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# Identification of Compounds by High-Content Screening That Induce Cytoplasmic to Nuclear Localization of a Fluorescent Estrogen Receptor α Chimera and Exhibit Agonist or Antagonist Activity In Vitro

**Angie B. Dull[1], Anuja A. George[2], Ekaterina I. Goncharova[3], Jason R. Evans[3], Antony Wamiru[1], Laura K. Cartner[4], Gordon L. Hager[2], and James B. McMahon[4]**

**Abstract**

We have completed a robust high-content imaging screen for novel estrogen receptor α (ERα) agonists and antagonists by quantitation of cytoplasmic to nuclear translocation of an estrogen receptor chimera in 384-well plates. The screen was very robust, with Z′ values >0.7 and coefficients of variation (CV) <5%. The screen utilized a stably transfected green fluorescent protein–tagged glucocorticoid/estrogen receptor (GFP-GRER) chimera, which consisted of the N-terminus of the glucocorticoid receptor fused to the human ERα ligand binding domain. The GFP-GRER exhibited cytoplasmic localization in the absence of ERα ligands and translocated to the nucleus in response to stimulation with ERα agonists and antagonists. The BD Pathway 435 imaging system was used for image acquisition, analysis of translocation dynamics, and cytotoxicity measurements. We screened 224,891 samples from our synthetic, pure natural product libraries, prefractionated natural product extracts library, and crude natural product extracts library, which produced a 0.003% hit rate. In addition to identifying several known ER ligands, five compounds were discovered that elicited significant activity in the screen. Transactivation potential studies demonstrated that two hit compounds behave as agonists, while three compounds elicited antagonist activity in MCF-7 cells.

**Keywords**

estrogen receptor, high-content screening, cell-based assay, nuclear translocation, cytotoxicity

## Introduction

Estrogen receptor α (ERα)–mediated signal transduction is a complex pathway, which regulates proliferation, differentiation, and reproductive physiology. Unliganded ERα exists in a heterocomplex of chaperone proteins that maintains proper folding of the protein and keeps the receptor in a repressed, nontranscriptional state in the absence of hormone. This heterocomplex consists of the ERα, an Hsp90 dimer, p23, and FKBP52.[1] This complex undergoes nucleocytoplasmic shuttling, but under steady-state conditions, it is localized predominantly in the nucleus. Ligand binding induces a conformational change in the receptor, which results in loss of chaperone binding, leading to coactivator binding, receptor dimerization, and binding to DNA at estrogen response elements (EREs) to initiate the transcription of ERα-regulated genes.[1] ERα can also alter transcription by indirectly interacting with DNA by a tethering mechanism mediated by protein-protein interactions with DNA-bound transcription factors, such as AP-1,[2,3] nuclear factor (NF)–κB,[4] and SP1.[5,6] Elevated estrogen levels can lead to initiation, promotion, and progression of breast tumors by several pathways in postmenopausal women. Estrogen production from the ovaries ceases following

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menopause, and the source of estrogen in postmenopausal women is conversion of androgens to estrogens in peripheral tissues, including the breast. ER signaling through the nucleus, mitochondria, and nongenomic signaling at the plasma membrane lead to rapid cell proliferation that may lead to elevated mutation rates, altered cell-cycle control, and inhibition of apoptosis, which perpetuate the growth and survival of the cancer cell. These mechanisms of carcinogenesis mediated through estrogen signaling illustrate the importance of targeting ERα for therapeutic intervention.

Discovery of compounds that target the estrogen receptor and exhibit reduced incidence of adverse effects are crucial for the treatment of breast cancer and hormone replacement therapy (HRT) for women. These adverse effects are related to the mixed agonistic/antagonist activity of a given drug, which is dependent on tissue, cell, and promoter, coactivator, or corepressor expression profiles. While HRT reduces menopausal symptoms, maintains bone mineral density, and decreases the risk of colon cancer, these drugs also elevate the risk for the development of breast cancer, coronary heart disease, stroke, and Alzheimer disease and blood clots.[7–9] Selective estrogen receptor modulators (SERMs) are drugs that elicit agonism or antagonism depending on tissue, cell, promoter, or coregulator expression. Tamoxifen is a SERM that is used for the treatment of hormone-responsive breast cancer, which is typically used as an adjuvant therapy after surgery and radiation. Tamoxifen is a competitive inhibitor, which competes with estradiol for binding to the ligand binding domain of the ER, which is followed by recruitment of corepressors, resulting in transcriptional inhibition. The levels of coactivators and corepressors that interact with ER play a role in determining whether ER that is bound to tamoxifen behaves as an antagonist or partial agonist.[10] While tamoxifen exhibits its ER antagonistic activity in the mammary tissue, this drug also exhibits partial agonistic activity in the uterine tissue, which increases the risk for the development of endometrial cancer and uterine sarcoma.[11] Tamoxifen mediated agonistic activity is also found in bone, liver, and the cardiovascular system. Agonist activity of tamoxifen in the skeletal tissues facilitates maintenance of bone density.

Nearly half of patients do not initially respond to tamoxifen treatment. Patients with metastatic disease are likely to develop tamoxifen resistance, while 30% to 50% of patients with early stage ER-positive breast cancer who are administered tamoxifen relapse with resistant disease.[12] Tamoxifen is metabolized to two known active antagonistic metabolites with similar affinities for ER: 4-hydroxy-N-desmethyl tamoxifen (endoxifen) and 4-hydroxytamoxifen (4-OHT).[13] CYP2D6 metabolizes tamoxifen to endoxifen, which is a more efficacious, clinically active metabolite than 4-OHT. Recent studies of patients treated with tamoxifen revealed that genetic polymorphisms of the cytochrome P450 CYP2D6 correlate with a higher risk of recurrence,[14,15] which may be responsible for intrinsic tamoxifen resistance. Further studies are needed to examine the role of genetic polymorphism regarding endocrine resistance. Tamoxifen has also been demonstrated to induce nonalcoholic steatohepatitis in humans,[16,17] which is a fatty acid disease that can develop into hepatocarcinoma or cirrhosis of the liver. Several studies in rats have demonstrated that tamoxifen is a potent genotoxic and hepatocarcinogenic in rats,[18] and its carcinogenesis is based on the ability of tamoxifen to be both a tumor initiator and tumor promoter in rat liver.[19,20]

The adverse effects and endocrine-resistant disease associated with current drug therapies illustrate the importance of targeting ERα for therapeutic intervention through novel approaches. In this article, we have presented the screening results for a high-content nuclear translocation imaging assay using a green fluorescent protein (GFP)–tagged estrogen receptor chimera in an effort to discover novel compounds that may function as therapeutic targets for improved HRT or breast cancer therapeutics. Five compounds were identified that demonstrated ERα agonist and antagonist activity in MCF-7 cells.

## Materials and Methods

### Chemicals and Reagents

*Reagents for high-content screening and subsequent dose-response experiments.* Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), and G418 were purchased from Mediatech (Manassas, VA). The penicillin/streptomycin solution, trypsin-EDTA, staurosporine, and 37% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Sodium butyrate was purchased from Millipore (Billerica, MA). The 384-well glass-bottom plates were acquired from Matrical (Spokane, WA). Hoechst 33342 was obtained from Invitrogen (Carlsbad, California). Characterized fetal bovine serum (FBS) and charcoal/dextran-treated FBS were obtained from Hyclone (Logan, UT). The following known ERα ligands were acquired from Sigma-Aldrich: 17-β estradiol (E2), estrone, 4-OHT, and estriol. Bortezomib was acquired from Millennium (Cambridge, MA).

*Reagents for luciferase reporter assays.* The DMEM, RPMI, penicillin-streptomycin, and Lipofectamine LTX reagents were purchased from Invitrogen (Grand Island, NY), and FBS was acquired from Atlanta Biologicals (Lawrenceville, GA). The charcoal-stripped fetal bovine serum was purchased from Equitech-Bio (Kerrville, TX). The Bright-Glo luciferase assay system was purchased from Promega (Madison, WI). Raloxifene, MG132, ICI

182,780, and RU486 used in luciferase reporter assays were acquired from Sigma-Aldrich. Bortezomib was acquired from Millennium.

### Cell Culture

The 6020 cells were cultured in DMEM and supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mg/mL G418, and 10% FBS. The C127 and MCF-7 cells were grown in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS. The cells were cultured in a humidified incubator at a 37 °C, 5% $CO_2$, 95% air environment.

### Library Plate Dilutions

Synthetic compound libraries were screened at 10 µM for pure compounds, and natural product extracts were screened at 10 µg/mL. Library dilution plates were made in complete media containing charcoal/dextran-treated FBS. Dilutions were made using the Biomek FX liquid-handling instrument (Fullerton, CA). Controls were added to columns 1 and 2 on each plate 384-well plate. DMSO (0.5% final concentration) served as the negative control, which was located in rows A through H, columns 1 and 2. Estradiol (5 µM final concentration), used for the positive control, was located in rows I through P in columns 1 and 2. Due to time constraints on the third day of the assay, dilution plates were made the day before and frozen overnight at −20 °C.

### Nuclear Translocation Assay

The 6020 cells were plated into 384-well Matrical glass-bottom black plates at 3500 cells/well and cultured in DMEM, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mg/mL G418, and 10% charcoal/dextran-treated FBS. Charcoal/dextran-treated FBS was used when the cells were plated into assay plates, which had been stripped of hormones or other growth factors, which may be ligands for the ER and stimulate nuclear translocation of the GFP-GRER receptor. The 6020 cells were seeded into 384 plates at 50 µL/well (or 100 µL/well for 96-well plates) using a Bio-Tek MicroFill microplate dispenser (Winooski, VT) and incubated at 37 °C, 5% $CO_2$, 95% air overnight. Twenty-four hours after seeding the cells into assay plates, cells were treated with 70 mM sodium butyrate (25 µL/well for 384-well plates) using a Beckman FX liquid-handling device (Fullerton, CA). Cells were treated with sodium butyrate for 24 h to induce expression of the GFP-GRER chimera, which is located in a silent region of the genome. The following morning, cells were treated with ER ligand dose-response plates or chemical library plates for 6 h. The Beckman FX was used to add 10 µL of the ER ligand dose-response plates or chemical dilution plates to the assay plates. After a 6-h treatment with dose responses or chemical library plates, the assay plates were subjected to fixation with 4% formaldehyde for 45 min. After fixation, the assay plates were washed 5 times with 100 µL DPBS using a Bio-Tek plate washer. The plates were stained with 0.3 µg/mL Hoechst 33342 in PBS, which was added (15 µL/well) using a Bio-Tek MicroFill microplate dispenser to the assay plates. The plates were stained overnight at 4 °C, and the next day, assay plates were washed two times with 100 µL DPBS using a Bio-Tek plate washer. Plates were sealed with aluminum sealing tape and barcoded with a Velocity-11 VCode Bar Code Label Print and Apply Station (Menlo Park, CA).

### Image Acquisition and Nuclear Translocation Analysis

Images were acquired using a BD Pathway 435 imaging system (Rockville, MD) integrated with a Thermo CRS catalyst-5 robotic arm (Waltham, MA), and a Symbol barcode reader (Schaumburg, IL) was used for unattended imaging. An Olympus 20× 0.75 NA objective was used for image acquisition, four sites (montages) were acquired per well, and GFP (180 ms, gain = 10) and Hoechst (30 ms, gain = 0) filters were used. Translocation dynamics were quantitated with BD Attovision software using a ring-based (two-output) algorithm, and a GFP threshold of 300 to 4095 gray values was used for segmentation of the cytoplasmic area. Nuclear areas were segmented by Hoechst staining, and GFP intensity was measured in that defined region of interest. The cytoplasmic region of interest (ROI) for GFP fluorescence was measured by a 20-pixel dilation from the nuclear boundary, and only thresholded GFP was quantitated in the cytoplasmic region. The nuclear/cytoplasmic ratios were measured, followed by application of a translocation threshold, and percent positive for translocation was reported. Images were written to a terabyte server during image acquisition. Attovision and Image Data Explorer software (BD Biosciences, Rockville, MD) were used for analysis of image data and data mining. SigmaPlot (San Jose, CA) software was used to generate dose-response curves and calculate $EC_{50}$ values based on a sigmoidal, logistic, four-parameter regression method.

### Hit Determination

The $Z'$ values were calculated as described in Zhang et al.[21] Compounds that elicited a translocation value of ≥72% were identified as primary hits, which is three standard deviations from the mean percent nuclear translocation with 5 µM estradiol. These hits were analyzed for average nuclear area, and compounds that generated a nuclear area below 380 pixels were eliminated as false positives due to

cytotoxicity. The hits were further evaluated for the number of cells or ROIs, and wells with less than 30 ROIs were eliminated. The remaining primary hits were cherry picked and retested in the 6020 cell line and in the parental C127 cell line. Cherry-picking experiments confirmed the activity in the 6020 cell line and also eliminated false positives caused by fluorescent compounds localized to the nucleus in the parental C127 cell line.

### *Luciferase Assays*

MCF-7 human breast cancer cells were maintained in DMEM supplemented with 10% FBS and 100 U/L penicillin/streptomycin. Prior to transfection, MCF-7 cells were transferred for 24 h to phenol red–free DMEM supplemented with 10% charcoal-stripped FBS and 100 U/L penicillin/streptomycin. MCF-7 cells were plated at a density of 100,000 cells/well in 96-well plates in phenol red–free DMEM with 10% charcoal-stripped FBS. On the following day, 200 ng of plasmid ERE-tk-luc was transfected per well using Lipofectamine LTX Invitrogen (Grand Island, NY) reagent, according to the manufacturer's instructions. The next day, the transfected cells were induced with 10 μM of indicated compounds for 24 h. For estradiol competition assays, cells were treated with estradiol alone (100 nM), hit compound alone (100 nM), and 100 nM estradiol that was challenged with 100 nM, 1 μM, or 10 μM of hit compound. Luciferase activity was measured by the Promega Bright-Glo luciferase assay system (Madison, WI) using the manufacturer's instructions and a FLUOstar Optima luminometer (Cary, NC).

## Results and Discussion

The ERα undergoes nucleocytoplasmic shuttling, yet the nuclear compartment is strongly favored even in the absence of hormonal ligands. Unlike many steroid/nuclear receptors, the glucocorticoid receptor (GR) is localized in the cytoplasm in the absence of GR ligands. Constitutive nuclear localization of the native ERα in the absence of ligand presented difficulty for the development of a nuclear translocation screen. Consequently, we used a cell line that stably expressed the GFP-GRER chimeric receptor, which consisted of the N-terminus, DNA binding domain, and hinge and partial ligand binding domain regions of the GR, which was fused with the human ERα ligand binding domain and tagged with GFP, which our group previously characterized.[22] Previous experiments have demonstrated that the GFP-GRER remains cytoplasmic in the absence of estradiol-like compounds and that binding of both ERα agonists and antagonists will cause nuclear translocation of the receptor, with the assay exhibiting specificity to ERα ligands.[23] Furthermore, past studies demonstrated that the GFP-GRER does not undergo cytoplasmic to nuclear translocation in response to stimulation with GR ligands.[22] We previously reported the development of this assay for high-throughput screening (HTS), which included determination of appropriate assay media (effects of phenol red and FBS), optimal compound treatment time points for GFP-GRER stimulation, dose response with known ERα agonists and antagonists, and ERβ ligands that were demonstrated not to induce nuclear translocation of the GFP-GRER chimera. In addition, the screen underwent validation using the Library of Pharmacologically Active Compounds (LOPAC), as well as inclusion of additional screening parameters to identify hit compounds, while minimizing identification of false positives, which included cytotoxicity filters and screening in the parental cell line that does not express the GFP-GRER chimera to eliminate compounds that fluoresce and localize to the nucleus.[23]

This assay was developed for high-content screening in 384-well plates, and the assay was very robust, with $Z'$ values >0.7 and CVs <5%, and exhibited specificity for ERα ligands.[23] Additional image analysis filters were incorporated during assay development to eliminate cytotoxic false positives and false positives caused by fluorescent compounds that localized to the nucleus. Hits were identified as compounds characterized with nuclear translocation values ≥72%, nuclear area >380 pixels, and ROIs >30.[23] We included the nuclear area and ROI filters to eliminate false positives from cytotoxic compounds. During assay development for the screen, we determined that toxicity from the crude natural product extracts was particularly troublesome for HTS. During development of the assay, we screened a crude natural product extract library that produced a 12% hit rate, which was excessive and not amenable for HTS.[23] We determined by visual inspection of the images that most of these hits were due to abnormal image processing due to toxicity. After inclusion of the nuclear area and ROI filters, there was a 75% reduction in false-positive hits due to cytotoxicity. Additional methods to eliminate false-positive compounds caused by compound fluorescence were used by screening the hit compounds in the parental cell line that does not express the GFP-GRER construct. Thorough assay development and validation are necessary to increase the specificity of the screen, which is essential for large primary HTS campaigns, and it is important to focus on pursuing and prioritizing compounds that exhibit specificity for the ERα pathway, rather than having a large number of hits that eventually are eliminated downstream.

The assay screened 224,891 samples for translocation of the GFP-GRER receptor from the cytoplasm to nucleus. The screening sample libraries consisted of synthetic, pure natural products: crude natural product extracts and prefractionated natural product extract libraries. The GFP-GRER was localized in the cytoplasm in the absence of ERα

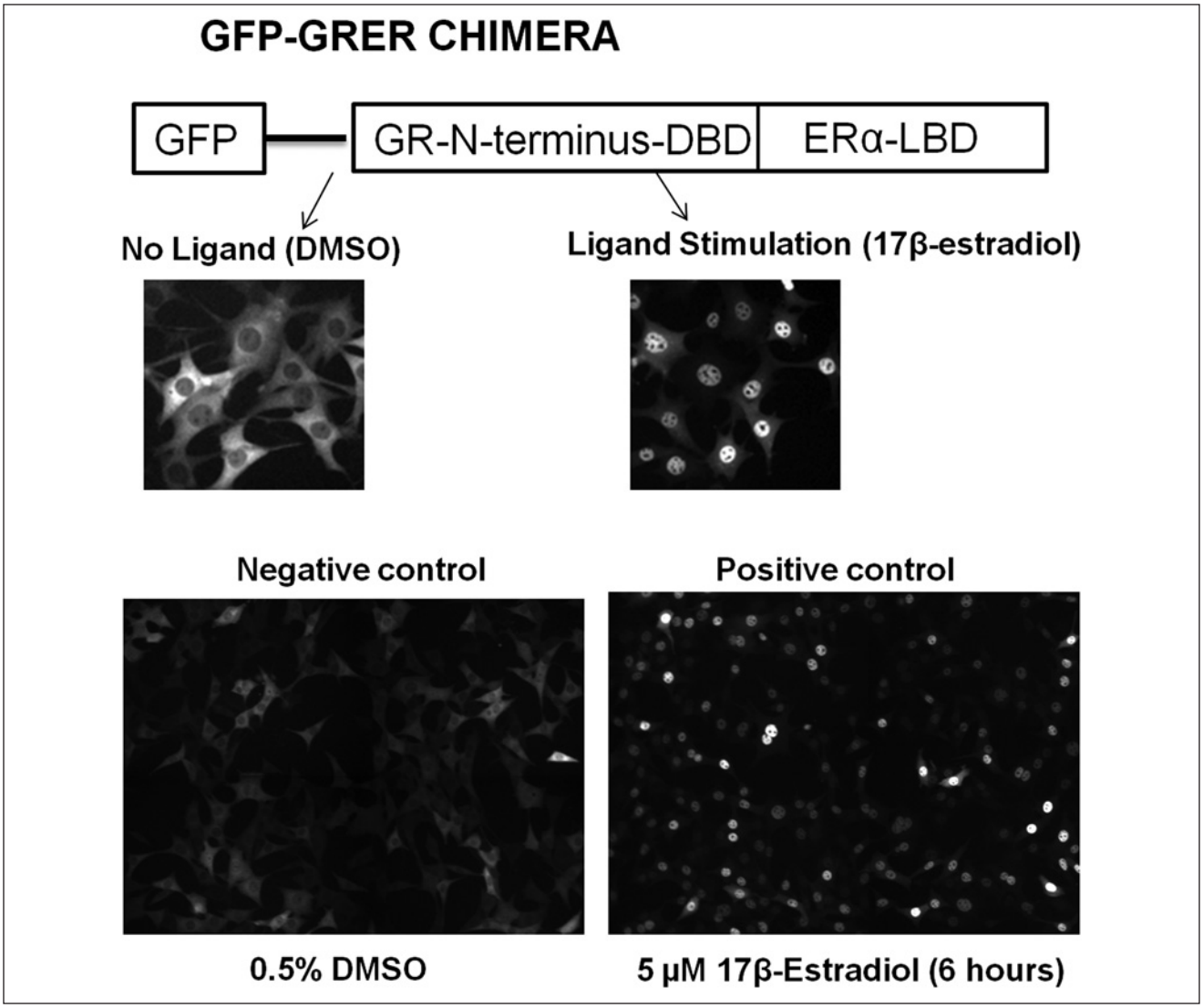


**Figure 1.** Localization of the green fluorescent protein (GFP)–glucocorticoid/estrogen receptor (GRER) chimera after treatment with assay controls. Fluorescent images depict the subcellular localization of the GFP-GRER in response to 0.5% DMSO or 5 μM 17β-estradiol treatments. ERα, estrogen receptor α; LBD, ligand binding domain.

ligands and translocated to the nuclear compartment upon ERα stimulation with 17β-estradiol (**Fig. 1**). Six known ER ligands were identified in the screen, and these compounds included ERα agonists and antagonists, as well as SERMs. Estradiol, estriol, estrone, raloxifene, tamoxifen, and 8-prenylnaringen were the known ERα ligands discovered during the primary screen (**Fig. 2**). 8-Prenylnaringen was purified and identified from a plant extract located in our prefractionated natural product extracts library. The extract was subjected to several rounds of bioassay-guided fractionation to elucidate the structure. We identified the compound as 8-prenylnaringenin, which is a known phytoestrogen derived from the flower of hops, and its taxonomy is *Macaranga bicolor*. This compound was also identified as a hit from another screen in our laboratory for ABCG2 inhibitors, and its isolation and structural elucidation have been previously described.[24] The identification of known ER ligands during the primary HTS further validated our assay and demonstrated that we were able to effectively identify the ER agonists, antagonists, and SERMs; furthermore, the assay was capable of identifying hit compounds from natural product extracts. The identification of 8-prenylnaringen from the prefractionated natural product extracts library and not from our crude natural product extracts library confirmed our theory that prefractionation of the crude natural product extracts library would improve hit identification through elimination of cytotoxic fractions from the crude extracts.

The screen had a 0.003% hit rate, and five hit compounds were identified that included four synthetic compounds and

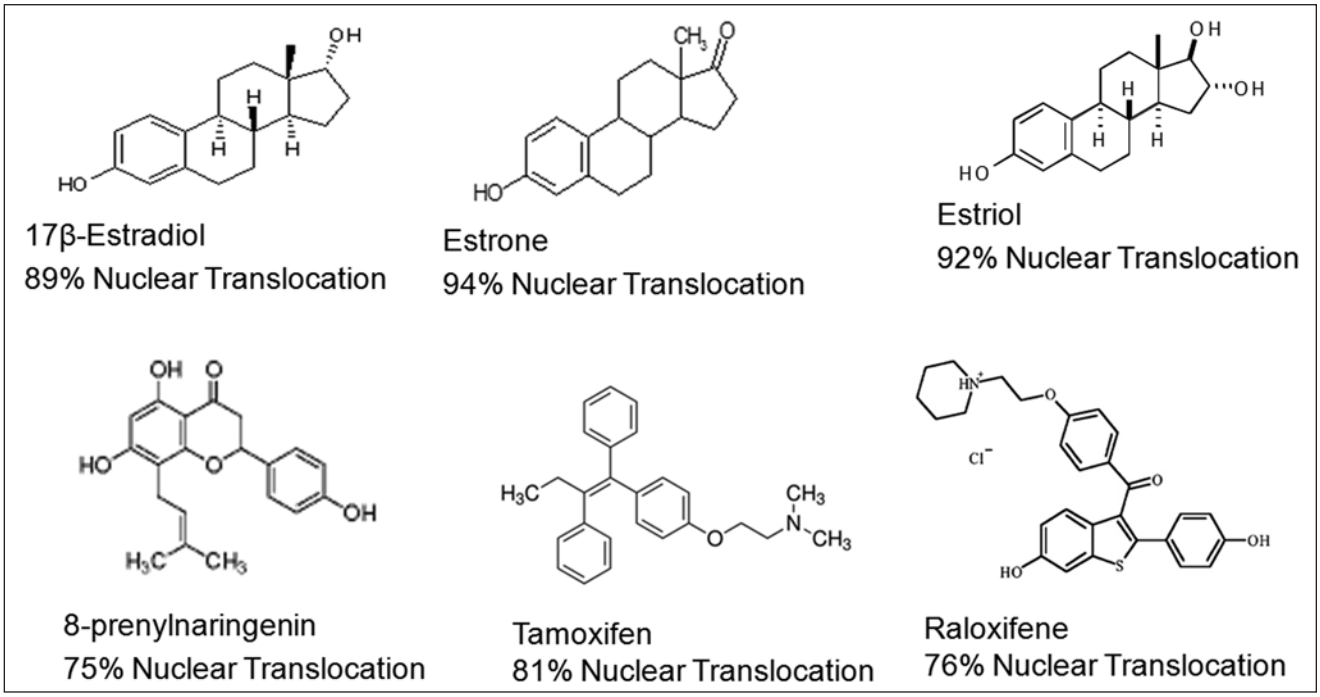


**Figure 2.** Structures of known estrogen receptor (ER) agonists and antagonists identified during the primary high-throughput screen, and corresponding percent nuclear translocation values are shown.

one pure natural product. These hits included MG132, bortezomib, CMLD 1845, DCIII-43, and NSC 333856, which exhibited nuclear translocation values of 84%, 87%, 85%, 90%, and 87%, respectively (**Fig. 3**). The GFP images from the BD Pathway 435 imaging system depict the translocation of the GFP-GRER to the nucleus in response to each of the hit compounds (**Fig. 3**). These hits were confirmed by cherry picking and retesting in quadruplicate in the 6020 assay cell line and in the C127 parental cell line (data not shown). Testing in the parental cell line was essential to eliminate potential false-positive fluorescent compounds that may bind DNA and localize to the nucleus. All five hits were confirmed and did not show fluorescence in the parental cell line. The structures of the five hit compounds are depicted in **Figure 4**. The CMLD 1845 hit is the most structurally similar to estradiol but differed by the presence of a methyl oxirane ether group at position 3 (**Fig. 4**).

Dose-response curves for GFP-GRER nuclear translocation were performed for each hit compound, and MG132, NSC 333856, DCIII-43, bortezomib, and CMLD 1845 hit compounds exhibited $EC_{50}$ values of 192 nM, 4 μM, 71 nM, 37 nM, and 14 μM, respectively (**Fig. 5**). We also compared the dose-response curves of the hits with curves for known ER ligands to compare the potencies of the compounds (**Fig. 5**). Bortezomib was second in potency after estradiol, and the remainder of the compounds were less potent than estradiol, 4-OHT, estrone, and estriol.

We evaluated the transactivation potentials of the hit compounds as compared with known ER agonists or antagonists in the MCF-7 human breast cancer cell line (**Fig. 6a**). The antiestrogens tamoxifen, raloxifene, RU486, and ICI 182,780 did not demonstrate any detectable agonist activity in MCF-7 cells and had transactivation potentials below vehicle controls, with fold increase activity of 1.15, 0.89, 0.4, and 0.24, respectively. MG132, bortezomib, and NSC 333856 demonstrated absence of agonist activity in the MCF-7 cell line, with fold transactivation induction levels of 0.61, 0.78, and 1.34, respectively. The DCIII-43 and CMLD 1845 compounds exhibited measurable agonistic activity, with transactivation potentials of 5.20 and 2.67, respectively.

DCIII-43 and CMLD 1845 compounds behaved as agonists, and MG132, NSC 333856, and bortezomib lacked agonist activity in MCF-7 cells. DCIII-43 and CMLD 1845 also exhibited agonist activity in ECC-1 cells, which is a human endometrial cancer cell line (data not shown). Competition transactivation assays with 17β-estradiol are necessary to declare the compounds with no detectable agonist activity as ERα antagonists. In an effort to further validate the compounds with no detectable agonist activity as antagonists, we performed estradiol competition transactivation luciferase reporter assays in MCF-7 cells (**Fig. 6b**) to elucidate whether these hit compounds elicited antagonism or if there was no effect on estradiol-mediated transactivation. Tamoxifen was evaluated in our competition assay to confirm the functionality of the assay and for comparison of the activity of our hit compounds to a known ERα antagonist. Cotreatment of 100 nM estradiol and 10 μM tamoxifen

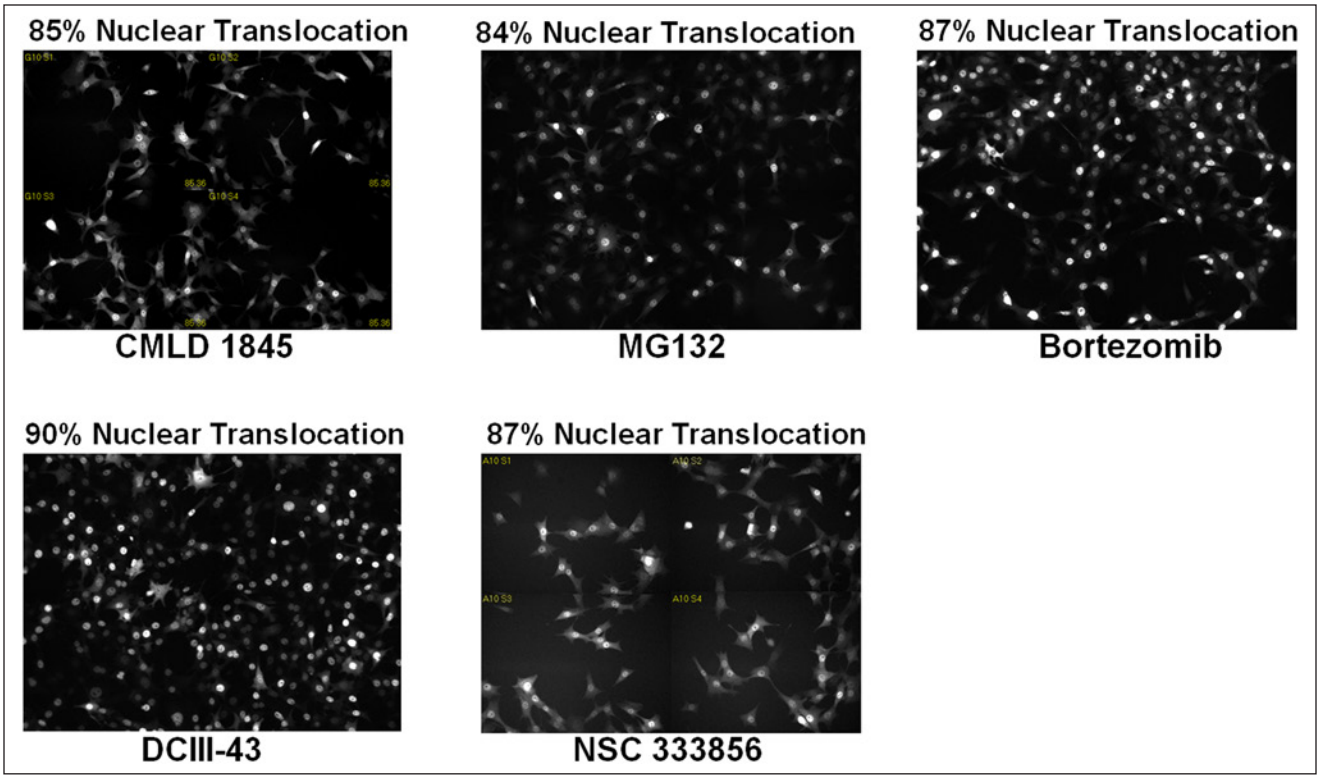


**Figure 3.** Fluorescent images of estrogen receptor (ER) nuclear translocation primary hit compounds. Five primary hit compounds were identified during the ER nuclear translocation screen of 224,891 synthetic, pure natural products and natural product extracts. Compounds were characterized as hits with the criteria of a nuclear translocation ≥72%, nuclear area >380, regions of interest >30. Fluorescent images depict the subcellular localization of the green fluorescent protein (GFP)–glucocorticoid/estrogen receptor (GRER). Nuclear translocation was quantitated, and the translocation values are indicated next to the GFP image.

resulted in a 51% decrease in estradiol-mediated transactivation potential and exhibited a dose-dependent inhibition of estradiol transactivation. We evaluated MG132, bortezomib, and NSC 333856 for antagonist activity in the presence of 100 nM estradiol, and these compounds decreased the fold induction of estradiol transactivation by 48%, 60%, and 44%, respectively (**Fig. 6b**). MG132 and NSC 333856 showed a dose-dependent fold decrease in estradiol-mediated transactivation, where the highest dose of 10 μM elicited the highest transactivation inhibition. Interestingly, bortezomib exhibited the largest fold inhibition of 60% at 100 nM and 54% at 1 μM, and it only depressed estradiol transactivation by 29% at 10 μM. Since CMLD 1845 exhibited weak agonist activity in MCF-7, we tested whether this compound elicited partial antagonist activity in the estradiol competition assay. CMLD 1845 did not exhibit any decrease in estradiol-mediated transactivation (data not shown) and was subsequently classified as a weak agonist. These data demonstrate that MG132, bortezomib, and NSC 333856 exhibit antagonistic activity in MCF-7 cells.

Hit pure natural product NSC 333856 is known as tetrocarcin A (TC-A), which is a Gram-negative antibiotic isolated from *Micromonospora chalcea* that has previously reported antitumor activity. Tetrocarcin A has been shown to induce apoptosis and inactivate the PI3K pathway in human breast cancer cell lines.[25] TC-A induces DNA fragmentation, condensed chromatin, and caspase activation without perturbing the plasma membrane. The PI3K pathway is inactivated by tetrocarin A treatment by dephosphorylation of Akt, PTEN, and PDK1, without affecting their protein expression levels. This demonstrates that tetrocarcin A induces apoptosis through inhibition of PI3K signaling.[25] Tetrocarcin has also been shown to induce apoptosis in lymphomas. Studies with B-chronic lymphocytic leukemia cells (B-CLL) have demonstrated that TC-A treatment induces a novel apoptotic pathway that involves induction of endoplasmic reticulum stress through a Bcl-2–independent pathway that involves Hsp70 upregulation, mitochondrial-dependent apoptotic pathways, and activation of caspase 12.[26] Tetrocarcin A may be an attractive therapeutic for B-CLL and useful to patients who exhibit resistance to standard chemotherapeutic agents since TC-A activates a novel apoptotic pathway. It has not been previously reported that tetrocarcin A (NSC 333856) has a direct effect on ERα activity (antagonism) or ERα subcellular localization, so it will be interesting to explore the biology of this compound

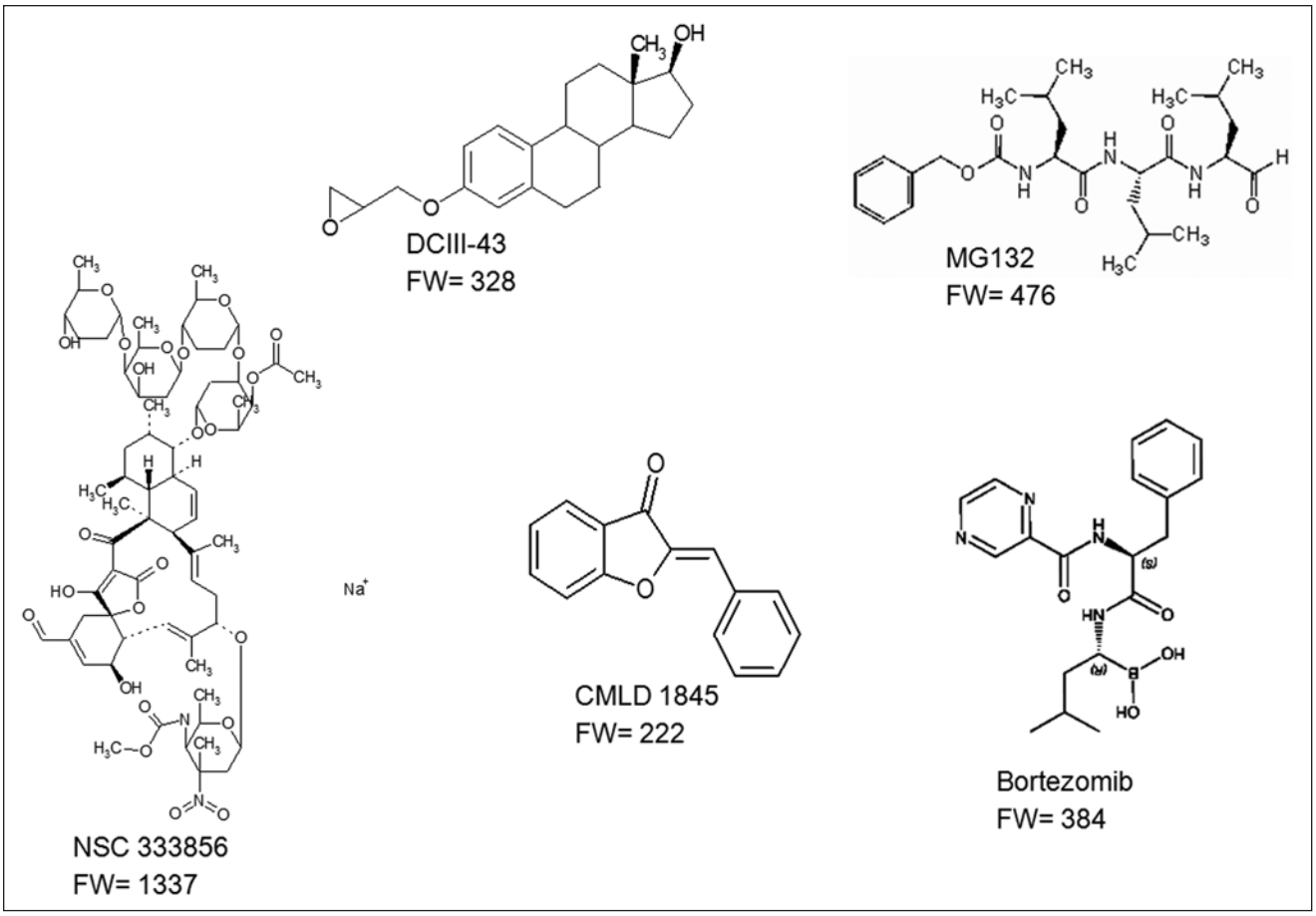


**Figure 4.** Structures of the five hit compounds identified by the estrogen receptor nuclear translocation primary screen. The formula weight (FW) for each compound is shown.

with respect to ERα signaling to determine if tetrocarcin A exhibits a novel mechanism on this pathway that may be a benefit therapeutically to patients with breast cancer. The data presented in this article have shown that NSC 33856 behaves as an antagonist in MCF-7 cells, and it will be interesting to further elucidate this function, perhaps in combination with other antagonists or SERMs, such as tamoxifen, which may be important therapeutically to minimize acquired disease resistance associated with monotherapy.

Studies have demonstrated that bortezomib increases the efficacy of fulvestrant by enhancement of cytoplasmic aggregation of ER.[27] Fulvestrant is a breast cancer therapeutic that targets the proteasomal degradation of ER in the nucleus and leads to aggregation of newly synthesized ER in the cytoplasm. Aggregated proteins are cytotoxic and eliminated through autophagy or proteasomal degradation. Fulvestrant resistance has been associated with the unfolded protein response (UPR) activation. Bortezomib increases fulvestrant-induced cytoplasmic ER aggregation and does not inhibit proteasomal degradation of ER in nucleus.[27] In addition, cotreatment of bortezomib and fulvestrant reverses tamoxifen resistance since studies have demonstrated that fulvestrant-bortezomib treatment promotes regression of breast cancer xenografts exhibiting tamoxifen resistance.[27] Perhaps these effects are related to our data, which demonstrated the antagonistic activity of bortezomib. Given these data, it will be interesting to explore the effects of cotreatment of bortezomib with the other hits identified in the screen—in particular, cotreatment of NSC 333856 and bortezomib or cotreatment of bortezomib or NSC 33856 with known ERα antagonists.

These hit compounds will undergo further biological evaluation to further explore the mechanism of action of these compounds on the ERα signaling pathway and to determine if these compounds may be useful candidates for HRT or breast cancer therapeutics. This primary screen identified compounds that translocated the GFP-GRER chimera from the cytoplasm to the nucleus, and our transactivation potential assays demonstrated compounds with agonist and antagonist activity. The CMLD 1845 and DCIII-43 compounds elicited agonist activity in both

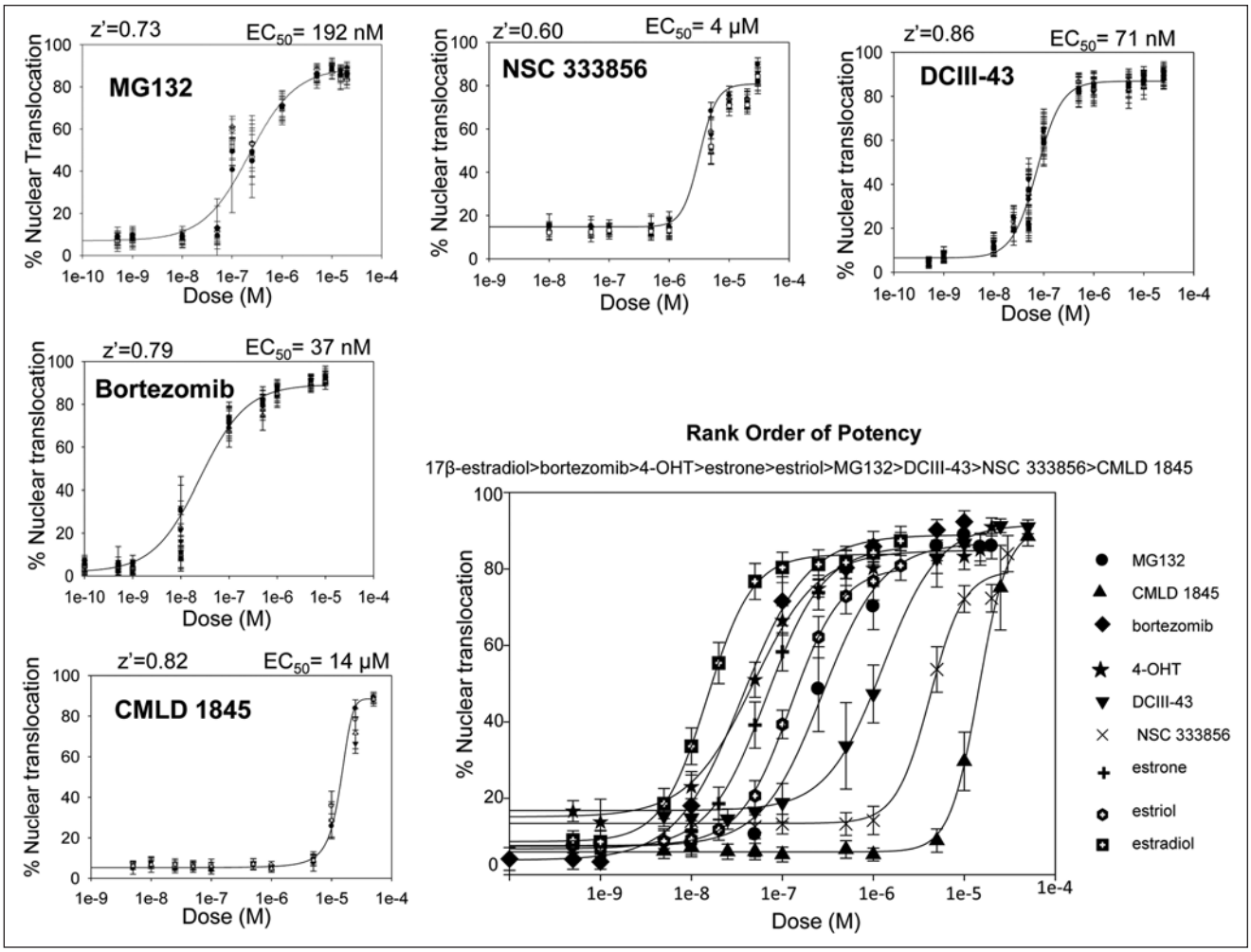


**Figure 5.** Nuclear translocation dose response of estrogen receptor (ER) primary hit compounds. Dose-response (DR) curves depicting the green fluorescent protein (GFP)–glucocorticoid/estrogen receptor (GRER) nuclear translocation dynamics of the five primary ER ligand hit compounds and potency comparison with known ligands. Dose responses were generated for MG132, NSC 333856, DCIII-43, bortezomib, and CMLD 1845. Comparison of potencies among the five primary hits and known ER agonists and antagonists was determined using dose-response curves depicting nuclear translocation dynamics. Experiments were performed as described in the Materials and Methods.

MCF-7 cells (**Fig. 6**) and in ECC-1 cells (data not shown). Our estradiol competition transactivation assays demonstrated that MG132, bortezomib, and NSC 333856 exhibit antagonist activity in MCF-7 cells.

When considering the potential mechanisms of action of the ERα primary hit compounds, many cellular processes may be targeted by the compounds identified in the GFP-GRER nuclear translocation screen, which do not necessarily involve direct binding to ER but may be therapeutically relevant regarding ERα signaling. In future studies, the mechanism of action of the hit compounds will be evaluated further to determine whether the compounds directly bind to ERα or exhibit therapeutic relevance to cancer or hormone therapy. The hit compounds may be increasing nuclear import through protein phosphorylation or protein-protein interactions, or compound binding to the ER may promote allosteric conformations that enhance nuclear import. Conversely, accumulation of the GFP-GRER in the nucleus could be the result of the compounds targeting the nuclear export machinery. Another mechanism may involve modification of chromatin or direct or indirect interactions with the DNA to regulate gene expression. Compounds may behave similar to aromatase inhibitors in blocking synthesis of ER ligands or affect proteasomal degradation of ER. The studies evaluating the effect of cotreatment of fulvestrant and bortezomib illustrate the importance of using multitargeted therapies instead of monotherapy in an effort to prevent or reverse drug resistance. Consequently, it will be interesting to evaluate the biological response from a combination of the hits or cotreatment of the hits

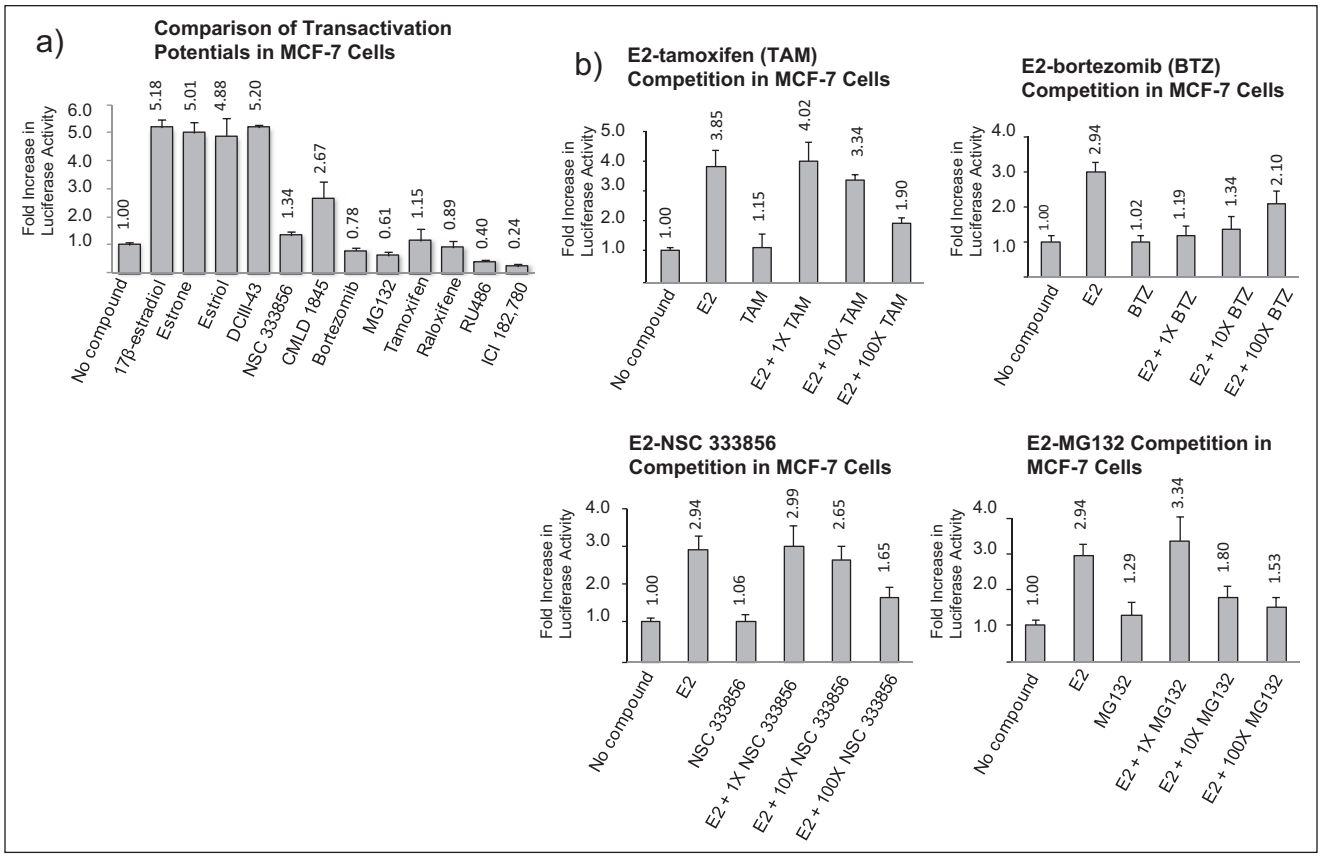


**Figure 6.** Transactivating potential of primary hits by luciferase assays in the MCF-7 human breast cancer cell line. (**a**) Transactivation potential of the hit compounds and known estrogen receptor (ER) ligands was evaluated in MCF-7 cells at 10 μM. (**b**) Compounds that lacked any detectable agonist activity were subjected to estradiol competition to determine if compounds exhibited antagonism or if there was no effect on transactivation. Estradiol and hit compounds were tested individually at 100 nM, followed by cotreatment of 100 nM estradiol with increasing doses of hit compounds. Hit compounds were treated at 1, 10, and 100 times that of estradiol concentration (100 nM, 1 μM, and 10 μM, respectively). Relative light units (RLU) were normalized to no compound added; assays were performed in triplicates and representative of three biological replicates.

with other known breast cancer therapeutics to determine if there is an additive or synergistic effect that would have the benefit of multidrug treatment to reduce the incidence of cancers exhibiting drug resistance.

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## Declaration of Conflicting Interests

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