format that parallels that of Fig. 1. Molecular models for the fit of these three rematched sets in the ligand binding pocket of their cognate ER α mutants, together with E wild type ER α complex, are also shown in Fig. 8. In the most effective pair, E ethyl ether (ligand **8**) with E353A ER α , the model shows how nicely the ethyl ether is accommodated by the enlarged region in the C(3) subpocket formed by mutating the glutamate residue back to alanine. The fit for the methyl furan (**21**) in this same pocket is also good. These two redesigned fits can be contrasted with that of E in wild type ER α , in which the much smaller hydroxyl group in E abuts its three hydrogen bonding partners in ER, with no evidence of extra space.

The carboxymethyl–E ligand **6**, which rematched rather well with E353S ER α , also shows a good fit into the enlarged pocket of the mutant receptor. However, despite all of our attempts at modeling (see Section 5), we find that

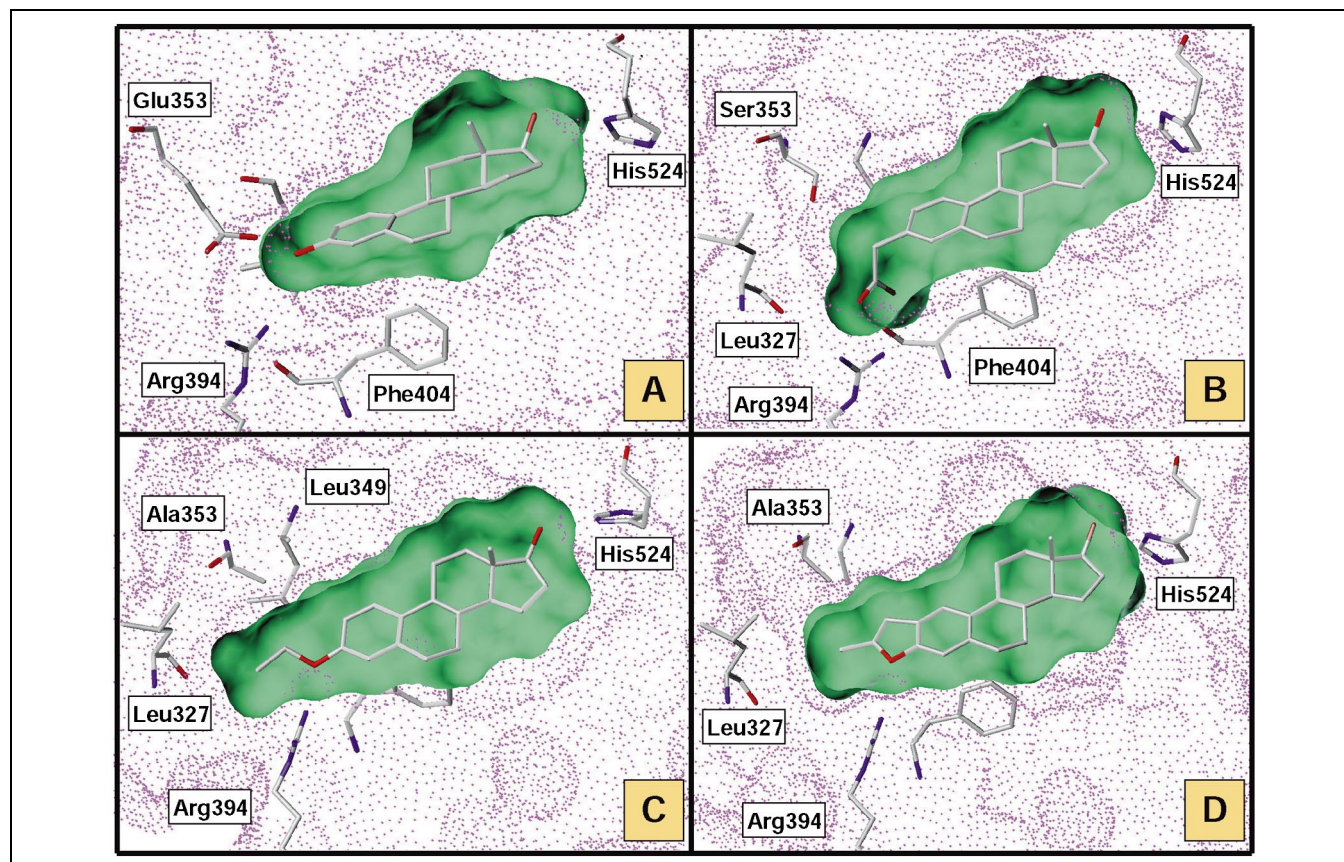


Fig. 8. Models of the ligands in the binding pocket of ER α . In each case, the surface of the ligand is shown in green and the surface of the ligand binding pocket as purple dots. A: E (**1**) in wild type ER α . B: Ligand **6** in E353S ER α . C: Ligand **8** in E353A ER α . D: Ligand **21** in E353A ER α .

the carboxyl group prefers to form a strong interaction with arginine 394, rather than serine 353. When one considers the energetic importance of a salt bridge in the interior of a protein and the limitations of modeling, this finding is perhaps not surprising. It does suggest, however, that there may be opportunities for further refinement of this interaction by placing additional substituents on the methylene group of this molecule to more optimally fill space that remains in the C(3) subpocket of this mutant.

In a number of other cases, coordinated changes in ligand–receptor pairs did not work, or worked only in part; deficiencies could be because either the receptor or ligand mismatches were insufficient or the rematch was too feeble. For example, mutations in ER α that did give poor receptor mismatches, that is, not reduce E potency sufficiently, were the E353D, L391A, and L428A mutations (Table 1). In the first case, shortening the acidic residue at 353 by only one carbon atom did not preclude productive interaction with the phenol of E. In the last two cases (L391A and L428A), the changes are being made at a site on ER where the ligand binding pocket is more flexible, so there are no polar interactions to enforce ligand–receptor contact. The more pronounced functional group changes, E353S and E353A, were effective in reducing the binding and potency of E, as was the L349A mutation.

The effect that changes in ligand structure had on affinity and potency towards wild type ER α (i.e., ligand mismatch) were, for the most part, understandable (Tables 2 and 3). The small change in ligand structure, phenol to benzylic alcohol (ligand **3**), designed to match this ligand with E353D, had only a minor effect on ligand affinity and potency on wild type ER α . However, the more profound changes in ligand structure had greater effects on ligand affinity and potency. The only unexpected findings in this regard were the fact that carboxy E (ligand **2**) still retained significant affinity and potency on wild type ER α , as did the simple furan (ligand **20**) and the 2-allyl derivative (ligand **13**). Aside from the successful ligand–receptor rematches noted at the start of the Discussion, there were a number of cases where rematching was surprisingly poor. In most of these, molecular modeling could be used, after the fact, to show the existence of unfavorable ligand–receptor contacts.

We believe that it is significant that we found the designed reengineering of reciprocal ligand–receptor contact sites worked best within the tight steric constraints of the A-ring binding pocket, where the polar interaction of the phenol with its hydrogen bonding partners is dominant. As far as we examined, designed reengineering worked less well outside of this region, where the ligand–receptor fit may be looser and the receptor more flexible [8,16]. In these latter regions, interactions that occur more deeply within the protein, that is, not directly at the ligand–receptor interface, are likely to be contributing to the stabilization energy, so reengineering by design here may prove to be more challenging.

3.2. *Hormones and nuclear receptors with unique specificities are useful in controlling gene expression*

Unique specificity regulatory systems are under active investigation not only as means to control the transcription of transgenes [10,18–20], but also to regulate enzyme activity [6,21,22], to probe the function of certain enzymes [21], and to regulate protein solubility [23]. There are a number of classical approaches to the regulation of gene expression with ligands. The metal-activated metallothionein system has more recently been replaced by various versions of the tetracycline-regulated system [24,25], and the use of nuclear hormone receptors from different phyla, such as using the ecdysone receptor from insects to regulate genes in mammalian cells, has seen wide application [26].

There are a number of well documented cases where nuclear receptor mutants were found that had useful shifts in hormone specificity, although these were not obtained by design (see Section 1). A potentially serious problem encountered with most of these systems is that the ligand being used to regulate the mutant receptor still has biological activity on endogenous receptors. Thus, in the language of this report, the activating ligands in these systems have not been sufficiently mismatched with their endogenous receptors for the specificity of the system to be unique.

In more recent cases, designed mutations made in the retinoid X receptor (RXR) system led to significant changes in hormone–nuclear receptor specificity, such that the mutants were more responsive to synthetic ligands than to the natural ligand 9-*cis* retinoic acid [27], but the synthetic ligands still activated the wild type RXR. A designed approach in the retinoic acid receptor system in which charge and size changes were made, also led to altered specificity, but this study did not address the effect of the natural ligand on the mutant receptors, that is, the issue of receptor mismatch [28].

In a number of studies on ER, random mutagenesis and phenotypic selection have been used to generate mutant ERs that are responsive to a ligand that has very low potency on wild type ER [29,30]. At least in these examples, one is starting with a ligand that is well mismatched with the wild type receptor, and ER mutants that show significantly enhanced responsiveness towards the novel ligand have been found in this fashion. However, typically, these mutant receptors still retain significant responsiveness to E, and thus are not effectively mismatched with the endogenous hormone, E.

Thus, despite some encouraging outcomes, most of these other nuclear hormone receptor systems with modified specificity are still not optimal, because of the unwanted activation of the parent receptor by the small molecule drugs, or residual sensitivity of the mutant receptors to the endogenous ligand. The new, more fully orthogonal specificities generated by redesigning molecule–protein in-

terfaces, as illustrated in this report, may prove to be better candidates for the development of regulated gene expression systems.

4. Significance

Our work represents a significant step in the use of structure-based design to achieve a pronounced shift in hormone–receptor specificity in nuclear receptor systems. A noteworthy feature of our approach is that we consider explicitly from the beginning, not only the goal of obtaining a new ligand–receptor specificity, both also the importance of mismatching the new ligand with the endogenous receptor and the mutant receptor with the endogenous hormone, E. This structure-based design approach could also be combined, in a sequential fashion, with random mutagenesis and phenotypic screening or selection methods (in vitro evolution), particularly when the latter would be applied both in a positive-selection manner (with the designed ligand) and a negative-selection manner (with the mutant receptors counter screened against endogenous estrogens). In fact, this in vitro evolution approach might be more effective if one starts with a system where structure-based design has already been used to create a system in which the desired ligand and receptor mismatches and the ligand–receptor rematch have been developed to a considerable extent, as we have demonstrated here for ER. Nevertheless, even with a fully optimized novel hormone–receptor specificity set, there are obviously additional challenges to be faced in using these molecular partners to regulate gene expression in vivo.

5. Materials and methods

5.1. Chemical synthesis

A complete description of the synthesis of Ligands **2–22** is given in the Supplementary Material, together with full experimental details and compound characterization.

5.2. Biological methods

5.2.1. Binding affinity determination

Determination of ligand affinity for the ER was done by a competitive radiometric binding assay using lamb uterine cytosol as a source of receptor and tritium-labeled E as tracer, with unbound free ligand being removed by adsorption onto charcoal-dextran, according to a previously described method [31]. Binding assays were done in duplicate, and the average is listed in Tables 2 and 3 as RBA values, where the affinity of E is considered to be 100%. This assay gives repeat RBA values with a coefficient of variation of 0.3.

5.2.2. Construction of ER mutants

Site-directed mutagenesis of the human ER α cDNA was performed by the method of Kunkel [32], as described previously [14]. Mutations were confirmed by restriction enzyme analysis, and by sequencing using the Big Dye sequencing kit from Perkin-Elmer. Details are given in the Supplementary Material.

5.2.3. Transfection assays

Transfections were done in ER-negative MDA-MB-231 human breast cancer cells and utilized the estrogen-responsive reporter 2ERE-pS2-CAT, pCMV5-wild type or mutant ER α expression vector, and the internal control plasmid pCMV β , expressing β -galactosidase as described [33], except that lipofectin-transferrin transfection reagent was used instead of calcium phosphate for DNA transfections, due to its improved efficiency. Cells were exposed to ligand for 24 h. CAT assays were performed and were normalized to the internal β -galactosidase activity [33]. Details are given in the Supplementary Material.

5.3. Supplementary material available

Experimental details regarding the synthesis of ligands **2–22**, the construction of ER mutants, and the transfection assays are available on <http://www.elsevier.com/locate/chembiol>.

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