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Functionally Orthogonal Ligand−Receptor Pairs for the Selective Regulation of Gene Expression Generated by Manipulation of Charged Residues at the Ligand−Receptor Interface of ERα and ERβ

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Abstract: The reengineering of protein-small molecule interfaces represents a powerful tool of chemical biology. For many applications it is necessary to engineer receptors so that they do not interact with their endogenous ligands but are highly responsive to designed ligand analogues, which in turn do not interact with endogenous proteins. The chemical design strategy used to reengineer protein−small molecule interfaces is particularly challenging for interfaces involving relatively plastic receptor binding sites and therefore presents a unique challenge in molecular design. In this study we explore the scope and limitations of a new strategy for manipulating polar/charged residues across the ligand receptor interface of estradiol (E2) and the estrogen receptor (ER). Carboxylate-functionalized E2 analogues can activate ERα(Glu353→Ala) and ERβ(Glu305→Ala) with very large selectivites, demonstrating that this design strategy is extendable to other members of the steroid hormone receptor family. Neutral E2 analogues were found to complement ERα(E353A) with similar potencies but with generally lower selectivities. This suggests that the high selectivity observed with ligand−receptor pairs generated by exchanging charged residues across ligand−receptor interfaces is only due in part to their complementary shapes and that appropriate introduction of charged functionality on the ligand can provide substantial enhancement of selectivity by decreasing the engineered ligands affinity for the endogenous receptor. Attempts to modify the cationic residues by complementing Arg394→Ala or Arg394→Glu were not successful.

Introduction

The ability to create ligands that can selectively bind and activate engineered proteins represents one of the most important tools of chemical biology.[1] Of particular interest has been the creation of enzyme−substrate or ligand−receptor pairs capable of selectively controlling signal transduction events or gene transcription. There are numerous examples of engineered proteins that uniquely respond to substrate or ligand analogues; however, there are relatively few examples of engineered ligand receptor pairs that can truly function independently of the endogenous systems, having ligand analogues that do not react with the endogenous proteins and engineered receptors that do not interact with endogenous ligands. Thus, the exploration of new design strategies to engineer potent and functionally orthogonal ligand−receptor pairs represents an important challenge in chemical design that would have a broad range of potential applications and may form the basis for developing new compounds which may restore activity to mutationally impaired receptors associated with genetic disease.[2−5]

Currently there are a relatively limited number of systems that allow for the remote, conditional regulation of eukaryotic gene expression.[3,4,6−10] Recently, we demonstrated that through the rearrangement of electrostatic partners within an existing protein salt bridge, potent and highly selective ligand−receptor pairs were generated from the estrogen receptor.[11] In this study we explore the scope and limitations of this ligand−receptor engineering strategy by illustrating that neutral ligands which complement the same loss-of-charge mutation can have similar potency as charged ligands but tend to have lower selectivity. Some of the designed ligand−receptor pairs show sufficient activity and selectivity to act as transcriptional regulators that are functionally orthogonal to both known ER subtypes. In

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addition we show that manipulations of the cationic partner of the same protein salt bridge was not a successful strategy for creating active ligand−receptor pairs, suggesting limitations to this general strategy.

Nuclear Hormone Receptors, Ligand-Dependent Transcriptional Regulators That Can Be Modified To Target Unique Sequences of DNA and Bind Unique Ligands. The nuclear and steroid hormone receptors, which function as naturally occurring ligand-dependent transcriptional regulators, may serve to act as unique inducible transcriptional regulators of selected gene targets if their ligand-binding and DNA-binding specificity could be appropriately modified. Several studies have already demonstrated that the ligand-dependent transactivation effects of the nuclear and steroid hormone receptors can be directed toward different DNA sequences (promoters) through modification of their DNA binding domains or through the construction of functional chimeras constructed with DNA binding domains of other proteins.[12−19] The ligand-binding domains of steroid hormone receptors have also been used to control the actions of site-specific recombinases in a ligand-dependent manner.[20−24] Therefore, the development of modified forms of steroid hormone receptor ligand-binding domains, which can uniquely respond to synthetic ligands, is of great interest for the selective and conditional control of transgene expression and recombination.

Whereas several early studies have shown that hormone receptor ligand-binding domains can be reengineered to preferentially mediate recombination in response to known hormone receptor antagonists, few studies have been directed toward the construction of modified receptors that mediate transcription with ligands that do not interact with endogenous receptors.[20−22,25] Miller and Wheland reported one of the first studies to create transcriptionally active receptors with altered ligand-binding specificity wherein a random mutagenesis/selection approach was used to generate mutant forms of the estrogen receptor, which are responsive to synthetic ligands that have very low activity with the "wild-type" receptor ERα(wt).[19,26] In these studies ERα mutants show significantly enhanced responsiveness toward the synthetic ligand, but still retain significant responsiveness to estradiol (E2). Subsequently, Peet and Koh used the reported crystal structures of the nuclear receptors retinoid X receptor (RXR) and retinoic acid receptor (RAR) to identify specific residues in RXR and RAR that could be modified to create receptors which preferentially respond to synthetic ligands that were discovered through screening or rational molecular design.[27−29]

The estrogen receptor is a member of the nuclear/steroid hormone receptor family that controls the expression of a specific set of genes involved in the growth, development, and maintenance of a diverse range of tissues in response to E2.[30,31] Two subtypes of the human estrogen receptors have been identified: hERα [32] and hERβ,[33] which display unique tissue distributions and have different patterns of tissue expression.[33−36] Recently, we and Tedesco et al. independently reported a rational, structure based design approach to engineer "coordinated changes" to the ligand−receptor interface at the A-ring of E2.[11,37] In these studies mutation of the same charged residue (Glu353) involved in receptor−ligand hydrogen bonds was used to create ligands with substantially reduced activity toward the natural ligand estradiol. Two different ligand design strategies, utilizing either neutral or anionic E2 analogues, were used to create high-affinity ligands for the mutant ERs. Interestingly, many E2 analogues having pendant groups attached to the A-ring of E2, retained significant affinity for wild-type ERs, suggesting that this region of the E2−ER interface may have significant plasticity. Here we explore the potential advantages of ligands of complementary charge to create selective ligand−receptor pairs that can act as functionally orthogonal transcriptional regulators.

Results and Discussion

Defining Functional Orthogonality, Receptor Selectivity, and Ligand Discrimination. An ideal reengineered ligand−receptor pair should be orthogonal to the endogenous system such that the modified receptor no longer responds to its natural ligand, and the synthetic ligand does not interact with the endogenous or "wild-type" form of the receptor.[1] In reality, engineered ligand−receptor pairs are rarely absolutely orthogonal but often show reduced or partial activity with their natural counterparts. The selectivity of the engineered ligand−receptor pair can be quantified as a ligand's receptor selectivity (RS), defined as the ratio of activities of the engineered ligand with the modified receptor to the activity of the same ligand in the wild-type receptor (eq 1). A receptor's ability to discriminate against its natural ligand can be quantified as the RS determined

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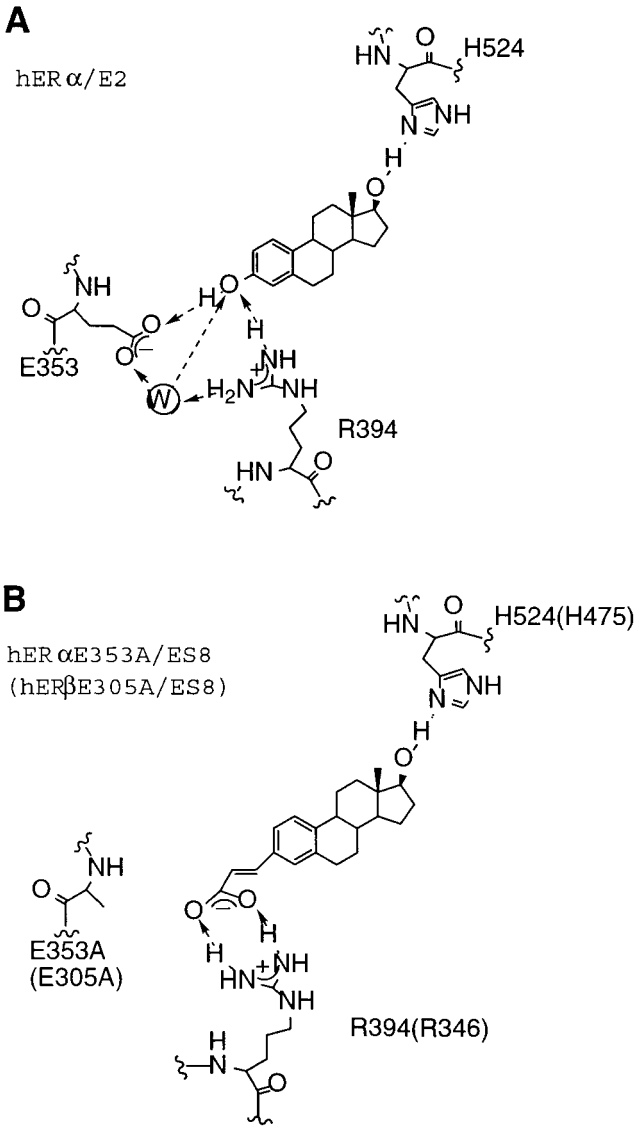
for the endogenous ligand. In practice, engineered ligand−receptor pairs need only be "functionally orthogonal" such that the modified receptor is not significantly activated by endogenous concentrations of the natural ligand and the synthetic ligand is capable of activating the modified receptor over a concentration range that does not activate endogenous receptors. By this functional definition of orthogonal reactivity, the comparison of the modified receptor's selectivity between the natural ligand and the ligand analogue is irrelevant so long as the modified receptor is nonresponsive to physiological concentrations of ligand.

$$\text{RS} = \text{receptor selectivity} = \text{EC}_{50}(\text{ligand+mutant})/\text{EC}_{50}(\text{ligand+wt}) \quad (1)$$

Carboxylate Functionalized Ligand ES8 Functionally Orthogonal to Both Known ER Subtypes. Recently, we reported a new engineered ligand−receptor pair composed of the ligand ES8 and a modified form of the ERα variant HEO, HEO(E353A), generated by a ligand−receptor design strategy termed polar-group exchange (Chart 1).[11,33,38] *For the purposes of this discussion we will use HEO to designate the point mutant ERα(G400V) and ERα to designate the true wild-type ERα.* The significant receptor selectivity observed with ES8 and HEO(E353A) suggested that ES8 may have sufficient receptor selectivity to act as an orthogonal ligand to both endogenous ER subtypes, a requirement necessary if this ligand−receptor pair were to be applied in vivo. We have evaluated the ability of carboxylate-functionalized ligands ES6, ES8, and ES9 (Figure 1) to activate both ERα(wt) and ERβ(wt) using luciferase reporter gene assays in transiently transfected HEK293 cells (Tables 1 and 2). In addition, we also constructed and evaluated the same Glu353→Ala mutation in the endogenous ERα, ERα(E353A). The ligands ES6 (EC_{50} = 3.9 nM), ES8 (EC_{50} = 1.0 nM), and ES9 (EC_{50} = 0.8 nM) are very potent agonists for ERα(E353A), having very high receptor selectivities, RS = 22, 95, and 53, respectively, compared to ERα(wt). Although, ES8 has greater activity in the endogenous receptor ERα (EC_{50} = 107 nM) than previously reported in the point mutant HEO (EC_{50} = >1000), ES8 is a much more potent agonist with ERα(E353A) (EC_{50} = 1.0 nM) than HEO(E353A) (EC_{50} = 60 nM; Figure 2). The ligand receptor pair composed of the ligand ES8 and ERα(E353A) has one of the largest receptor selectivites (RS = 95) of any transcriptionally active reengineered hormone receptor yet measured. In addition to its potency and large receptor selectivity observed with ES8 and ERα/ERα(E353A), ES8 is also a comparatively weak agonist of ERβ (EC_{50} = 170 nM), demonstrating that ES8 is a functionally orthogonal ligand capable of activating ERα(E353A) at concentrations that do not activate either of the endogenous ER subtypes.

While the ability of ES8 to select against binding the endogenous subtypes of ER is critical to the development of a functionally orthogonal transcriptional regulator, the modified receptor also needs to effectively discriminate against binding the endogenous ligand, E2. The modified receptor ERα(E353A) has significantly reduced response to E2 but still retains sufficient affinity with E2 (EC_{50} = 2.5 nM) that the modified receptor may be weakly activated by E2 at the upper limit of concentrations of E2 (ca. 1 nM) which might be expected in certain tissue types in vivo (Figure 2).[39] This suggested that it would be necessary to modify the receptor to reduce its activity with E2; however, obvious substitutions of Glu353 that could still accommodate the ligand ES8, Glu353→Thr and Glu353→Ser, showed even greater affinity for E2 than the Glu→Ala substitution at this position.[37,40] We therefore asked if similar modifications to ERβ might afford a receptor with lower responsiveness to E2 and still retain high affinity with ES8.

Chart 1. Key Polar Interactions between ER and Bound Ligands: (A) E2 with ERα Based on Reported Crystal Structure; (B) Proposed Interactions of Polar Group Exchange Modified Receptor ERα(E353A) and Carboxylate-Functionalized Ligand ES8[a]



[a] ES8/ERβ(E305A) is shown in parentheses.

Transcriptionally Active Ligand−Receptor Pair ES8/ERβ(E305A), a Highly Selective, Functionally Orthogonal Transcriptional Regulator. The three-dimensional structures of the ligand-binding domains (LBDs) of both hERα and hERβ complexed with a variety of receptor agonists and antagonists have been determined.[41−45] Several studies, including this one,

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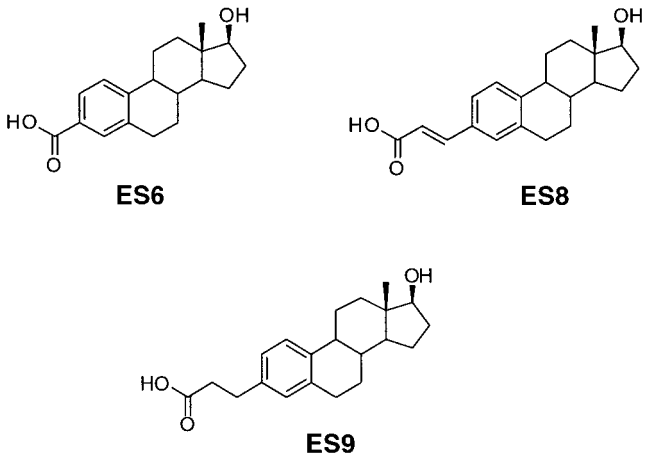


Figure 1. Carboxylate-functionalized estrogen analogues.

Table 1. Activities of Carboxylate Functionalized Estrogen Analogues with ERα(E353A) and Associated Selectivities

entry	receptor	ligand	EC_{50}[a] (nM)	K_d[b] (nM)	RS
1	hERα	E2	0.03 ± 0.01	0.15 ± 0.05	
2	hERα	ES6	86 ± 15	68 ± 20	
3	hERα	ES8	95 ± 15	107 ± 30	
4	hERα	ES9	45 ± 5.0		
5	hERα(E353A)	E2	2.5 ± 0.3	60 ± 20	1/83
6	hERα(E353A)	ES6	3.9 ± 0.4	10.8 ± 1.0	22
7	hERα(E353A)	ES8	1.0 ± 0.1	7.2 ± 0.5	95
8	hERα(E353A)	ES9	0.8 ± 0.2		56

[a] EC_{50}s reported as ±SEM of six independent experiments performed in triplicate. [b] K_ds reported as three independent experiments performed in triplicate. RS = EC_{50}(mutant)/EC_{50}(wt).

Table 2. Activities of Carboxylate Functionalized Estrogen Analogues with ERβ(E305A) and Associated Selectivities

entry	receptor	ligand	EC_{50}[a] (nM)	K_d[b] (nM)	RS
1	hERβ	E2	0.07 ± 0.01	0.6 ± 0.1	
2	hERβ	ES6	310 ± 30	850 ± 150	
3	hERβ	ES8	240 ± 30	170 ± 30	
4	hERβ(E305A)	E2	10.4 ± 1.0	140 ± 30	1/150
5	hERβ(E305A)	ES6	300 ± 30	1400 ± 300	1
6	hERβ(E305A)	ES8	0.6 ± 0.1	20 ± 4.0	400

[a] EC_{50}s reported as ±SEM of six independent experiments performed in triplicate. [b] K_ds reported as three independent experiments performed in triplicate.

demonstrate that Glu353 of ERα plays an important role in the binding of the phenol hydroxyl of the A-ring of E2.[46−48] The recently solved crystal structure of ERβ(LBD), complexed with the partial agonist genistein (GEN), reveals that ERβ has a striking structural conservation with ERα, despite only modest sequence homology (47%).[44] The phenol hydroxyl of GEN interacts with the side chains of Glu305, Arg346, and a buried

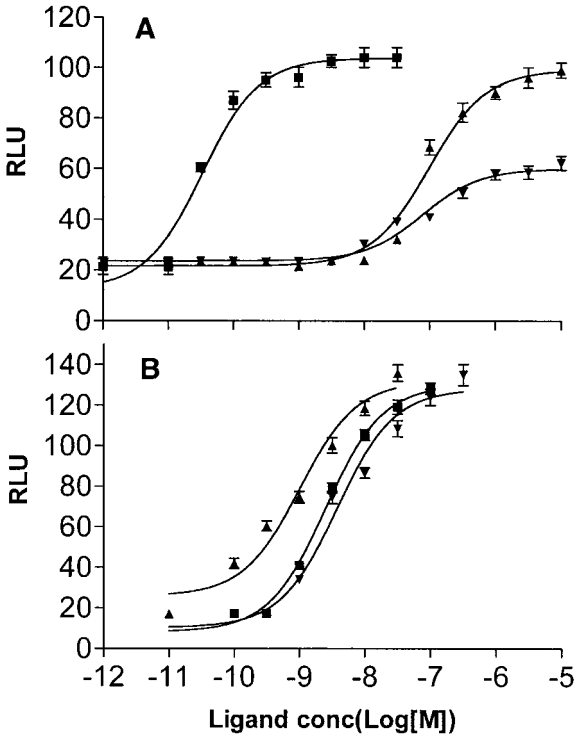
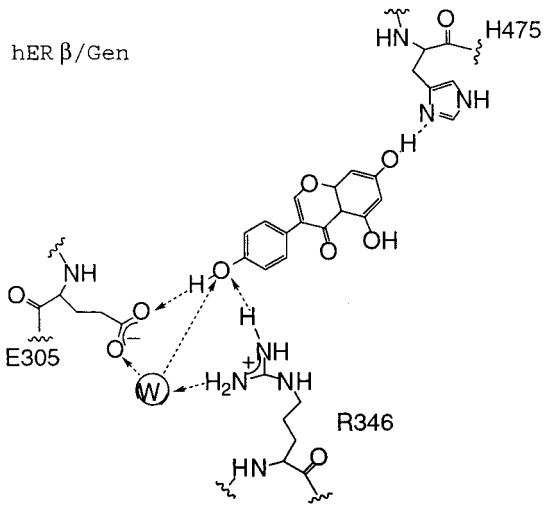


Figure 2. Dose−response curves for transactivation response of luciferase reporter by estrogen receptors with E2 (■), ES6 (▼), and ES8 (▲): (A) wild-type ERα; (B) mutant ERα(E353A). Data are mean ± SEM of six independent experiments run in triplicate. RLU = relative light units.

Chart 2. Key Polar Interactions in the Structure of Genistein (GEN) with ERβ



water molecule in ERβ in a fashion analogous to the phenol hydroxyl of the A-ring of E2 to Glu353 and Arg394 in ERα (Chart 2). It is therefore reasonable to assume that Glu305 and Arg346 of ERβ bind E2 in a fashion analogous to the binding of E2 to ERα and the analogue ES8 might similarly be able to activate ERβ(E305A) (Chart 1B).

The receptor ERβ(wt) and the mutant ERβ(E305A) were evaluated for their ligand-dependent transactivation response to ES6, ES8, and E2 in transiently transfected HEK 293 cells. E2 activates ERβ(wt) (EC_{50} = 0.07 nM) similarly to ERα(wt) (EC_{50} = 0.03 nM) (Table 2). However, the mutant ERβ(E305A) is 150 times less active toward E2 than ERβ(wt) (EC_{50} = 10.4 nM, RS= 1/150). Thus, ERβ(E305A) is five times more efficient

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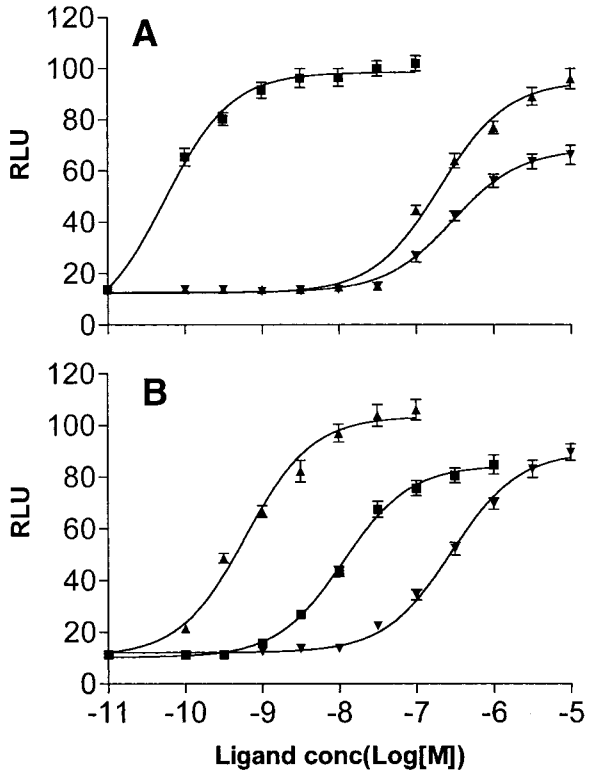

Figure 3. Dose−response curves for transactivation response of estrogen receptors by E2 (■), ES6 (▼), and ES8 (▲): (A) wild-type ERβ; (B) mutant ERβ(E305A). Data are mean ± SEM of six independent experiments run in triplicate.

at discriminating against E2 than ERα(E353A) and should not be significantly activated by in vivo concentrations of E2, which are typically significantly below 1 nM.[39]

The ligand ES6 shows only modest activity toward the modified receptor ERβ(E305A) (EC_{50} = 300 nM) and has essentially no selectivity compared to ERβ(wt) (RS = 1). The ligand ES8, however, is a very potent agonist toward ERβ-(E305A) and exhibits a very large selectivity for the modified receptor ERβ(E305A) over ERβ(wt) (EC_{50} = 0.6 nM, RS = 400) (Figure 3). These results suggest that our general polar-group exchange strategy may be extendable to the reengineering of other ligand receptor interfaces at least within the steroid hormone receptor family.

The ligand ES8 also shows a 158-fold preference for the mutant form of the β subtype ERβ(E305A) over the wild-type α subtype (ERα), demonstrating that ES8 and ERβ(E305A) also represents a highly selective transcriptional regulator, ES8 being functionally orthogonal to both known endogenous ER subtypes and the modified receptor ERβ(E353A) being functionally orthogonal to endogenous concentrations of E2.

In Vitro Ligand-Binding Assays Having Trends Similar to the Observed Activities. The ability of the ligands E2, ES6, and ES8 bind to the wild-type ERα, ERβ, and mutants ERα-(E353A) and ERβ(E305A) was determined by radio-ligand displacement assays using the receptor ligand-binding domains expressed in *E. coli* following standard methods.[11]

The in vitro ligand-binding data shows qualitatively similar trends to the observed transcriptional activities observed in culture (Tables 1 and 2). However, some quantitative differences can be noted in the receptor selectivities determined by cellular activity versus the intrinsic binding selectivities determined in vitro. For example, the association constants determined in vitro would suggest that the ES8 has only a 9-fold preference for ERβ(E305A) versus ERα(wt), whereas ES8's receptor selectivity (RS = 400) based on its cellular activity is greatly enhanced. Similar, but less dramatic, enhancement of binding selectivity is observed with ES8 in the α subtype, where the activity-based selectivity (RS = 95) was larger than the selectivity determined by binding (binding selectivity = 15). Because we are comparing the same ligand to different receptors, these differences cannot be simply ascribed to differences in intracellular availability of the ligand. Instead these results suggest that differential interactions with cellular accessory proteins can either enhance or reduce a ligand's receptor selectivity compared to its intrinsic binding selectivity measured in vitro.

The transactivation response of hormone receptors is mediated and therefore modulated by the interaction of heat-shock proteins (HSP90) with the unliganded receptor and by transcriptional coactivators with the liganded form of the receptor. The interactions of accessory proteins and coactivators have been shown to influence the dose response characteristics of hormone receptors.[49,50] Therefore, ligand-binding affinity need not exactly parallel the cellular activity. The ligand-dependent association of the ER with heat-shock proteins has been used extensively for the conditional control of recombination mediated by FLP or CRE−ER or chimeras.[21−24,51] In these studies, the ligand−receptor complex need not be transcriptionally active, as recombination can be mediated by either agonists or antagonists. The observed ligand-binding affinities suggest that these ligand−receptor pairs may find potential applications for mediating ligand-dependent recombination.

Complementing Glu353→Ala Mutations with Neutral Ligand Substitutions. The success of our "polar group exchange" modified ligand receptor pairs ES6, ES8, and ES9 with ERα(E353A) and ERβ(E305A) prompted us to more carefully evaluate the scope and limitations of this general ligand−receptor design strategy. There are numerous examples of ligand−receptor or enzyme−substrate pairs that have been reengineered by altering the complementary steric interactions between ligand and receptor. These "bump and hole" or shape-complementary ligand−receptor modifications have largely involved the modification of nonpolar groups which participate in hydrophobic interactions between protein and small molecule. In such cases it has often been observed that a "hole-modified" protein can still retain substantial affinity for its natural ligand (or substrate).[52−54] We sought to examine if the manipulation of polar groups involved in electrostatic interactions across the ligand receptor interface might impart greater receptor selectivity and ligand discrimination to engineered ligand receptor pairs. Our new design strategy involved exchanging a carboxylate group which normally forms an intramolecular protein salt bridge for a ligand-associated carboxylate capable of forming an intermolecular protein−ligand

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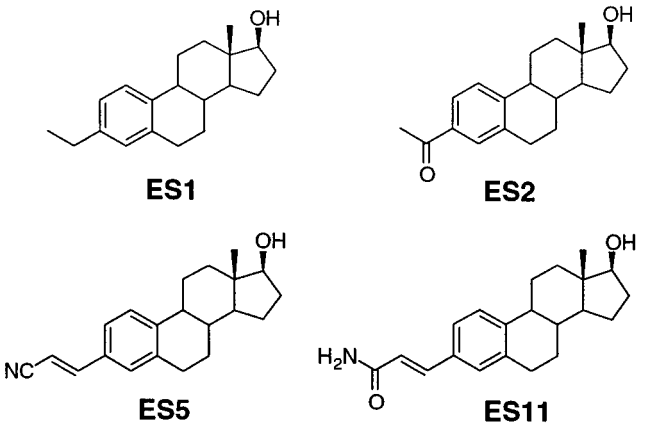
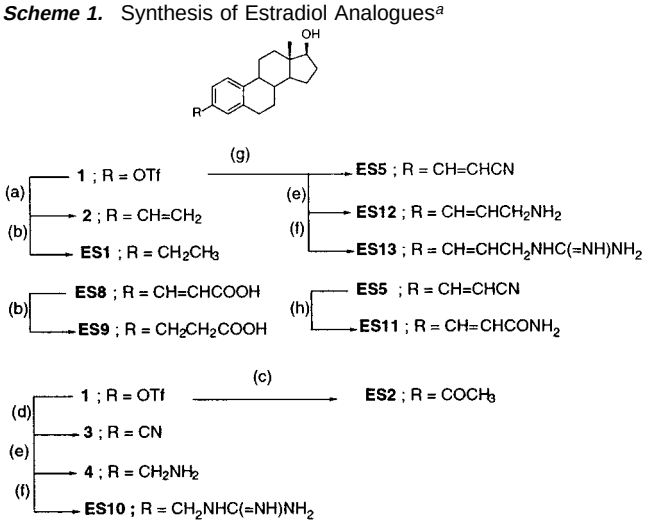


Figure 4. Neutral analogues of estradiol.

Scheme 1. Synthesis of Estradiol Analogues[a]



[a] (a) Vinyl tributyltin, $Pd(PPh_3)_2Cl_2$, LiCl, DMF, 90 °C /4 h; (b) H_2, 10% Pd/C, EtOH, room temperature/1 h; (c) CO, Me_4Sn, $Pd(Ac)_2$, dppf, LiCl, DMSO, 90 °C /7 h; (d) KCN, tris(dibenzylideneacetone) dipalladium(0)−chloroform, dppf, NMP, 60 °C/ 6 h; (e) $LiAlH_4/AlCl_3$, Et_2O, room temperature/2 h; (f) 1-*H*-pyrazole-1-carboxamidine hydrochloride, DIEA, DMF, room temperature/12 h; (g) acrylonitrile, $Pd(PPh_3)_2Cl_2$, LiCl, Et_3N, DMF, 90 °C/14 h; (h) H_2O_2–NaOH, MeOH−H_2O, 50 °C /1 h.

salt bridge with the modified receptor. The carboxylate-functionalized ligand would be expected to encounter repulsive electrostatic interactions with the remaining carboxylate of the endogenous receptor. While our goal was to demonstrate the advantages of manipulating electrostatic interactions across protein−ligand interfaces, implicit in our design is the same shape complementary modifications observed in bump and hole modified ligand−receptor pairs. To explore the advantages of using charge-substituted ligands to complement Glu353→Ala mutations, we designed and synthesized a series of estradiol analogues having neutral substitutions at C-3 (Figure 4 and Scheme 1). Ligands ES1, ES2, and ES5 and evaluated their ability to selectively activate ERα(E353A) and ERα in reporter gene assays using transiently transfected HEK293 cells. The three ligands ES1, ES2, and ES5 are all very potent agonists for the modified receptor ERα(E353A). ES1 and ES2 have EC_{50} values below 2 nM (Table 3). While these results suggest that neutral ligands can complement ERα(E353A) as effectively as the carboxylate-substituted, "polar-group exchanged" ligands ES6 and ES8, the neutral ligands often retain substantial activity with the wild-type receptor and therefore tend to possess modest

Table 3. Activities and Selectivites of Neutral Estrogen Analogues with ERα(E353A)

entry	receptor	ligand	EC_{50}[a] (nM)	RS
1	hERα	ES1	2.6 ± 0.3	
2	hERα	ES2	20.6 ±3.0	
3	hERα	(*E*)-ES5	23.5 ± 4	
4	hERα	(*Z*)-ES5	6.5 ± 1	
5	hERα(E353A)	ES1	2.0 ± 0.3	1.3
6	hERα(E353A)	ES2	1.8 ± 0.2	11.4
7	hERα(E353A)	(*E*)-ES5	14.5 ± 2	1.6
8	hERα(E353A)	(*Z*)-ES5	29.5 ± 5	4.5

[a] EC_{50}s reported as ±SEM of six independent experiments performed in triplicate. [b] K_ds reported as three independent experiments performed in triplicate. RS = EC_{50}(mutant)/EC_{50}(wt).

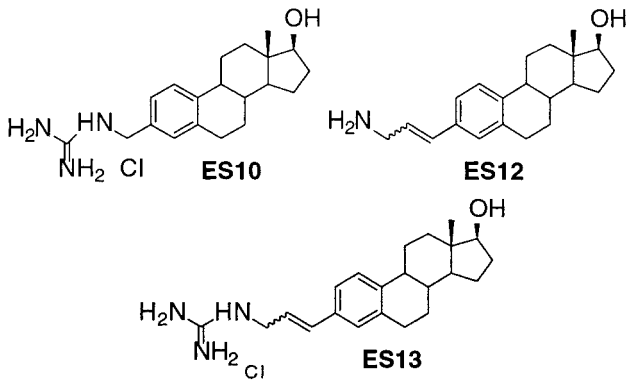


Figure 5. Basic (cationic) analogues of estradiol.

selectivites (RS < 12). The E-isomer of ES5 in fact has a slight preference for the wild-type receptor. The ability for wild-type ERα to accommodate these neutral "bumped" analogues of estradiol suggests that this portion of the E2−ER interface has sufficient plasticity to accommodate ligands of notably larger size than E2. Tedesco et al. similarly evaluated eight other estradiol analogues with neutral substituents at C-3 with ERα-(E353A). Out of the combined total of 11 ligands tested in ERα-(E353A) having greater than 1% of the potency E2 has with the wild-type ERα, no ligand had a receptor selectivity greater than 35. Whereas of the three carboxylate-fucntionalized, polar group exchanged ligands evaluated all three ligands were potent agonists with ERα(E353A) with significant receptor selectivities, two ligands (ES9 and ES8) having receptor selectivites greater than any neutral estradiol analogue yet reported. Within the limitations of comparing relatively small numbers of ligands, these results suggest that reengineering receptors by exchanging the covalent connectivity of polar/charged functional groups across the ligand−receptor interface can create ligand receptor pairs of similar affinity but of often greater selectivity than is generally attained by appropriately designed ligands having neutral substituents.

Exchange of Cationic Groups: Complementation of Arg394→Ala. The success achieved complementing Glu→Ala, "loss of carboxylate" mutations in ERα and ERβ with carboxylate-functionalized estrogen analogues suggested that the Arg394→Ala mutations in ERα might be similarly complemented by guanidine or amine functionalized estrogen analogues (Figure 5). On the basis of molecular models, we designed and synthesized the base functionalized ligands ES10, ES12, and ES13 and the mutant receptors HEO(R394A) and HEO(R394E) and evaluated their ability to activate reporter gene expression in transiently transfected HEK293 cells (Scheme 1). The

designed ligands as well as E2 show no activity whatsoever in HEO(R394A) and HEO(R394E) up to 1000 nM. Similar amphiphilic amine and guanidine functionalized ligands were found to activate modified forms of the nuclear receptor RAR, suggesting that these ligands should have sufficient cellular permeability to interact with hormone receptors but are unable to form a transcriptionally active complex with our HEO mutant. It is possible that the base-functionalized ligands do not effectively complement modified receptors HEO(R394A) and HEO(R394E). One might also expect that the desolvation of ligands on binding to be less favorable for guanidine than carboxyl groups.[54] Alternatively, it may also be possible that the mutant receptors are structurally too unstable to adopt a functionally folded form. These studies may suggest potential limitations to manipulating charged residues to alter ligand-binding specificity. (1) Unlike hydrophobic residues, the binding of ligands with polar functionality is generally associated with a greater energetic cost of desolvation, which must be adequately compensated for by the modified receptor in the ligand-bound complex. (2) With any ligand−receptor engineering strategy, one must carefully choose to modify a receptor is such a way as to provide specificity without grossly compromising the structural integrity of the protein.

Engineered Ligand−Receptor Pairs Accentuating Potential Differences between HEO, ERα(G400V), and the True Wild-Type ERα. In our initial work with ER, we used the modified receptor HEO, a Gly400→Val point mutant of ERα, to evaluate our designed ligand−receptor pairs. The Gly400→Val point mutation is located in a turn between helix-5 and sheet-1 of ERα and is 14 Å from E2 when bound in the ligand-binding pocket of ER over 14 Å from the ligand-binding pocket. HEO has been shown to respond similarly to E2 as ERα and has commonly been employed to evaluate ligand activities in culture due to its lower background activity and favorable dose−response characteristics.[33] During the course of our work, we have observed several instances where the behavior of ligands in ERα mutants differs substantially from their behavior with the analogous mutants of HEO. For example, we found that the carboxylate-functionalized ligand ES6 shows little activity below 1000 nM with HEO(E353A), binds with high affinity, and is a potent agonist for ERα(E353A) (EC_{50} = 3.9 nM, RS = 22). Similarly the ligand ES2 is more than 15 times less potent an agonist for HEO(E353A) but has almost the same activity as ES8 in ERα(E353A).

It is remarkable that these substantial differences in activity and ligand-binding selectivity are the result of a single point mutation which is located remote to the ligand-binding pocket. Others have also observed ligands displaying different behaviors in HEO and ERα.[38] These results suggest that mutations that do not directly contact the ligand may have significant effects on ligand-binding affinity, activity, and receptor selectivity. While it has been well-established that E2 similarly activates HEO and ERα in cultured cells with certain practical advantages, these observations illustrate that other synthetic ligands or modified forms of HEO and ERα may not and that caution should be used when using HEO to evaluate a ligand's activity with ERα.

Conclusions

The reengineering of protein−small molecule interfaces can provide powerful tools for chemical biology. The need for engineered ligand receptor pairs to be of high potency yet functionally orthogonal to endogenous ligand−receptor pairs presents a unique challenge in molecular design particularly in ligand−receptor pairs with relatively plastic receptor binding sites. When appropriately modified, polar/charged interactions across ligand−receptor interfaces can be modified to afford potent and highly selective ligand−receptor pairs. The ligand−receptor pair ES8/ERβ(E305A) constitutes perhaps the first engineered hormone receptor that can function as a unique ligand dependent transcriptional regulator, which is functionally orthogonal to both known receptor subtypes.

Experimental Procedures

Materials. Radiolabeled estradiol ([^{3}H]E2; [2,4,6,7- ^3H]estra-1,3,5,-(10)-triene-3,17-diol), 84.0 Ci/mmol, was obtained from Amersham Pharmacia Biotech UK Limited Corp. (Arlington Heights, IL). Unlabeled estradiol, butylated hydroxyanisole (BHA), isopropyl-D-thiogalactopyranoside (IPTG), and yeast extract were purchased from Sigma (St. Louis, MO); Hydroxyapetite (HAP; Bio-Rad, Hercules, CA) was prepared following the proceedure of Williams and Gorski.[55] BL21-(DE3)pLysS competent cells and pET15b were obtained from Novagen (Madison, WI). Dulbecco's modified eagle media (DMEM) was purchased from Mediatech, Inc. (Herndon,VA). Human Embryonic Kidney 293 (HEK 293) cells were obtained from ATCC (American Type Tissue Collection) and were maintained at the University of Delaware Cell Culture Core Facility. Transactivation response assays were performed using Dual-Luciferase Reporter Assay System (Promega No. E1960) following the manufacturer's protocol. The synthesis and characterization of 3-carboxyl-17-(hydroxyestra-1,3,5(10)-triene (ES6) and 3-acrylic acid 17-(hydroxyestra-1,3,5(10)-triene (ES8) were reported previously.[11] Full experimental details for the synthesis of ligands ES1, ES2, ES5, ES10, ES9, ES11, ES12, and ES13 are available in the Supporting Information.

Molecular Modeling. Molecular modeling was performed on a silicon graphics Octane using Flo98/QXP modeling software as described previously.[11,56] Site models were constructed from the reported crystal structure of ERβ complexed with genestein (PDB = 1QKM).[44]

Plasmid Constructs Plasmids. pSG5-hERα(E353A), pSG5-hERα-(E353S), pSG5-hERα(E353T), pSG5-hERβ(E305A), pSG5-HEO-(R394A), and pSG5-HEO(R394E) were constructed using oligonucleotide-directed single-stranded DNA mutagenesis by using the Quickchange method (Stratagene).

All mutants and constructs were sequenced over the entire coding region of the gene. pSG1-HEO(E353A) was prepared as described previously.[11]

Prokaryotic expression vectors pET15b-hERβLBD and pET15b-hERβLBD(E305A) were generated by subcloning of the wild-type and mutant hormone-binding domain sequences into the NdeI and BamHI sites of pET15b using cloning primers 5′-GGGAATTCCA-TATGGTGCTGCGGGAGCTGCTGCTGG-3′ and 5′-CGCCGGATC-CGCCCCCGTGATGGAGGACTTCG-3′.

The construction of pET15b-hERα and pET15b-hERα(E353A) were reported previously.[11] The identity of all constructs was confirmed by restriction mapping and DNA sequencing of the entire coding region of each receptor.

In Vitro Ligand-Binding Assay. Human ERαLBD (residues 304−554), hERβ (residues 255−509), and their mutant hERα(E353A) or hERβ(E305A) were expressed in *E. coli* BL21(DE3)pLysS cells and assayed using hydroxyapetite method following previously reported procedures.[11] The binding assays were performed in triplicate and reproduced in three independent experiments.

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Cell Culture, Transfection, and Luciferase Assay. Prior to transfection (24 h), HEK293 cells were seeded at a density of 40 000 cells/well in a 24 well culture plate and grown in DMEM (without phenol red), supplemented with 10% FBS; 3 h prior to transfection the medium was changed to DMEM supplemented with 10% charcoal-resin stripped FBS. Transfections were performed by $CaPO_4$ coprecipitate method using 0.02 μg of receptor, 0.03 μg of reporter ERE-Luc, and 0.14 μg of pRLbasic control (Promega). After the transfection (6 h), the medium was removed and replaced with DMEM with 10% charcoal-resin stripped FBS containing appropriate concentrations of ligand. The cells were allowed to incubate with the new medium for 30 h before harvesting by passive lysis. Reporter gene expression was measured by dual luciferase assay (Promega) using a Dynex luminometer following manufacturer's protocol.

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Supporting Information Available: Experimental procedures for the preparation of new compounds including characterization and protocols for biological assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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