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Research Paper

Selective regulation of gene expression by an orthogonal estrogen receptor–ligand pair created by polar-group exchange

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Abstract

Background: The nuclear and steroid hormone receptors function as ligand-dependent transcriptional regulators in eukaryotes. Hormone receptors have been engineered to selectively respond to synthetic ligands and used as remote regulators of gene expression for the study of gene function and as potential regulators of gene therapies.

Results: In this work, a new ligand–receptor engineering strategy called 'polar-group exchange' is used to create a mutant form of the estrogen receptor, ER(Glu353→Ala), which lacks a carboxyl group critical for high-affinity binding of estradiol, but is able to transactivate in response to nanomolar concentrations of a carboxylate-functionalized estrogen analog, ES8. ES8 activates ER(Glu353→Ala) at concentrations that do not appreciably activate the 'wild-type' receptor ER(wt). Two similar carboxylate-functionalized ligands, ES6 and ES7, do not induce transactivation function. Similar selectivities are observed in ligand-binding assays in vitro, which follow the trends predicted by molecular modeling.

Conclusions: Polar-group exchange is an effective strategy for rationally engineering ligand–receptor pairs. The ER(E353A)/ES8 ligand–receptor pair should constitute a unique and functionally orthogonal ligand-dependent transcriptional regulator.

Keywords: Gene expression regulation; Estrogen receptor; Polar-group exchange; Synthetic ligand

1. Introduction

As we enter the post-genomic era, the need to identify the functions of the tens of thousands of newly discovered genes presents a new and daunting challenge. Systems for conditionally controlling gene expression provide a powerful new tool for discovering the roles of specific genes in development, homeostasis and programmed cell death and as potential regulators of gene therapies [1–3]. Ligand-dependent transcriptional regulators, such as the tetracycline-inducible expression system [4–6], the ecdysone receptor [7,8], RNA aptamers [9], and chemical inducers of dimerization [10–12], allow for the precise temporal control of gene expression in eukaryotic systems. For the study of many genes, which may be expressed only transiently or which may be toxic, inducible expression systems provide a level of temporal control that is not generally achievable using transgenic 'knock-outs'.

1.1. The steroid/nuclear hormone receptors act as ligand-dependent transcriptional regulators

The steroid/nuclear hormone receptors function as natural ligand-dependent transcriptional regulators of specific genes involved in development and homeostasis [13–15]. The actions of steroid/nuclear hormone receptors are mediated primarily by two functional domains, a ligand-binding domain (LBD) which mediates ligand-dependent transactivation function [16–18], and a DNA-binding domain (DBD) which targets specific hormone response elements (HREs) [19,20]. Several examples of transcriptionally active chimeric receptors, created by fusing the LBDs of steroid/nuclear hormone receptors to DBDs of other proteins, suggest that in principle, the ligand-dependent transactivation functions of hormone receptors can be targeted to virtually any gene [13,21–27]. In the absence of ligand, steroid hormones are generally associated with

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heat-shock proteins (HSPs) [28] but dissociate from HSPs upon ligand binding, dimerize and bind to specific HREs. The liganded form of the receptor associates with transcriptional co-activators, which interact directly with the basal transcription machinery (Fig. 1).

1.2. Ligand–receptor engineering is providing unique tools for chemical biology

The ability to create ligands that can selectively bind and activate engineered proteins represents one of the most powerful tools of chemical biology. (For a recent review see [29].) Most reported examples of ligand–receptor engineering are based on the 'bump and hole' strategy first described by Schreiber for re-engineering the complex formed by cyclophilin A and cyclosporin A [30]. Many reported examples of engineered ligand–receptor pairs involve modification of non-polar ligand–receptor interactions and often these modified receptors still exhibit significant affinity for their natural ligands [29]. In this work a new strategy, we call 'polar-group exchange', is used to create a high-affinity ligand for a modified estrogen receptor (ER) by changing the covalent connectivity of polar groups involved in an intra-molecular protein salt-bridge. The new ligand–receptor pair can function as a unique transcriptional regulator and is functionally orthogonal to both the natural ligand and receptor.

2. Results and discussion

2.1. New transcriptional regulators from engineered hormone receptors

Hormone receptors have been modified by site-specific or random mutagenesis to selectively bind synthetic non-agonist ligands [3,31–34]. There are several examples of receptor–recombinase fusions that have been modified to selectively mediate recombination in response to known receptor antagonists [35–39]. However, relatively few examples of transcriptionally active ligand–receptor complexes have been reported and in many cases their selectivity is limited [33,34,40–42]. The high-resolution crystal structures of the LBDs of several members of the nuclear/steroid hormone receptor family have been solved [43–51] and provide the basis for the rational molecular design of receptors as they can be selectively activated by synthetic hormone analogs [31,52,53].

2.2. Manipulation of polar groups may provide greater ligand–receptor selectivity

Manipulation of key polar binding interactions, hydrogen bonds or salt-bridges, between ligand and receptor may help create receptors that can better discriminate against their natural ligand in preference for an appropriate synthetic analog. In addition, it is often desirable to add polar and/or charged functionality to ligands in order to alter their solubility and pharmaco-kinetic properties.

Salt-bridges and other polar interactions within a protein's core can enhance both the specificity and stability of the protein–ligand complex as well as the folded form of the protein. The introduction of polar groups into the hydrophobic core of proteins is typically associated with an unfavorable energetic cost of desolvation, which is generally offset by the formation of substantial hydrogen bonding, and dipolar interactions with the protein [54,55]. Ligand–receptor design strategies that alter charged polar interactions are often observed to afford ligand–receptor pairs of notably lower potency [53,56].

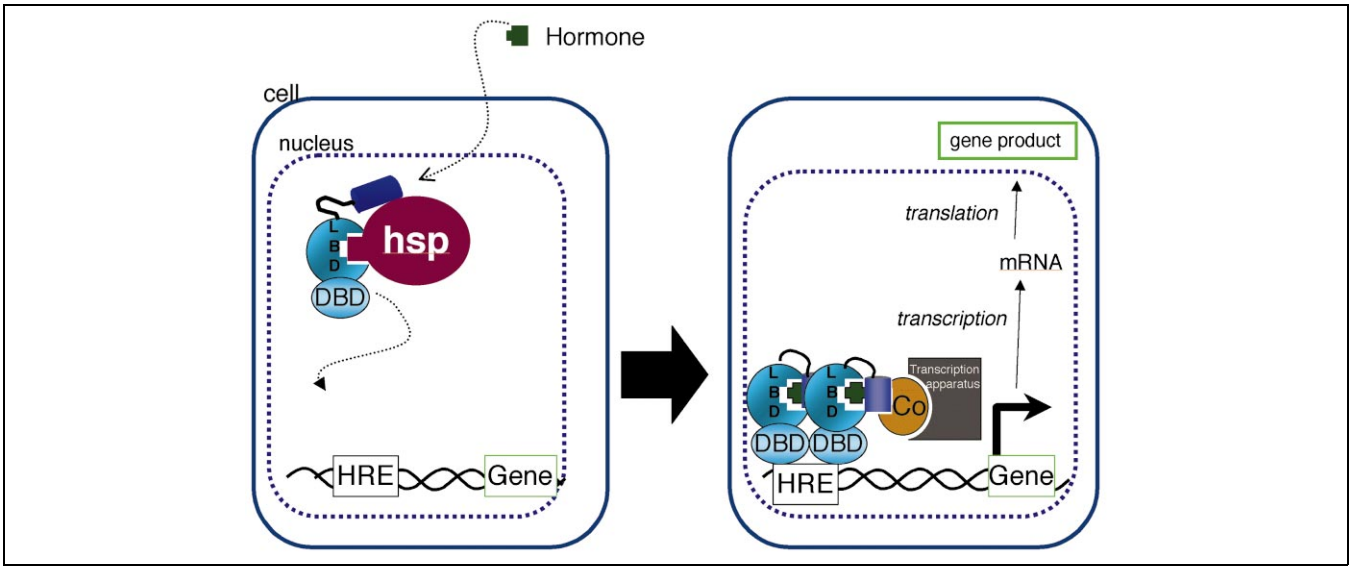


Fig. 1. Mechanism of steroid hormone receptor action. Upon ligand binding, steroid receptors dissociate from HSPs, dimerize and bind HREs and activate transcription through interaction with transcriptional co-activators (Co). (LBD = ligand-binding domain; DBD = DNA-binding domain.)

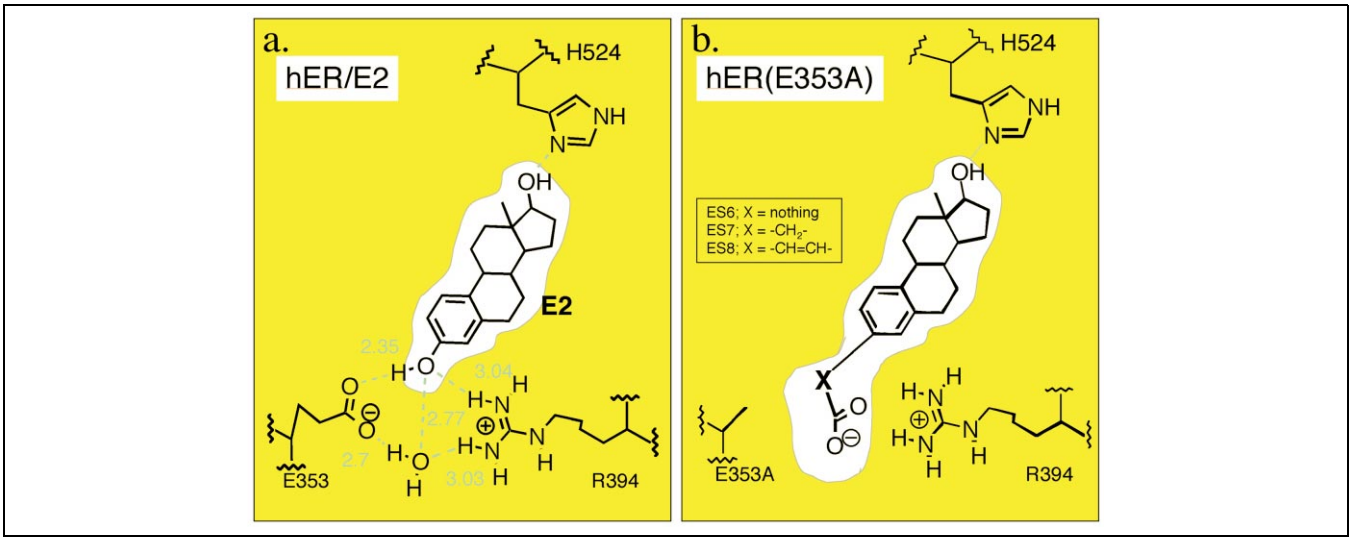


Fig. 2. (a) The intra-molecular protein salt-bridge between Glu353 and Arg394 provides key hydrogen bonding partners for the phenol hydroxyl of E2. (b) The mutation Glu353 → Ala causes loss of the anionic, carboxylate salt-bridge partner. Carboxylate-functionalized ligands may act in lieu of the removed carboxylate to form an inter-molecular, protein–ligand salt-bridge.

Previously we demonstrated that a neutral amide and a cationic guanidine-functionalized analog of retinoic acid could preferentially activate mutant forms of the retinoic acid receptor to which neutral or acidic (anionic) residues were introduced by mutagenesis [53]. These 'charge-neutralized' and 'charge-reversed' ligand–receptor pairs exhibit relatively poor potency and selectivity and therefore did not represent a practical tool for selective gene regulation.

The co-crystal structure of the estradiol (E2)-bound form of human ER (hER) shows that the 3-hydroxyl of estrogen is held via hydrogen bonds to an intra-molecular protein salt-bridge formed by Glu353 and Arg394, which are adjacent to the ligand-binding pocket (Fig. 2a) [47,48]. Glu353 and the 3-hydroxyl group of E2 have been shown to be important for the selectivity and affinity of E2 binding [42,57,58].

We envisioned that an orthogonal ligand–receptor pair could be generated by substituting the intra-molecular protein salt-bridge for an inter-molecular protein–ligand salt-bridge created by removing the carboxylate of Glu353 and replacing it with a carboxyl group attached to the ligand (Fig. 2b). Ideally, this new 'polar-group-exchanged' complex should retain a network of polar interactions similar to the native complex, and only differ in the covalent connectivity of key polar functional groups.

2.3. Ligand–receptor pairs designed on the basis of agonist-bound site models

We sought to test if carboxylic acid-functionalized analogs of E2 could bind to an ER mutant, hER(E353A), which has the anionic salt-bridge partner of Arg394 replaced by the small neutral side-chain of alanine. We designed three carboxylic acid-functionalized estrogen analogs to bind the modified receptor hER(E353A) by computer-aided molecular design using Flo/Qxp (Thistlesoft) [59].

Generally, hormone receptors undergo substantial structural reorganization upon ligand binding and undergo a series of inter-molecular events which include dissociation of HSPs, dimerization, DNA binding and association with transcriptional co-activators [15–17]. Clearly, the apparent association energies (E_{ass}s) calculated from constrained site models based on the active E2-bound complex cannot be expected to represent the overall energetics associated with ligand-dependent transactivation. However, it is reasonable to view a ligand's ability to bind the active conformation of the receptor as a prerequisite for ligand activity.

The compounds ES6, ES7 and ES8 were selected for synthesis and further study on the basis of their apparent E_{ass}s calculated for their association to constrained site models of hER(E353A) created from the coordinates of the reported E2–ER co-crystal structure (Fig. 3a). Monte Carlo docking simulations performed with the ER(E353A) site model show that the ligands ES6, ES7 and ES8 can bind with favorable energies and with minimized structures virtually superimposable with that of the reported structure of E2 bound in the native complex (Fig. 3b–d). These ligands were synthesized from the 3-triflate of E2 by palladium-mediated cross-couplings to create analogs with different carboxyl tethers (Scheme 1) [60–62].

2.4. Ligand activities in cell culture

Ligand-dependent transcription assays were performed using the modified ER HEO, which contains a Gly400 → Val mutation [65]. The modified receptor HEO is commonly employed in place of the 'true wild-type' receptor (HEGO) in transiently transfected cells to mini-

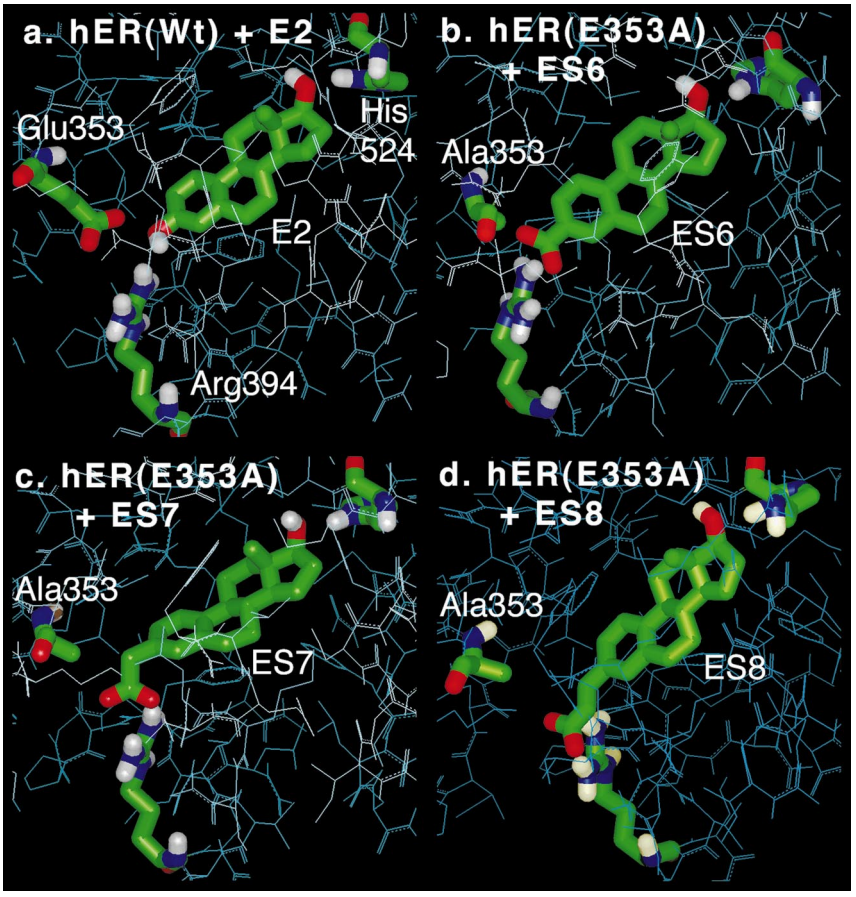
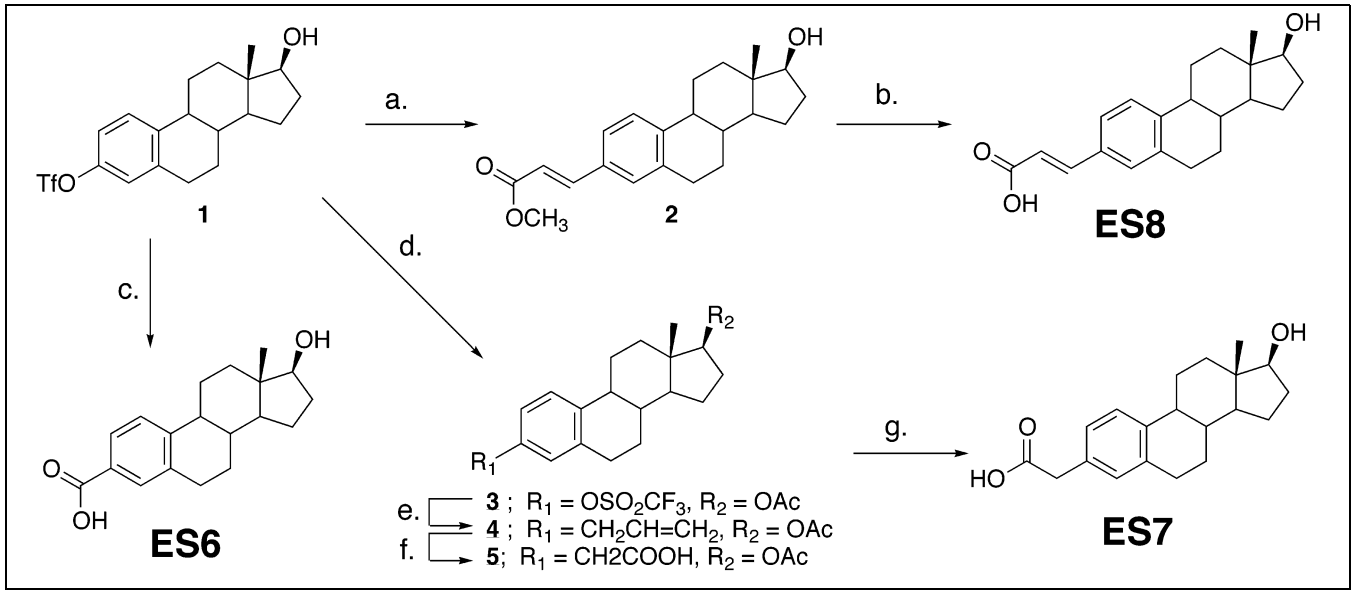


Fig. 3. Comparison of native ER–E2 complex (a) and modeled 'polar-group exchanged' ligand–receptor pairs ES6 (b), ES7 (c) and ES8 (d).



Scheme 1. Synthesis of carboxylate-functionalized ligands ES6, ES7 and ES8: (a) methyl acrylate, $Pd(PPh_3)_2Cl_2$, LiCl, DMF, 90–100°C/18 h [60]; (b) KOH, MeOH–H_2O; (c) CO, AcOK, $(AcO)_2Pd$, dppf, DMSO, 60°C/3 h [63]; (d) Ac_2O/Pyr, THF, 0°C; (e) allyltributyl tin, $Pd(PPh_3)_2Cl_2$, LiCl, DMF, 90°C/4 h [61]; (f) $NaIO_4/RuCl_3$–H_2O, CH_3CN–CCl_4–H_2O, RT/1 h [64]; (g) KOH, MeOH–H_2O, reflux/2 h.

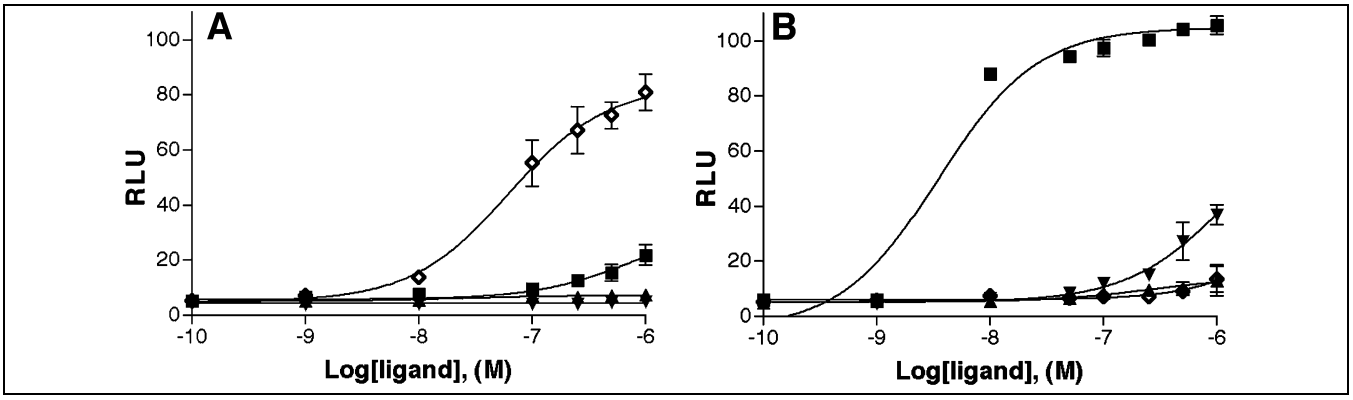


Fig. 4. Dose–response behavior of (A) HEO(E353A) and (B) 'modified wild-type' receptor HEO towards E2 (■), ES6 (▲), ES7 (▼), and ES8 (◇). Data are mean ± S.E.M. of six independent experiments run in triplicate.

mize high background activity associated with the wild-type in the absence of added ligand [66]. The three ligands, ES6, ES7 and ES8, were evaluated for their ability to activate reporter gene expression in human embryonic kidney 293 (HEK293) cells transiently cotransfected with an ER-responsive luciferase reporter (ERE-luc) and either the mutant HEO(E353A) or the 'modified wild-type' receptor HEO (Fig. 4). Ligands generally show similar selectivities (but activate at lower concentrations) when the true wild-type HEGO or HEGO(E353A) are employed, however EC_{50}s could not be accurately determined due to the high background activities observed for HEGO in the absence of ligand.

The dose–response data clearly show that cells expressing the mutant receptor HEO(E353A) show almost wild-type transactivation activity towards nanomolar concentrations of the ligand ES8 ($EC_{50} = 60$ nM). Only a very weak transactivation response is observed with HEO(E353A) at concentrations of E2 at or below 1000 nM, which is substantially higher than in vivo levels of E2 which are typically below 1.3 nM (Fig. 4A) [67]. The ligands ES6 and ES7 also show little to no activity at concentrations up to 1000 nM suggesting that the precise length and conformational preferences of the carboxyl tether are critical for activity towards the mutant receptor. Importantly, ES8 is unable to elicit a significant transactivation response in cells expressing the modified wild-type HEO at concentrations below 1000 nM whereas E2 is active at low nanomolar concentrations ($EC_{50} = 4.1$ nM) (Fig. 4B). ES8 also shows no activity towards cells transfected with reporter only. Together the data demonstrate that polar-group exchange can be an effective strategy for generating functionally orthogonal ligand–receptor pairs where the ligand can selectively activate the modified receptor at concentrations that do not activate the native receptor and the modified receptor is not activated by endogenous concentrations of natural ligand.

2.5. *Activity results are supported by in vitro ligand binding*

The observation that cells expressing HEO(E353A) show a strong transactivation response towards ES8 and not towards the structurally similar ligands ES6 and ES7, supports the notion that the activity of ES8 is related to its interactions with HEO(E353A) and not from non-specific interactions with the cell. The ability for the ligands E2, ES6 and ES8 to bind the 'true wild-type' HEGO(wt) and the mutant HEGO(E353A) was also assessed by radioligand displacement assays using the LBD of HEGO(wt) and that of HEGO(E353A) expressed in *Escherichia coli* following standard methods (Table 1) [68,69]. In vitro studies are consistent with the observed in vivo selectivity of ES8. Interestingly, ES6 binds HEGO(E353A) with high affinity ($K_d = 10.8$ nM) as was predicted by modeling stud-

Table 1
Comparison of in vitro ligand-binding constants (K_d), observed activities in cell culture (EC_{50}) and calculated apparent E_{ass}

Entry	Receptor	Ligand	E_{ass} (kJ/mol)	EC_{50} (nM)[a]	K_d (nM)[b]
1	hER(wt)	E2	−42.8	3.6 ± 0.4	0.15 ± 0.5
2	hER(wt)	ES6	−40.8	> 1000	68 ± 20
3	hER(wt)	ES7	−31.4	> 1000	n.d.
4	hER(wt)	ES8	−20.7	> 1000	107 ± 30
5	hER(E353A)	E2	−31.4	> 1000	60 ± 20
6	hER(E353A)	ES6	−52.2	> 1000	10.8 ± 1.0
7	hER(E353A)	ES7	−44.0	> 1000	n.d.
8	hER(E353A)	ES8	−53.7	60 ± 15	7.2 ± 0.5

[a] EC_{50}s determined using HEO with values reported as ± S.E.M. of six independent experiments performed in triplicate.
[b] K_ds determined using HEGO reported as ± S.E.M. of four experiments performed in triplicate.

ies, but evidently does not show activity in HEO(E353A) in the concentration range of our assay.

Although the apparent E_{ass}s calculated in the absence of explicit solvent cannot be expected to accurately compare structurally dissimilar ligands towards a specific receptor, it is noteworthy that the calculations predict the relative selectivities of E2, ES6 and ES8 towards ER(wt) and ER(E353A). In addition, modeling accurately predicted the relative affinities of the carboxylate-functionalized ligands ES6 and ES7 towards the mutant ER(E353A) and towards the native ER(wt). Taken together this suggests that site models of ligand-bound complexes provide a useful guide for rational molecular design of new receptor agonists.

3. Significance

This work describes a new rational design strategy called 'polar-group exchange' for engineering orthogonal ligand–receptor pairs. This strategy which involves altering the covalent connectivity of polar/charged groups adjacent to a ligand-binding pocket is used to create a mutant from the ER that selectively activates gene expression upon binding a rationally designed synthetic estrogen analog which does not activate the endogenous ER. The modification of receptor side-chains that are involved in ligand–receptor hydrogen bonds of the native complex provides a mechanism to actively discriminate against binding the natural ligand.

In this study we show that the mutant ER(E353A) has a 400-fold lower affinity for E2 and should not be responsive to endogenous concentrations of E2. However, ES8 can provoke a strong transcriptional response in cells expressing ER(E353A) at concentrations that do not activate cells expressing ER(wt). Apparent E_{ass}s calculated from site models based on the agonist-bound crystal structure are consistent with the results of in vitro ligand binding and cellular transactivation assays suggesting that molecular modeling of agonist-bound structures provides a useful guide to ligand–receptor design within the steroid hormone receptor family. With further refinement, this functionally orthogonal ligand–receptor pair should constitute a new tool for remote regulation of eukaryotic gene expression.

4. Materials and methods

4.1. General

All compounds were purchased from Aldrich unless otherwise noted. Tetrahydrofuran (THF) and ether were distilled from sodium benzophenone ketyl under nitrogen. Unless noted otherwise, all reactions were performed under a nitrogen atmosphere. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-400 spectrometer at the University of Delaware NMR facility. All chemical shifts are reported as δ (ppm) referenced to tetramethylsilane (TMS). Mass spectroscopy was performed at the University of Illinois mass spectrometry laboratory and the University of Delaware mass spectrometry laboratory. Oligonucleotides were purchased from The Great American Gene Company, Ransom Hill Bioscience Inc. Chromatography was performed using ICN SiliTech (60) flash silica. HEK293 cells (American Type Tissue Culture Collection) were maintained at the University of Delaware Cell Culture Core Facility. Transactivation response assays were performed using the Dual-Luciferase Reporter Assay System (Promega #E1960) following the manufacturer's protocol. Radiolabeled E2 ([^{3}H]E2) ([2,4,6,7-^{3}H]estra-1,3,5,(10)-triene-3,17-diol), 84.0 Ci/mmol, was obtained from Amersham Pharmacia Biotech UK (Arlington Heights, IL, USA). Unlabeled E2, butylated hydroxyanisole (BHA), isopropyl-D-thiogalactopyranoside (IPTG), leupeptin, aprotinin, pepstatin and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma; hydroxyapatite (HAP; Bio-Rad) was prepared following the procedure of Williams and Gorski [69]. The pET15b vector and BL21(DE3)pLysS cells were obtained from Novagen (Madison, WI, USA). Cell culture media was purchased from Mediatech (Herndon, VA, USA). Fetal bovine serum (FBS) was obtained from Summit Biotechnology (lot # FA0727).

4.2. Molecular modeling

Molecular modeling was performed on a silicon graphics Octane using Flo98/QXP modeling software (available from Thistlesoft, P.O. Box 227, Colebrook, CT 06021, USA; cmcma@ix.netcom.com) [59]. Site models representing all residues within 12 Å of bound E2, were constructed from published X-ray coordinates of Brzozowski et al. [47]. (Note: the HEO mutation G400V is remote to the ligand-binding site and is not included in the 12 Å model.) The side-chains of all residues that contacted the ligand were 'free' during all simulations. A site model for the mutant receptor ER(E353A) was generated by manually changing the side-chain of Glu353 and performing 10 rounds of 3 ps simulated annealing with E2 bound. Ligands were evaluated using the Monte Carlo docking (mcdock) algorithm of QXP (1000 steps) which minimizes the energy of the ligand-bound complex starting from random conformations and orientations of the ligand. Apparent E_{ass}s are determined as the difference between the energy of the minimized ligand-bound complex and the sum of the energies of the global minimum of the free ligand plus the local minimum of the receptor. All calculations are performed in the absence of explicit solvent using a distance-dependent dielectric (4.0r, Å).

4.3. Plasmid constructs

Mammalian expression vectors pSG1-HEO(E353A), pSG1-HEGO(E353A) (HEGO = ERα(wt), HEO = ERα(G400V)) were constructed from the parent plasmids pSG1-HEO and pSG1-HEGO [65], by oligonucleotide-directed mutagenesis using Quickchange (Stratagene) using the following oligonucleotides:

E353Af, CCAACCTGGCAGACAGGGCGCTGGTTCACATG-ATCAACTGG; E353Ar, CCAGTTGATCATGTGAACCAG-CGCCCTGTCTGCCAGGTTGG.

Prokaryotic expression vectors pET15b-ER and pET15-b(E353A) were generated by sub-cloning of the wild-type and mutant hormone-binding domain sequences into the *Nde*I and *Bam*HI sites of pET15b (Novagen, Madison, WI, USA) using cloning primers 5′-GGGAATTCCATATGAACAGCCTGGC-CTTGTCC-3′ and 5′-CGCCGGATCCGCGCTAGTGGGCG-CATGTAG-3′. All constructs were sequenced over the entire coding region of ER to confirm the presence of only the desired mutation.

4.4. Cell culture and transfections

All cell culture experiments were performed using phenol red-free Dulbecco's modified Eagle's medium (DMEM). Twenty-four h prior to transfection, HEK293 cells were seeded at a density of 40 000 cells per well in 24-well culture plates. Three h prior to transfection, the media was changed to media made with 10% charcoal-resin-stripped FBS [70]. Transfections were performed by the $CaPO_4$ method using 0.03 μg DR4−luc+, 0.14 μg pRLbasic and 0.08 μg pSG1-HEO or pSG1-HEO(E353A) per well. Six hours after transfection, the media was removed and replaced with DMEM+10% charcoal-resin-stripped FBS containing appropriate concentrations of ligand. The cells were allowed to incubate with ligand for 36 h before harvesting by passive lysis. Cell extracts were immediately assayed using the Dual-Luciferase Assay (Promega) using a Dynex luminometer. Activity is reported in relative light units (RLU) determined as the ratio of the firefly luminescence divided by the luminescence of the renilla luciferase control. Dose–response data were analyzed using non-linear regression analysis of Graphpad Prism (Graphpad Software).

4.5. In vitro ligand-binding assays

The LBDs (amino acids 304–554) of ER (HEGO or HEGO(E353A)) were expressed in *E. coli* according to the procedure of Katzenellenbogen [68]. Briefly, 1 l of 2XYT media was inoculated with overnight cultures (5 ml) of *E. coli* BL21(DE3)pLysS cells expressing either pET15bHEGO-LBD or pET15bHEGO(E353A)-LBD. The cultures were grown at 37°C to an $OD_{600\,nm}$ of 0.6–0.7. The cells were induced with IPTG and incubated at 30°C for 3 h. The cells were harvested by centrifugation (15 min at 6000×*g*, 4°C) and the cell paste resuspended in 10 ml cold lysis buffer (50 mM Tris (pH 7.5), 10% (v/v) glycerol, 0.1 mM BHA, 10 mM mercaptoethanol, 50 μg/ml pepstatin A and 0.2 mM PMSF). The suspension was sonicated (Branson sonicator with microprobe) for 3×10 s at 60% power. The cell debris and the pellet fraction were separated from the supernatant by centrifugation (10 000×*g*, 20 min, 4°C). The crude supernatant could be used directly or diluted to the desired concentration in binding buffer (50 mM Tris, pH 7.5, 10% glycerol, 0.1 mM BHA, 10 mM mercaptoethanol, 0.5% yeast extract). SDS–PAGE analysis confirms the induction of an approximate 35 kDa protein for both wild-type and mutant proteins as reported [68]. Preparations of HEO(E353A)-LBD bound E2 too weakly (or perhaps was too unstable) to determine dissociation constants for E2 or for the compounds ES6, ES7, and ES8 by competitive titration, therefore binding assays were performed on the true wild-type HEGO.

The ligand-binding assays were performed according to the procedure of Katzenellenbogen [68]. The ER was diluted to ~1.5–2 nM (or 5–10 nM for HEGO(E353A)-LBD) in binding buffer and incubated with various concentrations of [^{3}H]E2 in the absence or presence of a 200-fold excess of unlabeled ligand for 16–18 h at 4°C. The bound ligand was assayed by adsorption onto HAP for 30 min at 4°C, followed by three washes with 0.8 ml of cold 0.05 M Tris (pH 7.3). The HAP pellet was resuspended in 1 ml of EtOH and counted in 10 ml of scintillation fluid. The amount of specific ligand binding at each ligand concentration, determined as the difference between the total binding and the non-specific binding, was fit to the following equation by non-linear regression analysis using GraphPad InPlot (Graphpad software):

$$\mathrm{CPM} = \mathrm{CPM_i} + (\mathrm{CPM_f} - \mathrm{CPM_i}) \times \frac{(L_0 + P_0 + K_d) - \sqrt{(L_0 + P_0 + K_d)^2 - 4P_0L_0}}{2P_0}$$

where L_0 and P_0 are the total concentration of ligand and active ER, respectively; $\mathrm{CPM_i}$ and $\mathrm{CPM_f}$ are the amount of counted scintillation fluid at initial and saturated concentrations, respectively, and K_d is the dissociate constant of ligand and receptor.

The dissociation constants for the designed ligands ES6 and ES8 were determined by competitive titration. The tritiated tracer, [^{3}H]E2 (84.0 Ci/mmol), was used at 5 nM to give the assay greater sensitivity to measure the high affinity of the E2 to wild-type ER, while 20 nM was used for mutant due to the low affinity of E2 to mutant. The crude receptor was incubated with different concentrations of unlabeled ligand along with 5 nM [^{3}H]E2 (84.0 Ci/mmol) for HEGO-LBD or 20 nM [^{3}H]E2 for HEGO(E53A)-LBD, at 4°C for 16–18 h. The unlabeled competitors were added from stock solutions in dimethyl sulfoxide (DMSO) such that the final concentration of DMSO in the assay solution was 1%. Dissociation constants were determined by non-linear regression analysis using GraphPad Prism.

4.6. Analysis of ligand purity

Because of the potential for small amounts of E2 or estrogenic impurities to cause otherwise inactive compounds to exhibit activity, the purity of all ligands tested was rigorously established by high-performance liquid chromatography (HPLC). All samples used were demonstrated to be homogeneous and free of any detectable traces of E2 (to less than 0.01%) by HPLC under conditions where the two species were well resolved.

4.6.1. 3-Carboxyl-17β-hydroxy-estra-1,3,5(10)-triene (ES6)

A mixture of **1** (3-trifluoromethanesulfonyl-17β-hydroxy-estra-1,3,5(10)-triene) [71] (100 mg, 0.2 mmol), potassium acetate (0.392, 4 mmol), palladium(II) diacetate (20 mg, 0.05 mmol),

1,1-bis(diphenylphosphino)ferrocene (dppf) (100 mg, 0.2 mmol) in DMSO (3 ml) was purged with carbon monoxide for 5 min and stirred under a CO balloon at 60°C for 3 h. The reaction mixture was diluted with 10 ml water, acidified with 5 ml 1 N HCl and extracted with 3×15 ml CH_2Cl_2. The organic layer was washed with 2×10 ml water, 10 ml brine, dried with $MgSO_4$. The solvent was concentrated under reduced pressure to afford ES6 as a white powder which was recrystallized from MeOH to afford 55 mg of ES6 (0.18 mmol, 70.5%); mp >260°C; δ_H (400 MHz, CD_3OD) 0.72 (s, 3H), 1.18–1.59 (m, 7H), 1.71 (m, 1H), 1.89–2.34 (m, 5H), 2.84 (q, J = 4.2 Hz, 2H), 3.61 (t, J = 8.6 Hz, 1H), 7.32 (d, J = 8.2 Hz, 1H), 7.65 (s, 1H), 7.68 (d, J = 8.2 Hz, 1H); δ_C (100 MHz, CD_3OD) 11.77, 24.17, 27.33, 28.33, 30.79, 38.09, 39.99, 44.43, 46.18, 49.99, 51.53, 82.53, 126.68, 128.15, 129.17, 131.48, 138.26, 147.31, 170.39; HRMS (EI) calcd for $C_{19}H_{24}O_3$: 300.1725. Found: 300.1717.

4.6.2. 3-Methyl acrylic-17β-hydroxy-estra-1,3,5(10)-triene (2)

A solution of 240 mg (0.6 mmol) **1**, 114 mg (1.3 mmol) methyl acrylate, 112 mg (2.6 mmol) lithium chloride, a few crystals of 2,6-di-*tert*-butyl-methylphenol, and 1.0 ml triethylamine in 5 ml DMF was sparged with nitrogen for 10 min before 40 mg (0.2 mmol) bis(triphenylphosphine)palladium dichloride was added. The rapidly stirred suspension was then heated to 90°C for 24 h. The mixture was allowed to cool before 20 ml 10% HCl was added and the mixture was extracted with 3×30 ml ethyl acetate. The organic layer was washed with brine (2×20 ml), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica flash chromatography to give 170 mg of **2** (0.5 mmol, 83.8%); mp 153–155°C; δ_H (400 MHz, $CDCl_3$) 0.77 (s, 3H), 1.24–1.52 (m, 7H), 1.75 (m, 1H), 1.96–2.37 (m, 5H), 2.5 (brs, 1H), 2.84 (q, J = 3.5 Hz, 2H), 3.71 (t, J = 8.6 Hz, 1H), 3.78 (s, 3H), 6.40 (d, J = 16 Hz, 1H), 7.21 (s, 1H), 7.28 (d, J = 7.8, 1H), 7.65 (d, J = 16 Hz, 1H); δ_C (100 MHz, $CDCl_3$): 12.68, 23.55, 26.45, 27.45, 29.83, 30.78, 37.12, 38.82, 43.63, 44.99, 50.53, 52.12, 84.26, 117.06, 125.75, 126.37, 129.43, 132.04, 137.72, 143.72, 146.81, 168.15; HRMS (EI) calcd for $C_{22}H_{28}O_3$: 340.1962. Found: 340.2006.

4.6.3. 3-Acrylic acid-17β-hydroxy-estra-1,3,5(10)-triene (ES8)

To a solution of 50 mg (0.15 mmol) of **2** in 5 ml ethanol was added 2 ml of 1 M NaOH and the solution was heated to reflux for 1 h. After cooling to ambient temperature, the solution was acidified by addition of 1 M HCl and extracted with 2×10 ml EtOAc. The combined organic extracts were dried over $MgSO_4$ and concentrated under reduced pressure to afford a light yellow powder. The crude product could be further purified by recrystallization twice from MeOH to afford 35 mg (0.11 mmol, 73%) of pale yellow prisms: mp 238–240°C; δ_H (400 MHz, CD_3OD) 0.75 (s, 3H), 1.25–1.50 (m, 7H), 1.71 (m, 1H), 1.80–2.34 (m, 5H), 2.84 (q, J = 4.3 Hz, 2H), 3.63 (t, J = 8.6 Hz, 1H), 6.37 (d, J = 16 Hz, 1H), 7.24 (s, 1H), 7.30 (d, J = 8.6 Hz, 2H), 7.58 (d, J = 16 Hz, 1H); δ_C (100 MHz, CD_3OD) 11.28, 24.17, 27.38, 28.38, 30.81, 38.11, 40.14, 44.45, 45.02, 46.09, 51.25, 82.55, 118.23, 126.56, 127.18, 130.07, 133.16, 138.69, 143.82, 146.78, 170.80; HRMS (EI) calcd for $C_{21}H_{26}O_3$: 326.1882. Found: 326.1871.

4.6.4. 3-Trifluoromethanesulfonyloxy-17β-acetoxy-estra-1,3,5(10)-triene (3)

To a solution of triflate **1** (1.06 g, 2.5 mmol) in pyridine (3 ml) at 0°C was added acetic anhydride (1.0 ml, 10 mmol). The reaction was allowed to stir at ambient temperature for 24 h before water (100 ml) was added and the resulting mixture partitioned with ether (50 ml). The organic extract was washed with 20 ml 1 N HCl, 3×20 ml $CuSO_4$, 2×20 ml water, 20 ml brine and was dried over $MgSO_4$. The organic layer was evaporated under reduced pressure, and the residue was recrystallized from EtOH to afford 1.02 g (2.3 mmol, 90%) of fine, white needles: mp 56–58°C; δ_H (400 MHz, $CDCl_3$) 0.83 (s, 3H), 1.38–1.49 (m, 7H), 1.75 (m, 1H), 1.91 (m, 2H), 2.06 (s, 3H), 2.25 (m, 3H), 2.89 (dd, J = 8.8, 4.2 Hz, 2H), 4.69 (dd, 8.0, 7.8 Hz, 1H), 6.96, (d, J = 2.6 Hz, 1H), 7.01 (dd, J = 8.6, 1.6 Hz, 1H), 7.33 (d, J = 8.7 Hz, 1H); δ_C NMR (100 MHz, $CDCl_3$) 12.19, 21.37, 23.40, 26.13, 26.93, 27.69, 29.66, 36.93, 38.11, 42.98, 44.09, 49.95, 82.36, 117.31, 118.31, 121.32, 127.38, 139.69, 140.91, 147.64, 171.39; HRMS calcd for $C_{21}H_{25}F_3SO_5$: 446.1375. Found: 446.1397.

4.6.5. 3-Allyl-17β-acetoxy-estra-1,3,5(10)-triene (4)

A solution of 220 mg (0.5 mmol) of **3**, 0.17 ml (0.55 mmol) allyltributyl tin, 68 mg (1.6 mmol) lithium chloride and 1 mg 2,6-di-*tert*-butyl-4-methylphenol in 3 ml DMF was sparged with nitrogen for 10 min before 10 mg (0.05 mmol) bis(triphenylphosphine)palladium dichloride was added. The resulting suspension was then heated to 90°C for 4 h. The reaction mixture was allowed to cool to room temperature, and treated with 0.2 ml pyridine and 0.4 ml HF–pyridine. This mixture was allowed to stir at room temperature overnight. The mixture was then diluted with ethyl ether, filtered through a small pad of Celite, and washed with 10 ml water, 10 ml 10% HCl, 10 ml water and 10 ml brine. The organic layer was dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica flash chromatography to give 130 mg (0.42 mmol, 84% yield) of **4**: mp 101–102°C; δ_H (400 MHz, $CDCl_3$) 0.82 (s, 3H), 1.21–1.59 (m, 7H), 1.71 (m, 1H), 1.89 (m, 2H), 2.06 (s, 3H), 2.15–2.34 (m, 5H), 2.85 (m, 2H), 3.33 (d, J = 6.8 Hz, 2H) 4.68 (t, J = 8.1 Hz, 1H), 5.06 (m, 1H), 5.95 (m, 1H), 6.92 (s, 1H), 6.98 (d, J = 8.6 Hz, 1H), 7.23 (d, J = 8.6 Hz, 1H); δ_C (100 MHz, $CDCl_3$) 12.22, 21.39, 23.42, 26.13, 27.32, 28.02, 29.62, 37.05, 37.92, 38.48, 43.04, 44.28, 50.02, 82.88, 94.32, 103.89, 125.80, 126.99, 130.31, 131.86, 137.05, 139.20, 171.44.

4.6.6. 3-Carboxymethylene-17β-acetoxy-estra-1,3,5(10)-triene (5)

To a rapidly stirred solution of 70 mg (0.2 mmol) of **3** in 0.5 ml carbon tetrachloride and 0.5 ml acetonitrile was added a solution of 142 mg (0.68 mmol) sodium metaperiodate in 0.75 ml water and 1 mg (0.01 mmol) ruthenium trichloride hydrate. The resulting suspension was stirred at ambient temperature for 1 h and then diluted with 50 ml of methylene dichloride. The layers were separated, and the organic phase washed with 10 ml water, 10 ml brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel flash chromatography to give 35 mg (0.1 mmol, 50% yield) of **5**: δ_H (400 MHz, $CDCl_3$) 0.77 (s, 3H), 1.24–1.50 (m, 7H), 1.71 (m, 1H), 1.92 (m, 2H), 2.06

(s, 3H), 2.15–2.34 (m, 5H), 2.84 (m, 2H), 3.57 (s, 2H), 4.68 (t, $J = 8.1$ Hz, 1H), 7.00 (d, $J = 8.6$ Hz, 1H), 7.14 (s, 1H), 7.26 (d, $J = 8.6$ Hz, 1H); δ_C (100 MHz, $CDCl_3$) 14.41, 21.35, 23.46, 25.06, 26.14, 27.26, 28.06, 29.61, 37.02, 43.06, 45.56, 50.01, 67.81, 82.80, 125.82, 127.56, 130.98, 137.25, 139.77, 146.84, 171.54, 171.71.

4.6.7. 3-Carboxymethylene-17β-hydroxy-estra-1,3,5(10)-triene (ES7)

To a solution of 20 mg (0.06 mmol) of **5** in 5 ml ethanol was added 2 ml of 1 M NaOH and the solution heated to reflux for 1 h. The solution was allowed to cool to ambient temperature and acidified with 1 M HCl, which resulted in the formation of a white precipitate. The mixture was extracted with 2×10 ml EtOAc, and dried over $MgSO_4$. The solution was concentrated under reduced pressure to afford a light yellow powder. An analytical sample was obtained by recrystallization twice from MeOH to give 12 mg (0.04 mmol, 68%) of a white solid: δ_H (400 MHz, CD_3OD) 0.78 (s, 3H), 1.25–1.50 (m, 7H), 1.70 (m, 1H), 1.94 (m, 2H), 2.11–2.34 (m, 5H), 2.84 (m, 2H), 3.51 (s, 2H), 3.74 (t, $J = 8.1$ Hz, 1H), 6.97 (d, $J = 8.6$ Hz, 1H), 7.14 (s, 1H), 7.26 (d, $J = 8.6$ Hz, 1H); δ_C (100 MHz, CD_3OD) 11.77, 24.17, 27.34, 28.33, 30.08, 30.56, 30.81, 38.10, 40.01, 44.43, 46.19, 51.55, 82.60, 126.67, 128.14, 129.12, 131.47, 138.27, 147.34, 170.36; HRMS (EI) calcd for $C_{20}H_{26}O_3$: 314.1882. Found: 314.1876.

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