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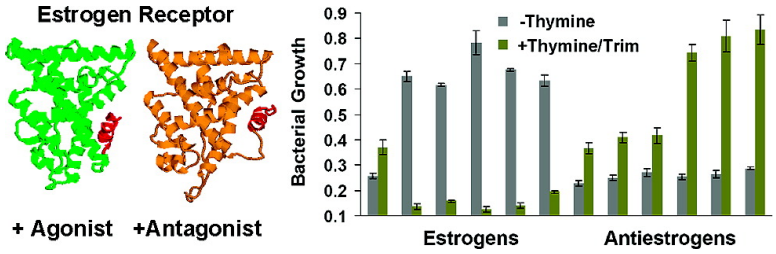

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Engineered Chimeric Enzymes as Tools for Drug Discovery: Generating Reliable Bacterial Screens for the Detection, Discovery, and Assessment of Estrogen Receptor Modulators

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Abstract: Engineered protein-based sensors of ligand binding have emerged as attractive tools for the discovery of therapeutic compounds through simple screening systems. We have previously shown that engineered chimeric enzymes, which combine the ligand-binding domains of nuclear hormone receptors with a highly sensitive thymidylate synthase reporter, yield simple sensors that report the presence of hormone-like compounds through changes in bacterial growth. This work describes an optimized estrogen sensor in *Escherichia coli* with extraordinary reliability in identifying diverse estrogenic compounds and in differentiating between their agonistic/antagonistic pharmacological effects. The ability of this system to assist the discovery of new estrogen-mimicking compounds was validated by screening a small compound library, which led to the identification of two structurally novel estrogen receptor modulators and the accurate prediction of their agonistic/antagonistic biocharacter in human cells. Strong evidence is presented here that the ability of our sensor to detect ligand binding and recognize pharmacologically critical properties arises from allosteric communication between the artificially combined protein domains, where different ligand-induced conformational changes in the receptor are transmitted to the catalytic domain and translated to distinct levels of enzymic efficiency. To the best of our knowledge, this is one of the first examples of an engineered enzyme with the ability to sense multiple receptor conformations and to be either activated or inactivated depending on the nature of the bound effector molecule. Because the proposed mechanism of ligand dependence is not specific to nuclear hormone receptors, we anticipate that our protein engineering strategy will be applicable to the construction of simple sensors for different classes of (therapeutic) binding proteins.

Introduction

The recent development of chimeric protein-based sensors for ligand binding may greatly simplify the detection of potentially valuable compounds with specific affinity for particular protein targets. These biosensors are typically constructed by fusing a target ligand-binding domain (LBD) to an easily assayed reporter protein. Properly designed fusions allow ligand-induced conformational changes in the target LBD to be transmitted to the reporter and allosterically modulate its properties. By selecting a suitable signaling protein, these systems can be tuned to report binding through detectable changes in phenotype. A recent example of a successful design of this type is a set of chimeric fusions of the maltose-binding protein (MBP) with β-lactamase.[1,2] *Escherichia coli* (*E. coli*) cells expressing these fusions are rescued on antibiotic-containing media only in the presence of ligands that bind to and change the structure of the MBP domain. Another example is based on fusions of calmodulin with circularly permuted variants of the green fluorescent protein.[3] In this case, the efficiency of chromophore formation depends on calcium binding, thus allowing the intracellular concentration of calcium ions to be conveniently reflected by cell fluorescence.

Drug discovery often relies on the identification of compounds with the ability to specifically bind to and modulate

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the function of a particular protein target. Simple biosensors that enable the reliable detection of protein−ligand interactions among large libraries of test compounds in a facile, high-throughput fashion could make an important contribution to the drug discovery process.[4] Attractive targets for this strategy are the nuclear hormone receptors (NHRs). They are the largest group of metazoan transcription factors and are involved in vital functions of the cell, such as development, differentiation, homeostasis, reproduction, and metabolism.[5,6] Hence, deregulation of their function is closely related to a number of pathological conditions, including carcinogenesis, infertility, obesity, inflammations, cardiovascular diseases, and osteoporosis.[5,6] Accordingly, NHRs comprise one of the largest classes of protein drug targets, with a pharmaceutical significance comparable to that of G protein-coupled receptors, kinases, ion channels, and other membrane transporters. Currently, about 4% of all marketed therapeutics interfere with the activity of these proteins.[7]

NHRs are modular proteins consisting of a DNA-binding domain and a C-terminal LBD, with some of these receptors also containing an N-terminal transactivation domain.[5,6] Approximately half of these transcription factors are activated by the binding of lipophilic small-molecule hormones or synthetic/natural hormone agonists. For example, signaling of the estrogen receptor (ER), the best characterized member of this superfamily, is typically initiated by the endogenous estrogen 17β-estradiol (E_2). This steroidal hormone binds to the two ER subtypes (ERα and ERβ) and induces a conformational change that allows ER homo- and heterodimers to form.[8] These can then recruit different coactivators and form complexes capable of regulating the expression of specific genes, e.g., by binding to regulatory regions of DNA termed estrogen response elements (EREs). Gene expression via ER can be activated by two transcription activation functions (AFs), one mediated by the N-terminal transctivation domain (AF-1) and one by the C-terminal region of the LBD (AF-2).[5,6]

Therapeutically valuable compounds with the ability to antagonize the effects of estrogen, such as 4-hydroxytamoxifen (the active metabolite of tamoxifen) and raloxifene, bind to ER with high affinity but cause a conformational change that is distinct from that induced by estrogen agonists.[5,6] In this case, ER can still dimerize, but is unable to recruit coactivators, and instead interacts weakly with corepressors. However, although antagonist binding results in transcriptional silencing through AF-2, the antagonist-bound ER complex may retain some ability to induce gene expression via AF-1.[5] Therefore, a number of known estrogen analogues, including tamoxifen and raloxifene, exhibit tissue-specific estrogenic or antiestrogenic responses and are therefore termed selective ER modulators (SERMs). SERMs sometimes also act as mixed agonists/antagonists in the same cell and via the same ER subtype depending on the absence/presence of E_2, respectively.[9,10] The specific response in a given tissue depends on a number of parameters, including the promoter of the estrogen target gene and the coactivator/corepressor complement of the cell.[5,8] However, the conformation that the binding domain adopts upon binding of a particular ligand is probably the most decisive factor determining the pharmacological responses.[11]

We have shown in previous work that engineered chimeric fusions of NHR LBDs with the very sensitive reporter enzyme thymidylate synthase (TS) yield simple sensors that report the presence of hormone-like compounds by changes in bacterial growth.[12] Our present work describes a dramatically advanced *E. coli*-based estrogen sensor with extraordinary reliability in detecting a wide variety of estrogenic compounds and in recognizing important aspects of their pharmacological profiles. By using this sensor, we were able to rapidly screen a small chemical library and identify structurally novel ER modulators, while predicting their agonistic/antagonistic biocharacter in human cell assays. Further, we provide strong evidence that our engineered chimeric sensor discriminates between agonistic and antagonistic effects by functioning as an allosteric enzyme, which presumably recognizes different ligand-induced conformational changes that occur in the LBD and translates them into distinct levels of TS activity.

Results

Construction of a Second-Generation Estrogen-Regulated Chimeric Enzyme. In previous work, we constructed two simple bacterial hormone-sensing prototypes by fusing the LBD of either the human ERα or the human thyroid hormone receptor β, in combination with a solubilization (MBP) and a stabilization domain (modified intein splicing domain), to the N terminus of the bacteriophage T4 TS enzyme.[12] In these constructs, the stabilization domain comprised the first 96 and the last 41 amino acids of a splicing-deficient variant of the *Mycobacterium tuberculosis* RecA (Mtu RecA) intein. These chimeric sensor proteins provided hormone-dependent TS activity, which was easily monitored by growth phenotype in TS-knockout *E. coli* cells. Subsequently, we showed that a second estrogen-sensing bacterial system can be constructed on the basis of a fusion of the human ERβ LBD with a more stable intein splicing domain comprising the first 110 and the last 58 residues of the Mtu RecA intein (plasmid pMIT::ERβ*; Figure 1A).[13,14] This work demonstrated that the ERα- and ERβ-based sensors allow the facile detection of subtype-selective ER ligands in the context of positive TS selection.[14] In the present work, we investigated the ability of the ERβ-based system to be used as an improved sensor for general estrogenicity, using both the positive and negative tunable selections provided by the TS reporter system (Figure 1B).[15]

As it had been observed with our ERα-based sensor,[12] TS-knockout *E. coli* cells transfected with pMIT::ERβ* and incubated in liquid thymine-free medium (−THY) at 34 °C were

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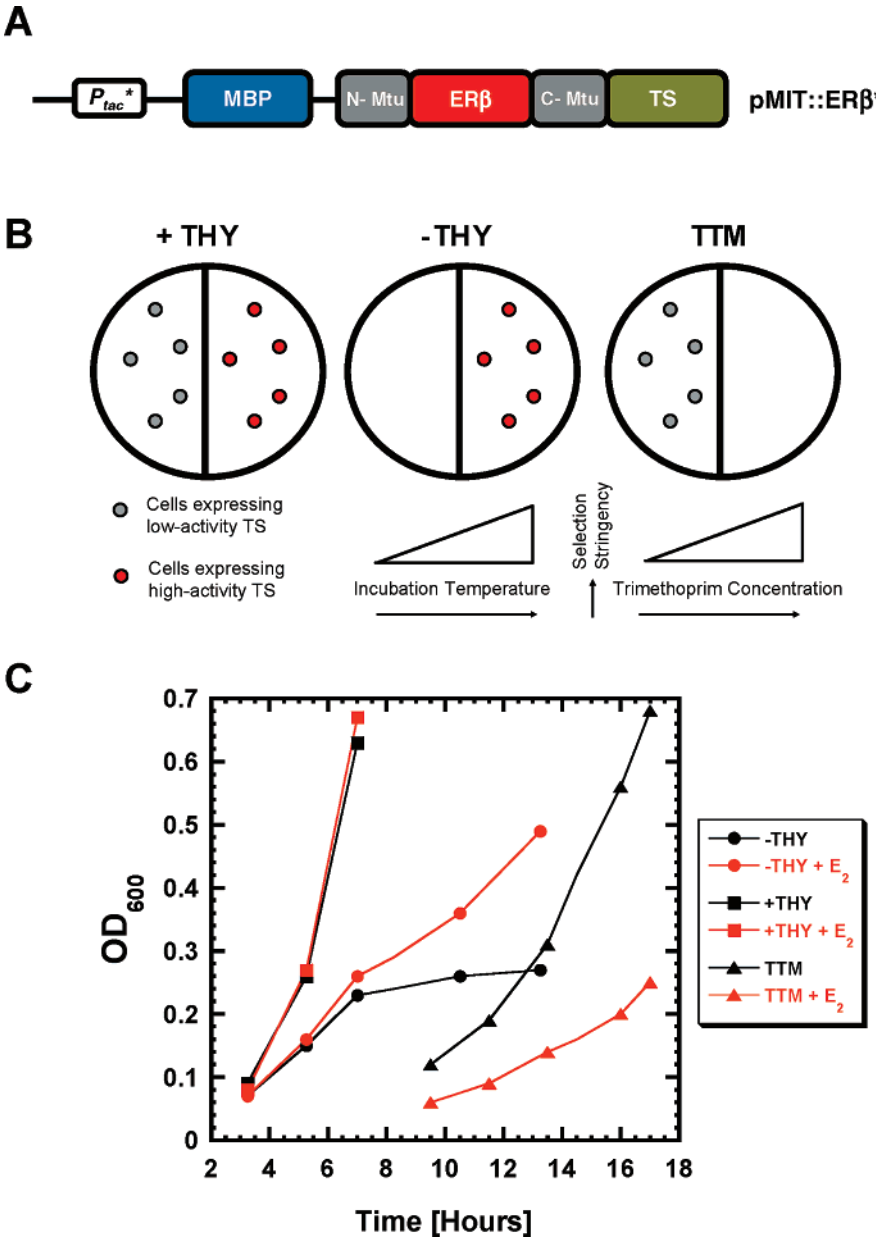

Figure 1. Design and associated growth phenotypes of the ERβ-based estrogen-sensing system. (A) Chimeric protein fusion used to couple estrogen binding via ERβ with the catalytic activity of thymidylate synthase. P_{tac}*, artificial *tac* promoter required for hormone-dependent phenotypes;[12] MBP, maltose-binding protein; N-Mtu, the first 110 residues of the *Mycobacterium tuberculosis* RecA intein (Mtu RecA intein) carrying a splicing-inactivating Cys1Ala mutation; C-Mtu, the last 58 residues of the Mtu RecA intein; ERβ, residues Arg254 to Lys504 of the human ERβ (encompasses the entire ligand-binding domain of the receptor); TS, bacteriophage T4 thymidylate synthase. (B) Schematic representation of the growth phenotypes associated with the TS genetic selection system. In a thymine-rich medium (+THY medium), cells do not require active TS for growth (nonselective). In the absence of thymine (−THY), cells are subjected to selection for high TS activity (positive selection). When both thymine and the dihydrofolate reductase inhibitor trimethoprim are added to the medium (TTM), selection against TS activity takes place (negative selection). The stringency of both modes of selection is tunable, in the case of the negative selection by varying the trimethoprim concentration, while positive selection becomes more stringent at elevated temperatures. (C) Effect of the addition of 10 μM estrogen (orange curves) on the growth rates of bacterial cells harboring the pMIT::ERβ* plasmid in liquid −THY medium at 34 °C (circles), and in liquid +THY (squares) and TTM media (triangles) at 37 °C. OD_{600}: Optical density at 600 nm.

able to grow only in the presence of estrogen.[14] More specifically, cells incubated in the presence of estrogen yielded saturated cultures after 15−20 h of incubation but were unable to grow beyond an $OD_{600} = 0.2-0.4$ in the absence of estrogen, even after 36 h of incubation (see below). The rapid initial background growth in the absence of estrogen occurs presumably due to small amounts of thymine and thymidine that are transferred to the selective medium with the inoculum. However, after incubation under suitable conditions of positive TS selection, the presence/absence of estrogen results in an apparent growth/no growth readout. As expected, in a nonselective thymine-rich medium (+THY), healthy growth was observed irrespective of the presence of estrogen. Interestingly, with the pMIT::ERβ* sensor, the impact of estrogen binding on TS activity could also be observed when negative selection was performed: the addition of E_2 to cells incubated in a thymine-rich medium supplemented with trimethoprim (TTM) resulted in an inhibitory effect on cell growth (Figure 1C).

Previously reported control experiments utilizing auxotrophic *E. coli* strains indicate that estrogenic compounds (including E_2) do not have a general impact on TS activity or bacterial growth.[12] This work included additional control experiments

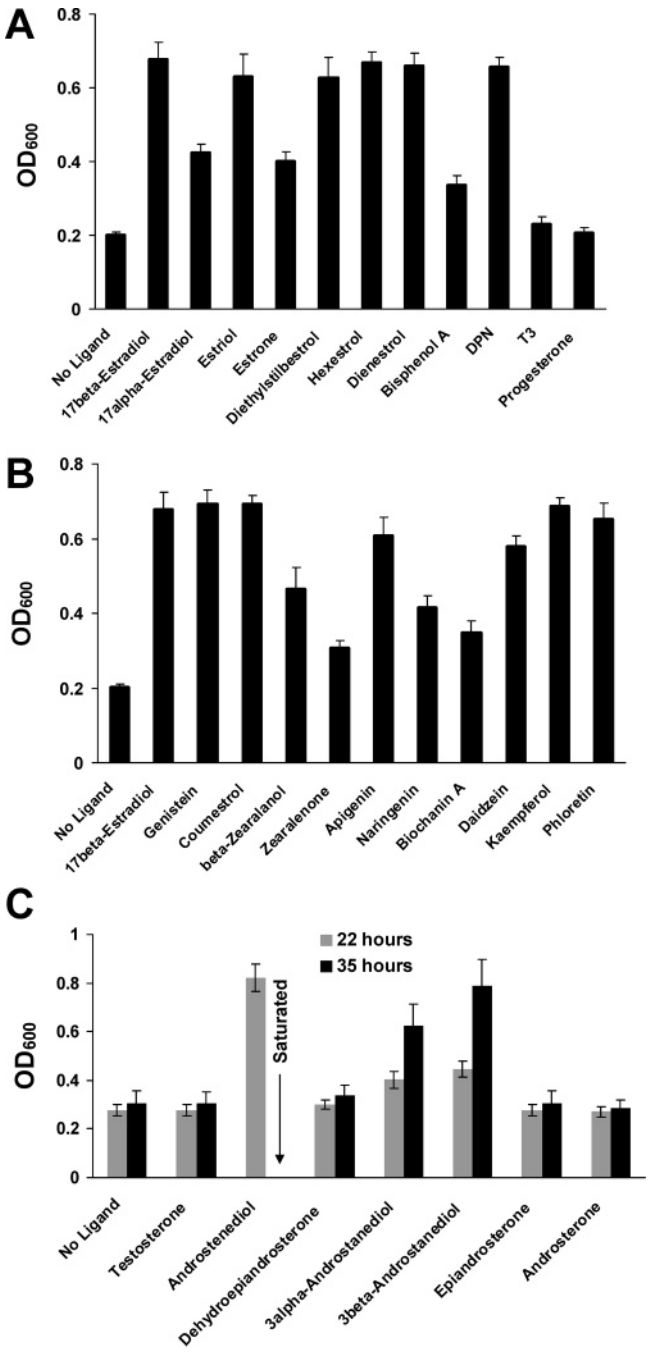

Figure 2. Reliable detection of estrogenic compounds: (A) synthetic estrogen analogues; (B) phytoestrogens; (C) testosterone and some of its metabolic precursors and metabolites. The relative binding affinity values of testosterone, androstenediol, dehydroepiandrosterone, 3α-androstanediol, 3β-androstanediol, epiandrosterone, and androsterone for ERβ are <0.01,[16] 13.84 ± 1.93, 0.090 ± 0.011, 0.288 ± 0.112, 2.608 ± 0.440, 0.013 ± 0.001, and 0.003 ± 0.002, respectively. 17β-Estradiol is arbitrarily set to 100. TS-knockout bacterial cells transfected with pMIT::ERβ* were grown in liquid −THY medium at 34 °C in the presence of 10 μM ligands for approximately 15 h in A and B and for 22 (gray) and 35 h (black) in C. Experiments were carried out in triplicate, and the error bars represent one standard deviation from the mean value.

with estrogen and thyroid-hormone sensing strains showing that growth enhancement in this system is only observed when an appropriate ligand is provided for a given sensor strain. Finally, the current observation that growth is enhanced in −THY medium in the presence of estrogen, but inhibited by estrogen in TTM medium, provides very strong evidence that ligand binding directly modulates TS activity in the chimeric sensor protein, and not through a more general growth effect. Thus, the effect of estrogen on TS activity and the resulting growth phenotypes is highly specific and acts through the ERβ LBD in our chimeric sensor protein.

Reliable Detection of Estrogenic Compounds. To evaluate the ligand-sensing performance of the ERβ-based sensor, cells harboring pMIT::ERβ* were incubated in liquid −THY medium at 34 °C in the presence of a small library of synthetic estrogen analogues. As observed previously (Figure 1C), these cells were unable to grow in the absence of ligand, but the addition of all the compounds that are known to possess estrogenic properties resulted in the induction of growth (Figure 2A). The control compounds 3,3′,5-triiodo-L-thyronine (T_3) and progesterone, the cognate ligands of the thyroid hormone and progesterone receptors, respectively, were unable to enhance growth.

We then exposed our sensor cells to a second library comprising estrogenic compounds originating from plants (phytoestrogens). These natural hormone analogues exemplify the dietary contribution to estrogen signaling and include compounds with considerable ERβ binding selectivity.[16] A number of these are thought to be promising natural products for use in hormone replacement therapy,[17,18] and in many cases their intake is associated with cardioprotective and anti-inflammatory properties, as well as a reduced risk for endocrine-related cancer.[19] As with the synthetic estrogen library, every phytoestrogen tested was able to enhance cell growth and could be easily detected (Figure 2B).

The ERβ-based sensor was also able to detect the binding of several steroids with low affinity for ER. These were selected from the metabolic precursors or metabolites of testosterone, and although some of them are classified as androgens, they are able to bind to ER weakly and induce estrogenic and other responses both in males and females.[20,21] Growth phenotypes in the presence of these compounds indicated that approximately half of the tested steroids could be detected, although some of them required longer incubation times (Figure 2C). The ligands that were unable to induce a growth response are known to exhibit extremely low binding affinities for ER (e.g., testosterone).[20] One of the detected steroids, the dihydrotestosterone metabolite 3β-androstanediol, has been proposed to function as the principal ligand of ERβ in the prostate,[22,23] where it may inhibit the migration of prostate cancer cells.[21]

Notably, a number of the detected compounds, such as bisphenol A and biochanin A, exhibit very low binding affinities for ER (1000- to 10000-fold lower than that of E_2).[16] This makes

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such compounds virtually undetectable by similar nontranscriptional assays in yeast or bacteria. For example, estrone, 17α-estradiol, and bisphenol A were unable to enhance cell growth in our prototype bacterial estrogen-sensing system,[12] while genistein, estriol, and other lower-affinity binders were found inactive in a similar yeast assay based on a chimeric fusion of ERα with dihydrofolate reductase.[24] All of these compounds, however, produced clear changes in growth in the pMIT::ERβ* sensor system.

The sensitivity of our biosensor was further evaluated by determining dose−response curves for various estrogenic compounds at 34 °C (Figure 3A). This test revealed a half-maximal effective concentration (EC_{50}) for E_2 of approximately 100 nM, about 100-fold lower than the concentration required by the ERα-based sensor.[12] In addition, all of the tested compounds could be detected at nanomolar concentrations and the higher-affinity ones at sub-nanomolar concentrations (Figure 3A). This sensitivity is comparable to that of previously reported chimeric sensors for estrogen binding in yeast[25,26] or in vitro,[27] and to the sensitivity of other simple screening systems, such as recently developed NHR microarrays of coactivator recruitment.[28] Interestingly, for low-affinity ligands, the sensitivity of our system converges with that of highly sensitive in vitro binding assays,[29] and with some transcriptional activation assays in genetically engineered yeast[30,31] and mammalian cells.[9,31,32] These features may render this system a particularly attractive tool for the rapid screening of samples and extracts derived from natural products or environmental sources. Because the EC_{50} values correlate well with binding affinity (Figure 3B), the intensity of a given ligand−receptor interaction can be estimated by comparing its EC_{50} value to those of known compounds.

Recognition of Pharmacological Properties. A general limitation of simple screening systems for NHR modulators is the inability to predict the pharmacological effect of a particular hormone mimic. For example, in vitro competitive binding assays without coactivator recruitment, as well as transcriptional activation assays in yeast, have been unable to discriminate between known NHR agonists and antagonists.[33] This is a very important characteristic for a screening system since most of the clinically valuable hormone analogues that target ER, such as tamoxifen, raloxifene, and ICI 182,780, exert their anticancer therapeutic effects through their ability to antagonize estrogen signaling.[11]

To investigate the ability of the ERβ-TS sensor to reliably differentiate between agonistic and antagonistic effects, our sensor strain was incubated in E_2-containing −THY medium at 34 °C, or TTM medium at 37 °C, and exposed to increasing concentrations of known estrogen agonists, SERMs, or pure estrogen antagonists (Figure 4). In these experiments, a subsaturating concentration of E_2 (500 nM as opposed to 10 μM used in Figure 2) was used to allow a clear determination of any growth enhancement arising from agonistic behavior of the test compounds. As it was previously observed with our prototype estrogen sensor,[12] the addition of all of the estrogen agonists in E_2-supplemented −THY medium had an additive effect on growth, while the addition of the antiestrogens tamoxifen, 4-hydroxytamoxifen, and clomiphene was found to have an inhibitory effect on growth (Figure 4A). In TTM medium, growth phenotypes were inverted: the presence of estrogen agonists suppressed growth, whereas antagonist addition enhanced bacterial growth (Figure 4B). Notably, with the ERβ sensor, raloxifene and ICI 182,780 were also able to antagonize E_2, although they could not be detected and/or correctly identified as antagonists by the ERα-based sensor.[12] However, the full antagonistic effect of ICI 182,780 could not be observed at the tested concentrations, presumably reflecting that its affinity of binding to ERβ, as determined using standard methodology (see Experimental Section for details), is 40-fold lower than that of E_2, whereas that of, e.g., 4-hydroxytamoxifen is only 4.5-fold lower. One cannot exclude, though, that secondary effects (e.g., low diffusivity through the *E. coli* membrane) might also modulate the inhibitory effect. Additionally, antagonistic effects in this system could be detected at concentrations above 5−10 μM with the pure estrogen antagonist ZK 164,015, as well as with the synthetic SERMs GW 5638, tetrahydrochrysene and PPT (data not shown). PPT exhibits a subtype-selective pharmacological profile, activating ER signaling via ERα, while acting as an estrogen antagonist through ERβ at high concentrations.[8,34] These observations, together with the fact that estrogen analogues have been shown not to interfere with cell growth in the absence of the ER sensor protein,[12] provide strong evidence that antiestrogens directly antagonize the ability of E_2 to bind to ERβ and enhance the TS activity of the chimeric sensor.

Molecular Basis of Agonist−Antagonist Discrimination. Crystallographic studies have revealed the structural basis of the functional differences between NHR agonists and antagonists. These studies suggest that agonist binding induces a structural shift in the LBD that allows the C-terminal helix 12 to close as a lid over the hormone-binding cavity and expose a surface of the receptor that can recruit coactivators and initiate signaling (Figure 5A).[5,6,8,35] Antagonistic compounds on the other hand, whose backbone structures resemble that of agonists but contain bulky side chains that protrude from the binding cavity, prevent helix 12 from closing properly over the binding pocket. Instead, they yield a LBD structure where helix 12 extends outward (Figure 5A) and prevents coactivator recruitment, while allowing weak interactions with corepressors.[5,6,8,35] Note that no crystal structure of the apo form of either ER subtype has yet been determined, and therefore the structural model of the unliganded form of ER shown in Figure 5A is conceptual and based on the determined structure of the apo LBD of the retinoid X receptor α.

It has been proposed that the hormone-regulated activities of steroid−LBD fusions with heterologous proteins expressed

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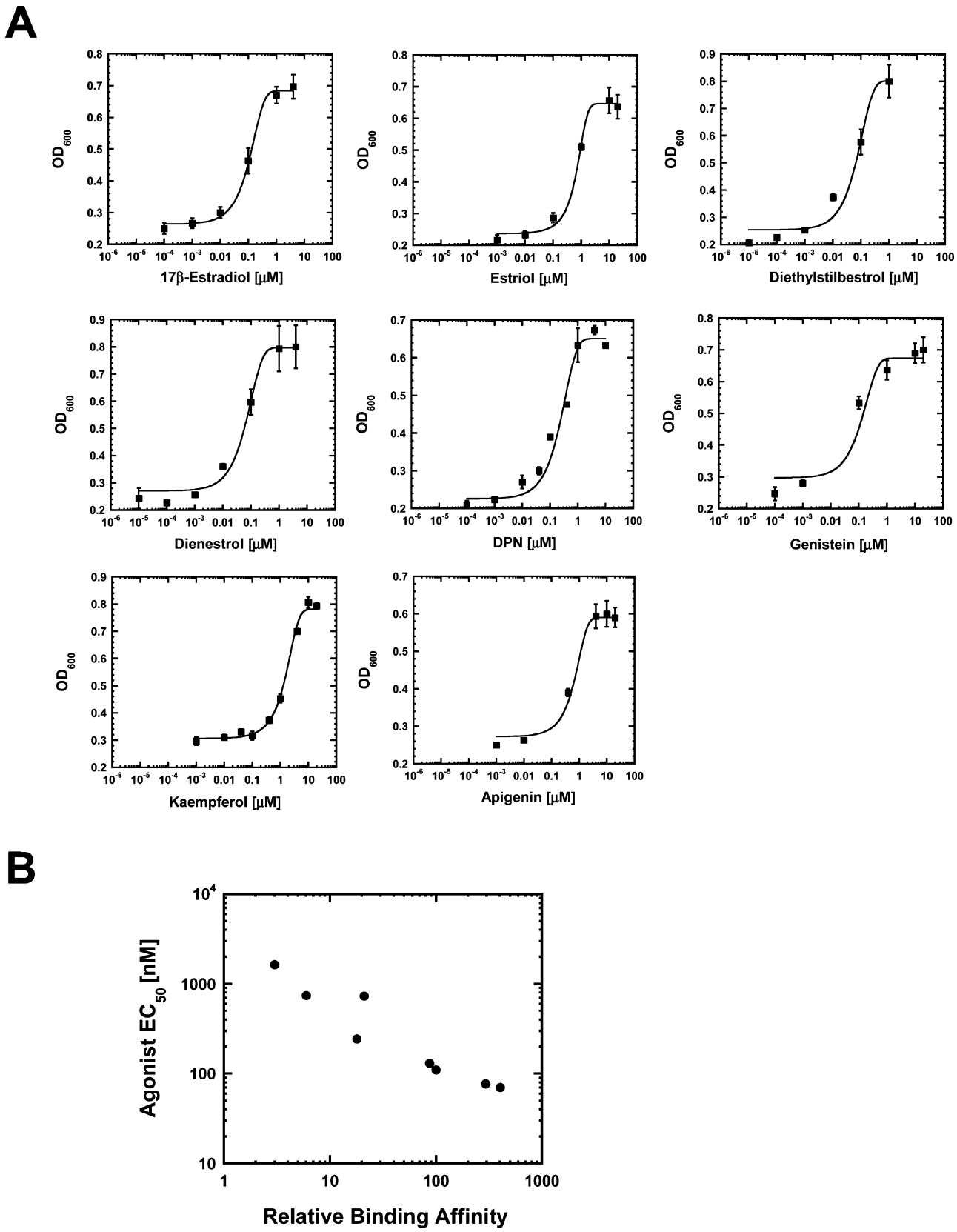

Figure 3. Sensitivity of the ERβ-TS sensor. (A) Dose−response curves of endogenous (17β-estradiol[14]), synthetic (estriol, diethylstilbestrol, dienestrol, DPN[14]), and plant-derived (genistein,[14] kaempferol, apigenin) estrogens. Cells carrying pMIT::ERβ* were grown in liquid −THY medium at 34 °C for 15−20 h. Experiments were carried out in triplicate and the error bars represent one standard deviation from the mean value. (B) Correlation between the EC_{50} values of the tested estrogen analogues determined from the generated curves in A, and the in vitro measurements of their relative binding affinities for ERβ as reported by Kuiper et al.[16,20] and Kim et al.[28]

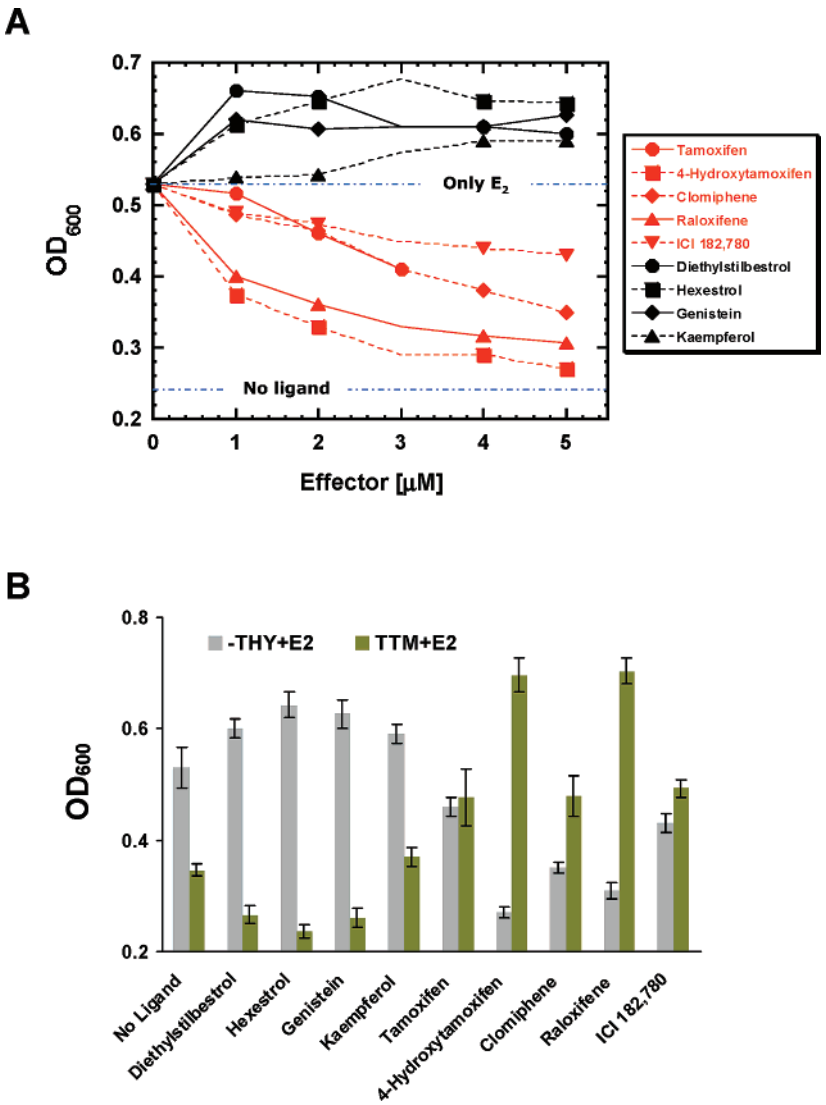

Figure 4. Recognition of the pharmacological properties of estrogen analogues. (A) Bacterial cells carrying the plasmid pMIT::ERβ* were exposed to increasing concentrations of known estrogen agonists (black) and antagonists (orange) in liquid −THY medium containing 500 nM E_2 at 34 °C for approximately 15 h. The upper blue line represents the level of cell growth in the presence only of 500 nM E_2, while the lower line the measured level of growth in the absence of E_2. (B) Cells transfected with pMIT::ERβ* and grown in E_2-enriched (500 nM) liquid −THY at 34 °C or TTM media at 37 °C were exposed to a 5 μM concentration of estrogen analogues (tamoxifen was added at a 2 μM concentration) for approximately 17 h. Experiments were carried out in triplicate, and the error bars in B represent one standard deviation from the mean value. E_2 = 17β-estradiol.

in eukaryotic hosts occur due to interactions with the molecular chaperone Hsp90.[36] In these chimeric fusions, chaperone binding to the partially unfolded LBD sterically blocks the activity of the fusion partner. Hormone binding induces folding of the LBD and dissociation of Hsp90, resulting in activation of the reporter protein. Estrogen-dependent activity of our ERβ-TS sensor could similarly be occurring due to ligand-induced dissociation of bacterial Hsp90 homologues such as HtpG, or other *E. coli* molecular chaperones.[37] An alternative mechanism for the observed phenotypes is that binding of estrogen analogues to the ERβ domain affects the overall thermodynamic or proteolytic stability of the ERβ-TS chimera. Such an effect would result in hormone-dependent amounts of the TS reporter in the *E. coli* cytoplasm, leading to apparent ligand-regulated TS activity. We believe that these mechanisms cannot account for all of the hormone-dependent activities of our NHR-TS fusions expressed in *E. coli*, however, as a number of our previously and presently described observations are not consistent with the mechanistic models mentioned above. Instead, we have proposed that the conformational changes that occur in the LBD upon ligand binding are communicated through the intein to the TS domain to allosterically modulate its catalytic efficiency.[12] This communication is thought to take place intramolecularly in a stably folded sensor protein, and to be independent of interactions with other proteins acting in trans.

We investigated this hypothesis by exposing our sensor strain to a number of known estrogen agonists and antagonists in −THY medium in the absence of E_2. As observed with the estrogen analogues in Figure 2, agonist addition resulted in enhancement of TS activity and induction of cell growth (Figure 5B). On the contrary, antagonist addition did not increase TS activity, although these compounds are known to bind to ER with high affinity.[20] The fact that antiestrogens can antagonize E_2-enhanced cell growth in our system even at relatively low

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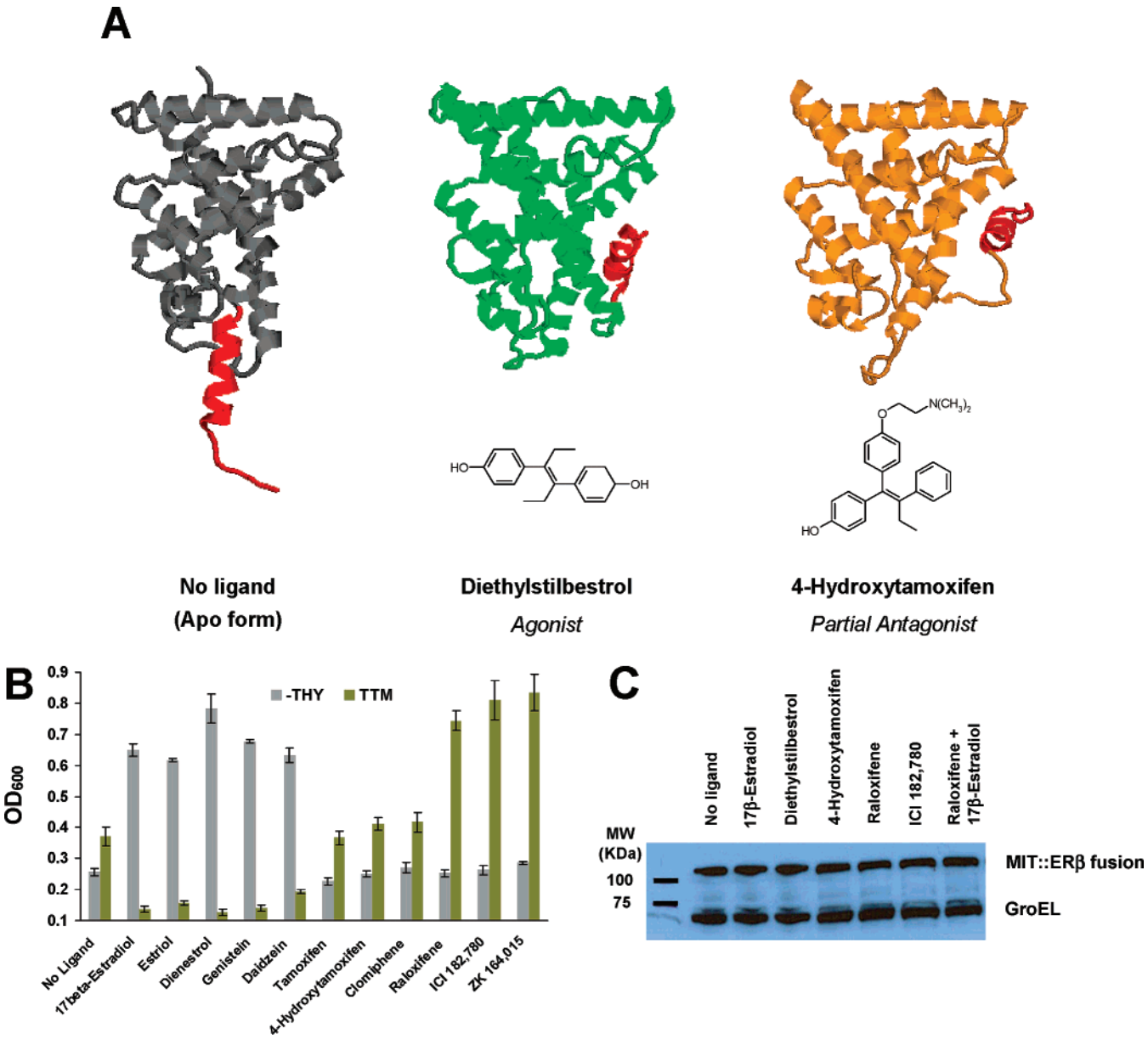

Figure 5. Molecular basis of agonist-antagonist discrimination. (A) Differential positioning adopted by the C-terminal helix 12 of NHRs (colored red) in the presence of pharmacologically distinct classes of hormone analogues. Crystal structures of the ligand-binding domains of the human retinoid X receptor α in the absence of ligand (gray)[58] and of the human ERα in the presence of the full agonist diethylstilbestrol (green) and the partial estrogen antagonist 4-hydroxytamoxifen (yellow).[35] (B) Cells harboring pMIT::ERβ* were grown in liquid −THY medium at 34 °C for 15 h (gray) and in liquid TTM medium at 37 °C for 16 h (green) in the presence of estrogen agonists and antagonsts. Ligands were added at a 4 μM concentration, apart from tamoxifen which was added at 2 μM. For the experiments in TTM media, raloxifene and ZK 164,015 were supplied at 40 μM and ICI 182,780 at a 30 μM concentration. Experiments were carried out in triplicate, and the error bars represent one standard deviation from the mean value. (C). Expression levels of the ERβ-TS sensor protein as revealed by Western blot analysis using an anti-MBP antibody. Estrogen analogues were added to a 5 μM concentration. The raloxifene + 17β-estradiol sample contained 0.5 μM 17β-estradiol and 5 μM raloxifene. GroEL expression probed with an anti-GroEL antibody as described in Experimental Section was used as a marker for equal loading.

ratios of antagonist/agonist concentrations (Figure 4) is an indication that the receptor domain retains the ability to bind these compounds tightly in the bacterial cytoplasm. Western blot analysis further revealed that the addition of estrogen agonists, antagonists, or combinations of these types of compounds does not affect the expression levels of the ERβ-TS sensor protein (Figure 5C). Taken together, these results imply that agonist and antagonist binding induce receptor conformations with distinct fingerprints on the enzymic efficiency of our chimeric sensor. The fingerprint corresponding to the antagonist-bound form of the receptor results in lower activity of the catalytic TS domain, thus allowing the ERβ-TS sensor to discriminate between agonistic and antagonistic effects.

As judged by growth phenotypes in −THY medium, the enzymic efficiency of the antagonist-bound forms of the ERβ-TS fusion closely resemble that of the apo form (Figure 5B). Although the apo and antagonist-bound forms of ER are similar in several aspects,[28,29] they are clearly distinguishable using conformation-sensitive fluorophores[38] or other sensitive sensors of receptor conformations.[27,39,40] To investigate the potential for

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detecting conformational differences between the apo and antagonist-bound forms of the ERβ domain in our system, ligand-dependent phenotypes were additionally evaluated under conditions of negative TS selection. As expected, in an estrogen-free TTM medium, cells could grow healthily, while agonist addition had an inhibitory effect on growth (Figure 5B). Most interestingly, the addition of the antiestrogens raloxifene, ICI 182,780, and ZK 164,015 at saturating or near-saturating concentrations resulted in significant growth enhancement. This implies that the antagonist-bound form of the receptor induces a state of lower TS activity than the apo form of the receptor. Tamoxifen, 4-hydroxytamoxifen, and clomiphene could not be fully evaluated for this effect, as they exhibited general toxicity effects in the 10−20 μM range.

The findings presented here provide further support *against* a mechanism where hormone-dependent effects occur due to simple ligand-dependent receptor stability or hormone-regulated chaperone dissociation. The antagonist-induced *inactivation* of the ERβ-TS chimera observed in this work strongly supports a mechanistic model based on intramolecular effects in a well-folded sensor protein. Although pure estrogen antagonists such as ICI 182,780 antagonize ER signaling by an additional mechanism that involves destabilization of the ER LBD and reduction of the receptor levels in the cell,[11,41,42] most antiestrogens do not possess an inherent ability to destabilize ER. Indeed, some of the studied estrogen antagonists are known to either not interfere (e.g., raloxifene) or to even increase ER stability (e.g., 4-hydroxytamoxifen).[11,42] In the context of the ERβ-TS fusion in *E. coli*, all of these antiestrogens were found not to interfere with the expression levels of our sensor protein. Furthermore, if the catalytic activity of the ERβ-TS fusion was regulated by interactions with molecular chaperones, antagonist binding would result (at least in some cases) in LBD folding and chaperone dissociation, leading to enhancement of TS activity. A consequence of this mechanistic model, where biologically relevant ligand effects are recognized on the basis of the conformations adopted by the LBD of the ERβ-TS fusion, is that SERMs and pure estrogen antagonists are simply classified as "antagonists" by our system. This has been observed both with this work (Figures 4 and 5) and in our previous work.[12]

Discovery of Novel Estrogen Receptor Modulators. To investigate the ability of the constructed biosensor to assist discovery of new ER modulators, a library of 20 structurally novel compounds was synthesized and screened for hormone-mimicking behavior in our system. We chose to synthesize mainly (*E*)-stilbenoids (Figure 6A) bearing hydroxy groups at ring positions identical (compounds **4a, 4c**) or similar (compound **4b**) to those of resveratrol, a plant-derived stilbenoid reportedly exhibiting mixed ER agonist/antagonist properties.[43] Studies on structure−activity relationships for ER binding suggest that the O−O distance between the two distant OH groups should be in the range of 9.7−12.3 Å to mimic the hydrogen bonding ability of the 3- and 17β-OH groups of E_2.[44] For the new stilbenoids we have estimated the O−O distances between pairs of distant OH groups in molecular mechanics (MM3) optimized geometries of these compounds to lie in the range of 9.3−12.5 Å. Several of the new stilbenoids were also endowed with bulky aliphatic (e.g., *tert*-butyl) substituents in the hope that they would behave as ERβ antagonists by a mechanism similar to that described for tetrahydrochrysene, an ERα agonist acting as an antagonist through ERβ (see Discussion).[45]

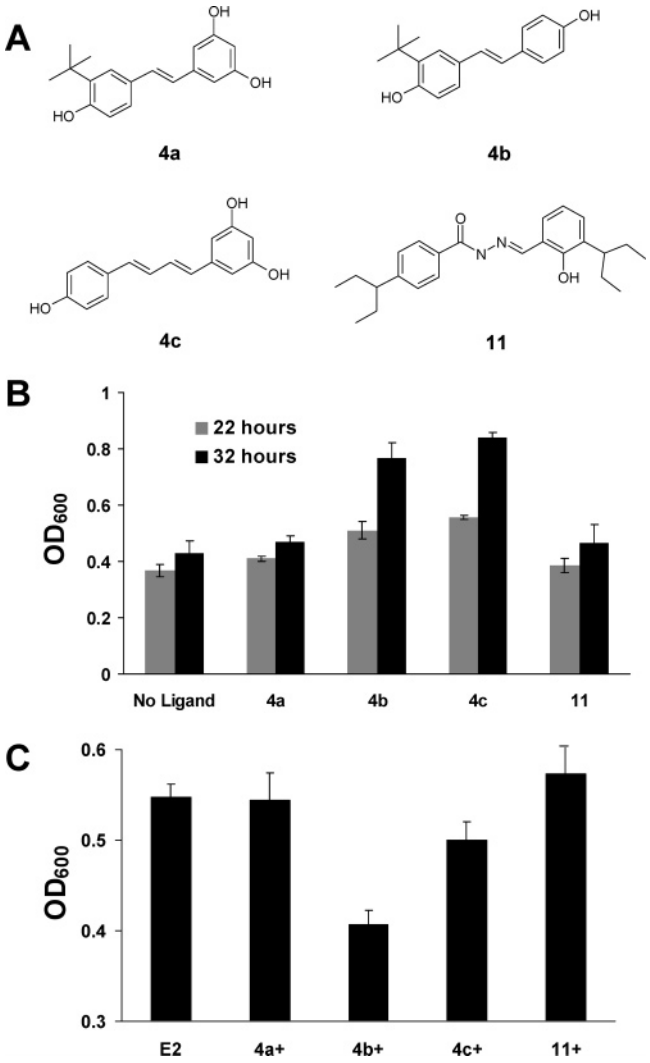

Figure 6. Discovery of novel compounds with the ability to bind to ERβ and differentially mediate ER signaling. (A) Chemical structures of some of the studied compounds. (B) Cells transfected with pMIT::ERβ* and grown in liquid −THY medium at 34 °C in the presence of 5 μM test ligands for 22 (gray) and 32 h (black). (C) Effects of the addition of a 5 μM concentration of some of the synthesized compounds on the growth phenotypes of cells carrying pMIT::ERβ* and incubated in liquid −THY medium containing 500 nM E_2 at 34 °C for 18 h. Experiments were carried out in triplicate, and the error bars represent one standard deviation from the mean value.

Incubation of our sensor strain in −THY medium with the new compounds revealed that most of them, such as the

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stilbenoid **4a** and the acylhydrazone derivative **11**, did not have a significant impact on cell growth (Figure 6B and data not shown). However, the stilbenoids **4b** and **4c** were able to enhance bacterial growth (Figure 6B), implying that they are ERβ binders and probably act as agonists as well. The fact that considerably longer incubation times were required before these ligands could be detected (compare Figures 2A,B and 3A with 6B) implies that **4b** and **4c** have much lower potencies than E_2 and other estrogenic ligands of even very low affinities/potencies. To look for potential ER antagonists within our chemical library, the impact of the new compounds on the growth phenotype of our sensor strain was also evaluated in E_2-containing −THY medium. Compounds **4c**, as well as **4a**, **11**, and all the other compounds that were inactive in the assay described in Figure 6B, did not exhibit a significant impact on cell growth (Figure 6C and data not shown). Most interestingly, however, the addition of **4b** partially suppressed growth (Figure 6C), indicative of ER antagonistic effects. Under conditions of negative TS selection in E_2-rich TTM medium, the addition of compound **4b** resulted in enhancement of bacterial growth (data not shown), thus demonstrating that the growth-suppressing ability of this compound in E_2-supplemented −THY medium does not occur due to general toxicity.

To validate the library screening data obtained with the bacterial sensor, we initially determined the relative binding affinities of the new compounds for human ERα (RBAα) and ERβ (RBAβ) using a fluorescence polarization assay described previously.[17,18] While **4a**−**c** could compete with the fluorescent estrogen ES2 for binding to ERα (Figure 7A) and ERβ (Figure 7B), **11** was unable to displace ES2 from either receptor. The RBAα values of **4a**−**c** were 0.29 ± 0.04, 0.59 ± 0.09, and 0.23 ± 0.03, respectively, whereas the corresponding RBAβ values were 0.17 ± 0.02, 1.94 ± 0.24, and 0.78 ± 0.10. This implies that **4b** and **4c** can fit in the binding cavity of ERβ better than **4a**. The fact that **11** does not appear to be an ER binder and that **4a** binds to ERβ with low affinity (600-fold lower than E_2 and 5−11-fold lower than **4b** and **4c**), provides a good explanation of why these compounds were not identified as "hits" by our bacterial sensor.

Next, we assessed the ability of the new compounds to induce ERE-dependent gene expression using HEK:ERβ cells, a clone of human embryonic kidney (HEK) 293 cells that was generated by stable transfection with both a human ERβ expression vector and a reporter plasmid carrying an ERE upstream of a minimal thymidine kinase promoter and the cDNA of luciferase. Reporter gene expression of HEK:ERβ cells is regulated by estrogens exclusively through the transfected ERβ, since parental HEK293 cells failed to respond to estrogens when transiently transfected with the reporter alone (data not shown). Treatment of HEK:ERβ cells with increasing concentrations of the stilbenoids **4a**−**c** induced luciferase expression in an ERβ-dependent manner, since induction was inhibited by the pure ER antagonist ICI 182,780 (Figure 7C and data not shown). Their potencies for a luciferase induction effect equal to 25% of that of E_2 ranked in the following order: **4b** $\approx$ **4c** > **4a**, in close agreement with the determined RBAβ values. Compound **11** was totally ineffective in mounting a luciferase response even at a 10 μM concentration. The induction efficacies of the stilbenoids at 10 μM differed from the maximal efficacy of E_2 significantly (*t*-test; $P < 0.05$) and in a manner consistent with weak (**4a**), partial (**4b**), or super agonism (**4c**) at this concentration (see Experimental Section for the classification of agonists and antagonists). However, at the stilbenoid concentration of 4 μM (where the bacterial sensor was challenged), **4a** was unable to induce luciferase expression through ERβ, whereas **4b** and **4c** exhibited partial and full agonism, respectively (Figure 7C and data not shown), in close agreement with the responses of our *E. coli* sensor. The induction efficacies of 0.1 nM E_2 alone (maximal-set equal to 100) or in combination with 10 μM stilbenoid or 10 nM ICI 182,780 (exhibits full antagonism under these conditions) were consistent with an inhibitory effect of **4b** equal to 78% of that of ICI 182,780 (Figure 7D). The stilbenoid **4b** was able to antagonize E_2 at 4 μM also (data not shown), in close agreement with the bacterial sensor.

The above effects were, to a large extent, reproduced by the stilbenoids using MCF-7:D5L cells, a clone of MCF-7 human breast cancer cells that has been generated by stably transfecting them with a reporter plasmid carrying an ERE upstream of a minimal globin promoter and the cDNA encoding for luciferase. The growth and gene expression of MCF-7:D5L cells are primarily regulated by estrogens through the endogenous ER (predominantly ERα), as it is the case also with the parental MCF-7 cells.[17,18] Treatment of these cells with the new compounds resulted in an ER-dependent induction of luciferase expression, as assessed using ICI 182,780 (Figure 7E and data not shown). The potencies for a luciferase induction effect equal to 25% of that of E_2 ranked in the following order: **4b** > **4a** $\approx$ **4c**, in close agreement with the determined RBAα values. While **4a**−**c** induced luciferase expression significantly at 10 μM, compound **11** was ineffective in this case also. The induction efficacies of the stilbenoids at 4 μM as compared to that of E_2 at $\geq$0.1 nM were consistent with weak (**4a**), partial (**4b**) and full agonism (**4c**). The induction efficacies of 0.1 nM E_2 alone or in combination with a stilbenoid or 10 nM ICI 182,780 were consistent with **4b** behaving as a partial antagonist at 4 (data not shown) and 10 μM (Figure 7F; the inhibitory effect of **4b** is 53% of that of ICI 182,780). Thus, while **4a** was largely inactive, **4c** behaved as an agonist and **4b** as a partial agonist/antagonist in MCF-7:D5L and HEK:ERβ cells alike.

Given that **4a** and **4b** have roughly similar backbone structures and molecular volumes, the ERβ selectivity of **4b** was somewhat unexpected. The different disposition relative to the backbone structure of the potentially H-bonding OH groups of **4a** and **4b** may allow the latter stilbene to assume a more favorable orientation for interacting with the amino acid side chains that line the ERβ binding cavity. This orientation may involve displacement of His475, which could render **4b** a more effective antagonist through ERβ than through ERα, as already described for tetrahydrochrysene.[45] Correct positioning of His475 (His524 in ERα) relative to helix 12 reportedly contributes to the stability of the agonist conformation of the receptor.[45] We recently reported on a similar possible displacement of this key His as probably accounting for the partial agonist properties of the isoflavone ebenosin.[18] Ebenosin [8-(1,1-dimethylallyl)formononetin] bears a bulky isoprenyl substituent vicinal to its 7-OH group. Docking calculations indicated that the isoprenyl moiety sterically hinders the interaction of 7-OH with Glu305 and Arg346 of ERβ, which in turn causes the 4′-methoxy group of ebenosin to engage His475 into a repulsive interaction.[18] Given that ERβ possesses a considerably smaller

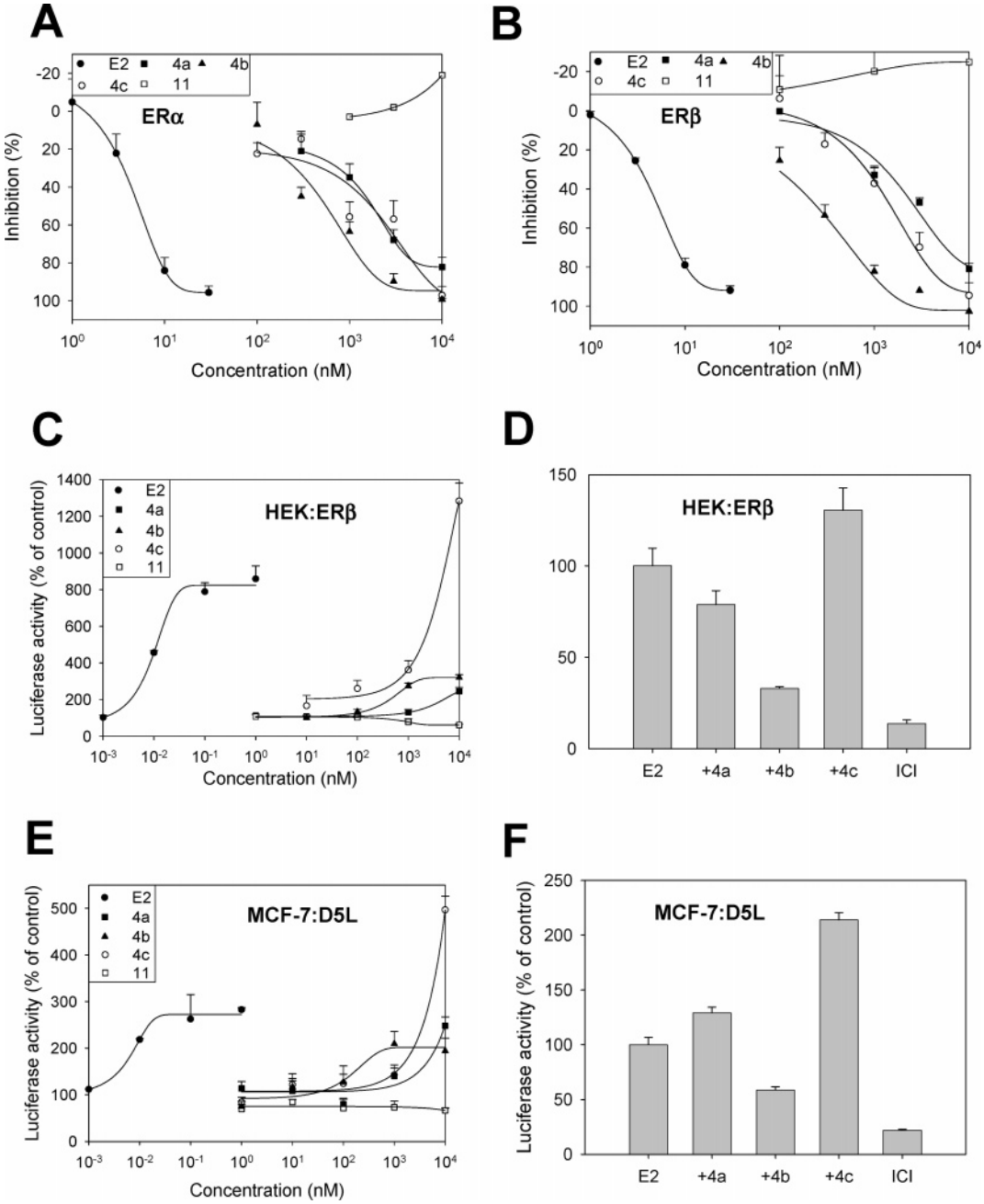

Figure 7. Demonstration of the ability of the bacterial sensor to reliably report on the signaling properties of compounds capable of binding to ER. (A) Dose−response curves of the displacement of the fluorescent estrogen ES2 from ERα by serial 1/3.3 dilutions of E_2 or the new compounds **4a**−**c** and **11**. (B) Dose−response curves of the displacement of the fluorescent estrogen ES2 from ERβ as in A. (C) Dose−response curves of the induction of luciferase expression in HEK:ERβ cells by serial 1/10 dilutions of E_2 and the new compounds. (D) Luciferase response of HEK:ERβ cells exposed to E_2 (0.1 nM) in the absence or presence (+) of **4a**, **4b**, or **4c** (10 μM), or the pure estrogen antagonist ICI 182,780 (10 nM). (E) Dose−response curves of the induction of luciferase expression in MCF-7:D5L cells by serial 1/10 dilutions of E_2 and the new compounds. (F) Luciferase response of MCF-7:D5L cells exposed to 0.1 nM E_2 (set equal to 100) in the absence or presence (+) of **4a**, **4b**, or **4c** (10 μM), or ICI 182,780 (10 nM). Dose−response experiments were carried out in triplicate, and the data are mean ± SEM of the three measurements. Data in the histograms are mean ± SEM of three independent experiments carried out in triplicate; ICI = ICI 182,780.

binding cavity than ERα,[46] the finding that **4b** is a less effective antagonist through ERα than through ERβ probably suggests that the repulsive interaction is weakened as the binding cavity becomes spacious enough to accommodate the bulky *tert*-butyl substituent of this stilbene.

The observation that **4b** and **4c** enhanced growth of our bacterial sensor strain in −THY medium (Figure 6B) is in accordance with their partial and full agonist behavior, respectively, in E_2−deprived mammalian cells (Figure 7C,E). Most interestingly, while **4c** failed to exhibit a significant impact on bacterial growth in E_2-containing −THY medium, **4b** suppressed growth under these conditions (Figure 6C), in full accordance with the finding that only the latter stilbenoid exhibited antagonist behavior in E_2−supplemented mammalian cells (Figure 7D,F). In spite of the low potencies and the pharmacological effects exhibited by the stilbenoids **4b** and **4c**, which most probably do not constitute them as potential hormone

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therapeutics, these results suggest that our *E. coli* system is a reliable sensor for the detection of ERβ modulators and that it can be used as a sensor of general estrogenicity as well. Our findings convincingly support the applicability of this simple system for screening large compound libraries in search of potentially useful ER modulators.

Discussion

This work describes a dramatically enhanced bacterial sensor for hormone binding that is appropriate for practical screening applications of potentially therapeutic compounds. An engineered fusion comprising the LBD of the human ERβ and the very sensitive reporter enzyme TS yielded an enzyme chimera with the ability to report ligand-induced conformational shifts of the receptor domain through changes in TS activity. Ligand-dependent enzymic activity could be readily detected by *E. coli* growth phenotypes in selective media. Due to the high sensitivity and reversibility of the TS genetic selection, this system is able to evaluate a wide repertoire of ligand-dependent effects. Demonstrated applications include the accurate detection of a large variety of known estrogen analogues, the discovery of structurally novel ones, and the ability to reliably report on important aspects of the pharmacological profile of a particular hormone mimic.

The binding of steroidal or nonsteroidal and of natural or synthetic estrogen agonists to the receptor domain enhanced the catalytic activity of the ERβ-TS chimera and promoted growth of our bacterial sensor strain, thus enabling rapid detection of agonist binding. We observed that the ligand-sensing capability of the ERβ-based fusion was dramatically enhanced compared to our prototype ERα-containing sensor and allowed the facile detection of nearly *every* estrogenic compound of the chemical library examined, including binders with very low affinities for either ER subtype. In addition, the sensitivity of this second-generation sensor for E_2 was also increased 100-fold compared to the prototype system.[12] We attribute these critical improvements to the inclusion of a more stable intein splicing domain and to the somewhat narrower binding pocket of ERβ compared to that of ERα,[46] which may be allowing for more fastidious ligand−receptor recognition. It is known that the "single-hormone receptors" such as ER, the thyroid hormone, and the glucocorticoid receptors possess smaller ligand-binding pockets than the "multiple-hormone receptors", e.g. the peroxisome proliferator-activated receptor.[47] This feature helps single-hormone receptors bind their cognate ligands with higher affinity and specificity and increases their sensitivity. We believe that in a nonnative environment such as the cytoplasm of *E. coli*, where the folding of these protein domains (natively found only in animals) is problematic, small variations in the stability of the chimeric fusion and in the molecular recognition efficiency can have a profound effect on the performance of the system.

Despite the large improvement in sensitivity, the potencies of E_2 and other high-affinity ER binders in our sensor (as measured by EC_{50}) remain 2−3 orders of magnitude lower than that of transcriptional activation assays in yeast[30,31] and mammalian cells.[9,17,28,31,32] This is potentially due to differences in transport and stability of the ligands in *E. coli* cultures, as well as differences in binding affinity for the ligands to the artificial sensor protein. However, it is also likely that this arises from the fact that a single tight binding event in our system leads to the activation of a single molecule of reporter protein, while in transcriptional activation systems a single transcriptional activation event (one mRNA molecule) can yield multiple copies of active reporter protein. Most interestingly, though, as the affinity of a particular hormone analogue for ER is decreased, the sensitivity of our system converges with that of the eukaryotic systems, and in some cases even surpasses it.[30,31] We attribute the latter, somewhat unanticipated, behavior to the nontranscriptional nature of this biosensor, where weak binding is presumably sufficient to slightly enhance the activity of the TS reporter proteins expressed in a given cell, even though most are unbound at any given moment. In a transcriptional system, however, weak binding may not be sufficient to elicit the more complex transcriptional initiation of the reporter gene, thus causing these compounds to be much more difficult to detect with these systems. Although the potencies of high-affinity "drug-like" estrogenic compounds in the bacterial screen do not quantitatively match those of mammalian transactivation assays, this system nonetheless holds great potential as a cheap, fast, and facile first-line detection system. Candidate compounds identified by this system, as with candidates identified by any initial screen, would be subjected to more sensitive and pharmacologically relevant mammalian assays and eventual animal studies before adoption as potential therapeutics. However, reliability in detecting all active compounds from an initial library is the primary desired characteristic of any screening system, and this system has demonstrated this capability for both known and unreported estrogenic compounds with highly varying activities. Further, the ability of this system to detect low-potency compounds is a great advantage, as many of these compounds are becoming toxicologically relevant, especially when metabolically stable and capable of accumulating in the body. In particular, persistent weakly active pollutant and environmental estrogens have been shown to have potential health impacts on human and animal populations.[48] Thus, there is a great need for highly sensitive systems to rapidly and cheaply evaluate thousands of commodity chemicals for potential estrogenic/antiestrogenic effects. Our system is particularly good at detecting low-affinity compounds of endocrine disrupting potential (unpublished results; see also Figure 2A, e.g., for bisphenol A).

The presence of estrogen antagonists did not enhance the catalytic activity of the ERβ-TS sensor, although these compounds are able to diffuse through the bacterial membrane and bind tightly to the recognition domain in the *E. coli* cytoplasm. Instead, antagonist binding was found to have an inactivating effect on the enzymic activity of the ERβ-TS fusion and was readily detectable under certain conditions (Figure 5B; TTM). This engineered enzyme can thus occupy three distinct states of catalytic efficiency: in the presence of an agonist it adopts a state of higher activity, in the absence of any ligand it provides intermediate activity, and in the presence of antagonists it exhibits lower TS efficiency. On the basis of this property, important features of the complicated phenomena that determine the pharmacological activity of a particular hormone analogue can be recognized by mere observation of bacterial growth.

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Nontranscriptional hormone-binding sensors based on artificial chimeric enzymes have great potential for the construction of assays with the ability to recognize pharmacologically critical properties. Previously constructed fusions of ERα with Flp recombinase expressed in yeast[49] or mammalian cells,[50] and of the glucocorticoid receptor with dihydrofolate reductase in yeast and mammalian cells[51] have been found capable of differentiating agonists from antagonists. These systems were the first to demonstrate that transcriptional processes may not be required for such purposes. The ability of our chimeric sensor to unravel biologically relevant activity in a prokaryotic environment, and issue a report by way of a trivial phenotypic assay, offers remarkable simplicity, speed, and potential for high-throughput applications. Some nontranscriptional in vitro screens, such as NHR microarrays of coactivator recruitment[28] and FRET-based sensors of receptor conformations,[27] can make these kinds of distinctions and are sufficiently simple for high-throughput screening formulations, but they lack the genetic tractability and potential for evolutionary approaches provided by bacterial selections.

We used our bacterial sensor to rapidly screen a small library of structurally novel compounds for potential estrogen-mimicking behavior and identified two new ERβ ligands (**4b** and **4c**). Compound **4c** was characterized as an estrogen agonist, while **4b** as an antagonist by our bacterial assay. The reliability of this sensor for the screening and detection of novel ER modulators was verified by confirming these results in ERβ- and ERα-mediated transcriptional activation assays in human cells. The stilbenoid **4b** contains a *tert*-butyl substituent that is considerably less bulky than the helix 12-interfering moieties of typical estrogen antagonists (e.g., tamoxifen and raloxifene). Nevertheless, **4b** was able to fully antagonize estrogen signaling via ERβ, and to a lesser extent through ERα. This was revealed by the full and partial antagonism exercised by 10 μM **4b** on E_2 induction of luciferase expression in HEK:ERβ and MCF-7:D5L cells, respectively. We believe that these antagonistic effects occur due to interference with the positioning of the helix 11 residue His475 of ERβ (and possibly to a lesser extent with His525 of ERα), which could displace helix 12 indirectly by a mechanism already described for tetrahydrochrysene.[45] Tetrahydrochrysene is perhaps the only known example of an "indirect" ERβ antagonist,[8] while no such antagonistic compounds have been reported for ERα yet.

Our group and others have argued previously that the hormone-regulated effects observed in (at least some) chimeric NHR fusion proteins reflect a mechanism involving intramolecular effects, where interactions with other proteins, such as molecular chaperones,[36] are not involved.[12,49−51] Specifically, we have proposed that the hormone-regulated catalytic activity of NHR-TS fusions occur due to the large conformational changes that occur at the receptor domain upon ligand binding.[12] These structural shifts, which primarily reflect the highly flexible, hormone-dependent repositioning of the C-terminal helix 12, but also involve modest shifts of the α-helices that confound the LBD, are presumably transduced allosterically through the intein hinge to the catalytic domain. The perturbed structure of the overall fusion most likely interferes with the ability of the intein to block homodimerization of the TS domain (required for its activity),[52] thus altering its enzymic efficiency. Evidence presented here provides further support for this model, where known structural differences in binding modes of the receptor are sensed by the catalytic domain, allowing for both the detection of hormone-like binding and the facile differentiation between agonistic and antagonistic effects.

The findings presented here strongly suggest that the ERβ-TS fusion functions as a new type of enzymic sensor for molecular conformations of ER. To the best of our knowledge, this is the first example of an engineered allosteric enzyme with the ability to be either activated or inactivated, depending on the pharmacological nature of a bound effector molecule. Lim and co-workers have engineered three-domain proteins with the ability to be activated by one ligand and inactivated by another, but these fusions do not exhibit catalytic activities themselves.[53] Other previously reported examples of chimeric enzyme switches have been able to sense a ligand-free "open" conformation and a ligand-bound "closed" form of the recognition domain and transduce a binary output signal ("on−off" or "higher−lower" activity of a reporter protein).[54] A particularly interesting case is the engineered MBP-β-lactamase fusion RG13, which recognizes two different closed conformations of MBP.[1] In this case, maltose and β-cyclodextrin binding to the MBP pocket are thought to induce different closure angles of the recognition domain that result in different levels of β-lactamase activation. The chimeric enzyme described here can sense and report on at least three distinct receptor domain conformations: an apo form, an agonist- and an antagonist-bound form, while the biocharacter of the effector molecule determines whether the activity of the reporter will be positively or negatively regulated. These results demonstrate further the potential of engineered chimeric proteins for sensing complex ligand-dependent functions and for the construction of regulatable enzymes with advanced ligand-switching behaviors.

On the basis of our proposed model for ligand sensing, we anticipate that the adopted protein engineering strategy will allow the construction of simple biosensors for a variety of ligand-binding target proteins, including those from other therapeutically relevant classes. Large conformational changes upon ligand binding may not be required for the construction of such chimeric sensors, as communication between artificially combined protein domains can be additionally enhanced through directed evolution techniques.[55] The highly sensitive and selectable TS phenotype makes this reporter ideal for these applications, while the genetic tractability of *E. coli* suggests additional combinatorial possibilities in the development of new drug candidates.

Conclusion

An optimized chimeric enzyme comprising the ERβ LBD, a stable intein splicing domain, and the TS reporter enzyme, allowed the construction of a dramatically enhanced estrogen sensor with the ability to report the presence of hormone-like

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compounds through changes in bacterial growth. This system is able to detect a wide repertoire of natural and synthetic estrogen analogues, and differentiate between their agonistic/antagonistic biocharacter in a very reliable manner. We used this simple sensor to screen a small library of structurally novel compounds and identified two new ER modulators, for which we were able to accurately predict their ability to function as estrogen agonists or antagonists in human cells.

Strong evidence was presented that our sensor protein functions as an allosteric enzyme, which senses pharmacologically relevant ligand-induced conformational changes in the receptor domain and translates them into distinct levels of enzymic efficiency. Due to the high sensitivity and the reversible selection capability of the TS genetic system, we found that our chimeric enzyme exhibits different catalytic efficiencies depending on the nature of the bound effector molecule: in the presence of an estrogen agonist it adopts a state of higher activity, in the absence of any ligand it provides intermediate activity, and in the presence of antagonists it exhibits lower TS efficiency. To our knowledge, this is the first example of an engineered chimeric enzyme that recognizes more than a ligand-bound and an unbound form of the recognition domain and demonstrates the potential of artificial protein chimeras for sensing complex ligand-dependent functions. Because the proposed mechanism of ligand dependence of our sensor is not specific to NHRs, we anticipate that the herein adopted protein engineering strategy will enable the construction of similar sensors for different classes of (therapeutic) ligand-binding proteins.

Experimental Section

Reagents. The estrogen analogues 17α-estradiol, 17β-estradiol, diethylstilbestrol, hexestrol, dienestrol, estriol, estrone, tamoxifen, 4-hydroxytamoxifen, raloxifene, tetrahydrochrysene ((*R*,*R*)-*cis*-diethyltetrahydro-2,8-chrysenediol), genistein, daidzein, kaempferol, coumestrol, phloretin, apigenin, naringenin, zearalenone, β-zearalanol, biochanin A, and the thyroid hormone 3,3′,5-triiodo-L-thyronine were purchased from Sigma. Clomiphene, progesterone, and bisphenol A (4,4′-isopropylidenediphenol) were obtained from ICN Biomedicals, while ICI 182,780, DPN, PPT, and ZK 164,015 were obtained from Tocris Cookson. The steroids testosterone, androstenediol (5-androsten-3β, 17β-diol), dehydroepiandrosterone (5-androsten-3β-ol-17-one), 3α-androstanediol (5α-androstan-3α,17β-diol), 3β-androstanediol (5α-androstan-3β,17β-diol), epiandrosterone (5α-androstan-3β-ol-17-one), and androsterone (5α-androstan-3α-ol-17-one) were purchased from Steraloids. GW 5638 was synthesized according to previously described procedures.[56] All hormone analogues were dissolved in ethanol to form 10 mM stock solutions, apart from progesterone and PPT which were prepared as 1 mg/mL and 5 mM solutions in ethanol, respectively. Daidzein was prepared as a 50 mM solution in 1:1 (v/v) ethanol: dimethylsulfoxide (DMSO), apigenin as a 5 mM solution in 3:1 (v/v) ethanol:DMSO, androstenediol as a 5 mM solution in 6:1 (v/v) ethanol: DMSO and tetrahydrochrysene as a 5 mM solution in DMSO.

Plasmids. The construction of the plasmid pMIT::ERβ* has been reported in previous work.[14] The expression vector pCDNA3.1-hERβ encoding hERβ was constructed by subcloning the BamHI fragment from the plasmid pSG5-hERβ into the BamHI site of pCDNA3.1/*myc*-HisB. Plasmid pCDNA3.1/*myc*-HisB containing the neomycin resistance gene is from Invitrogen. Plasmids pERE-tk-Luc, pSG5-hERα, and pSG5-hERβ have been described in previous work.[57]

Bacterial and Human Cell Lines. *E. coli* XL1-Blue cells (Stratagene) were used for plasmid constructions and the *E. coli* strain D1210Δ*thyA*::Kan^R [F$^-$Δ(*gpt-proA*)62 *leuB*6 *supE*44 *ara*-14 *galK*2 *lacY*1 Δ(*mcrC-mrr*) *rpsL2*0 (*Str*^{r}) *xyl*-5 *mtl*-1 *recA*13 *lacI*^{q}] was used for the determination of growth phenotypes.[13]

MCF-7:D5L cells were maintained as already described.[17,18] HEK-293 cells (ATCC) were maintained in Dulbecco's MEM (DMEM) medium (Sigma) supplemented with 10% fetal bovine serum (FBS). HEK:ERβ cells are HEK-293 cells stably transfected with the calcium phosphate co-precipitation method using 5 μg of pCDNA3.1-hERβ expression plasmid together with 20 μg of pERE-tk-Luc reporter plasmid. 18 h after transfection cells were washed with phosphate-buffered saline (PBS), fed with fresh medium, and 24 h later re-fed with medium containing 0.8 mg/mL geneticin. Cells were fed with fresh geneticin-containing medium every 2−3 days, and colonies were isolated 3 weeks later and tested for luciferase activity in the presence or the absence of 1 nM E_2. MCF-7:D5L and HEK:ERβ cells were cultured and subcultured as recommended by the supplier (ATCC) for the respective parental cells.

Bacterial Growth Phenotypes. Cells derived from three individual bacterial colonies were grown for approximately 12 h in ampicillin-containing LB medium and supplemented with 50 μg/mL thymine. These cultures were used with a 1:200 dilution to inoculate 5 mL of defined selective media[15] with 200 μg/mL ampicillin, and the specified concentrations of each of the hormone analogues in triplicate. Thymine-rich media contained at least 50 μg/mL thymine, and, in TTM media, trimethoprim was added to a 10 μg/mL concentration. In all bacterial growth experiments the concentration of organic solvents was kept below 0.2%. Levels of *E. coli* growth were measured as OD_{600} on a GENESYS 2 spectrophotometer.

Western Blotting. *E. coli* D1210 Δ*thyA* transfected with pMIT::ERβ* were grown in thymine-supplemented LB medium at 37 °C to early stationary phase ($OD_{600} \sim 1.0$) in the presence of the specified concentrations of estrogen analogues. Cells from 1 mL of culture were harvested by centrifugation and resuspended in 100 μL of lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0). Following boiling lysis, the total protein content of 2.5 μL of cell extract (corresponding to an equal number of cells as judged by OD_{600} measurements) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly(vinylidene fluoride) (PVDF) membranes. Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at 4 °C overnight. After washing with TBST, membranes were sequentially incubated with various dilutions of the following antibodies in TBST + 0.5% milk: mouse anti-maltose binding protein (Sigma), rabbit anti-GroEL (Sigma), peroxidase-conjugated anti-rabbit IgG (Sigma), and peroxidase-conjugated anti-mouse IgG (Biorad) at room temperature for approximately 1 h. After washing with TBST again, the probed proteins were visualized on X-ray film with SuperSignal West Pico chemiluminescent substrate (Pierce).

Induction of Luciferase Expression. Stilbenoid induction of ERE-dependent luciferase gene expression was assessed using HEK:ERβ as well as MCF-7:D5L cells as previously described.[17,18] Briefly, cells cultured and subcultured as reported above, were plated in flat-bottomed 96-well microplates at a density of 10 000 cells/well in phenol-red-free DMEM supplemented with 5% dextran coated charcoal (DCC)-treated FBS (DCC−FBS),[17,18] and 72 h later the cells were treated with the test compounds for 16 h. Following treatment, luciferase activity was assayed using the commercial Steady-Glo Luciferase Assay System (Promega). The number of viable cells was also determined using

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similarly treated parallel microcultures for measuring the conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) to colored formazan[17,18] as a means to normalize luciferase activity values. Full agonist (E_2 at ≥ 0.1 nM) and nonagonist (vehicle only) controls served to classify ER modulators as super, full, partial, weak and marginal agonists depending on whether their luciferase induction efficacy was significantly >100, 76−100, 26−75, 10−25, and 1−10% of the efficacy of E_2. Similarly, full suppression of the luciferase induction effect of 0.1 nM E_2 by ICI 182, 780 (at ≥ 10 nM) and nonsuppression (vehicle only) controls served to classify ER modulators as full, partial, weak, and marginal antagonists depending on whether their suppression of the effect of E_2 was 76−100, 26−75, 10−25, and 1−10% of the efficacy of ICI 182, 780. The significance of the difference in luciferase activity between control and stilbenoid-treated cells was determined using Student's *t*-test.

Binding to Isolated Recombinant Human ERα and ERβ. The RBA values were assessed as previously described.[17,18] Briefly, the concentrations of the stilbenoids that inhibited binding of 1 nM ES2 (a fluorescein-labeled estrogen from Invitrogen) to the isolated human ERα or ERβ (Invitrogen) by 50% (IC_{50}), was assessed using a Beacon 2000 Fluorescence Polarization Reader (Invitrogen). The RBAα and RBAβ values (mean ± SEM of at least three independent experiments) were calculated by [(IC_{50} estradiol/IC_{50} derivative) × 100]. IC_{50} values of E_2 for ERα and ERβ were 3.59 ± 0.12 and 4.13 ± 0.44, respectively. The RBAα and RBAβ values of E_2 were set equal to 100.

Statistics. Data were analyzed using the SPSS 10.0 statistical package for Windows and compared using an independent samples *t*-test for comparison of the means of at least three independent experiments each involving triplicate or more measurements. Differences were considered statistically significant for values of $P < 0.05$.

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Supporting Information Available: Detailed experimental procedures for the synthesis of compounds **4a**−**c** and **11** and analytical data for the final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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