

Directed evolution of estrogen receptor proteins with altered ligand-binding specificities

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Transcriptional activators that respond to ligands with no cellular targets are powerful tools that can confer regulated expression of a transgene in almost all biological systems. In this study, we altered the ligand-binding specificity of the human estrogen receptor α (hER α) so that it would recognize a non-steroidal synthetic compound with structural similarities to the phytoestrogen resveratrol. For this purpose, we performed iterative rounds of site-specific saturation mutagenesis of a fixed set of ligand-contacting residues and subsequent random mutagenesis of the entire ligand-binding domain. Selection of the receptor mutants and quantification of the interaction were carried out by exploiting a yeast two-hybrid system that reports the ligand-dependent interaction between hER α and steroid receptor coactivator-1 (SRC-1). The screen was performed with a synthetic ligand (CV3320) that promoted growth of the reporter yeast strain to half maximal levels at a concentration of 3.7 μ M. The optimized receptor mutant (L384F/L387M/Y537S) showed a 67-fold increased activity to the synthetic ligand CV3320 (half maximal yeast growth at 0.055 μ M) and a 10-fold decreased activity to 17 β -estradiol (E2; half maximal yeast growth at 4 nM). The novel receptor-ligand pair partially fulfills the requirements for a specific 'gene switch' as it responds to concentrations of the synthetic ligand which do not activate the wildtype receptor. Due to its residual responsiveness to E2 at concentrations (4 nM) that might occur *in vivo*, further improvements have to be performed to render the system applicable in organisms with endogenous E2 synthesis.

Keywords: estrogen receptor/ligand specificity/saturation mutagenesis

Introduction

The mammalian steroid receptors are ligand-dependent transcriptional activators that have been engineered to serve as specific 'gene switches' in heterologous systems such as yeasts, plants and mammals (Louvion *et al.*, 1993; Aoyama and Chua, 1997; Gallinari *et al.*, 2005). The basic strategy involves fusing the ligand-binding domain (LBD) of the receptor to a heterologous transcriptional activator which

binds to a specific target promoter that is not recognized by endogenous transcription factors. A typical example is the chimeric transcriptional activator GVG that consists of the DNA-binding domain of yeast transcription factor GAL4, the activation domain of *Herpes simplex* virus protein VP16 and the LBD of the rat glucocorticoid receptor (Aoyama and Chua, 1997). This protein activates transcription from a target promoter containing *GAL4* upstream activating sequences and a core promoter only in the presence of dexamethasone and related steroids and has been used for studying a number of biological processes in transgenic plants (Moore *et al.*, 2006). In mammalian cells, such an activator would respond to endogenous steroids, and—vice versa—endogenous genes would respond to the steroid applied to activate the transgene. Therefore, numerous studies have been initiated to alter the ligand specificity of various receptors so that it would bind to compounds with no cellular targets (Chockalingam *et al.*, 2005; Gallinari *et al.*, 2005). This concept is also valuable for agricultural purposes as side effects on other organisms could be avoided.

The estrogen receptor is particularly suited to design such a so-called functionally orthogonal gene switch because it can accommodate a variety of non-steroidal compounds in its binding pocket (Gallinari *et al.*, 2005). A minimal requirement is the distance between two terminal polar oxygen groups of the inducing molecule (Fig. 1 (Anstead *et al.*, 1997; Brzozowski *et al.*, 1997)) and an aromatic ring in the 'A' position, while the remaining part of the molecule can be quite different in structure. Indeed, more spacious chemicals like the antagonist tamoxifen or the agonists of the tetrahydrofluorenone-type fit into the pocket (Brzozowski *et al.*, 1997; Gallinari *et al.*, 2005).

Recently, a general method for the generation of specific receptor-ligand pairs was described (Chockalingam *et al.*, 2005). It involves the random replacement of each ligand-contacting residue by all possible 20 amino acids. The generated receptor mutants are selected for binding to a synthetic ligand using a high throughput yeast screening system. Subsequently, the site saturation mutagenesis is repeated with the resulting mutant. The ligand specificity can finally be enhanced by random point mutagenesis of the LBD and phenotypic screening for further improved mutants. Using the synthetic non-steroidal compound 4,4'-dihydroxybenzil (DHB), a mutant human estrogen receptor α (hER α) that showed a 50-fold enhanced binding activity to DHB and a 140-fold reduced affinity to its natural ligand E2 was selected after two rounds of mutagenesis. After three further rounds of mutagenesis and selection, a mutant receptor was identified that showed a 5-fold enhanced binding activity to DHB and an over 10^6 -fold reduced affinity to E2 when compared with the wildtype protein. Thus, the selectivity was enhanced by over 10^7 -fold.

Here we have applied this strategy to a class of synthetic non-steroidal compounds that are characterized by one aromatic ring with a hydroxyl group being connected to a

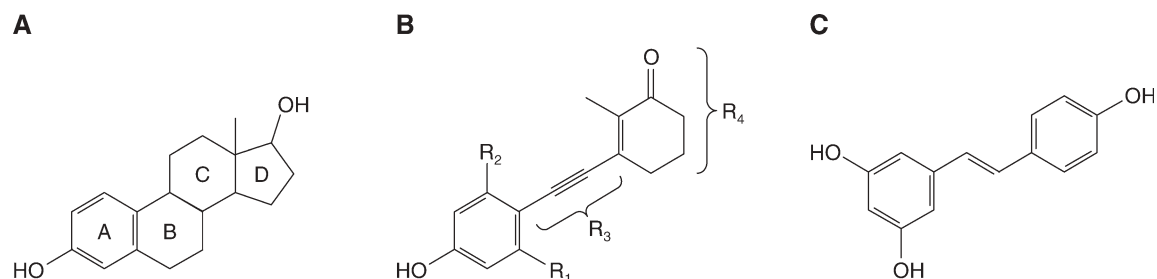


Fig. 1. Structures of 17 β -estradiol (A), the scaffold of synthetic ligands used in this study (B) and resveratrol (C). R₁ to R₄ represent variable parts. A complete list of synthetic ligands is given in Supplementary data available at *PEDS* online, Fig. S1.

substituted cyclohexenone via a C–C triple bond (Fig. 1B). The starting apparent affinity of the ligand to the wildtype receptor as determined by its effective concentration to promote half maximal growth (EC₅₀) of the yeast reporter strain (Chockalingam *et al.*, 2005) was 3.7 μ M and was enhanced to 0.055 μ M after three rounds of mutagenesis and selection. Conversely, the affinity of the mutant receptor to E2 was reduced by a factor of 10.

Methods

Synthesis of ligands

Estrogen analogs were synthesized using a Pd-catalyzed *Sonogashira*-reaction of an iodoarene derivative with propargylic alcohol followed by an oxidative elimination of the CH₂OH-group and a second Pd-catalyzed *Sonogashira* reaction with a 3-iodocyclopentenone or a 3-iodocyclohexenone (Tietze *et al.*, 2008). A broad range of substitution patterns was covered and a complete list of the compounds tested in this work is depicted in Supplementary data available at *PEDS* online, Figure S1.

Mutagenesis of the human estrogen receptor, selection of mutants, ligand dose-response assays and molecular modeling

A yeast two-hybrid system that reports the ligand-dependent interaction between hER α and human SRC-1 was used for the selection of hER α mutants and the dose-response assay (Chockalingam *et al.*, 2005). Briefly, the bait GBD-hER α contains most of the LBD (amino acids 303–553) and the F domain (amino acids 554–595) of hER α fused to the GAL4 DNA-binding domain (GBD). SRC-1 fused to the GAL4 activation domain (GAD) served as a prey (AD-SRC-1). The GBD-hER α /AD-SRC-1 complex, which is formed only after binding of agonist ligands to LBD, activates the selection marker gene *HIS3* enabling the yeast strain YRG2 to grow on histidine drop-out medium. Plasmids, mutant library creation and screening, ligand dose-response assays and molecular modeling were carried out as described previously (Chockalingam *et al.*, 2005). Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (Pettersen *et al.*, 2004).

Plasmids used in the yeast one-hybrid system

Plasmid pGBT-GEV is a pGBT9 (Clontech Laboratories Inc., Saint-Germain-en-Laye, France) derivative that contains the GBD (aa 1–92), the human estrogen receptor α ligand-

binding domain (hER α LBD; aa 282–595) and the VP16 activation domain (aa 423–490) as in pHCA/Gal4ERVP16 (Louvion *et al.*, 1993) behind the *ADHI* promoter. Derivatives of pGBT-GEV containing the wildtype or engineered LBDs were transformed into yeast strain PJ69-4A according to Gietz and Woods (Gietz and Woods, 2002). Quantitative β -galactosidase assays were performed as described (James *et al.*, 1996).

Results and discussion

Rationale for the synthesis of suitable ligands

Estrogen receptor agonists induce a specific conformational switch of cognate receptor proteins, which lead to the dissociation from the inhibitory protein HSP90 (Yamamoto *et al.*, 1988) and allow the association with the co-activator SRC-1 (Onate *et al.*, 1995). Therefore, it can be envisioned that the starting ligand for a strategy designed to obtain receptor proteins with altered ligand specificities should already weakly promote activation of hER α . Typically, estrogen receptor ligands comprise two hydroxyl groups separated by a rigid hydrophobic linker region consisting of four carbocyclic rings. The hydroxyl group at the ‘A’ ring forms hydrogen bonds with the polar amino acids arginine 394 (R394) and glutamic acid 353 (E353) of hER α , whereas the hydroxyl group at the D ring forms a hydrogen bond with histidine 524. This arrangement is important for the ligand-induced conformational change (Brzozowski *et al.*, 1997). Therefore, we designed chemicals (Fig. 1B and Supplementary data are available at *PEDS* online, Fig. S1) that contain two polar oxygen containing groups at a distance of 10–12 Å which is in the same range as the distance of the oxygen atoms in the estradiol (E2) molecule. The two aromatic rings of the synthetic compounds which correspond to the ‘A’ and ‘D’ rings of the E2 molecule are connected either by a linear two-carbon single, double or triple bond spacer rather than by the carbocyclic rings found in E2. The scaffold of the ligands has similarity to the phytoestrogen resveratrol found in red wine (Fig. 1C), which activates hER α at concentrations of 3–10 μ M (Gehm *et al.*, 1997). As phytoestrogens are phytoalexins with anti-fungal activity (Hain *et al.*, 1990), they do not necessarily have plant and mammalian targets when used at low doses, though beneficial activity through the interaction with sirtuins has been observed for these compounds (Howitz *et al.*, 2003). The aromatic ‘A*’ ring of the synthetic ligands carries the hydroxyl group in a position analogous to the hydroxyl group in E2. The ‘D*’ ring being either a 5- or a 6-membered ring carries a keto-group similar to the E2

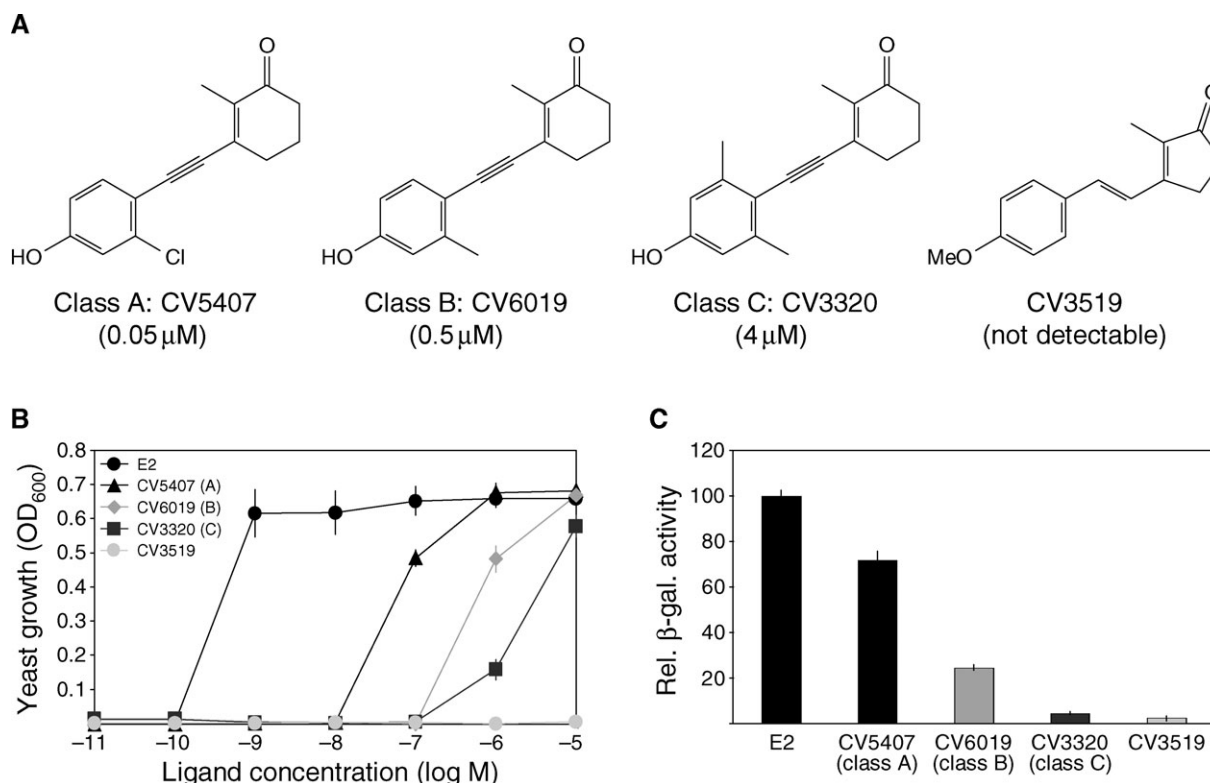


Fig. 2. Ligand classification based on their potential to mediate the interaction of the wildtype hER α with the co-activator SRC-1 in yeast. (A) Structures of different ligands representative for classes A to C. CV3519 does not represent a suitable ligand for hER α and serves as a negative control. The concentrations necessary to achieve half-maximal induction (EC_{50}) of the yeast growth response as determined in (B) is given below each ligand. (B) Dose-response growth curves of yeast clones expressing the ligand-binding domain of hER α fused to the GAL4 DNA-binding domain (GBD-hER α) and SRC-1 fused to the GAL4 activation domain (AD-SRC-1) in the presence of different ligands shown in (A). (C) Yeast one-hybrid system: *LacZ* reporter gene activity mediated by the chimeric activator consisting of the ligand-binding domain of hER α fused to the GAL4 DNA-binding domain and the VP16 activation domain (GEV). Relative β -galactosidase activities were calculated by setting the activity induced by the natural ligand E2 to 100%. Ligands were applied at 10 μ M concentrations. Values represent mean \pm standard deviation (SD) of three replicates.

homologue estron which renders the synthetic compounds more stable. The absence of the 'B' and the 'C' ring should lead to a reduced affinity to the wildtype receptor and the introduction of additional substituents to rings 'A*' or 'D*' should provide contact sites to mutant receptor proteins.

Selection of appropriate ligands for the creation of an orthogonal ligand-receptor pair

Twenty-seven different ligands of our compound library were grouped into three classes according to their capacity to promote yeast growth by facilitating the interaction between the wildtype receptor LBD and its co-activator SRC-1 in the yeast two-hybrid system (A: 0.01–0.09 μ M; B: 0.1–0.9 μ M; C: 1–9 μ M; Supplementary data are available at *PEDS* online, Fig. S1). All experiments were performed in 96-well plates that allow measuring cell density at OD_{600} . Cells expressing both the bait (GBD-hER α) and the prey protein (AD-SRC) showed the half-maximal growth at an effective concentration (EC_{50}) of 0.42 nM E2, which is in good agreement with the reported value for ligand-dependent transactivation by the full-length hER α in yeast and CHO cells (Wrenn and Katzenellenbogen, 1993). Class A contains only one compound (CV5407 (Fig. 2A)) which shows an EC_{50} of 0.05 μ M (Fig. 2B). Exchanging the chlorine of CV5407 to a methyl group decreased the EC_{50} by a factor of 10 (0.5 μ M; Fig. 2B). Addition of a further methyl group in R₂ generated a compound with an EC_{50} value of 4 μ M (class C ligand;

Fig. 2A and B). Like resveratrol (Fig. 1C; Gehm *et al.*, 1997), compounds containing a C₂-double bond or a C₂-single bond (R₃) had high EC_{50} values of 6 μ M (Supplementary data are available at *PEDS* online, Fig. S1). As expected, protection of the phenolic hydroxyl group at C-3 essentially abolished the interaction (Fig. 2A and B).

Classification of the ligands according to their affinities to the wildtype receptor was confirmed in the yeast one-hybrid assay. This system is based on a chimeric transcriptional activator (GBD-hER α -VP16) consisting of the yeast GBD, the hER α ligand-binding domain (LBD) (aa 282–576) and the VP16 activation domain. The yeast strain PJ69 encodes the β -galactosidase reporter genes under control of a target promoter containing *GAL4* upstream activating sequences (*GAL7:lacZ*) (Louvion *et al.*, 1993). Activation of this reporter gene by GBD-hER α -VP16 depends on the binding of E2 to LBD, which leads to the dissociation of the protein from the inhibitory protein HSP90, allowing translocation into the nucleus (Yamamoto *et al.*, 1988). As shown in Fig. 2C, the relative effective concentrations of the various ligands established with the two-hybrid system were reproduced. However, 20% of the maximal response was obtained at 10 μ M CV6019 with the yeast one-hybrid system, whereas 0.2 μ M were sufficient in the yeast two-hybrid system, indicating that the one-hybrid system is approximately 200-fold less sensitive than the two-hybrid system. This can be explained by the fact that basal amounts of GBD-hER α and

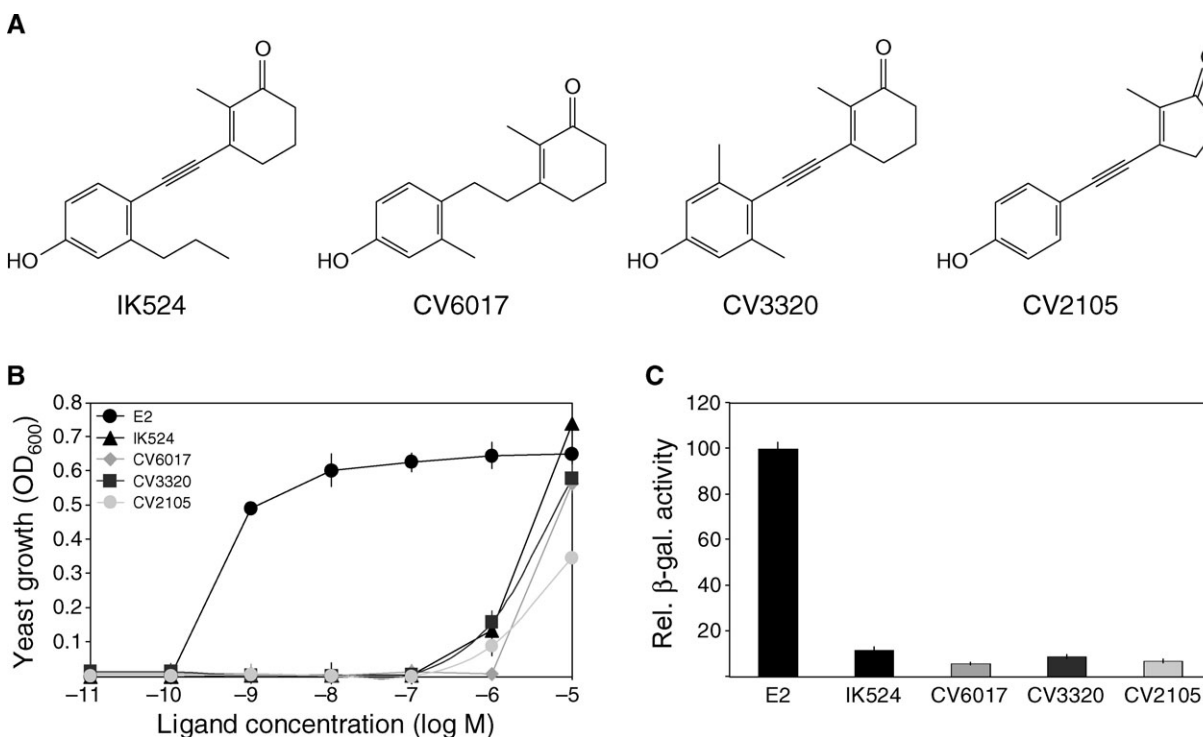


Fig. 3. Interaction of class C ligands with wildtype hER α . (A) Structure of ligands. (B) Dose-response growth curves of yeast clones expressing GBD-hER α and AD-SRC-1 in the presence of different ligands shown in (A). (C) *LacZ* reporter gene activities based on GEV binding to its target promoter in the yeast one-hybrid system. Relative β -galactosidase activities were calculated by setting the activity induced by the natural ligand E2 to 100%. Ligands were applied at 10 μ M concentrations. Values represent mean \pm SD of three replicates.

GBD-hER α -VP16 are in the nucleus even in the absence of the ligand, which in turn leads to higher background activities and thus to a reduced sensitivity of the GBD-hER α -VP16-based system.

We performed our directed evolution studies with four group C ligands (Fig. 3A), thus reducing the risk of activating wildtype hER α in the case of a future application. All four compounds activated the wildtype hER α LBD to half maximum activity at concentrations between 2 and 7 μ M in the yeast two-hybrid system, but did not increase background-level activity of the yeast one-hybrid strain even at a 10 μ M concentration (Fig. 3C).

Saturation mutagenesis

The saturation mutagenesis strategy involves the replacement of each amino acid residue that is expected to contact the ligand with the 19 possible alternatives followed by the selection using the yeast two-hybrid system. To identify residues likely to interact with our synthetic ligands, we exploited the known co-crystal structure of hER α LBD complexed with anti-estrogen diethylstilbestrol (DES) for molecular modeling studies (Shiau *et al.*, 1998) (Fig. 4). Docking of class C ligand CV3320 into the binding pocket of hER α LBD was performed using Molecular Operating Environment (MOE; Chemical Computing Group, Montreal, Canada) as described before (Chockalingam *et al.*, 2005). Twenty-one residues that are in direct contact (within 4.6 Å) were identified. Three of the 21 residues, namely E353, R394 and H524, were excluded from individual site saturation mutagenesis because of their known essential role in hydrogen bonding with the terminal polar oxygen atoms of E2 (Brzozowski *et al.*, 1997).

Each yeast library of receptor variants mutated at one of the 18 positions was plated onto minimal media agar plates that allowed selecting for both the prey and the bait plasmid. One hundred and ninety yeast colonies from each library were picked and subjected to the yeast two-hybrid screening by monitoring the growth on selective medium in a microtiter plate. The concentration of the synthetic ligands (CV2105, CV3320, CV6017 and IK524) was 0.5 μ M, which is about 10-fold lower than the EC₅₀ value determined with the wildtype receptor. Yeast cells expressing the non-mutagenized wildtype construct were included as controls in the screening step. Mutants were considered as promising candidates when yeast cells grew more vigorously when compared with the controls in the presence of the synthetic ligand and when the same mutant grew less vigorously in the presence of 0.5 nM E2.

In the first round of saturation mutagenesis, six putative candidate clones were identified with ligand CV3320 and two with ligand IK524. Sequence analysis revealed that the leucine residue at position 384 was exchanged to phenylalanine (L384F) in all eight clones. The clone showed a 10-fold improved activity towards CV3320, and a 90-fold weakened response towards E2 (Fig. 5A; Table I). The mutant protein also showed a 10-fold higher sensitivity towards IK524, but the sensitivity to CV6017 or CV2105 was not altered (data not shown). Next, the DNA of the mutant was taken as a template for performing further saturation mutagenesis in the remaining 17 positions. No clones with improved sensitivity towards CV3320 or IK524 could be obtained, but one of the mutants (L384F/L387M) revealed 8.6-fold reduced activity in combination with E2 (Fig. 5B; Table I). This double mutant also showed reduced sensitivity

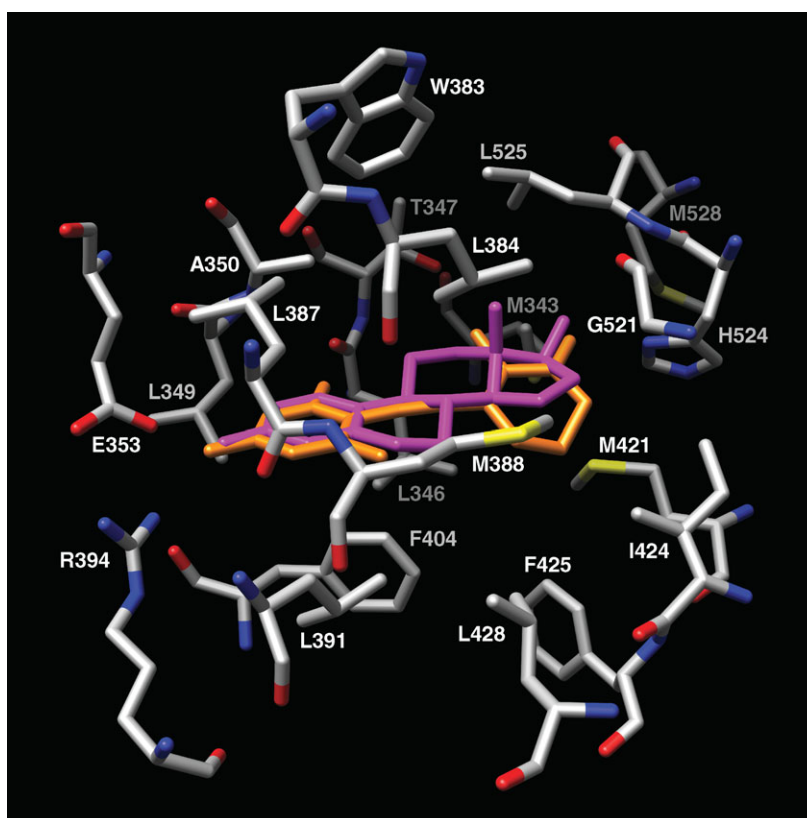


Fig. 4. Selection of residues for saturation mutagenesis. The hER α LBD crystal structure with diethylstilbestrol (DES) was loaded in Molecular Operating Environment (MOE), Chemical Computing Group, Montreal, and DES was replaced by CV3320 which was allowed to rotate freely in the energy-minimized state. Subsequently, docked CV3320 (orange) and E2 (pink) were superposed. Twenty-one residues were identified to be within 4.6 Å of the CV3320 ligand.

towards IK524 (data not shown). When performing a third round of saturation mutagenesis in the remaining 16 positions and a subsequent screen on CV3320, no further improvement was achieved.

Random mutagenesis

To improve the ligand-binding characteristics of the double mutant, error-prone PCR was performed to introduce random mutations throughout the entire LBD. Approximately 2×10^6 transformants were directly plated on selective medium containing 0.05 μM of ligand CV3320. Three hundred and seventy-eight clones were transferred into four 96-well master plates (minimal media selecting for the two plasmids) and incubated overnight at 30°C. Subsequently, colonies were inoculated into 96-well plates containing selective conditions for the two plasmids and for the transactivation (histidine drop-out medium). Four colonies that did not grow in the absence of the ligand and grew more vigorously than the original double mutant in the presence of 0.05 μM CV3320 were analyzed further. After plasmid rescue and retransformation, one clone showed a 5.8-fold increased affinity towards CV3320 compared to the L384F/L387M double mutant. This clone showed an 80-fold increased affinity towards E2 compared to the L384F/L387M double mutant (Fig. 5C; Table I). DNA sequencing revealed that tyrosine 537 was mutated into a serine (Y537S). The triple mutant (L384F/L387M/Y537S) finally served as a template for a second round of random mutagenesis. However, screening of a library of 3.2×10^6 variants did not result in a yeast colony with improved affinity towards CV3320 or decreased

affinity towards E2. Table I summarizes the results of the three successful rounds of mutagenesis as determined with the yeast two-hybrid system.

In the yeast one-hybrid system, the L384F/L387M/Y537S triple mutant receptor in combination with 10 μM CV3320 conferred $\sim 77\%$ of the effect elicited by E2 in combination with the wildtype receptor. In contrast, the improved features of the L384F/L387M double mutant (EC_{50} value of 0.32 μM) were not detected (Fig. 5D). This was unexpected, because the interaction of the L384F/L387M double mutant to E2 (EC_{50} value of 0.32 μM) was detectable in this system. It could well be that the CV3320-induced conformational switch of GBD-hER α has to be stabilized by the subsequent association with SRC-1, whereas 0.32 μM concentrations of this ligand are not sufficient for the efficient dissociation from HSP90. Thus, caution is required if a system that has been selected for improved ligand specificity with SRC-1 is applied in a context requiring dissociation from HSP90.

Accommodation of the synthetic ligand into the mutated receptor ligand-binding pocket

Though the size of the cavity (490 Å³) of hER α is nearly twice the volume of E2 (245 Å³), we chose less space-filling ligands of the resveratrol type for our approach. Our rationale was to decrease the affinity of hER α to the ligand by replacing rings 'B' and 'C' by an aliphatic spacer and to complement for the missing interactions by adding functional groups to ring 'A*' that would be recognized only by a mutated receptor protein. This strategy allowed us to test a compound library characterized by a common scaffold and variable substitutions.

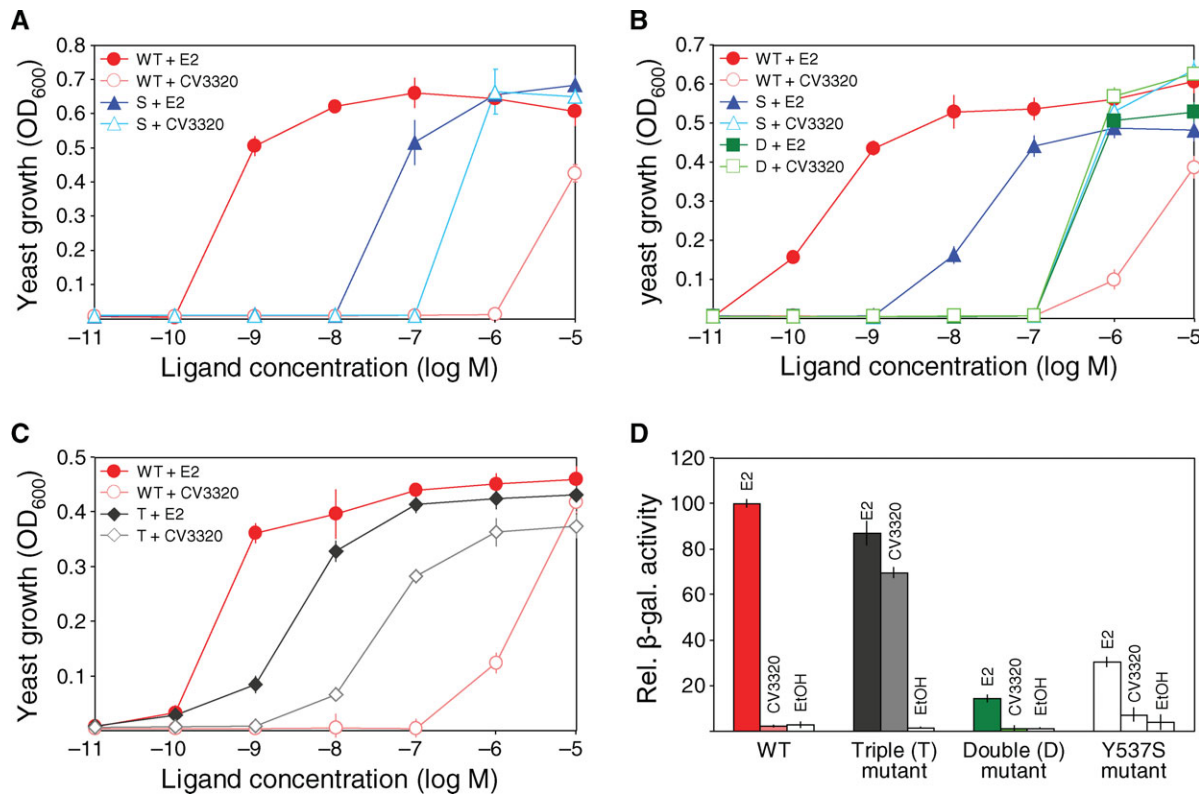


Fig. 5. Changing the binding specificity of hER α by stepwise mutagenesis. (A) Growth of yeast clones expressing wildtype and single mutant (S; L384F) GBD- hER α and AD-SRC-1 on different concentrations of either E2 or CV3320. (B) Growth of yeast clones expressing wildtype, single mutant (S; L384F) or double mutant (D; L384F/L387M) BD-ER α and AD-SRC-1 on different concentrations of E2 or CV3320. (C) Growth of yeast clones expressing wildtype and triple mutant (T; L384F/L387M/Y537S) BD- hER α and AD-SRC-1 on different concentrations of E2 or CV3320. (D) *LacZ* reporter gene activities based on wildtype and mutant GEV proteins binding to their target promoter in the yeast one-hybrid system. Relative β -galactosidase activities were calculated by setting the activity induced by the natural ligand E2 in combination with the wildtype GEV to 100%. Ligands were applied at 10 μ M concentrations. The same color code as in (A) to (C) is used. Values represent mean \pm SD of two or more experiments.

Indeed, one ligand (CV3320) with two methyl groups added to the 'A*' ring turned out to be a suitable candidate for the development of an orthogonal ligand-receptor pair. The saturation mutagenesis of amino acids lining the ligand-binding pocket (LBP) led to an exchange of L384 into F384 that caused increased affinity to CV3320. Figure 6 shows the relative position of this amino acid to CV3320 and E2. Due to the relative proximity of F384 to the methyl group of CV3320 (~ 2 Å), a transient asymmetry in the electronic charge might be created, leading to an increased van der Waal's interaction. In contrast, the interaction with E2 is weakened, presumably due to space restrictions. Position 384 has been shown before to be involved in ligand specificity: hER β , which differs from hER α by recognizing a different subset of so-called 'selective estrogen receptor modulators' (SERMs) encodes a methionine at this position (Hillisch *et al.*, 2004). Site-directed

mutagenesis and transactivation studies suggested that differences in the ligand specificities of hER α and hER β are due to this amino acid exchange when occurring in combination with other mutations (Kumar *et al.*, 2004).

The second round of mutagenesis did not yield a mutant with increased binding affinity to CV3320, indicating that no other favorable contacts to the ligand could be established within the LBP. However, the L387M mutation decreased the affinity to E2 and IK524, most likely due to space restrictions generated by the longer side chain of methionine when compared with leucine. Indeed, L387 contributes to the subpocket that accommodates the 'A' ring of E2 (Anstead *et al.*, 1997). The affinity to CV3320 is not reduced presumably because of the small size of the ligand. Further improvements of the ligand-binding selectivity for CV3320 could not be obtained using the stepwise saturation mutagenesis strategy.

Table I. Summary of results from two rounds of saturation mutagenesis and one round of random mutagenesis based on the yeast two-hybrid system in the presence of CV3320 or E2

Variant	EC ₅₀ , CV3320 (μ M)	EC ₅₀ , E2 (nM)	Selectivity	Fold improvement
WT	3.7 \pm 1.5	0.42 \pm 0.07	1.1 $\times 10^{-4}$	1
L384F	0.31 \pm 0.01	37 \pm 7	1.2 $\times 10^{-1}$	1000
L384F/L387M	0.32 \pm 0.18	320 \pm 160	1	10 000
L384F/L387M/Y537S	0.055 \pm 0.015	4 \pm 1.9	0.7 $\times 10^{-2}$	630

Mean values and standard deviations are calculated from the mean values of two to six independent experiments (WT and single mutant), or from one experiment with two or more replicates (single mutant and triple mutant). Selectivity is the quotient of EC₅₀, E2 divided by EC₅₀, CV3320.

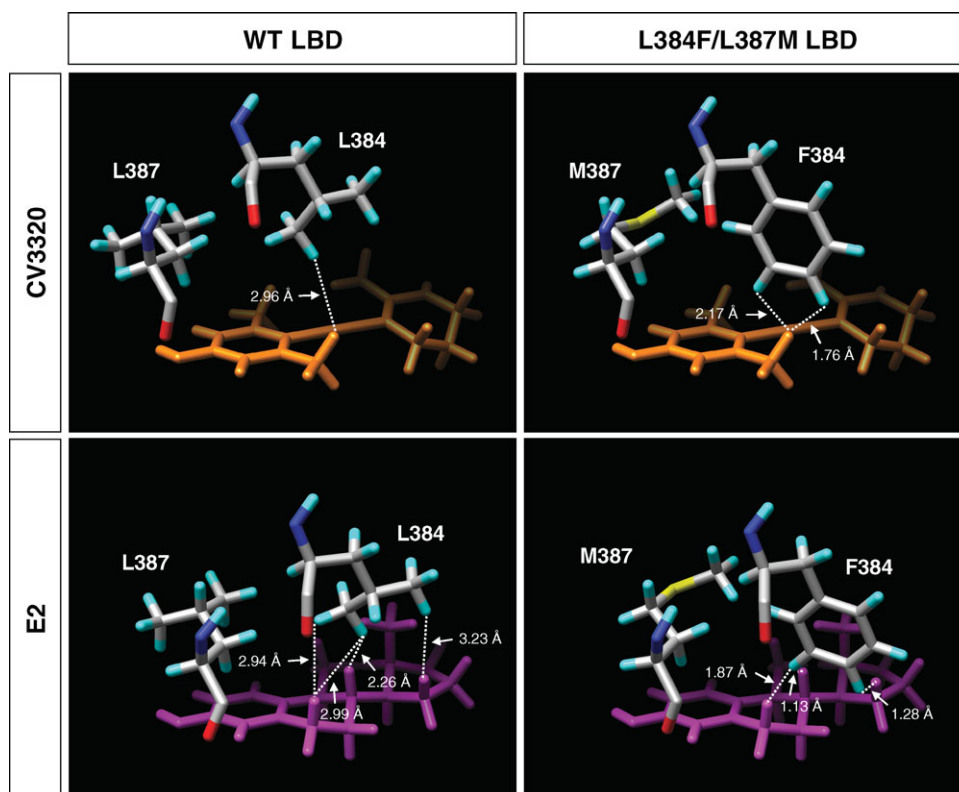


Fig. 6. Relative position of the two critical amino acids within the ligand-binding pocket that increase or decrease the affinity of the LBD of the human estrogen receptor α (hER α) to CV3320 and E2, respectively. Docking of the two ligands into the ligand-binding pocket of wt and mutant hER α was done as described (Chockalingam *et al.*, 2005).

Random mutagenesis of the LBD with the two point mutations L384F and L387M by error prone PCR yielded a mutant protein with a tyrosine to serine exchange at residue position 537, which is located 9 Å away from the ligand. The triple mutant (L384F/L387M/Y537S) showed increased affinity to CV3320 and E2 when compared with the double mutant. It has been reported that mutations of Y537 to N, A and S led to constitutive activation (Zhang *et al.*, 1997; Yudit *et al.*, 1999), which contrasts with our findings that activation of the triple mutant was still ligand-dependent. In our hands, the Y537S single mutant also did not lead to constitutive activation (Fig. 5D). It could well be that, when being part of a hybrid protein, this mutant does not flip into the active conformation though the conformational change seems to be facilitated upon binding of ligands.

In conclusion, we have shown that ligands containing rings corresponding to the A and the D ring of the steroid E2, which are connected by a C–C triple bond linker rather than by a rigid ring system, can be used as a scaffold for the development of a functionally orthogonal gene switch. The novel receptor-ligand pair responds to concentrations of the synthetic ligand (0.05 μ M) which do not activate the wildtype receptor minimizing pleiotropic effects. Likewise, the commercially available RheoSwitch (New England Biolabs Inc.), which is based on the ecdysone receptor, uses ligands at concentrations ranging from 0.02–0.1 μ M for optimum induction of target genes (Palli *et al.*, 2003; Kumar *et al.*, 2004).

Testing the system in mammalian or plant cells is necessary to further explore its potential applicability. As the affinity of the mutant hER α receptor to E2 is only decreased by a factor of 10, one problem in mammalian cells might be the

potential activation by endogenous E2 or other steroids. Likewise, in plants which lack endogenous E2 synthesis but—depending on the plant family—produce phytoestrogens (Hain *et al.*, 1990), pre-induction might occur. As phytoestrogens also interact with chromatin modifying proteins of the sirtuin family (Howitz *et al.*, 2003), it has to be tested whether CV3320 or related compounds affect sirtuin function at the concentrations used. Also, other questions like the half-life and mobility of the chemical in the foreign organism have to be addressed. Still, the structural motif of our non-steroidal ligands, i.e. the substitution of rings B and C of the original steroid framework by an alkene linker, has the potential to be applied in the future.

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