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A Set of Time-Resolved Fluorescence Resonance Energy Transfer Assays for the Discovery of Inhibitors of Estrogen Receptor-Coactivator Binding

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Abstract

Therapeutic block of estrogen action is typically achieved with conventional antagonists (CAs), compounds that displace estradiol from the estrogen receptor (ER) and induce formation of an ER conformation that cannot bind to coactivator proteins, such as the steroid receptor coactivators (SRCs). As an alternative mode for blocking estrogen action, we are seeking small molecules that act as coactivator binding inhibitors (CBIs), i.e., they compete directly with SRC3 for interaction with estradiol-bound ER. CBIs would be interesting mechanistic probes of estrogen action and might also provide an alternative, more durable endocrine therapy for hormone-responsive breast cancer, where cellular adaptations lead to resistance to CAs. We have designed and optimized a set of time-resolved fluorescence resonance energy transfer (TR-FRET) assays to monitor the interaction of ER with SRC3 and ligands, and we have used them in high-throughput screens to discover small molecule CBIs that are able to disrupt this interaction. These assays also distinguish CBIs from CAs. These robust and sensitive “mix and measure” assays use low concentrations of ER labeled with a europium chelate as FRET donor and a Cy5-labeled SRC as acceptor. This multiplexed protocol produces excellent signal to noise ratios (> 100) and Z' values (> 0.8).

INTRODUCTION

Blocking hormone action through the estrogen receptors (ERs), as is desirable in endocrine therapy for breast cancer and the stimulation of folliculogenesis, classically involves the use of estrogen antagonists.⁶ Antiestrogens, such as tamoxifen, displace the endogenous estrogen agonist, estradiol, and change the conformation of ER, thereby blocking many of its gene-regulatory functions. Antiestrogens, however, can have mixed agonist/antagonist activity, and their effectiveness in blocking ER activity in breast cancer can decrease with time, a phenomenon termed “hormone resistance.”¹³ Therefore, we seek compounds that would interfere with ER signaling, not at the ER-ligand interaction, but at the interaction of ER with its coactivator proteins.

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The transcription-regulating function of the ERs, ER α and ER β , relies on their interaction with coactivator proteins. The best studied coactivators are members of the p160 class of steroid receptor coactivators (SRCs) that functionally link ER with modification of chromatin structure and activation of the basal transcriptional machinery.¹⁶ The interaction of the SRCs with ER is regulated by ligand-induced conformations of the ER ligand-binding domain (LBD): Agonists position the C-terminal α -helix of the ER-LBD to create a hydrophobic groove that serves as a docking site for the SRCs, whereas antagonists position this helix so that it blocks coactivator binding (Figure 1A, center and left). Crystal structures reveal that the coactivator interacts with the hydrophobic groove in the LBD through nuclear receptor interaction boxes (NR boxes), which are LXXLL sequence motifs that adopt a two-turn amphipathic α -helical conformation.¹⁷ In this conformation, the first and last leucine residues extend downward into the hydrophobic groove, while the second leucine is positioned on a hydrophobic shelf next to the groove.

This interaction between the SRC helix and ER groove provides an alternate site for blocking estrogen action. In fact, some peptides having the LXXLL sequence can block transcription induced by hormone agonists,¹⁰ and small molecule analogs of these peptides could also have this net antagonistic effect. In this report, we will refer to *compounds that block coactivator binding directly* as coactivator binding inhibitors (CBIs; Figure 1A, center and right); by contrast, compounds that block coactivator binding indirectly by competing with estradiol at the ligand binding site and inducing a conformation that does not bind coactivator are termed conventional antagonists (CAs, Figure 1A, center and left). Although as yet untested, it is possible that CBIs might be effective in overcoming acquired resistance to CAs that can develop in endocrine therapies for breast cancer. This resistance is thought to involve the upregulation and modification of coactivators such that they can still bind to ER-CA complexes and thereby activate transcription.¹⁶

Although structure-based design has produced some examples of molecules that appear to act through an ER CBI mechanism,^{1,5,12,19} their potency as inhibitors of ER-coactivator binding has been relatively poor, as is also the case with the only reported compounds discovered through high throughput screening (HTS).¹⁴ Higher affinity CBIs are needed as effective mechanistic probes and as leads for potential therapeutics that work through this novel mechanism of inhibition.

Classical glutathione S-transferase (GST)-pull down and other related assays traditionally used to study receptor-coactivator interactions are labor-intensive and not adaptable to HTS.^{2,9} We¹² and others^{1,11} have described fluorescence polarization (FP) assays for monitoring the interaction of a fluorophore-labeled SRC LXXLL peptide with the ER LBD, but this assay is also not optimal: It has low dynamic range and requires high ER concentrations (200 nM), the latter of which also makes accurate determination of K_i 's difficult. Thus, we sought alternative assays based on fluorescence resonance energy transfer (FRET) that might be more amenable to a HTS format.

Others have reported FRET-based assays to examine nuclear receptor-coactivator interactions, but certain features complicate their use in a HTS format: blocking and washing steps, expensive lanthanide-conjugated antibodies,^{4,8,18} expensive biologic fluorophores,⁸ or fluorescence emission ranges that overlap with many inherently fluorescent test compounds.⁴ To overcome these problems, we have developed a time-resolved (TR) FRET assay. This assay monitors the interaction between the ER LBD labeled (via a streptavidin-biotin interaction) with a europium chelate and a Cy5-labeled sequence of the SRC3 coactivator protein (or in one case a Cy5-labeled ligand). The europium chelate functions as a long-lifetime (ca. millisecond) luminescent donor, and the organic fluorophore serves as the TR-FRET acceptor. By exciting the europium complex with

pulsed light and gating the emission with a 50- μ sec delay, background emission from the short-lifetime (nsec) organic fluorophore FRET acceptor can be eliminated. When properly optimized, the TR-FRET method gives low background signals and can be run in a homogeneous, mix-and-measure format with very low amounts of europium-labeled streptavidin and biotin-labeled ER-LBD. The choice of the europium-Cy5 FRET pair allows the monitoring of acceptor emissions at longer wavelengths than other commonly used assay fluorophores such as fluorescein. Autofluorescent compounds found in libraries typically emit at wavelengths shorter than 550 nm; thus, our choice of Cy5 minimizes false positives due solely to interfering emission patterns.

Here, we present details of the development, optimization, and validation of a set of three 384-well format TR-FRET assays to discover small molecule CBIs for ER (Assay A1) and to distinguish them from CAs (Assays A2 and A3). These assays are simple, sensitive and robust, and they are characterized by excellent Z' values and S:B ratios. Thus, they are amenable to HTS format and can be readily used for screening large chemical libraries.

MATERIALS AND METHODS

17 β -Estradiol and tamoxifen were purchased from Aldrich, and LANCETM Streptavidin-Europium Chelate from Perkin Elmer. Cyclononane-Cy5 was synthesized in the Katzenellenbogen laboratory, as was pyrimidine CBI, as previously reported.¹² The SRC1-Box II peptide was synthesized by the University of Illinois Protein Sciences Facility of the Carver Biotechnology Center.

Protein expression, purification, and labeling of ER α -417, ER β -369, and SRC3

N-terminally His-tagged constructs in pET15b plasmids for ER α -417, ER β -369 and SRC3 were prepared as previously described.¹⁵ The LBDs of ER α -417 (amino acids 304–554; C381,530S) and ER β (amino acids 256–505; C334,481S), each with a single reactive cysteine at C417 or C369, respectively, 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 hours at 28 °C, as previously reported.³

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 30000g, 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.^{3,15}

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 or ER β -369 LBDs were 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 tricarboxymethylphosphine, 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.^{3,15}

Fluorescence Resonance Energy Transfer (FRET) Measurements

Assay buffer (FRET buffer) contains 20 mM Tris, pH 7.5, 0.01% Nonidet P40, and 50 mM NaCl. Black 384-well microplates (Corning Costar, Cambridge, MA) were used and FRET signals were recorded on an Analyst HT plate reader (Molecular Devices, Sunnyvale, CA). The instrument Z height was set to 2 mm, and a 400 μ s integration time was used. Europium excitation was at 330/80 nm, and emission from europium donor (D) and Cy5 acceptor (A) was measured at 620/7.5 and 665/7.5 nm, respectively. All FRET signals were expressed as $A_{665}/D_{620} \times 10^4$. The FRET signal window was considered the difference between the maximal FRET value recorded for bound SRC3-Cy5/ER, run with agonist estradiol and SA-Eu, and the minimal FRET value (background), recorded in the absence of ER. Each assay was performed with both ER α -417 and ER β -369 with very similar results; only the assay performance with ER α -417 will be discussed.

Assay development and optimization

The primary CBI FRET assay (A1) was performed in black 384-well microplates with a total volume of approximately 20 μ L per well. To determine its binding affinity, SRC3-Cy5 was serially diluted into FRET buffer and mixed with biotin-labeled ER α -417 (0.3 nM), LANCETM Streptavidin-Europium Chelate (SA-Eu, 0.5 nM) and estradiol (1 μ M). A 20- μ L volume of this mixture was dispensed into the well, incubated at room temperature for 1 hour, and measured with the Analyst HT reader. The binding affinity of ER α -417 was determined by serial dilution into FRET buffer, mixing with SRC3-Cy5 (6 nM), SA-Eu (0.5 nM) and estradiol (1 μ M), and evaluating 20 μ L of this mixture as described above. To investigate the performance characteristics of the assay itself, without possible interference from test compounds, the signal-to-background ratio (S:B) and the Z' factor were calculated based on the following equations: $S:B = \mu_b/\mu_f$ where μ_b and μ_f are the FRET signals for bound (b) ER α -417/SRC3-Cy5 and free (f) SRC3-Cy5 alone, respectively. The difference between mean signals for bound and free was represented by $(\mu_b - \mu_f)$. The Z' factor was calculated using the following equation: $Z' = 1 - (3 \times SD_b + SD_f)/(\mu_b - \mu_f)$.¹¹

Assay stability was evaluated by monitoring SRC3-Cy5 recruitment to ER α -417 after incubation times from 10 minutes to 24 hours. The effect of DMSO on the maximal signal was evaluated by increasing solvent percentage up to 20% and measuring after a 2-hour incubation at room temperature. Microsoft Excel (Microsoft Corporation, Redmond, WA), Origin 6.1 (OriginLab Corporation) and Prism 4.0 (Graphpad Software, San Diego, CA) were used to evaluate data.

Competition FRET assays using known CBIs as positive control compounds

The assay was also evaluated using two positive-control CBIs, an unlabeled 15-mer SRC1 Box II peptide (NH₂-CLTERHKILHRLLE-CO₂H) and a small molecule CBI (pyrimidine CBI), previously reported by us.¹² These were dissolved in DMSO, serially diluted, and added to assay buffer containing 0.3 nM ER α -417, 2 nM SRC3-Cy5, 0.5 nM SA-Tb, and 1 μ M estradiol in a volume of 20 μ L. Vehicle (DMSO) controls were also included, and plates were incubated at RT for 10 min to overnight before measurement. The percent inhibition was calculated as: % of Inhibition = $(\text{FRET signal} - \text{background})_{\text{compound}} / (\text{FRET max. signal} - \text{background})_{\text{DMSO}} \times 100$ with FRET signal and background as defined above. Nonlinear regression analysis using Prism 4.0 (Graphpad software) gave IC₅₀ values for these positive controls.

High Throughput Assay Format and Validation

The suitability of the CBI FRET assay for HTS was determined by performing the assay in 260 384-well microplates, with each plate containing the appropriate controls, including 16

wells of free SRC3-Cy5 (6 nM), SA-Eu (0.5 nM) and estradiol (1 μ M); and 16 wells of bound ER α -417(0.3 nM)/SRC3-Cy5 (6 nM), SA-Eu (0.5 nM) and estradiol (1 μ M). The FRET signals were used to calculate S:B ratios and Z' factors. Assay buffer, mixed with 0.3 nM ER α -417, 2 nM SRC3-Cy5, 1 μ M estradiol, and 0.5 nM SA-Eu, were dispensed in 19- μ L quantities into each well before addition of library compounds as 0.5 μ L of 1 mM DMSO compound stocks, yielding a final compound concentration of 25 μ M and DMSO concentration of 2.6% (v/v). After 1 hour at room temperature, plates were measured using an Analyst HT plate reader; assay data were analyzed using CambridgeSoft, and the activity cutoff set as a percentage inhibition greater than 50%. Subsequent to a preliminary assay of a library of known pharmacologically active compounds, the LOPAC library, the concentration of SRC3-Cy5 used in the assay was decreased from 6 nM to 2 nM without any significant changes in assay parameters.

FRET Confirmatory Assay Format and Validation

Two FRET-based confirmatory assays using most of the original components were used to probe the mechanism by which tested compounds produced a decrease in FRET signal. First, to ascertain whether competition was occurring at the receptor-coactivator binding site, the primary CBI assay was repeated with 10-fold higher concentration of SRC3-Cy5 (final concentration 20 nM) (Assay A3). Second, to evaluate the affinity of the tested compounds for the ligand-binding site, ER α -417 and SA-Eu were used, but a fluorophore-labeled ligand, nonane-Cy5, replaced SRC3-Cy5 (Assay A2). In the second assay, the concentrations of ER α -417 and nonane-Cy5 that gave maximal signal were determined by titrating each component in the presence of fixed concentrations of the other constituents. Thus, nonane-Cy5 ligand was titrated in the presence of 0.5 nM SA-Eu and 0.3 or 1 nM ER α -417, and ER α -417 was titrated in the presence of 5 and 100 nM cyclononane-Cy5. K_d values of the ligand, as well as the Z' values and S:B ratios for these assays, were determined.

Non-Fluorescence-Based (ELISA) Confirmatory Assay Format and Validation

A non-fluorescence-based confirmatory ELISA was performed in 96-well microplates coated with Protein A/G (Pierce Biotechnology) in a total volume of 100 μ L per well using the FRET buffer. Full-length human estrogen receptor α and β proteins were purchased from Invitrogen. 17 β -estradiol (4 μ M; Sigma) was incubated with ER (~500 ng/mL) for 30 minutes on ice before addition of 25 μ L of diluted (80 ng) mouse anti-human estrogen receptor α (Cell Signaling) or mouse anti-human estrogen receptor β (Affinity Bioreagents) monoclonal antibody to the appropriate wells on the microplates. After 30 minutes, 25 μ L of the ER/estradiol mixture was added to the microplates containing the antibodies. After 25 μ L of diluted test compounds was added and allowed to incubate for 15 minutes, 25 μ L of 60 nM biotin-labeled SRC3 was dispensed into the wells. This complete reaction mixture was allowed to incubate for 90 minutes at room temperature. The microplates were then washed four times with Wash Buffer (1 $\times$ PBS, 0.05% TWEEN 20) before 50 μ L of a streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch) diluted 1:10,000 was added and incubated for 30 minutes. The microplates were then washed four times, and 100 μ L of tetramethylbenzidine was added. The reaction was stopped at 10 minutes by addition of 100 μ L of 0.1N sulfuric acid. The microplates were read at 450 nm on an EnvisionTM plate reader (Perkin Elmer).

RESULTS AND DISCUSSION

Development, Optimization, and Validation of a TR-FRET Estrogen Receptor/SRC3 Binding Assay

We replaced our original FP assay for CBIs,¹² which used wild type ER LBD, with a FRET assay by implementing an ER LBD construct, mutated to include only one reactive cysteine that could be singly labeled with a thiol-reactive biotin reagent.¹⁵ Labeling the protein with a streptavidin-europium (SA-Eu) conjugate provided the long-lifetime donor for the assay that produced high signals even at low nanomolar concentrations (Figure 2). We chose to use a streptavidin-europium chelate, in contrast to a more conventional antibody-conjugated europium chelate targeting an epitope tag, because the smaller molecular weight of the streptavidin-europium chelate reduces the perturbation of the system. Our interest in using these components was also based on extensive studies we have performed with site-specific ER mutants in which we have determined that neither the mutation in the ER LBD, nor the labeling with biotin and SA-Eu changed the biological activity of the protein in terms of its ability to bind ligand and coactivators.¹⁵ Measurements in previous studies have demonstrated the similarity in both ligand binding and kinetic measures between ER LBD (domain E) and full-length ER (domains A-F) to support our use of the shorter construct for these experiments.^{3,15} The results of GST pull-down assays have also shown that neither mutational changes nor labeling with a small molecule has any detrimental effect on coactivator recruitment behavior of the LBD as compared with the wild-type full-length receptor.¹⁵ No longer being restricted by the requirement of an FP assay for significant mass differences between components, we also changed the small labeled peptide previously used to a more biologically-relevant, labeled protein fragment of the SRC3 coactivator, encompassing around 200 amino acids, including three NR-boxes.

As shown in Figure 2, increasing concentrations of either the SRC3-Cy5 (Figure 2A) or the europium-labeled ER component (Figure 2B) resulted in increases in FRET signal and background values. The maximal assay window (FRET signal - background, as defined above) for the Cy5 titration reached ~6000, with a K_d of 1.1 nM, whereas the window for the ER titration extended to ~5000, with a K_d of 0.3 nM. As shown later, the signal associated with ER/SRC binding is blocked using a positive control unlabeled SRC1-Box II peptide (cf., Figure 4A). Thus, these results provide a simple, reliable, and robust assay for studying ER/SRC binding and discovering small molecule compounds that disrupt this interaction.

In both titrations, the quality of the assay dynamic range for HTS suitability was assessed by calculating S:B ratios and Z' values over the concentration range. In the SRC3-Cy5 titration, the signal window increased until ~50 nM (Figure 2A); maximal S:B ratios of 140 were obtained at concentrations less than 6.25 nM, which minimized high background measurements from excess fluorophore, while the Z' value for the assay remained fairly constant at around 0.95 for concentrations up to 400 nM SRC-Cy5 (Figure 2C). In the ER titration, the signal increased until ~0.6 nM (Figure 2B); the maximum S:B ratio was 140 at 0.625 nM ER, with a small decrease at higher concentrations, and Z' values were ~0.95 for the entire concentration range (Figure 2D). S:B ratios for these assays were higher than 27 at the lowest ER concentration tested (0.08 nM). The Z' factor of an assay incorporates both the dynamic range and variability of an assay into a single measurement, and it is considered that Z' should be greater than 0.5 for reliable and robust assays.¹¹ Both titration assays show consistent Z' factors of 0.95 over the concentration ranges, indicating that the assay is robust.

An assay suitable for HTS should also show good stability over reasonable incubation times. When we examined SRC3-Cy5 titration assays over times from 10 minutes to 24 hours, reproducible results with no change in signal intensity were seen up to 4 hours (Figure 3A);

thereafter, assay signal strength eventually decreased to 50%. This stability with time eliminates concern associated with dispensing large numbers of assay plates for scheduled readings during the high-throughput screen. HTS often employs compound libraries that are dissolved in solvents such as DMSO; therefore, the assay components must tolerate the presence of these solvents. The assay showed no appreciable decrease in signal with up to 8% DMSO and very little decrease with concentrations up to 20% (Figure 3B). The Z' values remained constant throughout the DMSO titration, and the S:B ratios decreased only slightly with above 8% DMSO (Figure 3C). Thus, the ER/SRC3 interaction assay is stable in the presence of at least 8% DMSO.

Validation of the CBI Assay with Known Peptide Antagonists and Small Molecule CBIs

Although the ER-SRC3 interaction assay validated well, it also needed to be evaluated in a competition mode, where peptides or small molecules must displace SRC3-Cy5, as this is the mode used for screening. Component concentrations selected (0.3 nM ER α -417 and 2 nM SRC3-Cy5), were shown by previous experiments to give good signal. The concentration of SRC3-Cy5 used is about 2 times higher than its K_d and provides a near maximal signal; using a low concentration of SRC3-Cy5 is also advantageous in that it does not preclude competition by new CBI compounds. The SA-Eu label at 0.5 nM is sufficient because a single streptavidin tetramer can bind up to four biotin-labeled ERs. The agonist ligand estradiol was used at 1 μ M, a concentration shown previously to fully saturate 0.3 nM ER (data not shown). The presence of detergent in the assay buffer, which is required to minimize non-specific interactions, raises the apparent K_d of estradiol for the ER-LBD; so, a high concentration of estradiol is required. This assay, run in a competition mode under these conditions, was designated Assay A1, to distinguish it from the two follow-up TR-FRET assays, A2 and A3 (The conditions for all three assays are summarized in Figure 6D).

The first positive control and pure inhibitor of coactivator interaction, an unlabeled SRC1-Box II peptide containing three LXXLL motifs, was expected to bind to ER with an IC_{50} ~720 nM, based on affinities of related peptides evaluated in fluorescence polarization assays.¹² The second positive control (pyrimidine CBI), a small molecule CBI we previously described,¹² disrupted the ER/SRC3 interaction with an IC_{50} of 29 μ M in an FP assay using a labeled SRC peptide as the tracer. In titration assays, these two positive controls demonstrated an expected dose-dependent decrease in FRET signal as they dissociated SRC3-Cy5 from ER. Assays run with incubation periods of 10 minutes to 24 hours showed reproducible maximal signals and IC_{50} s for up to 24 hours for both control compounds (Figure 4). The IC_{50} value for the unlabeled peptide after 1 hour, 0.70 μ M, was close to other published values (Figure 4A),⁵ but the pyrimidine small molecule control showed a IC_{50} somewhat lower than previously published using an FP assay (Figure 4B). This difference in assay response is attributed to the higher sensitivity of the FRET assay, which uses lower concentrations of ER and SRC3. These results validate the CBI assay in a competition mode and confirm the conditions necessary to run this HTS assay for small molecule inhibitors of the ER/SRC3 interaction.

Mechanistic Confirmation of Hits with FRET-Based Assays as CBIs

Antagonist ligand competition confirmatory assay (A2)—We have used a specific sequence of two related TR-FRET confirmatory assays (A2 and A3) to minimize false positive hits and verify CBI activity (The conditions for all three assays are summarized in Figure 6D). As mentioned earlier, conventional antagonists (CAs or antiestrogens) are expected to score as active in the ER-SRC3 interaction assay by competing with estradiol for binding to the ligand-binding pocket and inducing an antagonist conformation that is unable to bind coactivator (Figure 1A). Because a high concentration of estradiol (1 μ M) is used in the FRET CBI assay (A1), however, only very high-affinity antiestrogens should

show this false-positive activity. These compounds, although interesting in their own right, do not act through a CBI mechanism and must be distinguished from true-positive CBI hits.

To establish the relative ligand binding potential of putative CBIs, we developed a binding assay that compares the hits to a Cy5-labeled ligand with known receptor affinity for the ER. The ligand, cyclononane-Cy5 (Figure 5E), which we prepared expressly for this assay, binds to the ER ligand-binding pocket with approximately 23% the affinity of estradiol, as determined in a radiometric competitive binding assay (data not shown);³⁷ this corresponds to a K_i of 0.9 nM (estradiol K_d is 0.2 nM). Simply replacing the SRC3-Cy5 component with cyclononane-Cy5 and removing estradiol gives a FRET assay that measures direct competition of compounds for binding to the ligand binding pocket of ER, from which their affinity as CAs can be calculated (Figure 1B). Knowing this value, one can calculate the concentration of compound that would be required to out-compete the concentration of estradiol used in the screening assay (1 μ M), thereby giving a false positive signal as an apparent CBI in the ER-SRC3 interaction assay through a CA mechanism.

To develop this assay, we performed titrations of cyclononane-Cy5 or ER, while holding the other assay components constant. From titrations of cyclononane-Cy5 ligand with 0.3 or 1 nM ER, and 0.5 nM SA-Eu (Figure 5A), we obtained an assay signal window of approximately 6,000 using 1 nM ER or approximately 4,000 using 0.3 nM ER, giving respective K_d 's of 44.9 nM and 5.8 nM. The ten-fold difference in K_d determined from these titrations, however, implied that conditions were not optimal and ligand depletion was being observed when 1 nM ER was used. Thus, the lower concentration of ER, 0.3 nM, was used in all subsequent assays. The Z' values for each concentration of cyclononane-Cy5 were fairly steady through the titrations, never falling below 0.6 at the lowest concentrations when 0.02 nM ER was used (Figure 5B). The S:B ratios showed an increase with nonane-Cy5 concentration from 0 to 30 nM, then decreased at concentrations up to 200 nM, but never falling below 10 (Figure 5B). A titration of the ER component in the presence of 5 nM or 100 nM cyclononane-Cy5 gave the highest assay signal windows (~10,000), Z' values (~0.9), and S:B ratios (0.75 to 0.85) when 100 nM cyclononane-Cy5 was used with a concentration of ER near 0.3 nM (Figure 5C,D). Due to the nearly identical K_d values obtained from titrations at either 5 nM or 100 nM cyclononane-Cy5 (0.4 nM or 0.3 nM, respectively), concerns regarding receptor depletion were alleviated for conditions within this range, and a concentration of tracer that gave high Z' values and S:B ratios was chosen for further assays. Therefore, the recommended conditions for this assay were determined to be 0.3 nM ER, 50 nM of nonane-Cy5 and 0.5 nM SA-Eu. The ligand competition confirmatory assay, performed using these conditions, was designated as A2, and it was very valuable in identifying compounds that proved to be false positives in the CBI assay because they had conventional antagonistic activity (see below, cf. Figure 6).

SRC3 competition confirmatory assay (A3)—A simple modification of the primary TR-FRET assay was used as a second counterscreen to distinguish false positive from true positive CBI hits: Assays of positive CBI hits were repeated using two different concentrations of the SRC3-Cy5 (2 nM and 20 nM). The measured IC_{50} values for true CBIs increased at the higher SRC3-Cy5 concentration because SRC3-Cy5 competes directly with these compounds for binding at the coactivator binding site. By contrast, the IC_{50} values of false-positive CBIs that were actually functioning as conventional antagonists were unaffected by changes in the SRC3-Cy5 concentration, because they were competing against an unchanged concentration of the agonist ligand estradiol. The assay signal window, Z' factor, and S:B can be extrapolated from the results presented above, when run with identical components and a 10-fold higher concentration of SRC3-Cy5. This second confirmatory assay was designated as A3.

The combined use of Assays A1-A3 to identify CBIs and distinguish them from CAs—

The two positive controls, the SRC1-Box II peptide and the pyrimidine CBI, were titrated in the original assay of CBI activity (A1) and in both confirmatory assays, the ligand-binding assay (A2) and the CBI assay with 10-fold higher coactivator (A3) (Figure 6). The peptide showed the following activities: an IC_{50} of 0.6 μM in the primary CBI assay (A1), an IC_{50} of $>40 \mu M$ in the ligand binding assay (A2), and an IC_{50} of 28.7 μM in the 10-fold higher coactivator assay (A3). Thus, from the large (46-fold) increase in IC_{50} values in going from the CBI assay A1 to A3, the peptide appears to have good CBI activity. Also, as explained below, its potency in the ligand binding assay (A2) is actually very low; so, a CA mechanism cannot account for its CBI activity. The pyrimidine small molecule control showed somewhat lower potency as a CBI, displaying IC_{50} 's of 6.2 μM , 19.7 μM , and 69.9 μM in A1, A2 and A3, respectively. Again, the 11-fold increase in IC_{50} values between the assays A1 and A3 indicates that the pyrimidine has CBI activity, and its activity in the ligand binding assay (A2) cannot account for its CBI activity.

While both peptide and pyrimidine show activity in the ligand binding assay (A2) (IC_{50} values are $>40 \mu M$ and 19.7 μM , respectively), these values actually represent very low ligand binding affinities, because in the A2 assay, compounds are competing against only 50 nM of the ER ligand cyclononane-Cy5, whose affinity is only 23% that of estradiol, whereas, if they were functioning as CAs in the CBI assay, they would need to compete against 1 μM estradiol. Thus, the ligand-binding assay A2 is ca. 80 fold more sensitive to CA binding than are the CBI assays (A1 and A3); this factor derives from the 20-fold ligand concentration ratio (1 μM estradiol vs. 50 nM cyclononane-Cy5) and the 4-fold ligand affinity difference (100% for estradiol vs. 23% for cyclononane-Cy5).

From the IC_{50} values in the A2 assay above, the known K_i of cyclononane-Cy5 (0.9 nM) and its concentration in this assay (50 nM), we can estimate the K_i values for peptide and pyrimidine binding to the ligand binding pocket as ca. 200 nM, which is 1000-times lower affinity than estradiol (K_d 0.2 nM). Thus, for these compounds to work in the CBI assay by a CA mechanism, whereby they would need compete with 1 μM estradiol, one would expect them to have IC_{50} values of ca. 1000 μM . Because their IC_{50} values in both CBI assays are ca. 100-fold less than this, one can presume that they are functioning as true CBIs in these assays. These results support the use of the peptide and pyrimidine CBI as control compounds in these assays and established the expected profile for newly-discovered CBIs.

Furthermore, the ability of this assay set to identify and exclude false positive hits working through a CA mechanism was verified using a known ER ligand antagonist, tamoxifen (Figure 6C). As expected, this compound appeared to be a CBI in the A1 assay with an IC_{50} of 24.6 μM ; however, further examination of the hit profile confirmed that this compound was indeed a more potent ER ligand, having an IC_{50} in the A2 assay of 1.2 μM . The affinity of tamoxifen for the ligand binding site as determined by radiometric methods (data not shown) is 1% the affinity of estradiol. Performing the same calculations as those described above, one obtains an expected IC_{50} value for tamoxifen in the A2 assay of 1.3 μM , the same as the experimental results, 1.2 μM . Significantly, both CBI assays (A1 and A3) gave very similar IC_{50} values (24.6 and 33.9 μM , respectively). Because there was no significant shift in potency with increased coactivator concentration, this compound does not directly compete with coactivator for the coactivator binding groove. Thus, we find, as expected, that tamoxifen would appear in a screen as a false positive CBI by working through a CA mechanism, but would be identified as such by the combined use of the three TR-FRET assays, A1, A2, and A3. We implemented this approach to hit evaluation to identify false positive CAs for the remainder of the CBI hits generated from HT screens.

Non-Fluorescence Based Confirmatory ELISA

As a low-throughput confirmatory assay for CBI active compounds that does not rely on fluorescence, we adapted a standard enzyme-linked immunosorbent assay (ELISA) to detect the interaction between ER and SRC3 (Figure 7A). Different sources of estrogen receptor antibodies were tested to determine which would be suitable for ELISA development. The optimized conditions for the ER α (Figure 7B) and ER β (Figure 7C) ELISAs show minimal nonspecific binding with the ligand or the other assay components. When using approximately 500 ng/mL of either estrogen receptor protein, 80 ng monoclonal antibody and 60 nM biotin-labeled SRC3 NRD, the S:N ratios for the ER α and ER β ELISAs were 5 and 8, respectively. Under these conditions, the SRC1-Box II peptide (Figure 7D) and pyrimidine CBI (Figure 7E) positive controls were tested. The peptide was active with a IC_{50} of 0.07 μ M (ER α) and 0.6 μ M (ER β). Pyrimidine CBI showed inhibition with a IC_{50} of 23 μ M on ER α , but was not active on ER β at concentrations lower than 50 μ M.

High-Throughput Format Development and Validation in HTS format

The CBI FRET was validated for use in a HTS format by screening a total of 86,106 compounds from Molecular Library Screening Center Network. The well-to-well and plate-to-plate variation present in the assay was determined by conducting experiments on separate days. Each plate contained 16 wells of free 2 nM SRC3-Cy5 with 0.5 nM SA-Eu, 2.6% DMSO and 1 μ M estradiol (i.e., lacking ER α -LBD) to give the minimal FRET signal expected and 16 wells of bound ER α -417 (0.3 nM)/SRC3-Cy5 (2 nM) with 0.5 nM SA-Eu, 1 μ M estradiol and 2.6% DMSO as vehicle to give the maximal FRET signal expected. Each well contained a total volume of 19 μ L dispensed by an automated robotic system. These measurements also allowed calculation of expected fluorescence values that could be used as standards in eliminating false positive hits due solely to compound fluorescence interference. The donor counts measured at 615 nm for the maximum signal controls were recorded and compared to that of each tested compound. Compounds with fluorescence outputs greater than 3 standard deviations from the average fluorescence output of the controls were considered to interfere with the assay and were discarded. The average FRET signal per plate from ER/SRC-Cy5 wells was consistent across plates and from day to day (Figure 8A). The average FRET signal from 260 plates was 4191.9 with a standard deviation of 625.3. The S:B ratios, as determined from each plate, were consistently higher than 100, and the average S/B from 260 plates was 247.4 with a SD of 58.9 (Figure 8B). The Z' factor range from 0.7 – 0.9 with average Z' of 0.87 (Figure 8B), demonstrating a robust and consistent assay. Compounds that inhibit the binding of ER to SRC-Cy5 by more than 50% were considered potential positives, and the representative results for 15,000 compounds are shown in Figure 8C. From a screen of 86,106 compounds, a total of 1442 compounds were identified as potential positives, which resulted in a hit rate of 1.67%. This TR-FRET assay, built to discover compounds that inhibit the binding of ER to SRC3, is well-suited for HTS and has been used to screen large compound libraries. The results of these screens will be described in subsequent publications.

SUMMARY

These time-resolved fluorescence resonance energy transfer (TR-FRET) assays, all of which can be performed in a simple “mix and measure” format, have collectively been validated as a robust and reliable system for the discovery of small molecule compounds that disrupt the interaction between the estrogen receptor and steroid receptor coactivators. Agonist-bound estrogen receptor (labeled site-specifically on biotin with streptavidin-europium) recruits a coactivator protein (SRC3 fragment labeled with Cy5), and this interaction was monitored using TR-FRET. The disruption of this binding by both peptide controls and small molecule compounds could be followed by a decrease in FRET signal. Confirmatory TR-FRET assays

were developed and optimized to identify false positive hits, namely, conventional ER antagonists that preclude coactivator binding by an indirect mechanism in which the agonist ligand, estradiol, is displaced from the ligand binding pocket of ER. Additionally, a confirmatory assay using a colorimetric readout was optimized and implemented to eliminate any positive hits based purely on fluorescence interference. These assays, which demonstrated high stability, solvent tolerance and good performance with small volumes, were particularly well suited for HTS. The calculated Z' values and S:N ratios for the assays were also well above the minimal levels needed for a HTS format. The primary assay, which was used to screen approximately 86,000 compounds for CBI activity on both ER α and ER β , is flexible and could easily be modified to screen large chemical libraries to discover CBIs, as well as conventional antagonists, for other nuclear receptors. Full HTS and follow-up medicinal chemistry results will be described in forthcoming publications from our laboratories.

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Figure 1. Schematic representation of FRET-based coactivator binding inhibitor (CBI) assay: ER in presence of agonist estradiol (**A, center**), with a competing CBI (**A, right**), and with a competing conventional antagonist (CA; **A, left**). Schematic representation of FRET-based CA-binding assay (**B**).

Figure 2.

Optimization of TR-FRET CBI Assay. Cy5-labeled steroid receptor coactivator (SRC-Cy5) titrated into solution of biotinylated estrogen receptor (ER-Biotin), streptavidin-europium chelate (SA-Eu) and estradiol (**A**). ER-biotin titrated into solution of SRC3-Cy5, SA-Eu and estradiol (**B**). Z' and signal/background ratios (S/B) across SRC3-Cy5 (**C**) and ER-biotin (**D**) titrations.

Figure 3. Assay Stability. CBI FRET signal at various time points (A) and at various concentrations of DMSO (B). Z' and S/B ratios for assay with increasing DMSO concentration (C).

Figure 4. Effect of time and temperature on positive controls. Peptide positive control SRC1-Box II (**A** and **C**) and previously reported small molecule positive control pyrimidine CBI (**B** and **D**).

Figure 5. Optimization of CA-binding assay. Titration of cyclononane-Cy5 using 0.3 and 1 nM of ERα (A) and titration of ER using 5 and 100 nM of cyclononane-Cy5 (C). Z' and S/B ratios (B, D) for CA-optimization. Structure of fluorescent ligand, cyclononane-Cy5 (E).

Figure 6. Evaluation of positive controls in the primary assay and the confirmatory assays. 15-mer peptide (**A**), pyrimidine CBI (**B**), and conventional antagonist tamoxifen (**C**) evaluated in CBI (A1), antagonist ligand competition (A2) and coactivator competition (A3) assays. Summary table of concentrations used in each assay (**D**).

Figure 7. Non-fluorescence based confirmatory ELISA. Schematic diagram of colorimetric confirmatory assay (**A**) with evaluation of expected maximal and minimal assay values (**B** and **C**) for ER α and β , respectively. Positive control peptide, SRC1-Box II, (**D**) and small molecule, pyrimidine CBI, (**E**) were also evaluated in this format.

Figure 8.

High-throughput format assay validation. CBI assay validated for HTS format using 86,106 compounds from Molecular Library Screening Center Network. Average FRET signal (A) and Z' factor and S/B (B) from ER/SRC-Cy5-containing wells evaluated across 260 384-well plates. (C). Percent inhibition produced by each compound versus compound identification number (ID) for 15,000 representative compounds.