Preferential Estrogen Receptor β Ligands Reduce Bcl-2 Expression in Hormone-Resistant Breast Cancer Cells to Increase Autophagy

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Abstract

Acquired resistance to selective estrogen receptor (ER) modulators (SERM) and downregulators (SERD) is a significant clinical problem in the treatment of estrogen (E2) receptor-positive (ER⁺) breast cancers. There are two ER subtypes, ERα and ERβ, which promote and inhibit breast cancer cell proliferation, respectively. Although ERβ breast cancers typically express a high ratio of ERα to ERβ, the acquisition of SERM resistance in vitro and in vivo is associated with increased relative expression of the ERβ. On some gene enhancers, ERβ has been shown to function in opposition to the ERα in the presence of E2. Here, we demonstrate that two different ERβ agonists, WAY-20070 and a novel “A-CD” estrogen called L17, produce a marked reduction in G2-M phase correlated with effects on cyclin D1 and cyclin E expression in a SERM/SERD-resistant breast cancer cell line. ERβ agonists recruited both the ERα and ERβ to the Bcl-2 E2-response element strongly reducing Bcl-2 mRNA and protein in an ERβ-dependent manner. L17 recruited RIP140 to the Bcl-2 promoter in cells overexpressing ERβ. Exposure to the ERβ ligands also resulted in increased processing of LC3-I to LC3-II, indicative of enhanced autophagic flux. The coaddition of ERβ agonist and the autophagy inhibitor chloroquine resulted in a significant accumulation of sub-G1 DNA which was completely prevented by the addition of the caspase inhibitor Z-VAD-FMK. We propose that combined therapies with an ERβ agonist and an inhibitor of autophagy may provide the basis for a novel approach to the treatment of SERM/SERD-resistant breast cancers. Mol Cancer Ther; 13(7); 1882–93. ©2014 AACR.

Introduction

The estrogen receptors (ER) are ligand-dependent nuclear receptors that contain a DNA-binding domain, ligand-binding domain, an N-terminal transcriptional activating function AF-1, and a C-terminal AF-2 (1). There are two subtypes called ERα and ERβ which can either form homodimers or heterodimers to transactivate responsive genes in the presence of E2. Although the ERα has a strong ligand independent AF-1 region, the ERβ AF-1 function is weaker (2) and mediates a dominant negative effect on ERα as a result of an N-terminal repressor function (3). Chromatin immunoprecipitation (ChIP) on chip analysis suggests that there is considerable overlap in DNA response element binding between the two receptors (4). ERβ activity is not only cell type dependent, but also enhancer sequence and ligand dependent (5). Several groups have demonstrated that ectopic expression of the ERβ in ERα⁺ breast cancer cells results in growth inhibition (6, 7) and prevents xenograft formation in nude mice in response to E2 (7). ERβ regulates gene transcription in an E2-independent and dependent manner with downstream effects impacting on cell-cycle progression (8, 9). Overexpression of ERβ can activate p21 and p27 expression causing a G2 accumulation (3). Cyclin D1 is positively regulated by the ERα and negatively regulated by the ERβ in the presence of E2 (7).

In the normal human and rodent mammary gland, the ERβ is expressed at higher levels than the ERα. This ratio is typically reversed in ER-positive (ER⁺) breast cancer (10) although many breast cancers continue to express low levels of ERβ. Reduced ERβ expression in ER⁺ breast cancer cells is in part due to promoter methylation (11). The standard of endocrine treatment for ER⁺ breast cancer is the selective ER modulator (SERM), tamoxifen (TAM). Unfortunately, the vast majority of responsive tumors eventually develop SERM resistance. TAM-
resistant (Tam-R) cell lines remain sensitive to growth inhibition by selective ER downregulators (SERDs) including fulvestrant (ICI 182,780; refs. 12–15). About half of patients respond to second-line endocrine therapy including aromatase inhibitors and fulvestrant (16); however, fulvestrant-resistant cells emerge (17) representing the limits of current endocrine therapy.

SERM resistance can be either intrinsic to a subpopulation of breast cancer cells within a tumor or can be acquired (18, 19). Typically, SERM resistance is not associated with loss of the ER (estimated at less than 25%; ref. 20). SERM resistance is complex and involves changes in intracellular signaling through growth factors or activated oncogenes that is implicated in the ligand-independent activation of the ER (18, 21).

Tamoxifen is a pure antagonist at the ERα (22) and some reports have shown a correlation between lack of ERβ expression and de novo SERM resistance (23), while others found that SERM-resistant tumors have increased ERβ mRNA expression (24). Derivatives of MCF-7 ERα breast cancer cells have been selected for SERM/SERD resistance and demonstrate a net decrease in the expression of the ERα but not ERβ (25). One such cell line, called LCC9, was derived from MCF-7 cells after long-term culture in the presence of fulvestrant and displays cross-resistance to tamoxifen (17).

In previous work, we have designed and synthesized new ligands that preferentially activate the ERβ (26). The structure of L17 is based on the ABCD-ring structure of E2 but lacks the B ring. In the current study, we have investigated the impact of L17 and a second ERβ agonist, WAY-200070 (27) on SERD/SERM-resistant LCC9 cells. Our results show that ERβ agonists inhibit LCC9 cell growth and induce an autophagic response associated with reduced Bcl-2 expression.

Materials and Methods

Cell lines and cell culture

MCF-7/LCC1 and MCF-7/LCC9 and MCF-7CL parental cells (28) were obtained from Dr. Robert Clarke, Georgetown University (Washington, DC) at low passage and were routinely cultured in Dulbecco's Modified Eagle Medium (with or without phenol red) containing 5% unstripped or dextran charcoal-stripped FBS (CSS). The MCF-7 cell line was confirmed by DNA fingerprinting using genetic markers at nine different loci (CSF1PO, TPOX, TH01, vWA, D165539, D7S820, D13S317, D5S818, and the Y chromosome-specific amelogenin) in the Clarke lab. Cells were passaged a maximum of eight times from the point of receipt. Markers were not retested although SERM/SERD resistance of LCC9 cells was reconfirmed (Supplementary Data). MCF-7 cells (denoted MCF-7PL) were originally obtained from the American Type Culture Collection. They have not been retested for lineage markers but continue to express the luminal markers ERα, E-cadherin, pS2, and CK18. The MCF-7 (rTA tet-ON ERβ) subclone was obtained from Dr. Leigh Murphy, University of Manitoba (Winnipeg, Manitoba, Canada), and was derived from a clone of MCF-7 cells stably expressing reverse tetracycline transactivator (clone 89 rTA) transfected with doxycycline-inducible His-Xpress-ERβ1 expression (tagged-ERβ1; ref. 29; and refs therein). These clones were not tested for genetic markers before or after receipt but were used exclusively for ChIP analysis after acute treatment with ligands. All cells were shown to be free of mycoplasma contamination by PCR.

Chemicals

17β-estradiol (E2), 4-hydroxytamoxifen, WAY-200070, and chloroquine were purchased from Sigma. Ligand 17 (L17) was synthesized as previously described (26). Z-VAD-FMK was purchased from BD Biosciences.

Antibodies

Antibodies were: anti-ERα (HC-20), anti-cyclin D1 (A-12 SRC-3/AIB1 (sc-9119), RIPA1(sc-8997; Santa Cruz Biotechnology); anti-ERβ for immunoblot analysis (Thermo-Scientific PAl-311; immunoblot analysis; Fig. 1) anti-ERβ (Novus Biologicals NBP-04936; all other immunoblot analyses); anti-ERβ (GTX70182; GeneTex) for ChIP; anti-actin (A-2066; Sigma); anti-LC3 (Novus Biologicals), anti-Bcl-2 (BD Biosciences), anti-cyclin E (ab7959), Abcam, ChromPure rabbit immunoglobulin G (IgG), whole molecule (011-000-003), peroxidase-conjugated goat anti-rabbit IgG (H+L) goat anti-mouse IgG (H+L), goat anti-chicken IgY (H+L; Jackson ImmunoResearch Inc.).

ERβ knockdown and induction

LCC9 cells were transfected with 25 nmol/L siGenome SMARTpool human ESR2 siRNA (Dharmacon Cat# M-003402–04) by reverse transfection using Dharmafect (Dharmacon) according to the manufacturer's directions. MCF-7(rTA tet-ON ERβ1) was treated with 1 μg/mL doxycycline (BioBasic) to induce overexpression of ERβ and reverse transfected with 25 nmol/L ESR1 siRNA ON-TARGET plus (Dharmacon Cat# (L-003401-00-0005). Control transfections were done with siCONTROL nontargeting (NT) siRNA #1 (Cat# D-001210-01-05). For some proliferation assays, LCC9 cells were infected with retrovirus, pSUPER expressing previously verified ERβ short hairpin RNA (shRNA; pERβshRNA; Addgene plasmid #35561) or nontargeting GFP-shRNA (shNT) vector (Addgene plasmid #30519).

Cellular proliferation/viability assay

A total of 7 × 10^4 cells were plated in 60 mm dishes then treated with ethanol (vehicle) or varying concentrations of E2, L17, and WAY. Treatment was performed in triplicate and experiments repeated three times. Viable cells were enumerated using Trypan blue exclusion or a Vi-Cell XR cell viability analyzer (Beckman Coulter). In some experiments, chloroquine and Z-VAD-FMK were added at a final concentration of 33 and 100 μmol/L, respectively.
Figure 1. Relative ER expression and effects of ERβ agonists on LCC9 and MCF-7 cells. A, structures of E2, L17, and WAY200070. B, Western blot analysis showing the expression levels of ERα and ERβ in MCF-7 cells from our lab (MCF-7PL), two MCF-7 subclones that are hormone-independent (LCC1) and SERM/SERD-resistant (LCC9), and the MCF-7(LCC9) parental cell line obtained from the Clarke lab (MCF-7CL; lane 4). MCF-7 (C) and LCC9 (D) cells were grown in phenol red-free medium with CSS and were treated with increasing concentrations of E2 or L17. Cells were enumerated using Trypan blue exclusion after 3 and 5 days. Data represent the mean from three separate experiments each performed in triplicate; error bars indicate SE. E, MCF-7 and LCC9 cells were cultured in the presence of vehicle, 10 nmol/L E2, 10 nmol/L L17, or 10 nmol/L WAY for 3 days and viable cells were enumerated. Graph depicts percentage of vehicle-treated cell numbers ± SE. F, LCC9 cells infected with retrovirus expressing shERβ or shNT were treated with 10 nmol/L L17 or vehicle for 4 days and enumerated. Results shown are percentage of corresponding vehicle-treated cells. Bars are standard error of triplicate samples. The experiment was performed twice with similar results. G, anti-ERβ immunoblot analysis of LCC9 cell lysates 72 hours after infection with or pSUPER-shERβ or shNT. A cross-reactive band (*) is indicated. Actin was immunoblotted as a protein lysate loading control. Molecular weight marker (kDa) is shown on the left.
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Immunoblot analysis

Proteins were separated using SDS-PAGE and transferred to polyvinylidine difluoride membranes (Millipore). Immunoreactive bands were detected using substrate (Millipore) and quantified by densitometry using ImageJ v1.43.

Chromatin immunoprecipitation

ChIP assays were performed using a ChIP Assay Kit (Upstate Biotechnology) according to the manufacturer’s instructions with some modifications. Cells were treated with vehicle, 10 nmol/L E2, L17, or WAY for 30 minutes (ER ChIP) or 15 minutes (RIP140/SRC-3 ChIP). Chromatin was cross-linked with 1% formaldehyde then sonicated and protein–DNA complexes were immunoprecipitated with anti-ERα, anti-ERβ, anti-RIP140, anti-SRC-3, normal rabbit IgG, or no antibody (input) at 4°C overnight. Cross-links were reversed at 65°C for 4 hours. DNA was purified by phenol/chloroform extraction, precipitated, and subjected to PCR analysis. PCR primer sequences were the same as those used in quantitative real-time PCR (qRT-PCR). Of note, 5 μL of DNA (no antibody) was used for the input PCR reaction. Products were run on a 1% agarose gel and visualized by ethidium bromide staining. Bands were quantified by densitometry using ImageJ v1.43 and IgG subtracted from test samples then results graphed as a percentage of input DNA.

Additional Materials and Methods are included in Supplementary Files.

Results

ERβ agonists inhibit proliferation of LCC9 cells

Together with structural differences (Fig. 1A), L17 and WAY represent two different classes of ERβ agonists based on selectivity and binding affinity. WAY-200070 (WAY) is an aryl diphenoic azole ERβ agonist. The ERβ RBA of WAY is 133 (RBA ratio ERβ:ERα = 68; ref. 27), whereas the ERβ RBA of L17 is 1.73 (RBA ratio ERβ:ERα = 9.3; ref. 26). Because the ERβ has been shown to mediate repressive effects on proliferation, we determined whether ERβ agonists would differentially affect cells with high ERβ:ERα expression. Figure 1B shows the relative level of expression of ERα and ERβ protein in MCF-7 cells from our laboratory (MCF-7PL) in comparison with LCC1 cells, LCC9 cells, and MCF-7 cells that are parental to LCC9 and LCC1 (MCF-7CL). The expression of ERα is similar in both MCF-7 lines although the level of ERβ is reduced in MCF-7CL cells relative to MCF-7PL cells demonstrating significant variation across isolates of MCF-7 cells. ERα was reduced in LCC1 cells, whereas the ERβ was increased relative to parental MCF-7CL cells. LCC9 cells express substantially lower levels of ERα but maintain expression of ERβ at levels similar to LCC1 cells and higher than that in the parental MCF-7 cells. Thus the ratio of ERα to ERβ is much reduced in LCC9 cells compared with either MCF-7PL or MCF-7CL cells. The SERM/SERD sensitivity of the MCF-7 cells and resistance of the LCC9 cells used in this study were verified in proliferation assays after treatment with either tamoxifen or fulvestrant (Supplementary Fig. S1).

We next determined the effects of E2 and our novel ligand, L17, on proliferation of MCF-7 cells and LCC9 cells. We chose to use MCF-7PL cells because they express levels of the ERβ comparable with LCC9 cells and therefore serve as a more appropriate comparison than MCF-7CL cells. The results in Fig. 1C demonstrate that E2 increased MCF-7 viable cell numbers in a concentration-dependent manner while L17 had no significant effect. In contrast, E2 had little overall effect on LCC9 proliferation consistent with a previous report (30). Remarkably, L17 induced a dose-dependent decrease in viable cell numbers within 3 days which approached 40% within 5 days (Fig. 1D). WAY and L17 had a similar growth inhibitory effect on LCC9 cells at 10 nmol/L (Fig. 1E). To verify that growth inhibition induced by L17 was mediated through the ERβ, we treated LCC9 cells infected with a nontargeting retrovirus (shNT) or pSUPER-ERβ shRNA (shERβ; ref. 31) with L17 or vehicle. After 5 days, decreased proliferation was observed in L17-treated shNT-infected cells, while shERβ expression completely prevented growth inhibition (Fig. 1F). Verification of ERβ expression completely prevented growth inhibition (Fig. 1F).
knockdown is shown in Fig. 1G. These results are consistent with L17 signaling in an ERβ-dependent manner to inhibit LCC9 cell proliferation.

**ERβ agonists inhibit G1 and S phase exit in LCC9 cells**

We next assessed the impact of L17 and WAY on the cell cycle. After 3 days of culture in 10 nmol/L E2 MCF-7 cells demonstrated small reduction in G1 and increase in S phase cells (Fig. 2A and Supplementary Fig. S2A). As expected, tamoxifen produced a strong G1 arrest. Consistent with the results in Fig. 1, L17 and WAY had no significant effect on the cell-cycle distribution in MCF-7 cells.

E2 (10 nmol/L) induced a small (10%) decrease in the G1 and corresponding increase in the S phase of the cell cycle in LCC9 cells (Fig. 2B and Supplementary Fig. S2B), whereas tamoxifen had no effect. Remarkably, treatment of these cells with either L17 or WAY treatment resulted in a significant decrease in the G2–M phase with a corresponding increase in G1 and S phases of the cell-cycle accumulation suggesting that ERβ activation can inhibit both progression into and exit from S phase.

**Effects of ERβ agonists on cell-cycle proteins**

Cyclin D1 is induced by E2 via the ERα and is negatively regulated by the ERβ (7). E2 led to an increased expression of cyclin D1 in MCF-7 cells (Fig. 3C). In contrast, WAY and L17 induced a significant decrease in the expression of cyclin D1 in LCC9 cells (Fig. 3C). E2 also induced a decrease in the expression of cyclin E in both cell lines (Fig. 3D and E). WAY and L17 induced a significant increase in the expression of cyclin E in LCC9 cells (Fig. 3D and E).
of cyclin D1 and cyclin E that persisted up to 48 hours in MCF-7 cells, whereas L17 and WAY failed to elicit a similar response (Fig. 3A). Although a lower molecular weight 48 kDa species of cyclin E was present in response to E2 in LCC9 cells (Fig. 3B, D, and E), no induction of cyclin D1 was detected (Fig. 3B and C). Treatment of LCC9 cells with L17 or WAY had comparably little effect on the expression of cyclin D1 although increased cyclin E protein was observed after 48 hours of treatment with either L17 or WAY (Fig. 3B and D). Overall, the pattern of cyclin D1 and E expression is consistent with the observed effects on proliferation and cell-cycle distribution.

L17 and WAY inhibit expression of Bcl-2 and activate an autophagic response in LCC9 cells

Bcl-2 is a critical regulator of both apoptosis and autophagy. LCC9 cells have been shown to express higher basal levels of Bcl-2 relative to parental MCF-7 cells, and RNA interference (RNAi) for Bcl-2 induces an autophagic response in these cells (32). We previously reported that Bcl-2 is regulated in response to E2 (33) and consistent with this, E2 strongly induced Bcl-2 expression in MCF-7 cells (Fig. 4A and E). Both L17 and WAY transiently induced Bcl-2 within 24 to 48 hours and levels returned to baseline by 72 hours. Bcl-2 can inhibit autophagy through binding and inhibition of Beclin 1 (34). Phosphatidyethanolamine conjugation and subsequent cleavage convert LC3-I to II and can be used to assess autophagic flux (35). Immunoblot analysis of LC3 in MCF-7 cells showed that the overall basal level of LC3-I was high with little or no LC3-II. Although LC3-I levels remained constant following E2 treatment of MCF-7 cells, we noted a small increase in LC3-II at 48 hours post L17 treatment, whereas WAY had little effect on LC3-II (Fig. 4A and F). Consistent with previous reports (32), Bcl-2 is highly expressed in LCC9 cells and E2 treatment produced a small decrease in Bcl-2 protein observed after 24 and 48 hours (Fig. 4B and E). Remarkably, treatment with either L17 or WAY resulted in marked downregulation of the Bcl-2 protein. Unlike MCF-7 cells, baseline levels of LC3-II were detectable in LCC9 cells, however, LC3-II was strongly increased after treatment with E2, L17, or WAY (Fig. 4B and F). Knockdown of ERβ using siRNA showed that the L17-mediated decrease in Bcl-2 expression in LCC9 cells was ERβ dependent (Fig. 4C and D). Thus, activation of the ERβ, especially by ERβ-preferential agonists, has opposite effects on Bcl-2 expression in LCC9 and MCF-7 cells that may be a consequence of the high ERβ:ERα ratio in LCC9 cells.

ERβ agonists recruit both the ERβ and ERα to the Bcl-2 estrogen response element

The ability of ERβ ligands to reduce Bcl-2 expression in LCC9 cells might be the result of the association of ERβ agonist-bound homodimers or ERβ/ERα heterodimers to the Bcl-2 ERE. To address this question, we performed a ChIP analysis with antibodies against ERα and ERβ following a 1-hour exposure to each ligand. PCR using primers adjacent to Bcl-2 ERE in exon 2 (36). We used the pS2 gene as a second ERE-containing E2-responsive gene since the ERβ had previously been shown to interact with its promoter region (5). Neither ER was present on the Bcl-2 or pS2 promoter in vehicle-treated cells. Exposure to 10 nmol/L E2 for 1 hour resulted in recruitment of both the ERα and ERβ on the Bcl-2 gene in LCC9 cells (Fig. 5A and B and Supplementary Fig. S3A). Interestingly, ERα and ERβ were both present on the Bcl-2 ERE.
A  

MCF-7 ERα  

% Total signal relative to input  

Con  E2  L17  WAY  Con  E2  L17  WAY  

0  75  150  225  300  375  

B  

LCC9 ERα  

% Total signal relative to input  

Con  E2  L17  WAY  Con  E2  L17  WAY  

-10  0  10  20  30  40  

C  

MCF-7 ERβ  

% Total signal relative to input  

Con  E2  L17  WAY  Con  E2  L17  WAY  

0  50  100  150  200  250  

D  

LCC9 ERβ  

% Total signal relative to input  

Con  E2  L17  WAY  Con  E2  L17  WAY  

-10  0  10  20  30  40  

E  

Fold increase in Bcl-2 mRNA  

Veh  E2  L17  WAY  

1 h  

P < 0.01  

F  

Fold increase in pS2 mRNA  

Veh  E2  L17  WAY  

1 h  

P < 0.003  

G  

Fold increase in Bcl-2 mRNA after 24 h treatment  

Veh  E2  L17  WAY  

24 h  

P = 0.02  

H  

Fold increase in pS2 mRNA after 24 h treatment  

Veh  E2  L17  WAY  

24 h  

P = 0.015  

I  

RIP140 ChIP  

% Input  

siNT V  siNT E2  siNT L17  siERα V  siERα E2  siERα L17  

J  

SRC-3 ChIP  

% Input  

NT V  NT E2  NT L17  siERα V  siERα E2  siERα L17  

K  

U  ERα  

U  ERβ  

Actin  

55 kDa  

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following treatment with L17 or WAY in LCC9 cells with relatively equivalent band amplification for all agonists. In contrast, the ERα was robustly recruited to the Bcl-2 ERE in MCF-7 cells in the presence of E2, whereas L17 and WAY each recruited the ERα at significantly reduced levels relative to E2 (Fig. 5C and D and Supplementary Fig. S3B). The ERβ was present on the Bcl-2 promoter following E2, L17, and WAY treatment of MCF-7 cells although L17-induced recruitment was approximately two-thirds that of the other ligands (Fig. 5B). Overall, both L17 and WAY induced ERα and ERβ recruitment to the Bcl-2 and pS2 genes associated with transcriptional repression especially in LCC9 cells. In contrast, the strong induction of Bcl-2 transcripts by E2 in MCF-7 cells (see below) was correlated with a much greater fold increase in ERα recruitment to the Bcl-2 ERE by E2 relative to that induced by L17 and WAY. qRT-PCR analysis of transcripts induced after 1-hour exposure to ligands showed that E2 significantly induced Bcl-2 mRNA by 5-fold in MCF-7 cells, whereas L17 and WAY reduced Bcl-2 transcripts in both cell lines. Although the latter reduction within the 1-hour period was not highly significant, this may reflect the half-life of existing transcripts. E2 also reduced Bcl-2 mRNA in LCC9 cells although less than L17 and WAY (Fig. 5E).

For comparison with another E2-responsive gene, we analyzed the pS2 gene by ChIP and transcripts by qRT-PCR. All ligands recruited both ERα and ERβ to the pS2 promoter in LCC9 cells (Fig. 5A and B) which corresponded to weak induction (E2) or significant inhibition of expression (L17 and WAY) following a 1-hour treatment (Fig. 5F). Although all three ligands also recruited both the ERα and ERβ in MCF-7 cells (Fig. 5C and D), only E2 strongly increased pS2 mRNA (Fig. 5F).

We also analyzed mRNA levels 24 hours after treatment to assess the longer term effects of ligand activation on transcript levels. Both Bcl-2 and pS2 mRNA remained elevated after 24 hours in MCF-7 cells treated with E2, while the reduction in levels became less significant in L17 and WAY-treated MCF-7 and LCC9 cells and in E2-treated LCC9 cells (Fig. 5G). pS2 transcripts maintained a similar pattern of expression after 24 hours although a small rebound in pS2 mRNA was observed in L17-treated cells, possibly due to receptor desensitization which could alleviate repression (Fig. 5H).

Overall, these results suggest that L17 and WAY behave as inverse agonists wherein association of L17/WAY-bound ERβ mediates a reduction in basal transcription from the promoters regulated by these ERs. Transactivation of genes by the E2-bound ERα is facilitated by interaction with the p160 coactivators, while binding of antagonists results in the recruitment of corepressors (37). ChIP assays using antibodies against the RIP140 corepressor and SRC-3 coactivator were performed on Dox-treated MCF-7(rTA tet-ON ERβ) cells to increase the relative expression of ERβ. Cells were transfected with a nontargeting sequence (NT) or ERα siRNA as described in Materials and Methods and treated for 10 minutes with vehicle, E2, or L17. The results in Fig. 5I show that RIP140 was recruited to the Bcl-2 ERE by L17 in ERβ overexpressing cells coexpressing endogenous ERα or after ERα knockdown. In contrast, E2 reduced baseline (vehicle) levels of RIP140 in siNT-transfected cells but strongly recruited RIP140 when the ERα was reduced. Although E2 recruited SRC-3 onto the Bcl-2 ERE in the presence of endogenous levels of ERα, this occupancy was reduced in the siERα-transfected cells. By comparison, L17 did not recruit SRC-3 in either siNT- or siERα-transfected cells (Fig. 5I). Expression of ERβ mRNA and knockdown of ERα were confirmed as shown in Supplementary Fig. S4. Thus, the Bcl-2 ERE is negatively regulated by ligand-bound ERβ in conjunction with RIP140 recruitment.

Finally, we expressed the ERα or ERβ in HEK293 cells to compare the effects of WAY and L17 with E2 on ER proteins. Maximal activation of transcription by the ERα results in proteolytic degradation of the receptor (38). Consistent with this, the ERα protein was rapidly downregulated following E2-mediated transactivation (Fig. 5K). L17 has a low level of ERα-binding activity and resulted in a weak reduction in the ERα protein. The ERβ protein was only slightly reduced by both E2 and WAY, and L17 had no effect. This is consistent with the overall lack of transcriptional activation by the ERα in the presence of WAY and L17 as well as when all ligands bound to the ERβ.

Chloroquine converts L17/WAY-induced autophagy to apoptosis

Chloroquine (CQ) prevents the acidification of lysosomes to repress autophagy (39). Because WAY and L17 reduced Bcl-2 and increased levels of LC3-II in LCC9 cells,
we tested the possibility that chloroquine might convert this autophagic response to cell death. Figure 6A shows that a 5-day treatment with 33 μmol/L chloroquine to E2-free cultures caused an approximate 20% decrease in LCC9 cells and 35% in MCF-7 cells compared with vehicle-treated cells (Fig. 6B). To confirm that the effects of chloroquine in the presence of L17 were ERβ dependent, LCC9 cells infected with shERβ or control shNT were treated with L17, WAY, or vehicle in the presence or absence of chloroquine. Figure 6C shows that knockdown of ERβ prevented the effects of chloroquine both in the presence and absence of the ERβ ligands. This result suggests that the ERβ may act as both a ligand-dependent and -independent mediator of autophagy.

Strikingly, the combination of chloroquine and L17 or WAY induced a dramatic 80% decrease in cell numbers compared with control, which correlated to induction of sub-G1 DNA (Fig. 6D and Supplementary Fig. S5A).

Figure 6. Inhibition of autophagy induces apoptotic death when combined with ERβ agonists. LCC9 and MCF-7 cells were treated with vehicle, 10 nmol/L L17, or WAY, with or without chloroquine for a total of 5 days. LCC9 (A) and MCF-7 (B) cells were enumerated using Trypan blue exclusion. C, LCC9 cells were infected with pRS control virus or pSUPERshERβ for 48 hours then treated for 5 days with vehicle, 10 nmol/L L17, 10 nmol/L WAY with or without chloroquine (33 μmol/L). LCC9 (D) and MCF-7 (E) cells treated as in A and B were collected and sub-G1 DNA content was determined by flow cytometry. F, LCC9 cells were treated as in D except that in some samples 100 μmol/L Z-VAD-FMK was included in the medium. Data represent the mean of triplicate determinations. Bars, SE.
Chloroquine/ER\textsuperscript{b} agonist treatment also reduced MCF-7 viable cells in association with a sub-G\textsubscript{1} peak (Fig. 6F and Supplementary Fig. 5B) albeit not as dramatically as in LCC9 cells. Thus, SERD-resistant LCC9 cells expressing a high ratio of ER\textsuperscript{b}/ER\textsubscript{a} are preferentially sensitive to ER\textsuperscript{b} agonist combined with autophagy inhibition.

To distinguish whether the observed cell death was a function of conversion of autophagy to apoptosis, we repeated the experiment in LCC9 cells the presence or absence of the caspase inhibitor, Z-VAD-FMK (Fig. 6F and Supplementary Fig. 5C). Consistent with induction of apoptosis, Z-VAD-FMK almost completely blocked cell death in LCC9 cells induced by combination L17 and chloroquine.

Discussion

Previous studies have demonstrated that overexpression of the ER\textsuperscript{b} in MCF-7 breast cancer cells can inhibit cell growth in the presence of E2 (29) and prevent xenograft tumor formation (7). In transfected HeLa cells, the ER\textsuperscript{b} alone is unable to activate the transcription of the cyclin D1 gene in the presence of E2 and prevents E2 activation of cyclin D1 in the presence of coexpressed ER\textsuperscript{a} (40). Although ER\textsuperscript{b} activation has antiproliferative effects, the majority of human ER\textsuperscript{b} breast tumors do not express high levels of ER\textsuperscript{b} relative to ER\textsuperscript{a} at diagnosis. Remarkably, cell lines with acquired resistance to SERMs express an increased ratio of ER\textsuperscript{b} to ER\textsuperscript{a} (25).

In this study, we found that L17 and WAY had modulatory effects on cell-cycle proteins and either reduced 50 kDa cyclin E (MCF-7 cells) or weakly increased expression of 48 kDa cyclin E (LCC9 cells). Both ligands reduced cyclin D1 expression. Multiple phosphorylated residues on cyclin E are required for recognition by the SCF\textsuperscript{bw7} ubiquitin ligase which leads to degradation of cyclin E and progression through S phase (41). Further experiments are required to determine whether the 48 kDa cyclin E protein represents hypophosphorylated cyclin E, reflecting the lack of cell-cycle progression in E2 and ER\textsuperscript{b} ligand-treated LCC9 cells.

Results of ChIP-on-chip experiments have shown considerable overlap between the enhancers that bind the ER\textsuperscript{a} and the ER\textsuperscript{b} (42). This study is the first to demonstrate that ER\textsuperscript{b} agonists downregulate Bcl-2. Our results showed that both the ER\textsuperscript{a} and ER\textsuperscript{b} are recruited to the Bcl-2 ERE in both MCF-7 and LCC9 cells by both L17 and WAY. WAY treatment strongly recruited the ER\textsuperscript{b} on the Bcl-2 ERE in MCF-7 cells, consistent with its high affinity for this receptor. In contrast, L17 which has a lower affinity for ER\textsuperscript{b} than WAY (26, 27), and retains low affinity for the ER\textsuperscript{a}, recruited the ER\textsuperscript{a} more strongly than WAY. Regardless, both ligands decreased Bcl-2 mRNA in both cell lines. Ligand-ER\textsuperscript{b} conformation on a DNA sequence element determines transcriptional activation through coregulator recruitment. Our results suggest that, under conditions where the ratio of ER\textsuperscript{b}/ER\textsuperscript{a} is high, ER\textsuperscript{b} agonists recruit corepressors such as RIP140 to the Bcl-2 ERE resulting in reduced transcription of this gene. This result is consistent with recent report that RIP140 is the preferential coregulator of the ER\textsuperscript{b} (43). Because the liganded ER\textsuperscript{b} subtype determines chromatin binding (42), it seems that the Bcl-2 and pS2 genes are negatively regulated by ligands interacting with ER\textsuperscript{b}.

In LCC9 cells, E2 bound to ER\textsuperscript{a} and/or ER\textsuperscript{b} also reduced Bcl-2 transcription and protein expression. In fact, E2 can mediate growth inhibition and induce cell death in long-term hormone-deprived and antiestrogen-resistant breast cancer cells (44, 45). Notably, the Bcl-2 expression level was found to be a critical determinant of the ability of E2 to induce apoptosis in these cells (46). Indeed, it is possible that an increased ratio of ER\textsuperscript{b}/ER\textsuperscript{a} in SERM-resistant cells may play a significant role in mediating the fundamentally different response induced by E2 in SERM-resistant cells (47) and also why E2 fails to induce Bcl-2 in long-term E2-deprived breast cancer cells (48).

Although MCF-7 cells express a lower constitutive level of Bcl-2 relative to LCC9 TAM-R cells, they do not demonstrate the same autophagic flux seen in LCC9 cells which express a higher constitutive level of Bcl-2 protein. In LCC9 cells, autophagic flux is at least partially dependent on the presence of the ER\textsuperscript{b} as demonstrated by the reduction in chloroquine sensitivity following KD of ER\textsuperscript{b} in LCC9 cells. It is possible that ER\textsuperscript{b} regulates other critical components of the autophagic response in LCC9 cells. Interestingly, LCC9 cells have also previously been reported to contain a higher level of NF-\kappa B activity relative to parental cells (49) and NF-\kappa B activation can promote autophagy (50).

Clinical trials with agents which inhibit autophagy are being combined with chemotherapy and radiation to repress this prosurvival function (51). RNAi-mediated knockdown of Bcl-2 results in induction of autophagy in MCF-7 cells (52). On the basis of the dominant expression of the ER\textsuperscript{a} in most ER\textsuperscript{+} primary breast cancers, ER\textsuperscript{b} agonists may not be useful as first-line therapy. However, given that SERM/SERD-resistant cells often express a higher ER\textsuperscript{b}/ER\textsuperscript{a} ratio (24, 53), and tamoxifen fails to reduce Bcl-2 in LCC9 cells (49), our novel finding that ER\textsuperscript{b} agonists reduce Bcl-2 expression and promote autophagy in these cells suggests that combination ER\textsuperscript{b} agonists and autophagy inhibitors may represent a novel, relatively low toxicity therapeutic option in patients with acquired endocrine resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.A.C. Pratt

Development of methodology: S.C. Ruddy, L.C. Murphy

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Lau, M.A. Cabrita, B.C. McKay, L.C. Murphy

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.C. Ruddy, R. Lau, M.A. Cabrita, B.C. McKay, M.A.C. Pratt

Writing, review, and or revision of the manuscript: S.C. Ruddy, R. Lau, M.A. Cabrita, B.C. McKay, L.C. Murphy, M.A.C. Pratt
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.C. Ruddy, C. McGregor, L.C. Murphy

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Other (computational analysis and design of model ligands): J.S. Wright

Other (preparation of estrogen analogues): T. Durst

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References


## Molecular Cancer Therapeutics

### Preferential Estrogen Receptor β Ligands Reduce Bcl-2 Expression in Hormone-Resistant Breast Cancer Cells to Increase Autophagy

Samantha C. Ruddy, Rosanna Lau, Miguel A. Cabrita, et al.


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