Preferential estrogen receptor β ligands reduce Bcl-2 expression in hormone-resistant breast cancer cells to increase autophagy

Samantha C. Ruddy\textsuperscript{1}, Rosanna Lau\textsuperscript{1}, Miguel A. Cabrita\textsuperscript{1}, Chelsea McGregor\textsuperscript{1}, Bruce C. McKay\textsuperscript{2}, Leigh C. Murphy\textsuperscript{3}, James S. Wright\textsuperscript{4}, Tony Durst\textsuperscript{5}, and M.A. Christine Pratt\textsuperscript{1*}

1 University of Ottawa, Department of Cellular and Molecular Medicine
451 Smyth Road Ottawa ON Canada K1H 8M5

2 Cancer Therapeutics Program, Ottawa Hospital Research Institute and the Departments of Medicine, and Cellular and Molecular Medicine, University of Ottawa, 501 Smyth Road, Ottawa, ON, Canada, K1H 8L6.

3 Manitoba Institute of Cell Biology, University of Manitoba, 6020-675 McDermot Ave., Winnipeg, MB, Canada, R3E 0V9

4 Carleton University, Department of Chemistry, 1125 Colonel By Drive, Ottawa, ON, Canada, K1S 5B6.

5 University of Ottawa, Department of Chemistry, D’Iorio Hall, 10 Marie Curie Street, Ottawa, ON, Canada, K1N 6N5

* To whom correspondence should be addressed
Dr. M.A. Christine Pratt
Department of Cellular and Molecular Medicine
451 Smyth Road, Ottawa ON K1H 8M5
P: 613-562-5800 x8366-562-5636
Email: cpratt@uottawa.ca

Running Title: Targeting ERβ in SERM-resistant breast cancer

§ Supported by a grant from the Canadian Breast Cancer Foundation (Ontario Region) to M.A.C. Pratt
The authors disclose no potential conflicts of interest.
Word count: 5241
References: 53
Abstract

Acquired resistance to selective estrogen receptor (ER) modulators (SERMs) and downregulators (SERDs) is a significant clinical problem in the treatment of estrogen (E2) receptor positive breast cancers. There are two ER subtypes, ERα and ERβ, which promote and inhibit breast cancer cell proliferation respectively. While ER positive 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.
Introduction

The estrogen receptors (ERs) 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. While 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). 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+ breast cancer (10) although many breast cancers continue to express low levels of ERβ. Reduced ERβ expression in ER+ breast cancer cells is due in part to promoter methylation (11).

The standard of endocrine treatment for estrogen receptor positive (ER+) breast cancer is the selective estrogen receptor modulator (SERM), Tamoxifen (TAM). Unfortunately, the vast
The majority of responsive tumors eventually develop SERM resistance. TAM-resistant (Tam-R) cell lines remain sensitive to growth inhibition by selective ER down-regulators (SERDs) including fulvestrant (ICI 182,780) (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 the ER (estimated at less than 25%) (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).

TAM is a pure antagonist at the ERβ (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 ER+ 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 TAM (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, at low passage and were routinely cultured in DMEM (with or without phenol red) containing 5% unstripped or dextran-charcoal stripped FBS (CSS). The genetic relationship of the three cell lines with the original MCF-7 cell line was confirmed by DNA fingerprinting using genetic markers at nine different loci (CSF1PO, TPOX, TH01, vWA, D16S539, D7S820, D13S317, D5S818, and the Y chromosome–specific amelogenin in the Clarke lab. Cells were passaged a maximum of 8 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 ATCC. 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 ERβ1) subclone was obtained from Dr. Leigh Murphy, University of Manitoba, and were 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) (29 and refs therein). These clones were not tested for genetic markers prior to 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 (TAM), WAY-200070 and chloroquine were purchased from Sigma (Oakville, ON). Ligand 17 (L17) was synthesized as previously described (26). Z-VAD-FMK was purchased from BD Biosciences, Mississauga, ON.

Antibodies

Antibodies were: anti-ERα (HC-20), anti-cyclin D1 (A-12) SRC-3/AIB1 (sc-9119), RIP140 (sc-8997) (Santa Cruz Biotech, Santa Cruz, CA); anti-ERβ for immunoblot (Thermo-Scientific PAI-311 [immunoblot Figure 1]); anti-ERβ (Novus Biologicals NBP-04936 [all other immunoblots]); anti-ERβ (GTX70182) (GeneTex, Irvine, CA) for ChIP; anti-actin (A-2066) (Sigma); anti-LC3 (Novus Biologicals, Littleton, CO), anti-Bcl-2 (BD Biosciences), anti-cyclin E (ab7959), Abcam, Cambridge, MA), ChromPure rabbit 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., Westgrove, PA).

ERβ knockdown and induction

LCC9 cells were transfected with 25nM 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) were treated with 1µg/ml doxycycline (BioBasic) to induce overexpression of ERβ and reverse transfected with 25nM ESR1 siRNA ON-TARGET plus (Dharmacon Cat# (L-003401-00-0005). Control transfections were done with siCONTROL non-targeting (NT) siRNA #1 (Cat# D-001210-01-05). For some proliferation assays, LCC9 cells were infected with retrovirus, pSUPER expressing previously
verified ERβ shRNA (pERβshRNA) [Addgene plasmid #35561]) or non-targeting GFP-shRNA (shNT) vector [Addgene plasmid #30519].

**Cellular proliferation/ viability Assay**

7.0 x 10⁴ 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 3 times. Viable cells were enumerated using Trypan blue exclusion or a Vi-Cell XR cell viability analyzer (BeckmanCoulter). In some experiments chloroquine and Z-VAD-FMK were added at a final concentration of 33μM and 100μM respectively.

**Immunoblot**

Proteins were separated using SDS-PAGE and transferred to PVDF membranes (Millipore, Etobicoke, ON). Immunoreactive bands were detected using substrate (Millipore) and quantified by densitometry using Image J v1.43.

**Chromatin immunoprecipitation**

ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology, Charlottesville, VA) according to the manufacturer’s instructions with some modifications. Cells were treated with vehicle, 10 nM E2, L17, or WAY for 30 min (ER ChIP) or 15 min (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 immunoglobulin G (IgG) or no antibody (input) at 4°C overnight. Cross-links were reversed at 65°C for 4 h. DNA was purified by phenol/chloroform extraction, precipitated and subjected to PCR analysis. PCR primer sequences were the same as those used in qRT-PCR. 5μl 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 Image J
v1.43 and IgG subtracted from test samples then results graphed as a percentage of input DNA.
Additional Materials and Methods are included in supplemental files.

Results

ERβ agonists inhibit proliferation of LCC9 cells: Together with structural differences (Figure
1A), L17 and WAY represent two different classes of ERβ agonists based on selectivity and
binding affinity. WAY-200070 (WAY) is an aryl diphenolic azole ERβ agonist. The ERβ RBA
of WAY is 133 (RBA ratio ERβ:ERα=68) (27) while the ERβ RBA of L17 is 1.73 (RBA ratio
ERβ:ERα=9.3) (26). Since 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 while 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 was verified in
proliferation assays after treatment with either tamoxifen or fulvestrant (supplementary Figure
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 since they express levels of the ERβ comparable to LCC9 cells and therefore serve as a more appropriate comparison than MCF-7CL cells. The results in Figure 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 (Figure 1D). WAY and L17 had a similar growth inhibitory effect on LCC9 cells at 10nM (Figure 1E). In order to verify that growth inhibition induced by L17 was mediated through the ERβ, we treated LCC9 cells infected with a non-targeting retrovirus (shNT) or pSUPER-ERβ shRNA (shERβ) (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 (Figure 1F). Verification of ERβ knockdown is shown in Figure 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 10nM E2 MCF-7 cells demonstrated small reduction in G1 and increase in S phase cells (Figure 2A and supplementary Figure S2A). As expected, TAM produced a strong G1 arrest. Consistent with the results in Figure 1, L17 and WAY had no significant effect on the cell cycle distribution in MCF-7 cells.

E2 (10nM) induced a small (10%) decrease in the G1 and corresponding increase in the S phase of the cell cycle in LCC9 cells (Figure 2B and supplementary Figure S2B) while TAM 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 and cyclin E that persisted up to 48hr in MCF-7 cells while L17 and WAY failed to elicit a similar response (Figure 3A). While a lower molecular weight 48kDa species of cyclin E was present in response to E2 in LCC9 cells (Figures 3B,D,E) no induction of cyclin D1 was detected (Figure 3B,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 48hrs of treatment with either L17 or WAY (Figure 3B,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 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 (Figure 4A, E). Both L17 and WAY transiently induced Bcl-2 within 24 to 48 hrs and levels returned to baseline by 72 hrs. Bcl-2 can inhibit autophagy through binding and inhibition of Beclin1 (34). Phosphatidylethanolamine conjugation and subsequent cleavage converts 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. While LC3-I levels remained constant following E2 treatment of MCF-7 cells, we noted a small increase in LC3-II at 48hrs post L17 treatment while WAY had little effect on LC3-II (Figure 4A,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 hrs (Figure 4B,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 either E2, L17 or WAY (Figure 4B, F).

Knockdown of ERβ using siRNA showed that the L17-mediated decrease in Bcl-2 expression in LCC9 cells was ERβ-dependent (Figure 4C,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 chromatin immunoprecipitation (ChIP) analysis with antibodies against ERα and ERβ following a 1hr 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 10nM E2 for 1 hr resulted in recruitment of both the ERα and ERβ on the Bcl-2 gene in LCC9.
cells (Figures 5A,B and supplementary Figure S3A). Interestingly, ERα and ERβ were both present on the Bcl-2 ERE 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, while L17 and WAY each recruited the ERα at significantly reduced levels relative to E2 (Figures 5C,D and supplementary Figure 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 (Figure 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 1hr exposure to ligands showed that E2 significantly induced Bcl-2 mRNA by 5-fold in MCF-7 cells while L17 and WAY reduced Bcl-2 transcripts in both cell lines. Although the latter reduction within the 1 hr 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 (Figure 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 (Figure 5A,B) which corresponded to weak induction (E2) or significant inhibition of expression (L17 and WAY) following a 1hr treatment (Figure 5F). Although all three ligands also recruited both the ERα and ERβ in MCF-7 cells (Figure 5C,D), only E2 strongly increased
pS2 mRNA (Figure 5F).

We also analyzed mRNA levels 24hr after treatment to assess the longer term effects of ligand activation on transcript levels. Both Bcl-2 and pS2 mRNA remained elevated after 24hr 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 (Figure 5G). pS2 transcripts maintained a similar pattern of expression after 24hrs although a small rebound in pS2 mRNA was observed in L17-treated cells, possibly due to receptor desensitization which could alleviate repression (Figure 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 EREs. 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 in order to increase the relative expression of ERβ. Cells were transfected with a non-targeting sequence (NT) or ERα siRNA as described in Materials and Methods and treated for 10 min with vehicle, E2 or L17. The results in Figure 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. Whereas 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.
Expression of ERβ mRNA and knockdown of ERα were confirmed as shown in supplementary Figure S4. Thus, the Bcl-2 ERE is negatively regulated by ligand-bound ERβ in conjunction with RIP140 recruitment.

Lastly, 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 (Figure 5J). 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). Since WAY and L17 reduced Bcl-2 and increased levels of LC3-II in LCC9 cells, we tested the possibility that CQ might convert this autophagic response to cell death. Figure 6A shows that a 5 day treatment with 33μM CQ to E2-free cultures caused an approximate 20% decrease in LCC9 cells and 35% in MCF-7 cells compared to vehicle-treated cells (Figure 6B). To confirm that the effects of CQ 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 CQ. Figure 6C shows that knockdown of ERβ prevented the effects of CQ 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 CQ and L17 or WAY induced a dramatic 80% decrease in cell numbers compared to control, which correlated to induction of sub-G1 DNA (Figures 6D and supplementary Figure S5A). CQ/ERβ agonist treatment also reduced MCF-7 viable cells in association with a subG1 peak (Figures 6E and supplementary Figure S5B) albeit not as dramatically as in LCC9 cells. Thus, SERD-resistant LCC9 cells expressing a high ratio of ERβ:ERα are preferentially sensitive to ERβ agonist combined with autophagy inhibition.

To distinguish if 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 (Figure 6F and supplementary Figure S5C). Consistent with induction of apoptosis, Z-VAD-FMK almost completely blocked cell death in LCC9 cells induced by combination L17 and CQ.

**Discussion**

Previous studies have demonstrated that overexpression of the ERβ 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 ERβ 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 ERα (40). Although ERβ activation has anti-proliferative effects, the majority of human ER+ breast tumors do not express high levels of ERβ relative to ERα at diagnosis. Remarkably, cell lines with acquired resistance to SERMs express an increased ratio of ERβ to ERα (25).

In this study we found that L17 and WAY had modulatory effects on cell cycle proteins and either reduced 50kDa cyclin E (MCF-7 cells) or weakly increased expression of 48kDa cyclin E (LCC9 cells). Both ligands reduced cyclin D1 expression. Multiple phosphorylated
residues on cyclin E are required for recognition by the SCF$^{Fbw7}$ ubiquitin ligase which leads to degradation of cyclin E and progression through S phase (41). Further experiments are required to determine if the 48kDa cyclin E protein represents hypophosphorylated cyclin E, reflecting the lack of cell cycle progression in E2 and ER$\beta$ ligand-treated LCC9 cells.

Results of ChIP-on-chip experiments have shown considerable overlap between the enhancers that bind the ER$\alpha$ and the ER$\beta$ (42). This study is the first to demonstrate that ER$\beta$ agonists downregulate Bcl-2. Our results showed that both the ER$\alpha$ and ER$\beta$ 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$\beta$ 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$\beta$ than WAY (26,27), and retains low affinity for the ER$\alpha$, recruited the ER$\alpha$ more strongly than WAY. Regardless, both ligands decreased Bcl-2 mRNA in both cell lines. Ligand-ER conformation on a DNA sequence element determines transcriptional activation through coregulator recruitment. Our results suggest that, under conditions where the ratio of ER$\beta$:ER$\alpha$ is high, ER$\beta$ 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$\beta$ (43). Since the liganded ER subtype determines chromatin binding (42), it appears that the Bcl-2 and pS2 genes are negatively regulated by ligands interacting with ER$\beta$.

In LCC9 cells, E2 bound to ER$\alpha$ and/or ER$\beta$ 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 ERβ:ERα 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 ERβ as demonstrated by the reduction in CQ sensitivity following KD of ERβ in LCC9 cells. It is possible that ERβ 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-κB activity relative to parental cells (49) and NF-κB 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 knock-down of Bcl-2 results in induction of autophagy in MCF-7 cells (52). Based on the dominant expression of the ERα in most ER+ primary breast cancers, ERβ agonists may not be useful as first line therapy. However, given that SERM/SERD resistant cells often express a higher ERβ:ERα ratio (24,53), and TAM fails to reduce Bcl-2 in LCC9 cells (49), our novel finding that ERβ agonists reduce Bcl-2 expression and promote autophagy in these cells suggests that combination ERβ agonists and autophagy inhibitors may represent a novel, relatively low toxicity therapeutic option in patients with acquired endocrine resistance.

Acknowledgements: We thank Dr. Robert Clarke, Georgetown University, for the generous gift
of MCF-7/LCC9 cells and the parental MCF-7 control line.

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FIGURE LEGENDS

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). C, MCF-7 and D, LCC9 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 represents the mean from 3 separate experiments each performed in triplicate; error bars indicate standard error. E, MCF-7 and LCC9 cells were cultured in the presence of vehicle, 10nM E2, 10nM L17 or 10nM WAY for 3 days and viable cells were enumerated. Graph depicts percentage of vehicle-treated cell numbers ± standard error. F, LCC9 cells infected with retrovirus expressing shERβ or shNT were treated with 10nM 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 of LCC9 cell lysates 72 hrs 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.

Figure 2. Effects of ERβ agonists on the cell cycle in MCF-7 and LCC9 SERD/SERM-resistant cells. Graphs showing cell cycle distribution as a percentage of vehicle-treated cells for A, MCF-7 cells and B, LCC9 cells after 72 h of treatment with vehicle, 10 nM E2, TAM, L17 or WAY for 72 h before harvesting for cell cycle flow analysis. Results are representative of three independent experiments performed in triplicate.
Figure 3. Effects of ERβ ligands on cell cycle protein expression: Examples of immunoblots for cyclins and in lysates of A, MCF-7 and B, LCC9 cells treated with the indicated ligands over a 48hr time course. Actin was used as a protein loading control. Graphs showing mean densitometric values from 3 experiments for C, cyclin D1, D, 48kDa cyclin E and E, 50kDa cyclin E. Bars represent standard error; p values were calculated using a t-test.

Figure 4. ERβ agonists reduce Bcl-2 expression and increase autophagy in LCC9 cells. Immunoblot of Bcl-2 and LC3 in protein lysates from A, MCF-7 and B, LCC9 cells. Cells were treated with 10 nM E2, L17, WAY or vehicle for the indicated times. C, Bcl-2 immunoblot of lysate from LCC9 cells transfected with NT siRNA or ERβ siRNA and treated with vehicle or L17 for 48hrs. Actin was detected as a loading control. D, ERβ immunoblot of siRNA-transfected LCC9 cell lysates. A non-specific band (*) is indicated. Actin immunoreactivity was used as a loading control. Mean densities derived from 3 separate immunoblots for E, Bcl-2 and F, LC3-II are shown. Dashed lines indicate baseline expression level set at value of 1 after 72hrs (vehicle). Bars represent standard error; p values were calculated using a t-test.

Figure 5. Ligand-dependent recruitment of the ERα and ERβ and coregulators to the EREs of the Bcl-2 and pS2 genes. ChIP assays for ERs bound to the Bcl-2 and pS2 EREs after a 1 hr treatment with vehicle, E2, L17 or WAY. Bcl-2 exon 4 primers were used as a negative control. Densitometric analysis of Bcl-2 and pS2 ERE PCR products from an ERα ChIP expressed as a percentage of the input in A, MCF-7 cells and B, LCC9 cells. The same analysis using the anti-ERβ antibody in C, MCF-7 and D, LCC9 cells. The y-axis represents Image J quantification of
the amount of specific PCR product expressed as the percentage of antibody binding versus the
amount of PCR product obtained using a standardized aliquot of input chromatin. The signal in
the IgG lane was subtracted from each sample. The fold-induction over control of Bcl-2 and pS2
mRNA in MCF-7 and LCC9 cells following a 1hr (E and F) and 24hr treatment (G and H) with
each ligand was measured by qRT-PCR. Dashed lines indicate baseline expression level set at
value of 1 after 72hrs (vehicle). Data represents the mean from 2 separate experiments each
performed in triplicate; error bars indicate standard error; p values were calculated by t-test.
Darker bars are MCF-7 and light bars are LCC9. Dox-treated MCF-7 (rTA tet-ON ERβ1) were
transfected with siNT or siERα and treated with vehicle (Veh/V), 10nM E2 or L17 for 15 min.
ChIP was performed as described in Methods using; I, anti-RIP140 or J, anti-SRC3, followed by
PCR for the Bcl-2 ERE. PCR products quantified by Image J are expressed as a percentage of
input. The graph is representative of three separate experiments. K, HEK293 cells were
transfected with ERα or ERβ and treated for 3hr with the indicated ligands. ERα and ERβ
immunoblot of lysates is shown. Actin and a non-specific band (*) interacting with anti-ERβ
serve as loading controls. U; untransfected C; no treatment.

**Figure 6. Inhibition of autophagy induces apoptotic death when combined with ERβ
agonists.** LCC9 and MCF-7 cells were treated with vehicle, 10 nM L17 or WAY, with or
without CQ for a total of 5 days. A, LCC9 and B, MCF-7 cells were enumerated using trypan
blue exclusion. C, LCC9 cells were infected with pRS control virus or pSUPERshERβ for 48 hrs
then treated for 5 days with vehicle, 10nM L17, 10nM WAY with or without CQ (33µM). D,
LCC9 and E, MCF-7 cells treated as in A and B were collected and subG1 DNA content was
determined by flow cytometry.  

F, LCC9 cells were treated as in D except that in some samples 100µM Z-VAD-FMK was included in the medium. Data represents the mean of triplicate determinations. Bars indicate standard error.
Figure 1  Ruddy et al

A

17β-estradiol  L17  WAY200070

C

MCF-7

Concentration (nM)

Percent cell viability (relative to control)

Concentration (nM)

D

LCC9

Concentration (nM)

Percentage of cell viability (relative to control)

E

Test compounds (10 nM)

Viable cells (Percentage of control)

F

Cell number × 10^3

G

55kDa * ERβ actin

shNT  shERβ

shNT Veh  shNT L17  shERβ Veh  shERβ L17
Figure 2  Ruddy et al

A

MCF-7

DNA content (Percentage of control)

Test compounds (10 nM)

E2  TAM  L17  WAY

G1  S  G2/M

B

LCC9

DNA content (Percentage of control)

Test compounds (10 nM)

E2  TAM  L17  WAY

G1  S  G2/M
Figure 3 Ruddy et al

A

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MCF-7

- cyclin E
- cyclin D1
- actin

LCC9

B

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Cyclin D1

**p=0.1**  **p<0.08**

C

![Graph showing relative density of Cyclin D1](image)

D

Cyclin E (50kDa)

**p<0.01** **p<0.02**

E

Cyclin E (48kDa)

**p<0.05**

Vehicle 24h 48h

MCF-7  LCC9
Rudy et al Figure 4

A

**MCF-7**

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Bcl2

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**LCC9**

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Bcl-2

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siRNA | ERβ  | NT |
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Relative density (Normalized to actin)

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**LC3-II**

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Relative density (Normalized to actin)

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LCC9

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MCF-7 ERα

B

LCC9 ERα

C

MCF-7 ERβ

D

LCC9 ERβ

E

1hr

Fold increase in Bcl-2 mRNA

F

1hr

Fold increase in pS2 mRNA

G

24hrs

Fold increase in pS2 mRNA after 24 h treatment

H

24hrs

Fold increase in pS2 mRNA after 24 h treatment

I

RIP140 ChIP

J

LCC9 ERβ

K

ERα

ERβ

55kDa
Molecular Cancer Therapeutics

Preferential estrogen receptor $\beta$ ligands reduce Bcl-2 expression in hormone-resistant breast cancer cells to increase autophagy

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

*Mol Cancer Ther* Published OnlineFirst April 30, 2014.

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