Supplemental Material and Methods

Transfection

Cells were transfected with X-tremeGENE HP (Roche) according to the manufacturer's protocol. For experiments involving estrogen induction, cells were transfected in phenol red-free DMEM-F12 containing 10% charcoal-stripped FBS (hormone-depleted medium) (Invitrogen) and E2 was added 24 hours post-transfection.

Cloning and Mutagenesis

Point mutations were introduced into pcDNA-HA-ERα using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) as indicated in the manufacturer's instructions. Mutations generated are validated by Sanger sequencing using the following primers:

5'- GATTGGCCAGTACCAATGACA – 3'

5' - GATGATTGGTCTCGTCTGGCGCTCC – 3'

5' – CTGGAGACATGAGAGCTGCCAACCTT – 3'

Retroviral infection and generation of stable cell lines

All stable MCF7 cell lines with doxycycline inducible HA-ER WT and mutants were generated as stated in (1).

Immunoblotting

Cells were plated at 0.5 x 10^6 cells/well into 12-well culture plates (Costar 3513) in phenol red-free medium supplemented with 5% charcoal-stripped serum 24h before transfection or drug treatments. Cells were transfected according to the manufacturer's instructions. Cells were washed once with cold PBS, scraped off the plate, briefly

centrifuged to pellet down the cells and PBS was removed. The pellets were resuspended in RIPA lysis buffer (Pierce), supplemented with protease and phosphatase inhibitors (Pierce) and vortexed briefly. Lysates were cleared by centrifugation at 14,000 g for 10 minutes and protein concentrations of samples were determined using the BCA kit (Pierce). Thirty micrograms of each sample was run through 4-12% SDS-PAGE minigels (Invitrogen). Membranes were probed overnight with primary antibodies (ERa, Thermo Fisher Scientific SP1; PR, Dako PgR636; GAPDH, CST 2118, HA, CST 3724, Actin, CST 4970, phospho ER Serine118, Signalway Antibody 11072) followed by incubation with HRP-tagged secondary antibodies (CST 7074 or 7076) and visualized on a Syngene ChemiGenius with Super-Signal West Dura Chemiluminescence Substrate (Pierce).

Luciferase Assays

Cells were plated at a density of 4×10^6 per 10mm plates in hormone-depleted medium one day prior to transfection. Cells were transfected with 2.5 ug of HA-ERα wild type or mutants, 6.3 ug of 3x-ERE-TATA-Luciferase reporter and 1.2 ug of pRL-TK Renilla Luciferase plasmid per plate, using X-tremeGENE™ HP (Sigma-Aldrich). The cells were treated either with E2 or various antagonists as indicated a day after transfection for 24 hours and luciferase activities were determined using the Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's instructions. Luciferase bioluminescence measurements were performed with the Veritas™ Microplate Luminometer (Promega). All experiments were conducted in triplicate and the Firefly luciferase activity was normalized with the Renilla luciferase activity of each sample.

Quantitative RT-PCR

RNA was extracted from cells 48h post-transfection using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from 2 ug of RNA of each sample using the SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen) as per manufacturer's protocol. Synthesized cDNA was diluted with one volume of DEPC-treated water and 2 ul of the mixture was mixed with TaqMan® PCR Master Mix (Applied Biosystems) and primers. The relative quantification of each mRNA was performed using the comparative Ct method with a ViiA™ 7 Real-Time PCR system (Applied Biosystems). Samples were run in triplicate and normalized to the levels of *ACTB* for each reaction.

Proliferation assay with doxycycline-inducible HA-ER WT and mutant cell lines

Cells were plated at 1500 cells/well into 96-well culture plates in either hormone-deprived or regular media, treated with the indicated doses of doxycycline, fulvestrant or vehicle control, incubated in a humidified incubator with 5% CO_2 at 37^{o}C. They were assayed for cell proliferation at Day 0, 3, 5 and 7 using the Resazurin reagent (R&D Systems), which upon addition, culture plates were incubated at 37^{o}C incubator for 4 hours before their fluorescence were read at 560nm excitation and 590nm emission wavelengths with a microtiter plate reader (Molecular Devices).

Proliferation assay with parental and Y537S CRISPR knock-in cell lines

Cells were plated at 2000 cells/well into 96-well culture plates (Costar 3595), treated with test compounds or vehicle control (0.1% v/v) and incubated in the IncuCyte Zoom (Essen

Bioscience) in a humidified incubator with 5% CO_2 at 37 ^{o}C. They were monitored for % confluence using IncuCyte Zoom standard software over several days until vehicle control wells reached maximal confluency.

Time Resolved-FRET Assays

Protein Preparation for TR-FRET: Site-directed mutagenesis was used to generate the Y537S, D538G, S463P and E380Q mutations in the ligand binding domain (LBD) of the human estrogen receptor α (ERα amino acids 304-554). The WT and mutant ERα and the nuclear receptor domain (NRD) of human SRC3 encompassing three NR boxes (amino acids 627-829) were expressed in *E. coli*, using methods reported previously (2,3). ER LBDs of wild type and D538G were prepared as 6×His fusion proteins, with a single reactive cysteine at C417. While bound to the Ni-NTA-agarose resin (Qiagen Inc., Santa Clarita, CA), the ERs were labeled with MAL-dPEG4-biotin (Quanta BioDesign, Powell, OH), site-specifically at C417. The SRC3-NRD construct has 4 cysteines and was labeled non-specifically, also while on the resin, with 5-iodoacetamido fluorescein (Molecular Probes, Invitrogen, Eugene, OR). It was previously determined that an average of 1.8-2 cysteines are attached to the SRC3 NRD (4).

SRC titration: SRC3 was titrated into a fixed amount of ERα-LBD-biotin mixed with SaTb (streptavidin-terbium, Invitrogen, Grand Island, NY), on 96-well black microplates (Molecular Devices, Sunnyvale, CA) following previously determined methods (2). The time-resolved Förster resonance energy transfer (tr-FRET) measurements were performed with a Victor X5 plate reader (Perkin Elmer, Shelton, CT) with an excitation filter at 340/10 nm and emission filters for terbium and fluorescein at 495/20 and 520/25 nm,

respectively, with a 100 μs delay. Diffusion-enhanced FRET was determined by a parallel incubation without biotinylated ER-LBD and subtracted as a background signal. The final concentrations of reagents were: 1 nM ERα-417, 0.25 nM streptavidin-terbium, 1 μM ligand, SRC3-F1 coactivator titrated from 3.2×10^{-7} to 3.2×10^{-12} M. The data, representing 2-3 replicate experiments, each with duplicate points, were analyzed using GraphPad Prism 4 and are expressed as the EC_{50} in nM.

Ligand titration: Ligands were titrated into a constant amount of ER-LBD-biotin, SaTb, SRC3-F1. The final concentrations were 1 nM ER-LBD, 0.25 nM SaTb, 100 nM SRC3-fluorescein, and increasing ligand concentrations from 1×10-12to 1×10-6 M. Diffusion-enhanced FRET was determined by a parallel incubation without biotinylated ER-LBD and subtracted as a background signal. The tr-FRET was measured with a Victor X5 plate reader as outlined above. The data, representing 2–3 replicate experiments, each with duplicate points, was analyzed using GraphPad Prism 4, and are expressed as the EC50 in nM.

Plasma Bioanalysis - AZD9496

Each plasma sample (25 ml) was prepared using an appropriate dilution factor, and compared against an 11 point standard calibration curve (1-10000 nM) prepared in DMSO and spiked into blank plasma. Acetonitrile (100 ml) was added with the internal standard, followed by centrifugation at 3000 rpm for 10 minutes. Supernatant (50 ml) was then diluted in 300ml water and analyzed via UPLC-MS/MS.

Plasma Bioanalysis - Fulvestrant

Each plasma sample (25 ml) was prepared using an appropriate dilution factor, and compared against an 11 point standard calibration curve (1-10000 nM) prepared in DMSO and spiked into blank plasma. Water (20 ml) was added with the internal standard, followed by the additon of 500 uL MTBE. Samples were vortexed for 1 minute before centrifugation at 3000 rpm for 5 minutes. Supernatant (400 ml) was then removed to a clean tube and blown down under nitrogen at 35ºC. Samples were reconstituted in 200ml water, methanol, formic acid (90:10:0.1%) and analyzed via UPLC-MS/MS.

Trypsin proteolysis

Protein was prepared and labeled as described above for the time resolved-FRET assays. It was incubated in t/g buffer with or without 1 μM of ligand, at room temperature for 1 hr. Then, 1 μg trypsin per unit of protein was added for 10, 30, 60, and 300 min at room temperature according to previously established methods (5). FRET signal was measured using a Victor X5 plate reader as outlined above. The data, representing 2–3 replicate experiments, were analyzed using GraphPad Prism 4, and are expressed as half-lives (t½).

References

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