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The Selective Estrogen Receptor Modulator Bazedoxifene Inhibits Hormone-Independent Breast Cancer Cell Growth and Downregulates Estrogen Receptor α and Cyclin D1

Joan S Lewis-Wambi, Helen Kim, Ramona Curpan, Ronald Grigg, Mohammed A. Sarker, and V. Craig Jordan

Authors' Affiliations: *Women's Cancer Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania (J.S.L.W.); Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC (V.C.J., H.K.); Institute of Chemistry, Romanian Academy, Timisoara, Romania (R.C.); School of Chemistry, University of Leeds, Leeds, UK (R.G., M.A.S.)*

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b) **Address correspondence to:** Dr. Joan Lewis-Wambi, Women's Cancer Program, Fox Chase Cancer Center, 333 Cottman Ave, Philadelphia, PA 19111. Phone: 215-728-4094; Fax: 215-728-4333. Email: joan.lewis@fccc.edu

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d) **ABBREVIATIONS:** ER α , estrogen receptor alpha; ER β , estrogen receptor beta; SERM, selective estrogen receptor modulator; BZA, bazedoxifene acetate; TAM, tamoxifen; RAL, raloxifene; E2, 17 β -E2; FUL, fulvestrant; siRNA, small interfering RNA; 4OHT, 4-hydroxytamoxifen; ENDOX, endoxifen; PgR, progesterone receptor; FBS, fetal bovine serum; Luc, luciferase; ERE, estrogen response element; pRT-PCR, quantitative reverse transcriptase-PCR; DMSO, dimethyl sulfoxide; CHX, cycloheximide

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Abstract

Bazedoxifene (BZA) is a third generation selective estrogen receptor modulator (SERM) that was recently approved for the prevention and treatment of postmenopausal osteoporosis. It has antitumor activity; however, its mechanism of action remains unclear. In the present study, we characterized the effects of BZA and several other SERMs on the proliferation of hormone-dependent MCF-7 and T47D breast cancer cells and hormone-independent MCF-7:5C and MCF-7:2A cells and examined its mechanism of action in these cells. We found that all of the SERMs inhibited the growth of MCF-7, T47D, and MCF-7:2A cells, however, only BZA and FUL inhibited the growth of hormone-independent MCF-7:5C cells. Cell cycle analysis revealed that BZA and FUL induced G1 blockade in MCF-7:5C cells, however, BZA downregulated cyclin D1 which was constitutively overexpressed in these cells whereas FUL suppressed cyclin A. Further analysis revealed that siRNA knockdown of cyclin D1 reduced the basal growth of MCF-7:5C cells and it blocked the ability of BZA to induce G1 arrest in these cells. BZA also downregulated ER α protein by increasing its degradation and suppressing cyclin D1 promoter activity in MCF-7:5C cells. Lastly, molecular modeling studies demonstrated that BZA bound to ER α in an orientation similar to raloxifene; however, a number of residues adopted different conformations in the IFD docking poses when compared with the experimental structure of ER α -RAL. Together, these findings indicate that BZA is distinct from other SERMs in its ability to inhibit hormone-independent breast cancer cell growth and to regulate ER α and cyclin D1 expression in resistant cells.

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Introduction

Bazedoxifene acetate (BZA) is a new third generation selective estrogen receptor modulator (SERM) (Silverman et al., 2008) that is approved in Europe and is under regulatory review in the United States for the prevention and treatment of postmenopausal osteoporosis. In phase III clinical trials (Archer et al., 2009; Miller et al., 2008; Pinkerton et al., 2009) BZA (20 or 40 mg/daily) has been shown to prevent bone loss and to reduce bone turnover in postmenopausal women at risk for osteoporosis, with a favorable endometrial, ovarian, and breast safety profile. BZA also significantly reduces the risk of new vertebral fractures in postmenopausal women with osteoporosis compared to placebo (Silverman et al., 2008). In addition, recent studies indicate that BZA combined with conjugated estrogens relieves hot flashes and improves vulvovaginal atrophy and its symptoms (Kagan et al., 2010).

BZA is an indole-based ER ligand with unique structural characteristics with respect to tamoxifen (TAM) and raloxifene (RAL). It was assembled by using RAL as a template and substituting an indole ring for the benzothiophene core (Komm et al., 2005; Miller et al., 2001). BZA binds to both ER alpha ($ER\alpha$) and ER beta ($ER\beta$), with a slight higher affinity for $ER\alpha$, however, it is less $ER\alpha$ selective than RAL, with an affinity for $ER\alpha$ that is about 10-fold lower than 17β -estradiol (E2) (Miller et al., 2001). $ER\alpha$ is a well studied member of the steroid/nuclear receptor family of transcription regulators. $ER\alpha$ acts in the nucleus to regulate gene expression by binding to estrogen response elements (EREs) and related DNA sequences and through association with transcription factors bound at SP1 and AP-1 DNA binding sites. In response to high affinity estrogen binding, $ER\alpha$ dimerizes, binds to ERE DNAs, and undergoes a conformational change in the ligand binding domain that facilitates the recruitment of coactivators. In contrast, antagonist-occupied $ER\alpha$ recruits corepressors. While previous studies

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have reported that BZA antagonizes E2-dependent MCF-7 breast cancer cell proliferation *in vitro* (Komm et al., 2005), little is known about the actions of BZA on ER α expression and functionality. Also not known is whether BZA has antitumor activity in breast cancer cells that have acquired resistance to endocrine therapies.

We have previously reported the development of two ER α -positive human breast cancer cell lines; MCF-7:5C (Jiang et al., 1992; Lewis et al., 2005a) and MCF-7:2A (Lewis-Wambi et al., 2008b; Pink et al., 1995) which were clonally selected from hormone-dependent MCF-7 breast cancer cells following long term (> 1 year) estrogen deprivation. An interesting phenotype of MCF-7:5C and MCF-7:2A cells is that, unlike MCF-7 cells which require estrogen to grow and are inhibited by antiestrogens, they do not require estrogen to grow and they undergo apoptosis when exposed to physiologic levels of E2 (Jordan, 2008; Lewis-Wambi et al., 2008b; Lewis et al., 2005a). However, the effects of SERMs on MCF-7:5C and MCF-7:2A cells have not been fully examined. In this study, we investigated the effects of BZA, 4-hydroxytamoxifen (4OHT), endoxifen (ENDOX), raloxifene (RAL), and the pure antiestrogen fulvestrant (ICI 182,780) on the growth of MCF-7:5C and MCF-7:2A breast cancer cells and determined the mechanism of action of BZA in these cells. We found that all of the SERMs inhibited E2-stimulated MCF-7 and T47D breast cancer cell growth, however, only BZA and FUL significantly inhibited the hormone-independent growth of MCF-7:5C cells. The inhibitory effect of BZA was associated with cell cycle arrest and cyclin D1 and ER α downregulation which was reversed by siRNA knockdown of cyclin D1 and ER α . Interestingly, we found that FUL also inhibited MCF-7:5C cell growth, however, this compound partially downregulated cyclin D1. Together, these data show that BZA is distinct from the other members of the SERM family in its ability to inhibit the growth of breast cancer cells that are resistant to long-term estrogen deprivation.

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Materials and Methods

Reagents and cell culture. E2, 4-hydroxytamoxifen (4OHT; the active metabolite of TAM), and MG132 were purchased from Sigma Chemical Co. Fulvestrant (ICI 182,780, Faslodex) was a generous gift from Dr. A. E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, United Kingdom). Endoxifen (ENDOX) was a kind gift from Dr James Ingle of the Mayo Clinic (Rochester, Minnesota). Raloxifene (RAL) was a generous gift from Lilly Research Laboratories (Indianapolis, IN). Bazedoxifene acetate (BZA) was synthesized by Drs Ron Grigg and Mohammed Sarker of Leeds University using a previously described protocol (Miller et al., 2001). All of the compounds were dissolved in 100% ethanol except MG132 which was dissolved in dimethyl sulfoxide (DMSO). The compounds were added to the medium such that the total solvent concentration was never higher than 0.1%. An untreated group served as a control. The chemical structures of the compounds used in this study have been cited before (Jordan, 2007; Jordan, 2009; Komm et al., 2005) and are shown in Supplemental Figure 1.

MCF-7:WS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by E2 (Pink and Jordan, 1996), were used in all experiments indicating MCF-7 and T47D cells. Cells were maintained in estrogenized medium [phenol red RPMI 1640 plus 10% fetal bovine serum], but 3 days before all experiments, were cultured in steroid-free media as previously described (Lewis et al., 2005a; Lewis et al., 2005b; Pink and Jordan, 1996). MCF-7:5C (Jiang et al., 1992; Lewis et al., 2005a; Lewis et al., 2005b) and MCF-7:2A cells (Lewis-Wambi et al., 2008b; Pink and Jordan, 1996) were derived from the MCF-7 line by growth in estrogen-free media and two rounds of limiting dilution cloning and were maintained in phenol red-free RPMI 1640 medium containing 10% 3X dextran-coated charcoal treated FBS. MC2 cells were derived by stably transfecting ER-negative

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MDA-MB-231 breast cancer cells with the wild-type ER α (Jiang and Jordan, 1992) and these cells were grown in phenol red-free MEM supplemented with 5% 3 \times dextran-coated charcoal-treated calf serum, 0.5 mg/ml Geneticin. All cell culture reagents were from Invitrogen (Carlsbad, CA).

Cell proliferation assay. These procedures have been previously reported (Lewis et al., 2005; Lewis-Wambi et al., 2008). Briefly, MCF-7 and T47D cells were grown in fully estrogenized medium whereas MCF-7:5C and MCF-7:2A cells were grown in non-estrogenized media. Cells were seeded in 24-well plates (30,000/well) and after overnight incubation cells were treated with various concentrations of the tested compounds for 7 days. Media was changed on days 3 and 5 and the experiment was ended on day 7 and the DNA content of the cells was determined as previously described (Labarca and Paigen, 1980) using a Fluorescent DNA Quantitation kit (Bio-Rad Laboratories, Hercules, CA). Cell proliferation was also determined by cell counting using a hemocytometer.

Western blot analyses. Immunoblotting was performed using 30 μ g protein per well as previously described (Lewis et al., 2005a). Membranes were probed with primary antibodies against ER α , PgR, cyclin A, cyclin B1 or cyclin D1 (Santa Cruz Biotechnology) with β -actin (AC-15; Sigma Chemical Co.) used to standardize loading. The appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used to visualize the stained bands with an enhanced chemiluminescence (ECL) visualization kit (Amersham, Arlington Heights, IL). Bands were quantitated by densitometry using Molecular Dynamics Software (ImageQuant) and densitometric values were corrected for loading control.

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Cell cycle analyses. MCF-7 and MCF-7:5C cells were treated with E2 or BZA for 24 and 48 hours and then fixed using ice-cold 70% ethanol. Cell cycle distribution was determined by propidium iodide staining using a fluorescence-activated cell sorter (FACS; Becton Dickinson) as previously described (Ariazi et al., 2010). Data was analyzed using FlowJo 7.2.5 for Windows (Tree Star).

Knockdown of ER α and cyclin D1 by siRNA. MCF-7:5C cells were seeded at 1×10^5 per well in a 24-well plate overnight and then transfected with 100 nmol/L nonspecific, ER α , or cyclin D1 small interfering RNA (siRNA; Dharmacon) using Lipofectomine 2000 (Invitrogen), as previously described (Lewis et al., 2005a). Transfected cells were either harvested for Western blot analysis or reseeded for cell growth or cell cycle analysis.

Quantitative real-time PCR. The detail procedures have been previously reported (Lewis et al., 2005). MCF-7 and MCF-7:5C cells were treated with either E2 (10^{-9} mol/L) or BZA (10^{-7} mol/L) for 48 h and total RNA was isolated and then reverse transcribed to cDNA using the SuperScript II RNase H-reverse transcriptase system (Invitrogen, Life Technologies, Carlsbad, CA). Aliquots of the cDNA were combined with the SYBR green kit (Superarray) and primers, and assayed in triplicate by quantitative PCR over 40 cycles using a GeneAmp[®] 5700 Sequence detection system (Applied Biosystems), as previously described (Lewis et al., 2005a). Quantitation was done using the comparative CT method with 18S rRNA as the normalization gene, as previously described (Lewis-Wambi et al., 2008a). PCR primer sequences used were as follow : ER α forward 5'-GGAGGGCAGGGGTGAA-3', ER α reverse 5'-GGCCAGGCTGTTCTTCTTAGA-3'; cyclin D1 forward 5'-TCCTGTGCTGCGA AGTGGAAAC-3', cyclin D1 reverse 5'-AAATCGTGCGGGGTCATTGC; pS2 forward 5'-GAGGCCAGACAGAGACGTG-3, pS2 reverse 5'-CCCTGCAGAAGTGTCTAAAATTCA-3.

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Transient transfections and luciferase assays. Cells were cultured in estrogen-free RPMI 1640 media for 48 h prior to transfection. On the day of the experiment, cells were seeded in estrogen-free media at a density of 1.5×10^5 cells per well in 24-well plates. After 24h, cells were transfected with the firefly luciferase reporter plasmid pERE(5x)TA-ffLuc (containing 5 copies of a consensus ERE and a TATA-box driving firefly luciferase) and the pTA-srLuc Renilla luciferase plasmid (containing a TATA-box element driving renilla luciferase) (Promega) using LT1 (Mirus) transfection reagent, according to the manufacturer's protocol. After 24 hours, transfection reagents were removed and fresh media was added. Cells were then treated with ethanol (vehicle), 10^{-9} M E2, 10^{-8} M BZA, or E2 + BZA combined for 24 h. At the indicated time point, cells were washed, lysed, and ERE luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's recommendations. Samples were then read on a Mithras MB540 luminometer (Berthold Technologies, Oak Ridge, TN).

For the cyclin D1 promoter assay, MCF-7:5C cells were transiently transfected with the full length cyclin D1 promoter plasmid (-1745CD1-LUC) as previously described (Lewis et al., 2005c; Lewis et al., 2005d). The full length cyclin D1 plasmid (-1745CD1-LUC) (Albanese et al., 1995) was a gift from Dr Richard Pestell.

Molecular Modeling. The molecular modeling performed in this study has previously been described (Maximov et al., 2010). Briefly, the coordinates for the agonist and antagonist conformations of human ER α ligand binding domain co-crystallized with estradiol (E2), raloxifene (RAL) and 4-hydroxytamoxifen (4OHT) were extracted from the RCSB Protein Data Bank (PDB) (Berman et al., 2000) Entries 1gwr for E2 (Warnmark et al., 2002), 1err for RAL (Brzozowski et al., 1997) and 3ert for 4OHT (Shiau et al., 1998) were selected for further

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modeling and these structures were prepared for docking using the Protein Preparation Workflow (Friesner et al., 2004; Guallar et al., 2004) implemented in Schrödinger suite and accessible from within the Maestro 8.5 program. To study the molecular basis of interaction of bazedoxifene in the antagonist conformation of ER α , the ligands were docked into the binding site of the receptor co-crystallized with RAL (PDB code 1err). For comparison reasons, RAL was also docked in its native protein structure.

The input geometries of the ligands were generated with CORINA (online demo, http://www.molecular-networks.com/online_demos/corina_demo) and were further prepared for docking using the LigPrep2.2 utility (Friesner et al., 2004; Guallar et al., 2004). The prepared structure of ER α co-crystallized with RAL was used to generate the scoring grid for docking simulations. A grid box of 26 x 26 x 26 Å³ centered on the ligand was created, using the default parameters and without constraints.

Flexible ligand docking simulations were carried out with Glide 5.0 (Friesner et al., 2004; Guallar et al., 2004) using the default settings and the best 10 poses for each ligand were evaluated using Glide in Standard-Precision (GlideSP) and Extra-Precision (GlideXP) mode. The results obtained from the docking runs were compared and GlideXP docking poses were selected for analysis.

Statistical Analysis. All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean \pm S.D. Statistical significances between vehicle treatment versus drug-treatment were determined by one-way analysis of variance and the Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

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Results

BZA Inhibits the Growth of Hormone-Independent MCF-7:5C and MCF-7:2A Breast Cancer

Cells. We first compared the growth characteristics of hormone-dependent MCF-7 and T47D breast cancer cells to those of long-term estrogen deprived MCF-7:5C and MCF-7:2A cells in the presence of E2. Cells were grown in estrogen-free media and then treated with 10^{-14} M to 10^{-8} M E2 for 7 days and cellular DNA was measure as an index of growth. In parallel, cells were also treated with 10^{-9} M E2 for 2 to 12 days and then harvested and counted using a hemocytometer. Fig. 1A shows that E2 treatment stimulated the growth of MCF-7 and T47D cells in a concentration-dependent manner with maximum stimulation at 10^{-9} M, whereas, in MCF-7:5C and MCF-7:2A cells, E2 treatment had the opposite effect causing either complete growth inhibition in MCF-7:5C cells or partial growth inhibition in MCF-7:2A cells. This finding is consistent with our previous work (Lewis-Wambi et al., 2008b; Lewis et al., 2005a) which showed that physiologic concentrations of E2 induced programmed cell death (apoptosis) in MCF-7:5C and MCF-7:2A cells through activation of the mitochondrial death pathway and suppression of glutathione synthesis, respectively. Specifically, we found that E2 induced apoptosis in MCF-7:5C cells by activating proapoptotic proteins Bax, Bak, Bim, and p53 and by suppressing antiapoptotic proteins. E2 also downregulated survival proteins such as, NF κ B, phospho-Akt, and Her2/neu which were overexpressed in MCF-7:5C cells. In contrast, we found that MCF-7:2A cells underwent apoptosis after 10-12 days of E2 treatment and that these cells expressed elevated levels of the antioxidant glutathione (GSH) due to overexpression of glutathione synthetase (GS) and glutathione peroxidase 2 (GPx2), the two main enzymes involved in GSH synthesis. By selectively blocking the glutathione pathway in MCF-7:2A cells,

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we were able to sensitize these cells to E2-induced apoptosis which was mediated by activation of the JNK signaling pathway.

Next, we determined the inhibitory effects of BZA and other SERMs (see Supplemental Figure 1 for chemical structures) on MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells. For experiments, MCF-7 and T47D cells were grown in fully estrogenized media and MCF-7:5C and MCF-7:2A cells were grown in estrogen-free media and then treated with 10^{-12} M to 10^{-6} M BZA, RAL, FUL, 4OHT, or ENDOX for 7 days and cellular DNA was measured as an index of growth. Fig. 1B shows that all of the tested SERMs along with the pure antiestrogen FUL inhibited E2-stimulated growth in MCF-7 and T47D cells and hormone-independent growth in MCF-7:2A cells in a concentration-dependent manner, however, in MCF-7:5C cells, only BZA and FUL inhibited the growth of these cells with no effects observed with RAL, 4OHT, or ENDOX. BZA reduced the growth of MCF-7:5C cells in a concentration dependent manner causing an 80% reduction at 10^{-8} M whereas FUL reduced the growth by 55% at a similar concentration.

BZA Downregulates ER α Protein in MCF-7:5C and MCF-7:2A Cells. Since BZA dramatically reduced the growth of MCF-7:5C cells, we next determined whether BZA had actions similar to that of 4OHT or FUL at the level of ER α stability/degradation. We treated MCF-7:5C, MCF-7:2A, MCF-7, and T47D cells with 10^{-9} M E2 or 10^{-7} M FUL, 4OHT, RAL, or BZA for 24 hours and monitored ER α protein level. As shown in Fig. 2A, ER α protein was highly expressed in MCF-7:5C and MCF-7:2A cells compared to MCF-7 and T47D cells and treatment with BZA markedly downregulated ER α protein in MCF-7:5C and MCF-7:2A cells however it did not significantly reduce ER α levels in MCF-7 and T47D cells. The ability of BZA to downregulate ER α in MCF-7:5C and MCF-7:2A cells was greater than that of RAL and almost comparable to that of the pure antiestrogen FUL which strongly downregulated ER α in all of the cell lines. E2

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treatment also markedly downregulated ER α protein in all of the cell lines including MCF-7:5C (Fig. 2A), however, 4OHT stabilized ER α against degradation in MCF-7 and T47D cells, as previously reported (Pink and Jordan, 1996), with marginal stabilization observed in MCF-7:5C and MCF-7:2A cells (Fig. 2A). We also examined the effect of the tamoxifen metabolite, endoxifen (ENDOX), on ER α expression in the different cell lines and found that endoxifen did not down-regulate ER α in any of the tested cell lines (Supplemental Fig. 2). Our finding differs from that of Wu and coworkers (Wu et al., 2009) who reported that endoxifen degrades ER α in breast cancer cells.

We also performed dose response studies in MCF-7, MCF-7:5C, and MCF-7:2A cells to determine the optimal concentration at which BZA downregulated ER α protein. Fig. 2B showed that BZA reduced ER α protein level in MCF-7:5C cells in a concentration dependent manner with maximum inhibition at 10^{-6} M, whereas, in MCF-7 and MCF-7:2A cells, BZA only marginally reduced ER α protein in these cells. Notably, the inhibitory effect of BZA on ER α protein was less pronounced than that observed with E2 or FUL which almost completely reduced ER α protein level in MCF-7:5C cells. Time course studies revealed that BZA downregulated ER α protein as early as 2 h after treatment with maximum suppression at 24 h (Fig. 2C, top). BZA also downregulated ER α mRNA in MCF-7:5C cells to a level similar to that observed with E2 and FUL (Fig. 2C, bottom). To show that the decreased ER α protein by BZA was due to protein degradation, we used MG132 to inhibit the proteasome in MCF-7:5C and MCF-7 cells. We found that inhibition of proteasome activity completely blocked ER α degradation by BZA and E2 with partial reversal with fulvestrant (Fig. 2D). We further determined whether BZA might affect ER α protein expression by inhibiting its synthesis. We treated MCF-7:5C cells with 0.5 to 5 μ M cycloheximide (CHX) for 4 h to address this question.

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The impact of CHX on ER α protein expression was much less dramatic than that of BZA (data not shown), which suggest that BZA-induced down-regulation of ER α protein is not likely to involve protein synthesis inhibition. Together, these data show that BZA differs from the other SERMs in its ability to regulate cell growth and ER α protein expression in MCF-7:5C cells.

BZA Inhibits ER α Transcriptional Activity in MCF-7:5C Cells. To determine whether BZA blocks ER α function, we next examined the transcriptional activation of an estrogen response element (ERE) in MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells. Cells were transiently transfected with a 5X ERE-luciferase reporter plasmid and treated with 10^{-10} M E2, 10^{-8} M BZA, or E2 + BZA for 24 h. The results of these studies showed that basal ERE activity was elevated 5-fold in MCF-7:5C and 10-fold in MCF-7:2A cells compared with MCF-7 cells and treatment with BZA significantly reduced the basal ERE activity in these cells (Fig. 3A). E2 treatment further increased ERE activity in MCF-7:5C and MCF-7:2A cells by 1.5- and 2.5-fold, respectively, however, in MCF-7 and T47D cells the response was markedly more robust with a 12- and 20-fold increase, respectively (Fig. 3A).

To further test whether BZA is able to block ER α -regulated genes, we analyzed the expression level of pS2 mRNA in MCF-7:5C cells using qRT-PCR. The pS2 gene is often used as a prognostic marker in breast cancer cells and is frequently used in studies of ER action. Furthermore, it is suggested that estrogen regulates the expression of pS2 through an imperfect ERE in the pS2 promoter (Berry et al., 1989). Our results showed that basal pS2 mRNA level was ~3.5-fold higher in MCF-7:5C cells compared to wild-type MCF-7 cells and E2 treatment increased pS2 mRNA level by ~5.5-fold in MCF-7 cells and MCF-7:5C cells which was completely blocked by BZA (Fig. 3B). Notably, we also found that siRNA knockdown of ER α (Fig. 3C) significantly reduced the basal growth of MCF-7:5C cells and markedly reduced the

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inhibitory effect of BZA in these cells (Fig. 3C, bottom). In addition, suppression of ER α significantly reduced cyclin D1 protein in MCF-7:5C cells. Overall, these data indicate that in the absence of estrogen, the unliganded ER α drives the proliferation of hormone-independent breast cancer cells; however, in the presence of BZA, the ability to inhibit cell proliferation is dependent on receptor degradation.

BZA blocks cell cycle progression in MCF-7:5C cells and downregulates cyclin D1. Since BZA significantly reduced the growth of MCF-7:5C cells, we next examined its effect on cell cycle progression. For experiment, MCF-7 and MCF-7:5C cells were treated with 10^{-9} M E2, 10^{-8} M BZA, or E2 plus BZA for 48 h followed by propidium iodide staining and flow cytometric analysis. The results showed that in MCF-7:5C cells, E2 treatment significantly reduced the percentage of cells in S phase from 33% to 17% and marginally increased the percentage of cells in G1 phase from 60% (control) to 66%, whereas, BZA treatment increased the proportion of cells in the G1 phase from 60% to 81% and it reduced the proportion of S phase cells from 33% to 9% at 48 h. In MCF-7 cells, treatment with E2 increased the proportion of S-phase cells from 19% to 42% at 48 h with no effect observed with BZA alone (Fig. 4A). Notably, the inhibitory effect of BZA on cell cycle in MCF-7:5C cells was somewhat comparable to the pure antiestrogen fulvestrant; however, none of the other tested SERMs had any effect on cell cycle (data not shown).

Since BZA induced G1-phase cell cycle block in MCF-7:5C cells, we further investigated the G1-specific protein cyclin D1 in these cells. MCF-7 and MCF-7:5C cells were treated with BZA, E2, RAL, 4OHT or FUL for 24 h and lysates were prepared and analyzed by immunoblotting. Fig. 4B shows that cyclin D1 was undetectable in untreated MCF-7 cells, however, treatment

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with E2, and to a lesser extent 4OHT, markedly increased cyclin D1 protein in these cells. In contrast, we found that cyclin D1 protein was constitutively overexpressed in MCF-7:5C and MCF-7:2A cells and treatment with BZA completely reduced cyclin D1 protein in MCF-7:5C cells but not MCF-7:2A cells (Fig. 4B). Notably, none of the other SERMs inhibited cyclin D1 in MCF-7:5C cells, however, FUL significantly reduced cyclin D1 protein level at 96 h and it markedly reduced cyclin A protein in these cells (Supplemental Figure 3). Time course experiments revealed that BZA inhibited basal cyclin D1 protein in a time-dependent manner with measurable effects observed as early as 2 h after treatment and maximum reduction at 24 h (Fig. 4C, top). BZA also reduced cyclin D1 mRNA (Fig. 4C, bottom) and cyclin D1 promoter activity (Fig. 4C, top right) in MCF-7:5C cells. Lastly, we found that siRNA knockdown of cyclin D1 (Fig. 5A) significantly reduced the hormone-independent growth of MCF-7:5C cells (Fig. 5B) and it significantly reduced the ability of BZA to induce G1-blockade in these cells (Fig. 5C), thus confirming the importance of cyclin D1 in the inhibitory action of BZA in these cells.

Molecular modeling and docking of BZA into the ligand binding site of ER α . Molecular modeling and docking studies were carried out in an attempt to predict the bioactive conformation of BZA and to understand the molecular basis of interaction of this ligand with ER α . Using the available X-ray crystallographic data, the flexible docking of BZA into the ligand binding domain (LBD) of ER α co-crystallized with RAL was performed and for comparison reasons, FUL and RAL were also docked in their native protein structure. The superimposition of the docked solution and experimental structure of RAL shows that the docking model recapitulates the orientation of the native ligand in the active site and the same interactions with the key aminoacids of the binding cavity are formed with a ligand RMSD of

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0.362 when compared with the crystal structure (Fig. 6A). The experimental structure of ER α co-crystallized with E2 (PDB code 1gwr), the agonist conformation of the receptor, is displayed in Fig. 6B, while the experimental antagonist conformation of ER α bound to 4OHT and RAL are superimposed and presented in Fig. 6C. The docking results analysis reveal that BZA binds to ER α in an antagonist orientation similar with RAL (Fig. 6D) and has the tendency to form the same hydrophobic contacts with the aminoacids lining the binding cavity. In addition, the same complex H-bond network is formed with D351, E353, R394, H524 and a highly ordered water molecule, located in the vicinity of residues E353 and R394 (Fig. 6D). However, we should note that there are a number of residues that adopt different conformations in the IFD docking poses when compared with the experimental structure of ER α , 1err (Supplemental Figure 4). The most significant difference has been observed for Leu539 of helix 12. The larger ring of BZA causes the side chain of Leu539 to be pushed away from its original position by about 1Å. In all top ranked IFD structures (four poses having the composite score of 0.5 kcal/mol) Leu529 side chain is moved up from its original orientation, towards the ring of BZA to optimize the hydrophobic contacts between the ligand and residue side chain (Supplemental Figure 4). We also compared the docked structure of BZA with the binding mode of 4OHT to ER α (Fig. 6C) and superimposed it in the binding site of 4OHT-ER α complex (Fig. 6E). The 4OHT bound receptor shows that the H-bond between BZA and H524 is missing (Fig. 6E) due to the different orientation of this aminoacid in the binding site compared with the RAL-ER α complex (Fig. 6C). When FUL was docked to RAL-ER α complex (Fig. 7A), the H-bond network was recapitulated with one exception, the interaction with D351 is missing, while the flexible side chain of FUL fills the groove between helix 3 and helix 12 (Fig. 7B).

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Overall, these findings indicate that the alignment of BZA in the binding pocket of ER α predicted by the IFD is similar with that predicted via the rigid docking method (Glide) and with the alignment of RAL in the experimental structure, 1err. However, there are a few differences in the orientation of some residues in the binding site when the docking of BZA is performed with IFD protocol and these differences might help to explain the different biological effects of BZA versus RAL in our cell model.

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Discussion

In the present study, we report for the first time that BZA inhibits the growth of breast cancer cells that have acquired resistance to long-term estrogen deprivation (i.e. hormone-independent/aromatase inhibitor resistant). Specifically, we found that BZA at 10^{-8} M inhibited the growth of hormone-independent MCF-7:5C and MCF-7:2A breast cancer cells by 80% and 55%, respectively. The inhibitory effect of BZA in MCF-7:5C cells was associated with G1 arrest and cyclin D1 and ER α down-regulation whereas in MCF-7:2A cells BZA suppressed cyclin A with marginal effects on cyclin D1. The pure antiestrogen FUL also inhibited the growth of MCF-7:5C cells by inducing G1 arrest; however, it did not downregulate cyclin D1 until 96 hours which was 48 hours after its effect on cell cycle. Strikingly, RAL, 4OHT, and ENDOX failed to inhibit cyclin D1 expression in MCF-7:5C cells and these compounds did not have any growth inhibitory effect in MCF-7:5C cells. While it is not entirely clear why BZA was more potent than fulvestrant at inhibiting the growth of MCF-7:5C cells, one possibility might be due to the fact that BZA downregulated both ER α and cyclin D1 whereas FUL downregulated ER α and had marginal effects on cyclin D1 which was observed at 96 hours. Molecular modeling studies indicated that BZA bound the ligand binding domain of ER α in an antagonist orientation similar to RAL (Fig. 6D), but distinct from 4OHT (Fig. 6E) and fulvestrant (Fig. 7). However, a few differences were noticed in the orientation of some residues in the binding site when the docking of BZA was performed with the Induced Fit (IFD) protocol. The most significant difference was observed for Leu539 of helix 12. The larger ring of BZA caused the side chain of Leu539 to be pushed away from its original position by about 1Å. This alteration in the orientation of Leu539 side chain could trigger a conformational change of helix12 which in

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turn could lead to the recruitment of other proteins by the BZA-ER α compared to the RAL-ER α complex. Indeed, these findings help to further distinguish BZA from the other SERMs such as TAM and RAL and they support the concept that subtle but moderate structural differentiation can dramatically impact the ability of a ligand to regulate cell proliferation.

Previous research has indicated that deregulation of ER α expression is a driving force in the initiation and progression of estrogen-sensitive breast tumors (Garcia-Closas and Chanock, 2008; Garcia-Closas et al., 2008). It has been suggested that alterations in pathways leading to ER α synthesis and/or degradation underlie the deregulation of ER α and its consequent manifestations, including enhanced proliferation in breast tumors (Sommer and Fuqua, 2001). ER α is the predominant receptor isoform expressed in breast cancer cells, and increased numbers of ER α -expressing cells can be observed at the earliest stages of breast tumorigenesis. Previously, we have shown that ER α mRNA and protein levels are significantly elevated in breast cancer cells that have been adapted to grow in an estrogen-depleted environment (Lewis et al., 2005a; Murphy et al., 1990; Pink et al., 1996). This particular type of regulation in which ER α levels are increased following estrogen deprivation has been described as a Model I response (Pink and Jordan, 1996). A Model I response is characterized by an ER α that is expressed at high levels in the absence of estrogen and is subsequently down-regulated following estrogen binding, primarily through repression of the steady-state level of the mRNA. In the present study, we found that basal ER α protein levels were upregulated greater than 3-fold in hormone-independent MCF-7:5C and MCF-7:2A breast cancer cells compared to MCF-7 and T47D cells and treatment with BZA (10^{-8} M) induced proteasome-mediated degradation of ER α in these cells which was reversed by the proteasome inhibitor MG132. The ability of BZA to degrade

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ER α in MCF-7:5C cells was rapid and robust occurring as early as 4 h after treatment with maximum degradation at 24 h. Notably, BZA and fulvestrant were the only compounds that markedly reduced the growth of both MCF-7:5C and MCF-7:2A breast cancer cells and blocking BZA-induced ER α degradation with MG132 dramatically reduced its growth inhibitory effects on these cells (data not shown). The importance of ER α in mediating the antagonist effects of BZA in hormone-independent MCF-7:5C cells was further confirmed by siRNA knockdown experiments which showed a 60% reduction in the ability of BZA to inhibit the growth of these cells. Suppression of ER α also significantly reduced the basal growth of MCF-7:5C cells and E2-induced growth in wild-type MCF-7 cells, which is consistent with recent findings by Ariazi and coworkers (Ariazi et al., 2010). It should be noted; however, that degradation or suppression of ER α is not the only mechanism by which an antagonist can inhibit cell proliferation. For example, TAM has been shown to stabilize ER α protein against degradation in breast cancer cells (Murphy et al., 1990; Pink et al., 1996; Pink et al., 1995; Pink and Jordan, 1996), however, it is a potent antagonist in the breast with the ability to block E2-stimulated proliferation and E2-induced ERE activity in these cells.

Apart from ER α , BZA also significantly reduced cyclin D1 expression in hormone-independent MCF-7:5C breast cancer cells. Cyclin D1 is a breast cancer oncogene whose overexpression has been linked to poor prognosis in ER α and PgR-positive breast cancers (Lammie and Peters, 1991). It is a multifunctional G₁-phase cyclin whose regulatory effects are particularly important in breast development and cancer (Sutherland and Musgrove, 2004). Cyclin D1 is highly induced by estrogen (Said et al., 1997) and it contributes to poor treatment response of ER-positive tumors by acting downstream to promote hormone agonist- and

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antagonist-independent proliferation (Wilcken et al., 1997). We found that cyclin D1 protein was constitutively elevated by 3-to 5-fold in hormone-independent MCF-7:5C and MCF-7:2A cells compared to wild-type MCF-7 and T47D cells and treatment with BZA reduced it to an undetectable level in MCF-7:5C cells but not MCF-7:2A cells. In addition, we found that suppression of cyclin D1 in MCF-7:5C cells reduced the hormone-independent growth of these cells and it significantly reduced the ability of BZA to inhibit cell growth and induce cell cycle arrest in these cells. Suppression of cyclin D1 also significantly reduced ER α protein levels in MCF-7:5C cells with similar effects observed following ER α suppression, thus suggesting a link between cyclin D1 and ER α in these cells. Indeed, a connection between ER and cyclin D1 was previously demonstrated when cyclin D1 was shown to interact directly with the ligand-binding domain of ER and stimulate ER transactivation in a ligand-independent fashion (Zwijssen et al., 1997). More recently, cyclin D1 was shown to interact with coactivators of the SRC-1 family through a motif that resembles the leucine-rich coactivator binding motif of nuclear receptors. By acting as a bridging factor between ER and SRCs, it is thought that cyclin D1 can recruit SRC-family coactivators to ER in the absence of ligand. It is worth noting that hormone-independent MCF-7:5C cells express elevated levels of SRC1 protein compared to hormone-dependent MCF-7 cells and BZA treatment significantly reduces basal SRC-1 levels in these cells (data not shown).

Although cyclin D1 gene transcription is directly induced by estrogen, there is no estrogen response element in its. Instead, the cyclin D1 promoter contains multiple regulatory elements, including binding sites for AP-1, STAT5, NF- κ B, CRE, Sp1, and E2F. A fragment between -994 and -136 of the cyclin D1 promoter was previously shown to be estrogen-responsive and this region has binding sites for AP-1 and SP-1 (Altucci et al., 1996). More

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recently, we reported that estrogen-induced cyclin D1 transactivation in MCF-7 breast cancer cells was mediated by the cAMP response element (CRE) region which is known to bind activating transcription factor 2 (ATF-2) (Lewis et al., 2005c; Lewis et al., 2005d). A notable finding of our study was that basal cyclin D1 promoter activity was significantly elevated in hormone-independent MCF-7:5C cells compared to hormone-dependent MCF-7 cells and treatment with BZA completely reduced the promoter activity in these cells to the level seen in the untreated MCF-7 cells. In contrast, E2 did not induce cyclin D1 expression or promoter activity in hormone-independent MCF-7:5C cells whereas in hormone-dependent MCF-7 cells it increased cyclin D1 protein level by 3-fold and its promoter activity by 4-fold, which is consistent with its function as a proapoptotic agent in MCF-7:5C cells versus an agonist in MCF-7 cells.

In conclusion, it is clear from clinical data that BZA in combination with conjugated estrogens represents a new form of therapeutic agents for the treatment of postmenopausal symptoms and prevention of postmenopausal osteoporosis. The fact that it does not stimulate the breast or endometrium and is very effective at inhibiting the proliferation of endocrine-resistant breast cancer cells highlights its widespread therapeutic potential and demonstrates that not all SERMs are alike. Our data also suggest that the overexpression of ER α and cyclin D1 in MCF-7:5C cells might be driving the hormone-independent growth of these cells and that the ability of BZA to downregulate ER α and cyclin D1 is critical to treat and possibly reverse antihormone resistance in breast cancer.

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Authorship Contributions

Participated in research design: Lewis-Wambi and Jordan.

Conducted experiments: Lewis-Wambi and Kim.

Contributed new reagents or analytic tools: Grigg, Sarker, and Curpan

Performed data analysis: Lewis-Wambi.

Wrote or contributed to the writing of the manuscript: Lewis-Wambi and Jordan.

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Footnote

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Figure legends

Fig. 1. Effects of E2 and SERMs on the growth of hormone-dependent MCF-7 and T47D cells versus hormone-independent MCF-7:5C and MCF-7:2A cells. A, MCF-7 and T47D cells were grown in phenol red-free RPMI medium supplemented with 10% charcoal stripped FBS for 3 days prior to the start of the experiment. On the day of the experiment, all cell lines were seeded in phenol red-free RPMI medium supplemented with 10% charcoal stripped FBS at 30,000 per well in 24-well dishes and after 24 h were treated with 10^{-14} to 10^{-8} M E2 for 7 days, with retreatment every other day. At the conclusion of the experiment, cells were harvested and proliferation was assessed as cellular DNA mass ($\mu\text{g}/\text{well}$) using a DNA quantitation kit. B, the effects of antihormones on the growth of hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. Cells were seeded as described above except MCF-7 and T47D cells were grown in fully estrogenized media and then treated with 10^{-12} M to 10^{-6} M fulvestrant (FUL), bazedoxifene (BZA), raloxifene (RAL), 4-hydroxytamoxifen (4OHT), or endoxifen (ENDOX) for 7 days with retreatment on alternate days. Proliferation was assessed as cellular DNA mass ($\mu\text{g}/\text{well}$) as described in the methods section. Each point represents the mean of three determinations \pm SEM.

Fig. 2. Effects of SERMs on ER α expression and stability in hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. A, Western blot analysis of ER α protein levels in MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells in response to 24-h treatment with 10^{-9} M E2 or 10^{-7} M FUL, 4OHT, RAL or BZA. β -actin was used as a loading control. B, Western blot analysis of ER α protein levels in MCF-7, MCF-7:5C, and MCF-7:2A cells following treatment with 10^{-9} M to 10^{-6} M BZA for 24 h. For comparison, cells were also treated with 10^{-9} M E2 or 10^{-8} M FUL. C, Western blot analysis of ER α protein levels in MCF-

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7:5C cells in response to 10^{-8} M BZA treatment over a 24h time period. Quantitated protein levels were normalized to β -actin. Densitometric quantitation relative to the control is shown on the bottom of the immunoreactive bands. Also shown is ER α mRNA levels in MCF-7:5C cells following treatment with E2 (10^{-9} M), FUL (10^{-8} M), or BZA (10^{-8} M) for 24 hours. The amount of ER α mRNA was determined by real-time RT-PCR and normalized to the internal control 18S rRNA. Each data point represents the average of four biological replicates from three independent experiments. D, Western blot analysis of ER α protein levels in MCF-7 and MCF-7:5C cells pretreated with the proteasome inhibitor MG132 (4 μ mol/L) for 4 hours and then treated as indicated for 8 h. β -actin levels are shown as protein loading controls. Each point represents the mean of three determinations \pm SEM.

Fig. 3. BZA inhibits constitutive ER α transcriptional activity in hormone-independent and hormone-dependent breast cancer cells. A, ERE luciferase activity in hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. For experiment, cells were transiently transfected with a 5X ERE-luciferase reporter construct and treated with 10^{-9} M E2, 10^{-7} M BZA, E2+BZA, or nothing (control) for 24 h. Luciferase values for the treatment groups are reported as relative luciferase units (RLU). *, $p < 0.001$ compared with MCF-7 and T47D cells (control); **, $p < 0.0001$ compared with control for each cell line; #, $p < 0.01$ compared with untreated MCF-7:5C cells (control); †, $p < 0.05$ compared with untreated MCF-7:2A cells. B, real time RT-PCR analysis of pS2 mRNA gene expression in MCF-7 and MCF-7:5C cells after treatments with E2 (10^{-9} M), BZA (10^{-7} M), or E2+BZA for 24 h. Each data point represents the average of three biological replicates. *, $p < 0.01$ compared with untreated MCF-7 cells (control). **, $p < 0.001$ compared with untreated MCF-7 cells (control); †,

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$p < 0.001$ compared with untreated MCF-7:5C cells (control). C, MCF-7:5C cells were transfected with 100 nM nonspecific control or ER α siRNA for 48 h. Transfected cells were then harvested for Western blot analysis to detect ER α and cyclin D1 protein (top panel) or treated with 10^{-7} M BZA for an additional 4 days followed by cell counting using a hemocytometer (bottom panel). Data shown are representative of three independent experiments. *, $p < 0.001$ compared with untransfected control and nonspecific transfected cells; **, $p < 0.01$ compared with nonspecific transfected cells.

Fig. 4. Effects of BZA on cell cycle progression and cyclin D1 regulation in MCF-7 and MCF-7:5C cells. A, cell cycle distribution was determined by propidium iodide staining of DNA content and flow cytometry. Cells were treated with 10^{-9} M E2, 10^{-7} M BZA, or E2 plus BZA for 24 and 48h. Thirty-thousand cells per sample and three replicates per group were collected. Representative histograms are shown. B, Western blot analysis of cyclin D1 expression level in MCF-7 and MCF-7:5C cells following treatment with BZA and other SERMs. Prior to experiment, MCF-7 cells were switched from fully estrogenized media to estrogen-free media for 3 days and then treated with ethanol vehicle (control), 10^{-9} M E2 alone, or 10^{-9} M E2 plus FUL (10^{-7} M), RAL (10^{-7} M), 4OHT (10^{-7} M), or BZA (10^{-7} M) for 24 h. MCF-7:5C cells, however, did not require a media switch since they are hormone-independent and are routinely grown in estrogen-free media. MCF-7:5C cells were treated as described above for MCF-7 cells. Quantitated protein levels normalized to β -actin are indicated. C, BZA regulation of cyclin D expression and promoter activity in MCF-7:5C cells. Cells were treated with 10^{-7} M BZA for the indicated time points. Cyclin D1 protein and mRNA levels were determined by Western blot and quantitative RT-PCR, respectively with β -actin and 18S rRNA as internal controls. For cyclin

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D1 promoter activity experiment, MCF-7 and MCF-7:5C cells were cotransfected with a full-length cyclin D1 promoter plasmid (-1745CDLUC) and Renilla luciferase control plasmid overnight and then treated with 10^{-9} M E2, 10^{-8} M BZA, or E2 + BZA for 24 h. Luciferase activity was measured as described in materials and methods. Each point represents the mean of three determinations \pm SEM.

Fig. 5. Effect of cyclin D1 knockdown on proliferation and cell cycle in MCF-7:5C cells. A, Western blot analysis of cyclin D1 protein expression in MCF-7:5C cells transfected with 100 nM cyclin D1 siRNA or the nonspecific (NS)-control siRNA, as determined 72 hours post transfection. B, cell growth of transfected cells treated with 100 nM BZA or vehicle (control). Transfected cells (30,000/well) were seeded in 24-well dishes overnight and then treated with BZA for 5 days. After treatment, cells were collected and counted using a hemocytometer. Data is presented as percentage and is based on the mean from three independent experiments with duplicate (*, $p < 0.01$ versus nontarget transfected cells). C, cell cycle analysis of cyclin D1 siRNA-transfected and control-siRNA transfected MCF-7:5C cells following treatment with BZA for 48 hours. Data is based on the mean from three independent experiments with duplicate. *, $p < 0.01$; ** $p < .001$.

Fig. 6. Molecular modeling of ER α binding site with various ligands. A, comparison between the experimental (yellow sticks) and top ranked docking pose (cyan sticks) of raloxifene (RAL) to ER α binding site. The docking pose recapitulates very well the alignment of the co-crystallized ligand in the receptor binding site having a ligand RMSD of 0.36 Å. B, agonist conformation of ER α co-crystallized with E2; helix 12 is depicted in orange and lays over the

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binding site sealing the ligand inside it. The antagonist conformations of the receptor are shown in panels C, D, and E. X-ray structures of ER α co-crystallized with 4OHT (C), raloxifene (D), and bazedoxifene (E) docked into the ER α -raloxifene crystal structure. Helix 12 is depicted in magenta for 4OHT bound conformation and yellow for raloxifene and bazedoxifene. Also the key aminoacids lining the binding site are displayed and the network of hydrogen bonds in which they are involved with the ligands is shown in black dashed lines. Carbon atoms are colored in yellow for E2, orange for 4OHT, cyan for raloxifene and pink for bazedoxifene. These images show the differences between the agonist (B) and antagonist conformation (C, D, E) of ER α and present the alignment of bazedoxifene in the binding site of ER α which is similar with raloxifene's orientation and the same interactions with the key aminoacids of the binding cavity are encountered.

Fig. 7. Simplified representations of the ER α binding site with fulvestrant. A, cartoon representation of the ER α binding site with the best docking pose for fulvestrant (FUL, purple sticks). B, surface representation of ER α binding site accommodating FUL. Hydrophobic areas are mapped in purple while the hydrophilic parts are colored in light yellowgreen. The binding site accommodates very well the ligand which forms the H-bond contacts with the same aminoacids like E2 or RAL, while the aliphatic side chain protrudes out of the binding site and lies in the groove between helix 3 (orange cartoon) and helix 12 (purple cartoon). Only the key amino acids underlying the binding site are shown.

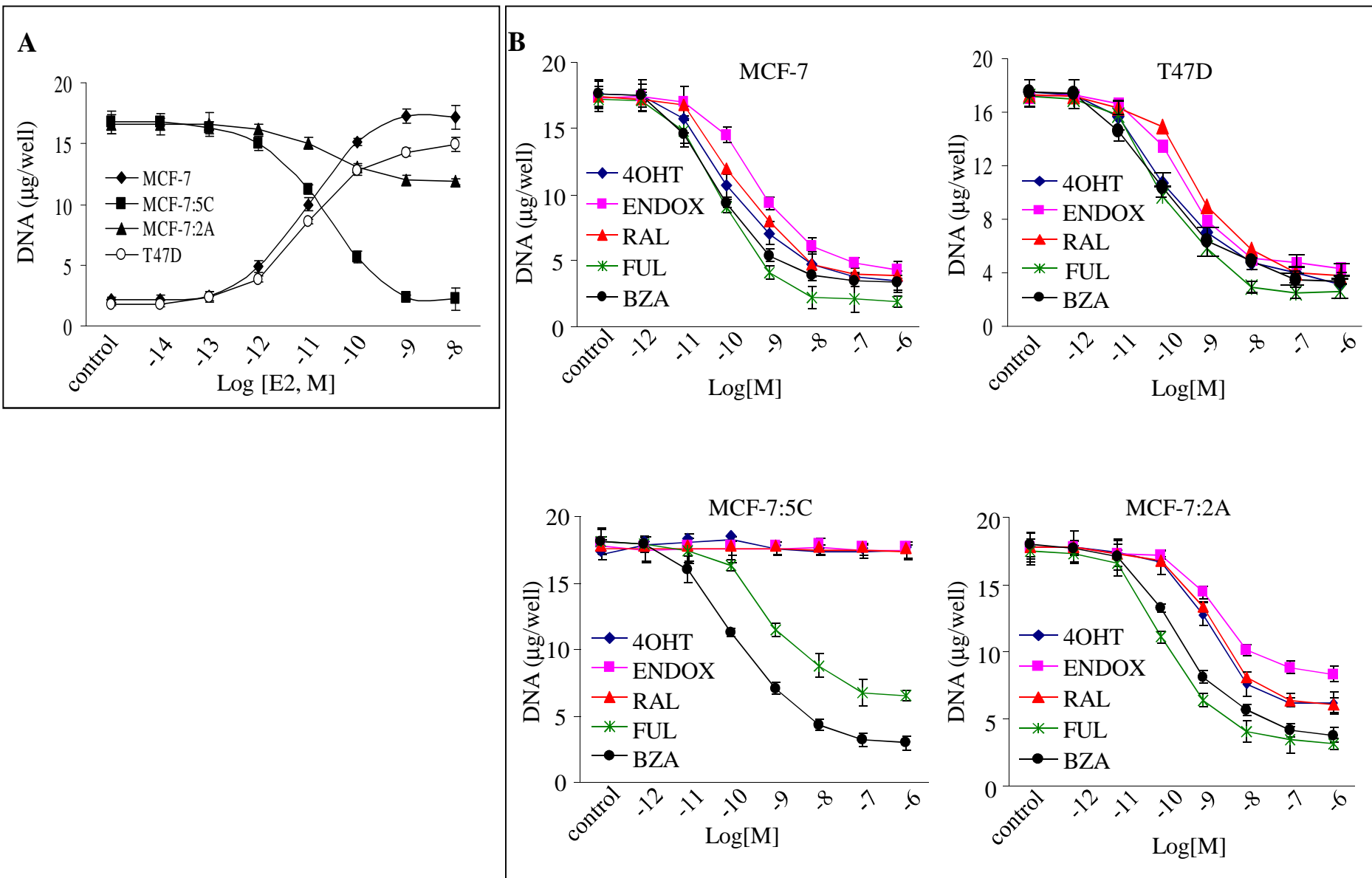


Figure 1

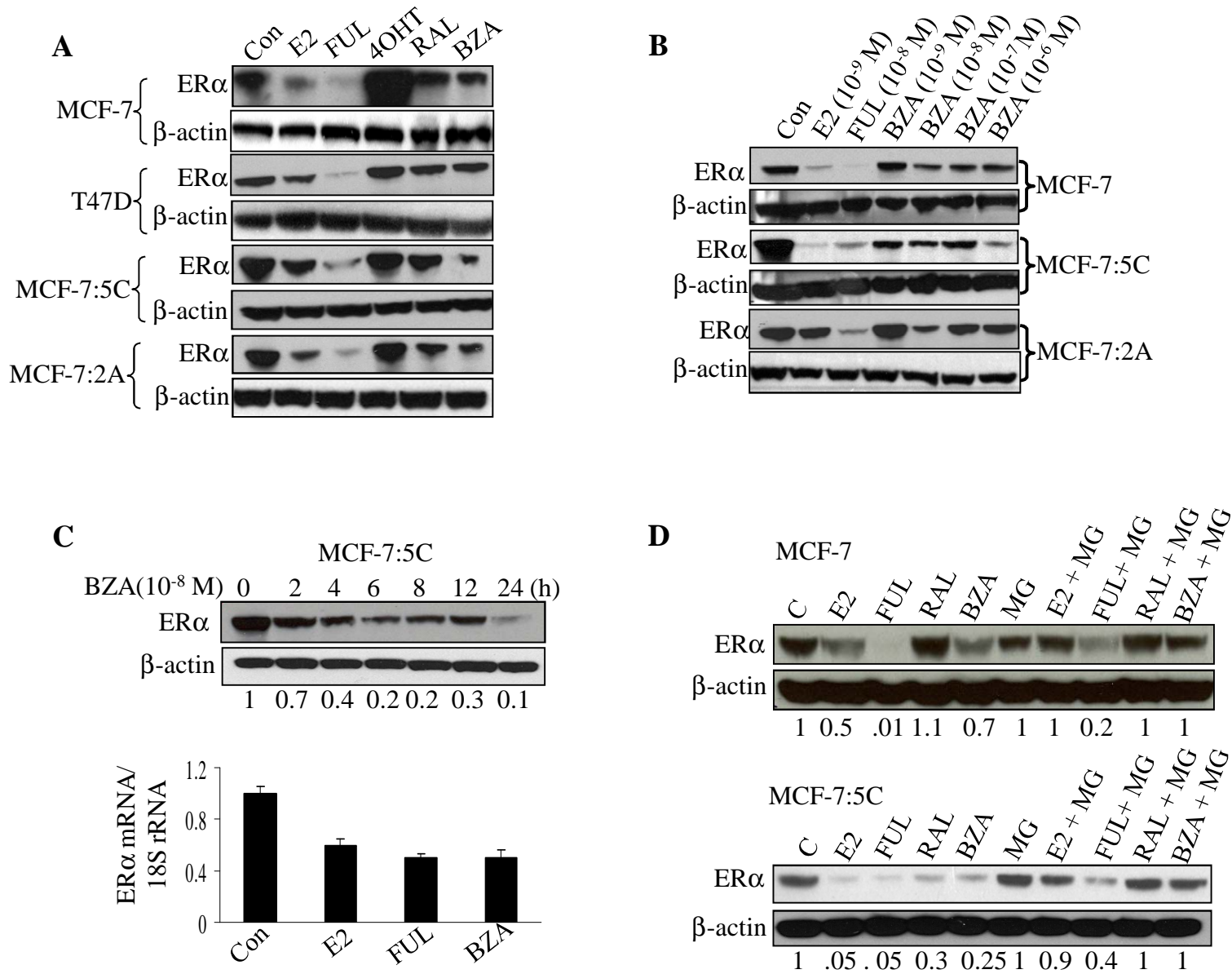


Figure 2

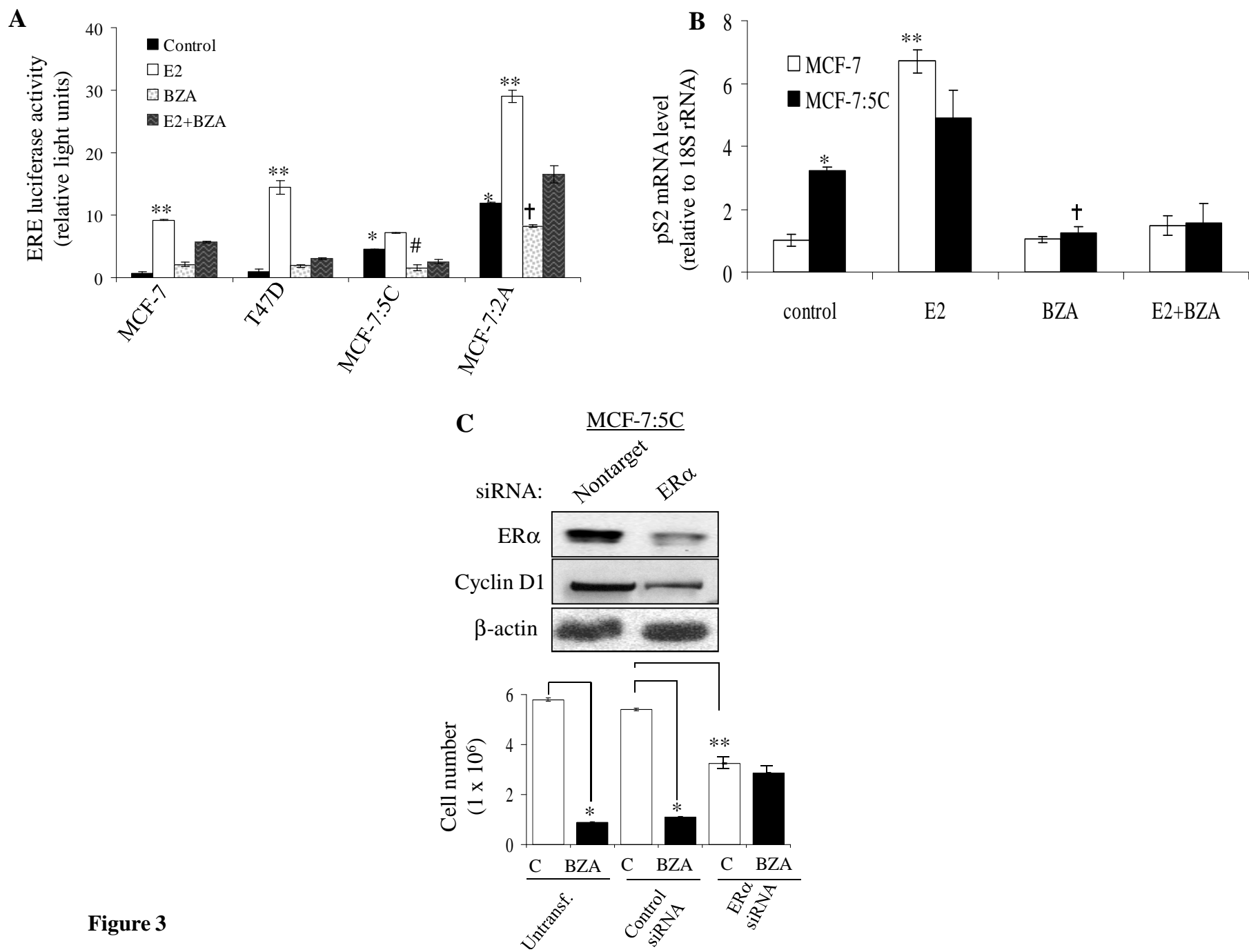


Figure 3

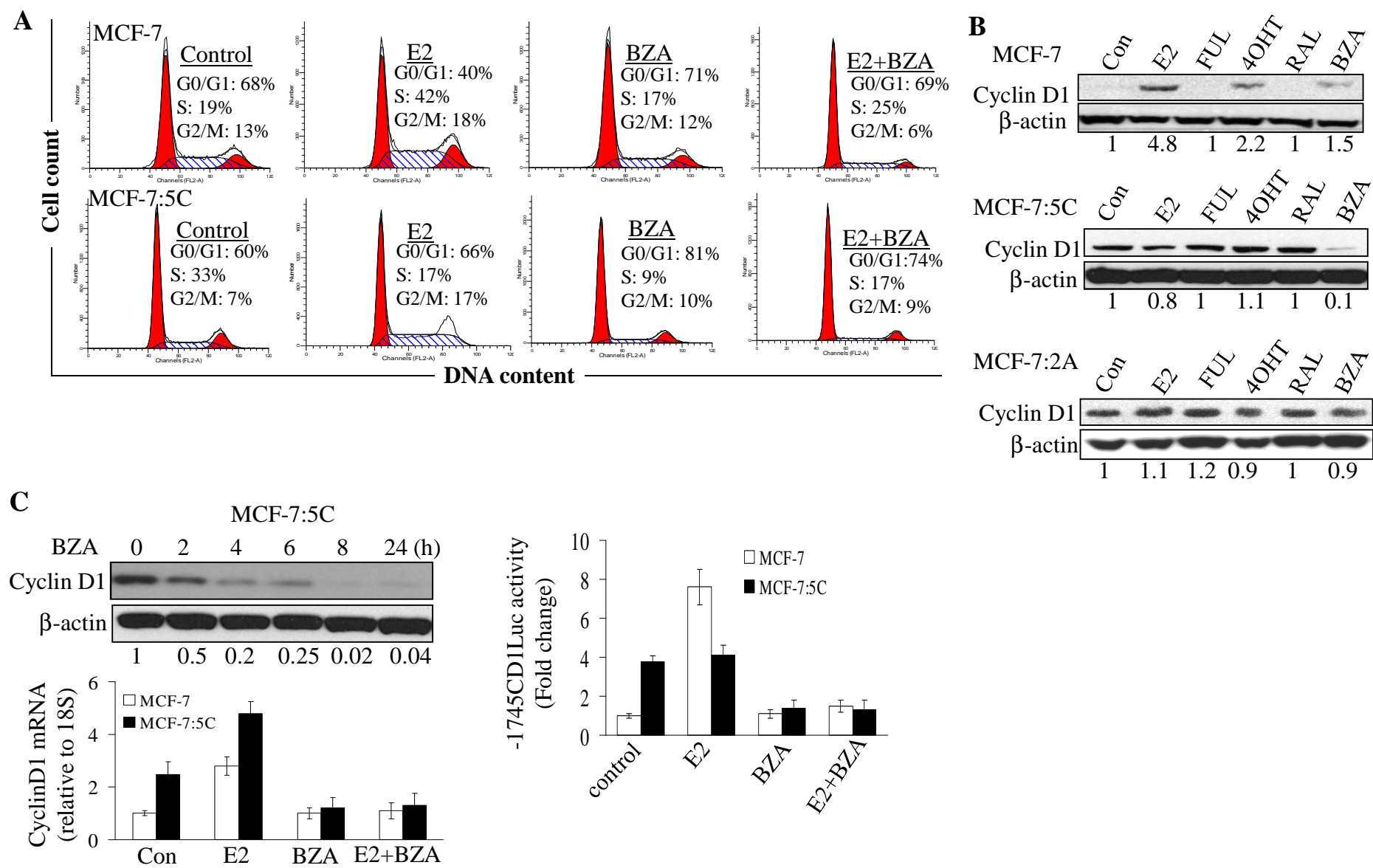


Figure 4

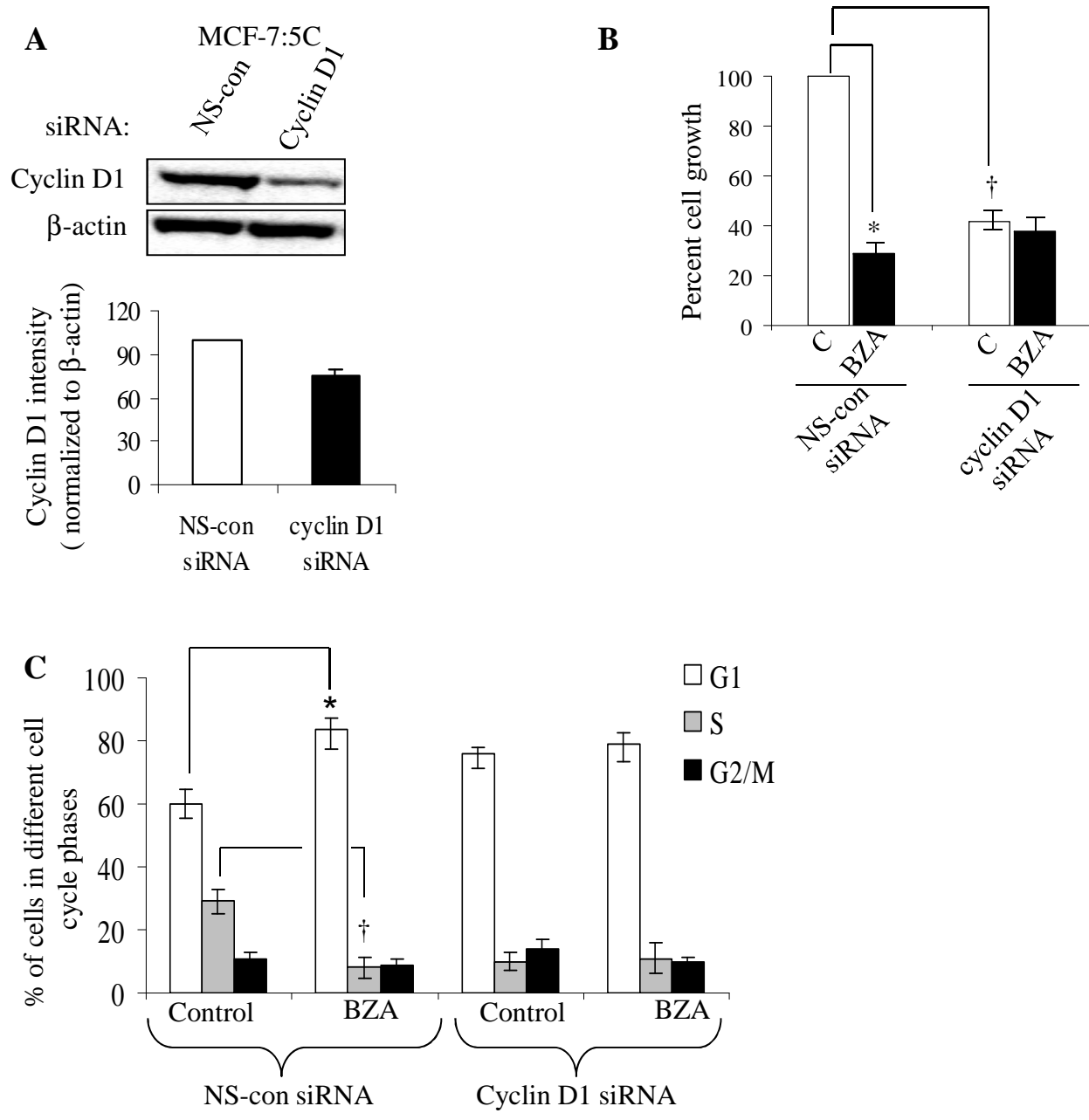
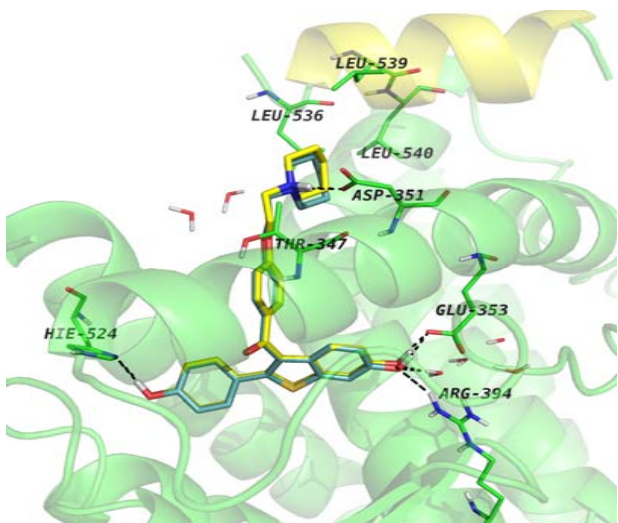
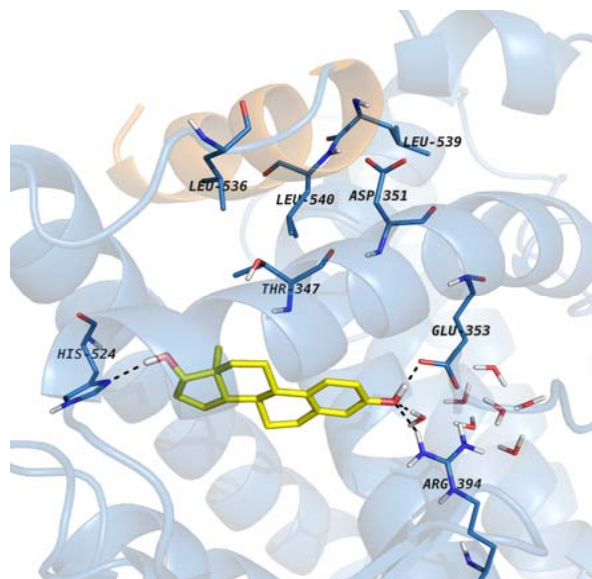


Figure 5

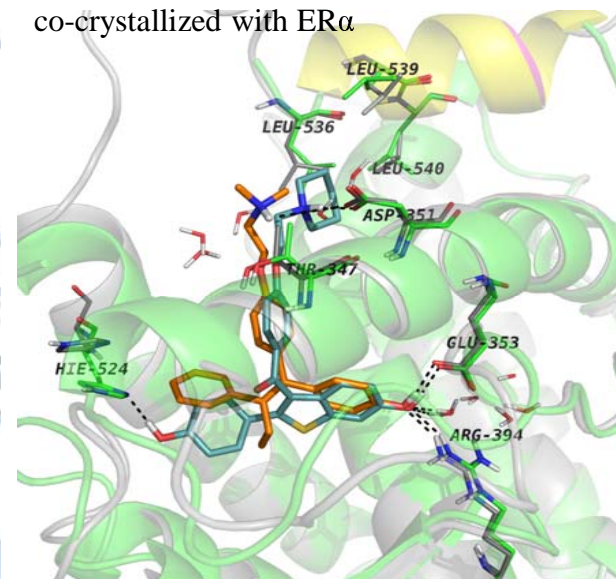
A. RAL docked to its native structure



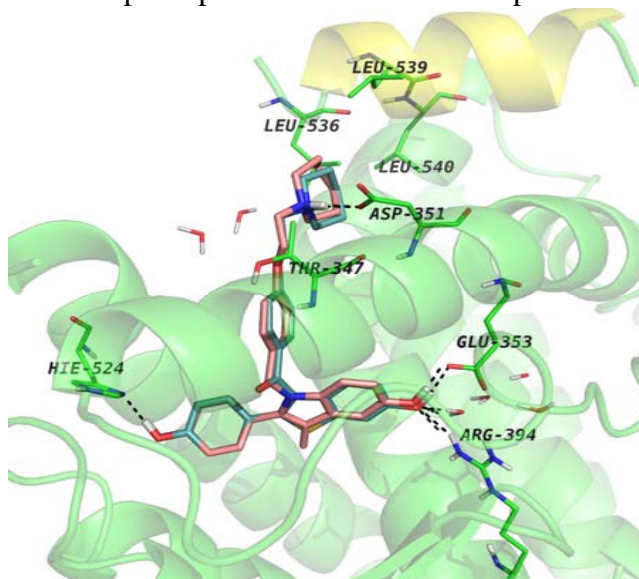
B. ER α co-crystallized with E2



C. Superimposed structures of 4OHT and RAL co-crystallized with ER α



D. BZA superimposed to RAL-ER α complex



E. BZA superimposed to 4OHT-ER α complex

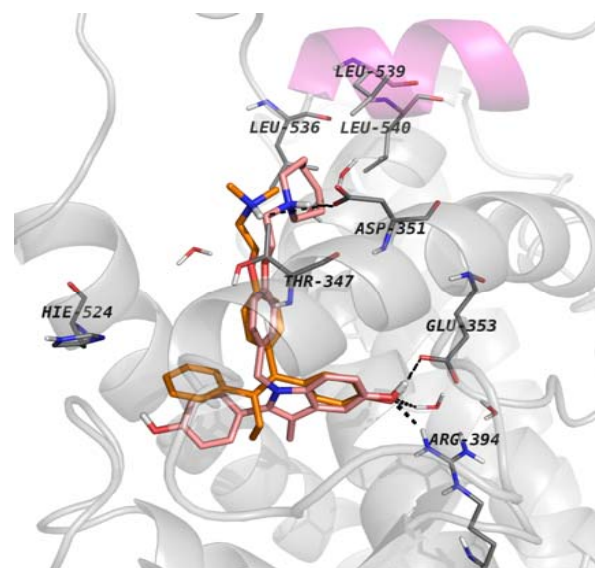


Figure 6

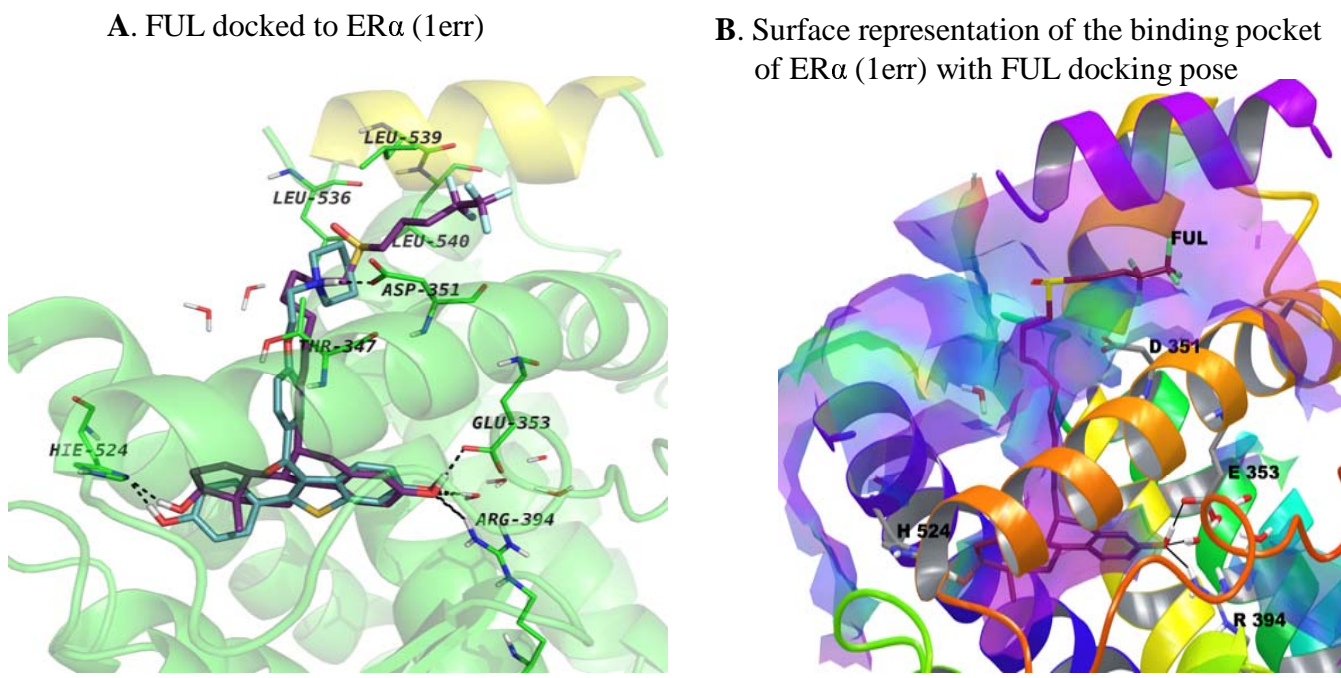
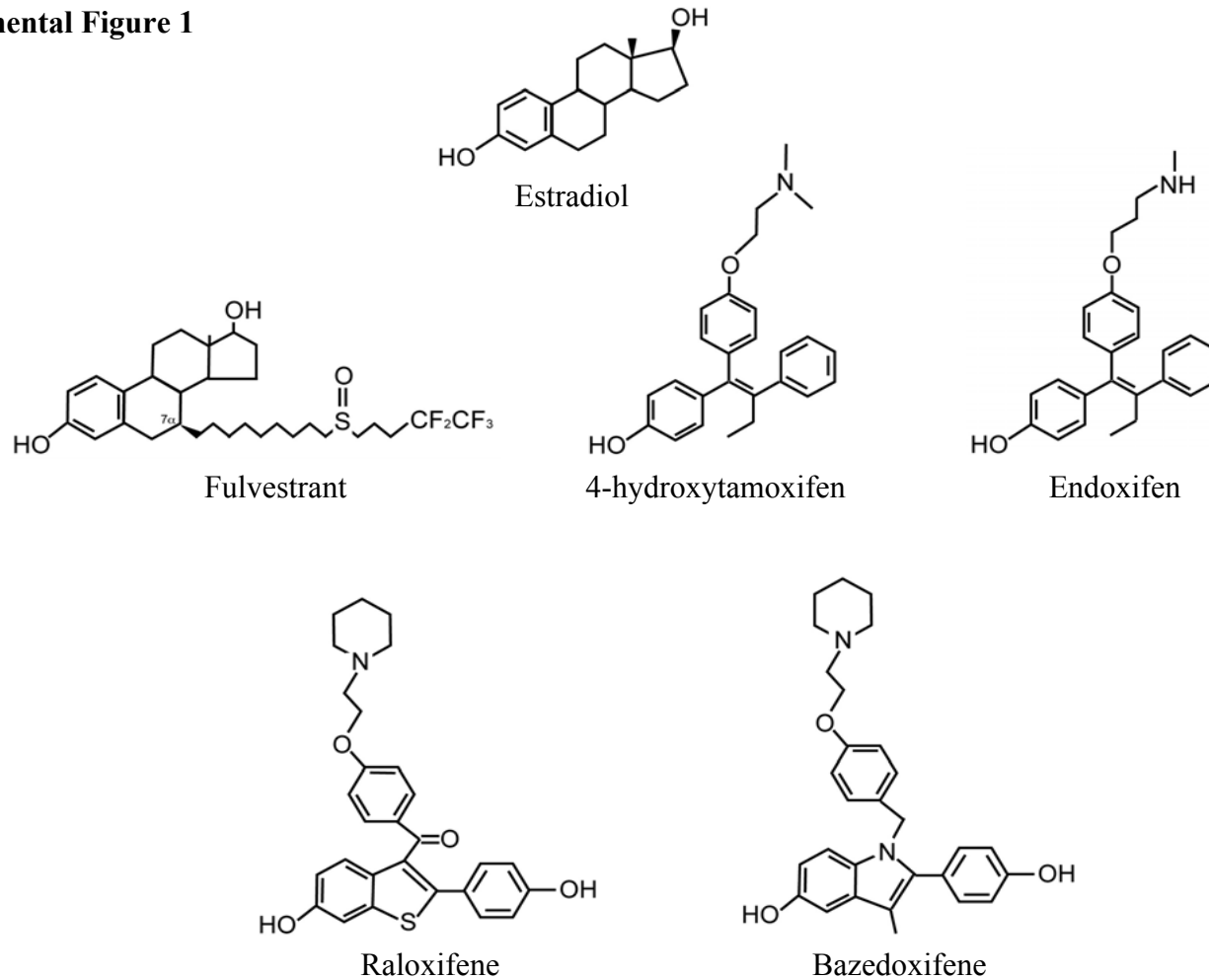


Figure 7

Supplemental Figure 1



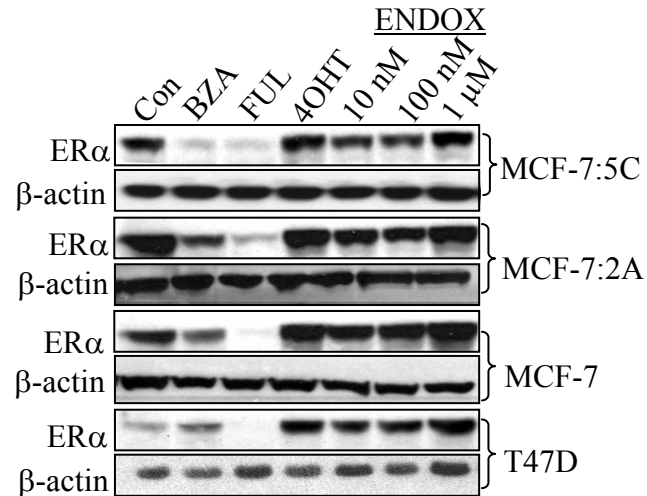
The Selective Estrogen Receptor Modulator Bazedoxifene Inhibits Hormone-Independent Breast Cancer Cell Growth and Downregulates Estrogen Receptor α and Cyclin D1.

Joan S Lewis-Wambi, Helen Kim, Ramona Curpan, Ronald Grigg, Mohammed A. Sarker, and V. Craig Jordan.

Molecular Pharmacology.

Chemical structures of the compounds used in this study. The agonist estradiol (E2) is shown, along with the pure antiestrogen fulvestrant (ICI 182,780), the hydroxylated metabolites of tamoxifen; 4-hydroxytamoxifen (4OHT) and endoxifen (ENDOX), and the related SERMs raloxifene (RAL) and bazedoxifene (BZA).

Supplemental Figure 2

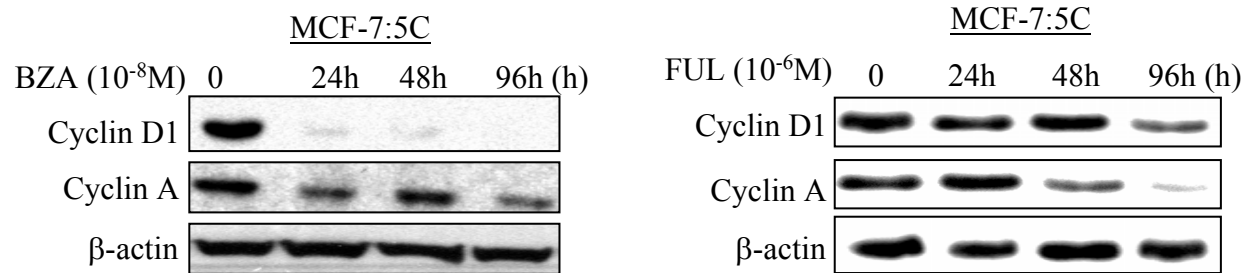


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Molecular Pharmacology.

Effect of endoxifen on ER α protein expression in MCF-7:5C, MCF-7, T47D, and MCF-7:2A breast cancer cells. The indicated cells were treated with 0.01, 0.1, and 1 μ M endoxifen for 24 hours and protein lysates were prepared and analyzed for ER α expression. For comparison, cells were also treated with 1 μ M BZA, FUL, or 4OHT for a similar time point. β -actin was used as an internal control to verify equal protein loading. Each blot is representative of 3 independent experiments and from a single exposure.

Supplemental Figure 3



The Selective Estrogen Receptor Modulator Bazedoxifene Inhibits Hormone-Independent Breast Cancer Cell Growth and Downregulates Estrogen Receptor α and Cyclin D1.

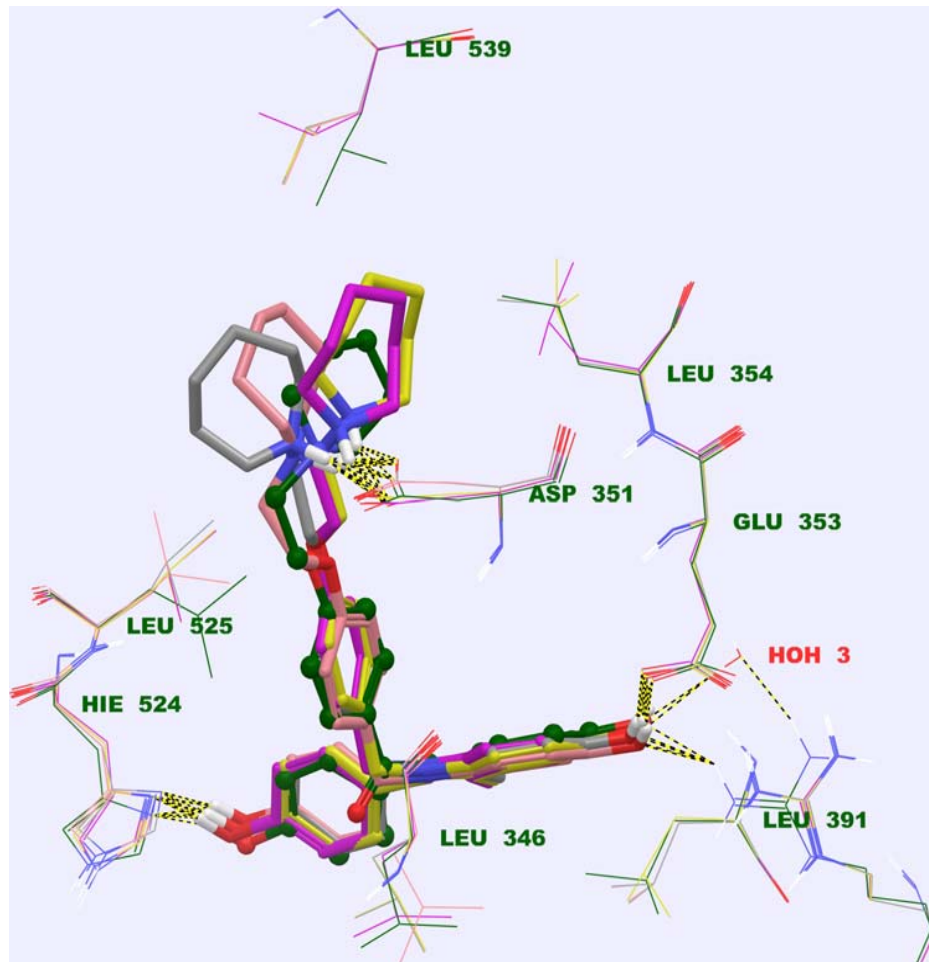
Joan S Lewis-Wambi, Helen Kim, Ramona Curpan, Ronald Grigg, Mohammed A. Sarker, and V. Craig Jordan.

Molecular Pharmacology

Effect of BZA and FUL on cyclin protein expression in

MCF-7:5C cells. Cells were treated with either 10⁻⁸ M BZA or 10⁻⁶ M FUL for 24, 48, and 96 hours and protein lysates were prepared and analyzed for cyclin D1 and cyclin A protein expression. β -actin was used as an internal control to verify equal protein loading. Each blot is representative of 3 independent experiments and from a single exposure.

Supplemental Figure 4



The Selective Estrogen Receptor Modulator Bazedoxifene Inhibits Hormone-Independent Breast Cancer Cell Growth and Downregulates Estrogen Receptor α and Cyclin D1.

Joan S Lewis-Wambi, Helen Kim, Ramona Curpan, Ronald Grigg, Mohammed A. Sarker, and V. Craig Jordan

Molecular Pharmacology. Flexible docking simulation performed with the Induced Fit protocol (IFD) method. The top ranked docking poses of BZA at the binding site of ER α are displayed. The experimental structure of ER α co-crystallized with RAL (1err) is depicted in dark green, while the top ranked IFD poses of the BZA-ER α complex are displayed with carbons colored in gray, light pink, yellow and purple, respectively