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A Functionally Orthogonal Estrogen Receptor-Based Transcription Switch Specifically Induced by a Nonsteroid Synthetic Ligand

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Summary

It is highly desirable to design ligand-dependent transcription regulation systems based on transactivators unresponsive to endogenous ligands but induced by synthetic small molecules unable to activate endogenous receptors. Using molecular modeling and yeast selection, we identified an estrogen receptor ligand binding domain double mutant (L384M, M421G) with decreased affinity to estradiol and enhanced binding to compounds inactive on estrogen receptors. Nonresponsiveness to estrogen was achieved by additionally adding the G521R substitution while introducing an "antagonistic-type" side chain in the compound, as in 4-hydroxytamoxifen. The triple-substituted ligand binding domain is insensitive to physiological concentrations of estradiol and has nanomolar affinity for the ligand. In this binary system, both receptor and ligand are, therefore, reciprocally specific. The mutated variant in the context of a chimeric transcription factor provides tight, ligand-dependent regulation of reporter gene expression.

Introduction

Several artificial systems that allow inducible expression of a transgene in response to small molecule ligands have been developed (reviewed in [1, 2]). These systems usually rely on two components. The first is a chimeric transcription factor obtained by fusing a DNA binding domain (DBD) to a transcription activation domain (AD) and to a regulatory domain that interacts with a small molecular weight compound that acts as an inducer drug. The second component is an artificial promoter consisting of multimeric binding sites for the DBD followed by a minimal promoter sequence. The chimeric transcription factor is recruited to (or in some cases released from) the specific target promoter upon interaction with the exogenously added drug: transcription of a transgene cloned downstream of this promoter can thus be modulated in vivo by drug delivery or withdrawal.

An ideal ligand-dependent transcription system must display a low basal activity and high inducibility: moreover, it should be activated in a dose-dependent manner by the inducer ligand. However, two additional criteria must be fulfilled for long-term gene therapy applications in humans. First, the chimeric activator should not be immunogenic; second, the inducer drug should display an appropriate safety profile and should not interfere with endogenous metabolic pathways [1].

Steroid receptors are composed of distinct and functionally independent domains with intrinsic DNA binding, transactivating, and ligand binding properties [3]. Inducible chimeric transactivators that function independently of endogenous steroids have been constructed by using mutant forms of the ligand binding domains (LBDs) of the progesterone receptor (PR) and of the estrogen receptor (ER) that are not responsive to their natural ligands but are induced by synthetic antagonists such as RU486 and 4-hydroxytamoxifen (4-OHT), respectively [4–8]. According to this strategy, we have recently generated a novel, to our knowledge, humanized gene switch specifically designed to work in muscle cells [9]. This system includes a 4-OHT-dependent chimeric transactivator, called HEA, composed of three elements derived from human proteins: the DBD of the human transcription factor hepatocyte nuclear factor-1 (HNF-1), a G521R mutant version of the human ERα-LBD, and the AD from the human transcription activator NF-kB p65. HEA binds and activates an artificial promoter containing multimeric HNF1α binding sites in a ligand-dependent manner. When delivered in vitro and in vivo to muscle cells that lack endogenous HNF-1, HEA displays a low basal activity and is efficiently activated after exposure to 4-OHT.

Built from components of human origin, the HEA system has a low potential to elicit an immune response in humans: however, prolonged administration of its inducer drug tamoxifen (TAM), the immediate precursor of 4-OHT, raises safety concerns for human gene therapy. TAM is currently used to prevent recurrence of breast cancer, and the HEA system is activated in mice by TAM dosages comparable to those used in clinical practice ([9] and references therein). Nonetheless, prolonged treatments with TAM are associated with a well-defined increased risk of endometrial cancer due to its partial agonist activity on endogenous ERα in tissues other than the mammary gland [10, 11]. Therefore, an inducer drug that does not show any binding to endogenous ERs would be appropriate for long-term gene therapy in humans.

These considerations prompted us to redesign the ER-LBD hormone interface in order to differentiate it from endogenous receptors and to identify cognate ligands that only bind this mutant LBD. Reengineering ligand-receptor pairs to generate functionally orthologous transcriptional regulators was proven to be feasible with the ER-LBD interface [12–14]. To this purpose, both groups made a limited number of coordinated changes into the full-length receptor and its natural ligand with a focus on the A ring of estradiol (E_2). Ideally, however, it would be important to generate a broad

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class of novel nonsteroidal ligands with enhanced specificity features.

To this aim, we have developed an alternative strategy. We started by identifying, within a series of active ER ligands, compounds inactive on both ERα and ERβ. By combining molecular modeling and screening of a library of ER mutants, we identified a novel, to our knowledge, class of ligands with high affinity and specificity for a triple-substituted ERα-LBD. Grafting this mutant into the HEA transcription factor allowed for highly selective and efficient transcriptional induction in mammalian cells.

Results

Rational Design and Construction of a Compound-Specific ER-LBD Mutant Library

Our goal was to modify the ER-LBD so that it was unable to bind natural ligands, such as E_2, but able to bind and be activated in the context of a chimeric transcription factor by a synthetic analog unable to bind the natural ER. In the search for candidate starting compounds, we looked at two essential criteria. First, the candidate should be inactive against both hERα and hERβ within an otherwise generally active series. Second, the structural features of the candidate should require only a limited modification around the ligand binding pocket of the hERα-LBD.

We synthesized CMP1 (Figure 1A), a tetrahydrofluorenone with a benzyl substituent at the 9a position. Tetrahydrofluorenones without the benzyl group, like CMP2 and 3 (Figure 1A), are otherwise nanomolar selective binders and activators of hERβ. Introduction of a large substituent such as benzyl at the C9a position has been found to result in a substantial loss of binding affinity for both hERα and hERβ ([15, 16]; R.R. Wilkening et al., personal communication; Table 1). The newly synthesized CMP1 indeed displayed a very poor binding affinity for both hERα and hERβ (IC_{50} values > 10^4 nM) and therefore represented a promising starting point around which a receptor mutagenesis strategy could be designed.

X-ray structures of agonist bound hERα- and hERβ-LBDs [17–19] were used to model CMP1 into the respective binding pockets by superimposing the tetrahydrofluorenone scaffold with that of the active analogs in the crystal structures. According to modeling of the free, unbound CMP1, three almost isoenergetic conformations of the 9a benzyl substituent were considered (Figure 1B). The superposition defined an area of the protein that clashes with the benzyl substituent of the tetrahydrofluorenone. Five residues within the ligand binding pocket were identified as the most likely candidate residues interfering with binding of CMP1: L391/L343, F404/F356, M421/I373, I424/I376, and L428/L380 of hERα/hERβ, respectively. A mutant library including these five positions should cover nearly 300° of conformational space of the 9a benzyl rotamer, including all three major conformations. Because the interference with binding of CMP1 is expected to arise from steric clashes between side chains and the 9a benzyl moiety, substitutions into smaller amino acids should remove this hindrance. At the same time, however, these changes could also destabilize the pocket, which would require some compensatory substitution at neighboring residues. Due to the generally hydrophobic character of both the binding pocket and the benzyl substituent, we therefore decided to use only G, A, C, V, I, L, M, F, Y, or W as possible substitutions at each of the five positions.

We have recently reported the generation of a 4-OHT-inducible, ERα-based humanized switch [9]. The selectivity of tetrahydrofluorenones for ERβ therefore constituted an additional issue to take into account for the generation of a new orthogonal switch based on the ERα-LBD. According to the crystal structures of hERα and hERβ [17–19], the tetrahydrofluorenones selectivity could be conferred by the two residues (individually or in combination) that differ in the ligand binding pockets of the two receptors, i.e., L384/M336 and M421/I373 in hERα/hERβ, respectively. M421 corresponded to one of the positions mutated in the library (see above), and inclusion of Ile as a possible substitution automatically introduced the wild-type (wt) hERβ residue into the library. For the second residue, we decided to generate the library in the context of a L384M-mutated hERα. To confirm our predictions, we performed in vitro radioligand displacement assays by using GST fusion constructs with single L384M or combined L384M/M421I substitutions in the hERα-LBD (hereafter called M-ERα-LBD and MI-ERα-LBD, respectively). The results show that both L384M and L384M/M421I mutants have significantly improved binding to the agonists CMP2 and CMP3 as compared to wt ERα (Table 1).

Generation and Characterization of a Yeast Library in the ERα Ligand Binding Pocket

In order to genetically select for CMP1-responsive variants, both wt and M-ERα-LBD were fused to the GAL4 DNA binding domain (DBD) and the VP16 transactivation domain (Figure 2A) to generate hybrid transcription factors. These were transfected in the yeast strain CG1945 to measure their β-galactosidase transactivating potential in the presence of E_2 or CMP2. While both constructs were equally sensitive to E_2, the responsiveness of the L384M chimera to CMP2 was, as expected, superior to that of the wt (Figure 2B). This test also demonstrated that tetrahydrofluorenones are able to cross the yeast membrane.

Generation of the library of M-ERα-LBD variants took advantage of the inherent yeast recombination system. Two oligonucleotides degenerate for ten different amino acids at each of the five library positions were synthesized by using a dinucleotide assembly strategy [20] that also included sufficiently long (>30 bp) constant flanking regions to direct the homologous recombination. To decrease the likelihood that clones with single or double mutations would be lost during synthesis, construction, or selection of the library, the concentration of wt codons was raised to 15% for each of the mutated positions. As a consequence, the relative abundance of 1 or 2 residue variants was significantly raised (by about 5- and 3-fold, respectively) compared to only a minor reduction in the relative abundance of 5 residue variants (to 0.8-fold) (see Experimental Procedures). This strategy was guided by modeling predictions that

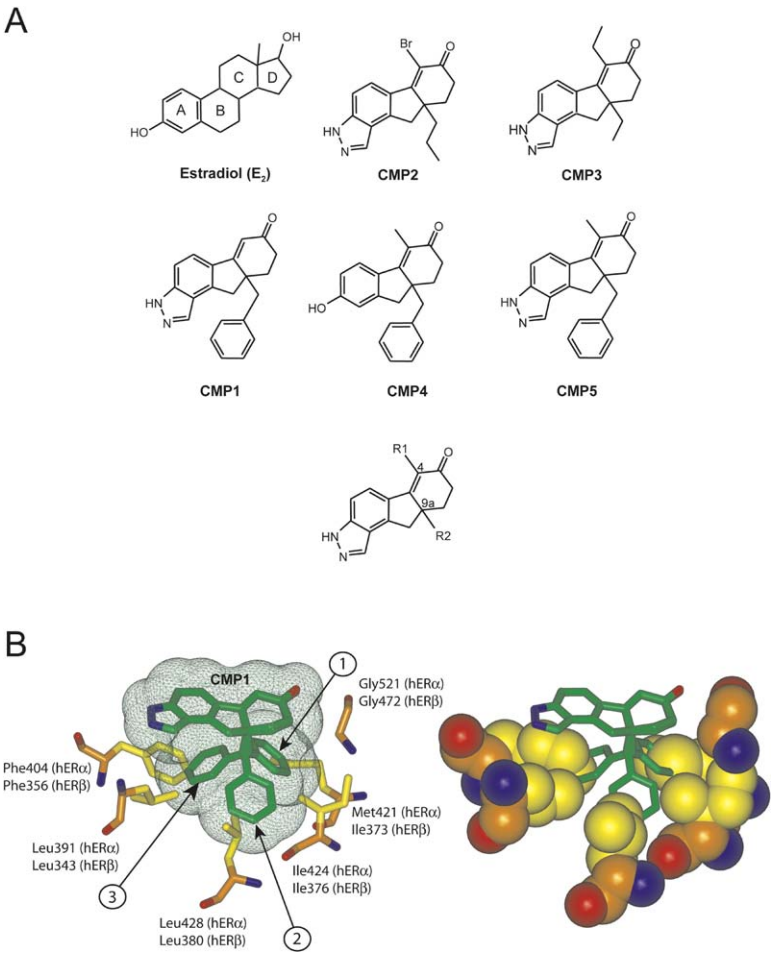


Figure 1. Design of a Compound-Specific ER-LBD Mutant Library

(A) Structures of estradiol and representative active and inactive tetrahydrofluorenone analogs. Positions 4 and 9a, where substituents have been introduced, are indicated.

(B) hERα-LBD mutant library. Molecular models of the three rotamers of CMP1 in the crystal structure of the hERα (PDB entry 1ERE) binding pocket. The five residues mutagenized in the library are shown together with G521. Left: solvent-accessible surface of CMP1. Right: space-filling representation highlighting the steric clashes between side chains and CMP1. Side chains of library residues are colored yellow.

suggested that predominantly 1 or 2 residue variants rather than multiple changes would be sufficient to accommodate the CMP1 ligand and, at the same time, be tolerated by the LBD structural framework.

Preparative amounts of mutated LBD fragments were synthesized by PCR amplification of the hERα-LBD template by using the degenerated oligonucleotide mix. The mutated fragment collection was then included in a scaled up cotransformation experiment together with a linearized recipient M-ERα-LBD vector. Approximately 10^6 colonies corresponding to a 10-fold library redundancy were plated, and 12 randomly chosen clones revealed the expected library complexity (data not shown). In order to probe the library for selection capacity, –His/–Trp growth-selective plates were treated with increasing amounts of the hERβ agonist CMP3. Sequencing of nine randomly picked clones indicated that, unlike with the nonselected clones, most of the CMP3-selected clones had either none or few amino acid substitutions at all five mutagenized positions; two of these positions, L391 and M421, are completely conserved (data not shown). It appears therefore that a very restricted number of mutations is tolerated in the hER binding pocket to maintain a productive interaction with a high-affinity ligand of the wt receptor. Interestingly, no clones containing the M421I

Table 1. Binding Specificities of E_2 and of Active and Inactive Tetrahydrofluorenones

	Binding (IC_{50}, nM)					
Compounds	wt hERα	wt hERβ	GST-wt ERα-LBD	GST-M ERα-LBD	GST-MI ERα-LBD	GST-MG ERα-LBD
E_2	1.6 (± 0.2)	1.4 (± 0.2)	2.3 (± 0.34)	5 (± 0.53)	0.7 (± 0.1)	46 (± 4.4)
CMP2	676 (± 82)	6 (± 0.8)	800 (± 85)	48 (± 5)	42 (± 3.75)	nd
CMP3	223 (± 30)	4 (± 0.45)	780 (± 103)	45 (± 5.5)	68 (± 8.4))	nd
CMP1	>10,000	>10,000	>10,000	>10,000	nd	3,900 (± 413)
CMP4	1,470 (± 197)	506 (± 63.3)	2,320 (± 225)	1,480 (± 133)	nd	12 (± 1.85)
CMP5	>10,000	1,247 (± 141)	>10,000	>10,000	nd	155 (± 18)

Average values of three or four experiments (± standard deviation); nd: not determined.

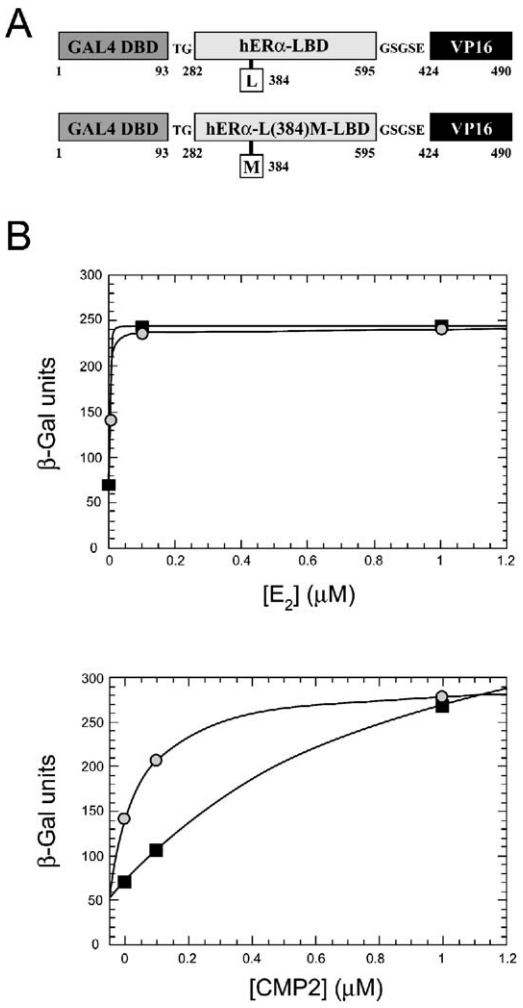


Figure 2. Comparison of Chimeric Transcription Factors Containing wt hERα-LBD or hERα-L(384)M-LBD

(A) Schematic representation of the GAL4DBD/hERα-LBD/VP16AD chimeric transcription factors.

(B) Transcriptional activation in yeast transformants expressing chimeras based on wt hERα-LBD (squares) or hERα-L(384)M-LBD (circles) by E_2 and the hERβ-specific compound CMP2. Dose-response curves of β-galactosidase activity in the presence of increasing concentrations of E_2 or CMP2 were obtained. EC_{50} values were determined as described in Experimental Procedures.

substitution were selected. This finding is in agreement with the observation that no further increase in binding affinity for CMP3 was observed by adding this second substitution to the L384M-mutated protein (Table 1).

Genetic Selection of ERα-LBD Variants

Having confirmed the expected composition and responsiveness of the library, we performed first growth selection in the presence of the inactive analog CMP1 at a concentration of 1 μM. A total of 1600 independent clones were subsequently screened against 1 μM CMP1 or DMSO as a control on –Trp/X-gal-containing plates. A total of 54 independent clones were judged positive for β-galactosidase transactivation, and 28 of these also retained positivity in the presence of 0.1 μM CMP1. A subsequent selection was carried out by using the same strategy but by performing the initial growth selection and first round of white/blue screening in the presence of a 10-fold higher compound concentration (10 μM). A total of 31 clones retained positivity when challenged with 0.1 μM CMP1 and were therefore selected for further characterization together with the initial 28 positive clones.

Table 2. DNA Sequence Analysis of Plasmids Rescued from the Genetically Selected Variants

L391	F404	M421	I424	L428	Number of Clones[a]
L	**F**	**G**	**I**	L	5
L	**W**	**G**	**I**	L	2
L	**F**	**G**	**M**	L	9
L	**W**	**G**	**M**	L	2
M	**W**	**G**	**M**	L	1
L	**F**	**G**	**V**	L	11
L	**W**	**G**	**V**	L	2
I	**F**	**G**	**V**	L	1
L	**F**	**G**	**L**	L	5
L	**W**	**G**	**L**	L	3
V	**W**	**G**	**L**	L	1
L	**W**	**A**	**L**	L	1
V	**W**	**A**	**L**	L	1

Three wt sequences and six mixed sequences were also identified but are not included. All amino acids found in selected variants that diverge from the wild-type ERα-LBD sequence are shown in bold.

[a] The number of independent clones in which the same mutation array was found is indicated.

DNA sequence analysis revealed a consensus sequence of the selected mutant variants (Table 2). The most prominent feature was the mutation of M421 into a smaller side chain residue (mostly into G and to a lesser extent into A) that occurred in 86% of the selected clones. Importantly, this mutation was never observed in clones selected by using the agonist CMP3 (data not shown), thus excluding a bias in the screening procedure. The isolated M421G mutation was found with a relatively low frequency and was more often present in combination with a substitution of I424 to M, V, or L. In addition, a third mutation of F404 to W was found in 28% of the mutated clones. Finally positions L391 and L428 were generally conserved. The consensus sequence present in most selected variants is consistent with a model in which the benzyl substituent of CMP1 is directed toward the positions originally occupied by M421 and I424 (Figure 1B, rotamer 1). Western blot analysis of yeast protein extracts from all representative clones shown in Table 2 by using a monoclonal antibody directed against VP16 AD did not show significant differences in the expression levels of the corresponding chimeric proteins (not shown).

A Yeast-Selected ER-LBD Variant Mutated in M421 Is Preferentially Bound and Transcriptionally Activated by Benzyl Tetrahydrofluorenones

Selected mutations shown in Table 2 were introduced in the pGEX-M-ERα-LBD prokaryotic expression construct, and the corresponding GST fusion protein variants were expressed in *E. coli*. Suitable aliquots of crude bacterial extracts containing comparable amounts of all dif-

ferent protein variants were tested for binding in vitro to increasing concentrations of ^{3}[H] E_2. All variants bound the natural ligand E_2 with reduced affinity compared to the M-ERα-LBD protein (data not shown). The mutant carrying the additional selected single substitution M421G (hereafter called MG-ERα-LBD) bound E_2 with a k_d value approximately 9-fold higher than that of M-ERα-LBD (46 nM and 5 nM, respectively) (Table 1). The k_d value for the hormone was in a range suitable to measure the affinity of MG-ERα-LBD for a series of benzyl-substituted tetrahydrofluorenones, CMP1, CMP4, and CMP5 (Figure 1A), in ^{3}[H] E_2 displacement assays. The glycine in position 421 conferred increased binding to all benzyl-substituted compounds tested (Table 1). The affinity for the phenolic tetrahydrofluorenone CMP4 was significantly higher than for the corresponding pyrazole analog CMP5. Furthermore, the binding affinity was higher for CMP5, with a methyl group in position 4, than for CMP1, possessing a hydrogen atom in the corresponding position. Interestingly CMP4 and E_2 showed reversed binding affinities for the double mutant MG-ERα-LBD as compared to wt ERα and ERβ receptors.

To confirm this partial selectivity at the transcriptional level, GAL4/VP16 chimeras containing M-ERα-LBD or MG-ERα-LBD were tested in ligand-dependent β-galactosidase transactivation experiments in the presence of E_2, CMP4, or CMP5. The results shown in Figure 3 confirm that only MG-ERα-LBD was activated preferentially by both CMP4 and CMP5, with EC_{50} values of 36 nM and 217 nM, respectively, comparable with the IC_{50} values measured in vitro. Its response to E_2 (EC_{50} = 1.5 nM) was approximately 8-fold higher than that associated with the parental clone (EC_{50} = 0.2 nM), a difference of the same order of magnitude of that measured in vitro (see Table 1).

M384 was backmutated to L in the context of the M421G-selected mutant, thus generating a single-substituted G-ERα-LBD. The transcriptional activity induced in yeast by CMP5 was strongly impaired in the absence of the L384M change (data not shown), indicating that both amino acid substitutions were necessary to define the ligand specificity of the selected mutant.

In conclusion, binding and transcriptional activity data taken together show that the introduction of only two amino acid substitutions, L384M/M421G, impart the desired orthogonality shift when combined with benzyl-substituted tetrahydrofluorenones. These two changes, however, are insufficient to reduce binding to E_2 to desirable levels.

Introduction of the G521R Mutation into the MG-ERα-LBD Causes Loss of Binding to E_2 and Selective Induction by "Antagonistic Chain"-Substituted Tetrahydrofluorenones

Estrogen receptors bearing the G521R substitution in their LBD exhibit low basal transcriptional activity, strongly reduced affinity for E_2, but intact binding and activation by 4-OHT [21, 22]. More generally, ligands such as tamoxifen or raloxifene, which have an "antagonistic tail," can apparently compensate for loss of affinity introduced by G521R in the ligand binding pocket.

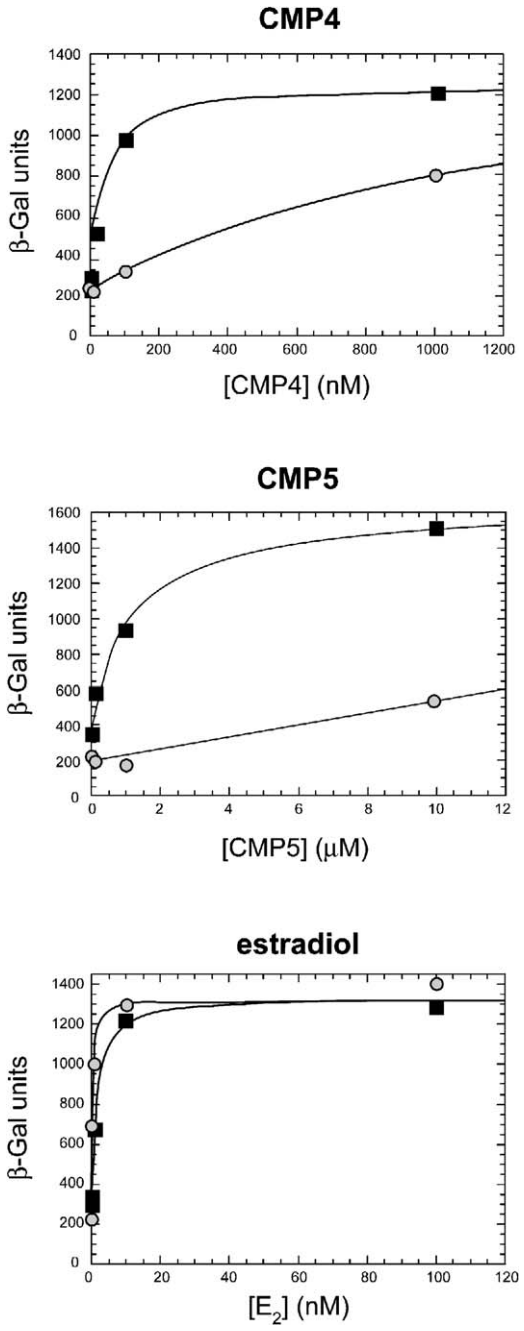


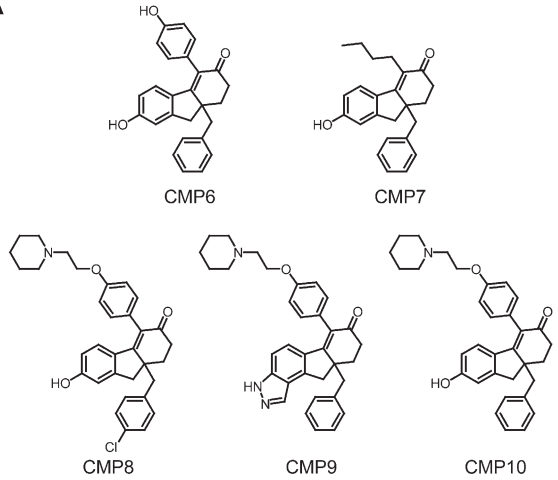
Figure 3. Ligand-Dependent Transcriptional Activity of the M(421)G-Selected Mutant in the *lacZ* Reporter Yeast Strain Y187

Dose-response curves of β-galactosidase activity in the presence of increasing concentrations of E_2 and the two tetrahydrofluorenone compounds CMP4 and CMP5 were determined for the L(384)M, M(421)G-selected mutant (squares) and for the L(384)M parental clone (circles). EC_{50} values were calculated as described in Experimental Procedures.

This property has been at the basis of the recent generation of a tamoxifen-dependent transcription switch [9].

A new series of compounds was generated by substituting the methyl group in position 4 with more bulky side chains mimicking an "antagonistic tail" ([15]; R.R. Wilkening et al., personal communication; Figure 4A).

A



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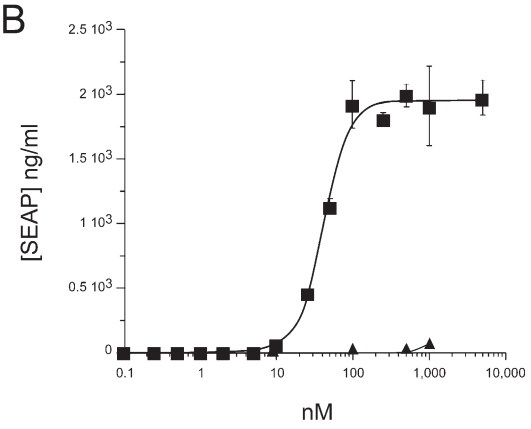

C

Compound	Ec50 (nM)
CMP6	11 (±3)
CMP8	41 (±2)
CMP9	294 (±40)
CMP10	14 (±2)

Figure 4. Orthogonal Gene Switch in Mammalian Cells

(A) Structures of representative "antagonistic chain"-substituted tetrahydrofluorenones.

(B) Representative dose-response curves of chimeric transcription factor HEA-MGR to CMP8 (squares) and E_2 (triangles).

(C) HEA-MGR response to "antagonistic chain"-substituted tetrahydrofluorenones.

Each data point represents the mean ± SD of three independent experiments.

Both pyrazole (CMP9) and phenol derivatives (CMP6, CMP7, CMP8, and CMP10) containing different side chains were challenged in competitive radiometric in vitro assays for binding to MG-ERα-LBD or to wt ERα-LBD (Table 3). All compounds showed nanomolar affinity for the double mutant (22–107 nM) and significant selectivity over wt LBD and both α and β full-length ER, albeit to different extents. Selectivity ranged between 50- and 150-fold, the differences being somewhat less pronounced on the full-length receptors. The highest selectivity was observed for CMP8, suggesting that substituents other than chloride at the benzyl para-position might be explored to further increase selectivity. Lack of the M421 side chain in the MG-ERα-LBD apparently generates enough space to also accommodate a substituted benzyl group. On the contrary, the observed loss in affinity of the wt ER-LBD for CMP10 was further accentuated with the Cl-substituted CMP8 (Table 3). Overall, these data indicate that the specificity conferred by the M421G substitution for the benzyl group in the 9a position of tetrahydrofluorenones (Table 1) is maintained in antagonistic chain-substituted ligands.

This evidence prompted us to generate a triple-substituted LBD carrying G521R in the context of MG-ERα-LBD (hereafter called MGR-ERα-LBD). We reasoned that if this mutant maintains high-affinity binding to "antagonistic chain"-substituted benzyl tetrahydrofluorenones, the system would show all of the desired properties for a functionally orthogonal ligand-dependent system, i.e., low affinity of the mutant receptor for E_2, and high-affinity binding of the same mutated receptor for a set of compounds with strongly decreased binding to the wt receptor. As expected, MGR-ERα-LBD does not bind to E_2 (data not shown). However, this property did not allow for measuring direct binding of CMP6 to CMP10 in the competitive radiometric in vitro assay. Hence, the selective response of MGR-ERα-LBD to the antagonist tetrahydrofluorenones was ascertained in yeast (Figure S1; see the Supplemental Data available with this article online).

To test the system directly in mammalian cells, we modified the ligand-dependent transcription regulator HEA-1 [9] by replacing the G521R mutant hERα-LBD with MGR-ERα-LBD. The new construct was cotransfected in HeLa cells along with the reporter plasmid 7xH1/CRP/SEAP [9]. Cells were treated with increasing concentrations of four antagonistic compounds (CMP6, CMP8, CMP9, and CMP10), and SEAP levels were measured in the culture medium after 24 hr of treatment (Figure 4C). The chimera was stimulated by all four compounds in a dose-dependent manner. A representative dose-response curve with CMP8 (the compound showing the highest degree of selectivity, Table 3) is shown in Figure 4B. In the absence of treatment, a basal SEAP level of 0.8 ± 0.2 ng/ml was measured, and this level is similar to that measured in the culture medium of cells transfected only with the reporter plasmid (data not shown). This indicated the absence of a significant basal activity in this experimental setting. About 1900 ng/ml SEAP was measured at the highest concentrations of CMP8, which corresponds to about a 2000-fold increase as compared to the uninduced condition. Similar induction levels were consistently observed in duplicate experiments and with all four compounds (data not shown). Figure 4B also shows that the chimera is minimally activated by E_2 only at the highest supraphysiological concentrations of the hormone (17-fold and 50-fold induction at 500 nM and 1 μM E_2, respectively). Consistent with the in vitro binding analysis (Table 3), CMP9 displayed the lowest activity with an EC_{50} of approximately 300 nM, while the

Table 3. Binding Specificity of "Antagonistic Chain"-Substituted Tetrahydrofluorenones

	Binding (IC_{50}, nM)				Selectivity (IC_{50} ratios)		
Compounds	GST-MG ERα-LBD	GST-wt ERα-LBD	wt hERα	wt hERβ	wt ERα-LBD/ MG-LBD	wt hERα/ MG-LBD	wt hERβ/ MG-LBD
CMP6	29 (± 3.5)	1371 (± 186)	223 (± 22)	96 (± 22)	47	7.7	3.3
CMP7	25 (± 3.5)	1306 (± 127)	259 (± 39)	307 (± 42)	52	10.4	12.3
CMP8	29 (± 2.8)	4436 (± 643)	1085 (± 121)	2232 (± 230)	153	37.4	77
CMP9	107 (± 11)	1126 (± 140)	622 (± 94.5)	125 (± 13)	11	5.8	1.2
CMP10	22 (± 3)	1272 (± 168)	375 (± 41)	184 (± 23)	58	17	8.4

Average values of three or four experiments (± standard deviation).

EC_{50} of the other compounds ranged from 11 to 40 nM (Figure 4C).

Discussion

Engineering of protein-ligand interfaces is emerging as an important approach for creating new tools to study and manipulate a variety of biological systems (reviewed in [23]). This concept is being applied in the design of enzymes with novel substrate specificities, to the control of processes such as apoptosis, recombination, and signal transduction, or to the provision of temporal control of gene expression. The purpose of our study was to broaden the repertoire of reagents capable of triggering ligand-induced transcription control of a transduced transgene, with the potential for an additional application in somatic gene therapy. The system we chose was the estrogen receptor LBD. Using a combination of rational design and yeast genetic selection, we generated an ERα-LBD variant specific for a lead compound not recognized by the wt receptor. Additional mutations led to the loss of recognition by the endogenous ligand and required the introduction of compensatory changes in the novel, to our knowledge, class of synthetic ligands.

Although the generation of functionally orthologous ligand-ER pairs had already been reported before [12–14], a major distinguishing feature of our work relies on the use of an entirely new, to our knowledge, class of compounds structurally unrelated to existing natural ligands. We applied a new, to our knowledge, strategy that employs structural information to identify a region of the receptor for modification, followed by generation of a library of mutants in this region and genetic selection in yeast. The ER is unique among steroid receptors in its ability to embrace a wide variety of nonsteroidal compounds. Although the "pincer-like" arrangement around the A ring imposes a general requirement for ligands to contain an aromatic ring, the remainder of the binding pocket can accept a variety of hydrophobic groups [17, 24, 25]. This promiscuity can be attributed to the size of the cavity, which has a probe accessibility of up to 450 A^3, which is nearly twice the volume of E_2 (245 A^3). From the various ER binders, we chose tetrahydrofluorenones as an emerging series of small ligands ([15, 16]; R.R. Wilkening et al., personal communication). Different from E_2, these compounds have a five-membered ring in position B and lack ring D. Although active compounds of this series are up to 400-fold more selective for ERβ (data not shown), a single amino acid change in the ERα ligand binding pocket (L384M) converted them into acceptable binders that could be used for further evolution.

Our initial strategy was based on a bump/hole approach that used an inactive compound carrying a bulky hydrophobic benzyl substituent combined with the strategy of generating additional space within the binding pocket. Modeling of this compound into the ER-LBD cavity led us to predict potential steric clashes with a restricted number of amino acid residues. Construction and selection of a library of mutated ERα-LBDs in yeast allowed us to explore simultaneously a large surface area around the bulky substituent and to identify M421 as a key residue. The substitution of M421 by amino acids with smaller side chains provided the highest gain in binding energy. This strongly validates the strategy that guided the library design confirming our prediction that the target compound should adopt only a few distinct conformations. Furthermore, through genetic screening of the mutant library, only one conformation was unambiguously selected, namely, the one having the benzyl substituent directed toward M421. The double-substituted LBD L384M/M421G represented a significant progress toward the desired shift in specificity since it proved to bind the cognate ligand CMP4 better than E_2. Conversely, CMP4 showed a 122- and 42-fold binding selectivity for the cognate receptor LBD L384M/M421G over wt ERα and ERβ, respectively.

The final step was the introduction of a well-characterized substitution in ERα, namely, G521R, which is known to strongly reduce binding to E_2 when present in the context of the wt receptor, while still allowing binding of compounds such as 4-OHT that carry a bulky side chain [21]. Crystal structures for 4-OHT and raloxifene bound to the wt receptor have shown that the bulky side chains are too long to be contained within the binding cavity [17, 18]. Instead, they protrude from the pocket between helix 3 and 11, displace helix 12, and establish additional contacts with a variety of residues. These additional contacts are believed to compensate for rearrangements that take place in the LBD as a consequence of the G521R substitution. We believe that the situation is similar with the "antagonistic" side chain at the 4 position of tetrahydrofluorenone derivatives CMP6–CMP10. Among these, all compounds with a hydroxyphenyl moiety bound MG-ERα-LBD with significantly high affinities (10^{-8}). CMP8 showed the highest selectivity for MG-ERα-LBD versus wt ERα or ERβ (37- and 77-fold, respectively).

The final assessment of the new orthogonal receptor-

ligand system was its capability to drive selective transcriptional induction of a reporter transgene in mammalian cells. This was done by grafting the MGR-ERα-LBD into the previously characterized hybrid transcription activator HEA-1 and transfecting it into HeLa cells. The results show that this new transcription switch is highly sensitive to low concentrations of CMP8 and is fully silent over a wide range of physiological and supraphysiological concentrations of E_2. Interestingly, association constants determined in vitro precisely correlate in our study with the observed EC_{50} values in HeLa cells for both the "antagonistic" side chain tetrahydrofluorenone derivatives and E_2. The same degree of correlation was not observed previously for a different ERα orthogonal ligand-receptor pair for which transactivation studies in cells were performed by using the entire receptor [14]. In that case, it was postulated that differential interactions with cellular accessory proteins can reduce the receptor selectivity of the ligand compared to its intrinsic binding selectivity measured in vitro. In our study, the use of a synthetic transactivator carrying only the ERα-LBD can minimize interaction with these accessory proteins. This in turn may render the in vitro and in vivo systems closer to each other and permit a better uniformity in the data.

Functionally orthologous ligand-receptor pairs have been generated in the past years for a variety of transcriptional switches useful in gene therapy [26–31]. Improvements in our knowledge of protein-protein and protein-ligand interactions are leading to constant enhancement in the selectivity of these systems, and the data presented in this paper provide further support to this concept. The final goal of these efforts is the generation of new compounds that combine selectivity with acceptable pharmacological properties, which would allow for oral delivery at acceptable doses but would not result in systemic toxicity. This will require further refinements and optimizations of the described lead compounds similar to those currently applied to standard drug discovery.

Significance

Controlled regulation of transcription by exogenous synthetic ligands acting on a specific transactivator has important applications in a large spectrum of biological systems, from in vitro cell culture to living organisms. A highly desired property of such a system is to minimize interference with the host system that could arise as a consequence of introducing an exogenous receptor and/or ligand. A binary system in which both receptor and ligand are reciprocally specific, recognizing only each other, would fulfill this requirement. However, in living organisms, introduction of an exogenous receptor will, in general, induce an immune response. Likewise, use of an endogenous receptor can affect accessory proteins through residual protein-protein interactions. Here, we describe, starting from the estrogen-receptor ligand binding domain, the stepwise design of a receptor-ligand system that requires only minimal modification of the endogenous receptor in order to become responsive to the synthetic ligand while nonresponsive to the endogenous estrogen ligand. Choice of a class of compounds inactive on the wt estrogen receptors isolates our system and avoids interference with endogenous estrogen activity. In order to minimize interaction with estrogen-receptor accessory proteins, we designed a modular synthetic transactivator combining the modified ligand binding domain with heterologous DNA binding and transactivation domains. The resulting chimeric transcription factor enabled tightly controlled ligand-dependent gene expression in mammalian cells. Further optimization of the described lead compounds with respect to their pharmacological properties should allow for oral delivery at acceptable doses in living organisms.

Experimental Procedures

Organic Chemistry

Synthesis methods for compounds CMP2 and CMP3 have been previously described in patents WO 200182923 and WO 200241835 [15, 16]. Compounds CMP1 and CMP4–CMP10 can be prepared according to synthesis methods described in patent WO 05/040212 [32]. These patents may be accessed free of charge at http://ep.espacenet.com.

Plasmid Constructs

Details on construction of all prokaryotic and eukaryotic expression vectors used in this work are included as Supplemental Data. Reporter plasmids 7xH1/CRP/SEAP and pCMV-Luc were described elsewhere [9].

Expression in Bacteria and Purification of GST-ER-LBD Fusion Proteins

Recombinant GST-ER-LBD fusion proteins have been produced by using the protocol detailed in the Supplemental Data.

In Vitro Ligand Binding Assays

Ligand affinities for the GST-ER-LBD polypeptides were determined by a competitive radiometric binding assay using tritium-labeled estradiol (^{3}[H]E_2) (Amersham, 158 Ci/mmol, 558 mCi/mg) as tracer. Microplate (Basic Flashplates, NEN) wells were coated for 12 hr at 4°C with 100 μl PBS containing anti-GST antibodies (Amersham) at a 5 μg/ml concentration. After three washes with 200 μl PBS, background was reduced by saturating with 200 μl PBS containing 1% BSA for 3 hr at 4°C. Wells were then washed three times with 200 μl lysis buffer containing 0.1 M NaCl and 0.2% n-Dodecyl β-D-maltoside (assay buffer). Suitable amounts of crude *E. coli* supernatants containing the GST-ER-LBD proteins (2–10 μl) or of purified polypeptides (8 nM) were bound to anti-GST antibody-coated wells for 1 hr at 23°C in 200 μl assay buffer with constant agitation. After three more washes with 200 μl assay buffer, the ligand binding reaction was set up in 195 μl assay buffer containing 2–10 nM ^{3}[H]E_2 and 5 μl DMSO or suitable dilutions of test compounds in DMSO. Incubation was for 2 hr at 23°C with constant shaking, followed by SPA radioactivity measurement using a microplate scintillation and luminescence counter (Top Count NXT, Packard). IC_{50} values were obtained by multiparameter logistic fitting of the experimental data with the aid of Kaleidagraph software.

Yeast Strains and Growth Conditions

CG-1945 (Clontech) was used as the yeast reporter host strain for display and screening of the library. It contains both *HIS3* and *lacZ* reporter genes under the control of a GAL4-responsive UAS integrated in the genome. Yeast strain Y187 (Clontech) was instead used for quantitative β-galactosidase reporter assays. It contains only the *lacZ* reporter gene, which is expressed at higher levels than in strain CG-1945. Both strains (gal4$^-$, gal80$^-$) were propagated at 30°C in YPD medium. Yeast strains transformed with pGBT9 plasmids were grown and stored in SD minimal medium (SD, Clontech) supplemented with –Trp amino acid mixture. Growth

selection for the nutritional reporter HIS3 was performed on agar plates in SD supplemented with a –His/–Trp amino acid mixture and either 15–30 mM (single plasmid transformants) or 70 mM (library clones pool) 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of HIS3 protein. In vivo β-galactosidase assays were performed on agar plates in SD supplemented with a –Trp amino acid mixture, X-gal (80 mg/L), and 1× BU salts (10× = 70 g $Na_2PO_4 \cdot 7H_2O$ and 30 g NaH_2PO_4 [pH 7]). Growth selective and X-gal plates were supplemented with DMSO, E_2, or tetrahydrofluorenone compounds at the indicated concentrations.

Transformation of Yeast Cells

Yeast cells were transformed by using the Li Ac procedure and the YEASTMAKER Yeast transformation kit (Clontech) and following the manufacturer's protocol. For single-plasmid, small-scale transformations, 0.1 μg DNA was used to transform 0.1 ml yeast competent cells, resulting in transformation efficiencies of approximately 10^5 colony-forming units (cfu) per μg of DNA. To set up transformation conditions for homologous recombination of pGBT9-GAL4DBD/ERα-LBD L(384)M/VP16AD with mutagenized PCR products, the expression vector was linearized by BglII digestion, and half of it was treated with Klenow polymerase prior to gel purification. A total of 100 ng of each of the two versions of the digested plasmid were then transformed as outlined with 200 ng, 300 ng, or 400 ng of a 160 bp PCR fragment obtained by using wt oligonucleotides corresponding to the degenerated oligonucleotides designed to construct the mutated library (see below). Cotransformation of 100 ng linearized blunt-ended vector with 300 ng PCR fragment showed an efficiency of approximately 10^5 per μg of DNA that was 100-fold higher than background with the recipient vector alone. Based on this condition, in the library-scale transformation procedure, 60 μg mutagenized fragment collection and 20 μg linearized blunt-ended recipient vector were used to transform 1 ml yeast cells with an estimated efficiency of 5×10^4 cfu per μg of DNA.

Library Design

Low-energy conformations of CMP1 were calculated by using Cerius2 software (Accelrys, San Diego, US). The three energy minimum CMP1 conformations were manually modeled into the superimposed crystal structures of human ERα-LBD (PDB entry 1ERE, [17]) and human ERβ-LBD (PDB entry 1QKM, [18]) by using the E_2 and raloxifene ligands as structural templates.

Library Construction and Selection

Degenerate oligonucleotide mixtures were synthesized by a split-pool strategy based on the following wt sequences: forward, 5′-GATCCTGATGATTGGTCTCGTCTGGCGCTCCATGGAGCACCCAGGGAAGCTACTGTTTGCTCCTAAC-3′; reverse, 5′-CATCATGCGGAACCGAGATGATGTAGCCAGCAGCATGTCGAAGATCTCCACCATGCCCTCTAC-3′. At each of the underlined positions (L391, F404, M421, I424, L428), the previously synthesized column material was split into ten individual pools, and the ten possible codons were synthesized separately and the aliquots were pooled together again. Codons used were: GGC(G), GCC(A), TGC(C), GTG(V), ATC(I), CTG(L), ATG(M), TTC(F), TAC(Y), and TGG(W). A slightly higher quantity of pooled column material (15% of total) was used to synthesize the wt codon, thus leaving 9.4% for each of the other nine remaining codons. This "wt spiking" increased the relative frequency of clones with a low number of substitutions (one or two) with respect to clones having four or five substitutions that represent the majority of the library clones. While in a homogeneous "10% library," the 45 possible single-substitution clones would have represented only 0.045% of the total library; the use of 15% wt codons increases this fraction to 0.2152%, i.e., the clones were about 5-fold as frequently represented in the library. Consequently, the fraction of clones with five substitutions (59,049 possible combinations) dropped from about 59% to about 44%.

Preparative amounts of mutated LBD fragments were synthesized by PCR amplification of 6 μg pGBT9-GAL4DBD/hERα-LBD/VP16AD DNA template by including 2.1 nmol of each of the degenerated oligonucleotide mixes in a total reaction volume of 6 ml containing 250 mM dNTPs, 5% DMSO, 600 μl Pfu 10× buffer, and 300 U Pfu polymerase (Stratagene). The PCR amplification consisted of 25 cycles at 95°C for 1 min, 65°C for 1 min, and 72°C for 2 min. A total of 60 μg of the mutagenized 160 bp product mixture was purified on QIAquick spin columns (Qiagen) and was used in a scaled up cotransformation experiment together with 20 μg linearized recipient vector as outlined. A total of 30 ml of the cotransformation reaction was spread on 20 23 cm × 23 cm –Trp-selective plates, colonies were harvested after 3 days of growth at 30°C, and 1 ml glycerol stocks of the amplified library were prepared. Suitable dilutions of the cotransformation mixture were spread onto 100 mm plates to control the efficiency of homologous recombination and to determine the library titer (5×10^4 cfu per μg of DNA). Glycerol stocks were also titered and contained 1.2×10^5 cfu per μl. A total of 1×10^5 cfus were spread on each of 20 100 mm –His/–Trp-selective plates containing 70 mM 3-AT and either 1 μM (first experiment) or 10 μM (second experiment) CMP1 (a total of 2×10^6 cfus per experiment were plated). After 6 days of growth at 30°C, the average number of His^+ colonies per plate was 200 in the first experiment and 60 in the second experiment. A total of 80 colonies out of 200 of the first screening and all colonies of the second screening were streaked out from each plate on X-gal 100 mm plates containing either DMSO or CMP1 at the concentration of 1 μM or 10 μM, respectively (a replica on –Trp master plates was also performed). After 3 days of growth at 30°C, all clones that were blue in the presence of the compound and white on control plates were streaked on X-gal plates containing either DMSO or CMP1 at a 10-fold lower concentration (here, a replica on –Trp master plates was also performed). This procedure was reiterated down to a compound concentration of 0.1 μM, at which 28 and 31 independent positive clones, respectively, were collected for further analysis.

Plasmid Rescue from Yeast Clones

Colonies corresponding to the mutants of interest were grown to saturation at 30°C for 16 hr in 2 ml of –Trp SD. Cells from a 1.3 ml culture fraction were collected by centrifugation and resuspended by vortexing in 0.2 ml protoplasting buffer (100 mM Tris-HCl [pH 7.5], 10 mM EDTA, 14.4 mM β-mercaptoethanol) containing 400 μl of a 40 U/μl Lyticase (Sigma) solution. After cell walls were dissolved by incubation for 2 hr at 37°C, 200 μl lysis solution (0.2 M NaOH, 1% SDS) was added, and samples were incubated at 65°C for 20 min, and then put rapidly on ice. Samples were mixed with 200 μl 3 M K-acetate (pH 5.4), incubated on ice for 15 min, and spun for 3 min at 13,000 rpm in an Eppendorf microcentrifuge. Plasmid DNA was recovered from supernatants by precipitation with 0.6 volumes of isopropanol. Rescued plasmids were both transformed in electro-competent *E. coli* DH12S cells and directly sequenced by PCR amplification of a 360 bp fragment using oligonucleotide primers (5′-CTGACCAACCTGGCAGACAG-3′, forward; 5′-GGACTCGGTGGATATGGTCC-3′, reverse) annealing 100 bp upstream and downstream, respectively, of the mutagenized insert. Amplified fragments were purified on QIAquick spin columns and subjected to automatic sequencing by using either of two sequencing primers (5′-GTTCACATGATCAACTGGGCG-3′ or 5′-GAGACTTCAGGGTGCTGGAC-3′) that annealed 70 bp from the mutagenized insert boundaries.

Yeast Protein Extracts and Western Blot Analysis

The urea/SDS method (Clontech Yeast Protocols Handbook) to prepare yeast protein extracts suitable to evaluate mutant protein expression by Western blot analysis by using anti-VP16 AD polyclonal antibodies (Santa Cruz) was followed.

Quantitative β-Galactosidase Assays

Single colonies were grown to saturation for 16 hr at 30°C in –Trp SD minimal medium; cells were collected by centrifugation and then diluted in YPD medium to an optical density of 0.04 at 600 nm. Subcultures of 5 ml volume were set up and allowed to grow for 7 hr at 30°C in the presence of DMSO or various ligand concentrations until they reached mid-log phase (OD_{600} = 0.4–0.5). Cells from 1.5 ml of culture (two duplicates per sample) were processed for quantitative β-galactosidase assays by following the Clontech Yeast Protocols Handbook procedure as described in the Supple-

mental Data. Dose-response data were analyzed by using a nonlinear regression analysis (Kaleidagraph software).

Cell Culture, Transfections, and SEAP Assays

All cell culture experiments were performed by using phenol red-free Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL) supplemented with 10% dextran charcoal-treated (Sigma) fetal bovine serum (FBS, GIBCO-BRL).

To evaluate the effect of compounds on the MGR-ERα-LBD chimera transcriptional activity, HeLa cells were seeded 18 hr before transfection in 6-well plates (3×10^5 cells/well) and transfected with 1 μg plasmid DNA per well (0.5 μg transactivator + 0.5 μg reporter) by using Lipofectamine (Invitrogen), according to manufacturer's instructions. A total of 100 ng of the luciferase reporter plasmid pCMV-Luc were included in the transfection mixture as an internal control for transfection efficiency. At 6 hr after transfection, culture medium was changed, and cells were left untreated or were treated with the various ligands. After an additional 24 hr, medium was harvested and analyzed for the activity of human-secreted alkaline phosphatase (SEAP) by a commercially available assay (Tropix Phospha-Light system) by following the manufacturer's guidelines. Reactions were performed in microplates, and SEAP activity was measured as light emission by using a microplate scintillation and luminescence counter (Top Count NXT, Packard). Values were subtracted of the background obtained by measuring endogenous alkaline phosphatase in untransfected cell medium and normalized against luciferase activity measured in cell extracts. Conversion to SEAP concentration values (ng/ml) was done by standard activity curves obtained with purified human placental AP (Sigma). Dose-response data were analyzed as outlined above.

Supplemental Data

Supplemental Data including an additional Experimental Procedures section and additional results are available at http://www.chembiol.com/cgi/content/full/12/8/883/DC1.

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