Preferential estrogen receptor β ligands inhibit proliferation and reduce Bcl-2 expression in Fulvestrant-resistant breast cancer cells

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ABSTRACT

Endocrine resistance 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 antiestrogen 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 exposure to two different ERβ agonists results in decreased cell viability, and produced a marked reduction in G2/M phase in antiestrogen resistant breast cancer cell line in conjunction with altered cyclin D1, and cyclin E expression relative to E2. ERβ agonists also strongly downregulated Bcl-2 expression and recruited both ERs to the Bcl-2 and pS2 E2-response elements resulting in a reduction in mRNA transcripts from both of these genes. Bcl-2 reduction correlated with increased lipidation of LC3-I to LC3-II, indicative of increased autophagic flux. Although ERβ agonist treatment alone did not induce apoptosis, remarkably, the coaddition of ERβ agonist and the autophagy inhibitor, chloroquine, resulted in robust cell death. Lastly, *in vivo* studies demonstrate that preferential-ERβ agonists are not estrogenic in the uterus or mammary gland.

Together, these observations suggest that combined therapies including an ERβ agonist and an autophagy inhibitor may provide the basis for a safe, novel approach to the treatment of antiestrogen-resistant breast cancers

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LIST OF ABBREVIATIONS

4-OHT	4-hydroytamoxifen
Adr	adriamycin
αERKO	estrogen receptor α knock out
AF-1	activation function-1
AF-2	activation function-2
AI	aromatase inhibitor
AIB1	amplified in breast cancer 1
AKT	protein kinase B
AP-1	activator protein 1
Atg	autophagy related genes
Bcl-2	B cell lymphoma/leukemia- 2
BECN1	beclin 1
βERKO	estrogen receptor β knock out
BH	Bcl-2 homology
BRCA1/2	Breast cancer gene 1/2
Cdk	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
cDNA	complementary deoxyribunecleic acid
ChIP	chromatin immunoprecipitation
CL	Clarke lab
CO_2	carbon dioxide
COOH	carboxyl functional group
CQ	chloroquine

CSS	charcoal stripped serum
DBD	DNA binding domain
DCC	dextran coated charcoal
DDT	dithiothreiotol
DMEM	Dulbecco's modification of Eagle's medium
DNA	deoxyribonucleic acid
E2	17β-estradiol / estrogen
EC50	half maximal effective concentration
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERα	estrogen receptor α
ERβ	estrogen receptor β
ERE	estrogen response element
ERK	extracellular signal-regulated kinases
ERT	estrogen replacement therapy
EtOH	ethanol
F	fluorine
FBS	fetal bovine serum
FDA	food and drug administration
FGF	fibroblast growth factor
g	grams
GH	growth hormone
GRIP1	glumatmate receptor interacting-protein 1

h	hour
H	hydrogen
H12	helix 12
HCL	hydrogen chlorida
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HER2	human epidermal growth factor receptor 2
HMW	high molecular weight
HRP	horse radish peroxidase
HRT	hormone replacement therapy
HSP	heat shock protein
IAP	inhibitor of apoptosis protein
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G
ILK-1	integrin-linked kinase 1
Kd	dissociation constant
kDa	kilodalton
KCL	potassium chloride
kg	kilogram
KH_2PO	potassium dihydrogen orthophosphate
Lamp-2	lysosomal associated-membrane protein-2
LBD	ligand binding domain
LC3-I/II	Microtubule-associated light chain 3-I/II
LC50	median lethal concentration
LiCl	lithium chloride

LMW	lower molecular weight
M	molar
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
mcl-1	induced myeloid leukemia cell differentiation protein
mg	milligram
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
$NAHCO_3$	sodium bicarbonate
Na_2HPO_4	disodium hydrogen orthophosphate
NEAA	non-essential amino acids
NFκB	nuclear factor kappa B
NCoR	Nuclear receptor co-repressor
nM	nanomolar
NP-40	Nonidet P-40
O_2	oxygen
OH	hydroxyl functional group
PBS	phosphate buffered saline
PCD	programmed cell death

PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEI	polyethyleneimine
PI-3	phosphatidyl inositol-3
PL	Pratt lab
PMSF	phenylmethylsulfonyl fluoride
PolII	polymerase II
PPT	propyl pyrazole triol
PR	progesterone receptor
PRF	phenol red free
PVDF	polyvinylidene fluoride
RBA	relative binding affinity
RIPA	radioimmunoprecipitation assay
RNAi	RNA interference
ROS	reactive oxygen species
Rpm	rotations per minute
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	serine
SERD	selective estrogen receptor downregulator
SERM	selective estrogen receptor modulator
siRNA	small interfering ribonucleic acid

SMRT	silencing mediator of retinoid and thyroid hormone receptors
SRC	steroid coactivator
Tam	Tamoxifen
TBS-T	tris buffered saline-tween
TE	tris-EDTA
TEB	terminal end bud
TGFβ	transforming growth factor β
TIF2	nuclear receptor coactivator 2
µg	microgram
µM	micromolar
µL	microliter
VEGF	vascular endothelial growth factor
XBP1	X-box binding protein-1

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CHAPTER I - INTRODUCTION

Breast Cancer

Breast cancer is the most common cancer among Canadian women, and each year nearly 1.3 million are diagnosed worldwide (Canadian Cancer Society, 2012). Breast cancer is a heterogeneous disease (Gallager, 1984) and many factors are implicated in its etiological pathogenesis. These include age, genetics, familial associations including mutations in the breast cancer genes BRCA1/2, diet, and obesity (Plu-Bureau et al, 1998; Soderqvist, 1998; Dumitrescu et al, 2005). Levels of endocrine factors, both exogenous and endogenous steroid hormones (ie. estrogen and progesterone) are also linked to the pathogenesis of breast cancer as a result of their significant effects on cell growth, differentiation, and function in the breast and other tissues (Malara et al, 2006). Breast cancers are categorized into four primary subtypes based on different genetic and epigenetic abnormalities and cell-type orgin (Cancer Genome Atlas Network, 2012). These intrinsic subtypes of breast cancer include: luminal A, luminal B, HER2-positive and basal-like or triple-negative breast cancer (Cancer Genome Atlas Network, 2012). Luminal breast cancer originates from the luminal cells of the mammary gland (Cancer Genome Atlas Network, 2012). 75% of luimnal breast cancers are less aggressive, positive for the estrogen receptor and/or the progesterone receptor, grow in response to estrogen and likely to respond to endocrine therapies, such as Tamoxifen or Fulvestrant (Clarke et al, 2001). HER2-positive breast cancer overexpresses the epidermal growth factor receptor, HER2, which stimulates its growth. It is more aggressive and fast growing and treated with a monoclonal antibody called Trastuzumab (or Herceptin) (Hicks et al, 2008). Basal-like or triple-negative breast cancer originates from the progenitor cells, typically do not express hormone receptors or HER2, and most are associated with the Breast cancer (BRCA)1 gene (Rakha et al, 2008). Overall these tumors have a poorer prognosis than other types, partially because no targeted therapies have been developed (Linn et al, 2009).

(i) Estrogen and Breast Carcinogenesis

Estrogens belong to a family of nine chemically different steroid hormones that all share a four-ringed, carbon atom backbone and are synthesized from cholesterol precursors (Simpson, 2003). The most abundant and biologically active natural estrogen produced in all vertebrates is 17β-estradiol (E2). E2 is produced primarily in the ovaries, but low levels of E2 are also obtained from aromatization of adrenal and ovarian androgens in the adipose tissue, bone, vascular endothelium, aortic smooth muscle, and brain (Simpson et al, 2001; Ali et al, 2002). These secondary sites become the major sources of estrogen synthesis in post-menopausal women, and play a role in breast cancer progression in this population (Clemons et al, 2001; Ali et al, 2002).

Estrogens are essential regulators of many physiological processes in both women and men. They play a major role in the development and differentiation of classical targets such as the reproductive tract and gonads, where they regulate proliferation and differentiation (Ali et al, 2002). E2 is also a key player in the functioning of non-reproductive tissues such as the skeletal, cardiovascular and central nervous system (Dupont et al, 2000; Emmen et al, 2003). Physiological E2 strongly influences several aspects of human physiology by eliciting different growth responses in reproductive and non-reproductive tissues based on the cell type, type of estrogen receptor present, dose and timing and duration of exposure (Okoh et al, 2011). Evidence of E2 involvement in breast cancer development derives from studies in which E2 induced breast cancer cell proliferation *in vitro*, and promoted mammary carcinogenesis in experimental animals (Lupulescu, 1995; Yue et al, 2003). Also inhibition of E2 synthesis with aromatase inhibitors in aging Sprague-Dawley rats inhibited the development of spontaneous breast tumors (Gunson et al, 1995).

(ii) Mechanisms of estrogen carcinogenesis

Considerable epidemiological and experimental data strongly implicate E2 in the development and growth of breast cancer (Lupulescu, 1995; Duffy et al, 2006). It is well accepted

that excessive lifetime exposure to E2, through early menarche, late menopause, oral contraceptives and/or estrogen replacement therapy (ERT), increases the risk for cancer in hormone-dependent organs, specifically breast and endometrium (Chang, 2011; Okoh et al, 2011). Studies in animal models including mice, rats and hamsters, have shown that the number of new cases cancer found in of the mammary gland, uterus, vagina, testis and kidney all increase following E2 administration (Duffy, 2006). These findings led the International Agency for Research on Cancer and National Toxicology Program to declare both endogenous and exogenous sources of steroidal estrogens as human carcinogens (WHO, 1999; NTP, 2002; Bolton et al, 2008). Although not fully characterized, E2 is a key factor in the initiation and promotion of breast carcinogenesis through various mechanisms (Yaghjyan et al, 2011).

Two major mechanisms are postulated to be involved in carcinogenic effects of estrogens: (1) stimulation of cell proliferation via estrogen receptor (ER)-mediated hormonal activity, and; (2) genotoxic effects by the metabolites and/or reactive oxygen species (ROS) generated during E2 metabolism leading to increased mutation rates.

E2 stimulates breast epithelial cell proliferation through nuclear ER-mediated signaling pathways (Bolton et al, 2008). Thus, the idea is that the proliferative stimulus provided by E2 leads to increased mitogenesis, thus increasing the risk of genetic errors and/or the generation of spontaneous mutations during DNA replication (Cavalieri, 2000; Yager, 2000). Secondly, an important aspect of E2 toxicology that has been implicated in breast carcinogenesis is its tissue-specific, cellular oxidative metabolism mediated by cytochrome P450 (cyp450) enzymes (Figure 1.1). This pathway generates reactive electrophilic estrogen o-quinone metabolites and ROS through redox cycling (Bolton, 1998; Yager, 2000). Several CYP450 isoforms, including CYP1A1/1A2, 1B1 and 3A4, selectively catalyze hydroxylation at the 2- and 4-positions on the aromatic ring of E2 giving

Figure 1.1. Metabolism of 17β-estradiol (E2) to form toxic quinones.
The main route of E2 metabolism is mediated by cytochrome P450 enzymes forming catechol estrogens, 4-hydroxy-estradiol (4-OH-estradiol) and 2-hydroxy-estradiol (2-OH-estradiol). The catechol estrogens are then further oxidized by CYPs to form toxic quinones, 3,4-estradiol quinone and 2,3-estradiol quinone.

rise to 2-hydroxy (OH)-estradiol and 4-OH-estradiol, respectively (i.e. catechol estrogens). Oxidation (by any oxidative enzyme or metal ion) of catechol estrogens further produces 2,3-estradiol-quinone and 4,3-estradiol quinone, respectively (i.e. o-quinones). O-Quinones are subject to enzymatic reduction and can undergo redox cycling with the semiquinone radical, mediated by CYP450 reductase, generating ROS including superoxide anion radicals and hydroxyl radicals (Chang, 2011). Reactive quinone metabolites can also directly damage DNA by forming DNA adducts (Clarke et al, 2003; Cavalieri et al, 2006; Fussell et al, 2011). Similarly, free radical toxicities such as DNA single strand breaks, chromosomal abnormalities, lipid peroxidation and protein oxidation, have been observed in both animals and humans treated with E2 (Boyd, 1991; Banerjee et al, 1994; Roy et al, 1999; Pisha, 2001; Russo, 2006; Bolton 2008; Nutter et al, 1991). Furthermore, breast cancer metastasis has been linked to the excessive generation of ROS (Malins et al, 1996; Malins et al, 2006). These studies support a role for estrogen in the development of breast cancer tumors via the enzymatic reduction of estrogen metabolites, which produce ROS, ultimately causing DNA damage (Gutierrez, 2000).

Estrogen Receptors

(i) Structure and Function

Most of the biological functions of E2 are mediated by the estrogen receptor (ER). The ER belongs to the nuclear steroid receptor superfamily of ligand-dependent transcription factors (Matthew et al, 2003). To date, two forms of the ER have been identified, the ERα and the ERβ (Figure 1.2). The ERα and the ERβ are encoded by different genes and located on different chromosomes (Marino et al, 2006). ERα, was initially isolated and characterized in the late 1950s (Toft et al, 1966; Jensen et al, 1968; Jensen et al, 1973), but it wasn't until 1986 that it was cloned and sequenced from MCF-7 breast cancer cells (Green et al, 1986). Ten years later the ERβ was discovered and cloned from a rat prostate cDNA library (Kuiper et al, 1996). ERα and ERβ are

expressed in many cell types at similar levels, however in some tissues or organs, one or the other ER subtype predominates. ERα is mainly expressed in the uterus, ovary, prostate (stroma), testes (Leydig cells), epididymis, bone, breast, liver, kidney, white adipose tissue, and in various regions of the brain (Enmark et al, 1997; Nilsson et al, 2010). On the other hand, ERβ is expressed primarily in the prostate (epithelium), testis, bone marrow, vascular endothelium, lung, bladder, intestinal epithelium, and certain parts of the brain (Kuiper et al, 1997, Nilsson et al, 2010).

The ERs are modular proteins containing evolutionarily conserved structurally and functionally distinct domains, labeled A/B, C, D, and E/F (Figure 1.2) (Matthews et al, 2003; Marino et al, 2006). The N-terminal A/B domain harbours the ligand-independent activation function-1 (AF-1), which shows promoter-and cell-specific activity. This domain which is constitutively active, shows the least homology between the two receptors (~17%), and mediates interactions with co-activator proteins. Two motifs located in the ER have been identified as important contributors to AF-1-ligand independent activity, namely "Box1", amino acids 41-64, and those that promote synergism with the LBD, "Box 2", amino acids 87-108 (Webb et al, 1998). Comparison of the AF-1 domains of both ERs has revealed that the AF-1 activity of ERβ is negligible compared to that of ERα (Hall et al, 1999; Matthews et al, 2003; Heldring et al 2007). In addition, the amino-termini of both ERs may also be phosphorylated by kinases in growth factor pathways, resulting in strong stimulation of AF-1 activity. Specific serine residues, including ser104, 106 and 118 have been identified as being phosphorylated by MAP kinases or cyclin-dependent kinases (cdks) (Trowbridge et al, 1997).

The central and most conserved domain (showing 96% sequence homology between the two ERs), the DNA-binding domain (DBD), plays a crucial role in the recognition and binding of the ERs to specific sequences on DNA. Also, this C-domain is also involved in receptor dimerization (Nilsson et al, 2010). Chip-on-chip analysis suggests that there is considerable overlap

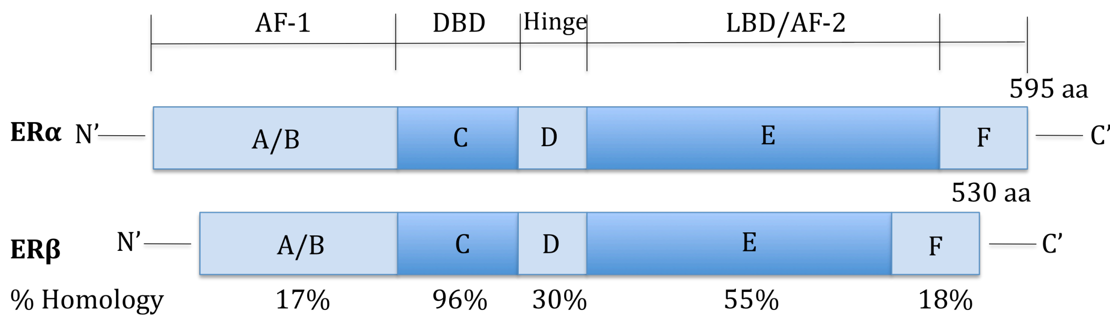


Figure 1.2. Schematic representation of the common structural and functional domains of ERα and ERβ. The domains of the receptors are indicated along the top as Activation function 1 (AF-1), DNA binding domain (DBD), the Hinge region, and the Ligand binding domain (LBD) and Activation function 2 (AF-2). The percentage of amino acid homology in these domains between ERα and ERβ is indicated. aa, amino acids. Adapted from: Klinge (2000).Steroids *65*, 227-51.

in DNA response element binding between the two receptors, however there are DNA regions that are preferentially bound by the ERα or the ERβ (Liu et al, 2008).

The D-domain is known as the hinge region, connecting the E/F domain with the C-region, and displays 30% sequence similarity between the two receptor subtypes. The hinge region is involved in nuclear localization of the receptor (Duffy, 2006) and has been shown to enhance DNA binding activity (Wang et al, 2001).

The E domain contains the ligand-binding domain (LBD) and the ligand-dependent activation function-2 (AF-2). This region is multifunctional and plays a role in ligand recognition and binding, homo/hetero dimerization, nuclear translocation, transcription activation, interacts with heat-shock proteins, and co-factor binding (Enmark et al, 1997). ERα and ERβ display 56% conservation in this region, suggesting that ligands may be developed that have different affinities for either receptor (Nilsson et al, 2010).

Finally, although the function of the C-terminal F domain is poorly understood, it is suggested that it plays a modulatory role for receptor transcription activity and recruitment of co-regulators (Sladek et al, 1999; Duffy, 2006). The F-domain has less than 20% amino acid identity between the ERα and the ERβ (Zhao et al, 2008). Even though the two ER subtypes show a high degree of sequence similarity in some of their domains, they differ in their ligand binding affinity, recruitment of co-regulators, and transcriptional response of ER-target genes (McInerney et al, 1998).

(ii) Estrogen Receptor Signaling

Various mechanisms for ER transcriptional regulation have been described and include both genomic and non-genomic pathways. The classical mechanism of ER action is dependent on ligand binding (Figure 1.3). In this model, the ER is sequestered in a multiprotein inhibitory complex, including heat shock proteins (HSPs), in the nuclei of target cells until hormone is available

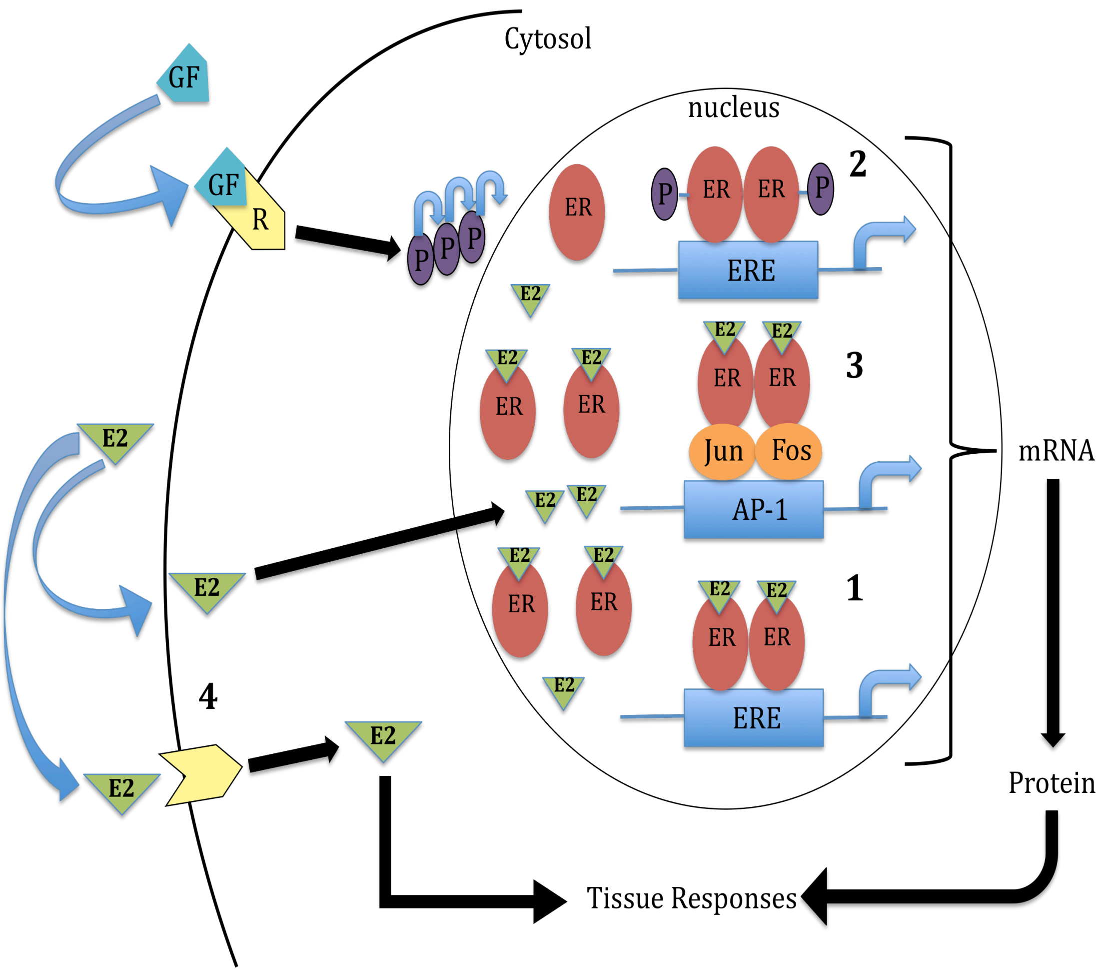


Figure 1.3. Schematic representation of the various mechanisms of estradiol (E2) and estrogen receptor (ER) signaling. The biological effects of E2 are mediated through at least four different pathways: **1)** Represents the classical ligand-dependent pathway where the ERs acts as nuclear transcription factors; **2)** Represents the ligand-independent activation of the ER which involves phosphorylation (P) of the ER and crosstalk with peptide growth factors (GF); **3)** Represents the ERE-independent genomic actions of the ER which involves tethering to other transcription factors (ie. Jun and Fos at the AP-1 enhancer), and **4)** Represents the non-genomic signaling of E2 which involves cytoplasmic and membrane-localized ER. Adapted from Hall et al. (2001). J Biol Chem.276, 36869-72.

(Rosenfeld et al, 2001; McDonnell et al, 2002). When E2 binds, the ER undergoes a conformational change that facilitates the dissociation of HSPs from the ER, triggering dimerization of the receptor (Hall et al, 2001; Leitman et al, 2010). As a dimer (either as homodimers (ERα:ERα or ERβ:ERβ) or heterodimers (ERα:ERβ)), the E2-bound ER can then bind to estrogen response elements (ERE), which are cis-acting enhancers located in the promoter regions of E2 target genes (Klinge, 2001; Heldring et al, 2007). Similar to other nuclear receptors, the ERs can either transactivate or silence promoters by binding to coactivator proteins which function to acetylate chromatin and link the receptor to the basal transcription complex, or to corepressors which function to prevent transcription. When an agonist binds, it induces the formation of a specific coactivator binding pocket within the LBD. Specifically, helix 12 (H12), is critical in regulating AF-2 activity (Heldring et al, 2007). Agonist ligands function by stabilizing the receptor conformation in order to allow optimal binding of coactivators, which will lead to transcriptional activation. In the "active" conformation, a hydrophobic groove is formed by helical elements within the receptor allowing the NR box (an α-helical leucine rich LXXLL motif) of some coactivators to interact with the nuclear receptor (Chang et al, 1999; Webb et al, 1998). H12 is then positioned across the ligand-binding pocket serving as an essential coactivator docking site (Heldring et al, 2007). In contrast, when an antagonist binds, the H12 positioning is interrupted. H12 is not stabilized, but instead H12 is reoriented to occlude the AF2 groove resulting in ER conformations in which the coactivator recruitment surface is physically blocked (Brzozowski et al, 1997; Shiau et al, 1998). Some of the key coactivators and corepressors known to interact with the ER include SRC1/SRC-3 (Onate et al, 1997), the p160's which include GRIP1/TIF2) (Hong et al, 1997; Voegel et al, 1998) AIB1, (Torchia et al, 1997) etc. (coactivators) and SMRT, NCoR, etc (corepressors).

Approximately one third of genes regulated by the ER in humans lack proximal ERE-like sequences (O'Lone et al, 2004). The molecular mechanisms by which the ER regulates transcription

at other response elements that do not contain EREs, such as at the AP-1 response element, is by tethering to and modulating the activity of other transcription factors through protein-protein interactions (Figure 1.3) (Gottlicher et al, 1998; DeNardo et al, 2005). The interaction of the ER with the activator protein-1 (AP-1) transcription factor complex has been most widely studied (Kushner et al, 2000). At certain AP-1-driven promoters, ERα and ERβ were shown to signal in opposite ways in the presence of E2, where ERα activated transcription meanwhile the ERβ repressed transcription (Safe et al, 2008; Kushner et al, 2000; Webb et al, 1995). Interestingly, anti-estrogens all increased AP-1 activity via the ERβ but not via the ERα (Webb et al, 1995; Paech et al, 1997; Kushner et al, 2000).

E2 can also exert so called non-genomic effects—rapid actions that occur outside the nucleus (Figure 1.3) (Nilsson et al, 2001; Marino et al, 2006). Common among other steroid hormones, these rapid responses can activate various protein-kinase cascades (Bjornstrom et al, 2005, Heldring et al, 2007). E2 can activate the mitogen-activated protein kinase (MAPK) and phopsphotidylinositol (PI)-3 signaling pathways in several cell types, including breast cancer (Migliaccio et al 1996; Kahlert et al, 2000; Song et al, 2002). Other rapid effects in response to E2 include increases in ion fluxes across membranes (ie. calcium and sodium), activation of adenylate and guanylate cyclase, and activation of nitric oxide synthase (Morley et al, 1992; Bjornstrom et al, 2005; Duffy, 2006). Although these effects have not been extensively studied, some authors have suggested that these non-genomic effects of E2 are mediated through a subpopulation of the ERs located at the plasma membrane (Pappas et al 1995; Razandi et al, 2004; Marino et al, 2006). In order to characterize the membrane form(s) of the ER and distinguish its function, additional research is required.

Finally, the activity of the ER can be regulated by extracellular signals in a ligand-independent manner in the absence of E2 (Figure 1.3). It is well accepted now that growth factors

that play a role in proliferation, differentiation and survival such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) can indirectly activate the ER leading to increased expression of ER target genes through the activation and phosphorylation of certain kinases (Smith et al, 1998; Hall et al, 2001). Growth factor signaling pathways provide an alternate stimulus for growth and survival and can greatly contribute to the development of a hormone-independent phenotype in some tumors (Shim et al, 2000; Coutts et al, 1998). Moreover, the activation of kinases by membrane-bound ERs contribute to breast cancer cell migration (ie. ERK and protein kinase B (AKT)) and invasion (ie. Src and ILK1 kinases), and are linked to breast cancer metastasis (Roy et al, 2012).

The ER in health and disease

(i) Role of the ERα and the ERβ in the normal and cancerous mammary gland

The significance of E2 in the growth and development of female mammary tissue is well documented in both rodents and humans (Hennighausen et al, 2005; Molyneux et al, 2007; Speirs et al, 2007). Several ER knockout mouse models for the study of E2 function have revealed the importance of these receptors *in vivo*, highlighting the distinct role that each receptor plays in breast development (Weihua et al, 2003). In the rodent mammary gland, ERβ is found in both the epithelial cells (ductal and lobular) and stromal cells (Lemmen et al, 1999). ERα is expressed in both the ductal epithelium and stroma, but not in the lobular epithelium (Saji et al, 2000). The mammary glands of female ERα knockout (αERKO) mice are rudimentary and cannot grow mammary gland tissue past the fetal stage (Couse et al, 1999; Weihua et al, 2003). Female ERβ knock out (βERKO) mice are morphologically indistinguishable from wildtype littermates as their mammary glands develop normally and they lactate normally (Krege et al, 1998; Forster et al, 2002). Based on these *in vivo* studies, ERα appears to be essential for normal mammary gland development and function.

(ii) ERα and ERβ may have opposing roles in the breast

It is estimated that ERα is expressed in only 7-10% of luminal epithelial cells in normal human mammary tissue (Petersen et al, 1987), whereas 80-85% of the same cells express ERβ (Roger et al, 2001; Heldring et al, 2007; Fox et al, 2008). In contrast, although ERβ expression predominates in the normal rodent and human breast tissue, several studies show that ERα mRNA is increased while ERβ mRNA is significantly reduced or lost in estrogen receptor-positive (ER+) breast carcinogenesis (Iwao et al, 2000). Similarly, compared to normal tissues, an increase in ERα:ERβ mRNA and protein ratios are increased in cancer (Bardin et al, 2004) leading the authors to propose a protective role of ERβ against the proliferative activity of E2 in mammary premalignant lesions (Bardin et al, 2004; Zhu et al, 2004). The decrease in ERβ expression is suggested to be a characteristic feature of breast carcinogenesis that is strongly associated with hypermethylation of one of two ERβ promoters observed in several cell lines and tumors (Garinis et al, 2002; Zhu et al, 2004; Skliris et al, 2008), which has led to the hypothesis that ERβ is a tumor suppressor (Zhu et al, 2004; Rody et al, 2005).

It is believed that the proliferative actions of E2 are mediated by the ERα, whereas ERβ is antiproliferative (Koehler at al, 2005) and has been shown to suppress ERα transcriptional activity and lower expression of ERα target genes including pS2, cyclin D1 and PR (Strom et al, 2004). Studies have confirmed that ERβ can inhibit the transcription of genes contributing to proliferation in breast cancer by RT-PCR, *in vitro* cell proliferation assays, and *in vivo* xenografts (Strom et al, 2004; Fox et al, 2008). Furthermore, ERβ has shown to promote anti-proliferative and pro-apoptotic functions as well as decrease motility and invasion of breast cancer cells (Lazennec et al, 2001). Since cell lines expressing high levels of endogenous ERβ are difficult to find, studies in which ERβ is expressed via gene transfer into ER+ MCF7 and T47D breast cancer cells show that ERβ inhibits their growth in response to E2 by upregulating antiproliferative genes ($p21^{Cip1}$ and $p27^{Kip1}$) and reducing the expression of proliferative and antiapoptotic genes (c-myc, cyclin A, and cyclin D1), thus causing G_2 cell cycle arrest (Paruthiyil et al, 2004; Strom et al, 2004; Fox et al, 2008). Due to its decreased

expression, ERβ's ability to modulate the transcriptional activity of ERα is altered during tumorigenesis and this may contribute to the pathogenesis of breast cancer.

(iii) ERα and ERβ as biomarkers of prognosis in breast cancer

The presence of the ERα in breast cancer tumors is a predictor of response to endocrine therapy (Ali et al, 2002; Murphy et al, 2006). The progesterone receptor (PR), a down-stream target of functional ER signaling, is also measured in breast cancer biopsies (Horwitz et al, 1978). Approximately 75% of all diagnosed breast cancers express the ERα, providing an established prognostic marker for response to treatment with endocrine therapy (Palmieri et al, 2002; Miller et al, 2011; Osborne et al, 2011). Furthermore, the expression of ERα is associated with low tumor grade, long disease-free survival and high overall survival. ERα positive tumors are also less invasive and show decreased motility leading to less aggressive cancers (Lazennec et al, 2001).

The significance of ERβ is still under debate and oftentimes, when considering a role for ERβ in human breast cancer, the literature is controversial. Approximately 70% of breast tumors express ERβ and most of these co-express both ERs. Some studies demonstrated that ERβ expression in tumors is associated with poorer prognosis, compared with tumors solely expressing ERα (Speirs et al, 1999). Other studies found that ERβ expression is correlated with an increase in cell proliferation markers including Ki67 and Cyclin A (Jensen et al, 2001). Overexpression of ERβ mRNA, both in vitro and in vivo, is also strongly associated with antiestrogen Tamoxifen (Tam)-resistant tumors compared to Tam-sensitive tumors (Speirs et al, 1999; De Cremoux, 2003), consistent with a requirement for a higher ERα:ERβ for Tam sensitivity. However, on the other hand, there are studies showing that ERβ expression in breast tumors confers a more favorable prognosis compared with tumors expressing only the ERα. Studies show that ERβ expression is significantly associated with negative axillary node status, low-grade tumors and low S-phase fraction (Jarvinen et al, 2000), with a greater disease-free survival (Leygue et al, 1998). The presence of ERβ in more than 10% of cancer

cells was associated with better survival in women treated with Tam (Mann et al, 2001). Overall, these studies support a role for ERβ as a tumor suppressor gene in breast cancer (Treeck et al, 2010). In the case of breast cancer, further investigation is necessary to determine whether ERβ offers any clinical value as a prognostic marker and its significance as a novel target in breast cancer therapy.

Endocrine therapy for breast cancer

(i) History

E2 supports the growth and tumorigenicity of approximately 50% of primary breast cancers (Nehra et al, 2010; Teixeira et al, 1995) and it is for this reason that endocrine manipulations are among the most effective, and least toxic, treatments for ER+, hormone-responsive breast tumors (Clark et al, 2001; Palmieri et al, 2002). Ovariectomy in premenopausal women is the oldest of these therapies (Beatson, 1896). Dr. George Beatson reported this more than a century ago, showing that the removal of the ovaries caused regression of the disease and improved the prognosis of approximately one-third of all patients (Beatson, 1896; Boyd, 1900). Although ovariectomy is still an effective therapy, the discovery of the hormone, estrogen (Allen et al, 1923), and the observation that estrogens promoted mouse mammary carcinogenesis, led Lacassagne (1936) to propose that breast cancer could be inhibited by the use of an estrogen antagonist. It wasn't until the discovery and cloning of the ER that the identification and design of other endocrine therapies targeting estrogen synthesis or estrogen signaling were established.

(ii) Antiestrogens and other treatment strategies for ER+ breast cancer

Currently, antiestrogenic drugs are administered to both pre- and post-menopausal women and are the most effective and widely applied endocrine manipulation (Clarke et al, 2001). Antiestrogens act primarily by competing with E2 for binding to the ER. They also exhibit cell and tissue specific agonist or antagonist effects. The action of antiestrogens is desirable in some tissues

like bone and the cardiovascular system, while highly unfavourable in tissues like the uterus or breast (Heldring et al, 2007). The standard and most prescribed endocrine treatment for ER+ breast cancer is a non-steroidal selective estrogen receptor modulator (SERM), Tamoxifen (Tam). Discovered in the late 1950's and used in the clinic for more than 40 years, Tam behaves as an ER antagonist in the breast but as an agonist in the uterus and bone. The unwanted agonistic effects of Tam in the uterus is linked to strong AF-1 activity of ERα (Jordan et al, 1987) and is also a function of cell-type specific set of coregulator proteins such as increased levels of co-activators like SRC-1 or SRC-3 (Smith et al, 1997; Martini et al, 2003; Smith et al, 2004). Although prolonged use of Tam can increase one's risk for developing thromboembolic disease and endometrial cancer, the usefulness and importance of Tam in breast cancer therapy is well established; Tam inhibits contralateral breast cancers, reduces the rate of disease recurrence by 50%, decreases the annual breast cancer death rate by one third (Clarke et al, 2003; Musgrove et al, 2009), and drastically increases the disease-free survival rate after 5 years (Chia et al, 2005; Jordan et al, 2007).

Raloxifene, a second generation SERM, is similar to Tam in that it acts as an E2 antagonist in the breast tissue and bone by competitive binding to the ER. Unlike Tam, Raloxifene does not induce uterine growth but unfortunately also increases the intensity and frequency of hot flashes in menopausal women (Harvey et al, 2006). Raloxifene is approved for breast cancer prevention, but not for treatment (Cummings et al, 1999) and has been approved by the Food and Drug Administration (FDA) to be used to prevent and treat osteoporosis in postmenopausal women.

ICI 182,780 (Fulvestrant) is a selective estrogen receptor downregulator (SERD). It is a pure steroidal ER antagonist exhibiting no agonistic properties when administered, thus it is characterized by antineoplastic activity in breast cancer and is devoid of uterotrophic effects (Howell et al, 2000). Fulvestrant binds to the ER, inhibits its action, and promotes the degradation of the ER through the ubiquitin-proteasome pathway (Fan et al, 2003; Marsaud et al, 2003) resulting in

complete inhibition of ER-mediated transcription inactivating both AF-1 and AF-2. Fulvestrant is often used as a second-line therapy in ER+ breast cancer patients who fail to respond to Tam (Patel et al, 2007). In a xenograft model with aromatase-transfected MCF-7 cells a combined therapy of an aromatase inhibitor plus fulvestrant was significantly more effective than either treatment alone, delaying the emergence of resistance (Jelovac et al, 2005; Macedo et al, 2008). Ultimately, Fulvestrant may be an effective option because it can eliminate ER+-dependent cells that start to operate in a ligand-independent fashion (Johnston, 2010). However, the use of pure antagonists is not without drawbacks; they may further exacerbate bone loss, a concern that also applies to aromatase inhibitors (Dowsett, 1997).

Aromatase inhibitors (AIs) are a therapeutic strategy in ER+ breast cancers for completely blocking estrogen signaling by inhibiting the enzyme aromatase that converts androgens to estrogens (Brodie et al, 2009). Current evidence suggests that AIs offer added benefits over Tam in postmenopausal women, especially in tumors expressing ER, SRC3 and HER2, in which Tam has been shown to act as an agonist and stimulates tumor growth (Strasser-Weippl et al, 2005). AI therapy also adds benefit in terms of improving disease free survival, and providing a different tolerability profile to long term Tam therapy (Johnston, 2010). Third generation AIs, including letrozole and anastrozole, appear most effective, even better than Tam, in the treatment of advanced disease. For postmenopausal women with ER+ metastatic breast cancer, AIs have become standard first-line therapy on the basis of randomized trials confirming enhanced efficacy over Tam (Smith et al, 2003). Although AIs are useful in adjuvant therapy and can reduce the risk of relapse, the long-term effects of estrogen suppression in post-menopausal women are unknown and careful monitoring for bone demineralization is essential (Smith et al, 2003).

Many human breast cancer cell lines have provided a renewable resource and are readily and commonly used as model systems for studying breast cancer cell biology. The MCF-7 breast

cancer cell line has become the leading model system for the study of estrogen receptor positive/hormone-dependent/antiestrogen sensitive breast cancer while MDA-MB-231 is an ER-negative/hormone-independent cell line (Shadeo et al, 2006). In addition, Clarke and colleagues have also generated the LCC series (MCF-7 derivatives) of breast cancer cell lines that model disease progression from an E2- and antiestrogen-sensitive phenotype to an E2-indepenent variant (MCF-7/LCC1) (Clarke et al, 1989; Brunner et al, 1993), to an E2-independent, antiestrogen-resistant phenotype (LCC9 cells) (Brunner et al, 1997). Ultimately, the intrinsic genetic differences between these cell lines will influence their biologic and pharmacologic response as an experimental model.

Although endocrine therapy has led to a significant improvement in outcomes for women with ER+ breast cancer, the efficacy of endocrine treatments has been impeded by the progression to a hormone-independent and metastatic phenotype. Despite the development of antiestrogens providing us with new therapeutic approaches for the management of breast cancer, they have also precipitated the phenomenon of antiestrogen resistance, which exists for all forms of endocrine therapy. Currently, substantial research is ongoing into combining targeted therapeutics with endocrine therapy to enhance responsiveness and delay resistance.

(iii) Antiestrogen Resistance

Despite the benefits of ER-targeted therapies in hormone-dependent breast cancer, many tumors will exhibit resistance to endocrine therapy presenting major limitations to controlling and managing the progression and metastasis of the disease (Clarke et al, 2001). Resistance can be classified as either intrinsic (de novo) or acquired resistance. Intrinsic resistance occurs *de novo* at the initial exposure to endocrine treatment whereas acquired resistance develops over time following an initial response to endocrine therapy (Moy et al, 2006; Massarweh et al, 2006; Musgrove et al, 2009). Clinically, the status of expression of the ER and progesterone receptor (PR) is the best predictors of response to endocrine therapy and is used to guide treatment decisions

(Bardon et al, 2003). 25% of ER+/PR+ tumors, 66% of ER+/PR- tumors and 55% of ER-/PR+ tumors initially fail to respond to Tam for reasons that are currently unknown (Nicholson et al, 1994; Clarke et al, 2003; Osborne et al, 2003). Several mechanisms have been suggested to play a role in this observed de novo resistance. First, the presence of ERβ has been implicated in Tam resistance as it not only exerts different transcriptional activity than ERα but can activate genes involved in proliferation and motility at AP-1 elements when bound to antiestrogens (Speirs et al, 1999; Johnston et al, 1999). For example, more than 30% of patients with early ER+ breast cancer relapse within 15 years after adjuvant therapy with Tam, and 17% of patients treated with an AI relapse within 9 years (EBRTCG, 2005). Second, the relative ratio of coactivator and corepressor proteins can also influence the response of the ER to Tam. For example, overexpression of coactivator SRC-1 amplifies the ER agonist response to Tam (Smith et al, 1997) while reduced expression of corepressor NCoR is linked to the manifestation of Tam resistance in breast cancer xenografts (Lavinsky et al, 1998).The absence of both ER and PR expression is the most common mechanism behind intrinsic resistance to Tam, but more recently the metabolism of Tam has also been implicated in *de novo* resistance (Clarke et al, 2003). In Tam-treated patients it has been reported that women carrying certain cytochrome P450 2D6 (CYP2D6) genotypes, specifically inactive alleles, fail to convert Tam to its active metabolite, endoxifen, and as a consequence, are less responsive to Tam and/or have higher risk of disease relapse (Rae et al, 2005).

In regards to acquired resistance, several mechanisms have been proposed based on clinical observations. Contrary to popular belief, mutations in ERα and/or loss of ER expression does not appear to be the primary mechanism driving acquired resistance and occurs only in less than 1% (Roodi et al, 1995) and ~20% respectively, in tumors of patients treated with endocrine therapy (Hull et al, 1983). Alternatively, crosstalk between receptor tyrosine kinases (RTKs) or growth factor pathways and ER signaling pathways are gaining a lot of attention for their central role in promoting

acquired resistance (Shou et al, 2004). The RTKs are responsible for persistent ER activity by activation of mitogen activated protein kinases (MAPK) and Akt, with subsequent phosphorylation and activation of the ERs. More importantly, these growth factor pathways become amplified in breast cancer cells and offer cells alternative proliferation and survival stimuli in the presence of effective inhibition of the ER signaling pathway (Kahlert et al, 2000; Simoncini et al, 2000; Song et al, 2002, Levin, 2003). For example, the epidermal growth factor receptor (EGFR) and HER-2 pathways become selectively up-regulated in breast cancer cells that acquire resistance to Tam during prolonged exposure (Moy et al, 2006). Hyperactivation of pathways through overexpression of the HER tyrosine receptor family and receptors for insulin/insulin growth factor 1 (IGF1), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF), can increase nonnuclear ER localization, enhancing cross-talk between the ER and growth factor pathways thereby contributing to resistance (Kern et al, 1994; Arpino et al, 2008; Chakraborty et al, 2010; Osborne et al, 2011).

Recently in the clinic it has been shown that the overexpression and hyperphosphorylation of co-activators, such as SRC3, leads to constitutive ER-activated transcription and may lead to patients showing reduced responsiveness to Tam (Osborne et al, 2003). In addition, increased transcriptional activity of transcription factors such as AP-1 and nuclear factor kappa B (NF-kB) are also associated with endocrine resistance (Zhou et al, 2007).

While considerable progress has been made to target E2 and the ERs in breast cancer, it is clear that endocrine resistance continues to be a major problem in the clinical management of the disease. Although the precise mechanisms of resistance remain to be established, there is an urgent need for developing new antiestrogen-based therapies to treat therapeutic resistant diseases, particularly those with reduced risk of increasing the incidence of endometrial carcinomas that may also be used for long-term HRT (Nehra et al, 2010).

Studying the molecular mechanisms underlying the development of endocrine resistance

has been greatly facilitated by the generation of resistant variants, most of which have been derived from the MCF-7 human breast cancer cell line. Table 1.1 describes antiestrogen-resistant cell lines, most of which retain ER expression and exhibit various patterns of resistance. Since we make use of the LCC variants in this study, I will briefly describe how this series was established. The LCC series started with a variant of MCF-7 cells called MCF7/MIII that was selected for growth in the absence of estrogen *in vivo* in ovariectomized nude mice (Clarke et al, 1989). MIII cells still express the ER, retain sensitivity to the growth inhibitory effects of antiestrogens, and will form tumors in mice (Clarke et al, 1989). The MCF-7/LCC1 variant, which still retains ER expression and is estrogen independent, was generated by passaging MCF-7 cells in ovariectomized nude mice (Clarke et al, 1989; Brunner et al, 1993). Subsequently, The MCF7/LCC1 cells were selected for resistance to the SERM Tam to produce a variant called MCF7/LCC2 that still retains sensitivity to the SERD Fulvestrant (Brunner et al, 1993). Finally, stepwise selections *in vitro* with Fulvestrant against MCF7/LCC1 cells were acheieved in order to obtain a stable Fulvestrant-resistant variant that was designated MCF7/LCC9 (Brunner et al, 1997). These cells retain expression of the ER at a level comparable to that of their parental MCF7/LCC1 cells. Therefore, the LCC1 variant represents an experimental model of progression from an estrogen-dependent to an estrogen-independent phenotype while the LCC9 cells provide a model of progression from an endocrine sensitive to an endocrine resistant, estrogen-independent phenotype.

The Cell Cycle

The cell cycle is an ordered series of events culminating into two major processes: DNA replication and the division of replicated chromosomes into two daughter cells each containing identical complete genomes (Schafer, 1998; Vermeulen et al, 2003). The stages of the cell cycle are commonly referred to as the G1, S, G2 and M phases (Figure 1.4). The cell cycle can be divided into separate chronological phases; interphase, the interlude between two M phases and encompasses

Parental	Variant	ER/PR[a]	Phenotype
MCF-7	LY2	+/-	E2-independent; TAM and ICI 164,384 cross-resistant
MCF-7	R27	+/?	TAM-resistant
MCF-7	RR	+/?	E2-independent; TAM-resistant
MCF-7	MCF-7/LCC1	+/+	E2-independent; Antiestrogen-responsive
MCF-7/LCC1	MCF-7/LCC2	+/+	E2-independent; TAM-resistant; ICI 182,780-responsive
MCF-7/LCC1	MCF-7/LCC9	+/+	E2-independent; TAM and ICI 182,780 cross-resistant
MCF-7	MCF-WES	+/+	E2-independent ;TAM-stimulated; ICI 182,780-resistant
ZR-75	ZR-75/LCC3	-/-	E2-independent; TAM and ICI 182,780 cross-resistant
ZR-75	ZR-75-9a1	-/-	E2-independent; TAM and ICI 182,780 cross-resistant
T47D	T47Dco	-/+	E2-independent; TAM and ICI 182,780 cross-resistant

Table 1.1. Representative antiestrogen-resistant human breast cancer variants derived from ER+/PR+ parental cells.
a indicates ER/PR expression in variant cell lines
? Indicates information is unknown or unclear
Adapted from Clarke et al. (2001). Pharmacol Rev. *53*, 25-71.

G1, S and G2, and mitosis (M), the process of nuclear division that includes prophase, metaphase, anaphase and telophase (Vermuelen et al, 2003).

The G1 and G2 phases represent the "gaps," or intermediate phases in the cell cycle that occur between the two major alternating processes, DNA synthesis and mitosis. In the G1 phase, the cell grows and prepares for chromosome replication (Schafer, 1998). During S phase, the cell synthesizes DNA and duplicates its centrosomes (Frouin et al, 2002). In G2, the cell prepares for mitosis. G1 and G2 phases serve as checkpoints for the cell ensuring that it is prepared to transition to the next phase (Schafer, 1998). Sometimes, cells in G1 phase can enter a resting state called, G_o in which they enter a quiescent state instead of actively cycling and committing to replicate DNA.

(i) Cell Cycle Regulation

Cell cycle control is critical for preventing constant cell division as well as in regulating the transition from one phase to the next at appropriate times. There are two primary mechanisms that are recognized to control cell cycle progress. The first mechanism involves a cascade of protein phosphorylations that relay a cell from one stage to the next (Vermeulen et al, 2003). Key players in this process make up a highly regulated family of serine/threonine protein and kinases called cyclin-dependent kinases (Cdks), each of which are activated at specific points in the cell cycle (McDonald et al, 2000; Malumbres et al, 2009). As their name suggests, Cdks require association with a second subunit called cyclins to be biologically activated. Cyclins bind to Cdks, creating an active complex with unique substrate specificity (Malumbres, 2007). For example, the transition from G1 into S phase in mammalian cells is regulated by at least two Cdks cyclin E/Cdk2 cyclin A/cdk2. Cyclin E/Cdk2 activity peaks in late G1, while cyclin A/cdk2 activity appears later, at the onset of DNA synthesis (Figure 1.4) (Pagano et al, 1992; McDonald et al, 2000). The synthesis and degradation of cyclins are important for cell cycle progression. During cell division, the cyclins are degraded by ubiquitin-mediated processes (Glotzer et al, 1991).The decision of a cell to exit one phase and

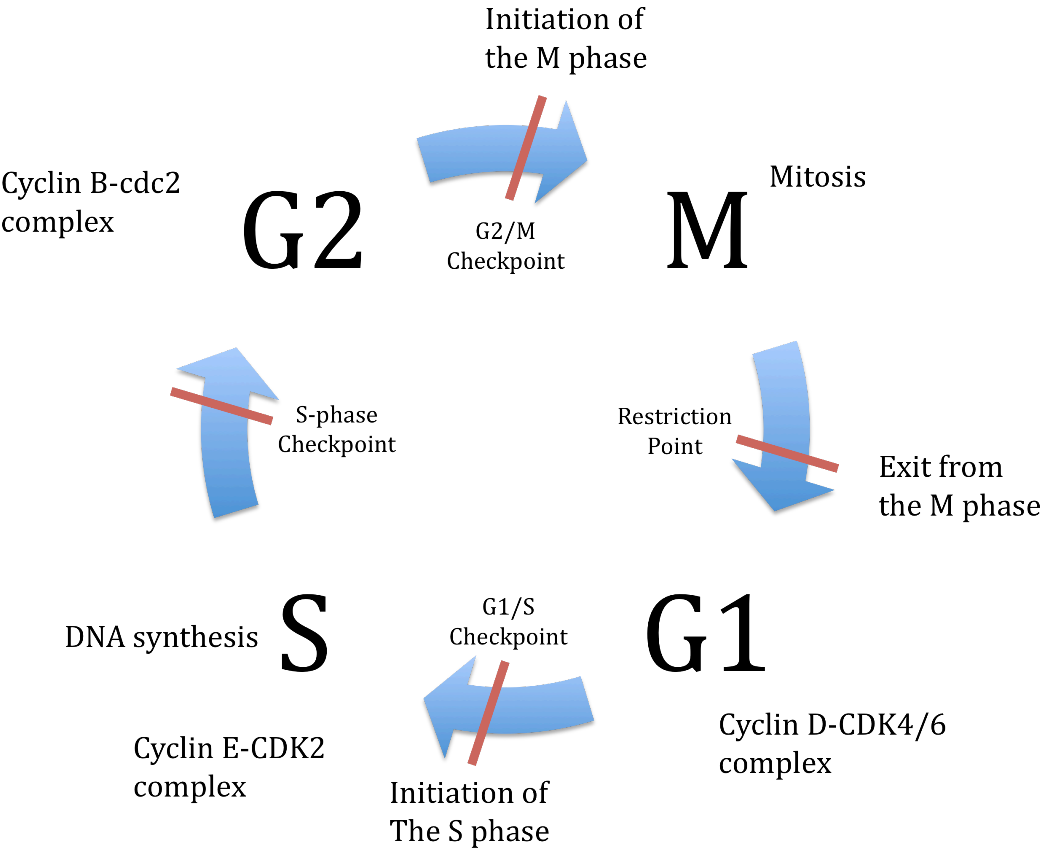


Figure 1.4. The cell cycle. Cell cycle progression is governed by cyclin-dependent kinases (cdks), the activities of which are regulated by binding of cyclins, by phosphorylation and by the cdk inhibitors. Adapted from Kong et al. (2003). Drugs Fut. *28*, 881-90.

transition into the next is in part the result of the balance between cyclin production and proteolytic degradation in the proteosome through ubiquitination (Glotzer et al, 1991).The active cyclin-Cdk complex serves as a signal to the cell to progress to the next cell cycle phase (Schafer, 1998; Malumbres, 2007). Eventually, the cyclin gets broken down, deactivating the Cdk, thus inhibiting the processes in a particular phase and signaling its completion.

Excessive genetic damage can occur if cells transition to the next phase of the cell cycle before it is safe to do so. Thus, the second mechanism of cell cycle regulation involves a set of checkpoints that arrests the progression through the cell cycle critical events or processes are successfully completed (Vermeulen et al, 2003; Bartek et al, 2004). Cell cycle checkpoints are responsible for detecting and responding to flaws in the accomplishments of critical events, such as DNA replication and chromosome segregation (Malumbres et al, 2009). When a checkpoint properly identifies damage, for example DNA strand breaks, signals will be sent to cell cycle progression machinery to delay transition through the cell cycle in order to provide time for repair and to induce the transcription of genes that facilitate repair until the danger posed by the mutation has been successfully averted. The tumor suppressor gene p53 is activated by DNA damage, arrests the cell cycle in G1 phase, and initiates DNA repair, making it a critical step against cancer formation (Hollstein, 1991). If the p53 gene is mutated or lost, the DNA-damaged cells will continue to proliferate. Cyclin dependent kinase inhibitors (CDKIs) are critical regulators of cell cycle progression. p21 and p27 in particular are negative regulators of cyclin/cdk activity and arrest cell cycle progression in response to a variety of stimuli (Hartwell et al, 1994). Mainly induced by p53, p21 mediates p53-induced G1 cell cycle arrest resulting from DNA damage (Koljonen et al, 2006). Similar to p21, p27 regulates cell cycle progression by inhibiting cyclin/cdk activity, however p27 is regulated by extracellular stimuli like transformed growth factor β (TGF-β) (Koljonen et al, 2006). There are additional checkpoints positioned before and after DNA replication called the G1/S and

G2/M checkpoint respectively (Schafer, 1998). Checkpoint failures produce a situation where the cell continues to divide despite damage to integrity resulting in genomic instability that has been implicated in the evolution of normal cells into cancer cells (Elledge, 1996).

(ii) The link between the cell cycle and cancer

Under normal circumstances, the balance between proliferation and programmed cell death, maintained by tight regulation of both processes, ensures the sustained integrity of the cells (Hartwell et al, 1994). When an imbalance between cell growth and cell division occurs, these orderly processes are disrupted and can lead to cancer (Collins et al, 1997).

Cancer is caused by the genetic imbalance between the proliferation and suppression mechanisms of normal cells resulting in their uncontrolled growth (Collins et al, 1997). Various types of DNA damage, when unrepaired, can cause mutations in genes involved in the mechanisms that control proliferation and cell death. These permanent mutations induce the activation of oncogenes that stimulate proliferation, as well as the inactivation of tumor suppressor genes that would normally inhibit inappropriate cell growth (Shafer, 1998; Hartwell et al, 1994). Thus, defects in the cell cycle regulatory system as well as mutations in the genes involved in the cell cycle checkpoint pathways and repair mechanisms, such as p53, p21 and p27, results in chromosomal instability, mutations to crucial genes in the cell cycle regulation, which can ultimately lead to cancer (Hanahan et al, 2000; Koljonen et al, 2006).

Autophagy

(i) Autophagy is a normal process

Autophagy is a homeostatic process that is primarily induced by nutrient limitation (Berardi et al, 2011; Rikiishi, 2012). It is an adaptive response as it allows cells to survive a variety of extracellular and intracellular stresses including nutrient deprivation, hormonal or therapeutic treatment, pathogen invasion, accumulation of damaged cellular organelles, and aggregated or

misfolded proteins (Periyasamy-Thandavan et al, 2009; Dalby et al, 2010; Wu et al, 2012). By self-cannibalizing and reusing the breakdown products of cellular components, the cell is able to survive periods of scarce nutrients. Autophagy is a tightly regulated process; it must be induced when necessary, but otherwise maintained at a basal level so as to maintain homeostasis in cellular differentiation, tissue remodeling, and growth control (Tanida et al, 2004; Meijer et al, 2004; Edinger et al, 2004; Lockshin et al, 2004; Debnath et al, 2005; Mizushima et al, 2008).

(ii) Types of autophagy

Overall, autophagy is a catabolic process that acts on intracellular components including lipids, proteins, polysaccharides, and organelles sequestered to lysosomes (Mizushima 2007; Fujishima et al, 2011). With the help of various digestive enzymes, lysosomes decompose these intracellular molecules and recycle their components (Mizushima, 2007). Several types of autophagy exist, and of them the three major types include microautophagy, chaperone-mediated autophagy, and macroautophagy (Mizushima, 2007; Periyasamy-Thandavan et al, 2009). For the purpose of this thesis I will only focus on describing macroautophagy and will hereafter refer to it as autophagy for the sake of simplicity. Macroautophagy is a large-scale catabolic process conserved across eukaryotes, and can be selective in targeting specific cargos such as organelles or invasive microbes or non-selective when engulfing a bulk of cytoplasm (Mizushima et al 2008; Chen et al, 2010; Mizushima et al, 2010). In macroautophagy, components of the cytosol and/or organelles are sealed in double-membraned autophagosomes, the intermediate vesicle for transport to lysosomes (Klionsky, 2007; Todde et al, 2009). When autophagosomes fuse with lysosomes, a new structure called the autolysosome is formed. In these autolysosomes, molecules will be degraded and eventually recycled (Yoshimori, 2004; Shitani et al, 2004).

(iii) Autophagy and Cancer

Autophagy is implicated in a number of diseases, including cancer. However, the role of autophagy in carcinogenesis is context dependent. Commonly referred to as type II cell death, autophagy has recently emerged as a novel mechanism through which cell death can be induced in carcinogenesis and is gaining attention as a potential drug target (Karantza-Wadsworth et al, 2007; Kim et al, 2007). Its connection to cancer is complex because autophagy can either promote cancer cell survival or it can inhibit the initiation of tumor growth (Karantza-Wadsworth et al, 2007; Levin, 2007; Dalby et al, 2010; Rikiishi 2012). Although there is much debate over autophagy's primary role in cancer, there is more of a consensus supporting autophagy as a promoter of cancer cell surivival, sustaining growth following chemotherapy or radiation (Berardi et al, 2011; Apel et al, 2008).

One way autophagy can suppress the growth of tumors is by inhibiting inflammation and promoting genomic stability (Dalby et al, 2010). The essential autophagy regulator beclin 1 is monoallelically deleted in 40-75% of cases of human breast, ovarian and prostate cancers (Liang et al, 1999; Aita et al, 1999). Likewise, disruption of beclin 1 in animal models decreases the capacity of autophagy, increases cellular proliferation and accelerates the growth of spontaneous tumors including lymphomas, lung carcinomas, hepatocellular carcinomas and mammary precancerous lesions (Qu et al, 2003; Yue et al, 2003; Dalby et al, 2010).

However, in the face of cellular stress, autophagy serves a protective function. Thus, in cancer cells, the autophatic pathway is becoming more frequently manipulated to enhance the response of tumor cells to the toxicity of cancer therapy (Mizushima et al, 2008; Rouschop et al, 2009). To this end, studies have shown that blocking the autophagic pathway in cancer cells may offer some advantage in certain contexts, as blocking autophagy can improve cancer cell sensitivity to different therapies, including DNA-damaging agents, anti-hormone therapies such as Tam, and radiation therapy (Kondo et al, 2005; Djavaheri-Mergny et al, 2007). Nevertheless, in certain circumstances, autophagy mediates the therapeutic effects of some anticancer agents. Thus, the

relation of autophagy to cancer development is complex, and depends on the genetic composition of the tumor cell and to which type of stress that cell is exposed. Taken together, modulation of autophagy is a very attractive novel approach for enhancing the efficacy of existing cancer therapy.

(iv) Autophagic Machinery

Originally identified in yeast, the autophagy-related (Atg) genes make up the core molecular machinery of autophagy (Xie et al, 2007; Periyasamy-Thandavan et al, 2009). The ATG proteins form distinct multi-subunit complexes, controlling different steps of autophagosome formation. The four characteristic stages of the autophagic pathway include: induction, autophagosome formation, autophagosome fusion and finally autophagosome breakdown (Figure 1.5) (Reggiori et al, 2002; Mizushima, 2007; Periyasamy-Thandavan et al, 2009). Although there are several external and internal stimuli to induce autophagy, the most typical trigger is nutrient starvation (Scott et al, 2004; Lum et al, 2005). Once induced, a master regulator of nutrient signaling and a negative regulator of autophagy, mTOR (mammalian target of rapamycin) is inhibited (Noda et al, 1998; Beck et al, 1999; Meijer et al, 2004). The inactivation of mTOR then leads to downstream dephosphorylation events activating the transcription of autophagy-related genes (Atgs) (Abeliovich et al, 2003) (Figure 1.5). In the first step of autophagosome formation, cytoplasmic constituents are sequestered by the elongating phagophore, a unique double membraned vesicle resulting in the formation of the autophagosome (Reggiori et al, 2005; Peryasamy-Thandavan et al, 2009) (Figure 1.5). Complexes of 18 Atg proteins coordinate this process (Klionsky et al, 2003; Suzuki et al, 2007). In addition, there are two key ubiquitin-like conjugation systems that function in this stage. Secondly, a lipid molecule called phosphatidylethanolamine (PE) gets conjugated to the carboxyl-terminal of the Atg8/LC3 (LC3) protein (Mizushima, 2007; Peryasamy-Thandavan et al, 2009). Initially, LC3 is cleaved by a cysteine protease, Atg4, forming the activated LC3-I (Ichimura et al, 2000). LC3-I is subsequently conjugated to PE giving rise to LC3-II, a lipidated form that gets recruited to autophagic membranes

(Peryasamy-Thandavan et al, 2009). Importantly, the relocation of LC3 from the cytosol to autophagic vesicles and the accumulation of LC3-II is a common marker for autophagic flux (Mizushima et al, 2007). The second conjugation event involves the covalent attachment of the carboxyl-terminal glycine of Atg12 to Atg5 (Mizushima et al, 2003; Peryasamy-Thandavan et al, 2009). This Atg5-Atg12 complex then forms polymers required for targeting and recruiting LC3 to the autophagosome (Peryasamy-Thandavan et al 2009). Next, the autophagosome fuses with the lysosome (Figure 1.5) releasing the sequestered cytoplasmic material for degradation by lysosomal proteases (Figure 1.5) (Reggiori et al, 2002; Mizushima, 2007). Finally, following the fusion stage, the autophagosome membrane is broken down by lysosomal proteases in which lysosomal associated-membrane protein-2 (Lamp-2) plays a key role (Reggiori et al, 2002; Yorimitsu et al, 2005). Lamp-2 is essential for the maturation of autophagosomes, while a deficiency in Lamp-2 results in the accumulation of autophagosomes (Jäger et al, 2004; Eskelinen et al, 2009).

(v) The role of the Bcl-2 protein in autophagy

A complex relationship between autophagy and cell death exists (Levine et al, 2008; Djavaheri-Mergny et al, 2010). Currently, the well-known antiapoptotic factor Bcl-2 is a key player in this context. As a cytoplasmic protein, Bcl-2 is overexpressed in 50-70% of breast cancers and as a consequence can lead to resistance to chemotherapy, radiation and hormone-induced apoptosis (Akar et al, 2008). The pool of mitochondrial Bcl-2 proteins is shown to only exert antiapoptotic functions (Pattingre et al, 2005; Levine et al, 2008, Hailey et al, 2010) against a wide range of inducers of apoptosis. Specifically, Bcl-2 neutralizes proapoptotic Bcl-2 family members, including Bid and Bax, etc., to prevent mitochondrial membrane permeabilization, caspase activation, and consequent cell death (Adams et al, 2007). In addition, Bcl-2 protein located at the endoplasmic reticulum (ER) negatively regulates the Beclin 1-dependent autophagy pathway by binding to and inhibiting Beclin 1 (BECN1) (Chang et al 2010; Strappazzon et al, 2011). Evidence shows that since

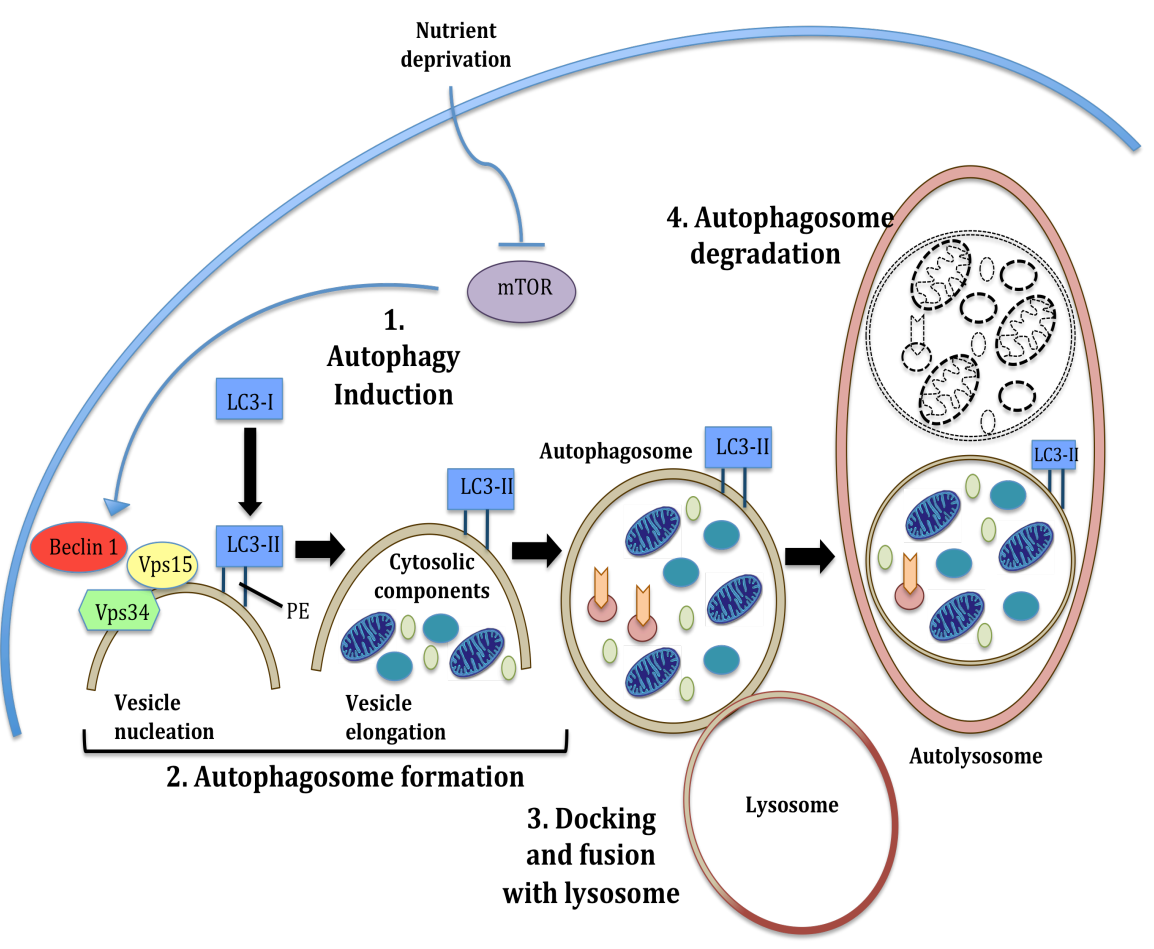


Figure 1.5. Schematic representation of the four stages in the autophagic pathway. 1) The main trigger for autophagy induction is nutrient deprivation. This stimuli inhibit mTOR, a negative regulator of autophagy followed by 2) the formation of the autophagosome which is an isolation membrane formed by vesicle nucleation and elongation. Once induced the autophagic machinery including Beclin 1, Vsp15 and Vsp34 is recruitmed to the isolation membrane. PE is conjugated to cytosolic LC3-I and converts it into lipidated LC3-II. LC3-II localizes to both the outside and the inside membranes of autophagosomes and therefore is often used as a marker. 3)The autophagosome, with the cytoplasmic material to be degraded fuses with the lysosome to form autolysosomes to facilitate degradation of the cytoplasmic components (4).Induction of autophagy, 2. Autophagosome formation, 3. Docking and fusion with the lysosome, and 4. Autophagosome degradation. Adapted from Lee et al. (2008). BMB Rep. *41*, 827-32.

Bcl-2 plays a central role in cell survival, silencing Bcl-2 in breast cancer cells that overexpress the protein, with specific siRNA, can cause autophagic cell death and may offer therapeutic benefits used alone or in combination with chemotherapy (Akar et al, 2008). However, because autophagy can be exploited as a survival pathway by cancer cells, downregulation of Bcl-2 as a means to induce autophagy may also work in favor of the cancer cells (Crawford et al, 2010). Therefore, another approach involving inhibition of protective auophagy with chloroquine (CQ), a lysosomotropic drug, for example, may break the resistance mechanism and lead to cell death (Dalby et al, 2010). Thus, the inhibition of autophagy may enhance efficacy of anticancer therapies (Abedin et al, 2007; Apel et al, 2008; Qadir et al, 2008).

(vi) The Role of autophagy in breast cancer treatment

Recently, there has been a tight connection between antiestrogens and autophagy. Studies show that endocrine therapy affects the autophagic pathway by modifying the number autophagosomes formed and by enhancing LC3 protein cleavage leading to an accumulation of LC3-II (Schoenlein et al, 2009; Crawford et al, 2010). It is still under debate whether apoptosis or autophagy is the primary mechanism through which breast cancer cells die or whether it is context and cell-dependent. A key study by Bursch et al (1996) demonstrated that, at high concentrations, antiestrogens such as Tam and Fulvestrant induced apoptosis, while at low concentrations (10^{-6}M) they induced autophagy, which could be rescued by E2 (Bursch et al, 1996). This observation stirred curiosity about whether autophagy as a survival mechanism can lead to drug resistance. Consistent with this finding, a recent study demonstrated that inhibition of autophagy of a Tam-resistant cell line did in fact resensitize these cells to Tam's antiestrogenic effects, implicating autophagy in antiestrogen-resistance (Quadir et al, 2008).

Bcl-2 protein

(i) Bcl-2, ER signaling and Antiestrogens

In normal mammary cells and breast cancer cells, ER signaling inhibits apoptosis through positively regulating Bcl-2 gene expression (Somai et al, 2003). *In vitro* studies in breast cancer cells have found that the altered expression and activity of Bcl-2 family members prevents cell death (Reed, 1994; Thompson, 1995). Specifically, E2 has been found to inhibit apoptosis in MCF-7 cells through the induction of Bcl-2 expression (Perillo et al, 2000). Previous studies have demonstrated that antiapoptotic Bcl-2 and proapoptotic Bax are associated with apoptosis in breast carcinoma, and the balance between Bax and Bcl-2 greatly influences a cells susceptibility to apoptosis (Heermeier et al, 1996; Jager et al, 1997; Metcalfe et al, 1999).

Research has shown that in antiestrogen-sensitive MCF-7 breast cancer cells, Tam induces time- and concentration-dependent down-regulation of Bcl-2 at both the mRNA and protein level which correlated with TAM-induced mitochondrial-dependent apoptosis (Zhang et al, 1999).

Although the deregulation of antiapoptotic Bcl-2 family members has been implicated in the progression of many different diseases (Adams et al, 1998), research concerning the role of anti-apoptotic Bcl-2 proteins in endocrine resistant breast cancers has received considerable attention. Studies have shown that initial expression of Bcl-2 correlates with ER expression, responsiveness to hormonal adjuvant therapy, and ultimately a good prognosis (Gee et al, 1994). Also, *in vitro* studies in MCF-7 cells have shown that overexpression of HER-2 increases antiapoptotic Bcl-2 and Bcl-xL proteins, leading to suppression of TAM-induced apoptosis and ultimately Tam-resistance (Kumar et al, 1996).

(ii) Bcl-2 Family Structure

There are 25 anti-apoptotic and pro-apoptotic members of the Bcl-2 family of proteins. These proteins are localized to mitochondria, smooth endoplasmic reticulum, and perinuclear membranes in hematopoietic cells (Kang et al, 2009). Bcl-2 proteins are distinguished by the presence of up to four distinct conserved sequence motifs called Bcl-2 homology (BH) domains (Reed, 2006)

designated BH1, BH2, BH3 and BH4 (Figure 1.6). All of the antiapoptotic family members, including Bcl-2, BclXL, Mcl-1, and Bcl-w, are homologous in all four BH domains. Meanwhile, the pro-apoptotic family members including Bax, Bak and Bok, display sequence homology between BH1-3 domains (Kroemer, 1997). A third subset of pro-apoptotic Bcl-2 family members exists and is collectively referred to as the BH3-only proteins. Some of these proteins include Bid, Bad, Bik, Bim, Puma and Noxa and share sequence homology only within an alpha helical segment in the BH3 domain which serves as a critical death domain (Figure 1.6) (Hanada et al, 1995; Danial, 2007).

The BH3 domain is critical for the pro-apoptotic and heterodimerization function of the Bcl-2 family of proteins and allows them to selectively bind to each other and regulate apoptosis (Danial, 2007). The antiapoptotic BH1, BH2, and BH3 domains bind proapoptotic family members by forming a hydrophobic cleft that can accommodate the hydrophobic face of the amphipathic α helical BH3 domain of a proapoptotic Bcl-2 protein (Kang et al, 2009). This interaction may be further stabilized by the presence of the BH4 domain within the antiapoptotic binding partner (Danial, 2007). Interestingly, mutational studies have also revealed that the BH4 domain is required for the function of antiapoptotic family members and may link Bcl-2 to other signaling pathways (Levine et al, 2008).

(iii) Bcl-2 and its role in regulating apoptosis

The family of apoptosis regulator proteins was founded by Bcl-2, which derives its name from *B-cell lymphoma 2* as it was initially described in chromosomal translocations in follicular lymphomas (Danial et al, 2007). This family of proteins represents one of the most biologically important classes of apoptosis-regulatory gene products (Kroemer, 1997). The relative ratio of proapoptotic and anti-apoptotic proteins greatly determines the cells sensitivity to apoptosis and will also be affected by the competitive heterodimerization of death-inducing and death-inhibitory members of the Bcl-2 family (Kroemer, 1997). Essentially, Bcl-2 family members protect the integrity

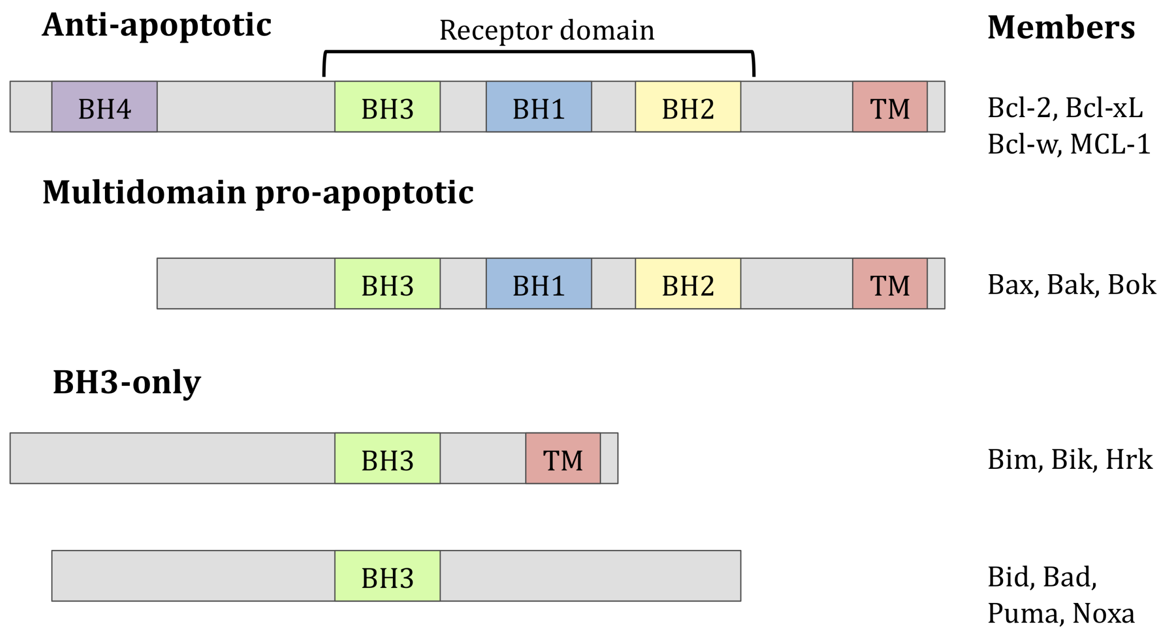


Figure 1.6. Classification of the members of the Bcl-2 family according to conserved domains. The organization of the four Bcl-2 homology (BH) domains are depicted among family members. Most members also have a carboxyl-terminal hydrophobic region that is thought to function as a transmembrane domain (TM) to facilitate association with intracellular membranes.
Adapted from Chan et al. (2004). Clin Exp Pharmacol Physiol. *31*,119-28.

of the mitochondria by preventing cytochrome c release and the subsequent activation of caspase-9 (Chan et al, 2004). However, once the proapoptotic proteins are activated, they translocate to the outer mitochondrial membrane, promote the release of cytochrome c from the mitochondria, and ultimately activate a caspase cascade that leads to increased mitochondrial mediated apoptotic cell death (Adams et al, 1998; Vander Heiden et al, 1999).

(iv) Therapeutic manipulation of the Bcl-2 pathway

Due to the prominent role that Bcl-2 family members play in regulating apoptosis it is no surprise that they are primary targets for therapies for several diseases characterized by dysregulated cell death (Kroemer, 1997). The first agent targeting Bcl-2 is a Bcl-2 antisense oligonucleotides called Oblimersen sodium, which has entered clinical trials (Kang et al, 2009). When Oblimersen is combined with standard chemotherapy in patients with chronic lymphocytic leukemia, it has shown chemosensitizing effects leading to improved survival (Rai et al, 2008). Other Bcl-2 antisense oligonucleotides are proving to be more effective than Trastuzumab (monoclonal antibody that interferes with the HER2/neu receptor) in modulating Tam sensitivity in HER-2 and Bcl-2-overexpressing breast cancer cell lines, ZR-75-1 and BT-474 cells (Kim et al, 2005). Small molecule inhibitors of Bcl-2 family members (ie. ABT-737, GX15-070) have also been developed and have entered clinical trials. They are designed to swap places with the BH3-only protein, thereby occupying the hydrophobic groove of anti-apoptotic Bcl-2 family members. “They can oligomerize Bax or Bak, which can subsequently depolarize the mitochondrial membrane potential thus releasing cytochrome c” (Kang et al, 2009). Thus, an understanding of the mechanisms of Bcl-2 action will provide clues for the development of cytoprotective drugs in addition to drugs that can exploit the Bcl-2 mode of action in order to induce apoptosis in tumor cells.

Statement of the problem

Despite the relative safety and success of antiestrogens, most initially responsive breast tumors acquire resistance, which continues to be a significant clinical problem in the treatment of ER+ breast cancers. Many initiatives have been undertaken to try and dissect the mechanisms that contribute to the development of endocrine resistance, in order to identify new therapeutic interventions for ER+ breast cancer. While ER+ breast cancers typically express a high ratio of ERα to ERβ, it has been suggested that the acquisition of SERM-resistance in vitro and in vivo is associated with increased relative expression of the ERβ (de Cremoux et al, 2003). The involvement of ERβ in antiestrogen resistance was first proposed by Speirs and colleagues (2000) who found high ERβ mRNA expression in tumors to be directly associated with resistance to Tam. Using quantitative real-time RT-PCR, De Cremoux's group (2003) investigated the expression of genes that could be expected to be involved in the development of antiestrogen resistance. They found that the antiestrogen resistant MCF-7/LCC9 cell line exhibited a 2.5-fold increase in ERβ mRNA and a 19-fold decrease in ERα transcript levels compared to sensitive MCF-7 control cells (de Cremoux et al, 2003). Although considerable progress has been made in elucidating the roles of both the ERα and the ERβ in breast cancer, there is an increasing amount of evidence associating the ERα with the proliferative actions of E2 while ERβ is linked to the anti-proliferative and anti-tumorigenic activity of this hormone. Targeting the ERβ in strategies toward developing a novel drug therapy for anti-estrogen resistant breast cancers requires that the ERβ agonist have low toxicity. Our group has developed a new ERβ –selective agonist called L17 that is based on the ABCD-ring structure of E2 but, unlike E2, L17 lacks the B ring and resists/retards the metabolism to mutagenic quinones (Wright et al, 2011). Based on the ability of ERβ expression to inhibit the growth of breast cancer cells we propose that ERβ agonists including our A-CD compound, L17, will inhibit the proliferation and reduce survival signaling in breast cancer cells that are resistant to selective estrogen receptor modulators and downregulators (SERMs/SERDs). Most studies in the literature test ERβ-selective

ligands in an artificial cellular environment by manipulating the ratio of the ER by various means. A unique feature in our studies is the use of a breast cancer cell line (LCC9) that endogenously overexpresses ERβ relative to ERα, which allowed us to test the efficacy of ERβ –specific agonists. Additionally, ERβ agonists will not induce proliferation in the normal breast epithelium. To explore ERβ function in antiestrogen resistant breast cancer cells we used our newly synthesized compound L17.

CHAPTER II - MATERIALS AND METHODS

Cell Culture

MCF-7 mammary epithelial cells and human embryo kidney 293 (HEK 293) cells were maintained in high glucose Dulbecco's modified eagle medium (DMEM) containing phenol red (Hyclone) and supplemented with 5% and 10% fetal bovine serum (FBS) (Hyclone) respectively, 1% non-essential amino acids (NEAA) (Invitrogen). Two endocrine-resistant MCF-7 cell line derivatives, MCF-7/LCC1 (partially estrogen-dependent) and MCF-7/LCC9 (fully estrogen insensitive and fulvestrant and tamoxifen resistant) (Clarke et al, 2001) were a gift from Dr. Robert Clarke (Georgetown University School of Medicine, Washington DC). They were routinely grown in phenol red free high glucose DMEM (Hyclone) supplemented with 5% stripped FBS (Hyclone), 1% NEAA and 1% sodium pyruvate. Serum was stripped of endogenous steroids by treatment with dextran-coated charcoal (DCC). Prior to experiments MCF-7 and HEK 293 cells were cultured for at least 5 days in phenol red free medium containing 5% or 10% stripped DCC, respectively, thereby reducing the degree to which exogenous estrogen is able to stimulate responses. For passaging, cells were washed twice with phosphate-buffered saline (PBS) (137 mM sodium chloride [NaCl], 2.7 mM potassium chloride [KCl], 10 mM disodium hydrogen orthophosphate [Na_2HPO_4], 1.76 mM potassium dihydrogen orthophosphate [KH_2PO_4], pH 7.0-7.4) prior to isolation. PBS was removed by aspiration and trypsin solution (0.25% trypsin, 1 mM ethylenediaminetetraacetic acid [EDTA]) (0.5% trypin-EDTA without phenol red was used on cells that were cultured in the phenol red free media) (Invitrogen) was added. Cells were incubated until cells detached, then resuspended in the trypsinizing solution, which was inactivated by addition of serum-containing media. Cells were then plated in complete medium at the desired density. To discern between apoptosis, necrosis, and autophagy, LCC9 cells were treated with chloroquine (33µM) in the presence or absence of 10 nM L17 or WAY, with or without the addition of 100 µM caspase inhibitor, Z-VAD-FMK. Following

incubation for 4 days, cells were collected and the rate of apoptosis was measured by flow cytometry as described below. Lastly, to determine cytotoxic effect of chemotherapeutic agent Adriamycin (Adr) in LCC9 cells, cells were treated with 200 μM Adr in the presence or absence of 10 nM L17 or WAY. After 72 h of incubation, cells were collected and cell viability was measured using trypan blue exclusion.

Preparation of dextran-coated charcoal (DCC) stripped serum

FBS was stripped of endogenous steroid hormones by treatment with DCC (0.25% Norit A, 0.0025% dextran in 0.01M Tris-HCl p.H. 8.0). The serum was incubated with the DCC for 45 min at 45°C after which point, the charcoal, along with the steroid hormones, was removed by centrifugation at 3000 rpm for 10 min at 4°C.

Compounds

17β-estradiol (E2), 4-hydroxytamoxifen (4OHT; the active metabolite of TAM), WAY-200070, and choloroquine were purchased from Sigma Chemical Co. Doxorubicin/ Adriamycin (Adr) was given to us by the Chemotherapy Pharmacy at the Ottawa Hospital Cancer Center (General Campus, Ottawa, ON). Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone), general caspase inhibitor was purchased from BD Biosciences Pharmingen. Ligand 17 (L17) was designed by Dr. Jim Wright and synthesized by Dr. Tony Durst of Carleton University and the University of Ottawa respectively, as previously described (Wright et al., 2011). All of the compounds were dissolved in 100% ethanol except choloroquine, which was dissolved in water.

Antibodies

The following primary antibodies were used for western blot analysis and ChIP: anti-ERα (HC-20) (Santa Cruz); anti-ERβ (Thermo-Scientific); anti-ERβ (Genetex); anti-actin (A-2066) (Sigma); anti-cyclin D1 (A-12) (Santa Cruz), anti-LC3 (Novus Biologicals), anti-cyclin E (Abcam ab7959), anti-

Bcl-2 (BD Biosciences) and ChromPure Rabbit IgG, whole molecule (Jackson Research Laboratories Inc. 011-000-003).

The following secondary antibodies were used for western blot analysis: peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson Research Laboratories Inc.); peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson Research Laboratories Inc.).

Transient Transfections and Luciferase Reporter Assays

Since HEK-293 cells are devoid of the ER they were used for the transient transfections. HEK-293 cells were plated into 6-well plates at 2 mL/well at a cell density of 1.2 $x10^5$ cells per well. Cells were immediately transfected with polyethyleneimine (PEI) (Polysciences Inc, PA), serum-free DMEM (Hyclone), and optimal amounts of the ERα or ERβ expression vector (pRS plasmid with T7 promoter) (0.75 μg each), 0.75 μg of estrogen response element (ERE)-tk-luciferase reporter plasmid, together with 0.5 μg of (pRL-TK vector (Promega) containing luciferase/ pRL-TK plasmid encoding *Renilla* luciferase (0.5μg). The cells were incubated for 24 h at 37°C, after which period media was changed and ligand was added. E2 was used as control and EtOH as vehicle (blank). The transfected HEK293 cells were treated with 10 nM of each ligand. 48 h post treatment cells were washed twice with PBS, and whole cell lysates were collected. Firefly and *Renilla* luciferase activities were measured consecutively using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's protocol. Light intensity was measured using a Lumat LB9507 (Berthold Technology) luminometer. Luciferase activity was divided by the *Renilla* activity (control reporter) to normalize for transfection efficiency. Each ligand dose was performed in triplicate and repeated three times.

Enumeration of viable cells

Media, including dead floating cells, was collected. Subsequently, cells were washed with 1x PBS and isolated by trypsinization and centrifugation. 100 μL of cell suspension was then stained with 10 μL 0.04% trypan blue (GIBCO) and viable cells enumerated using a haemocytometer as

determined by their ability to exclude trypan blue. For some experiments chloroquine was added at a concentration of 33µM.

Treatment was performed in triplicate and experiments were repeated 3 times.

Flow Cytometry

LCC9 and MCF-7 cells were treated for 72h or 120h with ethanol (vehicle) or 10 nM E2, TAM (1µM), L17, or WAY. Chloroquine was used at a final concentration of 33 µM. Both adherent and floating cells were harvested and fixed with 70% ethanol in -20°C for 24h. Cells were washed once with PBS and then resuspended in PBS-EDTA, treated with RNAse for 20 min then stained with propidium iodide. DNA content was analyzed using a Coulter Epics XL instrument from Beckman Coulter equipped with Expo 32 Acquisition software. Data was analyzed using the ModFit LT 3.2 program. All experiments were performed in triplicate, repeated at least 3 times, and yielded similar results.

SDS-polyacrylamide gel-electrophoresis and immunoblotting

Proteins were extracted from cells collected from 60mm plates. Cells were washed twice with PBS and scraped in 50 µL radioimmunoprecipitation assay (RIPA) buffer (1% Nonidet P-40 [NP-40], 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS] in PBS plus Complete Protease Inhibitor Cocktail tablet [Roche Applied Sciences] and PhosSTOP Phosphatase Inhibitor Cocktail tablet [Roche Applied Sciences]). The cell solution was incubated on ice for a minimum of 15 minutes, vortexed at maximum speed to rupture cells and centrifuged for 15 minutes at maximum speed at 4 °C to remove insoluble material. Resulting supernatants were stored at -20 °C. The Biorad D_c Protein Assay kit (Biorad) was then used to determine lysate protein concentrations according to the manufacturer's protocol. Following quantification, equal amounts of each sample (typically 10-20 µg of protein) were denatured in sample buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreiotol [DTT], 2% SDS, 0.1% bromophenol blue, 10% glycerol) at 100 °C for 5 minutes. Proteins were resolved on a

10-15% SDS polyacrylamide gel by electrophoresis (SDS-PAGE) (327 Laemmli,U.K. 1970). Gels were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) at 80-100 V for 2 hours or at 20 V overnight at 4°C. 5% non-fat skim milk powder dissolved in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 137 mM NaCl) containing 0.1% Tween-20 (TBS-T) was used to block the membrane for approximately 1 hr at room temperature. The membrane was next probed with the appropriate primary antibody diluted in blocking solution plus 0.02% sodium azide for 1 hour at room temperature or overnight at 4°C. The blots were washed twice in TBS-T for 10 minutes then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibody, diluted 1:10 000 in blocking solution for 1 hour at room temperature. This was followed by 4 further washes in TBS-T for 10 minutes each, after which the bands were detected using the Immobilon Western Chemiluminescent HRP Substrate (Millipore) and exposure to film. Bands were quantitated by densitometry using Image J v1.43 and densitometric values were corrected for loading control.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology, Inc., Charlottesville, VA) according to the manufacturer's instructions with some modifications. For each ChIP sample, 1.0 x 10^{6} cells were used. Following stimulation with 10 nm of E2, L17, WAY or Ethanol vehicle for 45 min, proteins were cross-linked to DNA by adding 1% formaldehyde directly to culture medium and incubating for 10 min at 37 °C. The cross-linking reaction was stopped by the addition of 2.5 M Glycine to a final concentration of 125 mM and incubating with shaking for 5 min at room temperature. Cells were then rinsed twice in ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL aprotinin and 1 µg/mL pepstatin A). 200 µL of SDS Lysis Buffer (1% SDS, 10 nM EDTA, 50 mM Tris, pH 8.1) containing protease inhibitors (1 mM PMSF, 1 µg/mL aprotinin and 1µg/mL pepstatin A) was added to cells and cells were scraped into 1.5 mL

Eppendorf tubes and incubated on ice for 10 min. The cells were then sonicated 5 x 15 seconds at 30% power output on a Branson sonifier 350 (Branson Sonic Power). These sonication conditions yielded an average DNA fragment length of approximately 500 base pairs as determined by agarose gel electrophoresis. Following sonication, cell debris was pelleted by contrifugation for 10 min at 13,000 rpm at 4 °C after which point the supernatant was transferred to a new 2 mL microcentrifuge tube and the pellet was discarded. The supernatant was diluted 10 fold with Dilution Buffer (0.01% SDS, 1.1% Triton-X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) including protease inhibitors as above, to reduce the SDS concentration. 1% of the diluted cell supernatant was aliquoted to be used as input/starting material for the PCR reaction. Lysates were pre-cleared twice with 30 µL Protein A Agarose/Salmon Sperm DNA (50% slurry)(1.5 mg BSA, 4.5 mg recombinant Protein A (provided as a 50% gel slurry), suspended in Tris-EDTA (TE) buffer, pH 8.0, containing 0.05% sodium azide) for 30 min at 4 °C with agitation. Agarose was then pelleted at 800 rpm at 4 °C for ~1 min and the supernatant fraction was collected. The immunoprecipitating antibody was added to the 2 mL supernatant fraction and incubated overnight at 4 °C with rotation. The following morning 30 µL of Protein A Agarose/Salmon Sperm DNA (50% slurry) was added to each immunoprecipitation and incubated for 1 hr at 4 °C with rotation to collect the antibody/chromatin complex. The agarose beads were then pelleted by centrifugation at 800 rpm at 4 °C for ~1 min and then washed for 5 min on a rotating platform with 1 mL of each of the following buffers: Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl) one wash, High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl) one wash, LiCl Immune Complex Wash Buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1) one wash, TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) two washes. 250 µL freshly prepared Elution Buffer (1% SDS, 0.1 M $NaHCO_3$) was then added to the pelleted protein

A/agarose/antibody/chromatin complex, vortexed briefly to mix and incubated at room temperature for 15 min with rotation. Agarose was then centrifuged and the supernatant fraction was transferred to another tube and the elution was repeated, after which the eluates were combined. The protein-DNA crosslinks were then reversed by adding 20 µL 5 M NaCl to the combined eluates (500 µL) and 1 µL 5 M NaCl to the 20 µL input samples which were aliquoted and saved earlier, followed by heating the samples at 65 °C for 4 hrs and stored at -20 °C overnight. The next day the combined eluates were incubated for 1 hr at 45 °C with 10 µL of 0.5 M EDTA, 20 µL 1 M Tris-HCl (pH 6.5) and 12.5 µL of 40X Pronase per 500 µL. DNA was then isolated by Phenol/chloroform extraction and ethanol precipitation. Pellets were then washed with 70% ethanol, air dried and resuspended in 50 µL TE buffer for PCR. 5 µL DNA (no antibody) was used for the input PCR. 5 µL ChIP DNA products were amplified (30 cycles) by PCR using primers specific to the ERE in Exon 2 of the Bcl-2 gene in addition to various controls. Primer sequences are indicated in Table 2.1. PCR 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 graphed as a percentage of input DNA.

Table 2.1 Primers for chromatin immunoprecipitation.

Primer Name	Sequence (5'-3')	Product Size
Bcl-2 ERE (F)	5'-AGGGGCTACGAGTGGGATG-3'	217 bp
Bcl-2 ERE (R)	5'-CGAGATGTCCAGCCAGCT-3'	
pS2 (F)	5'-TTCATGAGCTCCTTCCCTTC-3'	212 bp
pS2 (R)	5'-ATGGGAGTCTCCAACCT-3'	

RNA Extraction, reverse transcription and Quantitative-PCR

Cells were seeded in triplicate on 60 mm dishes and treated 24 hours later with vehicle or 10 nM E2, L17 or WAY for 1 or 24 hrs. Cells were trypsinized and collected by centrifugation and

total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions and RNA was eluted in 20 µL nuclease-free water. Bcl-2 and GAPDH cDNAs were amplified from 20 ng of total RNA that was reverse transcribed using the Bcl-2 and pS2-specific primers used for the ChIP experiment. All target transcripts were detected using the quantitative RT-PCR Quanti-Test SYBRGreen PCR Kit (Qiagen) on a Mastercycler Realplex device. The data were collected and analyzed using the comparative Ct (threshold cycle) method using GAPDH expression as the reference gene.

Animals

Immature female CD-1 mice (Charles River Laboratories, Wilmington, MA) were maintained in a temperature-controlled room on a 12 hr light: 12 hr darkness photoperiod cycle. Mice were fed Harlan Teklad 2018 rodent diet and water was supplied from glass bottles only *ad libitum*.

Uterotrophic assay

Female mice, at 21 days of age, were weighed and divided randomly into 5 experimental groups (n=6). Mice were administered L4, L33, L34, L17, E2 (positive control), or Saline (negative control) subcutaneously at a dose of 1 mg/kg of body weight once daily for 5 days. On the sixth day, the mice were weighed and sacrificed by cervical dislocation and their uteri were dissected out. The wet weight for each uterus was recorded and the data is expressed as a percentage of the control. The Ethics Board Committee for Animal Experiments at the University of Ottawa Animal Care Facility approved all the protocols for animal experiments.

Histological Analysis

The uteri from each animal were fixed in 10% formalin immediately after weighing, then dehydrated and embedded in paraffin. Serial 4-5 µm cross sections were made through the uterine horns, stained with hematoxylin and eosin, and observed using a Zeiss Axiophot Microscope.

Morphological Analysis

To evaluate the morphological changes induced by E2 or L17 exposure in the uterus, we focused on the proliferative state of the luminal epithelium along the uterine horns. Tissue sections from various regions of each uterine horn were evaluated for epithelial cell height in at least three different areas of the epithelial lining of the lumen along the uterus. The epithelial height was determined using the optical micrometer tool using the Northern Eclipse Program Version 7.0.

Whole mounts of normal mammary glands

Fourth inguinal mammary glands were extracted from mice and spread onto glass microscope slides. Glands were fixed overnight in Carnoy's fixative. Fixed glands were washed in 70%, 50%, 25% ethanol and finally water for 15 minutes each, then delipidated in acetone for 20 minutes three times. Glands were rehydrated in 100% then 95% ethanol for 20 minutes each then stained with hematoxylin for one hour at room temperature. Glands were rinsed clear in tap water then detained with acid alcohol (50% ethanol, 0.2% HCl) for 30 minutes twice. Lastly, glands were dehydrated in 70%, 95%, and 100% ethanol for 20 minutes each then stored in xylene.

Hepatotoxicity Assay

Adult male Sprague-Dawley rats, 250-300 g, were obtained from Charles River Canada Laboratories (Montreal, Canada), fed ad libitum, and allowed to acclimatize for 1 week. All animal treatment conformed to guidelines provided by the University of Toronto, Canada. Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldeus et al. 10 mL of isolated hepatocytes (106 cells/mL) was suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating round-bottomed flasks under an atmosphere of 95% O_2 and 5% CO_2 in a water bath (37°C) for 30 min. Stock solutions of the test compounds prepared in ethanol were added to the solution containing the hepatocytes to achieve the desired concentrations and incubated under the oxygen- rich atmosphere described above. The lethal concentration that killed 50% of the cells (LC50) after incubation for 2 h was determined by

counting the number of cells that excluded trypan blue (0.1% w/v). On this time scale reactive metabolites, e.g. catechols, apparentaly can form which then upset the cellular redox balance. It is also important to note that the LC50 values for the compounds range around 400μM, whereas physiological concentrations are in the nanomolar range (ie. 10 nM for E2). Thus, these concentrations are on the order of a million times the physiological concentrations and this hepatocyte toxicity assay functions as an accelerated toxicity test. All measurements were done in triplicate to obtain the stated ranges.

Binding Affinity Assays

Relative binding affinities (RBAs) to the estrogen receptors ERα and ERβ were determined by a competitive radiometric binding assay using 10 nM [^{3}H]estradiol as tracer and performed in the laboratory of Dr. John Katzenellenbogen (University of Illinois, Urbana, IL). Purified full-length human ERα and ERβ were purchased from PanVera (Madison, WI, U.S.). Incubations were done for 18-24 hrs at 0°C. Hydroxyapatite (BioRad, Hercules, CA) was used to absorb the ligand-receptor complexes, and free ligand was washed away. Binding affinities are expressed relative to the binding affinity of E2, which is set to 100%. E2 binds to ERα with a dissociation constant (Kd) of 0.2 nM and to ERβ with a Kd of 0.5 nM.

Statistical Analysis

All experiments were repeated at least three times and error bars indicate standard error. Significant differences between samples were evaluated using the Student t-test.

CHAPTER III - RESULTS

Assessment of transcriptional activity of L17 compared to Estrogen (E2) at the estrogen-response element (ERE)

In previous studies we have synthesized novel A-CD estrogens and tested these compounds for relative binding affinities (RBAs) to the ERα and ERβ relative to E2 (Wright et al, 2011). The consensus in the literature is that the presence and activation of the ERβ opposes the tumorigenic and proliferative effects mediated by the ERα and therefore, we chose the preferential ERβ binding A-CD compound, L17, for further analysis (Figure 3.1A). The relative binding affinities (RBA) for L17 were obtained in the laboratory of John Katzenellenbogen (University of Illinois) using competitive radiometric binding assays. RBAs are a measure of the binding affinity of L17 relative to that of the reference standard E2 for both estrogen receptors. L17 is naturally smaller than E2 and because the ligand binding domain of the ERβ is smaller than that of the ERα, L17 is better tolerated by the ERβ, hence why there is a preference for binding to the ERβ versus the ERα. The ERβ RBA of L17 is 1.73 (RBA ratio ERβ:ERα=9.3) (Wright et al, 2011). In some of the subsequent experiments we included another ERβ agonist called WAY-200070 (WAY) for analysis. WAY is an aryl diphenolic azole ERβ agonist developed by Wyeth (Figure 3.1A). The ERβ RBA of WAY is 133 (RBA ratio ERβ:ERα =68) (Malamas et al, 2004).

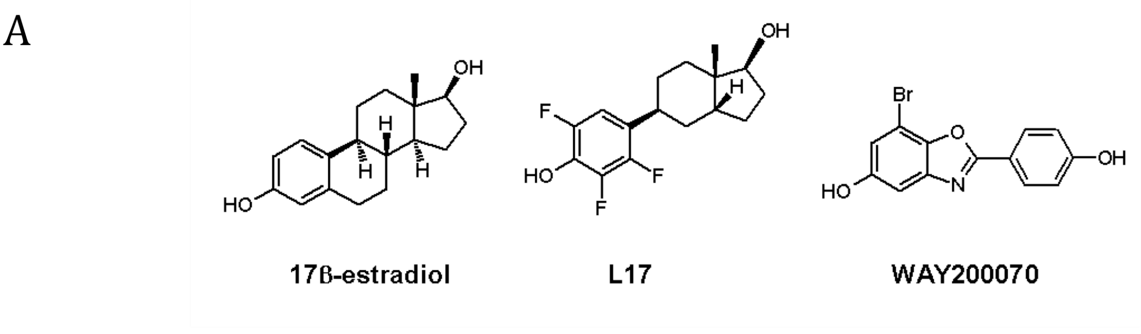
In order to determine the optimal concentration at which L17 activates the ERβ, we performed a dose-response experiment to assess the transcriptional potency of L17 compared to E2 using a synthetic ERE in HEK293 cells. We decided to use HEK293 cells because they are devoid of the estrogen receptor. Cells were transiently transfected with a hERα or a hERβ expression plasmid in addition to an ERE-luciferase reporter plasmid and treated with increasing concentrations of E2 or L17 for 48 h. Figure 3.1B shows dose responses of ERα and ERβ-mediated transcriptional activation demonstrating that E2 is approximately 10-fold more potent at the ERα than L17 at 10nM. In

contrast, both E2 and L17 similarly activated the ERβ on the same reporter gene and increased basal ERE activity at a concentration of 10 nM (Figure 3.1C). Although it is observed that L17 can induce the ERα to similar absolute levels as E2 at significantly higher concentrations, the concentration of L17 (10nm) is still low enough to favour ERβ activity. Figure 3.1D shows that L17 has an EC_{50E2} value approximately 400-fold greater than E2 (concentration required for 50% of the maximum E2 response) on the ERα while the EC_{50E2} for L17 on the ERβ is 40-fold higher than E2 (Figure 3.1E). Similar to L17, WAY was unable to activate ERα-mediated transcription at 10 nM (Figure 3.1F) however, it strongly induced the reporter gene via ERβ at the same concentration comparable to E2 and L17 (Figure 3.1G). Together with structural differences, L17 and WAY represent two different classes of preferential ERβ agonists based on selectivity and binding affinity.

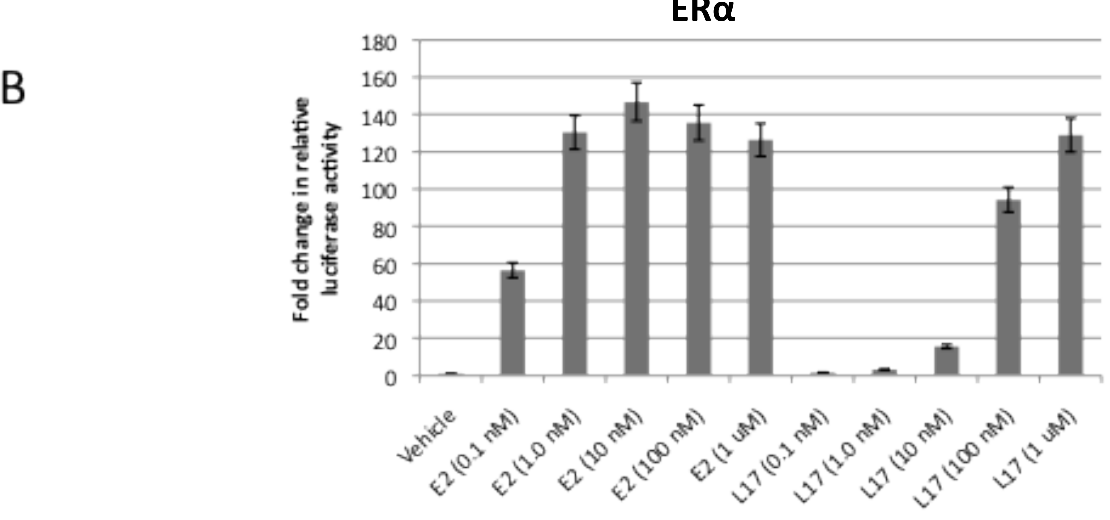
ERβ agonists inhibit proliferation of LCC9 cells

In the mammary gland, estrogens are potent mitogens, regulating growth, development and function of normal as well as cancerous cells. ERα mediates the proliferative effect of E2 in breast cancer, whereas ERβ seems to be anti-proliferative. E2 treatment of ERα + T47-D and MCF-7 cells implanted into mice has been shown to increase proliferation and tumor growth (Rochefort et al, 1984; Huseby et al, 1984). Since ERβ expression decreases or disappears during tumor development (Park et al, 2003; Myers et al, 2004), the question arose whether a reintroduction of ERβ or an overexpression of ERβ relative to ERα, a common property in cells that have acquired antiestrogen resistance, in ERα+ breast cancer cells could influence the tumor inhibitory properties. 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. To investigate this hypothesis, an MCF-7 breast cancer cell line derivative, LCC9 cells, with a higher ERβ:ERα ratio relative to their parental cell line, was used, using MCF7 cells as a control. Figure 3.2A shows the

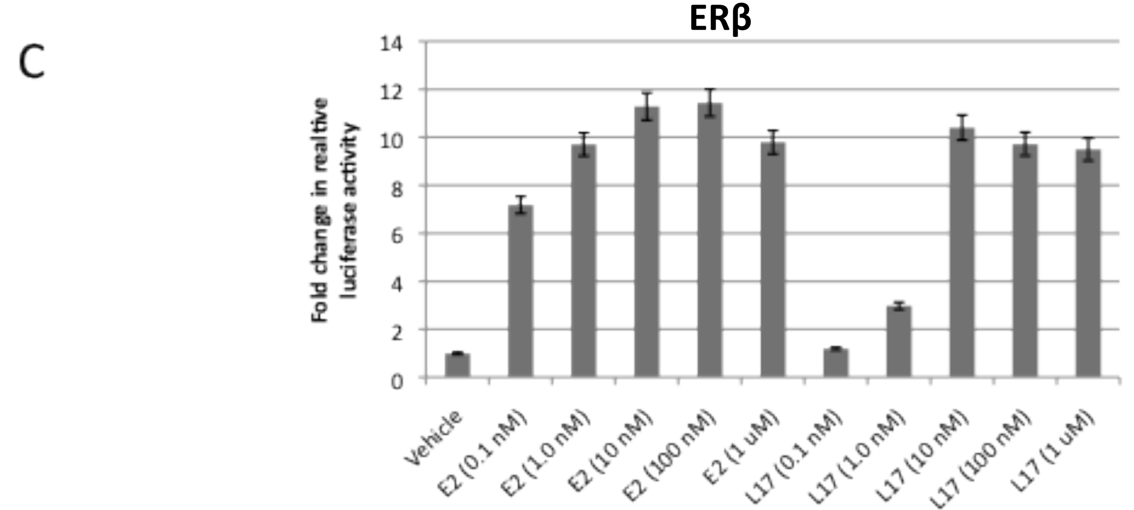
A

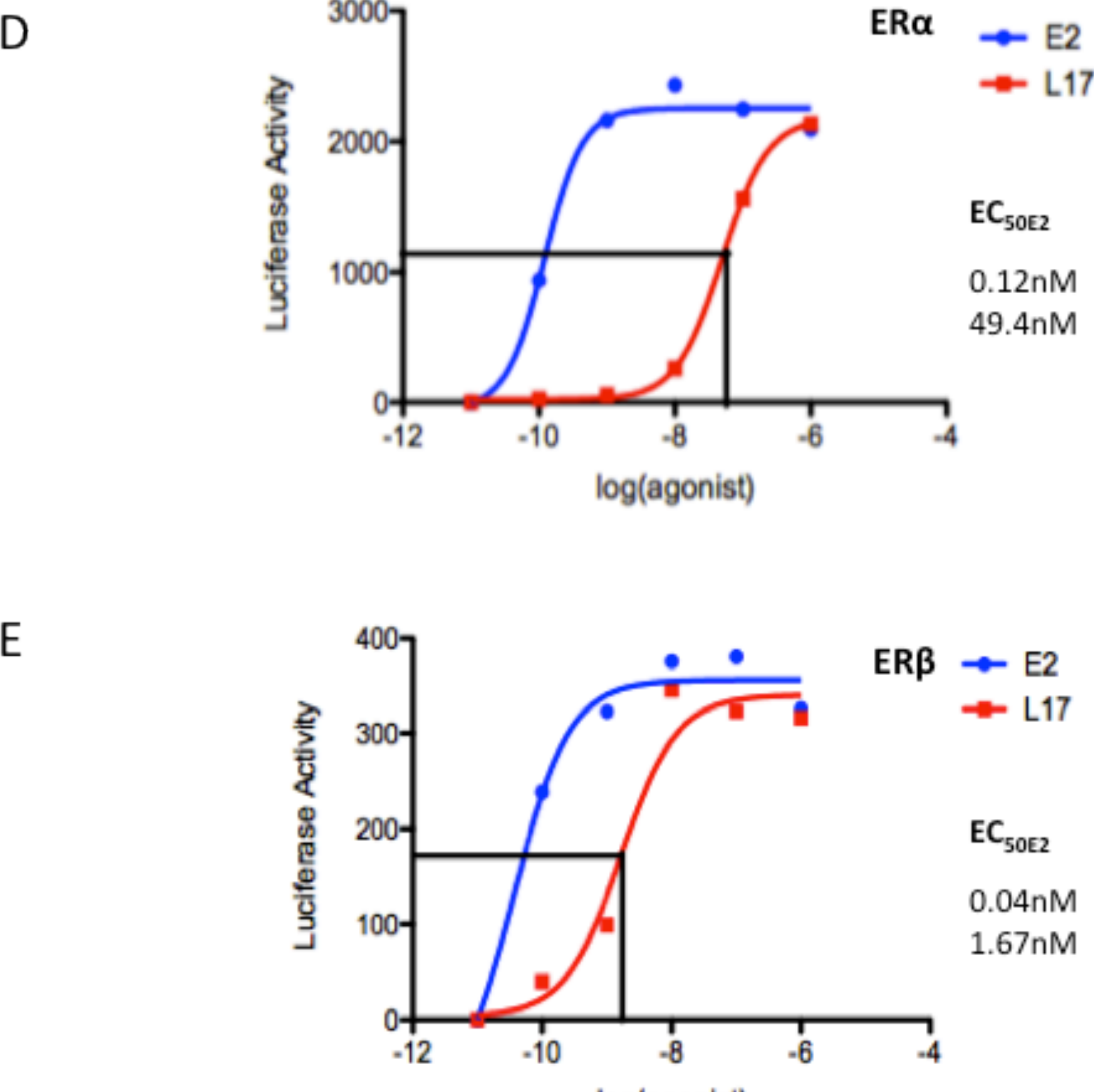


B

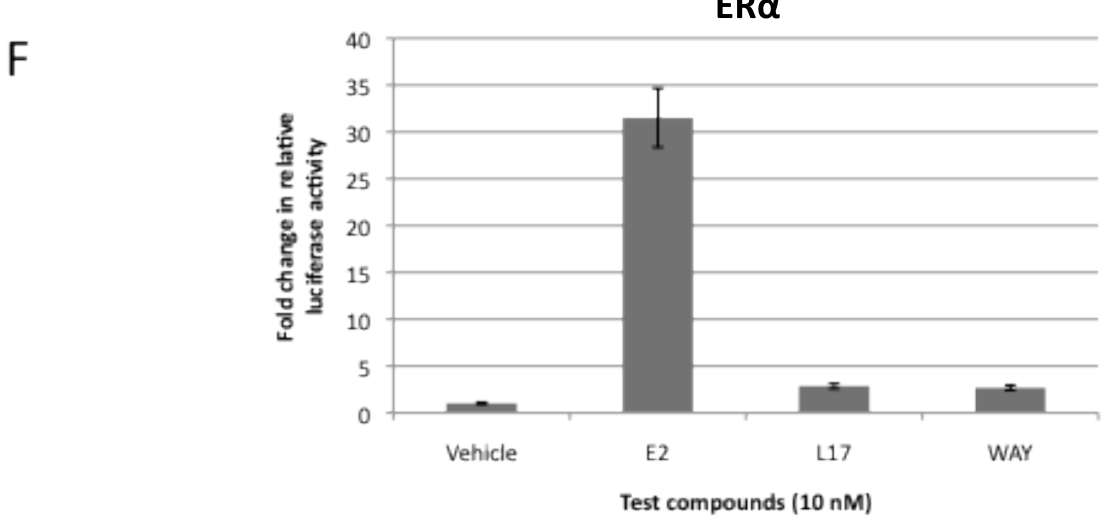


C




D
ERα
E2
L17
Luciferase Activity
3000
2000
1000
0
-12
-10
-8
-6
-4
log(agonist)
EC_{50E2}
0.12nM
49.4nM
E
ERβ
E2
L17
Luciferase Activity
400
300
200
100
0
-12
-10
-8
-6
-4
log(agonist)
EC_{50E2}
0.04nM
1.67nM

F



G

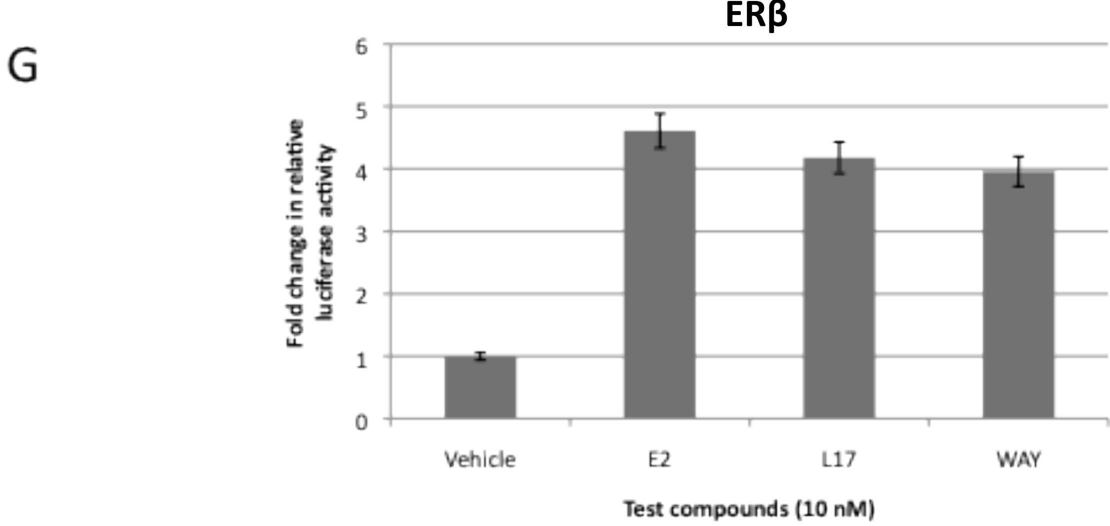


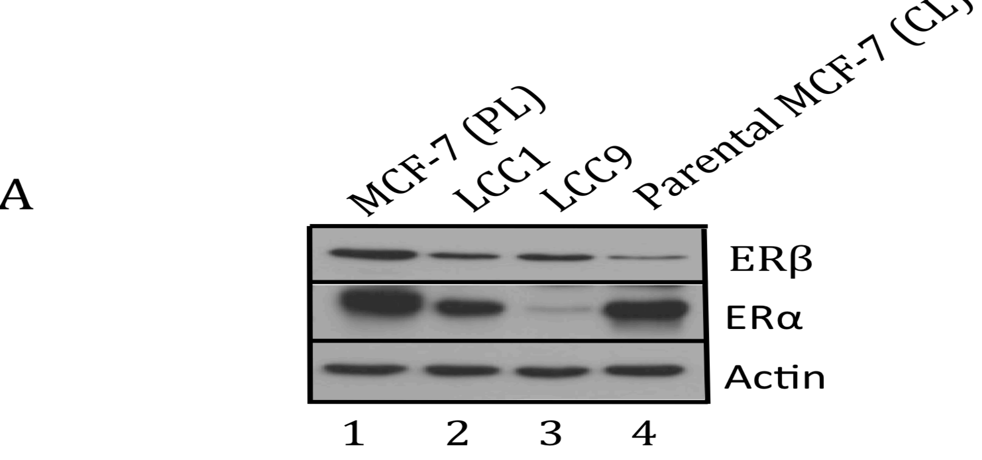
Figure 3.1. Characterization of the preferential ERβ agonist, L17.
A, Chemical structures of the compounds used in this study. **B,** HEK 293 cells were transiently transfected with human ERα or **C,** ERβ expression vectors and the ERE-Luc reporter plasmid and then treated with for 48 hours with vehicle control (EtOH), or increasing concentrations (ranging from 0.1 nM to 1µM) of E2 or L17. Cells were harvested after 48 hours and luciferase activity was assayed as described in Methods. Transfections were normalized to an internal *Renilla* luciferase control plasmid. Data represents the average of triplicate measurements of transcriptional activity of 3 independent experiments; error bars indicate standard error. **D,** EC_{50E2} were calculated for the ERα and **E,** the ERβ using Graph Pad Prism 3 software.**F,** HEK293 cells were transiently transfected with human ERα or **G,** ERβ expression vectors and the ERE-Luc reporter plasmid and then treated with for 48 hours with vehicle (EtOH) or 10 nM E2, L17 or WAY for 48 hours after transfection, harvested and luciferase activity was measured as described above.

relative level of expression of ERα and ERβ protein in MCF-7 cells from our laboratory (MCF-7PL) in comparison with LCC1 cells (E2-independent MCF-7 cells isolated in E2-free conditions) (Brunner et al, 1993), LCC9 cells (Fulvestrant and Tamoxifen resistant, E2- independent) 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 4-fold in MCF-7CL cells relative to MCF-7PL cells suggesting significant variation across stocks of

MCF-7 cells. ERα was reduced almost 2-fold in LCC1 cells while the ERβ was increased 2-fold relative parental MCF-7CL cells. LCC9 cells express strongly reduced levels of ERα (14-fold less) but maintain expression of ERβ at levels similar to LCC1 cells and approximately 3-fold higher than that in the parental MCF-7 cells (Figure 3.2A). Thus the ratio of ERα to ERβ is much reduced in LCC9 cells compared with either MCF-7PL or MCF-7CL cells.

We next determined the effects of E2, L17 or WAY on proliferation of MCF-7 cells and LCC9 cells. We chose to use MCF-7PL cells since they express significantly more ERβ than MCF-7CL cells and might be more sensitive to the effects of ERβ agonists. Cells were grown in phenol red free (PRF) media with charcoal stripped serum (CSS) and then treated with increasing concentrations of E2 or L17 for 3 and 5 days. Cells were then harvested and viable cells were counted using trypan blue exclusion. The results in Figure 3.2B demonstrate that treatment with E2 stimulated an increase in MCF-7 cell numbers in a concentration-dependent manner with maxiumum stimulation at 10 nM that reached a 70% increase in cell numbers compared to vehicle treated cells after a total of 5 days, while L17-treated cell numbers were similar to vehicle. In contrast, while E2 had little overall effect on LCC9 cell numbers, 10 nM L17 induced a marked dose-dependent decrease in viable cell numbers within 3 days, which approached 40% within 5 days (Figure 3.2C). At 10 nM, to WAY and L17 had similar effects on growth of MCF-7 and LCC9 cells (Figure 3.2D & E, respectively).

A



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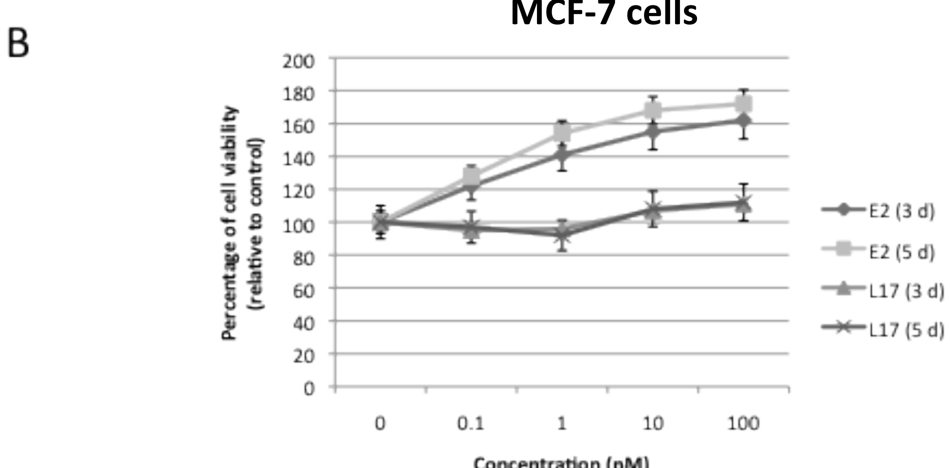

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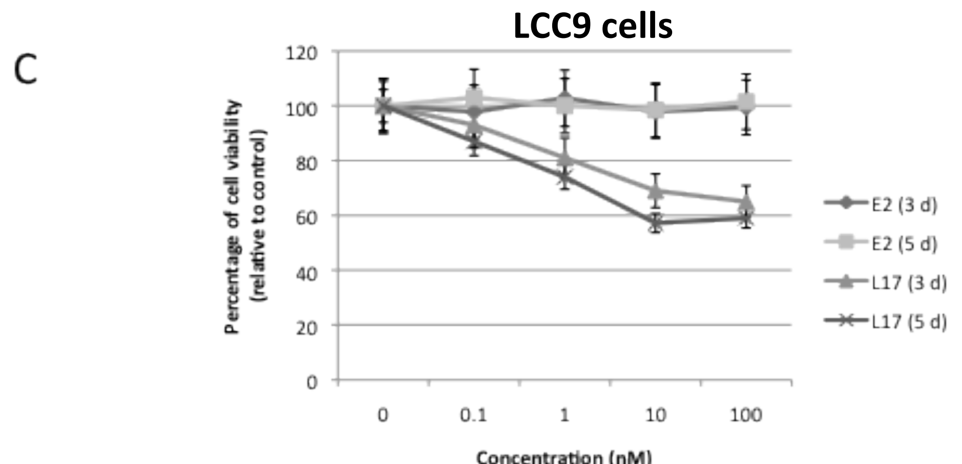

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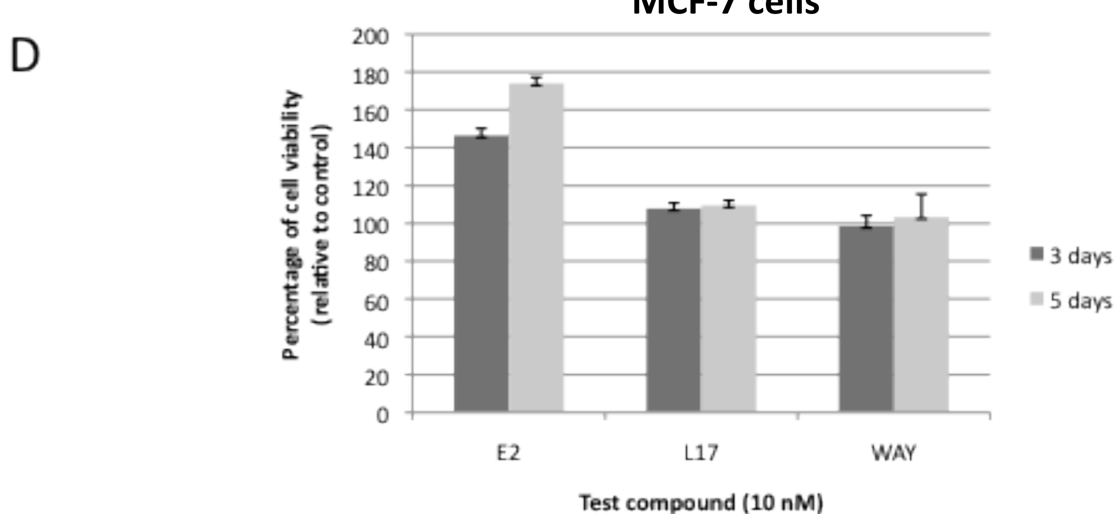


E

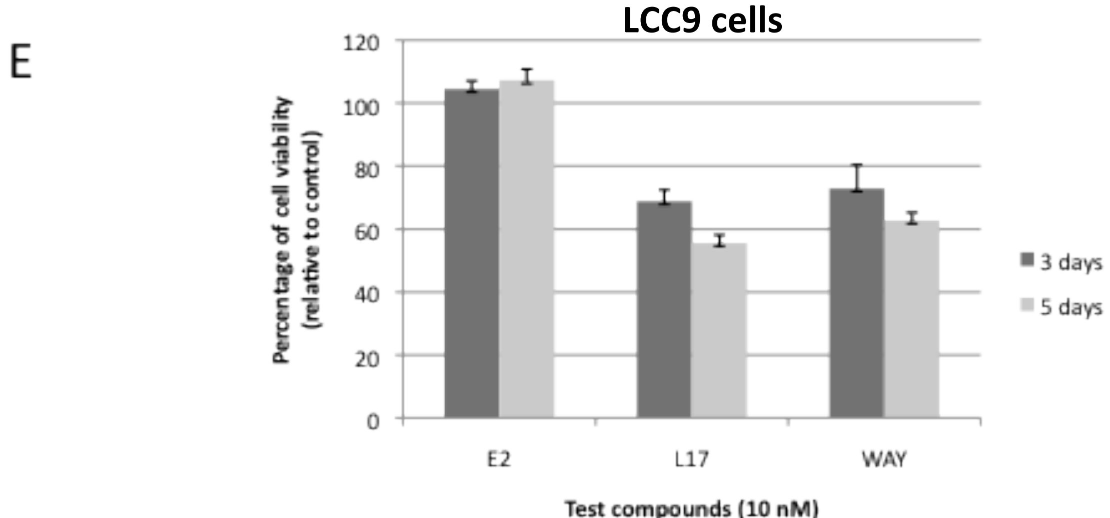


Figure 3.2. **Relative ER expression and effects of ERβ agonists on LCC9 and MCF-7 cells. A,** Two isolates of MCF-7 cells (lanes 1 and 4) and two MCF-7 subclones that are hormone-independent (LCC1) (lane 2) and SERM-resistant (LCC9) (lane 3) were harvested untreated and then protein extracts were immunblotted with antibodies against ERα and ERβ. MCF7 cells (lane 1) were used in all subsequent experiments. Actin was used as a loading control. **B,** MCF-7 and **C,** LCC9 cells were grown in phenol red free (PRF) media with charcoal stripped serum (CSS) and treated with vehicle (EtOH) or increasing concentrations of E2 or L17. Cell viability was assessed using trypan blue exclusion after 3 and 5 days and percentage of viable cells is expressed relative to vehicle treated cells. Data represents the mean from 3 separate experiments each performed in triplicate; error bars indicate standard error. **D,** MCF-7 and **E,** LCC9 cells were grown in PRF media with CSS and treated with vehicle (EtOH) or 10 nM E2, L17 or WAY for 3 and 5 days. Cell viability was then assessed using trypan blue exclusion and percentage of viable cells is expressed relative to vehicle treated cells. Data represents the mean from 3 separate experiments each performed in triplicate; error bars indicate standard error.

These findings show that the preferential ERβ agonists, L17 and WAY have strong antiproliferative effects on ERβ-high antiestrogen-resistant LCC9 breast cancer cells.

ERβ agonists inhibit G1 and S phase exit in LCC9 cells

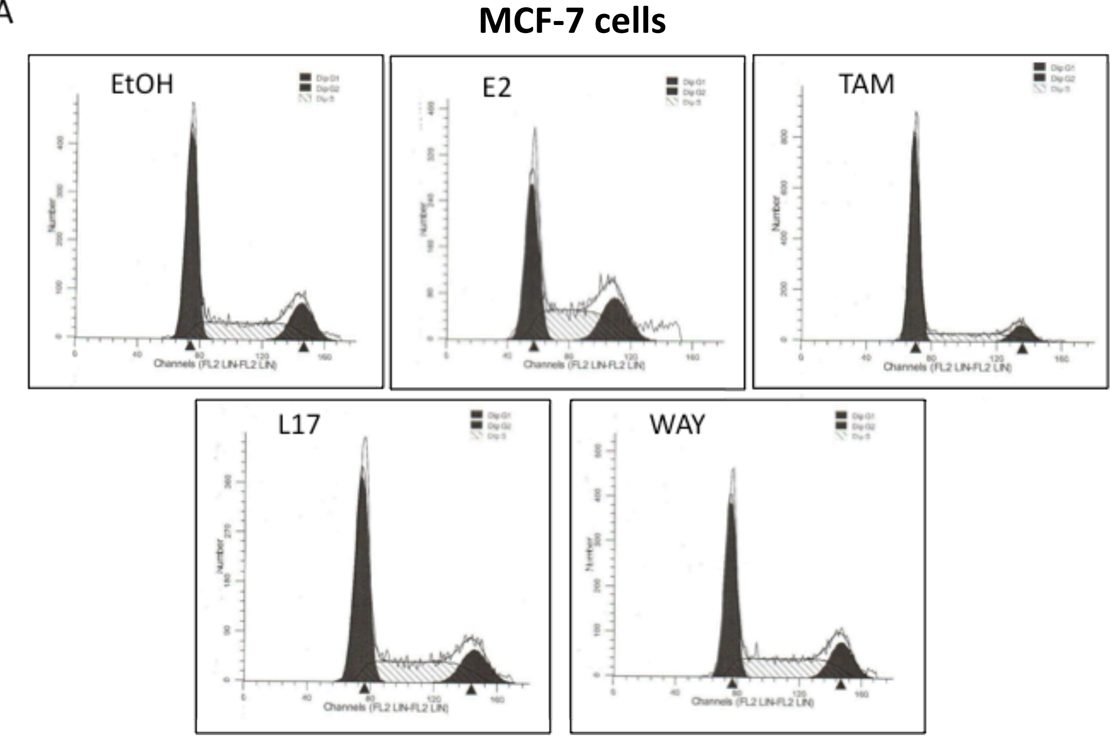
Since both L17 and WAY dramatically reduced the growth of LCC9 cells compared to MCF-7 cells, we next assessed the impact of L17 and WAY on cell cycle progression. MCF-7 and LCC9 cells were grown in PRF media with CSS and treated with vehicle control (EtOH) or 10 nM E2, L17, WAY or Tamoxifen (Tam) at an optimal concentration of 1 μM for a total of 72 h followed by staining with propidium iodide (PI) and analysis by flow cytometry. Figure 3.3A & C show representative flow cytometric histograms showing cell cycle distribution of MCF-7 and LCC9 cells respectively, after 72 h treatment with indicated ligands. The results in Figure 3.3B showed that after 3 days of culture in 10nM E2, MCF-7 cells demonstrated a 40% reduction in G1 and a corresponding 50% increase in S phase cells. As expected, TAM produced a strong G1 arrest, which is consistent with the literature (Ichikawa et al, 2008), while L17 and WAY had a similarly small but insignificant effect on cell cycle in MCF-7 cells (Figure 3.3B).

Previous studies of LCC9 cells have shown that they are relatively refractory to the proliferative effects of E2 (Klinge et al, 2010). In our experiments, E2 (10nM) induced a small (10%) decrease in G1 and corresponding 10% increase in S phase in LCC9 cells (Figure 3.3D) while TAM had no effect compared to vehicle treated cells. Remarkably, 72 h exposure to either L17 or WAY resulted in a decrease in the proportion of cells in G2/M phase by 50% compared to vehicle which is associated with a significant G1/S phase accumulation (Figure 3.3D) suggesting that ERβ activation can inhibit both progression into and exit from S phase.

Since L17 and WAY induced a G1/S phase cell cycle block in LCC9 cells, we further investigated the effects on the levels of G1 and S phase-specific proteins, cyclin D1 and cyclin E, respectively in the LCC9 cells compared to the MCF-7 cells following treatment with the ligands. To

accomplish this we did a time course experiment in which cells were grown in PRF media with CSS and treated with vehicle (EtOH) or 10 nM E2, L17 or WAY for up to 48 h. Cell lysates were then prepared and analyzed by immunoblotting. Cyclin D1, which is induced by E2 via the ERα, is negatively regulated by the ERβ (Paruthiyil et al, 2004). As expected, E2 induced cyclin D1 (2-fold) by 48 h in MCF-7 cells while, in contrast, L17 and WAY caused a weak (0.2-fold) induction that occurred only at 48h (Figure 3.3E). E2 weakly induced cyclin D1 in LCC9 cells (0.5-fold) over 48 h, although at a much reduced level relative to MCF-7 cells (Figure 3.3F). In contrast, both L17 and WAY transiently reduced cyclin D1 in LCC9 cells after 24 h and 48 h by 0.75 and 0.25-fold, respectively (Figure 3.3F).

Cyclin E:cdk2 complexes are critical for G1-S phase transition and DNA replication. Although cyclin E has not been shown to be a direct target of the ER, a recent report suggested that E2 may regulate the stability of cyclin E (Weroha et al, 2010). The known molecular weight of cyclin E is 53 kDa. However, tumor cells have the machinery for the proteolytic processing of full length cyclin E by an elastase-like protease into its lower molecular weight (LMW) forms thereby providing a growth advantage to cancer cells. We found that treatment with E2 elicited a profound induction of the 50 kilodalton (kDA) and 48 kDa cyclin E protein in MCF-7 and LCC9 cells by 1.5-fold (Figure 3.3E) and 9-fold (Figure 3.3F), respectively. Figure 4F also shows that both L17 and WAY also increased 50 kDa cyclin E expression in LCC9 (4 and 5-fold, respectively) (Figure 3.3E). The relative protein expression of cyclin D1, cyclin E high molecular weight (HMW) and cyclin E Low molecular weight (LMW) in both cell lines normalized to β-Actin expression is shown in Figures 3.3G,H, & I, respectively. These results suggest that preferential ERβ activation downregulates cyclin D1 and fails to strongly activate cyclin E expression in LCC9 cells which may then prevent cells from exiting both G1 and S phase. However further investigation using an ERβ- siRNA would be able to confirm whether these effects are in fact occurring through the activation of this ER.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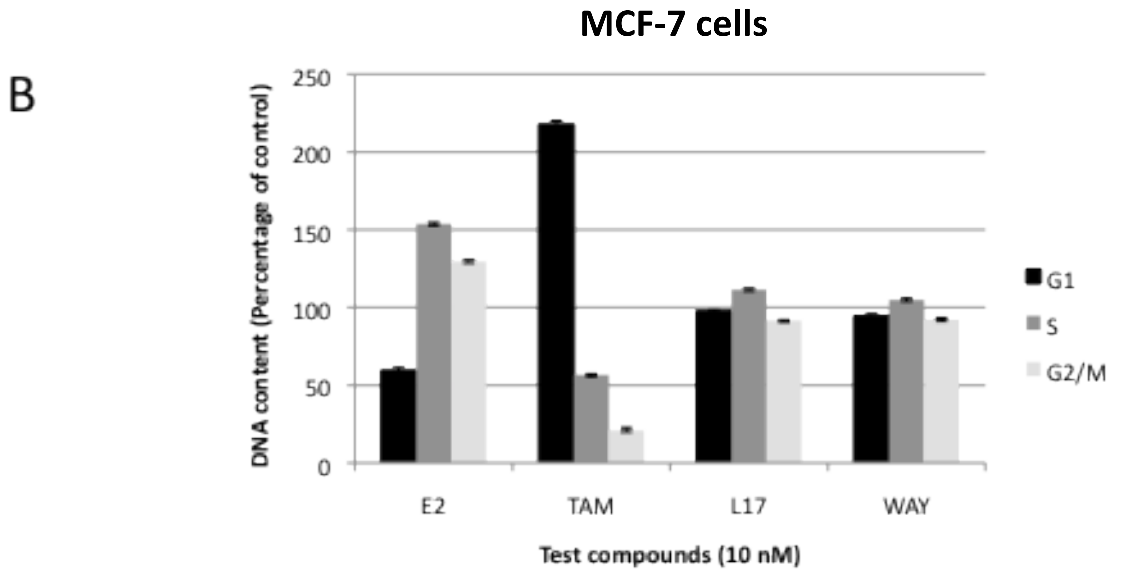
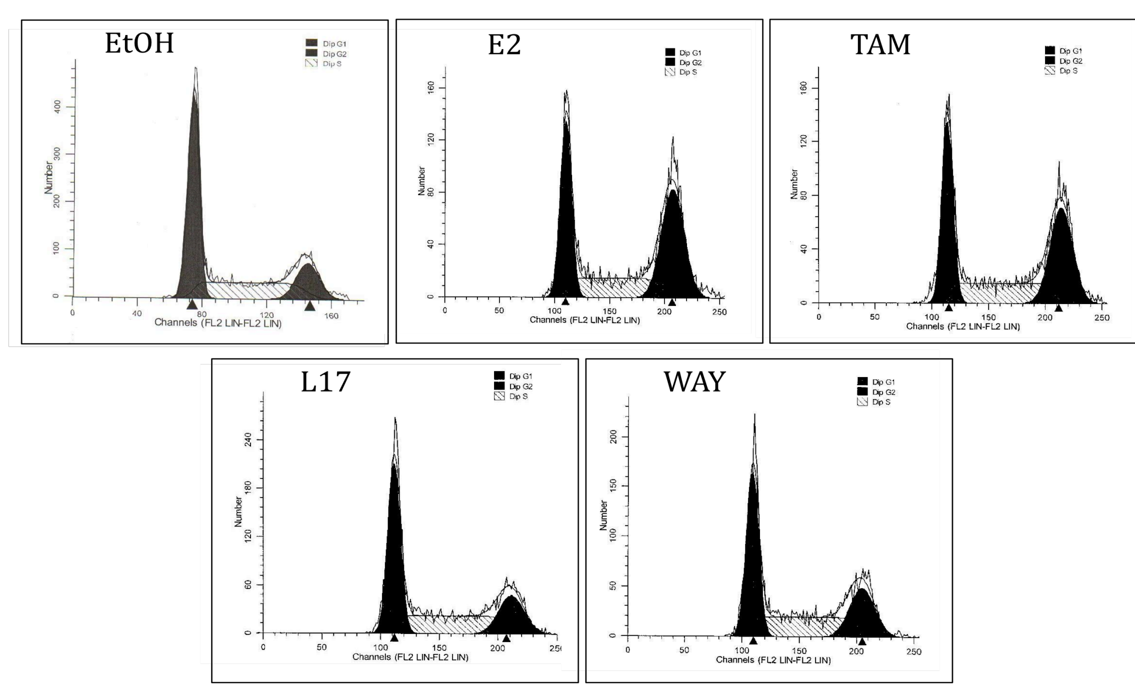
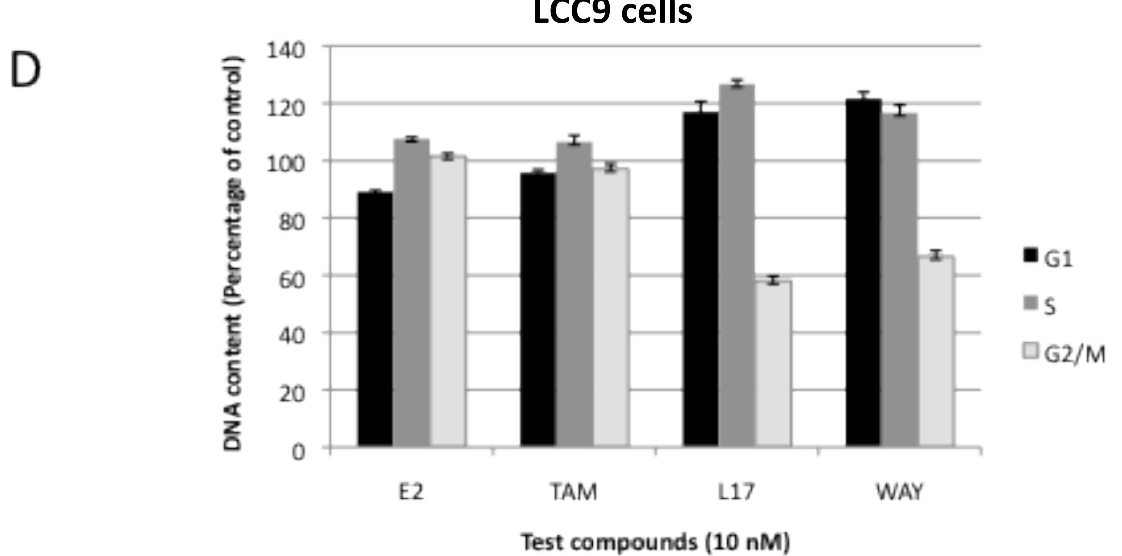
MCF-7 cells
EtOH
E2
TAM
L17
WAY
Channels (FL2 LIN-FL2 LIN)
Number


B
MCF-7 cells
DNA content (Percentage of control)
250
200
150
100
50
0
E2
TAM
L17
WAY
Test compounds (10 nM)
G1
S
G2/M

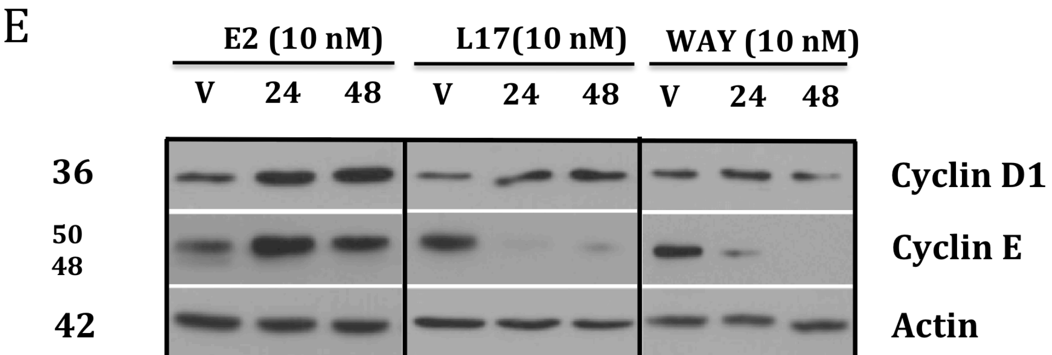
LCC9 cells

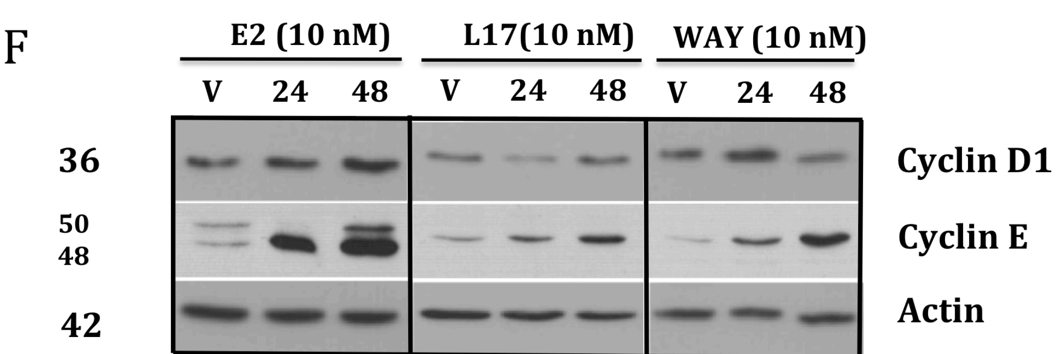
C

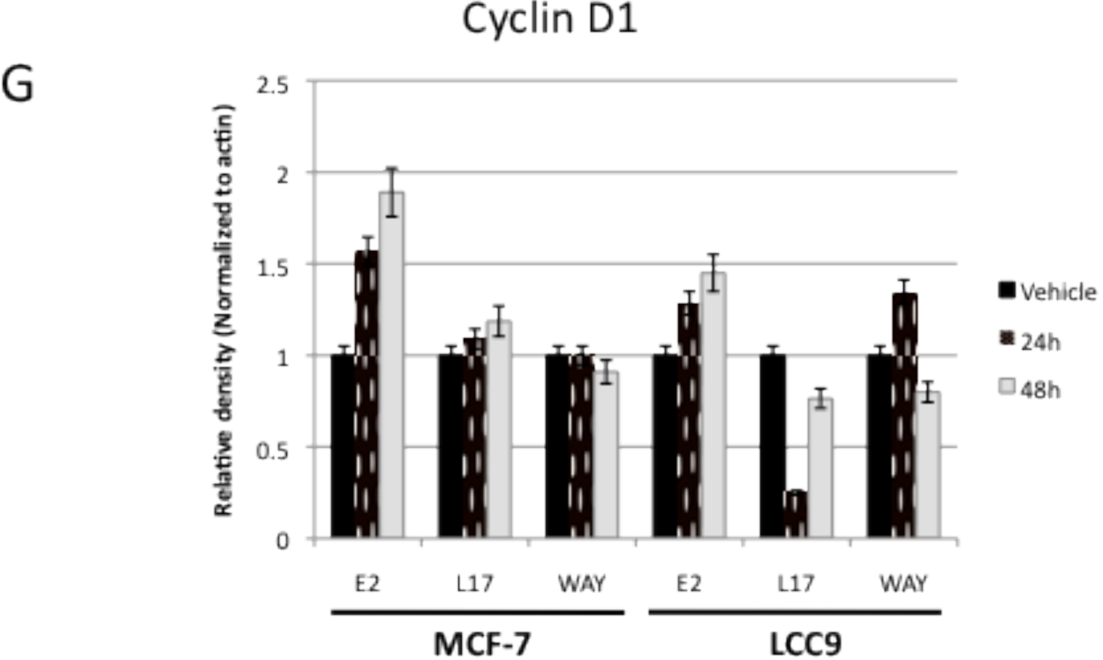


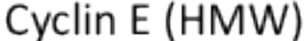


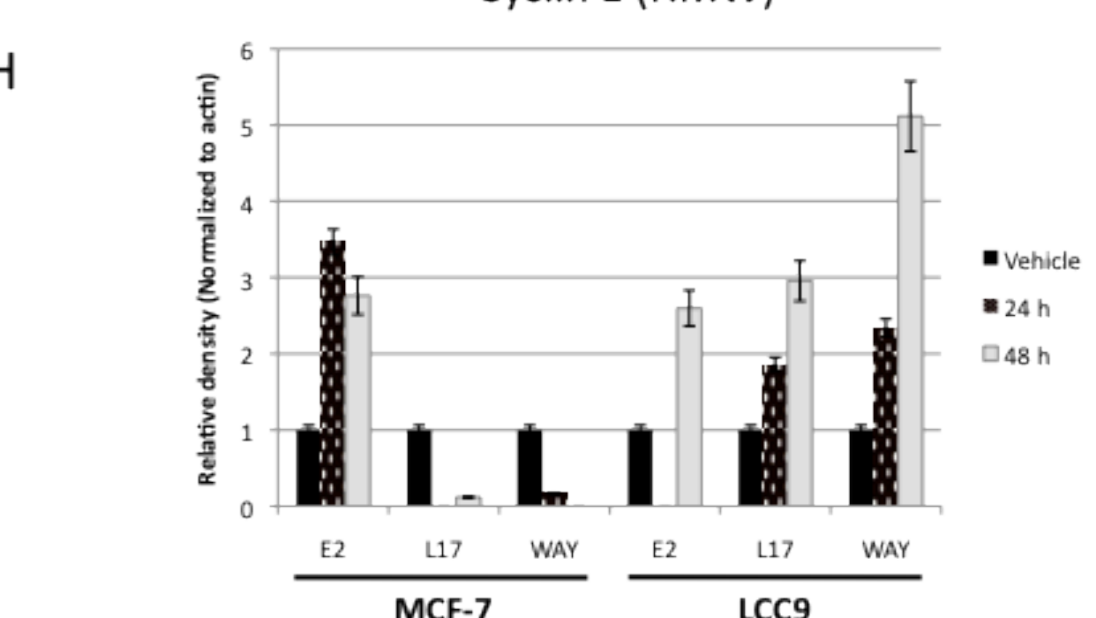
MCF-7 cells






G
Cyclin D1
Relative density (Normalized to actin)
0
0.5
1
1.5
2
2.5
E2
L17
WAY
E2
L17
WAY
MCF-7
LCC9
Vehicle
24h
48h


Cyclin E (HMW)


H
Relative density (Normalized to actin)
0
1
2
3
4
5
6
E2
L17
WAY
E2
L17
WAY
MCF-7
LCC9
Vehicle
24 h
48 h

I

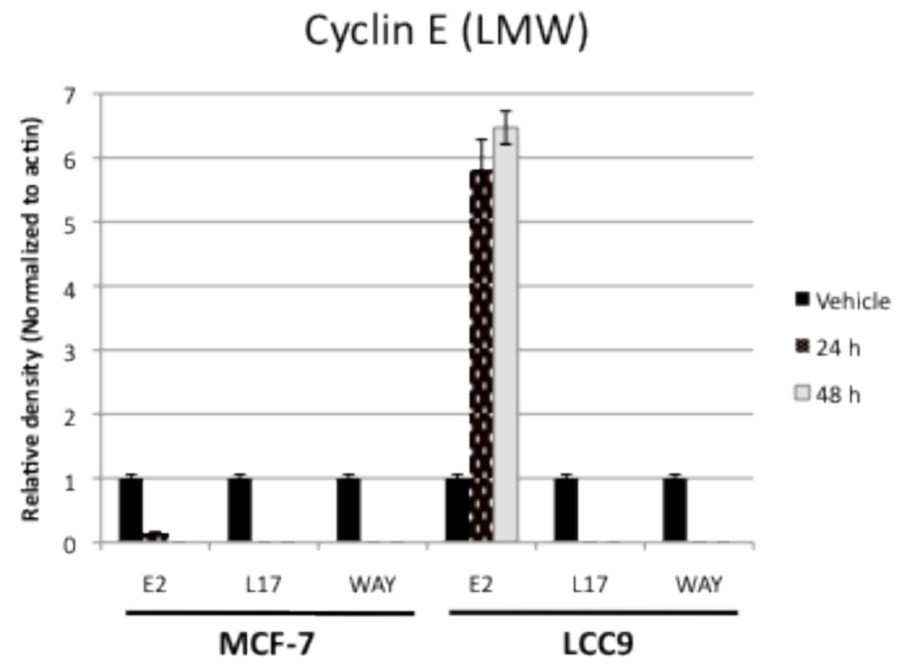


Figure 3.3. **Effects of ERβ agonists on the cell cycle in MCF-7 and LCC9 SERD-resistant cells.** Representative flow cytometric histograms showing cell cycle distribution of **A,** MCF-7 cells and **C,** LCC9 cells after 72 hour of treatment with 10 nM of the indicated ligand. Cells were grown in PRF media with CCS and then treated with vehicle (EtOH) or 10 nM E2, L17, WAY or 1 μM Tam for 72 h before harvesting for cell cycle flow analysis. DNA content results for **B,** MCF-7 cells and **D,** LCC9 cells from three independent experiments performed in triplicate were graphed as a percentage of vehicle-treated cells. Error bars indicate standard error. **E,** MCF-7 cells and **F,** LCC9 cells were grown in PRF media with CSS and treated with vehicle EtOH (V) or 10 nM E2, L17 or WAY at the indicated times. Protein extracts were immunoblotted for cyclin D1 and cyclin E. Actin was used as a loading control in all experiments. The numbers on the left indicate the molecular masses in kilodaltons. **G,** Bar graph shows the relative protein expression of cyclin D1, **H,** cyclin E (HMW) and **I,** cyclin E (LMW) in MCF-7 and LCC9 cells following exposure to the various ligands normalized to β-actin expression to minimize the effects of loading differences.

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

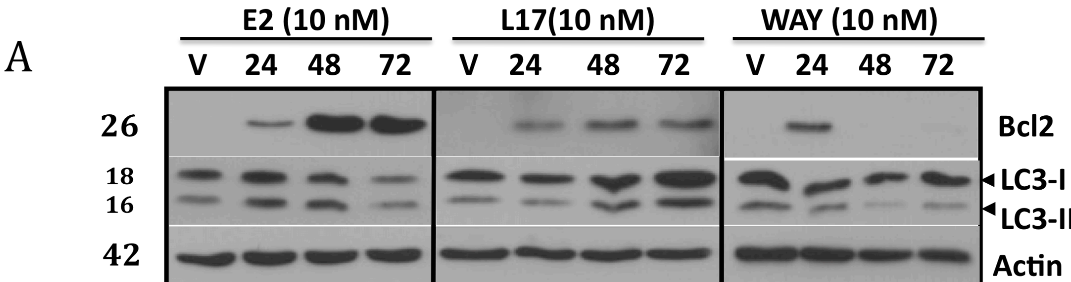
Since the Bcl-2 gene is induced by E2 (Teixiera et al, 1995) and is a critical regulator of both apoptosis and autophagy (Eisenberg-Lerner et al, 2009), we next assessed the regulation of the anti-apoptotic Bcl-2 gene by the ERβ agonists. We did a time course experiment in which MCF-7 and LCC9 cells were grown in PRF media with CSS and treated with vehicle (EtOH) or 10 nM of E2, L17 or WAY for up to 72 h after which point cell lysates were prepared and analyzed by immunoblotting. As expected, E2 strongly induced persistent Bcl-2 expression in MCF-7 cells 5-fold compared to control (Figure 3.4A). In addition, both L17 and WAY transiently induced Bcl-2 3-fold and 2-fold by 48 h and 24 h, respectively, after which point levels returned to baseline (Figure 3.4A). Bcl-2 can also inhibit autophagy through its inhibitory interaction with the evolutionarily conserved autophagy protein, Beclin 1 (Pattingre et al, 2005). When autophagy is induced, phosphatidylethanolamine (PE) conjugation converts LC3-I to II and can be used as an indicator of autophagic flux (Mizushima et al, 2007). Figure 3.4A also shows the immunoblot for LC3 in MCF-7 cells revealing that the overall basal level of LC3-I was high with little LC3-II. While LC3-I levels remained constant following E2 treatment of MCF-7 cells, we noted a 2-fold increase in LC3-II at 72 h post L17 treatment while WAY had little effect on LC3-II (Figure 3.4A).

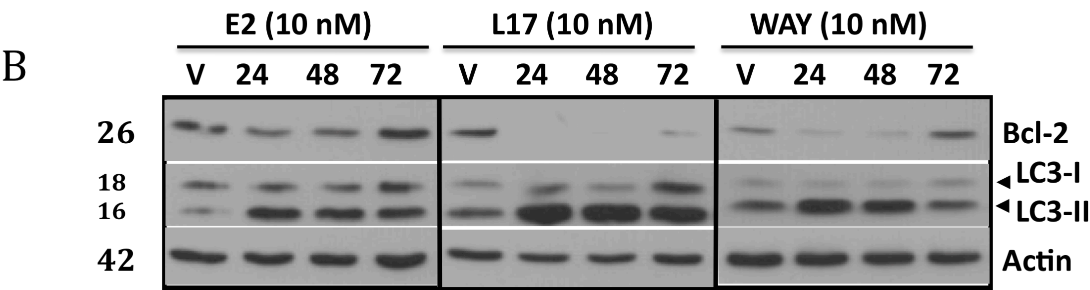
LCC9 cells have been shown to express higher basal levels of Bcl-2 relative to parental MCF-7 cells. This may be the result of the upregulation of NF-κB, a regulator of Bcl-2 in SERM-resistant breast cancer cells. In addition, studies have shown that down regulation of Bcl-2 can induce an autophagic response (Crawford et al, 2010). In agreement with this, we readily detected Bcl-2 in LCC9 cells and E2 treatment produced a small 0.2-fold decrease in Bcl-2 protein observed after 24 and 48 hrs (Figure 3.4B). Remarkably, both L17 and WAY treatment strongly downregulated Bcl-2 protein (Figure 3.4B). Baseline levels of LC3-II were consistently higher in LCC9 cells than in MCF-7 cells however, LC3-II was strongly increased 3-fold, 4-fold, and 3-fold after treatment with E2, L17 or

WAY, respectively (Figure 3.4B). Thus, activation of the ERβ especially by preferential ERβ 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. The relative protein expression of Bcl-2 and LC3-II in both cell lines normalized to β-Actin expression is shown in Figures 3.4C & 3.4D, respectively.

ERβ agonists recruit the ERβ and ERα to estrogen response elements

The inhibition of Bcl-2 expression is potentially a critical factor in L17 and WAY-mediated growth inhibition, and may actually sensitize LCC9 cells to other therapies. The ability of ERβ ligands to reduce Bcl-2 expression in LCC9 cells may be the result of the ERβ agonist bound homodimers or heterodimers of ERα and ERβ in which ligand is bound only to the ERβ to the Bcl-2 ERE. To address this question and assess the endogenous Bcl-2 ERE for occupancy by the ERα and ERβ we performed a ChIP. MCF-7 and LCC9 cells were grown in PRF media with CSS and treated with vehicle (EtOH) or 10 nM E2, L17 or WAY for 1 h. Chromatin was then crosslinked and samples were sonicated to shear DNA. Antibodies against ERα and ERβ were used to immunoprecipitate the complexes. Binding of the ERα and ERβ to the Bcl-2 ERE in exon 2 and the 5'ERE within the pS2 promoter was revealed by PCR. The pS2 gene is often used as a prognostic marker in breast cancer cells and is frequently used in studies of ER action. It is also suggested that E2 regulates the expression of pS2 through an imperfect ERE in the pS2 promoter (Berry et al, 1989). Neither ER was present on the EREs in vehicle-treated cells (Figure 3.5A & B). 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 3.5B, D & F). Interestingly, ERα and ERβ were both present on the Bcl-2 ERE following treatment with L17 or WAY in LCC9 cells. The ERα was recruited to the Bcl-2 ERE in MCF-7 cells in the presence of E2, and albeit to a lesser extent, L17 also recruited the ERα (Figures 3.5A & C). WAY also weakly recruited the ERα to the Bcl-2 ERE. The ERβ was also strongly associated with the Bcl-2 promoter following E2, L17 and WAY treatment in MCF-7 cells (Figure 3.5A, & E).


MCF-7 cells
A
E2 (10 nM)
L17(10 nM)
WAY (10 nM)
V 24 48 72
V 24 48 72
V 24 48 72
26
18
16
42
Bcl2
LC3-I
LC3-II
Actin


LCC9 cells
B
E2 (10 nM)
L17 (10 nM)
WAY (10 nM)
V 24 48 72
V 24 48 72
V 24 48 72
26
18
16
42
Bcl-2
LC3-I
LC3-II
Actin

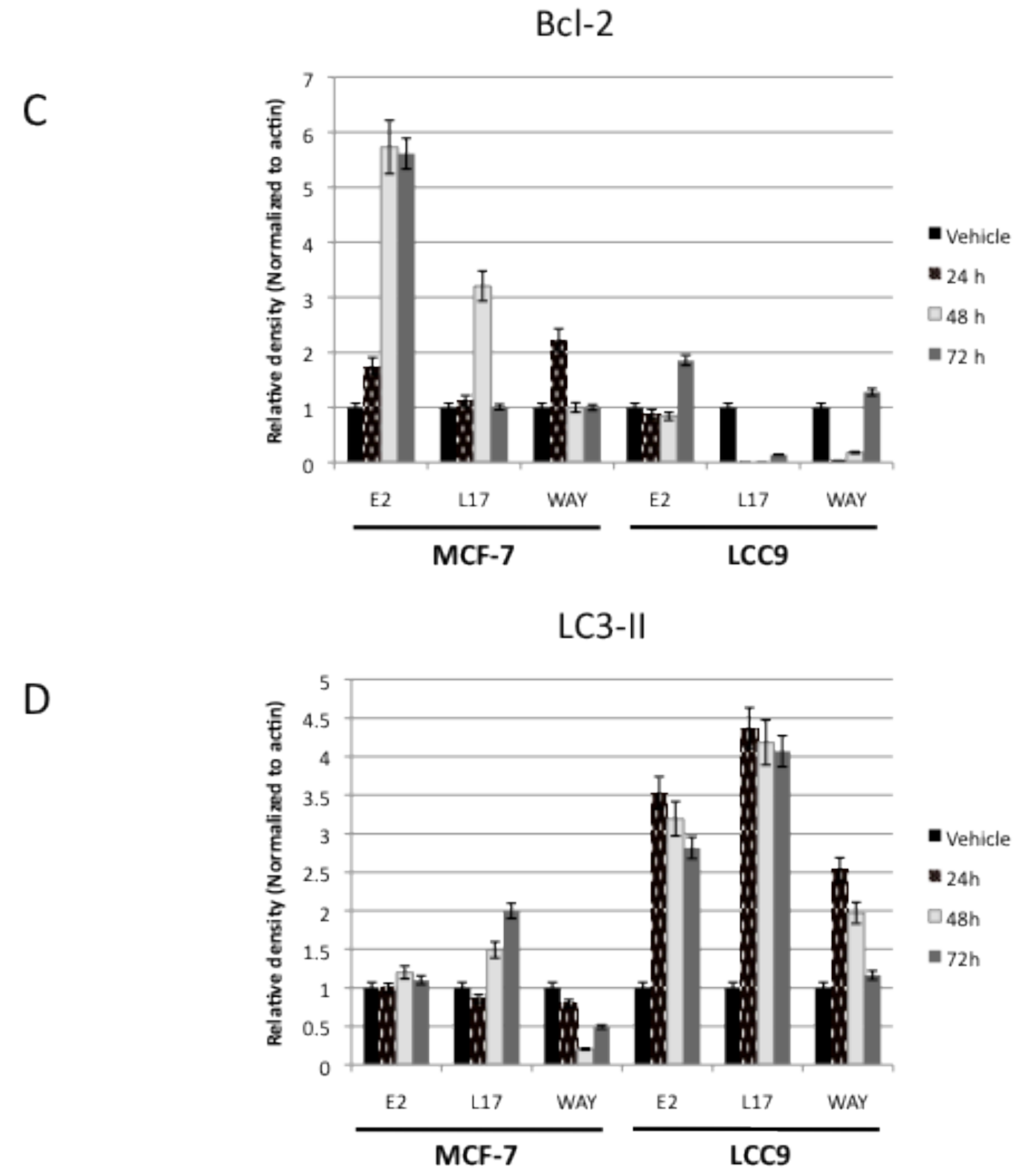


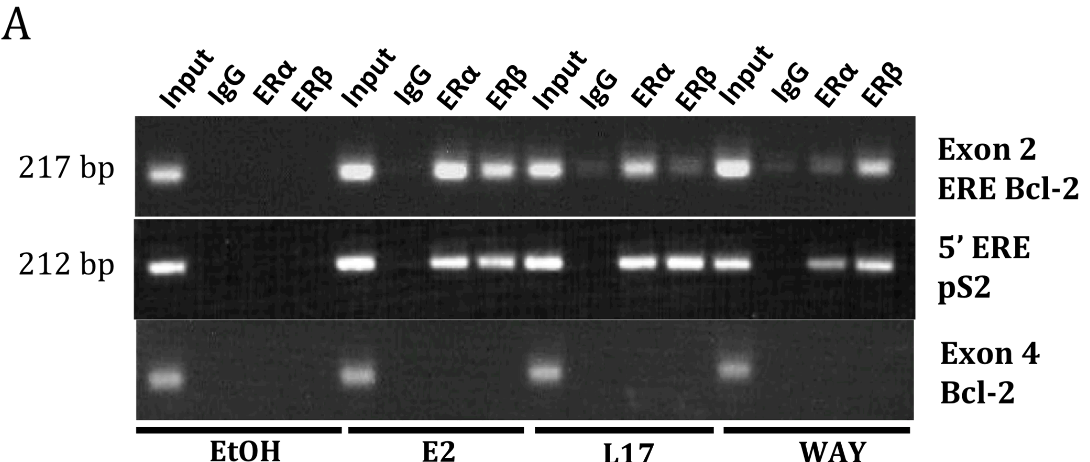
Figure 3.4. **L17 reduces Bcl-2 expression and activates an autophagy response in LCC9 cells. A,** MCF-7 cells and **B,** LCC9 cells were grown in PRF media with CSS and treated with vehicle EtOH (V) or 10 nM E2, L17 or WAY at the indicated times. Protein extracts were immunoblotted for Bcl-2 and LC3 I and II. Actin was used as a loading control in all experiments. The numbers on the left indicate the molecular masses in kilodaltons. **C,** Bar graph shows the relative protein expression of Bcl-2 and **D,** LC3-II in MCF-7 and LCC9 cells following exposure to the various ligands normalized to β-actin expression to minimize the effects of loading differences.

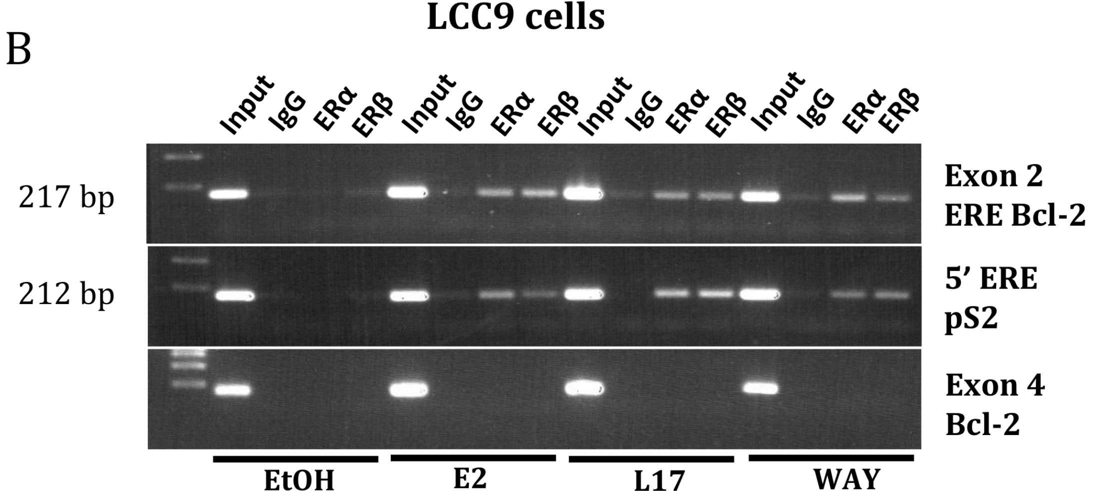
In addition, we analyzed the transcript level of Bcl-2 and pS2 mRNA in MCF-7 and LCC9 cells using qRT-PCR on the same time scale as the ChIP. Analysis of transcripts induced after the 1hr exposure to ligands showed that E2 strongly induced Bcl-2 transcripts 5-fold while L17 and WAY reduced Bcl-2 mRNA below basal levels compared to control in both cell lines (Figure 3.5G). For comparison we also analyzed the pS2 gene by ChIP and qRT-PCR. All ligands recruited both ERα and ERβ to the pS2 promoter in LCC9 cells (Figure 3.5B, D & F), which corresponded to weak induction (E2) (almost 2-fold) or inhibition of expression (L17 and WAY) (Figure 3.5H). Although all three ligands also recruited both the ERα and ERβ in MCF-7 cells (Figure 3.5A, C & E), only E2 strongly increased pS2 mRNA 6.5-fold (Figure 3.5H). We also analyzed mRNA levels 24hr following treatment to assess the longer term effects on transcript levels. Bcl-2 mRNA remained elevated after 24hr in MCF-7 cells treated with E2 however levels remained close to or below vehicle control in L17 and WAY-treated cells (Figure 3.5I). pS2 transcripts also maintained a similar pattern of expression after 24hrs although L17 induced a small (1.5-2-fold) increase in pS2 at this later time point in both cell lines (Figure 3.5J).

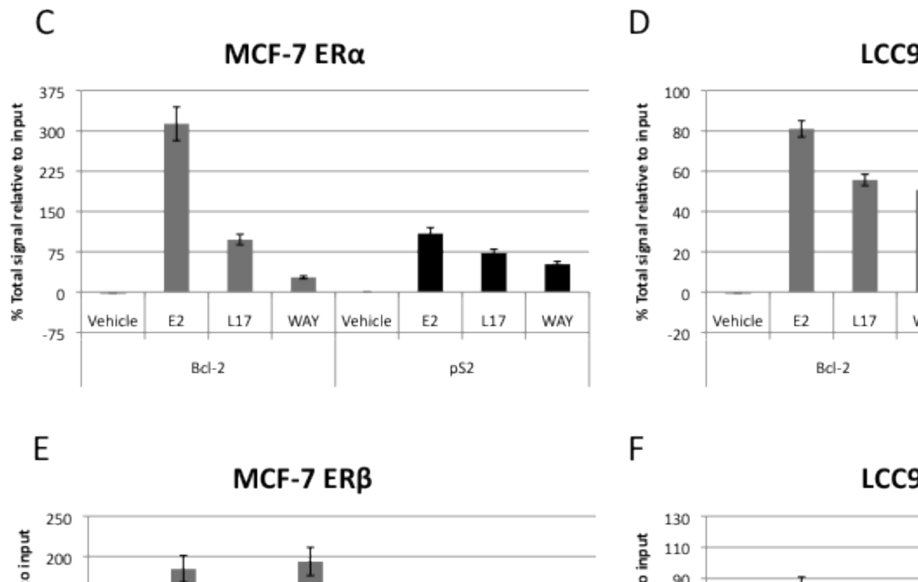
RNA Polymerase II (Pol II) forms a large transcription complex with general transcription factors and multiple other proteins (Boeger et al, 2005). Although measuring the mRNA transcript levels of Bcl-2 and pS2 genes was meant to determine the direction of transcription (up or down) after binding of the receptor to chromatin, Investigating the binding of RNA Pol II around transcription start sites on the Bcl-2 and pS2 genes may be another important indication of transcription of these genes.

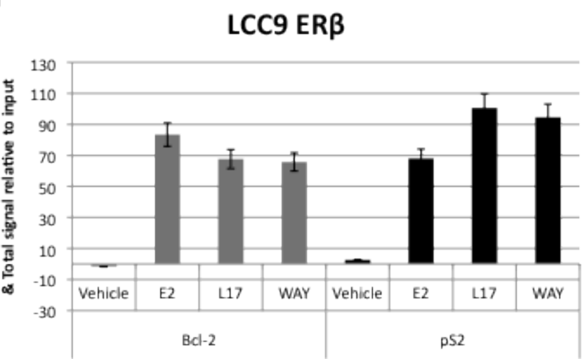
In summary, although both L17 and WAY induced recruitment of both the ERα and the ERβto the promoters of the Bcl-2 and pS2 genes transcription was repressed over the short term. These results suggest that L17 and WAY behave as inverse agonists mediating a reduction in basal transcription from the promoters regulated by these EREs. In order to address whether

MCF-7 cells


A
Input
IgG
ERα
ERβ
217 bp
212 bp
Exon 2 ERE Bcl-2
5’ ERE pS2
Exon 4 Bcl-2
EtOH
E2
L17
WAY


LCC9 cells
B
Input
IgG
ERα
ERβ
217 bp
212 bp
Exon 2 ERE Bcl-2
5’ ERE pS2
Exon 4 Bcl-2
EtOH
E2
L17
WAY


C
MCF-7 ERα
% Total signal relative to input
375
300
225
150
75
0
-75
Vehicle
E2
L17
WAY
Vehicle
E2
L17
WAY
Bcl-2
pS2
D
LCC9 ERα
% Total signal relative to input
100
80
60
40
20
0
-20
Vehicle
E2
L17
WAY
Vehicle
E2
L17
WAY
Bcl-2
pS2
E
MCF-7 ERβ
% Total signal relative to input
250
200
150
100
50
0
-50
Vehicle
E2
L17
WAY
Vehicle
E2
L17
WAY
Bcl-2
pS2


F
LCC9 ERβ
& Total signal relative to input
130
110
90
70
50
30
10
-10
-30
Vehicle
E2
L17
WAY
Vehicle
E2
L17
WAY
Bcl-2
pS2

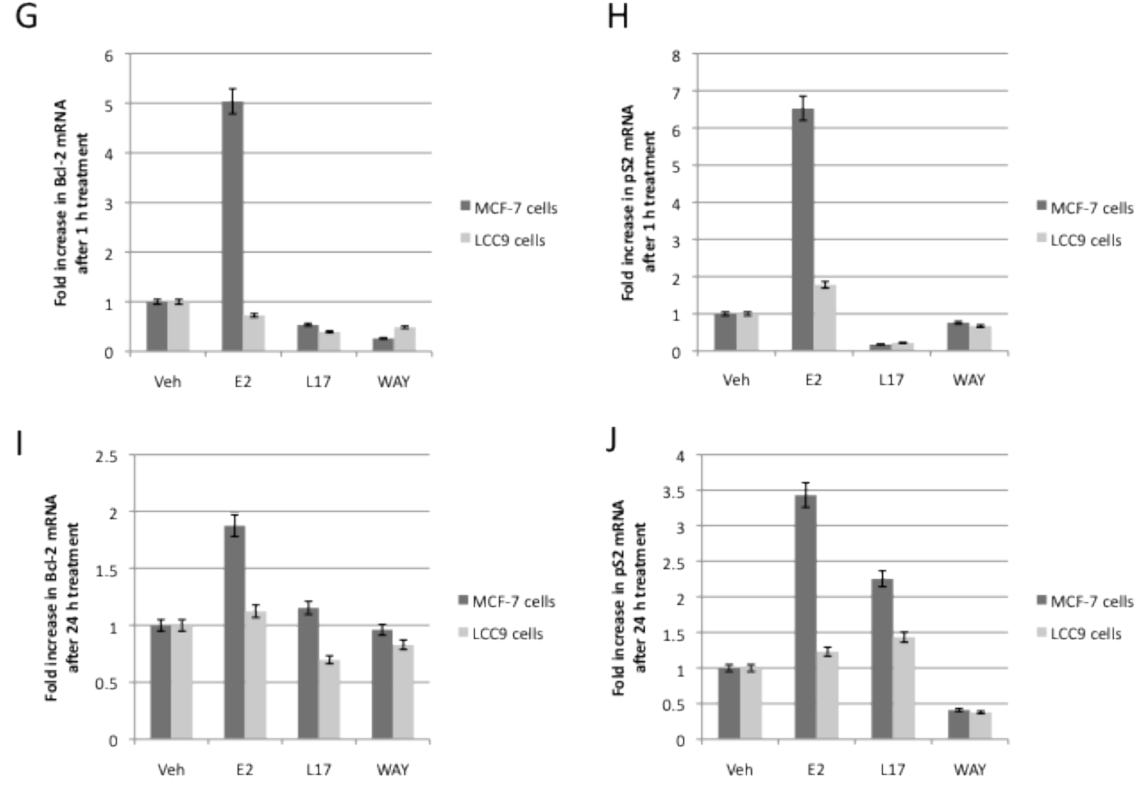


Figure 3.5. Recruitment of the ERα and β to the EREs of the Bcl-2 and pS2 genes in response to ligands. The status of the endogenous complex present on the estrogen response element in the Bcl-2 and pS2 genes was determined using chromatin immunoprecipitation. **A,** MCF-7 cells and **B,** LCC9 cells were grown in phenol red free media with charcoal stripped serum and treated for 1 hour with vehicle control, or 10 nM E2, L17 or WAY. Chromatin was then crosslinked and sonicated to shear DNA followed by immunoprecipitation was anti-ERα and anti-ERβ antibody. Following immunoprecipitation the crosslinks were reversed, the DNA was extracted and the interactions with ERα and ERβ were analyzed by PCR using specific primers targeting the ERE in exon 2 of the Bcl-2 gene and the ERE in the promoter region of the pS2 gene. A region from Exon 4 of the Bcl-2 gene was used as a negative control. Densitometric analysis of ChIP results using the anti-ERα antibody and PCR analysis of Bcl-2 and pS2 EREs in **C,** MCF-7 cells and **D,** LCC9 cells. Densitometric analysis of ChIP results using the anti-ERβ antibody in **E,** MCF-7 and **F,** LCC9 cells. The y- axis represents Image J quantitation 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 no-antibody (IgG) lane was subtracted from each sample as a nonspecific binding background. Total RNA was extracted from MCF-7 and LCC9 cells following a 1hour (**G** and **H,** respectively) and a 24 hour treatment (**I** and **J,** respectively) with vehicle or 10 nM E2, L17 or WAY. qRT-PCR was performed to determine Bcl-2 and pS2 transcript levels and expressed as a fold difference over cells treated with vehicle control (EtOH) and is indicated along the y-axis. . Data represents the mean from 2 separate experiments each performed in triplicate; error bars indicate standard error.

homodimers or heterodimers are recruited by L17 or WAY, a ChIP re-ChIP should be performed on a cell-by-cell basis. After the first immunoprecipitation with ERβ antibody, a sequential immunoprecipitation is accomplished with the addition of the ERα. If the second immunoprecipitation fails we know that ERβ homodimers are being recruited, however if the second immunopreciptation is successful we can confirm the presence of ERα:ERβ heterodimers on the ERE of the Bcl-2 gene.

ERβ agonists enhance the cytotoxic effect of chemotherapeutic agent Adriamycin in LCC9 cells

Since we have demonstrated that ERβ activation by L17 results in the downregulation of the key anti-apoptotic protein, Bcl-2, we examined the ability of ERβ agonists L17 and WAY to sensitize ER+ breast cancer cells to cytotoxic chemotherapeutics including the anthracycline, Adriamycin (Adr). Adr is one of the most widely used chemotherapeutic drugs in the treatment of cancer where it inhibits topoisomerase II causing DNA damage, subsequently inducing double strand breaks, and by forming DNA adducts (Tewey et al., 1984). The cytotoxic effects of the combination of L17 or WAY and Adr in LCC9 cells was evaluated after 72 h. Figure 3.6 shows that the simultaneous exposure of 200 uM chemotherapeutic agent Adr with 10 nM of either ERβ agonist, L17 or WAY, caused a significant further inhibition of LCC9 cell growth reducing proliferation by ~65% compared to control (Figure 3.6). These results show that the addition of an ERβ agonist enhanced the chemtherapeutic effect of Adr in these cells beyond that seen with the standard chemotherapeutic drug used along. The downregulation of Bcl-2, induced by L17 and WAY, may serve a role in sensitizing LCC9 cells to Adr, leading to cell death.

Chloroquine converts L17/WAY-induced autophagy to apoptosis

Chloroquine (CQ) is a 4-aminoquinoline drug that acts by inhibiting lysosomal acidification thereby impeding the fusion of autophagosomes with lysosomes and subsequent degradation, preventing autophagy (Maycotte et al, 2012). Since WAY and L17 reduced Bcl-2 and increased levels

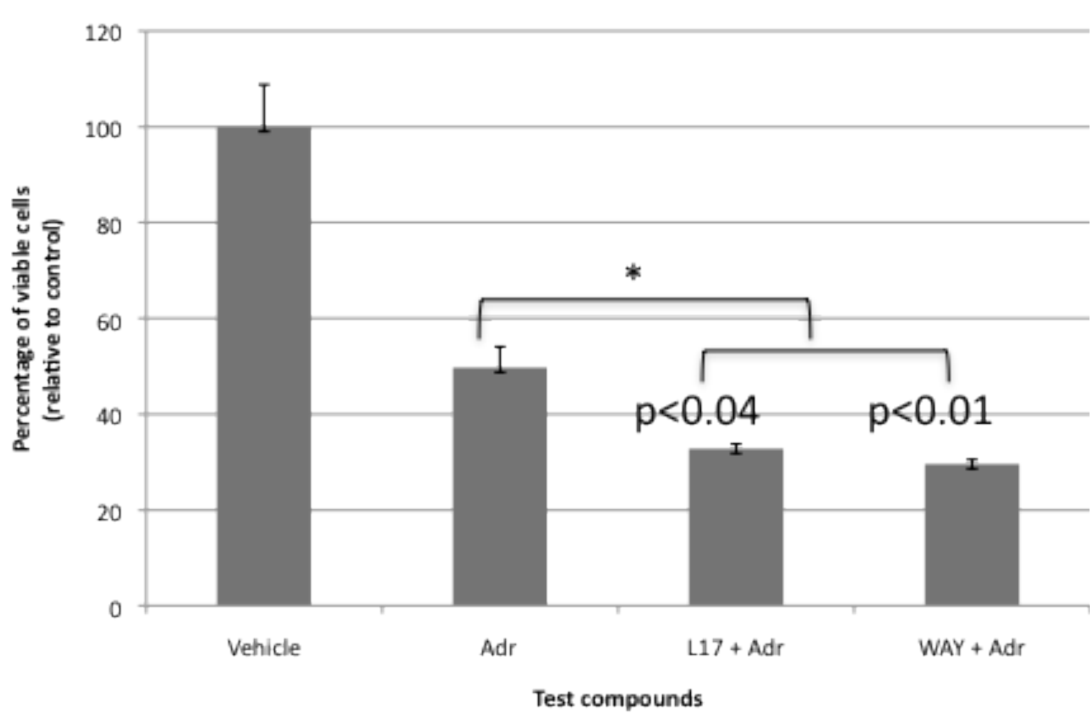
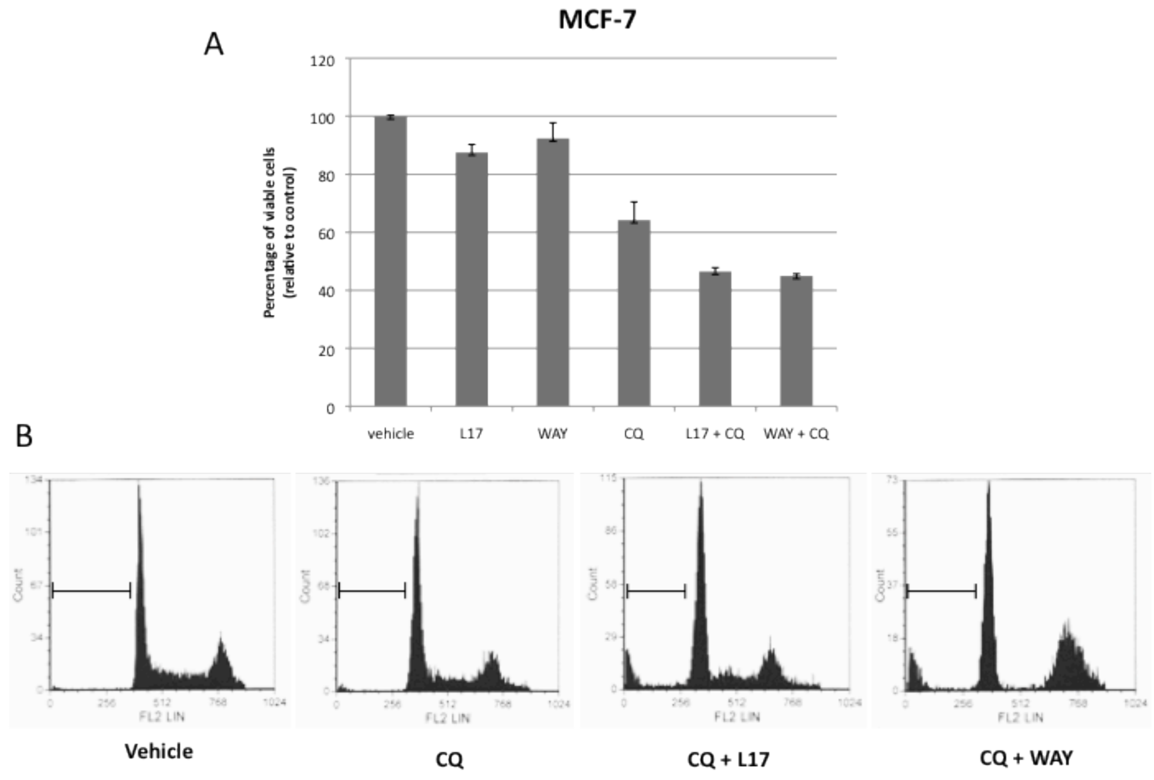


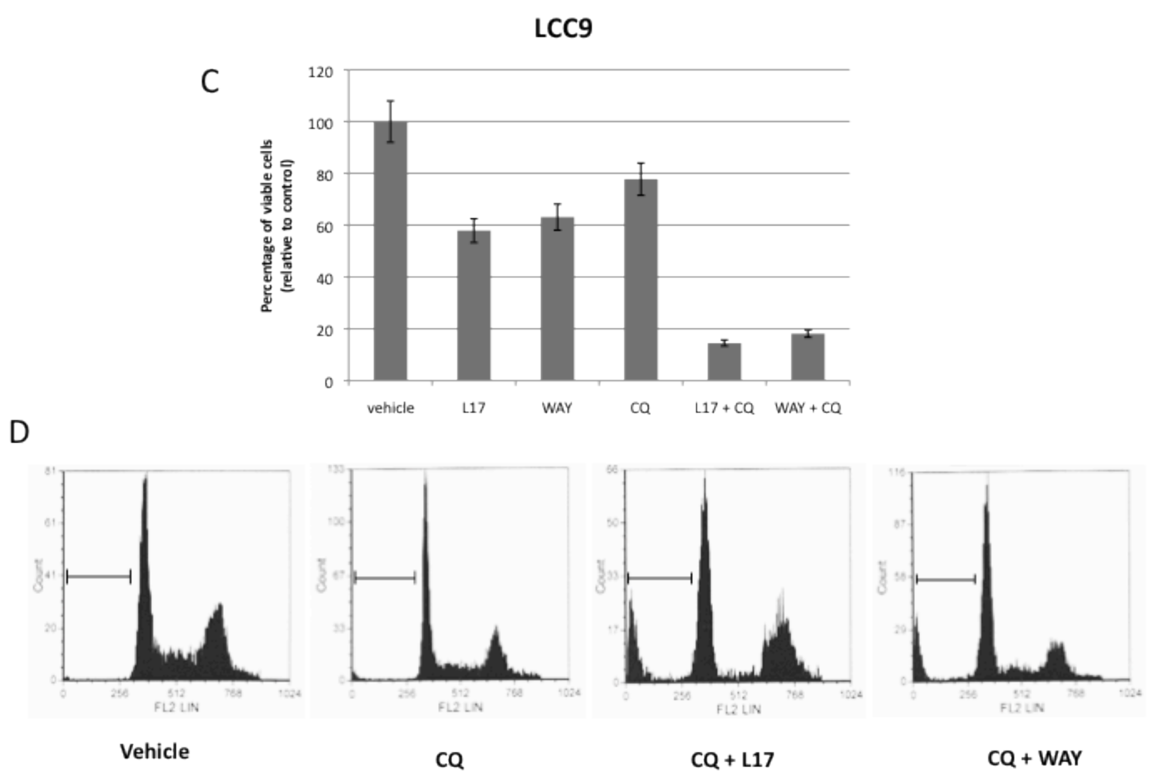
Figure 3.6. Effects of cotreatment with ERβ agonists and Adriamycin (Adr) in LCC9 SERD-resistant cells. Cells were grown in phenol red free with charcoal stripped serum and treated simultaneously with 10 nM L17 or WAY in combination with 200 nM Adr. After 3 days, viable cells were enumerated using the trypan blue exclusion test. Data represents the mean from 3 separate experiments each performed in triplicate; error bars indicate standard error.

of LC3-II particularly in LCC9 cells we tested the possibility that CQ might convert autophagy to apoptosis. MCF-7 and LCC9 cells were grown in PRF media with CSS and treated with vehicle (EtOH) or 10 nM E2, L17 or WAY in the presence or absence of 33 μM CQ. After 5 days the cells were collected and viable cells were counted using trypan blue. Subsequently, DNA was stained with PI and analyzed by flow cytometry. Figure 3.7A shows that a 5 day treatment with CQ alone in E2-free conditions caused an approximate 35% decrease in MCF-7 cell numbers and a 20% decrease in LCC9 cells compared to vehicle-treated cells (Figure 3.7C). Strikingly, the combination of CQ and L17 or WAY induced a dramatic 80% decrease in LCC9 cell numbers compared to control, which correlated to the induction of sub-G1 DNA (Figure 3.7D). The simultaneous combination treatment of CQ and the ERβ agonist also reduced MCF-7 viable cells in association with a subG1 peak (Figure 3.7B) albeit not as dramatically as in LCC9 cells due to L17 or WAY.

Apoptosis is a cell death process that relies on the activation of caspases (Egger et al, 2003). In order to confirm that the combination treatment of CQ plus an ERβ agonist results in apoptosis, we included Z-VAD-fmk in some cultures. The Z-VAD-fmk is a general caspase inhibitor that blocks apoptosis by crosslinking the fluoromethylketone (fmk) group to the cysteine in the catalytic site of all caspases, thus inhibiting their activation (Nicholson, 1999). Significantly, the inhibitory effect of L17 or WAY on Bcl-2 plus the inhibitory effect of CQ on the autophagic pathway was reversed by the addition of 100 μM caspase inhibitor Z-VAD-fmk (Figure 3.7E), based on the absence of sub-G1 DNA revealed by flow cytometric analysis (Figure 3.7F). Together these findings suggest the effect of L17 or WAY plus CQ is caspase-dependent ultimately leading to apoptosis.

Thus, SERD-resistant LCC9 cells expressing a high ratio of ERβ:ERα undergo marked apoptosis in the presence of ERβ agonists L17 or WAY and the autophagy inhibitor CQ. In future studies, Annexin V, labeled with a fluoresecent tag, such as FITC, used with flow cytometry may be a more accurate measure of apoptosis versus necrosis.


MCF-7
A
Percentage of viable cells (relative to control)
120
100
80
60
40
20
0
vehicle
L17
WAY
CQ
L17 + CQ
WAY + CQ
B
Count
FL2 LIN
Vehicle
CQ
CQ + L17
CQ + WAY


LCC9
C
Percentage of viable cells (relative to control)
120
100
80
60
40
20
0
vehicle
L17
WAY
CQ
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D
Count
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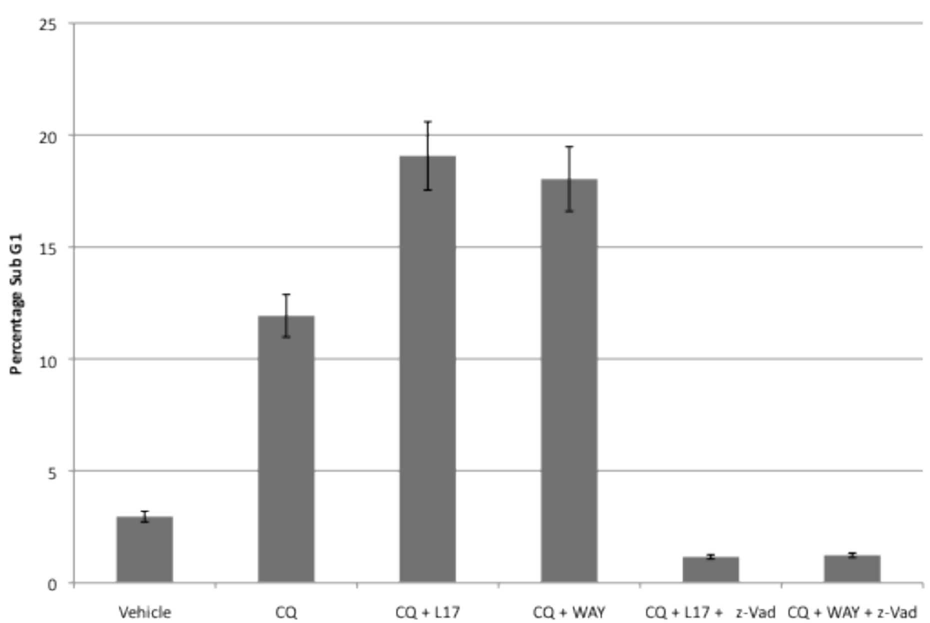
Vehicle
CQ
CQ + L17
CQ + WAY

E



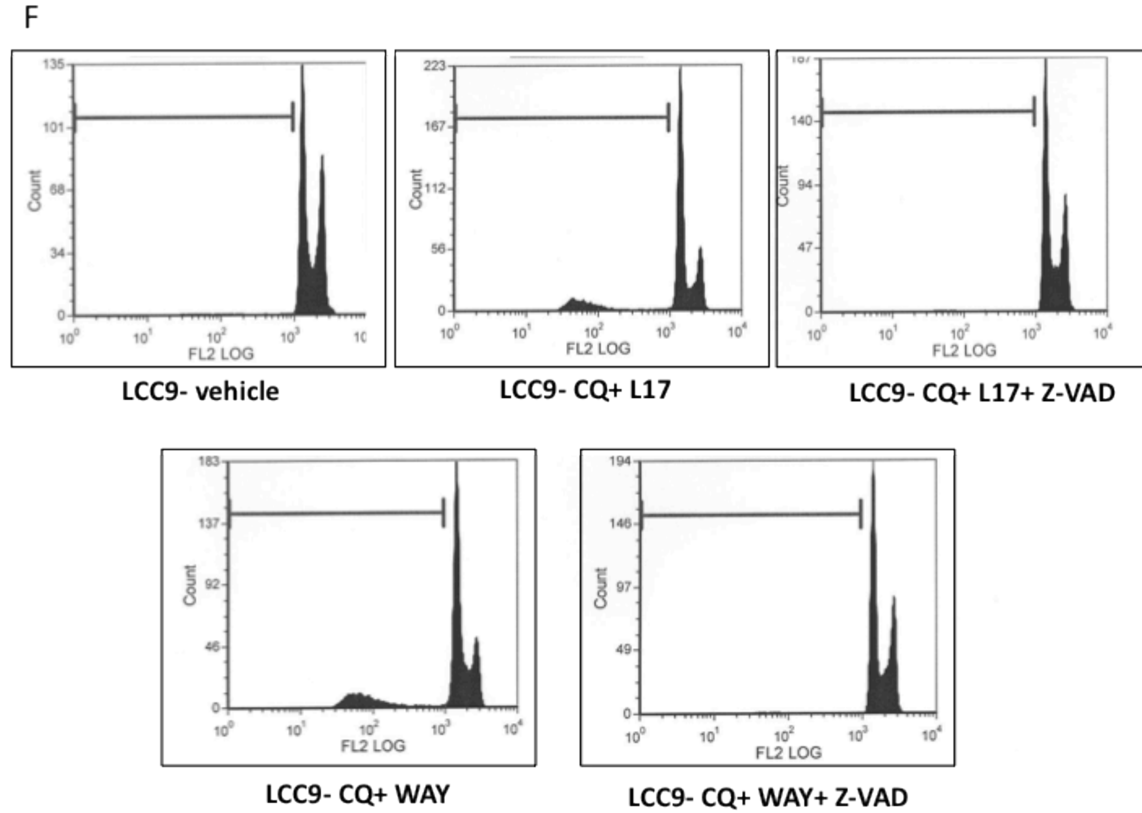


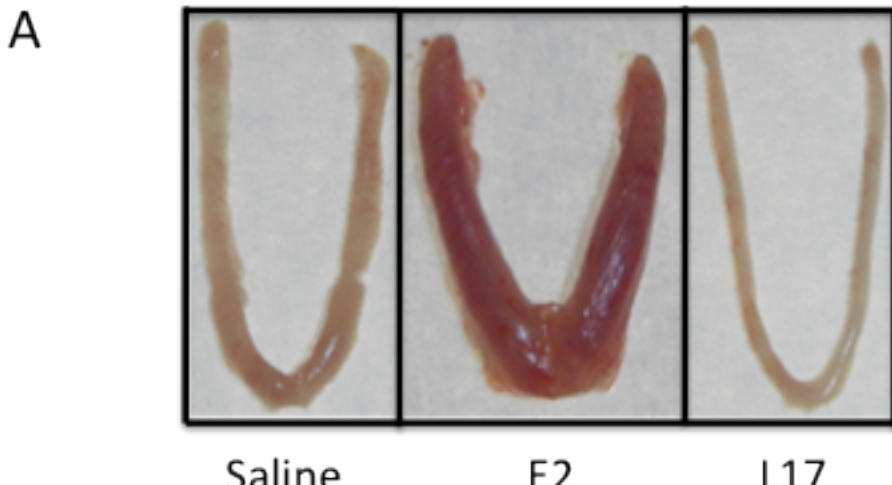
Figure 3.7. Inhibition of autophagy induces cell death when combined with ERβ agonists. MCF-7 and LCC9 cells were grown in PRF media with CSS and treated with vehicle, 10 nM L17 or WAY, with or without 33 µM chloroquine. **A,** MCF-7 and **C,** LCC9 cells were counted using trypan blue exclusion after a total of 5 days. Subsequently **B**, MCF-7 and **D**, LCC9 cells were then collected and DNA content was determined by staining with propidium iodide followed by flow cytometry to detect subG1 DNA. **E,** LCC9 cells were grown in PRF media with CSS and treated with vehicle, 10 nM L17 or WAY, 33 µM CQ, with or without 100 µM caspase inhibitor Z-VAD-fmk. **F,** Representative histograms from flow cyomtery analysis highlighting Sub-G1 DNA in the absence of Z-VAD-fmk. Data represents the mean of triplicate determinations. Bars indicate standard deviation.

L17 is not uterotrophic and does not induce proliferation in the rodent mammary gland.

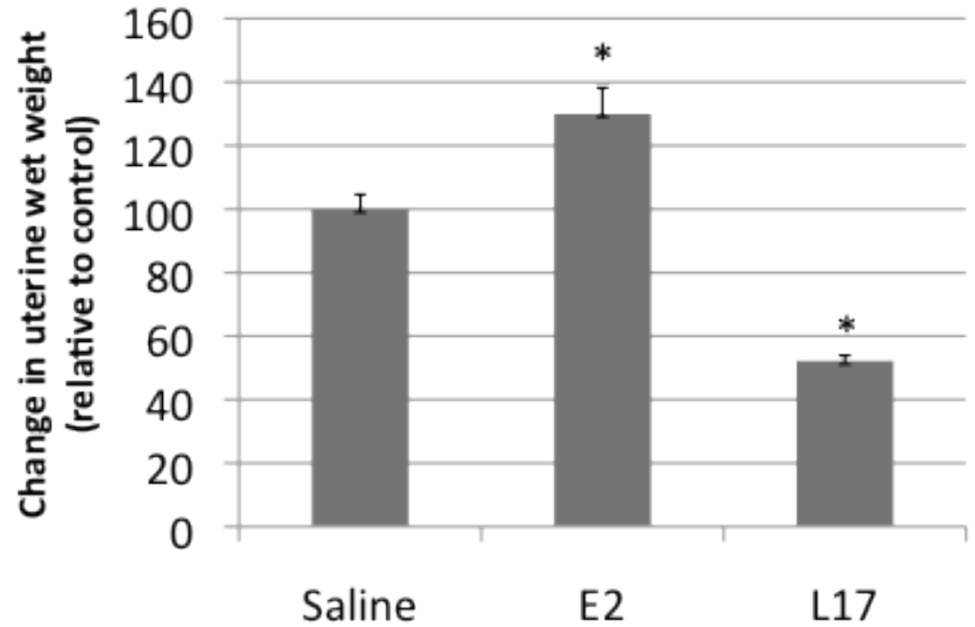
It is well established that estrogen therapy is associated with a proliferative effect on the uterus and an increased risk of endometrial cancer (Pinkerton et al, 2010). Therefore uterine safety must remain a central underlying criterion in the development of new alternate drugs, such as L17, that are to be used for long term therapies. One established method for determining the estrogenicity of a new chemical is the in vivo immature rodent uterotrophic assay which measures morphological endpoints such as the height of the luminal epithelium and uterine wet weight, two markers indicative of uterine response to estrogens (Reel et al, 1996). We accomplished this by injecting 3-week-old female CD-1 pre-pubertal mice subcutaneously with 1 mg/kg body weight E2, L17, or Saline vehicle control daily for 5 days. Uteri were then excised and weighed.

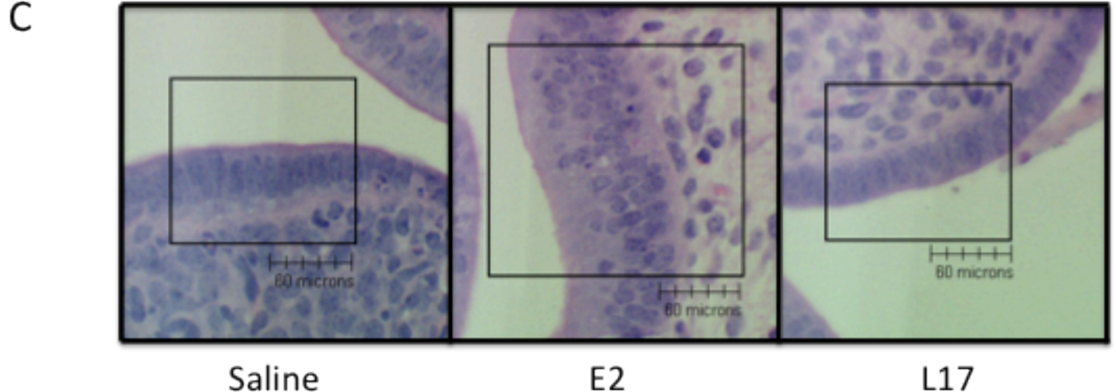
Figure 3.8A shows 4-week-old CD-1 uteri treated with the indicated ligand (1mg/kg) once daily for 5 days. Morphologically, the uteri from mice treated with E2 was more muscular and vascularized compared to vehicle- or L17-treated uteri which more thin and elongated. E2 induces uterine growth in immature female mice, which was confirmed by our study showing a 30% increase in uterine weight compared to control ($p<0.05$, by t-test) (Figure 3.8B). On the other hand, L17 did not have a stimulatory effect on the mouse uterus, but in fact, significantly decreased uterine wet weight by 50%. ($p<0.005$, by t-test) (Figure 3.8B). Figure 3.8C shows the effects of the indicated ligand on the epithelial lining of the mouse uterus after 5 days of treatment. Histological assessment of the effects of E2 on the uterus exhibited a thickening of the epithelial lining in

A



B





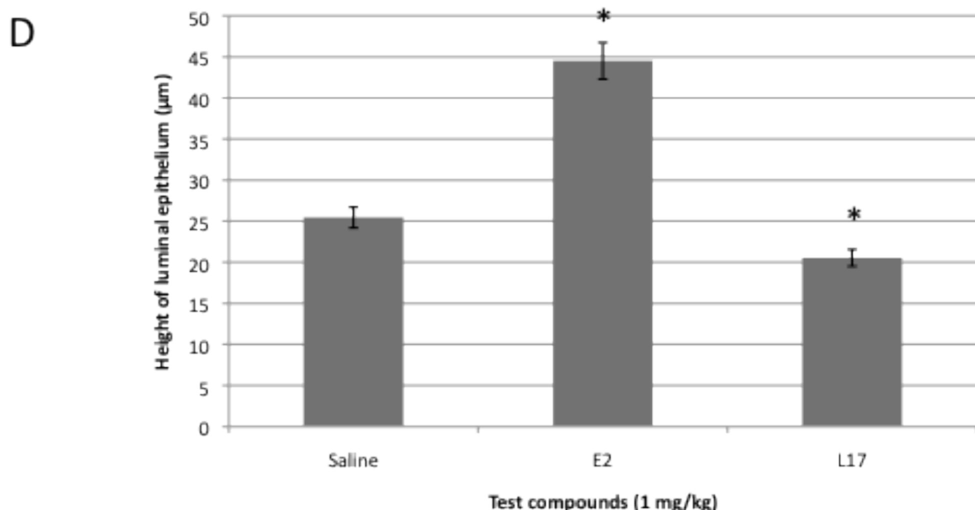


Figure 3.8. Morphological and histological assessment of the effects of L17 and E2 on mouse uteri. **A**, Morphological assessment of representative4-week old CD-1 mouse uteri following a 5 day treatment with the indicated ligand (1mg/kg). The uterus from mice treated with E2 was more muscular and vascularized. **B**, Mean uterine weights of prepubertal CD-1 mice after 5 days of treatment with indicated ligands (1mg/kg). Values represent the mean of 5 uterine weights from 5 mice per treatment group. Uteri from mice treated with the ERβ- agonist L17 resulted in a significantly reduced uterine wet weight relative to control (Saline). *Statistically significant differences between treated mice and the control mice using Student T-test ($p<0.05$). Error bars indicate standard error. **C,** Representative photomicrographs of hematoxylin and eosin stained 4 week old CD-1 mice uteri treated subcutaneously with vehicle control (Saline) or 1 mg/kg body weight E2 or L17 once daily for 5 days. E2 treatment elicited a uterotrophic effect whereas L17 did not. **D**, Mean height of the luminal epithelium of CD-1 mice after 5 days of treatment with indicated ligands. Values represent the mean of 5 measured heights of the luminal epithelium from 5 mice per treatment group. Uteri from mice treated with the ERβ- agonist L17 resulted in a significantly reduced uterine luminal epithelium cell height relative to control (Saline). *Statistically significant differences between treated mice and the control mice using Student T-test ($p<0.05$). Error bars indicate standard error.

response to 1 mg/kg body weight E2 ($p<0.05$), representing a 178% increase in luminal epithelial cell height (Figure 3.8D). Meanwhile, treatment with L17 elicited a small yet significant decrease ($p<0.05$) in luminal epithelium cell height compared to control, reflecting a 25% decrease (Figure 3.8D). Structural hypoplasia of the uterus and decreased responsiveness of the uterus to an estrogenic challenge following treatment with L17 was observed compared to E2-treated uteri. The mechanism underlying this effect is currently unknown however further investigation is required.

Figure 3.9 shows that that even after 5 days of treatment, a discernable difference in the overall density of the rodent mammary glands was observable. On a higher magnification (2x) ERβ-selective L17-treated mammary glands exhibit a decrease in side-branching and fewer terminal end buds compared to E2 treated glands (Figure 3.9). We can also observe in the mammary gland of mice treated with E2 the beginning of alveolar development in the duct, which appears to be lacking in control and L17-treated mammary glands. Together these results are consistent with a lack of proliferative activity of the ERβ selective ligands, and unlike E2, these data confirm that L17 does not induce proliferative effects in rodent uterine or mammary tissues.

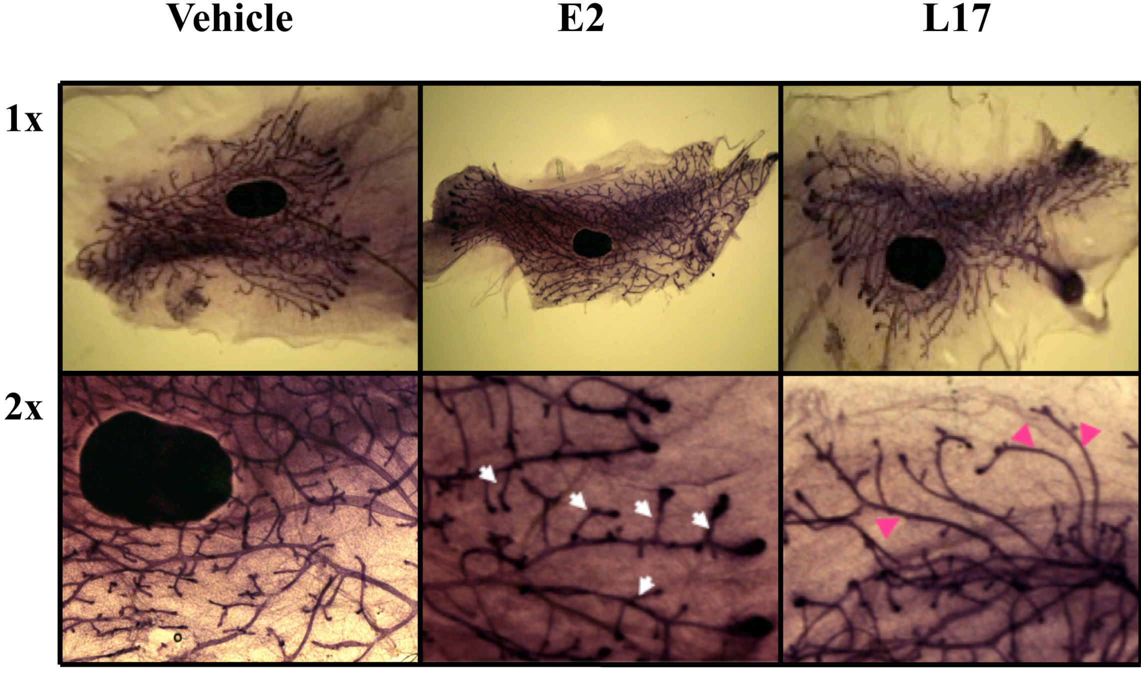


Figure 3.9. Morphological assessment of the effects of L17 and E2 on normal mammary gland development. CD-1 mice were treated subcutaneously with vehicle control (Saline) or 1 mg/kg body weight E2 or L17 once daily for 5 days. Representative mammary gland wholemounts of the fourth inguinal mammary glands were prepared as described in Materials and Methods and compared to detect changes in ductal branching, end bud and alveolar development (top panel; 1x magnification). At a higher magnification, white arrows show side branching in E2-treated glands while pink arrows point to regions in L17-treated glands that have relatively little side branching and fewer end bud formation (bottom panel; 2x magnification).

CHAPTER IV - DISCUSSION

Summary

A major barrier in the clinical treatment of breast cancer is the development of acquired resistance to endocrine therapies such as Tamoxifen (Tam) and Fulvestrant. The identification of a second estrogen receptor, ERβ, has sparked great interest in elucidating and characterizing its biological function from ERα and its potential therapeutic value in many diseases such as breast cancer. The use of ER-selective agonists have proven very effective in distinguishing physiological roles of both ER subtypes and preferential-ERβ agonists may be useful for a variety of clinical applications because they are not expected to induce the classic estrogenic side effects associated with the ERα such as uterine stimulation and have been shown to be anti-proliferative and anti-tumorigenic. While ER positive (ER+) breast cancers typically express a high ratio of ERα: ERβ, many endocrine resistant breast cancer tumors show a higher ratio of ERβ:ERα. The results in this study present a novel strategy for the treatment of endocrine-resistant breast cancer. A unique feature of this study is our use of an preferential-ERβ agonist, L17, in a cell line that naturally overexpresses ERβ relative to ERα, to achieve differential occupancy of ERβ, even when both ER subtypes are present. Previous studies oftentimes manipulate the cellular environment to overexpress the ERβ either through gene therapy or via treatment with a drug, like Fulvestrant that is known to downregulate and degrade ERα. Instead, we used an antiestrogen-resistant cell line, LCC9, that exhibits cross-resistance to Tam and Fulvestrant and inherently has a greater ERβ:ERα ratio. We show that ERβ agonists, L17 and WAY produce a marked reduction in G2/M phase of the cell cycle which correlates with effects on cyclin D1 and cyclin E expression in a Tam/Fulvestrant-cross resistant cell line. In addition, L17 and WAY suppress the expression of Bcl-2 protein and mRNA, which results in the activation of the autophagic pathway. This was demonstrated by an increase in LC3-II protein in Tam/Fulvestrant- cross-resistant breast cancer cells. Moreover, we demonstrate

that by inhibiting autophagy with an alysosomotropic drug, chloroquine (CQ), and simultaneously downregulating Bcl-2, results in apoptosis, indicated by the accumulation of sub-G1 DNA following treatment with the ERβ-agonists. Finally we show that combination treatment of LCC9 cells with ERβ agonists, L17 or WAY with the chemotherapeutic agent, Adriamycin (Adr), enhances the cytotoxic effect of the chemotherapeutic agent. Our *in vivo* studies also confirm that L17 is not uterotrophic or mammotrophic.

Characterization of L17

The discovery that estrogens act in target tissues by binding to two estrogen receptor (ER) subtypes, ERα and ERβ (Kuiper, 1997; Blesson et al, 2006) has resulted in major efforts to develop ligands that are selective agonists for either subtype, with additional attempts to classify certain ligands as selective estrogen receptor modulators (SERMs). Although both ER subtypes have many functional similarities in terms of substrate and ligand binding affinities, differences do exist in the mechanisms regulating their transcriptional activities (Gustafsson, 1997). For instance, when several studies have compared the activity of the AF-1 and AF-2 domains of ERα and ERβ, and have established that the AF-1 in the N-terminus of ERα is constitutively active enhancing ERα-mediation transcription, while the corresponding region in ERβ is negligible compared to that observed in ERα (Hall et al, 1999; Warnmark et al, 2002). In contrast, ERβ contains a repressor domain that significantly decreases its overall transcriptional activity (Hall et al, 1999). It is important to note that the respective AF-1 activities are cell and promoter specific. Additionally, the ERα-AF-1 and AF-2 domains act synergistically under most circumstances, whereas the AF-2 of the ERβ mainly functions autonomously (Hall et al, 1999; ed et al, 2000). This suggests that ERα and ERβ will display differences in their preferences for co-activators and co-repressors in certain target cell types (Hall et al, 1999). With the evidence showing that the principle role of ERα is to promote cell growth and survival while the ERβ is shown to be antiproliferative, targeting the ERβ using subtype-specific

ligands may be a valuable potential therapeutic target and prove relevant in the treatment of breast cancer.

To this end, we have developed a new ERβ-selective agonist, L17, which is based on the ABCD ring structure of estradiol (E2) but unlike E2 lacks the B ring (Figure 4.1) and strongly resists metabolism to quinones, which are potential mutagens. Research has shown that when E2 is metabolized it can trigger chemical events in cells that have marked disease potential (Bolton et al, 1998; Cavalieri et al, 2006). Unlike the vast majority of ERβ-selective ligands, which are structurally unrelated to E2, L17 is based on the structure of E2 and preliminary results suggest that it is less likely to result in toxicity (Tables A1 & 2). We reasoned that placing a fluorine (F) substituent at the ortho position physically blocks the site for OH addition to form the semi-quinone and then the quinone. The P450 hydroxylase enzyme needs an H-atom in order to substitute an OH. Thus, L17 has both ortho sites blocked.

If this compound is to be used in the endocrine treatment of breast cancer and administered over many months and in otherwise healthy individuals, it is critical that L17 has low toxicity. Therefore, we indirectly assayed for evidence of quinone formation and observed that L17 is highly stable compared with E2 (Table A1) which has the propensity to be oxidized and form quinones (Cavalieri et al, 2006). Consistent with this result, L17 also showed the highest LC50 value (the concentration at which a chemical causes 50% lethality/death) in a hepatocyte cytotoxicity assay (Table A2) indicating significantly reduced toxicity in hepatic cells, relative to E2. This is most likely due to the lack of enzymatic hydroxylation taking place when both ortho positions to the hydroxyl group on the phenol ring are occupied. E2 on the other hand has a unprotected phenol ring. Thus, the presence of unprotected ortho positions at R2, R4, and R5 in E2 (Figure 4.1), render it susceptible to quinone formation.

Parent A-CD Compound | E2 | L17

Figure 4.1. The chemical structure of the parent A-CD compound is presented highlighting the structural differences between E2 and L17. The B ring is lost in the A-CD compound L17 while it is still present in E2. Additionally, L17 has an A ring completely occupied by Fluorine substituents compared to E2 which has an unprotected phenol ring.

The ER acts on estrogen response elements (EREs) to either transactivate or transrepresss the expression of estrogen responsive target genes (Rivera-Gonzalez et al, 1998). In this study, we were interested in determining selective the effects of a new ligand, L17. L17 represents a new chemical entity that demonstrates a 10-fold higher binding affinity for ERβ over ERα. In order to confirm L17's selectivity for the ERβ we assessed the ligand induced transcriptional activation of a transfected synthetic ERE in human Hek293 cells in response to L17 compared to E2. Our results showed that L17 weakly induces ERα-mediated transcription at low concentrations (Figure 3.1B), but strongly activated the ERβ when compared to E2 on the same reporter gene at an optimal concentration of 10nM (Figure 3.1C). In addition to L17, we evaluated a second selective ERβ-agonist called WAY-200070 (WAY) (Figure 3.1A). WAY is an aryl diphenolic azole developed by Wyeth (Princeton, NJ). Similar to L17, WAY exhibits selectivity for the ERβ over the ERα, although with a much higher affinity (RBA = 68) (Harris, 2006), and shows similar transactivation properties as L17 on the ERα and the ERβ at 10 nM (Figure 3.1F & G). In this study, we focus on characterizing and elucidating how these two ERβ-agonists, L17 and WAY, exert their effects at the molecular level on different breast cancer cell lines. In addition, we will explore their potential clinical utility.

ERβ agonists inhibit proliferation of LCC9 cells

Numerous studies have shown that exposure of ERα-positive breast cancer cells to E2 promotes cell growth and survival while recent reports have demonstrated that expression of ERβ alone (Lazennec et al, 2001; Thomas et al, 2011) or co-expressed with ERα (Paruthiyil et al, 2004; Strom et al, 2004; Sotoca et al, 2008) inhibits cell proliferation following E2 treatment. It is well established that activated ERβ is capable of repressing the transcriptional activity of the ERα in heterodimeric complexes through a repressor domain within its AF-1 function (Hall et al, 1999; Gougelet et al, 2007). The ability of the two ER subtypes to heterodimerize when present in the same cell can have serious impacts on gene regulation (Chang et al., 2006). However, this biological

activity of ERβ that reduces E2 effects through ERα might be accounted for by factors beyond the formation of heterodimers. In fact, ERβ mRNA and protein is oftentimes decreased in tumor cells of various cancers including breast, ovarian, prostate and colon compared to normal tissues (Brandenberger et al, 1998; Pujol et al, 1998; Campbell-Thompson et al, 2001; Roger et al, 2001; Fixemer et al, 2003). Thus, the ERα:ERβ ratio appears to increase during carcinogenesis.

In vitro studies have shown that the relative ratio of ERα:ERβ in any given cell largely determines the response of that cell to estrogen and antiestrogens at the level of gene expression (Stossi et al, 2004; Monroe et al, 2005; Heldring et al., 2007). Further, the differential expression of ERα and ERβ in breast cancer cells has implications on proliferation, due to each of the receptor's respective roles. 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. Therefore scientists have made strides to manipulate the cellular environment by overexpressing ERβ protein relative to ERα through gene transfer techniques to determine the effects of ERβ activation in different breast cancer cells. For instance, Omoto et al (2003) and Paruthiyil and colleagues (2004) both reported that stably expressed ERβ in MCF-7 breast cancer cells has negative effects on proliferation, while Strom et al (2004) and Hartman et al (2006) demonstrated growth inhibition of E2-responsive T47D breast cancer cells following ERβ overexpression. These growth inhibitory effects were associated with repressed expression of a subset of DNA replication and cell-cycle related genes which include c-myc, cyclin D and cyclin A (Strom et al, 2004; Paruthyil et al, 2004), an increase in the expression of antiproliferative p21 (WAF1) and p27 (Kip1) (Paruthiyil et al, 2004), reduced cell motility and invasion (Platet et al, 1999; Lazennec et al, 2001) as well as reduced T47D tumor formation in a mouse xenograft model. Our data (Figure 3.2A) show that ERβ expression is upregulated in an antiestrogen-resistant breast cancer cell line (LCC9), which is in agreement other studies (Speirs et al, 1999) and is consistent with the hypothesis that ERβ plays a role in Tam resistance (de Cremoux

et al, 2003). These cell lines, most of which have been obtained *in vitro* following long term culture in the presence of SERMs have acquired resistance and have greatly helped facilitate research on acquired resistance (De Cremoux et al, 2003). The LCC series, which we use in these experiments, was established to further evaluate cross-resistance phenotypes and underlying mechanisms (Clarke et al, 2001). LCC9 cells express an increased ratio of ERβ to ERα compared to SERM-sensitive MCF-7 cells (Figure 3.2A) and exhibit cross –resistance to Fulvestrant and Tam (Brunner et al, 1997).

Our data show that treatment with an ERβ agonist, L17 or WAY, in SERM/SERD resistant LCC9 cells that express a higher ratio of ERβ:ERα, results in significantly reduced cell viability after a total of 5 days (Figure 3.2C). This demonstrates that the increase in ERβ:ERα in LCC9 cells may play a key role in conferring sensitivity to an ERβ agonist. Interestingly, we also show that in MCF-7 cells that are sensitive to the growth inhibitory effects of SERMS like Tam and Fulvestrant and express a higher ERα:ERβ ratio, L17 and WAY have little effect on cell proliferation (Figure 3.2B).

Antiestrogens such as Tam primarily affect ERα exhibiting partial agonist/antagonist function but behave as pure antagonists through ERβ (Osborne et al, 2000; Heldring et al, 2004). Consistent with this, the bulk of clinical studies show that patients whose tumors express ERβ positively respond to Tam in the adjuvant setting, meanwhile patients that co-express both ERs, respond better to Tam compared to patients with tumors expressing only the ERα (Herynk et al, 2004; Fox et al, 2008). Clinical findings (Speirs et al, 1999) alongside our *in vitro* data confirm that an increase in ERβ expression is associated with decreased cell survival in the presence of an ERβ agonist. Differences in ligand affinity, transcriptional activation, recruitment of distinct groups of cofactors, and heterodimerization all need to be considered as potential mechanisms contributing to the differential actions of ERα and ERβ (Klinge et al, 2004). The DNA binding domains of the ERs differ by one amino acid residue and ERα and ERβ homodimers and heterodimers can recognize and bind to the same ERE motifs (Cowley et al, 1997; Pace et al, 1997; Pettersson et al, 1997; Li et al,

2004). However, because ERα homodimers are more stable than ERα/ERβ heterodimers and even more so than ERβ homodimers, the fraction of heterodimers will be reduced, thus ERα dominance could influence the moderating effect that ERβ has on ERα gene regulatory activity (Charn et al, 2010). Therefore, in a cell line like LCC9, which has an inherent overexpression of ERβ, heterodimers are more likely to be formed, allowing ERβ to exert its modulating effect on ERα.

Furthermore, the ability of either ER to mediate repressor or stimulatory effects on any gene is determined by the ratio and combinations of co-factors recruited to the ERs evoked by different stimuli in a given cell (Heldring et al, 2007). Different ligands result in different ER conformations thus modulating the affinity for certain co-factors. Consequently, although the ERs can recognize most of the same response elements, they may exert different (sometimes opposing, effects in different tissues (Warner et al, 2010). For example, elevated NCoR gene expression is required for the ERβ-induced down-regulatory effects on ERα gene expression and cell proliferation thus blocking ERα's driving role on breast cancer cell growth (Bartella et al, 2012). The presence of ER isoforms may also lead to abberations in normal cell growth patterns. Humans express the ERβ splice variant, ERβcx. ERβcx does not bind to E2, and preferentially forms heterodimers with ERα and therefore exerts a dominant negative effect on ligand-dependent transactivation of E2-sensitive reporter genes (Warner et al, 2010). Since ERβcx inhibits ERα DNA binding, it has the capacity to render ERα non-functional in E2-dependent tissues, which can severely affect gene expression (Leung et al, 2006). Ultimately, the combinations of ERs and their splice variants will all contribute to the estrogenic outcome in a cell. As we have shown that L17 and WAY represent two different classes of ERβ agonists (Figure 3.1A) that have a higher affinity for the ERβ and induce ERβ-mediated transcription at optimal concentrations. Their anti-mitogenic effect on LCC9 SERM/SERD-resistant breast cancer cells offers the promise that ERβ-selective ligands may prove to be a useful

treatment for cancers that have failed standardized therapy and could offer a novel strategy in breast cancers that are rich in ERβ expression.

ERβ agonists inhibit G1 and S phase exit in LCC9 cells

E2 and the ER signaling pathways are centrally involved in the development of the mammary gland and mammary tumorigenesis by regulating the processes controlling the entry, progression through, and exit out of the cell cycle (Doisneau-Sixou et al, 2003). In contrast, an antiestrogen's antiproliferative activity, such as that mediated by Tam, is associated with inhibition of these processes and ultimately arresting cells in a state of quiescence (Hodges et al, 2003). Previous *in vitro* studies have demonstrated that estrogens are one of the main regulators of epithelial cell proliferation and differentiation in MCF-7 breast cancer cells by inducing the expression of hormone-responsive genes involved in the cell cycle through activation of their cognate receptor ERα (Musgrove et al, 1994; Prall et al., 1998; Sutherland et al., 1998; Doisneau-Sixou et al, 2003).

Specifically, E2 increases the expression and phosphorylation of cyclin D1, increases the activity of its catalytic partner, cdk4, and enhances the association between cyclin D1 and cdk4, which play fundamental roles in the G1/S checkpoint of the cell cycle (Dong et al, 2001). Liu et al (2002) have previously shown that in transfected HeLa cells, the ERβ expressed alone does not result in the activation of transcription of the G1-cyclin D1 gene in the presence of E2, and when the ERβ and ERα are co-expressed, it continues to prevent E2-activation of cyclin D1 (Paruthiyil et al, 2004). In fact, the cyclin D promoter is one site where ERβ opposes ERα-mediated activation and the ERβ-antiestrogen complex can stimulate transcription (Liu et al., 2002). Antiestrogen like Tam reduce cyclin D transcription, meanwhile cyclin D overexpression can lead to resistance to endocrine therapy (Hui et al, 2002; Hodges et al, 2003).

In our studies, we sought to determine the effects of ERβ-agonists L17 and WAY on the cell cycle in MCF-7 and LCC9 cells. Previous studies have demonstrated that overexpression of the ERβ in MCF-7 breast cancer cells can inhibit cell growth in the presence of E2 through repression of cyclin D1 and c-myc and induction of cdk inhibitors thereby preventing xenograft tumor formation (Paruthiyil et al, 2004). Although the majority of human ER+ breast tumors do not express high levels of ERβ relative to ERα at diagnosis, LCC9 cells express a high ratio of ERβ to ERα (Brunner et al, 1997), which make it a very useful model system for testing the efficacy of ERβ agonists. The results of our cell cycle analysis demonstrate that both L17 and WAY produced a decrease in G2/M phase in LCC9 cells (Figure 3.3D) following treatment while both also having little effect on the cell cycle in MCF-7 cells compared to vehicle treated cells (Figure 3.3B). The predominant effect of L17 and WAY was to induce a G1-S phase block, and cyclin E appears to be affected. Cyclin E, a critical protein in the G1/S phase transition of the cell cycle is abnormally regulated in a variety of human cancers (Akli et al, 2004). We observed that both L17 and WAY reduced 50 kDa cyclin E in MCF-7 cells (Figure 3.3E) or weakly increased expression of 48 kDa cyclin E relative to E2-treated LCC9 cells (Figure 3.3F). Since cyclin E binds to and activates cdk2 and is required to catalyze the exit from G1 and both for G1-S exit and S phase progression, a lack of induction as seen in MCF-7 cells following stimulation with the ERβ ligands could effectively retard cells in G1 and S phase. Interestingly, low molecular weight species of cyclin E have been associated with endocrine resistance (Charn et al, 2010). Likewise, the findings reported by Akli et al (2004) suggest that increased expression levels of low molecular weight isoforms of cyclin E may result in endocrine resistance due to their influence on cell proliferation and genetic instability. Ye et al (2004) have suggested one mechanism by which cyclin E retards cell cycle progression. Multiple phosphoryation residues on cyclin E are required for recognition by the SCF^{Fbw7} ubiquitin ligase, which eventually leads to degradation of cyclin E and thus progression through S phase—the less cyclin E is targeted for degradation, the longer the cell

cycle will be in stasis. Further experiments are required to determine if the 48 kDa cyclin E protein represents hypophosphorylated cyclin E, reflecting the lack of cell cycle progression in E2 and ERβ agonist-treated LCC9 cells.

Bcl-2 as a target for ERβ in antiestrogen-resistant cells

One proposed mechanism of resistance to SERMs is the change in the expression of genes or proteins involved in tumor cell survival (Clarke et al, 2001); however, the precise genes associated with regulating cell fate remain unclear. Bcl-2 is a well-characterized proto-oncogene that inhibits the mitochondrial apoptosis pathway (Emi et al, 2005). Bcl-2 prevents apoptosis by binding and inhibiting the activation of its pro-apoptotic family member Bax (Yang et al, 1995), thus inhibiting cytochrome c release from the mitochondria and resulting in the inhibition of caspase cascade activation (Esslimanni-Sahla et al, 2004). Bcl-2 expression is positively regulated by E2 (Sabourin et al, 1994; Teixeira et al, 1995; Gompel et al, 2000) and is frequently overexpressed in several diseases including breast cancer (Olopade et al, 1997).

When overexpressed in tumor cells, Bcl-2 prevents apoptosis and allows the cells to survive the effects of drug induced stresses (Riggins et al, 2005; Gomez et al, 2007). Thus, it has been hypothesized that the deregulation of Bcl-2 expression is a critical step in developing SERM resistance and may be a resistance mechanism to several chemotherapeutic drugs by allowing cells to evade cell death (Huang et al., 2000). However, evidence shows that small molecule Bcl-2 inhibitors, such as YC137 and antisense oligodeoxynucleotides, restore sensitivity to growth inhibition, activate apoptosis and increase sensitivity to cell death induced by chemotherapeutic drugs *in vitro* (Simoes-Wust et al, 2000; Xu et al, 2001). Therefore, our objective in this study was to determine the role of Bcl-2 in Tam-resistant breast cancer cells. Consistent with previous findings, we too showed that there is an overexpression of Bcl-2 in SERM/SERD-resistant breast cancer cells compared to their sensitive MCF-7 cell controls (Figure 3.4B). This study is, however, the first to

demonstrate that ERβ agonists L17 and WAY downregulate the Bcl-2 protein and are able to do so in SERM/SERD-resistant breast cancer cells (Figure 3.4B), at the level of transcription (Figure 3.5G & I). Because of the prominent role that Bcl-2 plays in the regulation of apoptosis and autophagy, the effects induced by L17 and WAY on the regulation of Bcl-2 in LCC9 cells may present a novel strategy to induce cell death in these endocrine-resistant cells.

The activity of ERα or ERβ on various estrogen response elements (ERE) is influenced by several factors including the chemical structure of the estrogenic ligand, its affinity for the receptor, transcriptional activation, and interactions with cofactors or heterodimerisation (Katzellenbogen et al, 1996; Heldring et al, 2007; Riggs et al, 2003; Charn et al, 2010; Warner et al, 2010). In this study, we report the identification of both the ERα and the ERβ binding DNA regions in MCF-7 and LCC9 cells, two cell lines that express varying levels of the two ER subtypes, using the chromatin immunoprecipitation (ChIP) assay. Whether or not these are heterodimers or homodimers still needs to be determined by Chip-re-ChIP. Results of ChIP-on-chip experiments have shown considerable overlap between the enhancers that bind the ERα and the ERβ when they are present alone in cells, however, when they are co-expressed, fewer chromatin binding sites are shared and new sites become occupied (Liu et al, 2008; Charn et al, 2010; Vivar et al, 2010). The ERα and ERβ can form homodimers or heterodimers in the presence of E2 and SERMs to induce transcriptional activation of target genes (Monroe et al, 2005; Marino et al, 2006), although the exact role of ERα/β heterodimers in estrogen signaling still remains elusive (Papoutsi et al, 2009).

The mechanism by which ERβ agonists downregulate Bcl-2 in these cells is unknown; however, there are at least two possible explanations. The first is the formation of ERβ homodimers interacting directly with the ERE in the Bcl-2 coding region. The second is the formation of ERα and ERβ heterodimers in which the ligand is bound only to the ERβ on the Bcl-2 ERE. In either case, the formation of such complexes may render them inactive when bound to DNA by preventing the

interaction of essential co-factors or the basal transcription machinery, and thus preventing transcription of the Bcl-2 gene. There are two EREs in the Bcl-2 coding region (Perillo et al, 2000), but we targeted the ERE that is demonstrated to have the highest transcriptional activity demonstrated in vitro by reporter gene assays. Consistent with the literature, our results also demonstrate the binding of ERα/ERβ heterodimers on the pS2 promoter (Figure 3.5A,C, & E), a well-characterized promoter to which both ERα and ERβ bound in MCF-7 cells (Zhao et al, 2007).

Using a ChIP assay, we demonstrate that both L17 and WAY recruit ERα and ERβ as heterodimers to form complexes on the Bcl-2 ERE. These complexes appeared in both MCF-7 and LCC9 cells (Figure 3.5A & B). Nonetheless, the ability of different enhancer elements to bind to ERα/β heterodimers may depend of a variety of factors. Evidence shows that functional ERα and ERβ heterodimers are able to bind to DNA with an affinity comparable to that of ERα homodimers, which is the preferred complex, but greater than that of ERβ homodimers (Cowley et al, 1997; Pace et al, 1997; Pettersson et al, 1997). They then interact with cofactor proteins to influence the transcription of estrogen-responsive genes (Cowley et al, 1997; Pettersson et al, 1997; Monroe et al, 2005). The relative abundance of either ER subtype present in the cell will also influence the extent to which ERα and ERβ form heterodimers (Li et al, 2004). Our findings reveal that WAY treatment strongly recruited the ERβ on the Bcl-2 ERE in MCF-7 cells (Figure 3.5E), consistent with its high affinity for this receptor. In contrast, L17, which has a lower affinity for ERβ than WAY, and retains low affinity for the ERα, recruited the ERα more strongly than WAY in the same cell line (Figure 3.5C).

When co-expressed, the presence of ERβ can negatively affect the transcriptional activity of ERα (Chang et al, 2006). When ERβ is the dominant subtype, the predominant mechanism of ERβ action might be negative modulation of ERα, whereas the formation of ERβ homodimers in cells expressing little or no ERα may exert an alternate function (Hall et al, 1999; Peng et al, 2003; Ogawa

et al, 2008) To verify this in our cell system, and to confirm the transcriptional deregulation of Bcl-2 expression, mRNA levels were also evaluated in the SERM/SERD-cross-resistance model and in the E2-responsive MCF-7 breast cancer cell line. Our data showed that in both instances, heterodimer recruitment of both ERs was associated with a decrease in Bcl-2 mRNA in both cell lines when ERβ was expressed (Figure 3.5G & I). This pattern was also observed in pS2 mRNA levels (Figure 3.5H & J). This result is in accordance with that observed by Papoutsi et al (2009), who showed that the decrease of pS2 mRNA levels in MCF-7 cells following overexpression of the ERβ was associated with a decrease of PolII recruitment to the pS2 promoter. Ligand-ER conformation determines transcriptional activation through coactivator recruitment (Charn et al., 2010); Our results suggest that L17-bound and WAY-bound ERs may stably recruit corepressors on this response element, functioning essentially as inverse agonists which could be investigated via ChIP-re-ChIP using antibodies against specific co-repressors following the immunoprecipitation of the ERβ. Webb et al (2003) showed that when bound to an agonist like E2 and Genistein, ERβ is strongly associated with corepressors, such as -CoR and SMRT. This observation could suggest that in the presence of our ERβ agonists, L17 and WAY, ERβ may also be binding to corepressors to exert the inhibitory actions that we have observed. Moreover, since the liganded ER subtype determines chromatin binding (Charn et al, 2010), liganded ERβ even as a heterodimer with ERα may be capable of negatively regulating Bcl-2 and pS2 genes.

Interestingly, enhancer regions of E2-repressed genes in cells co-expressing ERα and ERβ were three times more likely to have chromatin binding sites unique to ERβ than to ERα, suggesting that ERβ homodimers might be recruited more strongly than ERα homodimers to the EREs in some E2-repressed genes, especially in cells with a higher ERβ:ERα (Charn et al, 2010).

Interestingly, E2 can mediate growth inhibition and induce cell death in long-term hormone-deprived and antiestrogen-resistant breast cancer cells (Lewis et al, 2005; Jordan et al, 2005).

Moreover, although paradoxical, high dose E2 (estrogen replacement therapy; ERT) has successfully been used to treat breast cancers that have developed resistance to several successive antiestrogenic therapies, including several AIs (Lonning et al, 2001). Although the mechanism by which E2 eventually loses its ability to promote cell survival and becomes antitumorigenic is currently unknown, we observed that the Bcl-2 expression level was found to be a critical determinant of the ability of E2 to induce apoptosis in these cells (Song et al, 2005). 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 (Swaby et al, 2008) and also why E2 fails to induce Bcl-2 in long term E2-deprived breast cancer cells (Lewis-Wambi et al, 2009).

Chemotherapy is an important first –line therapy in the initial treatment of many cancers, including breast cancer (Levin et al, 2006). Not only is resistance to endocrine therapy a major problem in the clinic, but resistance to chemotherapeutic drugs that act mainly to induce cell death continues to present treatment obstacles (Lima et al, 2004). Consequently, resistance to cytotoxic chemotherapy agents such as Adriamycin (Adr), or doxorubicin, may be the result of resistance to apoptosis (Igney et al, 2002). One mechanism whereby cancer cells evade apoptosis is through overexpressing anti-apoptotic proteins, such as Bcl-2 or certain inhibitor of apoptosis proteins (IAPs) that may act downstream of Bcl-2 by inhibiting caspases (Huang, 2000; Cory et al, 2002; Salvesen et al, 2002). These proteins have been shown to be responsible for inducing resistance to apoptosis stimulated by cytotoxic drugs (LaCasse et al, 1998; Holcik et al, 2001; Verhagen et al, 2001; Salvesen et al, 2002). Common strategies, including downregulation of Bcl-2 expression by antisense oligonucleotides (Wang et al, 2000; Shangany et al, 2003) and small interfering RNAs (siRNAs) (Sharp, 2001; Tuschl, 2001) have been explored and proven successful mechanisms to overcome the apoptotic resistance of some tumor populations.

Since we observed a downregulation of Bcl-2 expression following treatment with ERβ agonists, L17 and WAY, we decided to investigate if this specific downregulation could sensitize LCC9 cells to the chemotherapeutic drug, Adr. Our study is the first to report that in antiestrogen-resistant LCC9 cells, simultaneous treatment with an ERβ agonist L17 or WAY and Adr, reduced the total number of viable cells by ~20% (Figure 3.6). These results suggest that downregulation of Bcl-2 protein by L17 and WAY may be sensitizing LCC9 cells to the cytotoxic effects of Adr. In order to prove this, a knock-down of Bcl-2 followed by treatment with L17 or WAY would need to be performed.In MCF-7 cells, Cory et al, (2002) Bcl-2 downregulation increased spontaneous apoptosis, which was reflected in an increase of free Bax and Bak with subsequent translocation of these proapoptotic proteins to the mitochondrial membrane. Moreover, our lab (Teixeira et al, 1995) demonstrated that in the presence of E2 (an inducer of Bcl-2 transcription), MCF-7 cells expressing Bcl-2 antisense transcripts are twice as sensitive to doxorubicin cytotoxicity as a control clone. These results, however, are not unexpected due to the prominent role that Bcl-2 plays in the regulation of apoptosis. Our study is the first to report that the efficacy of standard cancer therapies could be improved if tumors were sensitized with an ERβ-agonist prior to treatment with chemotherapy targeting multiple signaling pathways and thus enhancing cytotoxicity. Ultimately, the downregulation of Bcl-2 is proving to be a useful complementary anticancer therapeutic strategy. By downregulating Bcl-2 expression, L17 and WAY are able to synergize with Adr to overcome apoptotic resistance, suggesting a promising new therapeutic strategy rendering tumor cells more susceptible to cell death.

Chloroquine converts L17/WAY-induced autophagy to apoptosis

It is thought that autophagy may function as a survival mechanism for cancer cells in response to a variety of stresses, including nutrient deprivation, toxic stimuli and chemotherapy (Levine et al, 2004; Kroemer et al, 2005; Kondo et al, 2005). On this premise, clinical trials of agents

which inhibit autophagy are being combined with chemotherapy and radiation to repress this prosurvival function (Chen et al, 2011). Although the precise relationship between autophagy and apoptosis is uncertain, several anti-apoptotic Bcl-2 family members, like Bcl-2, can bind to and inhibit the activity of a well-conserved autophagy protein called Beclin-1 (Maiuri et al, 2007). RNAi-mediated knockdown of Bcl-2 results in induction of autophagy in MCF-7 cells (Akar et al, 2008). *In vitro* studies using ER+ MCF-7 breast cancer cells have shown that the inhibition or downregulation of Bcl-2 can induce the activation of the autophagic pathway (Kessel et al, 2007; Akar et al, 2008). Consistent with these findings, we show in this study that downregulation of Bcl-2 activates autophagy following treatment with an ERβ agonist as indicated by the increase in LC3-II protein observed following treatment with L17 and or WAY in LCC9 cells (Figure 3.4B). With the evidence linking the anti-apoptotic Bcl-2 protein to the regulation of the pro-survival autophagic pathway, we decided to inhibit autophagy in L17/WAY-treated LCC9 cells with the addition of an aslysosomatropic agent, chloroquine (CQ).
As previously mentioned, inhibition of autophagy can promote apoptosis in antiestrogen-resistant breast cancer cells (Ullman et al, 2008; Qadir et al, 2008). Our data shows that Bcl-2 inhibition by ERβ agonists L17 and WAY combined with CQ markedly induced apoptotic cell death in LCC9 cells (Figure 3.7C) even when compared to SERM sensitive MCF-7 cells (3.7A). These findings strongly suggest that Bcl-2 inhibition by ERβ agonists L17 and WAY may improve therapy in SERM resistant breast cancer cells by activating autophagy, which can be converted to apoptosis when inhibited by CQ.

In our study we determined that apoptosis is increased following Bcl-2 inhibition by L17 or WAY, and autophagy inhibition by CQ in LCC9 cells (Figure 3.7C & E). 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 may express a higher ERβ:ERα ratio

(Speirs et al, 1999; Shaw et al, 2006), our novel finding that ERβ agonists reduce proliferation and Bcl-2 expression and increase autophagic flux in these cells suggests that combination of ERβ agonists and autophagy inhibition may represent a novel, relatively low toxicity, therapeutic option in patients who have developed endocrine resistance.

Safety considerations for ERβ agonists

Selective estrogen receptor modulators (SERMs) were first developed with the expectation of effectively treating menopausal symptoms and osteoporosis without the unwanted estrogenic stimulation of the uterus and breast (Pinkerton et al, 2010), however, to date, there is no SERM that completely fits this desired profile. In order to successfully achieve such a compound, a balance of ER agonist and antagonist activity must be attained such that the endometrium is protected, breast stimulation is minimal and ER agonist activity is retained in the brain, vagina and skeleton (Peano et al, 2009).

It appears that uterine stimulation is the one endpoint that differentiates most SERMS currently being developed or preclinically screened. The uterus may be the most sensitive organ to the effects of SERMS which is why uterine safety should be one of the most important goals in the clinical development of these compounds (Pinkerton et al, 2010). Preclinical data show that Tam stimulates the human and rat uterus, which is associated with an increase in endometrial thickness and increases the risk of endometrial cancer in women with breast cancer 2-7.5-fold relative to placebo (Fisher et al, 1994). Raloxifene treatment is also associated with an increase in endometrial thickness but not to the extent where it is considered clinically significant (Fugere et al, 2000; Goldstein et al, 2000). A similar study showed that ERα-selective ligand, propyl pyrazole triol (PPT), fully stimulates uterine wet weight increase. ERβ in contrast, is not the principal ER expressed in the uterus which makes it a promising drug target (Couse et al, 1997). That being said, the activities of E2 and SERMs in a partiular tissue are in part determined by the relative expression ratio of the ER

subtypes (McDonnell, 2004).

To assess the biological function of ERβ *in vivo* and evaluate its utility as a drug target, we tested our ERβ agonist, L17, in the uterotrophic assay. We chose to use sexually immature mice to maximize our ability to detect estrogen-dependent responses in the uterus and mammary gland. The sexually immature rodent uterus provides a standard and sensitive bioassay for E2 action in which wet weight gain and histological changes, such as changes in epithelial cell height, are typical endpoints.

In the mouse, as expected, we showed that administration of 1 mg/kg E2 caused a 30% increase in organ weight compared to vehicle treated mice (Figure 3.8B). However, administration of 1 mg/kg L17 did not have any stimulatory effect on uterine weight, but in fact decreased uterine wet weight by half relative to vehicle treated animals (Figure 3.8B). This assay proves that E2 is uterotrophic, inducing a significant increase in wet weight and an increase in the height of the luminal epithelial cells (Figure 3.8D), reflecting E2's strong mitogenic capacity. On the other hand, our ERβ agonist, L17, demonstrated no agonist activity on the rodent uterus, in terms on wet weight and no increase in epithelial cell height (Figure 3.8D) was observed following a 5-day exposure to the ligand.

Through its cognate receptors, ERα and ERβ, E2 is also involved in normal development and differentiation of mammary tissues. In addition to evaluating the effects of L17 on the mouse uterus, we were interested in evaluating the effects of L17 on noncancerous tissue in order to gain knowledge about the effects of our ERβ agonist on the morphology and development of the rodent mammary gland. Considering E2 and E2-dependent hormones, such as prolactin, are key promoters of breast carcinogenesis (Mujagic et al, 2009), it is important that we try to prevent tumor development on cancerous or potentially cancerous cells (Kotoula et al, 1993).

Female reproductive hormones including E2, progesterone, and prolactin are necessary for complete development of the mammary glands in mice (Brisken et al, 2010). Hormone action is required for elongation and branching of the ductal structure, which occurs during puberty, as well as for the differentiation and development of lobuloalveolar end buds during pregnancy (Bocchinfuso et al, 2000; Shyamala et al, 2002; Hinck et al, 2005). Prior to birth, the mammary gland forms a rudimentary 'tree-like' ductal strcuture (Hinck et al, 2005). Under the influence of E2, this epithelial structure undergoes ductal elongation and invasion into the fatty stroma. This type of ductal arrangement allows secretory alveoli to differentiate and invade the stroma within the lobules, a process that is induced by the onset of pregnancy (Robinson et al, 1995; Hinck et al, 2005).

Mammary terminal end buds (TEBs) are bulbous, epithelial structures, located at the tips of the mammary ducts that elongate into the fat pad and are extremely sensitive to the growth stimulatory effect of E2 and other hormones (Hinck et al, 2005). In rodents, the mammary gland is slow to develop within the first few weeks of life, however once puberty hits, an extensive growth period begins. Not only is there rapid formation of TEBs, but an intricate network of branched ducts is established, ultimately differentiating into 'terminal ductal structures' (Russo et al, 2004; Kleinberg et al, 2009).

E2 is required for outgrowth of the epithelial ducts until they reach the margins of the stroma (Hewitt et al, 2005). Our whole mounts of the fourth inguinal mammary glands of prepubertal CD1 mice reveal that with the administration of 1 mg/kg E2 for 5 days induces terminal end buds at the periphery accompanied by a small number of alveoli within a developing duct (Figure 3.9). Treatment with ERβ agonist, L17, also reveals terminal end buds at the periphery, although fewer in density and appear similar to control mice (Figure 3.9). Unlike E2, L17 does not provide any early growth stimulus to the mammary gland ductal structure. The morphological

effects that we observe in the rodent mammary gland with L17 may parallel the effects of Tam which inhibits noncancerous mammary gland growth by altering stromal and epithelial differentiation which are reversible after treatment discontinuation (Kotoula et al, 1993). However, because these mice are still sexually immature (4 weeks), it is too soon for the duct to reach advanced alveolar development or prominent lobulo-alveolar development and thus, further study of longer exposure times would be necessary to clarify the consequences of exposure to these ligands.

These results are consistent with the hypothesis that ERβ activation is anti-proliferative, demonstrating a lack of mitogenic activity of the ERβ selective ligands at 1mg/kg, and unlike E2, these data confirm that L17 does not have the capacity to induce proliferative effects in rodent uterine or mammary tissues.

Conclusion

In this thesis the aim was to gain more insight into the molecular and cellular mechanisms of estrogen signaling in breast cancer, focusing on the anti-tumorigenic effects of ERβ. Although the clinical significance of ERβ in breast cancer is still largely under debate, the studies presented in this thesis have provided some insights into the inhibitory functions of ERβ and define a novel potential approach to overcome or help treat acquired cross-resistance to Tam and Fulvestrant with combination drug therapy.

The role of endocrine resistance remains a challenging obstacle in the clinical treatment of hormone-positive breast cancer. Regardless of the beneficial outcomes of endocrine therapy in the early stages of treatment, resistance to these drugs inevitably arises. Therefore understanding the molecular mechanisms behind resistance to each endocrine agent are crucial, as well as the knowledge of mechanisms that would increase the sensitivity to endocrine therapy.

The discovery of antiestrogen resistance has fuelled the search for alternate therapies and for targets that can interfere with signaling pathways involved in endocrine resistance. These may lead to new strategies for combating ER positive breast cancers. In this respect ERβ may be a good alternative candidate.

Based on the relatively weak effects of ERβ agonists, L17 and WAY, on MCF-7 cells, and the dominant expression of the ERα in most ER+ primary breast cancers, ERβ agonists are not likely to be useful as first line therapy. However, given that SERM/SERD resistant cells may express a higher ERβ:ERα ratio (Speirs et al, 1999; Shaw et al, 2006), our novel finding that ERβ agonists reduce proliferation and Bcl-2 expression and increase autophagic flux in these cells suggests that ERβ agonists combined with autophagy inhibition may represent a novel therapeutic option in patients who have developed endocrine resistance (Figure 4.2). To our knowledge, no studies have reported these molecular effects of ERβ, marking it not only an intriguing target but also a possible marker of choice for endocrine therapy. Further, given the complexity of ER signaling pathways and the cross talk that exists to regulate many physiological processes, many of the proteins and processes thought to be modulated by ERβ are most likely subject to regulation by other pathways. Therefore it is of importance to consider that the response to ERβ may depend on the activity of these other signals and cell-context-dependent, an important consideration during tumor progression and therapy. The work presented in this thesis highlights the possibility of using ERβ-agonists in combination therapy, in the context of endocrine-resistant breast cancers. Further, given the complexity of ER signaling pathways and the cross talk that exists to regulate many physiological processes, many of the proteins and processes thought to be modulated by ERβ are most likely subject to regulation by other pathways. Therefore it is of importance to consider that the response to ERβ may depend on the activity of these other signals and cell-context-dependent, an important consideration during tumor progression and therapy. The work presented in this thesis highlights

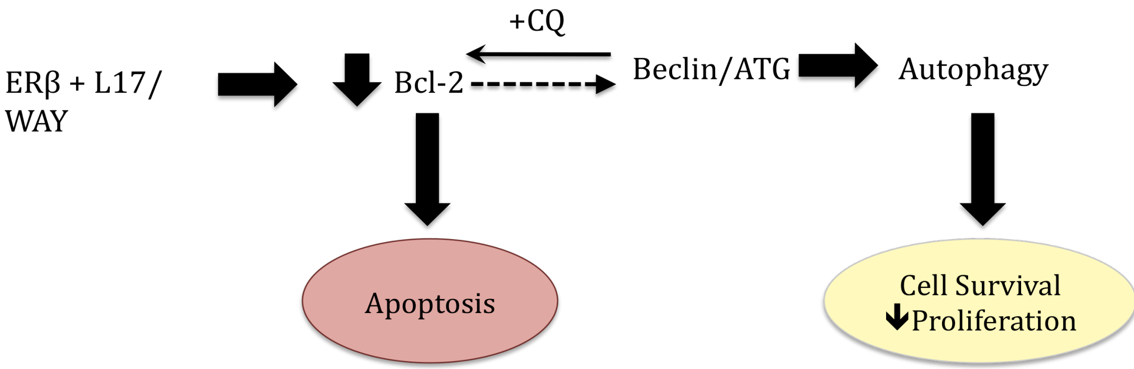


Figure 4.2. Schematic illustrating the effects of Bcl-2 inhibition and autophagy inhibition on SERM/SERD- cross-resistant breast cancer cells. SERM, Selective estrogen receptor modulator; SERD, Selective estrogen receptor downregulator; CQ, chloroquine.

the possibility of using ERβ-agonists in combination therapy, in the context of endocrine-resistant breast cancers.

Although these results are encouraging, more work remains to be done. Future *in vitro* work may include investigating the effects of these ERβ agonists in several other SERM-resistant cell lines, as well as testing the combination of CQ with other Bcl-2 antagonists to determine if the co-treatment can also induce apoptosis in antiestrogen-resistant cells. Future studies will also need to address the molecular mechanisms of transcriptional modulation through ERβ through ChIP re-ChIP experiments, as well as through ERβ gene knockdown to determine if a decrease in Bcl-2 mRNA expression is in fact induced solely by binding to the ERβ. In order to optimize the preclinical development process of L17 it would also be ideal to establish a xenograft tumor model of SERM/SERD resistance in nude mice and test the efficacy of the combination therapy of an ERβ agonist plus CQ *in vivo.*

REFERENCES

Abedin, M.J., Wang, D., McDonnell, M.A., Lehmann, U., and Kelekar, A. (2007). Autophagy delays apoptotic death in breast cancer cells following DNA damage. Cell Death Differ. *14*, 500-10.

Abeliovich, H., Zhang, C., Dunn, W.A.Jr., Shokat, K.M., and Klionsky, D.J. (2003). Chemical genetical analysis of Apg1 reveals a non-kinase role in the induction of autophagy. Mol Biol Cell. *14*, 477-90.

Adams, J.M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science. *281*, 1322-6.

Adams, J.M., and Cory, S. (2007). Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr Opin Immunol. *19*, 488–496.

Aita, V.M., Liang, X.H., Murty, V.V., Pincus, D.L., Yu, W., Cayanis, E., Kalachikov, S., Gilliam, T.C., and Levine, B. (1999). Cloning and genomic organization of beclin1, a candidate tumor suppressor genes on chromosome 17q21. Genomics. *59*, 59-65.

Akar, U., Chaves-Reyez, A., Barria, M., Tari, A., Sanguino, A., Kondo, Y., Kondo, S., Arun, B., Lopez-Berestein, G., and Ozpolat, B. (2008). Silencing of Bcl-2 expression by small interfering RNA induces autophagic cell death in MCF-7 breast cancer cells. Autophagy. *4*, 669-79.

Akli, S., and Keyomarsi, K. (2004). Low-molecular-weight cyclin E: the missing link between biology and clinical outcome. Breast Cancer. *6*, 188-91.

Ali, S., and Coombes, R.C. (2002). Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer. *2*, 101-12.

Allen, E., and Doisy, E. (1923). An ovarian hormone: preliminary report on its localization, extraction and partial purification and action in test animals. J Am Med Assoc. *81*, 819-821.

Apel, A., Herr, I., Schwarz, H., Rodemann, H.P., and Mayer, A. (2008). Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res. *68*, 1485-94.

Arpino, G., Wiechmann, L., Osborne, C.K., and Schiff, R. (2008). Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev. *29*, 217-33.

Banerjee, S.K., Banerjee, S., Li, S.A., and Li, J.J. (1994). Induction of chromosome aberrations in Syrian hamster renal cortical cells by various estrogens. Mutat Res. *3*, 191-197.

Bardin, A., Boulle, N., Lazennec, G., Vignon, F., and Pujol, P. (2004). Loss of ERbeta expression as a common step in estrogen-dependent tumor progression. Endocr Relat Cancer. *11*, 537-51.

Bartek, J., Lukas, C., and Lukas, J. (2004). Checking on DNA damage in S phase. Nat Rev Mol Cell Biol. *5*, 792-804.

Bartella, V., Rizza, P., Barone, I., Zito, D., Giordano, F., Giordano, C., Catalano, S., Mauro, L., Sisci, D., Panno, M.L., Fuqua, S.A., and Ando, S. (2012). Estrogen receptor beta binds Sp1 and recruits a corepressor complex to the estrogen receptor alpha gene promoter. Breast Cancer Res Treat.

Beatson, G.T. (1896). On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet. *2*, 104 –107.

Beck, T., and Hall, M.N. (1999). The TOR signalling pathway controls nuclear localisation of nutrient-regulated transcription factors. Nature. *402*, 689-692

Behrends, C., Sowa, M.E., Gygi, S.P., and Harper, J.W. (2010). Network organization of the human autophagy system. Nature. *466*, 68-76.

Berardi, D.E., Campodonico, P.B., Diaz Bessone, M.I., Urtreger, A.J., and Todaro, L.B. (2011). Autophagy: friend or foe in brest cancer development, progression, and treatment. Int J Breast Cancer. *2011*, 1-7.

Berry, M., Nunez, A.M., and Chambon, P. (1989). Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. Proc Natl Acad Sci USA. *86*, 1218-22.

Bjornstrom, L., and Sjoberg, M. (2005). Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol. *19*, 833-42.

Blesson, C.S., Awasthi, S., Kharkwal, G., Daverey, A., and Dwivedi, A. (2006). Modulation of estrogen receptor transactivation and estrogen-induced gene expression by ormeloxifene-a triphenylethylene derivative. Steroids. *7*, 993-1000.

Bocchinfuso, W.P., and Korach, K.S. (1997). Mammary gland development and tumorigenesis in estrogen receptor knockout mice. J Mammary Gland Biol Neoplasia. *2*, 323-34.

Boeger, H., Bushnell, D.A., Davis, R., Griesenbeck, J., Lorch, V., Stratten, J.S., Westover, K.D., and Komberg, R.D. (2005). Structural basis of eukaryotic gene transcription. FEBS Lett. *579*, 899-903.

Bolton J.L., and Thatcher, G.R.J. (2008). Potential mechanisms of estrogen quinone carcinogenesis. Chem Res Toxicol. *21*, 93-101.

Bolton, J.L., Pisha, E., Zhang, F., and Qiu, S. (1998). Role of quinoids in estrogen carcinogenesis. Chem. Res. Toxicol. 11, 1113-1127.

Boyd, S. (1900). On oophorectomy in cancer of the breast. BMJ. *2*, 1161-1167.

Boyd, N.F., and McGuire, V. (1991). The possible role of lipid peroxidation in breast cancer risk. Free Radic. Biol. Med. *10*, 185-190.

Brandenberger, A.W., Tee, M.K., and Jaffe, R.B. (1998). Estrogen receptor alpha (ER-alpha) and beta (ER-beta) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: fown regulation of ER-beta in neoplastic tissues. J Clin Endocrinol Metab. *83*, 1025-8.

Brodie, A., Macedo, L., and Sabnis, G. (2010). Aromatase resistance mechanisms in model systems in vivo. J Steroid Biochem Mol Biol. *118*, 283-7.

Brunner, N., Frandsen, T.L., Holst-Hansen, C., Bei, M., Thompson, E.W., Wakeling, A.E., Lippman,

M.E., and Clarke, R. (1993). MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. Cancer Res. *53*, 3229-32.

Brunner, N., Boysen, B., Jirus, S., Skaar, T.C., Holst-Hansen, C., Lippman, J., Frandsen, T., Spang-Thomsen, M., Fuqua, S.A., and Clarke, R. (1997). MCF7/LCC9: an antiestrogen-resistant MCF-7 variant which acquired resistance to the steroidal antiestrogen ICI 182,780 confers cross-resistance to the nonsteroidal antiestrogen tamoxifen. Cancer Res. *57*, 3486-93.

Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature. *389*, 753-8.

Bursch, W., Ellinger, A., Kienzl, H., Torok, L., Pandey, S., Sikorska, M., Walker, R., and Hermann, R.S. (1996). Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. Carcinogenesis. *17*, 1595-607.

Canadian Cancer Society's Steering Committee on Cancer Statistics. Canadian Cancer Statistics 2012. Toronto, ON: Canadian Cancer Society; 2012.

Cancer Genome Atlas Network. (2012). Comprehensive molecular portraits of human breast tumors. Nature. *490*, 61-70.

Cavalieri, E., Frenkel, K., Liehr, J.G., Rogan, E., and Roy, D. (2000). Estrogens as endogenous genotoxic agents—DNA adducts and mutations. J Natl. Cancer Inst. Monogr. *27*, 75-93.

Cavalieri, E., Chakravarti, D., Guttenplan, J., Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan, E., Russo, J., Santen, R., and Sutter, T. (2006). Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochim. Biophys. Acta. *1766*, 63-78.

Chakraborty, A.K., Welsh, A., and Digiovanna, M.P. (2010). Co-targeting the insulin-like growth factor I receptor enhances growth-inhibitory and pro-apoptotic effects of anti-estrogens in human breast cancer cell lines. Breast Cancer Res Treat. *120*, 327-35.

Chan, S.L., and Yu, V.C. (2004). Proteins of the bcl-2 family in apoptosis signaling: from mechanistic insights to therapeutic opportunities. Clin Exp Pharmacol Physiol. *31*, 119-28.

Chang, C., Norris, J.D., Gron, H., Paige, L.A., Hamilton, P.T., Kenan, D.J., Fowlkes, D., and McDonnell. (1999). Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol. *19*, 8226-39.

Chang, E.C., Frasor, J., Komm, B., and Katzenellenbogen, B.S. (2006). Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. Endocrinology. *147*, 4831-42.

Chang, N.C., Nguyen, M., Germain, M., and Shore, G.C. (2010). Antagonism of Beclin 1-dependent autophagy by BCL-2 at the endoplasmic reticulum requires NAF-1. EMBO J. *29*, 606–618.

Chang, M. (2011). Dual roles of estrogen metabolism in mammary carcinogenesis. BMB Reports. *44*, 423-434.

Charn, T.H., Liu, E.T., Chang, E.C., Lee, Y.K., Katzenellenbogen, J.A., and Katzenellenbogen, B.S., (2010). Genome-wide dynamics of chromatin binding of estrogen receptors alpha and beta: mutual restriction and competitive site selection. Mol Endocrinol. *24*, 47-59.

Chen, Y., and Klionsky, D.J. (2011). The regulation of autophagy- unanswered questions. J Cell Sci. *124*, 161-70.

Chia, S., Bryce, C., and Gelmon, K. (2005). The 2000 EBCTCG overview: a widening gap. Lancet. *365*, 1665-6.

Clarke, R., Brunner, N., Katzenellenbogen, B.S., Thompson, E.W., Norman, M.J., Koppi, C., Paik, S., Lippman, M.E., and Dickson, R.B. (1989). Progression of human breast cancer cells from hormone-dependent to hormone-independent growth both in vitro and in vivo. Proc Natl Acad Sci USA. *86*, 3649-53.

Clarke, R., Skaar, T.C., Bouker, K.B., Davis, N., Lee, Y.R., Welch, J.N., and Leonessa, F. (2001). Molecular and pharmacological aspects of antiestrogen resistance. J Steroid Bichem Mol Biol. *76*, 71-84.

Clarke, R., Liu, M.C., Bouker, K.B., Gu, Z., Lee, R.Y., Zhu, Y., Skaar, T.C., Gomez, B., O'Brien, K., Wang, Y., and Hilakivi-Clarke, L.A. (2003). Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. Oncogene. *22*, 7316-7339.

Clemons, M., and Goss, P. (2001). Estrogen and the risk of breast cancer. N Engl J Med. *344*, 276-85.

Collins, K., Jacks, T., and Pavletick, M.P. (1997). The cell cycle and cancer. PNAS. *94*, 2776-2778.

Cory, S., and Adams, J.M. (2002). The BCL2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer. *2*, 647-56.

Couse, J.F., Lindzey, J., Grandien, K., Gustafsson, J.A., and Korach, K.S. (1997). Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology. *138*, 4613-21.

Coutts, A.S., and Murphy, L.C. (1998). Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. Cancer Res. *58*, 4071-4.

Cowley, S.M., Hoare, S., Mosselman, S., and Parker, M.G. (1997). Estrogen receptors alpha and beta form heterodimers on DNA. J Biol Chem. *272*, 19858-62.

Crawford, A.C., Riggins, R.B., Shajahan, A.N., Zwart, A., and Clarke, R. (2010). Co-inhibition of BCL-W and BCL2 restores antiestrogen sensitivity through BECN1 and promotes an autophagy-associated necrosis. PLoS One. *5*, 1-11.

Cummings, S.R., Eckart, S., Krueger, K.A., Grady, D., Powles, T.J., Cauley, J.A., Norton, L., Nickelsen, T., Bjarnason, N.H., Morrow, M., Lippman, M.E., Black, D., Glusman, J.E., Costa, A., and Jordan, V.C.

(1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA. *281*, 2189-97.

Dalby, K.N., Tekedereli, I., Lopez-Berestein, G., and Ozpolat, B. (2010). Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. Autophagy. *6*, 322-9.

Danial, N.N. (2007). BCL-2 family proteins: critical checkpoints of apoptotic cell death. Clin Cancer Res. *13*, 7254-63.

De Cremoux, P., Tran-Perennou, C., Brockdorff, B.L., Boudou, E., Brunner, N., Magdelenat, H., and Lykkesfeldt, A.E. (2003). Validation of real-time RT-PCR for analysis of human breast cancer cell lines resistant or sensitive to treatment with antiestrogens. Endocr Relat Cancer. *10*, 409-18.

Debnath, J., Baehrecke, E.H. and Kroemer, G. (2005). Does autophagy contribute to cell death? Autophagy *1*, 66–74.

DeNardo, D.G., Kim, H.T., Hilsenbeck, S., Cuba, V., Tsimelzon, A., and Brown, P.H. (2005). Global gene expression analysis of estrogen receptor transcription factor cross talk in breast cancer: identification of estrogen-induced/activator protein-1-dependent genes. Mol Endocrinol. *19*, 362-78.

Dechering, K., Boersma, C., and Mosselman, S. (2000). Estrogen receptors alpha and beta: two receptors of a kind? Curr Med Chem. *7*, 561-76.

Djavaheri-Mergny, M., Amelotti, M., Mathieu, J., Besancon, F., Bauvy, C., and Codogno, P. (2007). Regulation of autophagy by NFkappaB transcription factor and reactives oxygen species. Autophagy. *3*, 290-2.

Djavaheri-Mergny, M., Maiuri, M.C., and Kroemer, G. (2010). Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1. Oncogene. *12*, 1717–1719.

Doisneau-Sixou, S.F., Sergio, C.M., Carroll, J.S., Hui, R., Musgrove, E.A., and Sutherland, R.L. (2003). Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocr Relat Cancer. *10*, 179-86.

Dong, Y., Sui, L., Sugimoto, K., Rai, Y., and Tokuda, M. (2001). Cyclin D1-CDK4 complex, a possible critical factor for cell proliferation and prognosis in laryngeal squamous cell carcinomas. Int J Cancer. *95*, 209-15.

Dowsett, M. (1997). Aromatase inhibitors come of age. Ann Oncol. *8*, 631-2.

Duffy, M.J. (2006). Estrogen receptors: Role in breast cancer. Critical reviews in clinical laboratory sciences. *43*, 325-347.

Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., and Mark, M. (2000). Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development. *127*, 4277-91.

Dumitrescu, R.G., and Cotaria, I. (2005). Understanding breast cancer risk—where do we stand in 2005? J Cell Mol Med. *9*, 208-21.

Early Breast Cancer Trialists' Collaborative Group (EBCTCG). (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomized trials. The Lancet. *365*, 1687-1717.

Edinger, A.L., and Thompson, C.B. (2003). Defective autophagy leads to cancer. Cancer Cell. *4*, 422-4.

Edinger, A. L., and Thompson, C.B. (2004). Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol. *16*, 663– 639.

Egger, L., Schneider, J., Rheme, C., Tapernoux, M., Hacki, J., and Borner, C. (2003). Serine proteases mediate apoptosis-like cell death and phagocytosis and phagocytosis under caspase-inhibiting conditions. Cell Death Differ. *10*, 1188-203.

Eisenberg-Lerner, A., Bialik, S., Simon, H.U., Kimchi, A. (2009). Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ. *16*, 966-75.

Elledge, S.J. (1996). Cell cycle checkpoints: preventing an identity crisis. Science. *274*, 1664-1672.

Ellis, P.A., Smith, I.E., Detre, S., Burton, S.A., Salter, J., A'Hern, R., Walsh, G., Johnston, S.R., and Dowsett, M. (1998). Reduced apoptosis and proliferation and increased Bcl-2 in residual breast cancer following preoperative chemotherapy. Breast Cancer Res Treat. *48*, 107-16.

Emi, M., Kim, R., Tanabe, K., Uchida, Y., and Toge, T. (2005). Targeted therapy against Bcl-2-related proteins in breast cancer cells. Breast Cancer Res. *7*, 940-52.

Emmen, J.M., and Korach, K.S. (2003). Estrogen receptor knockout mice: phenotypes in the female reproductive tract. Gynecol Endocrinol. *17*, 169-76.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J.A. (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab. *82*, 4258-65.

Eskelinen, E.L., and Saftig, P. (2009). Autophagy: a lysosomal degradation pathway with a central role in health and disease. Biochem Biophys Acta. *179*, 664-73.

Esslimani-Sahla, M., Simony-Lafontaine, J., Kramer, A., Lavalli, R., Mollevi, C., Warner, M., Gustafsson, J.A., and Rochefort, H. (2004). Estrogen receptor beta (ER beta) level but not its ER beta cx variant helps to predict tamoxifen resistance in breast cancer. Clin Cancer Res. *10*, 5769-76.

Fan, M., Bigsby, R.M., and Nephew, K.P. (2003). The NEDD8 pathway is required for proteasome-mediated degradation of human estrogen receptor (ER)-alpha and essential for the antiproliferative activity of ICI 182,780 in ERalpha-positive breast cancer cells. Mol Endocrinol. *17*, 356-65.

Federal Report on Carcinogens, National toxicology program (NTP), National toxicology program (NTP), 2002, pp.177283-77285.

Fisher, B., Costantino, J.P., Redmond, C.K., Fisher, E.R., Wickerham, D.L., and Cronin, W.M. (1994). Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst. *86*, 527-37.

Fixemer, T., Wissenbach, U., Flockerzi, V., and Bonkhoff, H. (2003). Expression of the Ca2+-selective cation channel TRPV6 in human prostate cancer: a novel prognostic marker for tumor progression. Oncogene. *22*, 7858-61.

Forster, C., Makela, S., Warri, A., Kietz, S., Becker, D., Hultenby, K., Warner, M., and Gustafsson, J.A. (2002). Involvement of estrogen receptor beta in terminal differentiation of mammery gland epithelium. Proc Natl Acad Sci USA. *99*, 15578-83.

Fox, E.M., Davis, R.J., and Shupnik, M.A. (2008). ERbeta in breast cancer—onlooker, passive player, or active protector? Steroids. *73*, 1039-51.

Frouin, I., Montecucco, A., Biamonti, G., Hubscher, U., Spardari, S., and Maga, G. (2002). Cell cycle-dependent dynamic asscoaition of cyclin/Cdk complexes with human DNA replication proteins. EMBO J. *21*, 2485-95.

Fugere, P., Scheele, W.H., Shah, A., Strack, T.R., Glant, M.D., and Jolly, E. (2000). Uterine effects of raloxifene in comparison with continuous-combined hormone replacement therapy in postmenopausal women. Am J Obstet Gynecol. *182*, 568-74.

Fujishima, Y., Nishiumi, S., Masuda, A., Inoue, J., Nguyen, N.M., Irino, Y., Komatsu, M., Tanaka, K., Kutsumi, H., Azuma, T., and Yoshida, M. (2011). Autophagy in the intestinal epithelium reduces endotoxin-induced inflammatory responses inhibiting NF-kappa B activation. Arch Biochem Biophys. *506*, 223-35.

Fussell, K.C., Udasin, R.G., Smith, P.J.S., Gallo, M.A., and Laskin, J.D. (2011). Catechol metabolites of endogenous estrogens induce redox cycling and generate reactive oxygen species in breast epithelial cells. Carcinogenesis. *32*, 1285-1293.

Gallager, H.S. (1984). Pathologic types of breast cancer: their prognoses. Cancer. *53*, 623-9.

Garinis, G.A., Patrinos, G.P., Spanakis, N.E., and Menounos, P.G. (2002). DNA hypermethylation: when tumour suppressor genes go silent. Hum Genet. *111*, 115-27.

Gee, J.M., Robertson, J.F., Ellis, I.O., Willsher, P., McClelland, R.A., Hoyle, H.B., Kyme, S.R., Finlay, P., Blamey, R.W., and Nicholson, R.I. (1994). Immunocytochemical localization of BCL-2 protein in human breast cancers and its relationship to a series of prognostic markers and response to endocrine therapy. Int J Cancer. *59*, 619-28.

Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. Nature. *349*, 132-8.

Goldstein, S.R., Scheele, W.H., Rajagopolan, S.K., Wilkie, J.L., Walso, B.W., and Parsons, A.K. (2000). A 12-month comparative study od raloxifene, estrogen, and placebo on the postmenopausal endometrium. Obstet Gynecol. *95*, 95-103.

Gomez, B.P., Riggins, R.B., Shajahan, A.N., Klimach, U., Wang, A., Crawford, A.C., Zhu, Y., Zwart, A., Wang, M., and Clarke, R. (2007). Human X-box binding protein-1 confers both estrogen and antiestrogen resistance in breast cancer cell lines. FASEB J. *21*, 4013-27.

Gompel, A., Somai, S., Chaouat, M., Kazem, A., Kloosterboer, H.J., Beusman, I., Forgez, P., Mimoun, M., and Rostene, W. (2000). Hormonal regulation of apoptosis in breast cells and tissues. Steroids. *65*, 593-8.

Gottlicher, M., Heck, S., and Herrluch, P. (1998). Tramscriptional cross-talk, the second mode of steroid hormone receptor action. J Mol Med. *76*, 480-9.

Gougelet, A., Mueller, S.O., Korach, K.S., and Renoir, J.M. (2007). Oestrogen receptors pathways to oestrogen responsive elements: the transactivation function-1 acts as the keystone of oestrogen receptor (ER)beta-mediated transcriptional repression of ERalpha. J Steroid Biochem Mol Biol. *104*, 110-22.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, P., and Chambon, P. (1986). Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature. *320*, 134-9.

Gunson, D.E., Steele, R.E., and Chau, R.Y. (1995). Prevention of spontaneous tumours in female rats by fadrozole hydrochloride, an aromatase inhibitor. Br J Cancer. *72*, 72-5.

Gustafsson, J.-A. (1997). Estrogen receptor beta—getting in on the action? Nat Med. *3*, 493-4.

Gustafsson, J.A., and Dahlman-Wright, K. (2007). Estrogen receptor beta2 negatively regulates the transactivation of estrogen receptor alpha in human breast cancer cells. Cancer Res. *67*, 3955-62.

Gutierrez, P.L. (2000). The metabolism of quinone-containing alkylating agents: free radical production and measurement. Frontiers Biosci. *5*, 629-38.

Hailey, D.W., Rambold, A.S., Satpute-Krishnan, P., Mitra, K., Sougrat, R., Kim, P.K., and Lippincott-Schwartz, J. (2010). Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell. *4*, 656–667.

Hall, J.M., and McDonnell, D.P. (1999). The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology. *140*, 5566-78.

Hall, J.M., Couse, J.F., and Korach, K.S. (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem. *276*, 36869-72.

Hanada, M., Aime-Sempe, C., Sato, T., and Reed, J.C. (1995). Structure-function analysis of Bcl-2 protein. Identification of conserved domains important for homodimerization with Bcl-2 and heterodimerization with Bax. J Biol Chem. *270*, 11962-9.

Hanahan, D., and Weinberd, R.A. (2000). The hallmarks of cancer. Cell. *100*, 57-70.

Harris, H.A. (2007). Preclinical characterization of selective estrogen receptor beta agonists: new insights into their therapeutic potential. Ernst Schering Found Symp Proc. *1*, 149-61.

Hartman, J., Lindberg, K., Morani, A., Inzunza, J., Strom, A., and Gustafsson, J.A. (2006). Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts. Cancer Res. *66*, 11207-13.

Hartwell, L., and Kastan, M.B. (1994). Cell cycle control and cancer. Science. *266*, 1821-1828.
Chang, E.C., Frasor, J., Komm, B., and Katzenellenbogen, B.S. (2006). Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. Endocrinology. *147*, 4831-42.

Harvey, H.A., Kimura, M., and Hajba, A. (2006).Toremifene: an evaluation of its safety profile. Breast. *15*, 142-57.

Heermeier, K., Benedict, M., Li, M., Furth, P., Nunez, G., and Hennighausen, L. (1996). Bax and Bcl-xs are induced at the onset of apoptosis in involuting mammary epithelial cells. Mech Dev. *56*, 197-207.

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Strom, A., Treuler, E., Warner, M., and Gustafsson, J.A. (2007). Estrogen receptors: how do they signal and what are their targets. Physiol Rev. *87*, 905-31.

Hennighausen, L., and Robinson, G.W. (2005). Information networks in the mammary gland. Nat Rev Mol Cell Biol. *6*, 715-25.

Hewitt, S.C., Harrell, J.C., and Korach, K.S. (2005). Lessons in estrogen biology from knockout and transgenic animals. Annu Rev Physiol. *67*, 285-308.

Hicks, D.G., and Kulkarni, S. (2008). HER2+ breast cancer: Review of biologic relevance and optimal use of diagnostic tools. Amer J Clin Path. *129*,263-273.

Hinck, L., and Silberstein, G.B. (2005). Key stages in mammary gland development: the mammary end bud as a motile organ. Breast Cancer Res. *7*, 245-51.

Hodges, L.C., Cook, J.D., Lobenhofer, E.K., Li, L., Bennett, L., Bushel, P.R., Aldaz, C.M., Afshari, C.A., and Walker, C.L. (2003). Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. Mol Cancer Res. *1*, 300-11.

Holcik, M., and Korneluk, R.G. (2001). XIAP, the guardian angel. Nat Rev Mol Cell Biol. *2*, 550-56.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991). P53 mutations in human canacers. Science. *253*, 49-53.

Hong, H., Kohli, K., Garabedian, M.J., and Stallcup, M.R. (1997). GRIP1, a transcriptional coactiavtor for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Mol Cell Biol. *17*, 2735-44.

Horwitz, K.B., Koseki, Y., and McGuire, W.L. (1978). Estrogen control of progesterone receptor in human breast cancer: role of estradiol and antiestrogen. Endocrinology. *103*, 1742-51.

Howell, A., Osborne, C.K., Morris, C., and Wakeling, A.E. (2000). ICI 182,780 (Faslodex): development of novel, “pure” antiestrogen. Cancer. *89*, 455.

Huang, Z. (2000). Bcl-2 family proteins as targets for anticancer drug design. Oncogene. *19*, 6627-6631.

Hui, R., Finney, G.L., Carroll, J.S., Lee, C.S., Musgrove, E.A., and Sutherland, R.L. (2002). Constitutive overexpression of cyclin D1 but not cyclin E confers acute resistance to antiestrogens in T-47D breast cancer cells. Cancer Res. *62*, 6916-23.

Hull, D.F.3rd., Clark, G.M., Osborne, C.K., Chamness, G.C., Knight, W.A.3rd., and McGuire, W.L. (1983). Multiple estrogen receptor assays in human breast cancer. Cancer Res. *43*, 413-6.

Huseby, R.A., Maloney, T.M., and McGrath, C.M. (1984). Evidence for a direct growth-stimulating effect of estradiol on human MCF-7 cells in vivo. Cancer Res. *44*, 2654-9.

Ichikawa, A., Ando, J., and Suda, K. (2008). G1 arrest and expression of cyclin-dependent kinase inhibitors in tamoxifen-treated MCF-7 human breast cancer cells. Hum Cell. *21*, 28-37.

Ichimura, Y., Kirisako, T., Tahao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000). A ubiquitin-like system mediates protein lipidation. Nature. *408*, 488-492.

Igney, F.H., and Krammer, P.H. (2002). Death and anti-death: tumor resistance to apoptosis. Nat Rev Cancer. *2*, 277-88.

Iwao, K., Miyoshi, Y., Egawa, C., Ikeda, N., and Noguchi, S. (2000). Quantitative analysis of estrogen receptor-beta mRNA and its variants in human breast cancers. Int J Cancer. *88*, 733-6.

Jager, R., Herzer, U., Schenkel, J., and Weiher, H. (1997). Overexpression of Bcl-2 inhibits alveolar cell apoptosis during involution and accelerates c-myc-induced tumorigenesis of the mammary gland in transgenic mice. Oncogene. *15*, 1787-95.

Jager, S., Bucci, C., Tanida, I., Ueno, T., Kominami, E., Saftig, P., and Eskelinen, E.L. (2004). Role for Rab7 in maturation of late autophagic vacuoles. J Cell Sci. *117*, 4837-48.

Jarvinen, T.A., Pelto-Huikko, M., Holli, K., and Isola, J. (2000). Estrogen receptor beta is coexpressed with ERalpha and PR and associated with nodal status, grade, and proliferation rate in breast cancer. Am J Pathol. *156*, 29-35.

Jelovac, D., Sabnis, G., Long, B.J., Macedo, L., Goloubeva, O.G., and Brodie, A.M. (2005). Activation of mitogen-activated protein kinase in xenografts and cells during prolonged treatment with aromatase inhibitor letrozole. Cancer Res. *65*, 5380-9.

Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W., and DeSombre, E.R. (1968). A two-step mechanism for the interaction of estradiol with rat uterus. Proc Natl Acad Sci USA. *59*, 632-8.

Jensen, E.V., and DeSombre, E.R. (1973). Estrogen-receptor interaction. Science. *182*, 126-34.

Jensen, E.V., Cheng, G., Palmieri, C., Saji, S., Makela, S., Van Noorden, S., Wahlstrom, T., Warner, M., Coombes, R.C., and Gustafsson, J.A. (2001). Estrogen receptors and proliferation markers in primary and recurrent breast cancer. Proc Natl Acad Sci USA. *98*, 15197-202.

Johnston, S.R., Lu, B., Scott, G.K., Kushner, P.J., Smith, I.E., Dowsett, M., and Benz, C.C. (1999). Increased activator protein-1 DNA binding and c-Jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. Clin Cancer Res. *5*, 251-6.

Johnston, S.J., and Cheung, K.L. (2010). Fulvestrant- a novel endocrine therapy for breast cancer. Curr Med Chem. *17*, 902-14.

Jordan, V.C., and Robinson, S.P. (1987). Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc. *46*, 1870-4.

Jordan, V.C., Lewis, J.S., Osipo, C., and Cheng, D. (2005). The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. Breast. *14*, 624-30.

Jordan, V.C., O'Malley, B.W. (2007). Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol. *25*, 5915-24.

Kahlert, S., Nuedling, S., van Eickels, M., Vetter, H., Meyer, R., and Grohe, C. (2000). Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. J Biol Chem. *275*, 18447-53.

Kang, M.H., and Reynolds, C.P. (2009). Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. Clin Cancer Res. *15*, 1126-1132.

Karantza-Wadsworth, V., and White, E. (2007). Role of autophagy in breast cancer. Autophagy. *3*, 610-3.

Katzenellenbogen, B.S. (1996). Estrogen receptors: bioactivities and interactions with cell signaling pathways. Biol Reprod. *54*, 287-93.

Kern, F.G., McLeskey, S.W., Zhang, L., Kurebayashi, J., Liu, Y., Ding, I,Y., Kharbanda, S., Chen, D., Miller, D., and Cullen, K. (1994). Transfected MCF-7 cells as a model for breast cancer progression. Breast Cancer Res Treat. *31*, 153-65.

Kessel, D., and Arroyo, A.S. (2007). Apoptotic and autophagic responses to Bcl-2 inhibition and photodamage. Photochem Photobiol Sci. *6*, 1290-5.

Kim, R., Emi, M., Matsuura, K., and Tanabe, K. (2005). Therapeutic potential of antisense (AS) Bcl-2 as a chemosensitizer for patients with gastric and breast carcinoma. Gan To Kagaku Ryoho. *32*, 1540-5.

Kim, I., Rodriguez-Enriquez, S., and Lemasters, J.J. (2007). Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys. *462*, 245-53.

Kleinberg, D.L., Wood, T.L., Furth, P.A., and Lee, A.V. (2009). Growth hormone and insulin-like growth factor-I in the transition from normal mammary development to preneoplastic mammary lesions. Endocr Rev. *30*, 51-74.

Klinge, C.M. (2000). Estrogen receptor interaction with co-activators and co-repressors. Steroids. *65*, 227-51.

Klinge, C.M., Riggs, K.A., Wickramasinghe, N.S., Emberts, C.G., McConda, D.B., Barry, P.N., and Magnusen, J.E. (2010). Estrogen receptor alpha 46 is reduced in tamoxifen resistant breast cancer cells and re-expression inhibits cell proliferation and estrogen receptor alpha 66-regulated target gene transcription. Mol Cell Endocrinol. *323*, 268-76.

Klionsky, D.J., Cregg, J.M., Dunn, W.A.Jr., Emr, S.D., Sakai, Y., Sandoval, I.V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., and Ohsumi, Y. (2003). A unified nomenclature for yeast autophagy-related genes. Dev Cell. *5*, 539-45.

Klionsky, D.J. (2007). Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol. *8*, 931-7.

Koehler, K.F., Helguero, L.A., Haldosen, L.A., Warner, M., and Gustafsson, J.A. (2005). Reflections on the discovery and significance of estrogen receptor beta. Endocr Rev. *26*, 465-78.

Koljonen, V., Tukianen, E., Haglund, C., and Bohling, T. (2006). Cell cycle control by p21, p27, and p53 in Merkel cell carcinoma. Anticancer Res. *26*, 2209-12.

Kondo, Y., Kanzawa, T., Sawaya, R., and Kondo, S. (2005). The role of autophagy in cancer development and response to therapy. Nat Rev Cancer. *5*, 726-34.

Kondo, Y., Kanzawa, T., Sawaya, R., and Kondo, S. (2005). The role of autophagy in cancer development and response to therapy. Nat Rev Cancer. *5*, 726-34.

Kong, N., Fotouhi, N., Wovkulich, P.M., and Roberts, J. (2003). Cell cycle inhibitors for the treatment of cancer. Drugs Fut. *28*, 881-90.

Kotoula, V., Karkavelas, G., Economou, L., Sionga, A., Boutis, L., and Kerameos-Foroglou, C. (1993). Effects of tamoxifen and CV 205502 on the morphology and the evolution of the noncancerous mouse mammry gland. Histol Histopathol. *8*, 627-36.

Krege, J.H., Hodgin, J.B., Couse, J.F., Enmark, E., Warner, M., Mahler, J.F., Sar, M., Korach, K.S., Gustafsson, J.A., and Smithies, O. (1998). Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci USA. *95*, 15677-82.

Kroemer, G. (1997). The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med. *3*, 614-20.

Kroemer, G., and Jaattela, M. (2005). Lysosomes and autophagy in cell death control. Nat Rev Cancer. *5*, 886-97.

Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA. *93*, 5925-30.

Kuiper, G.G., and Gustafsson, J.A. (1997). The novel estrogen receptor-beta subtype: potential role in the cell- and promoter-sepcific actions of estrogens and anti-estrogens. FEBS Lett. *410*, 87-90.

Kuiper, G.G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J.A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology. *138*, 863-70.

Kumar, R., Mandal, M., Lipton, A., Harvey, H., and Thompson, C.B. (1996). Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. Clin Cancer Res. *2*, 1215-9.

Kunapuli, S., Rosanio S., and Schwarz, E. R. (2006). How do cardiomyocytes die? Apoptosis and autophagic cell death in cardiac myocytes. Journal of Cardiac Failure. *12*, 381-391

Kushner, P.J., Agard, D.A., Greene, G.L., Scanian, T.S., Shiau, A.K., Uht, R.M., and Webb, P. (2000). Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol. *74*, 311-7.

Lacassagne, A. (1936). Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer. *27*, 217-225.

LaCasse, E.C., Baird, S., Korneluk, R.G., and MacKenzie, A.E. (1998). The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene. *17*, 3247-59.

Lavinksy, R.M., Jepsen, K., Heinzel, T., Torchia, J., Mullen, T.M., Schiff, R., Del-Rio, A.L., Ricote, M., Ngo, S., Hilsenbeck, S.G., Osborne, C.K., Glass, C.K., Rosenfeld, M.G., and Rose, D.W. (1998). Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc Natl Acad Sci USA. *95*, 2920-5.

Lazennec, G., Bresson, D., Lucas, A., Chauveau, C., and Vignon, F. (2001). ER beta inhibits proliferation and invasion of breast cancer cells. Endocrinology. *142*, 4120-30.

Lee, J.A., and Gao, F.B. (2008). ESCRT, autophagy, and frontotemporal dementia. BMB Rep. *41*, 827-32.

Leitman, D.C., Paruthiyil, S., Vivar, O.I., Saunier, E.F., Herber, C.B., Cohen, I., Tagliaferri, M., and Speed, T.P. (2010). Regulation of specific target genes and biological responses by estrogen receptor subtype agonists. Curr Opin Pharmacol. *10*, 629-36.

Lemmen, J.G., Broekhof, J.L., Kuiper, G.G., Gustafsson, J.A., van der Saag, P.T., and van der Burg, B. (1999). Expression of estrogen receptor alpha and beta during mouse embryogenesis. Mech Dev. *81*, 163-7.

Leung, Y.K., Mak, P., Hassan, S., and Ho, S.M. (2006). Estrogen receptor (ER)-beta isoforms: a key to understanding ER-beta signaling. Proc Natl Acad Sci USA. *103*, 13162-7.

Levin, E.R. (2003). Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol. *17*, 309-17.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell. *6*, 463-77.

Levine, B., Sinha, S., and Kroemer, G. (2008). Bcl-2 family members: dual regulators of apoptosis and autophagy. *Autophagy. 4*, 600–606.

Lewis, J.S., Meeke, K., Osipo, C., Ross, E.A., Kidawi, N., Li, T., Bell, E., Chandel, N.S., and Jordan, V.C. (2005). Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst. *97*, 1746-59.

Lewis-Wambi, J.S., and Jordan, V.C. (2009). Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit. Breast Cancer Res. *11*, 206.

Leygue, E., Dotzlaw, H., Lu, B., Glor, C., Watson, P.H., and Murphy, L.C. (1998). Estrogen receptor beta: mine is longer than yours? J Clin Endocr Metab. *83*, 3754-3755.

Li, X., Huang, J., Yi, P., Bambara, R.A., Hilf, R., and Muyan, M. (2004). Single-chain estrogen receptors (ERs) reveal that the ERalpha/beta heterodimer emulates functions of the ERalpha dimer in genomic signaling pathways. Mol Cell Biol. *24*, 7681-94.

Li, Y., Wang, J.P., Santen, R.J., Kim, T.H., Park, H., Fan, P., and Yue, W. (2010). Estrogen stimulation of cell migration involves multiple signaling pathway interactions. Endocrinology. *151*, 5146-56.

Liang, X.H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature. *402*, 672-6.

Lima, R.T., Martins, L.M., Guimaraes, J.E., Sambade, C., and Vaconcelos, M.H. (2004). Specific downregulation of bcl-2 and xIAP by RNAi enhances the effects of chemotherapeutic agents in MCF-7 human breast cancer cells. Cancer Gene Ther. *11*, 309-16.

Linn, S.C., and Van't Veer, L.J. (2009). Clinical relevance of the triple negative breast cancer concept: genetic basis and clinical utility of the concept. Eur J Cancer. *45*, 11-26.

Liu, M.M., Albanese, C., Anderson, C.M., Hilty, K., Webb, P., Uht, R.M., Price, R.H.Jr., Pestell, R.G., and Kushner, P.J. (2002). Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem. *277*, 24353-60.

Liu, Y., Gao, H., Marstrand, T.T., Strom, A., Valen, E., Sandelin, A., Gustafsson, J.A., and Dahlman-Wright, K. (2008). The genome landscape of ERalpha- and ERbeta-binding DNA regions. Proc Natl Acad Sci USA. *105*, 2604-9.

Lockshin, R.A., Zakeri, Z. (2004). Apoptosis, autophagy, and more. Int J Biochem Cell Biol. *36*, 2405–2419.

Lonning, P.E., Taylor, P.D., Anker, G., Iddon, J., Wie, L., Jorgensen, L.M., Mella, O., and Howell, A. (2001). High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat. *67*, 111-6.

Lum, J.J., DeBerardinis, R.J., and Thompson, C.B. (2005). Autophagy in metazoans: cell survival in the land of plenty. Nat Rev Mol Cell Biol. *6*, 439-48

Lupulescu, A. (1995). Clinical science review: estrogen use and cancer incidence: a review. Cancer Investigation. *13*, 287-295.

Lyons, W.R. (1958). Hormonal synergism in mammary growth. Proc R Soc Lond B Biol Sci. *149*, 303-25.

Macedo, L.F., Sabnis, G.J., Goloubeva, O.G., Brodie, A. (2008). Combination of anastrozole with fulvestrant in the intratumoral aromatase exnograft model. Cancer Res. *68*, 3516-22.

Maiuri, M.C., Zalckvar, E., Kimchi, A., and Kroemer, G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. *8*, 741-52.

Malamas, M.S., Manas, E.S., McDevitt, R.E., Gunawan, I., Xu, Z.B., Collini, M.D., Miller, C.P., Dinh, T., Henderson, R.A., Keoth, J.C.Jr., and Harris, H.A. (2004). Design and synthesis of aryl diphenolic azoles as potent and selective estrogen receptor-beta ligands. J Med Chem. *47*, 5021-40.

Malara, N.M., Leotta, A., Sidoti, A., Lio, S., D'Angelo, R., Caparello, B., Munao, F., Pino, F., and Amato, A. (2006). Ageing, hormonal behaviour and cyclin D1 in ductal breast carcinomas. Breast. *15*, 81-9.

Malins, D.C., Polissar, N.L., and Gunselman, S. J. (1996). Progression of human breast cancers to the metastatic state is linked to hydroxyl radical-induced DNA damage. Proc. Natl. Acad. Sci. USA. *93*, 2557-2563.

Malins, D.C., Anderson, K.M., Jaruga, P., Ramsey, C.R., Gilman, N.K., Green, V.M., Rostad, S.W., Emerman, J.T., and Dizdaroglu, M. (2006). Oxidative changes in the DNA of stroma and epithelium from the female breast: potential implications of breast cancer. Cell Cycle. *5*, 1629-1632.

Malumbres, M. (2007). Cyclins and related kinases in cancer cells. J BUON. *12*, 45-52.

Malumbres, M., and Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer. *9*, 153-66.

Mann, S., Laucirica, R., Carlson, N., Younes, P.S., Ali, N., Younes, A., Li, Y., and Younes, M. (2001). Estrogen receptor beta expression in invasive breast cancer. Hum Pathol *32*, 113-8.

Matthews, J., and Gustafsson, J.A. (2003). Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol Interv. *3*, 281-92.

Marino, M., Galluzzo, P., and Ascenzi, P. (2006). Estrogen signaling multiple pathways to impact gene transcription. Curr Genomics. *7*, 497-508.

Marsaud, V., Gougelet, A., Maillard, S., and Renoir, J.M. (2003). Various phosphorylation pathways, depending on agonist and antagonist binding to endogenous estrogen receptor alpha (ERalpha), differentially affect ERalpha extractability, proteasome-mediated stability, and transcriptional activity in human breast cancer cells. Mol Endocrinol. *17*, 2013-27.

Martini, P.G., and Katzenellenbogen, B.S. (2003). Modulation of estrogen receptor activity by selective coregulators. J Steroid Biochem Mol Biol. *85*, 117-22.

Massarweh, S., and Schiff, R. (2006). Resistance to endocrine therapy in breast cancer: exploiting estrogen receptor/growth factor signaling crosstalk. Endocr Relat Cancer. *13*, 15-24.

Maycotte, P., Aryal, S., Cummings, C.T., Thorburn, J., Morgan, M.J., and Thorburn, A. (2012). Chloroquine sensitizes breast cancer cells to chemotherapy independent of autophagy. Autophagy. *8*, 200-12.

McDonald, E.R. 3rd., and El-Deiry, W.S. (2000). Cell cycle control as a basis for cancer drug development (review). Int J Oncol. *16*, 871-86.

McDonnell, D.P., and Norris, J.D. (2002). Connections and regulation of the human estrogen receptor. Science. *296*, 1642-4.

McDonnell, D.P. (2004). The molecular determinants of estrogen receptor pharmacology. Maturitas. *48*, 7-12.

McInerney, E.M., Rose, D.W., Flynn, S.E., Westin, S., Mullen, T.M., Krones, A., Inostroza, J., Torchia, J., Nolte, R.T., Assa-Munt, N., Milburn, M.V., Glass, C.K., and Rosenfeld, M.G. (1998). Determinants of coactivator LXXLL motif specofocoty in nuclear receptor transcriptional activation. Genes Dev. *12*, 3357-68.

Meijer, A.J., and Codogno, P. (2004). Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol. *36*, 2445-62.

Metcalfe, A.D., Gilmore, A., Klinowska, T., Oliver, J., Valentjn, A.J., Brown, R., Ross, A., MacGregor, G., Hickman, J.A., and Streuli, C.H. (1999). Developmental regulation of Bcl-2 family protein expression in the involuting mammary gland. J Cell Sci. *112*, 1771-83.

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996). Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF07 cells. EMBO J. *15*, 1292-300.

Mizushima, N. (2007). Autophagy: process and function. Genes Dev. *21*, 2861-73.

Mizushima, N., and Yoshimori, T. (2007). How to interpret LC3 immunoblotting. Autophagy. *3*, 542-5.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature. *451*, 1069-75.

Mizushima, N., Yoshimori, T., and Levine, B. (2010). Methods in mammalian autophagy research. Cell. *140*, 313-326.

Molyneux, G., Regan, J., amd S,alley, M.J. (2007). Mammary stem cells and breast cancer. Cell Mol Life Sci. *64*, 3248-60.

Monroe, D.G., Secreto, F.J., Subramaniam, M., Getz, B.J., Khosla, S., and Spelsberg, T.C. (2005). Estrogen receptor alpha and beta heterodimers exert unique effects on estrogen- and tamoxifen-dependent gene expression in human U2OS osteosarcoma cells. Mol Endocrinol. *19*, 1555-68.

Morley, P., Whitfield, J.F., Vanderhyden, B, C., Tsang, B.K., and Schwartz, J.L. (1992). A new, nongenomic estrogen action: the rapid release of intracellular calcium. Endocrinology. *131*, 1305-12.

Moy, B., and Goss, P.E. (2006). Estrogen receptor pathway: resistance to endocrine therapy and new therapeutic approaches. Clin Cancer Res. *12*, 4790-3.

Mujagic, Z., Srabovic, N., and Mujagic. (2009). The role of prolactin in human breast cancer. Biochem Med. *19*, 235-49.

Murphy, E., and Korach, K.S. (2006). Actions of estrogen and estrogen receptors in nonclassical target tissues. Ernst Schering Found Symp Proc. *1*, 13-24.

Musgrove, E.A., and Sutherland, R.L. (1994). Cell cycle control by steroid hormones. Semin Cancer Biol. *5*, 381-9.

Musgrove, E.A., and Sutherland, R.L. (2009). Biological determinants of endocrine resistance in breast cancer. Nat Rev Cancer. *9*, 631-43.

Myers, E., Fleming, F.J., Crotty, T.B., Kelly, G., McDermott, E.W., O'higgins, N.J., Hill, A.D., and Young, L.S. (2004). Inverse relationship between ER-beta and SRC-1 predicts outcome in endocrine-resistant breast cancer. Br J Cancer. *91*, 1687-93.

Nehra, R., Riggins, R.B., Shajahan, A.N., Zwart, A., Crawford, A.C., and Clarke, R. (2010). BCL2 and CASP8 regulation by NF-kappaB differentially affect mitochondrial function and cell fate in antiestrogen-sensitive and –resistant breast cancer cells. FASEB J. *24*, 2040-55.

Nicholson, R.I., McClelland, R.A., Gee, J.M., Manning, D.L., Cannon, P., Robertson, J.F., Ellis, I.O., and Blamey, R.W. (1994). Epidermal growth factor receptor expression in breast cancer: association with response to endocrine therapy. Breast Cancer Res Treat. *29*, 117-25.

Nilsson, S., and Gustafsson, J.A. (2011). Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther. *89*, 44-55.

Noda, T. and Ohsumi, Y. (1998). Tor, a Phosphatidylinositol Kinase Homologue, Controls Autophagy in Yeast. J Biol Chem. *273*, 3963-3966.

Nutter, L.M., Ngo, E.O., and Abul-Hajj, Y. J. (1991). Characterization of DNA damage induced by 3,4-estrone-o-quinone in human cells. J. Biol. Chem. *266*, 16380-16386.

Ogawa, S., Inoue, S., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. (1998). Cross-inhibition of both estrogen receptor alpha and beta pathways by each dominant negative mutant. FEBS Lett. *423*, 129-32.

Okoh, V., Deoraj, A., and Roy, D. (2011). Estrogen-induced reactive oxygen species-mediated signalings contribute to breast cancer. Biochimica et Biophysica Acta. *1815*, 115-133.

O'Lone, R., Frith, M.C., Karlsson, E.K., and Hansen, U. (2004). Genomic targets of nuclear estrogen receptors. Mol Endocrinol. *18*, 1859-75.

Olopade, O.I., Adeyanju, M.O., Safa, A.R., Hagos, F., Mick, R., Thompson, C.B., and Recant, W.M. (1997). Overexpression of Bcl-x protein in primary breast cancer is associated with high tumor grade and nodal metastases. Cancer J Sci Am. *3*, 230-7.

Omoto, Y., Eguchi, H., Yamamoto-Yamaguchi, Y., and Hayashi, S. (2003). Estrogen receptor (ER) beta1 and ERbetacx/beta2 inhibit ERalpha function differently in breast cancer cell line MCF7. Oncogene. *22*, 5011-20.

Osborne, C.K., and Schiff, R. (2011). Mechanisms of endocrine resistance in breast cancer. Annu Rev Med. *62*, 233-47.

Osborne, C.K., and Schiff, R. (2003). Growth factor receptor cross-talk with estrogen receptor as a mechanism for tamoxifen resistance in breast cancer. Breast. *12*, 362-7.

Pace, P., Taylor, J., Suntharalingam, S., Coombes, R.C., and Ali, S. (1997). Human estrogen receptor beta binds DNA in a manner similar to and dimerizes with estrogen receptor alpha. J Biol Chem. *272*, 25832-8.

Paech, K., Webb, P., Kuiper, G.G., Nilsson, S., Gustafsson, J.A., Kushner, P.J., and Scanian, T.S. (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science. *277*, 1508-10.

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. EMBO J. *11*, 961-71.

Palimieri, C., Cheng, G.J., Saji, S., Zelada-Hedman, M., Warri, A., Wiehua, Z., Van Noorden, S., Wahlstrom, T., Coombes, R.C., Warner, M., and Gustafsson, J.A. (2002). Estrogen receptor beta in breast cancer. Endocr Relat Cancer. *9*, 1-13.

Papoutsi, Z., Zhao, C., Putnik, M., Gustafsson, J.A., Dahlman-Wright, K. (2009). Binding of estrogen receptor alpha/beta heterodimers to chromatin in MCF-7 cells. J Mol Endocrinol. *43*, 65-72.

Pappas, T.C., Gametchu, B., and Watson, C.S. (1995). Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. FASEB J. *9*, 404-10.
Park, M.-T., and Lee, S.-J. (2003). Cell cycle and cancer. Journal of biochemistry and molecular

biology. *36*, 60-65.

Park, B.W., Kim, K.S., Heo, M.K., Ko, S.S., Hong, S.W., Yang, W.I., Kim, J.H., Kim, G.E., and Lee, K.S. (2003). Expression of estrogen receptor-beta in normal mammary and tumor tissues: is it protective in breast carcinogenesis? Breast Cancer Res Treat. *80*, 79-85.

Paruthiyil, S., Parmar, H., Kerekatte, V., Cunha, G.R., Firestone, G.L., and Leitman, D.C. (2004). Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. Cancer Res. *64*, 423-8.

Patel, R.R., Sharma, C.G., and Jordan, V.C. (2007). Optimizing the antihormonal treatment and prevention of breast cancer. Breast Cancer. *14*, 113-22.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. *122*, 927–939.

Peano, B.J., Crabtreet, J.S., Komm, B.S., Winneker, R.C., and Harris, H.A. (2009). Effects of various selective estrogen receptor modulators with or without conjugated estrogens on mouse mammary gland. Endocrinology. *150*, 1897-903.

Peng, B., Lu, B., Leygue, E., and Murphy, L.C. (2003). Putative functional characteristics of human estrogen receptor-beta isoforms. J Mol Endocrinol. *30*, 13-29.

Perillo, B., Sasso, A., Abbondanza, C., and Palumbo, G. (2000). 17beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. Mol Cell Biol. *20*, 2890-2901.

Periyasamy-Thandavan, S., Jiang, M., Schoenlein, P., and Dong, Z. (2009). Autophagy: molecular machinery, regulation, and implications for renal pathophysiology. Am J Physiol Renal Physiol. *297*, 244-56.

Persad, S., and Dedhar, S. (2003). The role of integrin-linked kinase (ILK) in cancer progression. Cancer Metastasis Rev. *22*, 375-84.

Petersen, O.W., Hoyer, P.E., and van Deurs, B. (1987). Frequency and distribution of estrogen receptor-positive cells in normal, nonlactating human breast tissue. Cancer Res. *47*, 5748-51.

Pettersson, K., Grandien, K., Kuiper, G.G., and Gustafsson, J.A. (1997). Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha. Mol Endocrinol. *11*, 1486-96.

Pinkerton, J.V., and Goldstein, S.R. (2010). Endometrial safety: a key hurdle for selective estrogen receptor modulators in development. Menopause. *17*, 642-53.

Pisha, E., Liu, X., Constantinou, A.I., and Bolton, J.L. (2001). Evidence that a metabolite of quinine estrogens, 4-hydroxyequilenin induces cellular transformation in vitro. Chem Res. Toxicol. *14*, 82-90.

Platet, N., Cunat, S., Chalbos, D., Rochefort, H., and Garcia, M. (2000). Unliganded and liganded estrogen receptors protect against cancer invasion via different mechanisms. Mol Endocrinol. *14*, 999-1009.

Plu-Bureau, G., and Thalabard, J.C. (1998). Hereditary risks of breast cancer. Interaction of genetic factors and hormonal factors. Ann Endocrinol. *59*, 465-9.

Poliseno, L., Bianchi, L., Citti, L., Liberatori, S., Mariani, L., Salvetti, A., Evangelista, M., Bini, L., Pallini, V., and Rainaldi, G. (2004). Bcl2-low-expressing MCF7 cells undergo necrosis rather than apoptosis upon staurosporine treatment. Biochem J. *379*, 823-32.

Polson, H.E., de Lartigue, J., Rigden, D.J., Reedijk, M., Urbe, S., Clague, M.J., and Tooze, S.A. (2010). Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. Autophagy. *6*, 506-522.

Prall, O.W., Rogan, E.M., and Sutherland, R.L. (1998). Estrogen regulation of cell cycle progression in breast cancer cells. J Steroid Biochem Mol Biol. *65*, 169-74.

Pujol, P., Rey, J.M., Nirde, P., Roger, P., Gastaldi, M., Laffargue, F., Rochefort, H., and Maudelonde, T. (1998). Differential expression of estrogen receptor-alpha and –beta messenger RNAs as a potential marker of ovarian carcinogenesis. Cancer Res. *58*, 5367-73.

Qadir, M.A., Kwok, B., Dragowska, W.H., To, K.H., Le, D., Bally, M.B., and Gorski, S.M. (2008). Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. Breast Cancer Res Treat. *112*, 389-403.

Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Trozel, A., Rosen, J., Eskelinen, E.L., Mizushima, N., Ohsumi, Y., Cattoretti, G., and Levine, B. (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest. *112*, 1809-20.

Rae, J.M., Goetz, M.P., Hayes, D.F., Ingle, J.N., Li, L., Storniolo, A.M., Stearns, V., and Flockhart, D.A. (2005). CYP2D6 genotype and tamoxifen response. Breast Cancer Res. *29*, E6.

Rai, K.R., Moore, J., Wu, J., Novick, S.C., and O'Brien, S.M. (2008). Effect of the addition of oblimersen (Bcl-2 antisense) to fludarabine/cyclophosphamide for relapsed/refractory chronic lymphocytic leukemia (CLL) on survival in patients who achieve CR/nPR: Five-year follow-up from a randomized phase III study. J Clin Oncol. *26*, 7008.

Rakha, E.A., Reis-Filho, J.S., and Ellis, I.O. (2008). Basal-like breast cancer: A critical review. J Clin Oncology. *26*, 2568-2581.

Razandi, M., Pedram, A., Merchenthaler, I., Greene, G.L., and Levin, E.R. (2004). Plasma membrane estrogen receptors exist and function as dimers. Mol Endocrinol. *18*, 2854-65.

Reed, J.C. (1994). Bcl-2 and the regulation of programmed cell death. J Cell Biol. *124*, 1-6.

Reed, J.C. (2006). Proapoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, and therapeutic opportunities. Cell Death Differ. *13*, 1378-86.

Reel, J.R., Lamb, I.V.J.C., and Neal, B.H. (1996). Survey and assessment of mammalian estrogen biological assays for hazard characterization. Fundam Appl Toxicol. *34*, 288-305.

Reggiori, F. and Klionsky, D.J. (2002). Autophagy in the Eukaryotic Cell. Eukaryot Cell. *1*, 11-21.

Reggiori, F., Klionsky, D.J. (2005). Autophagosomes: biogenesis from scratch? Curr Opin Cell Biol.*17*, 415-22.

Riggins, R.B., Zwart, A., Nehra, R., and Clarke, R. (2005). The nuclear factor kappa B inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)-induced apoptosisin antiestrogen-resistant breast cancer cells. Mol Cancer Ther. *4*, 33-41.

Riggs, B.L., and Hartmann, L.C. (2003). Selective estrogen receptor modulators—mechanisms of action and application to clinical practice. N Engl J Med. *348*, 618-29.

Rikiishi, H. (2012). Novel insights into the interplay between apoptosis and autophagy. Int J Cell Biol. *2012*, 1-14.

Rivera-Gonzalez, R., Petersen, D.N., Tkalcevic, G., Thompson, D.D., and Brown, T.A. (1998). Estrogen-induced genes in the uterus of ovariectomized rats and their regulation by droloxifene and tamoxifen. J Steroid Biochem Mol Biol. *64*, 13-24.

Rochefort, H., Bardon, S., Chalbos, D., and Vignon, F. (1984). Steroidal and nonsteroidal antiestrogens in breast cancer cells in culture. J Steroid Biochem. *20*, 105-10.

Rody, A., Holtrich, U., Solbach, C., Kourtis, K., von Minckwitz, G., Engels, K., Kissler, S., Gatje, R., Karn, T., and Kaufmann, M. (2005). Methylation of estrogen receptor beta promoter correlates with loss of ER-beta expression in mammary carcinoma and is an early indication marker in premalignant lesions. Endocr Relat Cancer. *12*, 903-16.

Roger, P., Shla, M.E., Makela, S., Gustafsson, J.A., Baldet, P., and Rochefort, H. (2000). Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. Cancer Res. *61*, 2537-41.

Roodi, N., Bailey, L.R., Kao, W.Y., Verrier, C.S., Yee, C.J., Dupont, W.D., and Parl, F.F. (1995). Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer. J Natl Cancer Inst. *87*, 446-51.

Rosenfeld, C.S., Roberts, R.M., and Lubahn, D.B. (2001). Estrogen receptor- and aromatase-deficient mice provide insight into the roles of estrogen within the ovary and uterus. Mol Reprod Dev. *59*, 336-46.

Rouschop, K.M., and Woulters, B.G. (2009). Regulation of autophagy through multiple independent hypoxic signaling pathways. Curr Mol Med. *9*, 417-24.

Roy, D., and Liehr, J.G. (1999). Estrogen, DNA damage and mutations. Mutat. Res. *424*, 107-115.

Roy, S., Chakravarty, D., Cortez, V., De Mukhopadhyay, K., Bandyopadhyay, A., Ahn, J.M., Raj, G.V., Tekmai, R.R., Sun, L., and Vadlamudi, R.K. (2012). Significance of PELP1 in ER-negative breast cancer metastasis. Mol Cancer Res. *10*, 25-33.

Russo, J., and Russo, I.H. (2004). Development of the human breast. Maturitas. *49*, 2-15.

Russo, J., and Russo, I.H. (2006). The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol. Biol. *102*, 89-96.

Sabourin, J.C., Martin, A., Baruch, J., Truc, J.B., Gomepl, A., and Poitout, P. (1994). Bcl-2 expression in normal breast tissue during the menstral cycle. Int J Cancer. *59*, 1-6.

Safe, M.S., and Kim, K. (2008). Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. J Mol Endocrinol. *41*, 263-75.

Saji, S., Jensen, E.V., Nilsson, S., Rylander, T., Warner, M., and Gustafsson, J.A. (2000). Estrogen receptors alpha and beta in the rodent mammary gland. Proc Natl Acad Sci USA. *97*, 337-42.

Salvesen, G.S., and Duckett, C.S. (2002). IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol. *3*, 401-10.

Schafer, K.A. (1998). The cell cycle: a review. Vet Pathol. *35*, 461-78.

Schoenlein, P.V., Periyasamy-Thandavan, S., Samaddar, J.S., Jackson, W.H., and Barrett, J.T. (2009). Autophagy facilitates the progression of ERalpha-positive breast cancer cells to antiestrogen resistance. Autophagy. *5*, 400-3.

Scott, R.C., Schuldiner, O., and Neufeld, T.P. (2004). Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev Cell. *7*, 167-78.

Shadeo, A., and Lam, W.L. (2006). Comprehensive copy number profiles of breast cancer cell model genomes. Breast Cancer Research. 2006, *8*, R9.

Shangary, S., and Johnson, D.E. (2003). Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family. Leukemia. *17*, 1470-81.

Sharp, P.A. (2001). RNA interference-2001. Genes Dev. *15*, 485-90.

Shaw, L.E., Sadler, A.J., Pugazhendhi, D., and Darbre, P.D. (2006). Changes in oestrogen receptor-alpha and –beta during progression to acquired resistance to tamoxifen and fulvestrant (Faslodex, ICI 182,780) in MCF7 human breast cancer cells. J Steroid Biochem Mol Biol. *99*, 19-32.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., and Greene, G.L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell. *95*, 927-37.

Shim, W.S., Conaway, M., Masamura,S., Yue, W., Wang, J.P., Kmar, R., and Santen, R.J. (2000).

Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology. *14*, 396-405.

Shintani, T., and Klionsky, D.J. (2004). Autophagy in health and disease: a double-edged sword. Science. *306*, 990-995.

Shou, J., Massarweh, S., Osborne, C.K., Wakeling, A.E., Ali, S., Weiss, H., and Schiff, R. (2004). Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst. *96*, 926-35.

Shyamala, G., Chou, Y.C., Louie, S.G., Guzman, R.C., Smith, G.H., and Nandi, S. (2002). Cellular expression of estrogen and progesterone receptors in mammary glands: regulation by hormones, development and aging. J Steroid Biochem Mol Biol. *80*, 137-48.

Simoes-Wust, A.P., Olie, R.A., Gautshi, O., Leesh, S.H., Haner, R., Hall, J., Fabbro, D., Stahel, R.A., and Zangemeister-Wittke, U. (2000). Bcl-xl antisense treatment induces apoptosis in breast carcinoma cells. Int J Cancer. *87*, 582-90.

Simoncini, T., Hafezi-Moghadam, A., Brazil, D.P., Ley, K., Chin, W.W., and Liao, J.K. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature. *407*, 538-41.

Simpson, E.R., Clyne, C., Speed, C., Rubin, G., and Bulun, S. (2001). Tissue-specific estrogen biosynthesis and metabolism. Ann N Y Acad Sci. *949*, 58-67.

Simpson, E.R. (2003). Sources of estrogen and their importance. J Steroid Biochem Mol Biol. *86*, 225-30.

Skliris, G.P., Leygue, E., Watson, P.H., and Murphy, L.C. (2008). Estrogen receptor alpha neative breast cancer patients: estrogen receptor beta as a therapeutic target. J Steroid Biochem Mol Biol. *109*, 1-10.

Sladek, R., and Giguere, V. (2000). Orphan nuclear receptors: an emerging family of metabolic regulators. Adv Pharmacol. *47*, 23-87.

Smith, C.L., Nawaz, Z., and O'Malley, B.W. (1997). Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol. *11*, 657-66.

Smith, C.L., Nawaz, Z., and O'Malley, B.W. (1998). Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol. *11*, 657-66.

Smith, I.E., and Dowsett, M. (2003). Aromatase inhibitors in breast cancer. N Engl J Med. *348*, 2431-42.

Smith, C.L., and O'Malley, B.W. (2004). Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev. *25*, 45-71.

Smith, C.L. (1998). Cross-talk between peptide growth factor and estrogen receptor signaling pathways. Biol Reprod. *58*, 627-32.

Soderqvist, G. (1998). Effects of sex steroids on proliferation in normal mammary tissue. Ann Med. *30*, 511-24.

Somai, S., Chaouat, M., Jacob, D., Perrot, J.Y., Rostene, W., Forgez, P., and Gompel, A. (2003). Antiestrogens are pro-apoptotic in normal human breast epithelial cells. Int J Cancer. *105*, 607-12.

Song, R.X., Santen, R.J., Kumar, R., Adam, L., Jeng, M.H., Masamura, S., and Yue, W. (2002). Adaptive mechanisms induced by long-term estrogen deprivation in breast cancer cells. Mol Cell Endocrinol. *193*, 29-42.

Sotoca, A.M., van den Berg, H., Vervoort, J., van den Saag, P., Strom, A., Gustafsson, J.A., Rietjens, I., and Mruk, A.J. (2008). Influence of cellular ERalpha/ERbeta ratio on the ERalpha-agonist induced proliferation of human T47D breast cancer cells. Toxicol Sci. *105*, 202-11.

Speirs, V., Malone, C., Walton, D.S., Kerin, M.J., and Atkin, S.L. (1999). Increased expression of estrogen receptor beta mRNA in tamoxifen-resistant breast cancer patients. Cancer Res. *59*, 5421-4.

Speirs, V., and Merin, M.J. (2000). Prognostic significance of oestrogen receptor beta in breast cancer. Br J Surg. *87*, 405-9.

Speirs, V., and Walker, R.A. (2007). New perspectives into the biological and clinical relevance of oestrogen receptors in the human breast. J Pathol. *211*, 499-506.

Strappazzon, F., Vietri-Rudan, M., Campello, S., Nazio, F., Florenzano, F., Fimia, G.M., Piacentini, M., Levine, B., and Cecconi, F. (2011). Mitochondrial Bcl-2 inhibits AMBRA1-induced autophagy. EMBO J. *30*, 1195-1208.

Strasser-Weippl, K., and Goss, P.E. (2005). Advances in adjuvant hormonal therapy for postmenopausal women. J Clin Oncol. *23*, 1751-9.

Stossi, F., Barnett, D.H., Frasor, J., Komm, B., Lyttle, C.R., and Katzenellenbogen, B.S. (2004). Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ERbeta in human osteosarcoma cells: distinct and common target genes for these receptors. Endocrinology. *145*, 3473-86.

Strom, A., Hartman, J., Foster, J.S., Kietz, S., Wimalasena, J., and Gustafsson, J.A. (2004). Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci USA. *10*, 1566-71.

Sutherland, R.L., Prall, O.W., Watts, C.K., and Musgrove, E.A. (1998). Estrogen and progestin regulation of cell cycle progression. J Mammary Gland Biol Neoplasia. *3*, 63-72.

Suzuki, N.N., and Inagaki, F. (2006). Structual biology of Atg conjugation systems. Tanpakushitsu Kakusan Koso. *51*, 1537-41.

Swaby, R.F., and Jordan, V.C. (2008). Low-dose estrogen therapy to reverse acquired antihormonal resistance in the treatment of breast cancer. Clin Breast Cancer. *8*, 124-33.

Tanida, I., Ueno, T., and Kominami, E. (2004). LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol. *36*, 2503–2518.

Teixeira, C., Reed, J.C., and Pratt, M.A. (1995). Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Res. *55*. 3902-7.

Tewey, K.M., Rowe, T.C., Yang, L., Halligan, B.D., and Liu, L.F. (1984). Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science. *226*, 466-8.

The National Toxicology Program (NTP). 2002. Federal report on carcinogens. Pp. 177283-177285.

Thomas, C.G., Strom, A., Lindberg, K., and Gustafsson, J.A. (2011). Estrogen receptor beta decreases survival of p53-defective cancer cells after DNA damage by impairing G2/M checkpoint signaling. Breast Cancer Res Treat. *127*, 417-27.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science. *267*, 1456-62.

Todde, V., Veenhuis, M., and van der Kiel, I.J. (2009). Autophagy: principles and significance in health and disease. Biochem Biophys Acta. *1792*, 3-13.

Toft, D., and Gorski, J. (1966). A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci USA. *55*, 1574-81.

Torchia, J., Rose, D.W., Inostroza, J., Kamel, Y., Westin, S., Glass, C.K., and Rosenfeld, M.G. (1997). The transcriptional co-activator p/CIP bind CBP and mediates nuclear-receptor function. Nature. *387*, 677-84.

Treech, O., Lattrich, C., Springwald, A., and Ortmann, O. (2010). Estrogen receptor beta exerts growth-inhibitory effects on human mammary epithelial cells. Breast Cancer Res Treat. *120*, 557-65.

Trowbridge, J.M., Rogatsky, I., and Garabedian, M.J. (1997). Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex. Proc Natl Acad Sci USA. *94*, 10132-7.

Tuschl, T. (2001). RNA interference and small interfering RNAs. Chembiochemistry. *2*, 239-45.

Ullman, E., Fan, Y., Stawowczyk, M., Chen, H.M., Yue, Z., and Zong, W.X. (2008). Autophagy promotes necrosis in apoptosis-deficient cells in response to ER stress. Cell Death Differ. *15*, 422-5.

Vander Heiden, M.G., and Thompson, C.B. (1999). Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat Cell Biol. *1*, 209-16.

Verhagen, A.M., Coulson, E.J., and Vaux, D.L. (2001). Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. Genome Biol. *2*, 3009.1-10.

Vermeulen, K., Van Bockstaele, D.R., and Bernemanm Z.N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. *36*, 131-49.

Voegel, J.J., Heine, M.J., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998). The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and –independent pathways. EMBO J. *17*, 507-19.

Wang, J.L., Liu, D., Zhang, Z.J., Shan, S., Han, X., Srinivasula, S.M., Croce, C.M., Alnemri, E.S., and Huang, Z. (2000). Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. Proc Natl Acad Sci USA. *97*, 7124-9.

Wang, C., Fu, M., Angeletti, R.H., Siconolfi-Baez, L., Reutens, A.T., Albanese, C., Lisanti, M.P., Katenzellenbogen, B.S., Kato, S., Hopp, T., Fuqua, S.A., Lopez, G.N., Kushner, P.J., and Pestell, R.G. (2001). Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. J Biol Chem. *276*, 18375-83.

Warner, M., and Gustafsson, J.A. (2010). The role of estrogen receptor beta (ERbeta) in malignant diseases—a new potential target for antiproliferative drugs in prevention and treatment of cancer. Biochem Biophys Res Commun. *396*, 63-6.

Webb, P., Lopez, G.N., Uht, R.M., and Kushner, P.J. (1995). Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol. *9*, 443-56.

Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M.P., Chen, D., Huang, S.M., Subramanian, S., McKinerney, E., Katzenellenbogen, B.S., Stallcup, M.R., and Kushner, P.J. (1998). Estrogen receptor activation function 1 works by binding p160 coactivator proteins. Mol Endocrinol. *12*, 1605-18.

Webb, P., Nguyen, P., Valentine, C., Lopez, G.N., Kwok, G.R., McInerney, E., Katzenellenbogen, B.S., Enmark, E., Gustafsson, J.A., Nilsson, S., and Kushner, P.J. (1999). The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol. *13*, 1672-85.

Webb, P., Valentine, C., Nguyen, P., Price, R.H.Jr., Marimuthu, A., West, B.L., Baxter, J.D., and Kushner, P.J. (2003). ERbeta binds N-CoR in the presence of estrogens via an LXXLL-like motif in the N-CoR C-terminus. Nucl Recept. *1*, 4.

Weihua, A., Andersson, S., Cheng, G., Simpson, E.R., Warner, M., and Gustafsson, J.A. (2003). Update on estrogen signaling. FEBS Lett. *546*, 17-24.

Weroha, S.J., Lingle, W.L., Hong, Y., Li, S.A., and Li, J.J. (2010). Specific overexpression of cyclin E:CDK2 in early preinvasive and primary breast tumors in female ACI rats induced by estrogen. Horm Cancer. *1*, 34-43.

WHO 1999. Hormonal Contraception and post-menopausal hormonal therapy; in IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, pp. 399-530, IARCPress, Lyon, France.

Wright, J.S., Shadnia, H., Anderson, J.M., Durst, T., Asim, M., El-Salfiti, M., Choueiri, C., Pratt, M.A., Ruddy, S.C., Lau, R., Carlson, K.E., Katzenellenbogen, J.A., O'Brien, P.J., and Wan, J. (2011). A-CD

estrogens. I. Substituent effects, hormone potency, and receptor subtype selectivity in a new family of flexible estrogenic compounds. J Med Chem. *54*, 433-48.

Wu, W.K.K., Coffelt, S.B., Cho, C.H., Wang, X.J., Lee, C.W., Chan, F.K.L., Yu, J., and Sung, J.J.Y. (2012). The autophagic paradox in cancer therapy. Oncogene. *31*, 939-953.

Xie, Z., and Klionsky, D.J. (2007). Autophagosome formation: core machinery and adaptations. Nat Cell Biol. *9*, 1102-9.

Xu, Z., Friess, H., Solioz, M., Aebi, S., Korc, M., Kleeff, J., and Buchler, M.W. (2001). Bcl-x(L) antisense oligonucleotides induce apoptosis and increase sensitivity of pancreatic cancer cells to gemcitabine. Int J Cancer. *94*, 268-74.

Yager, J.D. (2000). Chapter 3: endogenous estrogens as carcinogens through metabolic activation. Journal of the National Cancer Institute Monographs. *27*, 67-73.

Yaghjyan, L., Colditz, G.A., Collins, L.C., Schnitt, S.J., Rosner, B., Bachon, C., and Tamimi, R.M. (2011). Mammographic breast density and subsequent risk of breast cancer in postmenopausal women according to tumor characteristics. J Natl Cancer Inst. *103*, 1179-89.

Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B., and Korsmeyer, S.J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell. *80*, 285-91.

Ye, X., Nalepa, G., Welcker, M., Kessler, B.M., Spooner, E., Qin, J., Elledge, S.J., Clurman, B.E., and Harper, J.W. (2004). Recognition of phosphodegron motifs in human cyclin E by the SCF(Fbw7) ubiquitin ligase. J Biol Chem. *279*, 50110-9.

Yorimitsu, T., and Klionsky, D.J. (2005). Autophagy: molecular machinery for self-eating. Cell Death Differ. *12*, 1542-52.

Yoshimori, T. (2004). Autophagy: a regulated bulk degradation process inside cells. Biochem Biophys Res Commun. *313*, 453-8.

Yue, W., Santen, R.J., Wang, J.P., Li, Y., Verderame, M.F., Bocchinfuso, W.P., Korach, K.S., Devanesan, P., Todorovic, R., Rogan, E.G., and Cavalieri, E.L. (2003). Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. J Steroid Biochem Mol Biol. *86*, 477-86.

Yorimitsu, T., and Klionsky, D.J. (2005). Autophagy: molecular machinery for self-eating. Cell Death Differ. *12*, 1542-52.

Yue, Z., Jin, S., Yang, C., Levine, A.J., and Heintz, N. (2003). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci USA. *100*, 15077-82.

Zhang, G.J., Kimijima, I., Onda, M., Kanno, M., Sato, H., Watanabe, T., Tsuchiya, A., Abe, R., and Takenoshita, S. (1999). Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-X(L), without alteration of p53 protein levels. Clin Cancer

Res. *5*, 2971-7.

Zhao, C., Matthews, J., Tujague, M., Wan, J., Strom, A., Toresson, G., Lam, E.W., Cheng, G., Gustafsson, J.A., and Dahlman-Wright, K. (2007). Estrogen receptor beta2 negatively regulates the transactivation of estrogen receptor alpha in human breast cancer cells. Cancer Res. *67*, 3955-62.

Zhao, C., Dahlman-Wright, K., and Gustafsson, J.A. (2008). Estrogen receptor beta: an overview and update. Nucl Recept Signal. *6*, 1-10.

Zhou, Y., Yau, C., Gray, J.W., Chew, K., Dairkee, S.H., Moore, D.H., Eppenberger, U., Eppenburger-Castori, S., and Benz, C.C. (2007). Enhanced NF kappa B and AP-1 transcriptional activity associated with antiestrogen resistant breast cancer. BMC Cancer. *3*, 59.

Zhu, X., Leav, I., Leung, Y.K., Wu, M., Liu, Q., Gao, Y., McNeal, J.E., and Ho, S.M. (2004). Dynamic regulation of estrogen receptor-beta expression by DNA methylation during prostate cancer development and metastasis. Am J Pathol. *164*, 2003-12.

APPENDIX

Supplementary Tables

Experimental data included in the Appendix was collected by the OICR MedChem Group (Toronto) and by the lab of Dr. PJ O'Brien (Faculty of Pharmacy, University of Toronto)

Compound	Percent Remaining (Phase I) 30 min	Metabolic Stability Classification	Percent Remaining (Phase I/II) 30 min	Metabolic Stability Classification
E2	2 %	Unstable	5 %	Unstable
L17	70 %	Moderate	34 %	Unstable

Table A1. A liver microsome assay to detectoxidation/quinone formation. Assays were performed by the OICR MedChem Group as described in Biochem Pharm 48:22147-56 (1994) and Drug Metab Dispos 28:560-6 (2000).

Ligand	Structure	Hepatic Cell LC_{50} (μM)
E2		400-450
1a		320-400
1c		250-280
1i		155-200
L17		>600

Table A2. Hepatocyte toxicity, showing LC50 for 2 h exposure. This assay was performed in the lab of Dr. PJ O'Brien (Faculty of Pharmacy, University of Toronto) as described in Chemico-Biological Interactions 167: 184-92 (2007). Table was modified from the data presented Journal of Medicinal Chemistry 54 (2): 433-448 (2011).