Published in final edited form as:

Bioconjug Chem. 2010 October 20; 21(10): 1880–1889. doi:10.1021/bc100266v.

Probing the Topological Tolerance of Multimeric Protein Interactions: Evaluation of an Estrogen/Synthetic Ligand for FK506 Binding Protein Conjugate

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Abstract

Bivalent small molecules composed of a targeting element and an element that recruits endogenous proteins have been shown to block protein-protein interactions in some systems. We have attempted to apply such an approach to disrupt the interaction of the estrogen receptor α with either its associated coactivators or with its dimerization partner (i.e., another estrogen receptor). We show here that a conjugate capable of simultaneously binding both the estrogen receptor and a recruited protein (FK506 Binding Protein 12 kDa) is, however, incapable of disrupting the multimeric estrogen receptor dimer/coactivator complex both *in vitro* and in cell-based reporter gene assays. We postulate why it may not be possible to disrupt this particular protein-protein complex—as well as other systems having high topological tolerance—with such bivalent inhibitors.

INTRODUCTION

The estrogen receptor α (ER α) is a ligand-activated transcription factor whose activity in regulating gene expression relies on two key molecular interactions: homodimerization of the ER itself and interaction of the ER dimer with its associated coactivators (e.g., the steroid receptor coactivators (SRCs)). The monomers of ER α are linked as homodimers by hydrophobic interactions among residues on two long α -helices arranged in parallel at the dimer interface, flanked across the dimer interface by both hydrophobic and polar interactions between other helical elements. The binding affinity of ER α dimerization has been estimated to be subnanomolar (1). In the interaction of ER with the coactivator, one molecule of an SRC binds to an ER dimer by placing two turns of an amphipathic α -helix into a hydrophobic groove on the surface of the ligand-binding domain of each monomer of the agonist-bound ER dimer. These α -helical elements contain three conserved leucine residues arranged in an LXXLL motif (L is leucine; X is typically a polar amino acid). The affinity of each LXXLL motif for ER α is characterized by a K_D of ca. 700 nM, with the bivalent interaction once estimated to have an avidity below 30 nM (2) but more recently found to be ca. 1 nM, with affinity being dependent to some extent on ligand structure (M. Jeyakumar and J. A. Katzenellenbogen, unpublished). ER α interaction with SRC has strict ligand dependence; it requires that the ERs be occupied by an agonist ligand, and it is

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Supporting Information Available: Synthesis of the long oligoethyleneglycol tether is described in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

disabled when the ER is either unliganded or liganded, in most circumstances, by an antagonist ligand. By contrast, ER α dimers form *in vitro* whether the ER is unliganded or liganded with either an agonist or antagonist ligand, although ligand binding modulates dimerization affinity to some degree (1).

We and others have postulated that these two protein-protein interaction “hot-spots”—the ER/ER dimer interface or the ER/SRC interface—could serve as therapeutic targets for cancers in which the estrogen receptor is upregulated, but that are non-responsive to traditional antagonist regimens, as is the case in antiestrogen-resistant breast cancer (3–6). In fact, it is the ER/SRC interaction that is targeted in conventional ER antagonism with antiestrogens, although this inhibition proceeds through an allosteric mechanism whereby antagonist binding in the internal ligand binding pocket induces a conformational change on the receptor surface that prevents SRC binding (7). Thus, targeting this interaction with small molecule inhibitors is well-validated, although those that act by a direct mechanism of inhibition are much less developed (3,8,9). Oftentimes, however, protein-protein interactions of this type are viewed as intractable targets in drug discovery, because the interactions typically occur over large surface areas, as is the case with the ER dimer interface, or may be highly dynamic, as is the case with the ER/SRC interaction (10,11). Because small molecules are, by definition, low molecular weight-compounds, they may lack sufficient steric bulk to inhibit the interaction.

Gestwicki et al. (12) have elaborated an interesting “Trojan Horse” (or heterobivalent ligand) approach to this general problem: It involves tethering a weak protein-protein interaction inhibitor to a second ligand molecule that, after gaining access to the cell, would recruit extra steric bulk in the form of a ubiquitous, endogenous protein, thereby increasing the effective size and, consequently, the potency and/or efficacy of the inhibitor (13). The group demonstrated this concept by disrupting the aggregation of the A β peptide that leads to formation of β -amyloid, a polymer implicated in the pathologic fibrillogenesis of Alzheimer’s disease. They tethered Congo Red, which, in turn, binds rather poorly to β -amyloid (i.e., IC₅₀ = 2 μ M), to SLF (Synthetic Ligand for FK-506 binding proteins [FKBPs]). In the presence of the ubiquitous and abundant cellular protein FKBP12, some of these conjugates (e.g., I, Chart 1) inhibited the aggregation of β -amyloid with IC₅₀ values of 50 nM, a 40-fold increase in potency compared to Congo Red. The effect was not seen in the absence of FKBP12, suggesting that the mechanism of inhibition was dependent on the steric hindrance of FKBP12 that followed from its recruitment by the SLF element in the Congo Red conjugate. This finding was a landmark because it suggested a perhaps generalizable mechanism for inhibiting protein-protein interactions.

We saw an opportunity to use this technology in a conceptually similar yet mechanistically distinct manner to develop a ligand that would have context-dependent estrogenic properties—that is, a molecule that would allow ER to recruit its dimerization partner and coactivator, and thus function as an *agonist, in the absence of FKBP*, but that would function as an *antagonist in the presence of FKBP*. In addition, this novel approach to inhibition of estrogen signaling might prove more robust against the development of acquired resistance which typically develops following endocrine therapy of breast cancer with standard antagonists such as tamoxifen (14).

The native agonist for the estrogen receptor, 17 β -estradiol (E2), is known to tolerate substitution at the 17 α position without experiencing substantial loss in affinity, while retaining agonist activity for the ER (15,16). Thus, we examined whether an estradiol-SLF conjugate linked through the 17 α position would by itself function as an ER agonist, engendering formation of the ER/SRC assembly, but, upon recruitment of FKBP, would place ER in an antagonized state resulting from FKBP disruption of the multimeric ER/SRC

assembly. Curiously, what we have found is that the ER, when liganded with an estradiol-SLF conjugate, was capable of binding not only its dimerization partner and a coactivator fragment, but also was able to recruit FKBP, thus indicating that this multimeric protein complex exhibits a high topological tolerance. We describe the design, synthesis and extensive biological evaluation of these compounds, and hypothesize why inhibition of this interaction might not be amenable to this Trojan horse-type strategy.

EXPERIMENTAL PROCEDURES

General Considerations

Reagents were obtained from Aldrich Chemical Company, TCI America, or Fisher Scientific and were used without further purification. Anhydrous solvents were purchased from Aldrich or obtained from a Solvent Dispensing System fabricated by J.C. Meyer, based on a design published by Pangborn et al. (17). Anhydrous DMF was vacuum-distilled over molecular sieves.

Reaction progress was monitored using thin-layer chromatography (TLC) on silica Gel 60 F₂₅₄ glass backed plates from EM Science. Visualization was achieved by UV light (254 nm) or phosphomolybdic acid or potassium permanganate indicator. Flash column chromatography was performed with Woelm silica gel (0.040–0.063 mm) packing (18).

¹H and ¹³C NMR spectra were recorded on either 400 or 500 MHz Varian FT-NMR spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) by reference to proton resonances resulting from incomplete deuteration of the NMR solvent. High and low resolution electrospray ionization mass spectra were obtained on either a Micromass Quattro or Micromass Q-ToF Ultima mass spectrometer. Melting points were measured using a Thomas Hoover capillary melting point apparatus and are uncorrected.

Chemical Synthesis

SLF-OH was synthesized according to a known procedure (19,20). The ethynylestradiol precursor was prepared (Scheme 1) by reacting 17α-ethynylestradiol with methyl 4-iodobenzoate using Sonagashira reaction conditions, to give, after saponification of the ester, an acid-functionalized estrogen. The acid was converted to the succinimid-1-yl ester **1** using dicyclohexylcarbodiimide as the dehydrating agent.

The estrogen prepared above was tethered to the SLF moiety by adding a mono-protected diamine to give, after deprotection of the remaining amine, amides **2** and **3**. The amines were coupled to the acid functionality of **SLF-OH**, to give the conjugates **4** and **5**, by activating the acid as either the acyl chloride or an activated ester.

Succinimid-1-yl 3-(3,14-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[A]phenanthren-17-ylethynyl)-benzoate (**1**)

17α-Ethynylestradiol (1.00 g, 1.0 eq) was dissolved in anhydrous THF (10 mL). To this solution was sequentially added CuI (96 mg, 0.15 eq), piperidine (0.842 mL, 2.0 eq), methyl *p*-iodobenzoate (883 mg, 1.0 eq), and PdCl₂(PPh₃)₂ (236 mg, 0.10 eq). The solution was allowed to stir at RT for 12 h, and was then heated to reflux for an additional 4 h. The crude reaction mixture was filtered through celite, and then purified using silica gel column chromatography (20% EtOAc/hexanes to 30% EtOAc/hexanes) to give a dark solid, which was recrystallized from boiling benzene to give 1.40 g (96% yield) of white flakes. ¹H NMR (400 MHz, CDCl₃) δ: 8.00 (d, *J*=8.1 Hz, 2 H), 7.50 (d, *J*=8.1 Hz, 2 H), 7.18 (d, *J*=8.6 Hz, 1 H), 6.67 (d, *J*=8.6 Hz, 1 H), 6.57 (s, 1 H), 4.78 (s, 1H), 3.93 (s, 3H), 2.93–2.79 (m, 2 H),

2.51–2.32 (m, 2 H), 2.30–1.69 (m, 8 H), 1.63 (s, 1 H), 1.58–1.30 (m, 5 H), 0.95 (s, 3 H); HRMS calcd for $C_{28}H_{30}O_4Na$, 453.2042; found, 453.2037.

The methyl ester (1.22 g, 1.0 eq) prepared above was dissolved in 25 mL methanol and cooled to 0 °C. LiOH (5 mL of 2.1 M; 3.6 eq) was added, and the solution was allowed to come to RT and was stirred for 72 h. The methanol was removed under reduced pressure, and 10 mL more water was added. HCl (1 M) was added dropwise until no more solid precipitated from solution. The white solid (1.04 g, 88% yield) was filtered and dried. 1H NMR (400 MHz, $CDCl_3$) δ : 8.04 (d, $J=8.1$ Hz, 2 H), 7.52 (d, $J=8.1$ Hz, 2 H), 7.16 (d, $J=8.6$ Hz, 1 H), 6.65 (d, $J=8.6$ Hz, 1 H), 6.58 (s, 1 H), 2.86–2.79 (m, 2 H), 2.49–2.30 (m, 2 H), 2.29–1.68 (m, 8 H), 1.59–1.31 (m, 5 H), 0.95 (s, 3 H); HRMS calcd for $C_{27}H_{28}O_4Na$, 439.1885; found, 439.1887.

The acid prepared above (1.00 g, 1.0 eq), *N*-hydroxysuccinimide (1.38 g, 5.0 eq), dicyclohexylcarbodiimide (0.90 g, 1.8 eq) and 4-(*N,N*-dimethylamino)pyridine (50 mg) were dissolved in CH_2Cl_2 and were stirred at RT. After 4 h, the solution was filtered, and the filtrate was evaporated. After dissolving the crude material in EtOAc (50 mL) and filtering again, the organic layer was washed with water (3 $\times$ 50 mL), dried ($MgSO_4$), filtered and concentrated to give 1.23 g (99% yield) of a viscous, colorless liquid that was used without further purification. 1H NMR (400 MHz, $CDCl_3$) δ : 8.05 (d, $J=8.06$ Hz, 2 H), 7.54 (d, $J=8.06$ Hz, 2 H), 7.13 (d, $J=8.55$ Hz, 1 H), 6.62 (dd, $J=8.55$, 2.69 Hz, 1 H), 6.55 (d, $J=2.69$ Hz, 1 H), 6.31 (t, $J=6.23$ Hz, 1 H), 5.76 (br. s., 1 H), 2.98–2.70 (m, 6 H), 2.48–2.31 (m, 3 H), 2.27–1.63 (m, 8 H), 1.54–1.31 (m, 2 H), 0.94 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ : 169.30, 169.17, 161.33, 153.63, 138.04, 131.99, 131.85, 130.35, 129.81, 126.42, 124.15, 115.20, 112.69, 97.50, 84.88, 80.38, 49.87, 47.73, 43.55, 39.41, 38.97, 37.30, 33.79, 33.08, 29.57, 27.17, 26.38, 25.62, 25.52, 25.40, 24.82, 22.91, 12.86; HRMS calcd for $C_{31}H_{31}NO_6Na$, 536.2049; found, 536.2047.

EE2-Amine 2

The previously prepared NHS ester **1** (200 mg, 1.0 eq) was dissolved in dry THF (0.3 mL), and diisopropylethylamine (90 μ L, 1.3 eq) was added to the solution, followed by *N*-Boc-ethylenediamine (75 mg, 1.2 eq) in 0.5 mL dry THF. The solution was stirred at RT for 13 h, at which point another equivalent of *N*-Boc-ethylenediamine was added, because the reaction was not finished. The reaction appeared complete within 10 min after the addition of the second molar equivalent of amine. The THF was evaporated, and the gummy residue left was extracted from water (25 mL) with ethyl acetate (3 $\times$ 25 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated; HRMS calcd for $C_{34}H_{43}N_2O_5$, 559.3172; found, 559.3176. The product was dissolved in ether, and then precipitated with hexanes and filtered. Then, 1 mL 4M HCl in dioxane was added, and the product was allowed to stand at RT for one h. The solvents were evaporated, to give 44 mg (20% yield) of a white solid that was used without further purification. 1H NMR (500 MHz, CD_3OD) δ : 7.80 (d, $J=8.3$ Hz, 2 H), 7.50 (d, $J=8.3$ Hz, 2 H), 7.08 (d, $J=8.5$ Hz, 1 H), 6.54 (dd, $J=8.2$, 2.1 Hz, 1 H), 6.47 (d, $J=1.5$ Hz, 1 H), 3.44 (t, $J=6.3$ Hz, 2 H), 2.83 (t, $J=6.2$ Hz, 2 H), 2.80 – 2.72 (m, 2 H), 2.39 – 2.31 (m, 2 H), 2.19 – 1.66 (m, 8 H), 1.50 – 1.26 (m, 4 H), 0.91 (s, 3 H); ^{13}C NMR (125 MHz, CD_3OD) δ : 169.83, 156.13, 138.91, 135.05, 132.61, 132.50, 128.59, 128.12, 127.42, 116.21, 113.92, 97.19, 85.77, 81.00, 68.25, 51.37, 49.03, 45.31, 43.75, 42.11, 41.29, 40.06, 34.89, 34.58, 30.87, 28.78, 27.91, 26.88, 26.20, 24.01, 13.67; HRMS calcd for $C_{29}H_{35}N_2O_3$, 459.2648; found, 459.2647.

EE2-SLF Conjugate 4

SLF-OH (11.5 mg, 1.0 eq) was converted to the acid chloride by dissolving in dry dichloromethane (0.2 mL), cooling to 0 °C, and adding thionyl chloride (3.5 mg, 1.3 eq).

After 5 min, the solvents were evaporated, and 0.2 mL more dry dichloromethane was added, the solution was cooled to 0 °C, and thionyl chloride (3.5 mg, 1.3 eq) was again added. The reaction was allowed to come to RT and then to sit for 10 h, at which point the solvents were evaporated. Then, a solution of amine **2** (11 mg, 1.2 eq) and diisopropylethylamine (10.7 μ L, 3.0 eq) in 0.3 mL dry DMF was added to the acid chloride, and the solution was stirred for 5.5 h at RT. The reaction mixture was then applied to a preparative thin-layer chromatography plate and eluted (CH₂Cl₂:methanol 90:10), and the topmost band was scraped from the plate. The product was extracted from the silica gel using dichloromethane/methanol (90:10) to give 4.5 mg (22% yield) of a white solid. ¹H NMR (500 MHz, CDCl₃) δ : 7.68 (d, *J*=8.4 Hz, 2 H), 7.46 (d, *J*=8.4 Hz, 2 H), 7.42 – 7.19 (m, 3 H), 7.15 (d, *J*=8.6 Hz, 1 H), 6.95 – 6.60 (m, 4 H), 6.57 (d, *J*=2.4 Hz, 1 H), 5.75 (dd, *J*=8.0, 5.5 Hz, 1 H), 5.26 (d, *J*=5.6 Hz, 1 H), 4.51 (d, *J*=4.9 Hz, 2 H), 4.12 (d, *J*=8.8 Hz, 1 H), 3.84 (s, 3 H), 3.84 (s, 3 H), 3.67 – 3.58 (m, 4 H), 3.35 (d, *J*=12.0 Hz, 1 H), 3.18 – 3.12 (m, 1 H), 2.85 – 2.78 (m, 2 H), 2.63 – 2.48 (m, 2 H), 2.46 – 2.30 (m, 3 H), 2.14 – 1.23 (m, 24 H), 1.20 (s, 3 H), 1.19 (s, 3 H), 0.95 (s, 3 H), 0.87 (t, *J*=7.4 Hz, 3 H); LR-ESI-MS: [M + H]⁺ = 1024.5.

EE2 Amine 3

A prepared amine (32 mg, 1.3 eq; see supporting information) was dissolved in 0.2 mL dry THF, and diisopropylethylamine (12 μ L, 1.4 eq) was added to this solution. Then NHS ester **1** (23 mg, 1.0 eq) was added, and the solution was stirred at RT for 6 h. The reaction mixture was then applied to a preparative thin-layer chromatography plate and eluted (90:10 acetonitrile:methanol). The topmost band was scraped from the plate, and the silica gel was extracted with acetonitrile/methanol (90:10) to give 20 mg (46% yield; HRMS calcd for C₅₁H₇₆N₃O₁₃, 938.5378; found, 938.5367) of product, which was then subjected to 0.4 mL 4M HCl in dioxane (6 h), which, after evaporation of the solvents, gave 15 mg (83% yield) of amine **3**. ¹H NMR (500 MHz, CD₃OD) δ : 7.79 (d, *J*=7.3 Hz, 2 H), 7.09 (d, *J*=8.6 Hz, 1 H), 6.53 (d, *J*=6.6 Hz, 1 H), 6.46 (d, *J*=1.9 Hz, 1 H), 3.84 – 3.32 (m, 30 H), 3.12 (br. s., 2 H), 2.76 (br. s., 2 H), 2.54 – 2.28 (m, 4 H), 2.22 – 1.71 (m, 8 H), 1.53 – 1.18 (m, 7 H), 0.92 (s, 3 H); LR-ESI-MS: [M + H]⁺ = 838.6.

EE2-SLF Conjugate 5

SLF-OH (17 mg, 1.0 eq), *N*-hydroxysuccinimide (7 mg, 2.0 eq), and dicyclohexylcarbodiimide (8 mg, 1.2 eq) were dissolved in 0.5 mL dichloromethane and were stirred together for 6 h. The product was then passed through a short plug (ca. 1 inch) of silica gel using 100% ethyl acetate as the eluant to give, after evaporation of solvents, 16 mg (80% yield) of a solid which was used immediately in the next step. The previously formed amine **3** (15 mg, 1.0 eq) and the previously formed NHS ester **1** were dissolved in 0.2 mL dichloromethane and diisopropylethylamine (16 μ L, 3.5 eq) was added to the solution. The mixture was stirred for 20 h, and the reaction mixture was transferred to a preparative thin-layer chromatography plate using acetonitrile/methanol (90:10) as eluant. The topmost band was scraped off of the plate, and the compound was extracted from the silica gel with acetonitrile/methanol (90:10) to give, after evaporation of the solvents, 5 mg (21% yield) of the conjugate **5**. ¹H NMR (500 MHz, CDCl₃) δ : 7.78 (d, *J*=8.5 Hz, 2 H), 7.49 (d, *J*=8.5 Hz, 2 H), 7.35 – 7.28 (m, 2 H), 7.20 – 6.54 (m, 8 H), 5.83 – 5.73 (m, 1 H), 5.36 – 5.28 (m, 1 H), 4.51 (s, 2 H), 4.35 – 4.22 (m, 1 H), 3.93 – 3.83 (m, 8 H), 3.76 – 3.34 (m, 32 H), 3.23 – 3.14 (m, 1 H), 2.97 – 1.29 (m, 31 H), 1.23 (s, 3 H), 1.21 (s, 3 H), 1.19 – 1.06 (m, 2 H), 0.94 (s, 3 H), 0.89 (t, *J*=7.4 Hz, 3 H); HR-ESI-MS: calcd for C₇₈H₁₀₇N₄O₁₉, 1403.7530; found, 1403.7504.

Estrogen Receptor Ligand-Binding Domain Expression and Labeling

N-terminally His-tagged constructs in pET15b plasmids for ER α -417 and SRC3 were prepared as previously described. The ligand binding domain of ER α -417 (amino acids 304–554; C381,530S) with a single reactive cysteine at C417 or the nuclear receptor domain of SRC3 encompassing three nuclear receptor (NR) boxes (amino acids 627–829) were transformed into *E. coli* BL21(DE3)pLysS, grown at 37 °C to OD₆₀₀ ~0.5, induced with 1 mM IPTG, and grown for 4 h at 28 °C, as previously reported (21).

For protein isolation, a cell pellet was suspended in 5 mL buffer (50 mM Tris buffer, pH 7.5, 10% glycerol, 0.1 mM TCEP) per gram and sonicated (Vibra cell sonicator with a micro probe; Sonic Materials, Inc., Danbury, CT) for 10 s at 60% power. After centrifugation for 30 min at 30000 g, the supernatant was purified to near-homogeneity by batchwise adsorption onto a nickel-charged nitrilotriacetic acid-agarose resin (Ni-NTA-agarose; Qiagen Inc, Santa Clarita, CA), following standard protocols (1,21).

Site-specific ER labeling was accomplished using 30 equivalents of a thiol-reactive biotin derivative (MAL-dPEG4-biotin, Quanta BioDesign) while His₆-tagged ER α -417 LBD was immobilized on the Ni-NTA resin. The SRC3-NRD was likewise labeled using the recommended equivalents of a thiol-reactive Cy5 derivative (CyTM5 Maleimide Mono-Reactive Dye Pack, Amersham Biosciences). Labeling reactions were incubated overnight at 4 °C in Tris-glycerol buffer (50 mM Tris pH 7.0, 10% glycerol, 0.1 mM tris(2-carboxyethyl)phosphine, TCEP). Excess fluorophore was removed by washing the protein-bound resin with wash buffer (50 mM Tris buffer, pH 7.5, 10% glycerol, and 10 mM mercaptoethanol) before eluting the labeled receptor using a solution of 100 mM EDTA, 0.5 M NaCl, and 20 mM Tris pH 8.0 (1,21).

FKBP12 Expression and Labeling

A stab culture of *E. coli* that had been transformed with the pGEX-2T plasmid for GST-FKBP12 was obtained from the laboratory of Dr. Jie Chen at the University of Illinois at Urbana-Champaign. A 5-mL culture on a standard LB growth media supplemented with 100 μ g/mL ampicillin was started by inoculating single *E. coli* BL21 colony, and agitated for approximately 16 h at 37 °C. The bacteria were diluted (1:100) into standard LB medium and grown at 37 °C until OD_{600 nm} ~0.7. Protein expression was induced with 0.1 mM IPTG. After 4 h of incubation at 37 °C, all cells were harvested by centrifugation at 5000 $\times$ g for 15 min at 4 °C. The pellet was resuspended in 5 mL 1 $\times$ PBS buffer (pH 7.4) and 5 mM DTT. After addition of a 10 mg lysozyme (Sigma) per 1 g of bacteria, the resuspension was sonicated ten times for 2 sec on ice. The fusion protein in the clear supernatant was batch purified using a 1-mL Glutathione Sepharose 4B (Amersham Biosciences), equilibrated in 5 mL ice-cold binding buffer (1 $\times$ PBS, pH 7.3) containing 5 mM DTT. The protein was allowed to bind for 4 h at 4 °C before elution with 300 μ L 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione and 5 mM DTT. The presence and purity of eluted GST-FKBP12 was evaluated by SDS-PAGE (22).

To produce fluorescein-labeled FKBP12, the protein was labeled non-specifically with iodoacetamide-fluorescein (Invitrogen) while on the glutathione resin using the same protocol for labeling ER and SRC proteins as described above.

FRET Assay of Estradiol-SLF Binding to ER by Recruitment of Fluorescein-SRC3

Purified biotin-ER α -417 and fluorescein-SRC3-NRD were used in time-resolved FRET assays previously described (23) and adapted for the ER/FKBP system. A stock solution (5 μ L) of ER α -417 (8 nM) and LanthaScreenTM Streptavidin-Terbium(Invitrogen) (2 nM) in TR-FRET buffer (20 mM Tris, pH 7.5, 0.01% NP40, 50 mM NaCl) was placed in separate

wells of a black 96-well Molecular Devices HE high efficiency microplate (Molecular Devices, Inc., Sunnyvale, CA). In a second 96-well Nunc polypropylene plate (Nalge Nunc International, Rochester, NY), a solution of each tested compound (0.001 M stock in DMF) was serially diluted in a 1:10 fashion into DMF. Each concentration of compound was then diluted 1:10 into TR-FRET buffer, and 10 μ L of this solution or vehicle was added to the stock ER solution in the 96-well plate. After a 2-min incubation, 5 μ L of 200 nM fluorescein-SRC3-NRD was added to each well. This mixture was allowed to incubate for 1 h at RT in the dark. TR-FRET was measured using an excitation filter at 340/10 nm, and emission filters for terbium and fluorescein at 495/20 and 520/25 nm, respectively. The final concentrations of the reagents were as follows: ER α -417 (2 nM), streptavidin-terbium (0.5 nM), test compound (0–50 μ M), SRC3-Fl (50 nM) (9).

FRET Assay of Estradiol-SLF Binding to FKBP

Purified FKBP12-GST and a fluorescein-labeled SLF compound were used for this time-resolved FRET assay. A stock solution (5 μ L) of FKBP12-GST (20 nM) and LanthaScreenTM anti-GST-terbium antibody (Invitrogen) (20 nM) in TR-FRET buffer (20 mM Tris, pH 7.5, 0.01% NP40, 50 mM NaCl) was placed in separate wells of a black 96-well microplate. The test compounds were prepared as described above and added to the protein solution. After a 2-min incubation, 5 μ L of 400 nM fluorescein-SLF was added to each well. This mixture was allowed to incubate for 1 h at RT in the dark. TR-FRET was measured as described above. The final concentrations of the reagents were as follows: FKBP12-GST (5 nM), anti-GST-terbium (5 nM), test compound (0–50 μ M), and SLF-Fl (100 nM).

FRET Assay of Agonist-SLF binding to both ER and FKBP

This assay was conducted according to the protocol used for the above experiment “*FRET Assay of Estradiol-SLF Binding to ER by Recruitment of Fluorescein-SRC*” replacing the 50 nM SRC3-Fl with 50 nM FKBP12-Fl. All other concentrations and measures remained identical.

FRET Assay of ER-SRC Binding in the Presence of FKBP

This assay was performed in the same format as the experiment above “*FRET Assay of Estradiol-SLF Binding to ER by Recruitment of Fluorescein-SRC*” with the following changes. Unlabeled FKBP12 and SRC3 were each titrated into TR-FRET buffer and added to the well of a microplate. A stock solution of ER α -417 (8 nM), compound **4** (25 μ M), and streptavidin terbium (2 nM) was incubated for 10 min, and 5 μ L of this solution was then added to each well of protein. Finally, 5 μ L of 200 nM SRC3-Fl was added to each well and allowed to incubate for 20 min before the fluorescence was read.

FRET Assay of ER Dimerization in the Presence of FKBP

This assay was performed according to the protocol “*FRET Assay of ER-SRC binding in the presence of FKBP*” with the exchange of 50 nM SRC3-Fl for 50 nM ER α -Fl in the final assay concentrations. Also, both compound **2** and estradiol were used as test compounds in this assay to provide additional positive and negative controls.

Luciferase Reporter Gene Assay

Human endometrial cancer cells were maintained in culture as described and transfected in 24 well plates (24). A mixture of HBSS (50 μ L/well), Holo-transferrin (Sigma T1408) (20 μ L/well), and lipofectin (Invitrogen #18292-011) (5 μ L/well) were incubated at RT for 5 min. The DNA mixture was made by adding 200 ng of pCMV β -galactosidase as internal control, 500 ng of the estrogen responsive reporter gene plasmid 2ERE Luc, and 100 ng of

full-length ER α expression vector with 75 μ L HBSS per well and, after addition to the first mixture, allowed to incubate for 20 min at RT. The cell media was changed to Opti-MEM (350 μ L/well) and 150 μ L of the transfection mixture was added to each well. The cells were incubated at 37 °C in a 5% CO₂ containing incubator for 6 h. The medium was then replaced with fresh medium containing 5% charcoal-dextran-treated calf serum and the desired treatments of compounds. Reporter gene activity was assayed at 24 h after ligand addition. Luciferase activity, normalized for the internal control β -galactosidase activity, was assayed as described (24).

RESULTS

Conjugate Design and Synthesis

The recruiting element/protein partner pair we chose, SLF/FKBP, was the same one used in the previously reported β -amyloid study (12), because of the advantages the pair confers: (a) FKBP is ubiquitously expressed in various mammalian tissues, including breast cancer cells (e.g., FKBP52 makes up 1.0–1.5% of the total protein concentration in ER+ breast cancer cells (25)); (b) SLF, which is effectively one structural half of the natural product FK506, binds to FKBP with high affinity ($K_d \approx 20$ nM) (19,26) but is incapable of recruiting calcineurin, the interaction partner for FKBP, and, therefore, should have no confounding biological effects; (c) accessible methods have been described for the synthesis of SLF (19,20); and finally, (d) because the SLF/FKBP pair proved effective in blocking protein-protein interactions in the β -amyloid case, we were hopeful that it would be a fruitful starting point for this project.

The position on the estradiol skeleton chosen for derivatization was the 17 α position, an alteration typically known to give agonists (15). The NHS ester **1** was prepared by reacting 17 α -ethynylestradiol with methyl 4-iodobenzoate using Sonogashira conditions, to give, after saponification of the methyl ester and activation, the NHS ester **1**. This activated ester **1** was reacted with mono-protected diamines, which after deprotection, gave the amine functionalized estrogens **2** and **3**. Reaction of these amines with SLF electrophiles, prepared according to known methods (19,20), gave the desired estrogen-SLF conjugates **4** and **5**.

Assay Design

In following the different protein-protein or protein-small molecule interactions in our studies described below, we have used various versions of a time-resolved fluorescence resonance energy transfer (TR-FRET) assay (23). In these assays, one of the protein partners, appropriately functionalized, is labeled with a FRET donor in one of two ways—by binding either a terbium streptavidin chelate (SA-Tb) or a terbium-labeled anti-GST antibody. The other partner, either a protein or small molecule, is labeled with the FRET acceptor fluorescein. In these experiments, an increase in FRET corresponds to a complex wherein both FRET donor and acceptor are in close proximity, meaning that both proteins are within the same complex. For further details, see our previous report (23).

EE2-SLF Conjugate **4** Binds ER and Recruits SRC3

As seen in Figure 1A, the short-tether EE2-SLF conjugate **4** was able to recruit a fluorescein-labeled SRC3 (SRC3-Fl) fragment to an ER α -LBD/SA-Tb complex, as judged by the dose-dependent increase in FRET signal seen with increasing concentrations of **4**. This result suggests that, as we had hoped, the conjugate **4** acts as an agonist, since antagonists are incapable of recruiting SRC to the ER in reconstituted *in vitro* systems. The potency of compound **4** is in general agreement with the results from a radiometric binding assay (21, 27), routinely carried out in our laboratories, which showed the relative binding

affinity of **4** to be 1.5% that of estradiol, which corresponds to a K_D of 13 nM (data not shown).

Interestingly, conjugate **5**, having a longer tether joining the SLF and EE2 portions of the molecule, was much less capable in recruiting the SRC3 fragment to the ER α -LBD. We believe this may be due to the non-specific interactions between the long tether and the proteins in the system that prevent the ligand from accessing the binding site.

EE2-SLF Conjugates Bind FKBP12

In a related TR-FRET experiment (Figure 1B), we showed that conjugate **4** is also capable of binding FKBP12, as judged by the dose-dependent competition of the ligand with a fluorescein-labeled SLF (SLF-FI). In this experiment, FKBP12-GST was labeled with an anti-GST-Tb chelate. A decrease in FRET is expected if the molecule competes with SLF-FI (K_D = 30 nM, data not shown) for binding to the FKBP12-Tb complex. The most potent FKBP12 binder was the unsubstituted SLF-OH (IC_{50} = 200 nM). The conjugates **4** and **5** bound to FKBP somewhat less well, with IC_{50} values of 1.7 and 1.8 μ M, respectively.

EE2-SLF Conjugate **4** Binds ER and FKBP12 Simultaneously

Having established that each half of the conjugate **4** is able to independently bind to its respective partner, it was then important to establish that the conjugates were capable of binding both protein partners simultaneously. In a TR-FRET assay (Figure 1C), increasing concentrations of conjugate **4** were able to recruit fluorescein-labeled FKBP12 (FKBP12-FI; labeled nonspecifically through available cysteine residues) to the Tb-ER complex. In this experiment, the negative control (the unfunctionalized **SLF-OH**) and the longer-tethered **5** were incapable of recruiting FKBP12 to the ER-Tb complex. This is in good agreement with the previous section, wherein **5** was shown to be a poor ER binder (Figure 1A). Importantly, the results of this experiment establish that the bivalent ligand **4**, EE2-SLF, is able to bind to both of its intended protein targets, ER α and FKBP12, and bring them into one complex.

SRC3 Is Recruited to the ER/Conjugate **4**/FKBP12 Complex

Conjugate **4** was capable of binding both ER and FKBP12 (Figure 1C); therefore, if it is incapable of recruiting SRC3 to ER in the presence of FKBP12, we can assume that this lack of recruitment is due to the recruited steric bulk of FKBP12. As seen in Figure 2A, ER-Tb saturated with conjugate **4** (25 μ M) was capable of recruiting SRC3-FI (high FRET), and this binding was reversible in the presence of increasing concentrations of unlabeled SRC3 (solid triangles). There was, however, no dose-dependent blockade of ER/SRC in the presence of increasing concentrations of unlabeled FKBP12 (open triangles). At the highest concentration of FKBP12 used (50 μ M), no reduction is seen in the FRET signal, even though this concentration was shown to be adequate in saturating the conjugate in the previous experiment (Figure 1B). Thus, it seems that when the ER is bound with the conjugate **4**, it can accommodate both SRC3 and FKBP12: The former is recruited due to the agonist character of the ethynylestradiol ligand (Figure 1A) and the latter can bind through the ligand component.

Conjugate **4** Is Also Incapable of Inhibiting ER Dimer Formation

In the previous experiment, ER was shown to bind a number of different proteins, but it was unclear whether a dimer or monomer of ER was responsible for this array of binding events. Based on structural studies of ER, dimer disruption might be more feasible than SRC inhibition, because X-ray crystallographic structures show that the 17 α -phenylethynyl group of the conjugates likely exits from the binding pocket on the side of ER opposite to that of the coactivator binding groove, but nearer the long helix 11, where ER dimerization takes

place (28). If helix 11 were to be sufficiently perturbed by the recruitment of FKBP12, this could have an effect on the ability of ER to dimerize.

To probe this question, the ER-Tb complex (1 nM) was incubated with conjugate **4** (10 μ M) and fluorescein-labeled ER (ER-Fl; 50 nM), such that a decrease in FRET would occur if dimer formation were to be disrupted (Figure 2B). In the positive controls, ER dimerization was disrupted (observed by a dose-dependent decrease in FRET) in the presence of increasing concentrations of unlabeled ER using either estradiol or conjugate **4**. These compounds do not, however, give this characteristic decrease in FRET in the presence of increasing concentrations of FKBP12 (open diamonds). Therefore, FKBP12 does not inhibit the formation of the ER dimer.

Conjugate **4** Is Incapable of Downregulating an ER-regulated Reporter Gene

Regardless of its activity in *in vitro* assays, we thought that the conjugate might have unexpected activity in cell-based assays of ER-mediated transcription. When we treated transiently transfected HEC-1 cells with conjugate **4** (24), we saw, however, a dose-dependent increase in luciferase expression that was almost identical to the negative control **2**, which lacks an SLF group (Figure 3). Thus, both of these compounds behave as conventional ER agonists that are unaffected by the presence of FKBP and consistent with their ability to recruit SRC (cf. Figure 1B). When competing against 1 nM estradiol, the conjugate **4** showed no significant dose-dependent decrease in luciferase production. The small decrease in signal from -9 (1 nM) to -8 (10 nM) is most likely due to the lesser efficacy of compound **4** in comparison to estradiol as the ligands are exchanged, rather than true inhibition based on recruitment of FKBP. These data lead us to conclude that the ability of conjugate **4** to bind FKBP does not affect its efficacy, in agreement with the *in vitro* experiments detailed above.

DISCUSSION

The experiments detailed above yield a rather surprising finding: the ER can serve as a nexus for a remarkable number of binding events and still retain its activity—namely, binding of SA-Tb, conjugate **4**, SRC3, FKBP12, and another ER monomer occurs simultaneously and without antagonistic consequence. While this is not the finding for which we had hoped, it is an interesting one nevertheless, given that the ER-LBD is only 31 kDa, yet is able to recruit and bind approximately 145 kDa of other protein fragments simultaneously (FKBP12-GST (38 kDa), SRC3-NRD (24 kDa), streptavidin (53 kDa)). Thus, the estrogen receptor appears to be a protein with a high topological tolerance in its interactions with protein binding partners and thereby appears to be quite resistant to the disruption of its normal, functional interactions by recruitment of FKBP12 using heterobifunctional ligand-SLF conjugates.

There are a number of differences between the system we have investigated and the β -amyloid system on which this project was founded (12), and a comparison is instructive. Perhaps most salient is that we have not used this method to fortify the affinity of a weak, direct protein-protein interaction inhibitor, such as coactivator binding inhibitors we have studied (8,9). Rather, we have based our heterobifunctional conjugates on estradiol, a high affinity ligand that binds to the ligand binding pocket. We were hopeful that the higher affinity of estradiol for the ligand-binding pocket of the receptor, compared to the relatively weak affinity of the known direct ER/SRC inhibitors (8,9), might serve as a better starting point for developing these types of inhibitors. Our inability to block either of these interactions with an inhibitor that binds to the ER with low nanomolar affinity, as does ours, would bode especially poorly for approaches that construct heterobifunctional conjugates

from molecules that bind to the ER with only micromolar affinity, as do the currently available direct inhibitors of coactivator binding (8,9,29,30).

An additional difference between the A β system and ours is the size of the components involved. An oligomer of A β weighs only ca. 1 kDa, and its oligomerization is being inhibited by a recruited FKBP twelve times its mass (12). In an *in vitro* sense, our system uses a 31-kDa construct of the ER-ligand binding domain and a 24-kDa SRC3 fragment. These are of comparable size to the FKBP-GST fusion protein used in our assays. Thus, it is conceivable that although the FKBP-GST is being recruited, it is just too small to block the interaction of the larger-sized proteins. Some breast cancer cell lines, such as MCF-7, express larger FKBP (e.g., FKBP51 or 52) that could be implemented in experiments similar to those described in this report (25); however, we do not believe that the steric bulk of these proteins would be significantly greater than the FKBP12-GST fusion protein (MW 38 kDa) that we have used here.

It is known that the affinities of the ER for its dimerization partner and SRC fragment are both very high ($K_d \approx <1$ nM for both). It is not clear from the literature what the affinity of A β is during oligomerization, but various groups have demonstrated that appreciable oligomerization occurs at concentrations of 1–5 nM (31,32), although higher concentrations (70 μ M – 2 mM) are typically used when larger assemblies, such as prefibrillar and fibrillar oligomers and fibrils, are formed (33). The SLF-Congo Red conjugate described by Gestwicki was capable of inhibiting formation of aggregates up to ca. 28 nm² in size in the presence of FKBP, suggesting that the compound is not capable of disrupting the bimolecular A β /A β interaction, but rather the formation of these larger, possibly lower-affinity assemblies (12).

The position chosen for tethering SLF to the estradiol skeleton (17 α) is an appropriate position for blocking ER dimerization, as the dimer interface occurs along the axis of helix 11 (28). Thus, it is surprising that the ER is able to bind its dimeric partner and FKBP simultaneously. By contrast, because SRC binds on a different face of the receptor, it might be difficult to access the ER/SRC interaction site with these types of inhibitors. It was for this reason that we created the longer-tethered conjugate **5**, in the hope that it might be able to extend further around the receptor to block the ER/SRC interaction. Unfortunately, we found that including this longer tether reduced the affinity of the molecule for the ER to levels that precluded it from being examined in our assays.

Another option in designing estrogen-SLF conjugates would be to supplement the activity of an antiestrogen by appending an SLF moiety; in fact, such a design has been reported for the androgen receptor (34). We have attempted to create such compounds for the estrogen receptor, but we have had difficulty in synthesizing one that has sufficient ER affinity to be examined in our assays.

More recently, heterobivalent molecules linking SLF with other ligands have been prepared (13,35). These conjugates bind either to FKBP12 or to the other target protein (dihydrofolate reductase or cytochrome P450, respectively) when the two proteins are present separately, but they do not bind to both simultaneously when both are present. The steric occlusion encountered with these heterobivalent ligands is different from the behavior of the ligands, originally reported by Gestwicki et al. (12), that bind to FKBP and at the same time are able to interfere with the polymerization of A β . Our heterobivalent ligands too are able to bind to both ER and FKBP, but they do not prevent ER from interacting with other proteins (SRC or itself).

Of the heterobivalent ER ligands that have been reported in the literature, most are cytotoxic estrogens that typically contain alkylating agents tethered to estrogens (36–39); as such, they

are not particularly germane to this discussion. The one exception of which we are aware is a geldanamycin-estradiol conjugate designed and synthesized by Danishefsky and coworkers (40). Meant to inhibit Hsp90 function and selectively degrade ER, the conjugate does seem to be selective for ER over other targets of Hsp90 inhibition (i.e., Raf-1, IGF1R) at very high concentrations (1 mM); however, no mechanistic studies were done to determine how the conjugate functions at a molecular level. It would be interesting to know whether this conjugate is also capable of simultaneously binding ER and Hsp90, whether simultaneous binding is necessary for its biological activity, and whether interruption of ER-associated protein-protein interactions contributes to its activity.

There have also been heterobivalent estrogen conjugates reported in the literature that are used in yeast-three hybrid assays, implying that the estrogen receptor is capable of binding to two different proteins simultaneously, although the interaction partner in this assay is streptavidin. This conjugate would presumably have no biological activity arising from multivalent interactions, as mammalian cells do not express avidins (41). We acknowledge a number of other factors (e.g., targeting element, recruiting element, tether length and composition, substitution site, enhanced affinity) one might change in creating inhibitors of the type mentioned here, but we wanted to share here our experience thus far—that a conjugate capable of binding both FKBP and ER simultaneously is incapable of inhibiting two different ER-associated protein-protein interactions.

Obviously, all of these factors merit careful consideration in the design of heterobivalent ligands, but beyond this, the overall topology of the protein-protein interaction is likely to be a critically important determinant of the feasibility and potential success of the Trojan horse approach to disruption of protein-protein interactions. The importance of the Congo Red-SLF conjugates is undeniable; however, the ER, long known as a proof-of-principle target in chemical biology experiments because of its therapeutic importance and well-studied biology, appears to have considerable topological tolerance in its interaction with its functional partner proteins (e.g., SRC). Therefore, it may not be the next logical conceptual target for this type of inhibition, and our experience suggests ways in which this Trojan horse approach may be limited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Kathryn Carlson for performing radiometric ligand-binding assays, Jie Chen for providing the FKBP12 constructs, Sung Hoon Kim for obtaining mass spectral data, and Manuel Rodriguez for assistance in synthesis. This research was supported by a grant from the National Institutes of Health (PHS 5R37 DK015556 to J.A.K.). J.R.G. received additional support from a David Robertson Fellowship, an NIH Fellowship NRSA 1 F30 ES016484-01, and Training Grant NRSA 5 T32 GM070421.

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Figure 1. Time-resolved fluorescence resonance energy transfer assays. (A) Titration (ER-Tb/SRC3-Fl) of **2** (red squares), **4** (blue triangles), and **5** (black triangles) showing SRC recruitment to ER when liganded with conjugate **4**. (B) Titration (FKBP-Tb/SLF-Fl) of SLF-OH (green squares), **4**, and **5** showing increasing concentrations of compounds competing for SLF-Fl. (C) Titration (ER-Tb/FKBP12-Fl) of SLF-OH, **4**, and **5** showing recruitment of FKBP12-Fl to ER when liganded with **4**.

Figure 2.

Time-resolved fluorescence resonance energy transfer assays. **(A)** Titration (Conjugate 4/ER-Tb/SRC3-FI) of either FKBP12 (open triangles) or SRC3 (closed triangles) showing that unlabeled SRC3 displaces SRC3-FI, but FKBP12 does not. **(B)** Titration (ER-Tb/ER-FI) of conjugate 4 (blue) or estradiol (E2; purple) showing that unlabeled ER (closed circles) is able to disrupt dimer formation, but FKBP12 (open diamonds) is not.

Figure 3. Luciferase reporter gene assay with **4** (closed blue triangles), **2** (closed red squares), and **4** + 1 nM E2 (open blue triangles). Increasing concentrations of **4** activate the ER-regulated luciferase gene, rather than downregulating luciferase expression.

Bioconjug Chem. Author manuscript; available in PMC 2011 October 20.

Chart 1.
Structures of SLF, Congo Red and SLF-Congo Red Conjugate I.