Activity of Estrogen Receptor β Agonists in Therapy-Resistant Estrogen Receptor-Positive Breast Cancer

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Background: Among women, breast cancer is the leading cause of cancer-related death worldwide. Estrogen receptor α-positive (ERα+) breast cancer accounts for 70% of all breast cancer subtypes. Although ERα+ breast cancer initially responds to estrogen deprivation or blockade, the emergence of resistance compels the use of more aggressive therapies. While ERα is a driver in ERα+ breast cancer, ERβ plays an inhibitory role in several different cancer types. To date, the lack of highly selective ERβ agonists without ERα activity has limited the exploration of ERβ activation as a strategy for ERα+ breast cancer.

Methods: We measured the expression levels of ESR1 and ESR2 genes in immortalized mammary epithelial cells and different breast cancer cell lines. The viability of ERα+ breast cancer cell lines upon treatments with specific ERβ agonists, including OSU-ERb-12 and LY500307, was assessed. The specificity of the ERβ agonists, OSU-ERb-12 and LY500307, was confirmed by reporter assays. The effects of ERβ agonists on cell proliferation, cell cycle, apoptosis, colony formation, cell migration, and expression of tumor suppressor proteins were analyzed. The expression of ESR2 and genes containing ERE-AP1 composite response elements was examined in ERα+ human breast cancer samples to determine the correlation between ESR2 expression and overall survival and that of putative ESR2-regulated genes.

Results: In this study, we demonstrate the efficacy of highly selective ERβ agonists in ERα+ breast cancer cell lines and drug-resistant derivatives. ERβ agonists blocked cell proliferation, migration, and colony formation and induced apoptosis and S and/or G2/M cell-cycle arrest of ERα+ breast cancer cell lines. Also, increases in the expression of the
INTRODUCTION

Breast cancer is the most prevalent cancer among women globally (1). It is the second leading cause of cancer-related deaths among women. In 2020, there were 2.3 million new breast cancer cases and 685,000 breast cancer deaths worldwide. Despite advances in diagnostic procedures and improved therapies, globally breast cancer-related morbidity and mortality are on the rise. The majority of breast cancer-related deaths occur due to distant metastasis. About 60% of metastatic breast cancers (MBC) are estrogen receptor positive (ER+). About 60% of metastatic breast cancers (MBC) are estrogen receptor positive (ER+) and human epidermal growth factor receptor 2 non-amplified (HER2-) (2). Although the development of effective estrogen blocking agents and cyclin-dependent kinase 4/6 inhibitors (CDK4/6i) has doubled development of effective estrogen blocking agents and cyclin-dependent kinase 4/6 inhibitors (CDK4/6i) has doubled progression-free survival on first-line therapy of ERα+/HER2-MBC, endocrine and CDK4/6i resistance emerges causing disease progression. Appropriate post-CDK4/6i therapy is poorly defined due to an incomplete understanding of CDK4/6i resistance, lack of effective agents, and lack of clinical trials that address this important issue.

While augmented signaling through receptor tyrosine kinases, NFI loss, C-MYC amplification, and activating mutations in the ESR1 gene result in endocrine resistance, alterations of cell-cycle genes cause CDK4/6i resistance (3–5). Due to redundancy and cross talk in these signaling pathways, attempts to counter therapeutic resistance by focusing on a single target have been mostly ineffective. Thus, there is an urgent need to develop novel therapeutic options in the second-line setting to improve the survival and response rate for aggressive endocrine and CDK4/6i-resistant MBC.

Estrogens play a vital role in breast tumorigenesis (6, 7). The stimulatory or repressive effects of estrogens are mediated through ERα and ERβ, which are gene products of ESR1 and ESR2, respectively, and the G protein-coupled estrogen receptor (GPRC30). Unlike ERα, which has a clear oncogenic role in ERα+ breast cancer, ERβ behaves like a tumor suppressor in many biological contexts. For example, the tumor-suppressive function of ERβ was demonstrated through its knockdown in ERα+ cell lines, which induced an invasive phenotype, increased anchorage-dependent cell proliferation, and elevated EGF-R signaling (8). In the presence of estradiol, ERβ overexpression reduced cell proliferation in vitro and tumor formation in vivo, effects that are in contradistinction to those of ERα (9, 10).

In these experiments, ERβ was also shown to repress the expression of oncogenes such as c-myc and cyclin D1 (CCND1).

The transcriptional function of ERs involves their binding to estrogen response elements (ERE) within promoters and enhancers (11). There are multiple conformations of EREs in the human genome, including consensus and non-consensus EREs, single and multiple binding sites, and composite EREs consisting of ERE half-sites in combination with binding sites for other transcription factors such as AP1 and Sp1. Although both the receptors exhibit transcriptional activity, they differ in their modes of transcriptional activation (12). Studies demonstrated that on certain E2-responsive ERE-AP-1 composite promoters, ERβ actually antagonizes the effects of ERα (13). For example, the CCND1 promoter, containing a cAMP response element and an AP-1-binding site, is activated by estradiol in cells overexpressing ERα but is inhibited in cells overexpressing ERβ (13).

ESR2 was discovered more than 20 years ago (14), but its clinical application was limited by the lack of highly selective ERβ agonists. Although both ERα and ERβ are activated by binding to endogenous estrogens, the development of several highly selective synthetic ligands of ERα or ERβ has uncovered new avenues to probe the function of these receptors (15).

In the present study, we investigated the effects of a novel and highly selective ERβ-selective agonist, OSU-ERb-12 (16), to inhibit preclinical models of ERα+ breast cancer and to counter endocrine and CDK4/6i resistance in vitro. We found that treatment of ERα+ breast cancer cell lines with OSU-ERb-12 caused apoptosis, induced cell-cycle arrest (at S phase), and decreased cell proliferation, colony formation, and cell migration. FOXO1 and FOXO3a protein expression was significantly increased in cells treated with OSU-ERb-12, a potential mechanism for its tumor-suppressive effects (17).

MATERIALS AND METHODS

Chemicals, Drugs, Plasmids, Antibodies, Primers, and Synthesis of MCSR-18-006

OSU-ERb-12 was synthesized in the Drug Development Institute (DDI) at OSU according to the procedure outlined before (16). LY500307 was also obtained from DDI, OSU. AC186 (cat# 5053), WAY200076 (cat# 3366), diarylpropionitrile (DPN; cat# 1494), 4-hydroxy-tamoxifen (Tam; cat# 3412/10), fulvestrant
commercially available reagents and solvents. Details of the reactions were carried out under argon atmosphere using corresponded with calculated patterns. Unless otherwise noted, the theoretical isotopic pattern was described. Measured patterns obtained mass resembling the most intense peak of the mass spectrometer. For carborane-containing compounds, the source and TLC plate express or using a Thermo LTQ Orbitrap Model S Compact Mass Spectrometer equipped with an APCI source and a DRX400 NMR spectrometer or a Bruker AV300NMR, fi3XERE TATA luc (lucerase reporter that contained three copies of vitellogenin estrogen response element) was a kind gift from Donald McDonnell (Addgene plasmid # 11354; http://n2t.net/addgene: 11354; RRID: Addgene_11354). Plasmids pcdNA3 (Ohu23619C; pcdNA3.1+; RRID: Addgene_10842), ERβ (Ohu25562C; pcdNA3.1+), ERβ (Ohu25562D; pcdNA3.1+c(K) DKY), c-Flag ERα (Ohu26868D; pcdNA3.1+c(K) DKY), and c-Flag ERβ (Ohu25562D; pcdNA3.1+c(K) DKY) were obtained from Promega. pRLTK plasmid was obtained from Promega. 3XERE TATA luc (lucerase reporter that contained three copies of vitellogenin estrogen response element) was a kind gift from Donald McDonnell (Addgene plasmid # 11354; http://n2t.net/addgene: 11354; RRID: Addgene_11354). Plasmids pcdNA3 (Ohu23619C; pcdNA3.1+; RRID: Addgene_10842), ERβ (Ohu25562C; pcdNA3.1+), ERβ (Ohu25562D; pcdNA3.1+c(K) DKY), c-Flag ERα (Ohu26868D; pcdNA3.1+c(K) DKY), and c-Flag ERβ (Ohu25562D; pcdNA3.1+c(K) DKY) were obtained from Promega. The following primers were used for the corresponding mRNAs. ESR2 full length: forward (5′-CTCCAGATCTCTTG TCTGGAACGGAT-3′), reverse (5′-GGTGGAGGAATGTTC CATGCCCCGTGTA-3′); ESR2 all isoforms: forward (5′-ACA CACCTTACCTGTAAACAGAGAG-3′), reverse (5′-GGG AGCCACACTTCCACATC-3′); ESR1: forward (5′-CCGCC GGCATTCJACAGGGC-3′), reverse (5′-GAAGAAGGCCGT TCACGG-3′); GAPDH: forward (5′-GGCTGTATTGGGCGG CTGTC-3′), reverse (5′-TT TGGAGGGATCTCGCTC-3′).

1H-NMR spectra were recorded using a Bruker AV300NMR, AVIII400HD NMR spectrometer or a DRX400 NMR spectrometer at The Ohio State University College of Pharmacy. Chemical shifts (δ) are specified in ppm from chemical reference shifts for internal deuterated chloroform (CDCl3) set to 7.26 ppm. Coupling constants are defined in Hz. Mass spectra were obtained using an Advion Expression Model S Compact Mass Spectrometer equipped with an APCI source and TLC plate express or using a Thermo LTQ Orbitrap mass spectrometer. For carbon-containing compounds, the obtained mass resembling the most intense peak of the theoretical isotopic pattern was described. Measured patterns corresponded with calculated patterns. Unless otherwise noted, all reactions were carried out under argon atmosphere using commercially available reagents and solvents. Details of the procedure for the synthesis of MCSR-18-006 are provided in Supplemental Data.

Cell Culture, Cell Viability, and Generation of Resistance
Immortal mammary epithelial cells MCF10A (ATCC Cat# CRL-10317, RRID: CVCL_0598) and breast cancer cell lines MCF7 (ATCC HTB-22), T47D (ATCC HTB-133; NCI-DTP Cat# T-47D, RRID: CVCL_0553), ZR-75-1 (ATCC CRL-1500), MDA-MB-231 (ATCC HTB-26, RRID: CVCL_0062), MDA-MB-468 (ATCC HTB-132, RRID: CVCL_0419), and HEK-293T (ATCC Cat# CRL-3216, RRID: CVCL_0063) were obtained from ATCC. All the cells were grown according to the supplier’s recommendation in a humidified atmosphere containing 5% CO2 at 37°C. Cells were passaged and media changed every 2 days. Mycoplasma contamination of the cells were checked monthly using the MycoAlert Plus Mycoplasma Detection Kit (cat# LT07-703) (Lonza, Walkersville, MD, USA) following the manufacturer’s protocol. For routine experiments, parental and drug-resistant cells of MCF7 and T47D were cultured in phenol red-free basal medium (DMEM) media, containing charcoal-stripped fetal bovine serum (10%), L-glutamine (2 mM), Na-pyruvate (1 mM), penicillin (100 units), and streptomycin (100 units).

Resistant MCF7 (MCF7-TamR and MCF7-FasR) cells were gifts from Dr. Kenneth Nephew (18). In addition, the MCF7 cell line that overexpresses CDK6 (MCF7-CDK6 O/E), which has previously been described and is resistant to abemaciclib, was a gift from Sarat Chandralapaty, Memorial Sloan Kettering Cancer Center (19). T47D cells were treated at gradually increasing concentrations with 4-hydroxy-tamoxifen (Tam), fulvestrant/Faslodex (Fas; estrogen receptor antagonist) or abemaciclib (cyclin-dependent kinase 4/6 inhibitor; CDK4/6i) to generate resistant cell lines (T47D-TamR, T47D-FasR, and T47D-CDK4/6iR). Similarly, MCF7 cells were treated with increasing concentrations of abemaciclib to generate MCF7-CDK4/6iR cells. Control cells were treated with the vehicle DMSO. The starting concentrations of the drugs ranged from 25 to 50 nmol/l and increased stepwise every 2–3 weeks. To evaluate the development of resistance, cells (both control and drug-treated) were examined for viability every 4 to 6 weeks with the CellTiter-Glo assay (Promega). Unless stated otherwise, cell viability was measured in quadruplicates by seeding the cells (2,000 to 3,000 per well in 96-well plate), followed by addition of Tam, Fas, or abemaciclib at different dilutions or DMSO (vehicle control) after 24 h. Seventy-two hours later, luminescence was measured after the addition of CellTiter-Glo reagent following the manufacturer’s protocol. Cell viability was calculated as percentage relative to vehicle controls (100%). Sigmoidal dose-response curves were plotted using GraphPad Prism software (GraphPad Prism, RRID: SCR_002798). Upon manifesting resistance, cells were maintained with continued drug exposure at concentrations to which they were resistant.

Immortal mammary epithelial MCF10A cells as well as MCF7 and T47D breast cancer (parental and respective resistant) cells were allowed to grow overnight followed by treatment with OSU-
ERb-12, LY500307, DPN (diarylpropionitrile), AC186, and WAY200070 (WAY) at varying concentrations as indicated. The fresh medium and drugs were replaced every alternate day. Cell viability was assessed after 7 days of initial drug exposure using CellTiter-Glo Luminescent Cell Viability Assay, and the viability curves were plotted as mentioned above.

**Reverse Transcription Polymerase Chain Reaction, Western Blot Analysis, Estrogen Response Element Luciferase Reporter Assays, and Messenger RNA Expression Analysis of Patient Samples**

Total RNA was isolated from cells using TRIzol reagent (cat# 15596026) (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, treated with DNase I, and reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time RT-PCR (qRT-PCR) was performed using 0.01–0.03µg cDNA with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) in an Applied Biosystems thermocycler. The fold difference in target gene mRNA levels normalized to GAPDH was calculated using the ΔΔCT method. Semiquantitative PCR was performed using the same set of primers as in qRT-PCR and visualized after electrophoretic separation to confirm the identity of the amplicons. The primers were designed spanning the exon–exon junction to avoid non-specific amplification of genes.

Whole-cell extracts were prepared in cell lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA, 1% SDS, and 1% IGEPAL CA-630; cat# 18896, Sigma–Aldrich) followed by sonication and centrifugation at 14,000 rpm for 10 min at 4°C. Protein concentrations in the extracts were measured using the bicinchoninic acid (BCA) method using BSA as the standard. Equivalent amounts of protein from whole-cell lysates were mixed with 4x Laemml’s buffer, boiled for 5 min at 97°C, separated by SDS-polyacrylamide (10%) gel electrophoresis (Thermo Fisher Scientific), transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL), and probed with the antibodies described above. Membranes were incubated overnight at 4°C with the primary antibody, washed, and blotted for an hour with secondary anti-mouse/rabbit (HRP-conjugated) antibodies. The enhanced chemiluminescence substrate detection system (Millipore-Sigma) was applied to detect bound antibody complexes and visualized by autoradiography. The loading control was GAPDH. The intensity of the protein bands was quantified using Image Studio (LiCor).

HEK293T cells (7.5 × 10⁴/well) seeded in a 24-well plate were transfected for 12 h with ERE-Luc, pRLTK (internal control, Promega), and c-Flag pcDNA3/ERα/ERβ plasmids using Lipofectamine 3000 (Thermo Fisher Scientific) following the manufacturer’s protocol. The media were changed with phenol-red free DMEM containing 10% charcoal-stripped FBS and insulin (6 ng/ml). Six hours later, cells were treated with OSU-ERb-12 or LY500307 at varying concentrations as indicated. DMSO was used as a vehicle control. Luciferase activity was assessed after 72 h of transfection using Dual-Luciferase Assay System (Promega).

Patients treated at The Ohio State University Comprehensive Cancer Center – Arthur G. James Cancer Hospital and Richard J. Solove Research Institute since 1998 with a diagnosis of metastatic ERα+ and HER2-negative (ERα+/HER2-) breast cancer and confirmed RNA sequencing analysis were eligible for this retrospective clinical correlation. Following IRB approval (OSU protocol No: 2018C0211), the list of patients fulfilling the previous criteria was obtained from the Ohio State University Medical Center and James Cancer Registry. 118 medical record were reviewed, and 37 patients had RNA sequencing performed through the Oncology Research Information Exchange Network (ORIEN) and were deemed eligible.

Data for the 37 eligible patients were initially queried and obtained from The Ohio State University Information Warehouse and from ORIEN-AVATAR and uploaded into REDCap (REDCap, RRID : SCR_003445). Data missing from the initial query were populated using a manual review of each patient’s electronic medical record.

Total RNA was sequenced with minimum 20 million reads and >65% reads aligned identified for subsequent processing to transcript abundance values (FKPM; fragments per kilobase per million reads) following the ORIEN standard pipeline: STAR aligner (STAR, RRID : SCR_004463), Star-fusion, and RSEM (RSEM, RRID : SCR_013027) with genome GRCh38 alignment/annotation.

**Cell Proliferation, Cell Cycle Analysis, Apoptosis, Clonogenic Survival, and Cell Migration Assays**

MCF7 and T47D cells were plated at 5 × 10⁵ cells per plate in phenol red-free complete DMEM supplemented with charcoal-stripped FBS. Twenty-four hours later, the cells were treated for 72 h with OSU-ERb-12 (0.5 and 10 µmol/l) or LY500307 (MCF7: 0.5 and 3 µmol/l; T47D: 0.5 and 7 µmol/l). Differing concentrations were used to test complete loss of viability. DMSO and fulvestrant (0.5 µmol/l) were used as negative and positive controls, respectively. The cells were harvested and stained as per the protocol for the Click-it EdU Alexa Fluor 647 Kit (Invitrogen; cat# C10424). The stained cells were analyzed via flow cytometry (BD FACSCalibur Flow Cytometer).

For cell-cycle analysis, MCF7 and T47D cells were plated and treated for 72 h with OSU-ERb-12 or LY500307 at the indicated concentrations. DMSO was used as vehicle control. The cells were harvested, fixed in 70% ethanol, and stained with propidium iodide. The stained cells were analyzed via flow cytometry on a BD FACSCalibur Flow Cytometer.

Breast cancer MCF-7 and T47D cells were plated and treated 24 h later with OSU-ERb-12 (0.5 and 10 µmol/l) or LY500307 (MCF7: 0.5 and 3 µmol/l; T47D: 0.5 and 7 µmol/l) for 48 h. Cells were collected and processed according to the manufacturer (TUNEL Assay Kit - BrdU-Red (cat# ab66110) (Abcam, Cambridge, MA, USA)). Processed breast cancer cells were analyzed on a BD FACSCalibur Flow Cytometer to determine the percentage of apoptotic cells in each treatment group.

MCF7 and T47D cells were plated in 60-mm dishes (~1,000–2,000 cells). Twenty-four hours after plating, cells were treated with OSU-ERb-12, LY500307, or vehicle (DMSO) for 7–10 days. The fresh medium and drugs were replaced every other day. Next, cell colonies were washed with PBS, fixed with paraformaldehyde (4%),
and stained with crystal violet solution (0.05%). Colonies were then washed with water and air-dried. Visible colonies were counted manually.

For cell migration assay, MCF7 cells were seeded, treated with DMSO (control), OSU-ERb-12, or LY500307 and allowed to grow until confluence. Confluent monolayers were scratched using a sterile pipette tip, washed, and incubated in complete medium containing DMSO or the drugs. Plates with similar scratch were selected by examination under microscope and used for further analysis. Images were captured immediately after scratch (0 h) and 24 h post-scratch. Migration of cells from the edge of the groove toward the center was monitored at 24 h (×40 magnification). To calculate the fraction of the gap covered by the cells in a 24-h period, the width of the scratch was measured at 0 h and at 24 h. The mean fraction of the filled area was determined, and data presented were normalized to the control cells.

Statistical and Bioinformatics Analyses
Viability, proliferation, apoptosis, and cellular mRNA expression were analyzed using student’s t-test. For each dose, linear mixed models were fit for log-transformed viability with fixed effects for regimen (4-hydroxy-tamoxifen, OSU-ERb-12, and 4-hydroxy-tamoxifen+OSU-ERb-12) and random effects accounting for the within-batch correlation of replicates. Predictions and standard errors for viability of the 4-hydroxy-tamoxifen+OSU-ERb-12 combination under a hypothesized Bliss independence model were computed from estimated mean viabilities under 4-hydroxy-tamoxifen and OSU-ERb-12 alone via the formula

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\text{Log Viability (Bliss)} = \text{Log Viability (4-hydroxy-tamoxifen)} + \text{Log Viability (OSU-ERb-12)}
\]

Interaction at each dose was selected by examination under microscope and used for further analysis. Images were captured immediately after scratch (0 h) and 24 h post-scratch. Migration of cells from the edge of the groove toward the center was monitored at 24 h (×40 magnification). To calculate the fraction of the gap covered by the cells in a 24-h period, the width of the scratch was measured at 0 h and at 24 h. The mean fraction of the filled area was determined, and data presented were normalized to the control cells.

RESULTS
Selection for Drug-Resistant MCF7 and T47D Cell Lines
We cultured the T47D cell line in the presence of DMSO (control), 4-hydroxy-tamoxifen, fulvestrant, or the CDK4/6i abemaciclib and MCF7 with abemaciclib, at gradually increasing concentrations to select for acquired resistance. With extended exposure of about 8 months, both the cell lines demonstrated decreased sensitivity to the drugs compared with the corresponding parental controls (Supplemental Figure 1). As shown in Supplemental Figure 1, the resistant cells maintained in drugs containing media and, after several passages over 8 months, demonstrated about 15–110-fold higher IC50 values compared with the corresponding control (DMSO) cells. However, in some cases (MCF7-TamR and T47D-TamR) even at high concentrations of the drugs, we did not observe a loss of viability and, therefore, we could not calculate an IC50 value. Three resistant lines T47D-TamR, T47D-FasR, and, T47D-abemaciclibR cells were maintained in culture with the corresponding drugs at 0.5 μmol/l, and MCF7-abemaciclibR was maintained in abemaciclib at 0.2 μmol/l. Chemical structures of the drugs/inhibitors used in this study are provided in Supplemental Figure 2. Lack of activation of the ERE-luciferase reporter vector by overexpressed ERα and ERβ proteins in 293T cells treated with the inactive chemical analog of OSU-ERb-12 and MCSR-18-006, is shown in Supplemental Figure 3. The lack of binding affinity of MCSR-18-006 for ERα and ERβ proteins as measured by radiolabeled estradiol competition binding assays is shown in Supplemental Figure 4.

ESR2 and ESR1 Genes and Their Protein Products Are Differentially Expressed in Various Breast Cancer Cell Lines, and ERβ Agonists Significantly Enhance ERβ-Driven ERE-Luciferase Promoter Activity
We assessed the basal expression levels of ESR2 and ESR1 in three ERα+ breast cancer cell lines (MCF7, T47D, and ZR-75-1) and the derivative endocrine-resistant and CDK4/6i-resistant lines (of MCF7 and T47D) and compared them with those of immortalized mammary epithelial cells (MCF10A) (Figure 1) using primers that selectively amplified only the full-length, canonical ESR2 transcript or that amplified all known splice variants of ESR2 (Supplemental Figure 5A), as well as primers that specifically amplify full-length ESR1. The p-values and 95% confidence interval (CI) of corresponding expression data are shown in Supplemental Table 1. qRT-PCR data demonstrated a comparable expression of full-length ESR2 in MCF7 and MCF10A lines (Figure 1A, Supplemental Table 1). While MCF7-FasR and MCF7-CDK6-O/E cells displayed no significant increase in full-length ESR2 expression relative to the control MCF10A cells, MCF7-TamR and MCF7-CDK4/6iR cells showed 3.6-fold (p = 0.0035) and 6-fold (p = 0.0001) higher expression levels, respectively (Figure 1A and Supplemental Table 1). On the other hand, T47D exhibited a 4.8-fold (p = 0.0265) higher expression of ESR2 compared to MCF10A cells. A significantly higher expression of full-length ESR2 in T47D-TamR (5.1-fold, p = 0.0009) and T47D-CDK4/6iR (5.1-fold, p = 0.0075) compared to MCF10A was noted (Figure 1A and Supplemental Table 1). ZR-75-1 cells displayed the highest level of full-length ESR2 RNA expression (~19-fold higher than MCF10A; p < 0.01) (Figure 1A and Supplemental Table 1). Both the TNBC lines had a significantly higher expression of full-length ESR2 compared with MCF10A (MDA-MB-231: 4.4-fold, p < 0.05; MDA-MB-468: 5.2-fold, p < 0.01), and these levels were comparable to those in the ERα+ MCF7 and T47D breast cancer cell lines.

When we measured expression levels using primers that amplified all the splice isoforms of ESR2, the expression levels were significantly higher than MCF10A in most of the cells tested.
FIGURE 1  |  (A–C)  ESR1 and ESR2 genes are differentially expressed in ERα+ parental, respective endocrine-resistant, and triple-negative breast cancer cell lines. (A, B) Expression of ESR1 and ESR2 in immortalized mammary MCF10A, transformed ERα+ MCF7 and T47D, endocrine-resistant MCF7-TamR, MCF7-FasR, T47D-TamR, and T47D-FasR, CDK6 overexpressing MCF7 (MCF7-CDK6 OH/E), CDK4/6 inhibitor-resistant MCF7 (MCF7-CDK4/6IR) and T47D (T47D-CDK4/6IR), ZR-75-1, and triple-negative breast cancer (TNBC; MDA-MB231, MDA-MB-468, Hs578t) cell lines. Total RNA was isolated from the established cell lines using TRIzol. The expression of each gene was assessed by quantitative RT-PCR (qRT-PCR) performed with the DNase-treated RNA samples using gene-specific primers spanning exon–exon junctions that include large introns in the corresponding genomic sequence to avoid genomic DNA amplification. Gene expression was calculated by the ∆∆Ct method using GAPDH as an internal control. The expression of each gene is shown as the fold change relative to MCF10A. All reactions were done in triplicate, and the experiment was repeated twice. Data were plotted as mean ± SD. (A) ESR2 genes; full length (left) and all isoforms (right). (B) ESR1. (C) Whole-cell lysates were extracted, and immunoblot analyses were performed for ERβ and GAPDH (loading control) (upper panel), and ERα and GAPDH (lower panel). The intensity of the protein bands was quantified using Image Studio (LiCor) software. Numbers under the lanes of each cell line represent normalized values of the corresponding protein band (ERα or ERβ). The normalized band intensity of MCF10A was considered as 1. Immunoblot analyses were repeated twice with corresponding biological replicates. Reproducible results were obtained in each independent experiment. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. For ERβ (upper panel), two different exposures were provided; low exp. = low exposure; high exp. = higher exposure of the blot. (D) ERE-luciferase-driven promoter activity upon treatment with selective ERβ agonists is significantly higher in ectopically expressing cells with ERβ compared to that of ERα. HEK293T cells were transfected with c-Flag pcDNA3 (vector control), c-Flag ERIx or c-Flag ERβ in combination with ERE-Luciferase (reporter) and TK-Renilla (pRLTK; internal control) plasmids (as described in Materials and Methods). Forty-eight hours after treatment of the cells with ERβ-specific agonists Renilla and Firefly, luciferase activities were measured using the dual-luciferase reporter assay system. Firefly luciferase was normalized to Renilla Luciferase. Treatment with: OSU-ERb-12 (0–10 µmol/l) (left) and LY500307 (0–10 µmol/l) (middle). Each assay was performed in triplicate with three experimental replicates (mean ± SD, *p < 0.05, **p < 0.01). The right panel shows equal expressions of ERα and ERβ as determined by Western blot analysis using the anti-flag antibody. Intensity of Flag-ERα/ERβ was normalized to GAPDH. The numbers under the corresponding protein band represent normalized values of the corresponding protein band intensity.
except MCF7, MCF7-FasR, and the TNBC line MDA-MB-468 (Figure 1A and Supplemental Table 1). About 5,000 (p < 0.05) and 12,000-fold (p < 0.05) increased ESR1 expression was noted in MCF7 and T47D cells, respectively, compared to MCF10A (Figure 1B and Supplemental Table 1).

To check the specificity of the primers to amplify the correct PCR products, we performed agarose gel electrophoresis with the samples of qRT-PCR. Our data showed a single band (Supplemental Figure 5B) with correct PCR products that were confirmed by sequencing.

Next, we performed Western blot analyses to evaluate the expression of full-length ERβ and ERα proteins with the cell lysates (Figure 1C). We tested antibodies raised against ERβ from different sources including Developmental Studies Hybridoma Bank (CWK-F12), Invitrogen (PPZ0506), and Sigma (clone 68-6-4). Of these tested antibodies while CWK-F12 and PPZ0506 were specific but only sensitive to the overexpressed (positive control) ERβ protein, the antibody from Sigma was specific as well as sensitive to ERβ protein expressed at endogenous levels. As shown in Figure 1C (upper panel), all the parental and resistant ERα+ cell lines, TNBC lines, and immortalized mammary epithelial cells expressed full-length ERβ. As expected, our data demonstrated that all the ERα+ parental cell lines but none of the TNBC cell lines expressed ERα protein. MCF7-TamR cells expressed more ERα protein than the parental MCF7 cells while MCF7-FasR had no detectable ERα expression. Similarly, T47D-FasR and T47D-CDK6/6IR cells had a lower expression of ERα than the parental T47D cells.

In summary, full-length ERβ mRNA and protein is expressed in ERα+ breast cancer cell lines at levels that are comparable to expression levels in TNBC cell lines, and its expression is preserved in all the resistant derivative cell lines.

To determine the specificity of ERβ agonists, we treated HEK293T cells with OSU-ERb-12 or LY500307 (known selective ERβ agonist) at increasing concentrations following co-transfection with plasmid 3XERE TATA luc, pRLTK, FLAG-ERα, or FLAG-ERβ (please see Materials and Methods for details) and measured luciferase reporter activity (Figure 1D). The expression of FLAG-ERα and FLAG-ERβ proteins was similar as measured by immunoblot for FLAG performed on lysates from the vehicle-treated 293T cells transfected with the corresponding expression plasmids (Figure 1D, right panel). Comparison of the induction of luciferase activity demonstrated that ERα exhibited full activity in the presence of 30 nmol/l OSU-ERb-12 and 10 nmol/l LY500307 treatment. Our data showed that luciferase activation by OSU-ERb-12 was significantly increased in the ERβ-expressing cells as compared to those that expressed ERα. For example, at 30 nmol/l of OSU-ERb-12 there was a ~4-fold (p < 0.05) and ~40-fold (p < 0.05) increase in luciferase activity in ERα and ERβ transfected cells, respectively, compared to their corresponding vehicle-treated cells (Figure 1D, left panel). There was 10-fold (p = 0.0059) higher ERE-LUC activity in ERβ-overexpressing cells compared to that of ERα by OSU-ERb-12 at 30 nmol/l (Supplemental Table 2). Similarly, for LY500307 at 10 nmol/l there was 2.1-fold (maximum induction; p < 0.05) activation by ERα and 84-fold (p < 0.05) activation by ERβ compared to the corresponding vehicle-treated samples (Figure 1D central panel, Supplemental Table 2). At this concentration of LY500307, ERβ demonstrated 40-fold higher activity (p = 0.0038) compared to ERα.

**ERβ Agonists Are Cytotoxic to ERα+ Breast Cancer Cell Lines and They Synergize With ERα+ Antagonists**

Next, we assessed the viability of parental, endocrine-resistant, CDK4/6i-R MCF7 and T47D, and MCF7-CDK6 O/E cell lines following treatment with ERβ agonists OSU-ERb-12 and LY500307 (Figure 2 and Supplemental Table 3). We assessed cell viability after 7 days of initial drug exposure using CellTiter-Glo Luminescent Cell Viability Assay. This duration is consistent with that used for toxicity assays with other endocrine agents such as fulvestrant (20, 21). We compared the viability of the drug-treated transformed cell lines to that of MCF10A cells. The IC50 values for T47D cells (OSU-ERb-12: 10.43 μmol/l—Figure 2C; LY500307: 7.29 μmol/l—Figure 2D), tamoxifen- and fulvestrant-resistant MCF7 cells, tamoxifen- and fulvestrant-resistant T47D cells, CDK6-overexpressing MCF7 cells, abamacilicib-resistant MCF7 cells, and abamacilcib-resistant T47D cells were significantly lower than that of MCF10A cells (OSU-ERb-12: 13.96 μmol/l; LY500307: 30.53 μmol/l; Figure 2 and Supplemental Table 3). Compared to the parental MCF7 cell line, all the resistant lines except MCF7-CDK6 O/E had significantly lower IC50 values for OSU-ERb-12 (Figure 2A). Similarly, all three resistant T47D lines displayed significantly higher sensitivity toward OSU-ERb-12 compared to their parental counterpart (Figure 2C and Supplemental Table 3).

Despite a high degree of selectivity, we saw some activation of ERα by both ERβ agonists in our reporter assay (Figure 1D). We also observed an increase in viability of ERα+ breast cancer cell lines when exposed to low concentrations of both ERβ agonists. We hypothesized that combining ERβ agonists with an ERα antagonist would increase their activity and eliminate their stimulatory effects at low concentrations. We tested several ERα antagonists, namely, 4-hydroxy-tamoxifen (selective estrogen receptor modulator), fulvestrant, elacestrant (both selective estrogen receptor degraders/SERDs), and MPP (selective ERα antagonist), at concentrations that fully block ERα, in combination with OSU-ERb-12. As shown in Figures 3A, B, in T47D cells, all these ERα antagonists caused a significant reduction in the IC50 of OSU-ERb-12 and eliminated its stimulatory effects at low concentrations. Of the tested drugs, 4-hydroxy-tamoxifen, when used at a concentration of 0.5 μmol/l, displayed the highest efficacy leading to the reduction of IC50 for OSU-ERb-12 to 1 μmol/l from 14.10 μmol/l (Figure 3A and Supplemental Table 4). We further analyzed the validity of the combination treatment of OSU-ERb-12 and 4-hydroxy-tamoxifen using the Bliss independence model (please see Materials and Methods for details). Our data demonstrated a significant dose-response with synergy (Figure 3C and Supplemental Table 4). There was evidence of synergy (the ratio being 1 or above) at all doses for the combination of OSU-ERb-12+Tam. There was no evidence of antagonism at any dose.
We next determined whether OSU-ERb-12 effects are specifically mediated by the ERβ receptor by comparing the OSU-ERb-12-induced decreases in cell viability to that of an inactive chemical analog MCSR-18-006 that differs at two atoms from OSU-ERb-12 (Supplemental Figure 2). As shown in Figure 3D, in T47D cells, OSU-ERb-12 demonstrated an IC50 value of 10.41 µmol/l that was 3.24-fold lower than for MCSR-18-006 (p < 0.01). However, in the presence of 4-hydroxy-tamoxifen (0.5 µmol/l) the IC50 of OSU-ERb-12 was 1.02 µmol/l, which was 38.5-fold lower than that of MCSR-18-006 combined with 4-hydroxy-tamoxifen (Figure 3D, right figure; Supplemental Tables 5, 6).

We then tested the viability of both MCF7 and T47D cell lines upon treatment with three other less selective ERβ agonists, namely, DPN (diarylpropionitrile) (15), AC186 (22), and WAY200070 (23). Our data demonstrated that none of these ERβ agonists (Supplemental Figure 6) exerted any significant cytotoxic effect on any of the ERα+ cell lines.

**Selective ERβ Agonists Exert Anti-Proliferative and Proapoptotic Effects on ERα+ Breast Cancer Cell Lines and Results in Increased Expression of FOXO 1/3 Proteins in ERα+ Breast Cancer Cell Lines**

Since both the ERβ agonists reduced the viability of ERα+ cell lines, we further examined the mechanism of reduced viability. Both OSU-ERb-12 and LY500307 reduced cell proliferation, induced S phase arrest, and increased apoptosis of MCF7 and T47D cells (Figure 4).

Cell proliferation was reduced by OSU-ERb-12 (10 µmol/l) and LY500307 (3 µmol/l) in MCF7 cells by 19% (p = 0.016) and 27% (p = 0.0028), respectively (Figure 4A and Supplemental Figure 7, Supplemental Table 7). Similarly, in T47D cells OSU-ERb-12 (10 µmol/l) and LY500307 (7 µmol/l) reduced proliferation by 31% (p = 0.0074) and 15% (p = 0.015), respectively (Figure 4A and Supplemental Figure 7, Supplemental Table 7). However, the observation that the ERβ agonists either significantly increased or did not decrease proliferation at the lower concentration (0.5 µmol/l) in both the cell lines explains the increased cell viability observed at lower doses in earlier experiments (Figure 2).

Cell-cycle analysis demonstrated that OSU-ERb-12 treatment (0.5 µmol/l) reduced the G0/G1 phase (8.7% decrease p = 0.02) and increased the S-phase fraction (6.4% increase, p = 0.0347) of MCF7 as well as in T47D cells (G0/G1: 6.6% decrease, p = 0.0036; S-phase: 5.2% increase, p = 0.0015) (Figure 4B, Supplemental Figure 8, Supplemental Table 8). Similarly, LY500307 at 0.5 µmol/l caused a significant reduction in the G0/G1 phase (13% decrease, p = 0.019) and an increase in the S-phase (7.1% increase, p = 0.049) of MCF7 as well as T47D cells (G0/G1: 7.7% decrease, p = 0.0018; S-phase: 6.2% increase, p = 0.0004) (Figure 4B, Supplemental Figure 8, Supplemental Table 8). However, at a higher dose (around IC50) OSU-ERb-12 demonstrated no significant decrease in the G0/G1 phase nor arrest at the S-phase—in both the cell lines—an observation that needs further explanation. Nevertheless, in T47D cells, LY500307 at higher doses (7 µmol/l) exhibited a dramatic decrease (34%, p = 0.0079) in the G0/G1 phase, an increase in apoptotic cells at SubG0 (5.6%, p = 0.0068), and an arrest at S (12.8% increase, p = 0.006) and G2/M (7.6% increase, p = 0.0135) phases. Altogether, these data suggest that treatment with ERβ agonists causes cell cycle arrest in S and/or G2/M phases.

We observed a significant increase in apoptosis of LY500307-treated (7 µmol/l) MCF-7 cells (7.7% apoptotic cells, p = 0.01) compared to the vehicle-treated control (4.2% apoptotic cells).
We did not observe a statistically significant increase in apoptosis of MCF7 cells treated with OSU-ERb-12. We noticed a significant increase in apoptosis of T47D cells treated with 10 µmol/l OSU-ERb-12 alone, 4-hydroxy tamoxifen alone, and OSU-ERb-12 in combination with 4-hydroxy tamoxifen. OSU-ERb-12 alone, 4-hydroxy tamoxifen alone, and OSU-ERb-12 in combination with 4-hydroxy tamoxifen. OSU-ERb-12-12+0.5µmol/l, 4-OHTam, 1.0µmol/l OSU-ERb-12+0.25 µmol/l, Fas,4.47µmol/l OSU-ERb-12+1µmol/l, Elacostrant, 6.18µmol/l OSU-ERb-12+0.75µmol/l MPP (Figure 4C, Supplemental Figure 9, Supplemental Table 9). In comparison with vehicle-treated cells, OSU-ERb-12 suppressed colony formation of MCF7 cells by 14% (p = 0.05) and 44% (p = 0.002) and LY500307 by 79% (p = 0.003) and 100% (p = 0.0007) at 3 and 5 µmol/l, respectively. Similarly, the reduction in colony formation in T47D with OSU-ERb-12 was 64.5% (5 µmol/l; p = 0.011). With LY500307, colony formation was reduced by 19.9% (3 µmol/l; p = 0.015) and 95% (5 µmol/l; p = 0.005). However, there was no significant reduction of colony formation in T47D...
FIGURE 4 | Cell proliferation, cell cycle, and apoptosis are affected upon treatment of ERα+ breast cancer cells with ERβ-specific agonists, OSU-ERβ-12, and LY500307. MCF7 and T47D cells (0.5 × 10⁶) were seeded on 100-mm dishes in phenol red free DMEM containing charcoal-stripped FBS and treated with the drugs as indicated. (A) A representative diagram of the cell proliferation profile in drug-treated cells. Cells were treated with DMSO (control), FAS (fulvestrant; negative control), OSU-ERβ-12, or LY500307 for 72 h, harvested, and stained following protocol for the Click-iT EdU Alexa Fluor 647 Kit (Invitrogen C10424). Cell proliferation was analyzed via flow cytometry on a BD FACSCalibur Flow Cytometer. Each assay was performed in triplicate and repeated twice. Data were plotted as mean ± SD (*p < 0.05, **p < 0.01). (B) A representative diagram depicting the cell-cycle profile in drug-treated cells. Cells treated with DMSO (control), OSU-ERβ-12, or LY500307 for 72 h at the indicated concentrations were harvested on ice, fixed, washed, and incubated with propidium iodide and RNase A followed by cell-cycle analysis on a flow cytometer. Each assay was performed in triplicate and repeated twice. Data were plotted as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001). (C) A representative diagram depicting the apoptosis profile in drug-treated cells. Cells treated with DMSO (control), OSU-ERβ-12, or LY500307 for 48 h at the indicated concentrations were harvested on ice, washed, and processed according to the manufacturer’s protocol (TUNEL Assay Kit-BrdU-Red; Abcam) followed by analysis on a BD FACSCalibur Flow Cytometer. Each experiment was repeated twice. Data presented are mean ± SD (*p < 0.05, **p < 0.01). In all assays, results shown are pooled averages across biological repeats.
treated with 3 µmol/l OSU-ERb-12 (Figure 5A and Supplemental Table 10).

We then performed wound healing assays to investigate whether OSU-ERb-12 and LY500307 treatments could lead to the reduction of migratory properties of breast cancer cells. As shown in Figure 5B, there was a significant decrease in the cell motility in the MCF7 cell line in the presence of both the agonists. Treatment with OSU-ERb-12 inhibited MCF7 cell migration by 34.7% (5 µmol/l; p = 0.0004) and 42.9% (10 µmol/l; p = 0.0026) and LY500307 by 70.2% (5 µmol/l; p < 0.0001) and 91.9% (10 µmol/l; p < 0.0001) (Figure 5B and Supplemental Table 11).

To elucidate the underlying mechanism of ERβ agonist-mediated cell death, we measured the levels of activated
executioner caspases by Western blot analysis. As MCF7 cells do not express caspase 3 (24), we measured caspase 7 levels in this cell line. Robust activation of the effector caspases 7 (MCF7) or 3 (T47D) resulted within 12 h of treatment of cells with both the agonists. The effect persisted at least up to 48 h (Figure 5C). In contrast, in vehicle-treated cells increased caspase cleavage was not detected. A similar increase in the proteolysis of their substrate PARP-1 was noted in ERβ agonist-treated cells (Figure 5C).

It has been demonstrated that ERβ suppresses tumor growth and induces apoptosis by augmenting the transcription of the tumor-suppressor genes FOXO1 and FOXO3 in prostate cancer (25). Therefore, we determined their expression levels in ERβ agonist-treated breast cancer cells. As shown in Figure 5D, both FOXO1 and FOXO3a protein levels were increased in OSU-ERb-12- and LY500307-treated MCF7 and T47D cell lines.

**ERβ Expression in Human Breast Cancer Samples**

Previous studies suggested that, distinct from ERα, ERβ inhibits transcription from promoters that incorporate estrogen response-tetradecanoyl phorbol ester (ERE-AP1) composite response elements (13). We hypothesized that the ERβ/ESR2 mRNA expression levels in ERα+ human breast cancer samples would negatively correlate with those of genes with promoters that contain ERE-AP1 response elements and that there would be a positive association between ESR2 mRNA expression levels and overall survival.

Thirty-seven patients with metastatic ERα+/HER2- breast cancer were included in this study. Demographic and clinical characteristics are displayed in **Supplemental Table 12**. All the patients in this cohort were female with a median age of 56 years (range 27–78). The patients were predominantly Caucasian (35, 95%), and most women were postmenopausal (23, 66%).

The objective was to determine the mRNA expression levels of the genes which are targets of ER-AP1-mediated transcription and AP1-independent ER mediated transcription including CCND1, MYC, IGF-1, Bcl-2, MMP-1, FN1, IGFBP-4, E2F4, CXCL12, PGR, (ER-AP1 dependent) EBAG9, and TRIM25 (canonical palindromic ERE dependent) and to correlate these with ESR1 and ESR2. We found by RNA-seq analysis that the expression of the cyclin D1 gene, the classic target of estrogen-stimulated transcription through an AP1 response element, negatively correlated with that of ERβ/ESR2 as measured using the Spearman correlation coefficient (rho = -0.45, p = 0.005) (**Figure 6A** and **Supplemental Table 13**). ERβ/ESR2 expression was also negatively correlated with that of ERα/ESR1 (rho = -0.35, p = 0.033). However, ERβ/ESR2 mRNA expression positively correlated with that of IGFBP4 (rho = 0.58, p < 0.001) and CXCL12 (rho = 0.54, p < 0.001) (**Figure 6B** and **Supplemental Table 13**). The univariate Cox proportional hazard estimate for overall survival by ESR2 expression was 0.54 (95% CI 0.06, 5.22), suggesting a positive trend that did not reach statistical significance in this numerically limited cohort (**Figure 6C**). The RNA-seq data reported in this paper are available at the Gene Expression Omnibus database (accession no. GSE198545) at: https://urldefense.com/v3/__https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE198545__;!!KGKeukY1kY3_5pg7Oz9dTTPBWXvXq1t1PTXXZYo7hndoLq0XVgXcesakiudG7GxZuDQamvwLNorY$.}

**DISCUSSION**

The ERα subtype constitutes 70% of all breast cancers. Annually about 600,000 breast cancer-related death occurs worldwide (1). Although metastatic ERα+ breast cancer is initially treated with estrogen deprivation or ERα blockade, endocrine resistance eventually entails a change of therapy. The advent of CDK4/6 inhibitors such as palbociclib (26, 27), ribociclib (28), and abemaciclib (29, 30) has doubled progression-free survival when used in combination with endocrine agents. However, resistance to CDK4/6 inhibitors is an increasing clinical challenge (31). Also, the duration of response to second-line endocrine therapies is generally short. After the exhaustion of endocrine treatment, chemotherapy remains the only treatment option. Therefore, there is an urgent need for tolerable therapies to prolong overall survival with better quality of life for advanced ERα+ breast cancer patients.

Accumulating evidence suggests that while ERα is prooncogenic in the mammary gland, ERβ plays a tumor-suppressor role in different cancers including breast cancer (32, 33). The efficacy of selective ERβ agonists such as LY500307 has been previously described in preclinical models of TNBC (34), melanoma (34), glioblastoma multiforme (35), and prostate cancer (36). However, there has been limited study of the role of ERβ in estrogen receptor α-positive breast cancer. One reason is that for this particular indication a high degree of selectivity for ERβ over ERα would be required. Our institution recently developed a highly selective ERβ agonist: OSU-ERb-12 (16). We confirmed the selectivity of this compound using ERE-luciferase promoter assays showing 10-fold higher induction upon treatment of ERβ-overexpressing cells.

Although previous preclinical studies have mostly focused on TNBC, we observed that ERβ was expressed (both RNA and protein level) in ERα+ breast cancer cell lines at levels that were not significantly different from those in TNBC cell lines (**Figures 1A–C**). Endocrine and CDK4/6 resistant derivatives of these ERα+ cell lines had comparable or higher expression compared to the parental cell lines. These observations, therefore, are in line with the potential for efficacy in ERα+ breast cancer.

We showed that OSU-ERb-12, like the control compound LY500307, exerted significant cytotoxicity toward MCF7 and T47D ERα+ breast cancer cell lines with IC50 values that were lower compared to immortal mammary epithelial cells (MCF10A). Furthermore, OSU-ERb-12 exhibited cytotoxicity toward the corresponding endocrine- and CDK4/6 inhibitor-resistant derivative lines of MCF7 and T47D with either similar or even significantly lower IC50 values, demonstrating its therapeutic efficacy toward both treatment-naïve and -resistant ERα+ breast cancer cells. Furthermore, we demonstrated that these effects are ERβ specific using a close structural analog that
lacks ERβ agonist activity and was many-fold less cytotoxic than the active compound. Also, we showed that less specific ERβ agonists have much lower potency for inhibiting ERα+ cell lines. The reason for this is unclear but could be due to off-target activation of ERα.

At lower concentrations of OSU-ERb-12 and LY500307, there was an increase in cell viability. We hypothesized that this may be due to ERα activation, given the large molar excess of ERα receptors over ERβ receptors in ERα+ breast cancer cell lines. This prompted us to investigate the cytotoxic efficacy of OSU-ERb-12 in combination with clinically available potent ERα antagonists. In the combination studies, tamoxifen showed maximum inhibitory effect with a 14-fold reduction of IC50 value compared with OSU-ERb-12 alone. Using the Bliss Independence model, we found a synergistic interaction between tamoxifen and OSU-ERb-12 at all the doses used in the study. These data would suggest that if clinically used, OSU-

ERb-12 should be administered in combination with tamoxifen or other selective estrogen receptor modulators. The strategy of using an endocrine agent, such as tamoxifen, with a sensitizing targeted agent in the endocrine-resistant setting has been used successfully in clinical trials (37).

Of note, the cellular 50% inhibitory concentrations were many-fold higher than the cellular 50% effective concentration for activation of a canonical palindromic ERE response element. There are many potential explanations for this. Firstly, inhibition of viability may only be achieved when the majority of available receptors are activated by the ligand, for example possibly at the EC90–100 concentration range. Secondly, the EC50 concentration represents transcriptional activation at a palindromic estrogen response element with optimal configuration and spacing of the half binding sites. Depending on the configuration of the ERs in promoters, EC50 may be higher. Of note, ligand–ER–DNA interactions, including the stoichiometry and affinity of the
The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MAC and JD conceived the project. JD, CCC, BR, and MAC designed the experiments. BR, ML, DGS, SDS, and MAC helped recruit the patients to the protocol under which the patient data were collected. JD, NW, JMM, PS, MS, JJD, DGS, and MK performed the experiments and analyzed the data. JD and MAC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.857590/full#supplementary-material

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