Mcl-1 Drives Resistance of Estrogen Receptor-α Positive Breast Cancers to Targeted Therapies

By

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I dedicate this thesis to my sources of support and inspiration: my parents, grandparents, and loving fiancé.

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ABBREVIATIONS

3D - 3-dimesional

AI – Aromatase Inhibitor

AKT – Protein Kinase B

ATCC – American Tissue Type Collection

Bad – BCL2 Associated Agonist of Cell Death

Bak – BLC2 Agonist/Killer 1

Bax – BCL2 Associated X

Bcl2-A1 – BCL2 Related Protein A1

Bcl-2 – B-Cell CLL/Lymphoma 2

Bcl-xL – BCL2 Like 1

Bcl-w – BCL2 like 2

Bid – BH3 Interacting Domain Death Agonist

Bik – BCL2 Interacting Killer

Bim – BCL2 Like 11

BH3 – Bcl-2 Homology-3

CCLE – Cancer Cell Line Encyclopedia

CGH – Comparative Genomic Hybridization

CHX - Cycloheximide

CLL – Chronic Lymphocytic Leukemia

c-Myc – Proto-Oncogene c-Myc

DCIS – Ductal Carcinoma *In Situ*

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

DUB - Deubiquitinase

E_2 - Estrogen

EGFR – Epidermal Growth Factor Receptor

ERα+ - Estrogen Receptor-alpha Positive

FFPE – Formalin Fixed Paraffin Embedded

HER2 – Human Epidermal Growth Factor Receptor 2 (also known as ErbB2)

Hrk – BCL2 Interaction Protein

IC50 – Half Maximal Inhibitory Concentration

IP - Immunoprecipitation

LUC - Luciferase

LTED – Long Term Estrogen Deprivation

MAPK – Mitogen-Activated Protein Kinase

MEC – Mammary Epithelial Cells

Mcl-1 – Myeloid Cell Leukemia 1

MOMP – Mitochondrial Outer Membrane Permeabilization

mTOR – Mechanistic Target of Rapamycin

mTORC1 – mTOR-Complex 1

NAC – Neoadjuvant Chemotherapy

Noxa – PMA-Induced Protein 1

OMM – Outer Mitochondrial Membrane

PI3K – Phosphatidylinositol-4,5-Bisphosphate 3-Kinase

PLA – Proximity Ligation Assay

Puma – BCL2 Binding Component 3

RNA – Ribonucleic Acid

RNAi – RNA-interference

RT-qPCR – Real Time-Quantitative Polymerase Chain Reaction

SE – Standard Error

SERM – Selective Estrogen Receptor Modulator

SERD – Selective Estrogen Receptor Downregulator

si-NP – siRNA-Loaded Nano-Particles

TAg – Large T-Antigen

TCGA – The Cancer Genome Atlas

TEB – Terminal End Bud

TNBC – Triple Negative Breast Cancer

TUNEL – Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

Ubq - Ubiquitinase

WAP – Whey Acetic Protein

CHAPTER I

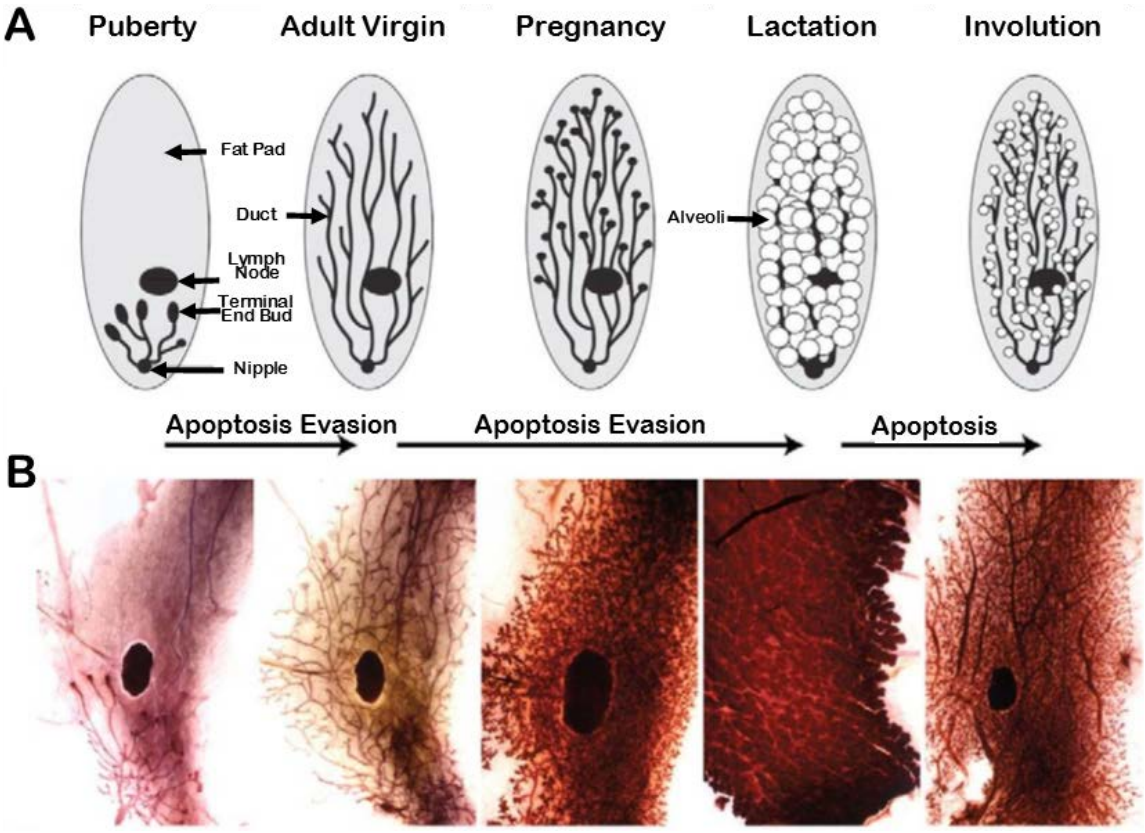
Introduction

Text adapted from: Williams, MM and Cook RS. Bcl-2 Family Proteins in Breast Development and Cancer: Could Mcl-1 Targeting Overcome Therapeutic Resistance? *Oncotarget.* February 2015.

Overview

The mammary gland is a dynamic tissue that undergoes many developmental stages, including puberty, pregnancy, lactation, post-lactational involution, and lobular (menopausal) involution. Normal progression through these stages is regulated by numerous intracellular signaling cues that regulate the balance of mammary epithelial cell (MEC) proliferation and cell death (1). During puberty and pregnancy, MEC proliferation in the ductal and alveolar MEC lineages, respectively, outweighs cell death, allowing for expansion of each MEC population as needed (**Figure 1**). In contrast, cell death predominates over proliferation to cull milk-producing MECs once offspring are weaned, and to support lobular involution at menopause. In the breast, as in many other tissues, the cellular commitment to apoptosis (a form of programmed cell death) is regulated by the intrinsic apoptotic pathway, which is comprised of the Bcl-2 family of proteins.

Two main subclasses of Bcl-2 proteins exist: anti-apoptotic (Bcl2-A1, Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) and pro-apoptotic (Bad, Bak, Bax, Bid, Bik, Bim, Hrk, Noxa, and Puma). Complex interactions among pro- and anti-apoptotic family members integrate signaling information to regulate cell death decisions. Due to the dynamic nature of the breast, characterized by sequential cycles of cell growth and cell death, Bcl-2 proteins are essential for development and homeostasis. Dysregulation of Bcl-2 proteins can impede development at several key stages.


A
Puberty
Adult Virgin
Pregnancy
Lactation
Involution
Fat Pad
Duct
Lymph Node
Terminal End Bud
Nipple
Alveoli
Apoptosis Evasion
Apoptosis Evasion
Apoptosis
B

Furthermore, sustained Bcl-2 family dysregulation contributes to evasion of cell death, a hallmark of cancer. In the context of breast cancers, Bcl-2 dysregulation promotes innate or acquired treatment resistance. This introduction will summarize the role of Bcl-2 family members in breast development, tumorigenesis, and therapeutic resistance, and will highlight one member, Mcl-1, as a promising therapeutic target in breast cancers.

Intrinsic apoptotic pathway

The intrinsic apoptotic pathway is characterized by mitochondria outer membrane permeabilization (MOMP), resulting in release of cytochrome-c from the mitochondria to the cytoplasm, thus triggering a caspase cascade resulting in cell death. This apoptotic pathway is governed by hierarchical interactions between pro-apoptotic and anti-apoptotic Bcl-2 proteins. Every Bcl-2 family member contains at least one of the four Bcl-2 homology (BH) domains, termed BH1, BH2, BH3, and BH4, that are highly conserved from *Caenorhabditis elegans* to humans (**Figure 2**). BH3 domains facilitate and stabilize protein-protein interactions within the Bcl-2 family, which is critical for regulating MOMP (4, 7).

Although not fully understood, most proposed mechanisms of MOMP suggest that Bcl-2 family effectors (Bak and Bax) heterodimerize to form higher-order oligomers, creating pores in the outer mitochondrial membrane (OMM) permitting cytochrome-c to escape from the mitochondria into the cytoplasm (8, 9) (**Figure 3A**). Bak/Bax harbor BH1-3 domains required for interaction and oligomerization (**Figure 2**). However, Bak/Bax cannot interact until they undergo a dramatic conformational change induced by binding of pro-apoptotic Bcl-2 activators (Bim, Puma, and Noxa) (10). Activators only harbor BH3 domains and are thus referred to as 'BH3-only' proteins. Further, activity of Bak/Bax is highly regulated by subcellular localization. Bak is associated with the OMM and sustains base-line activity of the intrinsic apoptotic pathway (11).

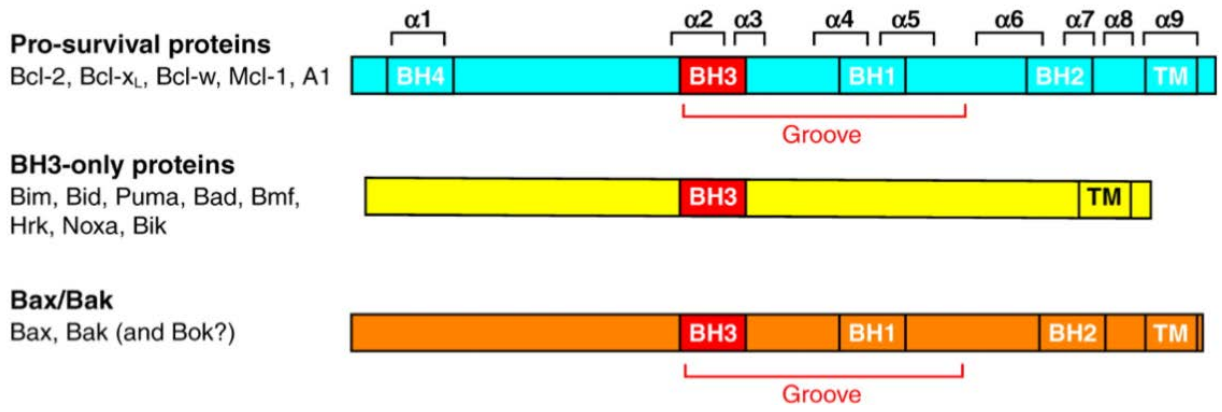


Figure 2. Structural homology of Bcl-2 family proteins.

Depicted are the structures of mammalian Bcl-2 family proteins. BH1-4 domains are required for interaction between Bcl-2 family proteins. BH3-motifs in BH3-only proteins bind within the hydrophobic ‘groove’ (denoted in red) of BH1-3 containing family members. From (4).

Meanwhile, Bax shuttles from the cytoplasm to the mitochondria based on apoptotic cues, suggesting that Bax localization adjusts the sensitivity of cells to apoptotic stimuli. A cell with high Bak/Bax expression at the OMM is more 'primed' for cell death through the intrinsic apoptotic pathway.

Other Bcl-2 proteins block BH3-only activators from interacting with Bak/Bax oligomers, and are termed 'anti-apoptotic' Bcl-2 proteins (Bcl2-A1, Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) (12-14). Anti-apoptotic Bcl-2 proteins contain BH1-4 domains, which generate a hydrophobic pocket that tightly binds with BH3 domains in the BH3-only activators and in Bak/Bax (15, 16). The BH3-binding motif of BH3-only activators is central to their function, and is the key to drug development strategies aimed at inhibiting anti-apoptotic Bcl-2 family members as a means to re-activate the intrinsic apoptotic pathway in cancer cells.

A final subclass of Bcl-2 proteins, the Bcl-2 sensitizers, is comprised of BH3-only proteins Bad, Bik, Hrk, and Noxa that bind to BH1-4 domains of anti-apoptotic Bcl-2 proteins tightly, but do not bind to Bcl-2 effectors (17). While sensitizers cannot activate Bak/Bax directly, their abundance can neutralize the inhibitory effects of anti-apoptotic proteins, thus rendering cells more 'sensitive' to apoptotic stimuli. Thus, BH3-only proteins are pro-apoptotic forces that cooperate to enhance Bak/Bax-mediated MOMP. Importantly both BH3-only activators and sensitizers are rapidly upregulated in response to oxidative stress, DNA damage, endoplasmic reticulum stress, or other insults that may provoke cell death through varying molecular mechanisms, including transcriptional regulation, subcellular localization and phosphorylation (18-21).

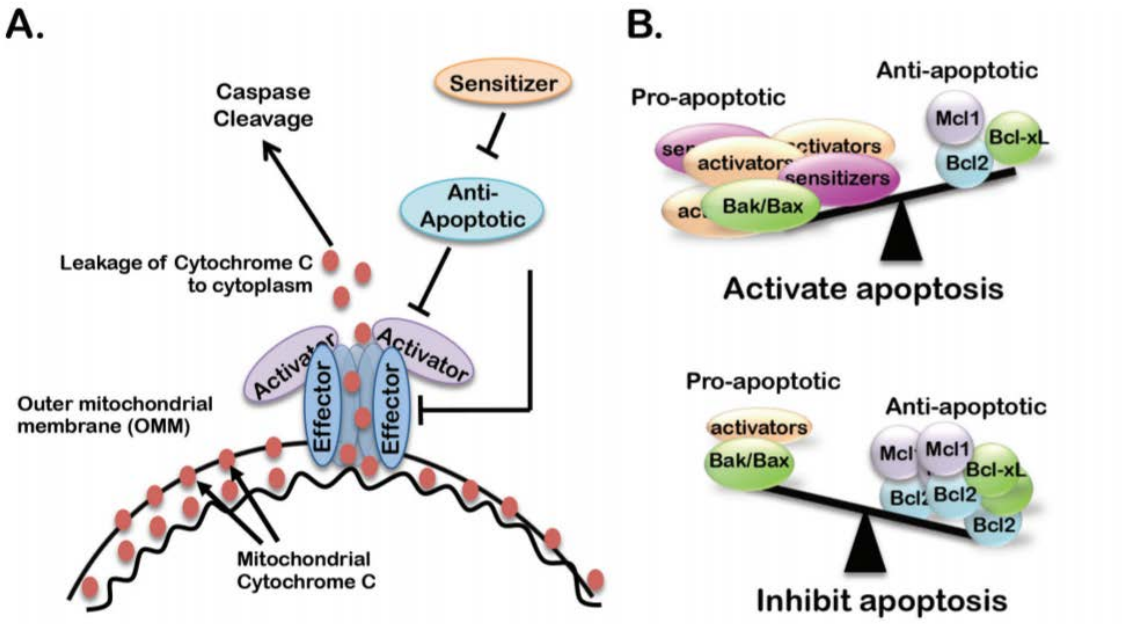


Figure 3. Intrinsic apoptotic pathway.

A. Bcl-2 family member effector proteins (Bax and Bak) oligomerize and permeabilize the outer mitochondrial membrane (OMM) upon activator protein (Bid, Bim, and Puma) binding. Cytochrome-c is able to escape the mitochondria through Bak/Bax pores, leading to caspase cleavage and apoptosis. Anti-apoptotic Bcl-2 family members (Bcl2-A1, Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) inhibit apoptosis by preventing effector protein oligomerization or Bak/Bax activation. Sensitizer proteins (Bad, Bik, and Noxa) counteract anti-apoptotic members by sequestering anti-apoptotic proteins. **B.** When the balance of active Bcl-2 proteins favors pro-apoptotic over anti-apoptotic family members, apoptosis is initiated.

Because commitment to cell death occurs only when the complex interplay between pro- and anti-apoptotic Bcl-2 classes favors apoptosis (**Figure 3B**), the fluctuation in expression/localization/activation of BH3-only proteins in response to cellular cues liken the BH3-only proteins to a cellular rheostat, constantly adjusting and tuning the sensitivity of cells to apoptosis.

Apoptosis is required in normal mammary gland development

The intrinsic cell death pathway is essential to several developmental phases of the mammary gland (**Table 1**). In response to hormonal stimuli during puberty, MECs within the terminal end buds (TEBs), the club-shaped structures at the distal mammary epithelial tips (**Figure 1**), proliferate and collectively invade the surrounding stroma. While differentiation of epithelial progenitors in the TEB populates the ducts with solid cords of mature luminal MECs, programmed cell death canalizes the ducts, resulting in lumen formation. In mouse models, transgenic overexpression of anti-apoptotic BCL-2 impaired TEB structure and disrupted ductal lumen formation (22), while genetic ablation of anti-apoptotic MCL-1 increased cell killing in TEBs, resulting in reduced ductal elongation (23). Similarly, genetic ablation of the BH3-only activator BIM in mice delayed lumen formation in mammary ducts due to reduced apoptosis in TEBs (24). Although some differences exist between human and mouse mammary gland development, the molecular events governing ductal development are heavily conserved. Taken together these studies demonstrate that Bcl-2 family proteins govern ductal lumen formation in the developing mammary gland during puberty.

Once the ductal epithelium is established, other MECs throughout the breast, the alveolar progenitor cells, undergo subtle rounds of hormonally-induced growth during the menstrual cycle (in humans) or estrus cycle (in rodents and other mammals). Alveolar expansion during the menstrual/estrus cycle prepares the MEC population for rapid pregnancy-induced growth of the alveolar (milk-producing) epithelium (**Figure 1**).

Class	*Bcl-2 Protein*	*Transgenic Model*	*Developmental Phenotype*	*Breast Cancer Phenotype*	*Ref.*
Effectors ↓	**BAK**	na	na	na	na
	BAX	WAP-*bax*	Impairs alveologenesis, induces premature involution	na	(25)
		MMTV-*myc bax*$^{+/-}$	na	Reduces tumor multiplicity	(26)
		bax$^{-/-}$	Normal involution	Not highly tumorigenic	(27)
		C3(1)/Tag-*bax*$^{-/+}$	na	Reduces apoptosis at preneoplastic stage	(28)
Activators ↓	**BIM**	*bim*$^{-/-}$	TEB filling	na	(24)
	BID **PUMA**	na	na	na	na
Sensitizer ↓	**BAD** **BIK** **NOXA**	na	na	na	na
Inhibitors ↓	**BCL-2**	WAP-*bcl2*	Reduces apoptosis in TEBs, increases cell survival/decreases cell death during involution	Accelerates MMTV-Myc-induced mammary tumorigenesis, delays DMBA*-induced breast tumor formation	(22, 27, 29, 30)
		WAP-TAg;WAP-*bcl2*	na	Reduces tumor latency	(31)
	BCL-XL	WAP-Cre-*bclxl fl/fl* MMTV-Cre-*bclxl fl/fl*	Promotes cell survival involution	na	(32)
	MCL-1	MMTV-Cre-*mcl1 fl/fl* K5-Cre-*mcl1 fl/fl*	Increases apoptosis TEBs, impaired alveolar expansion	na	(23)
		WAP–icre-*mcl1 fl/fl*	Precocious involution	na	(23)
	A1 **BCL-W**	na	na	na	na

Table 1. Transgenic misregulation of Bcl-2 family proteins in mouse models of mammary development and breast tumorigenesis. Adapted from (5), na = not available.

However, in the absence of pregnancy, the expanded alveolar progenitor population is removed by programmed cell death. Thus, apoptosis within the breast occurs with each hormonal cycle throughout a woman's reproductive lifespan.

In the event of pregnancy, massive expansion of the alveolar (milk-producing) epithelium occurs in preparation for nursing. The milk-producing cells are maintained while offspring continue to nurse. Once weaning occurs, the alveolar epithelium undergoes massive cell death in a tissue-remodeling event termed post-lactational involution. During this short period of time (7-10 days in mice, 6-12 months in women), up to 80% MECs undergo cell death. Mouse model-based evidence of Bcl-2 family member regulation of the lactation-to-involution switch is abundant. High expression levels of the anti-apoptotic proteins BCL-2, BCL-W, and MCL-1 during lactation were rapidly down-regulated during post-lactational involution (23, 33), allowing for induction of cell death. Disruption of the Bcl-2 family balance through transgenic BAX overexpression resulted in cell death during lactation instead of during involution, causing premature involution (25). Furthermore, genetic BCL-XL ablation in the mammary epithelium dramatically increased cell death during post-partum involution (32), while deletion of MCL-1 at different time points during mammary gland development impaired alveolar cell expansion or initiated precocious involution (23). Collectively, these studies demonstrate that Bcl-2 family proteins are key regulators of cell survival during lactation and are critical for inducing cell death of the milk producing epithelium once lactation ceases.

Less is known regarding the Bcl-2 family in lobular (menopausal) involution of the breast. However, increasing evidence suggests that the extent to which lobular involution occurs is directly proportional to a woman's risk of developing post-menopausal breast cancer. Nearly 70% of all breast cancers occur in post-menopausal women. It is therefore important to understand the molecular mechanisms contributing to lobular involution. Given the importance of Bcl-2 proteins

in other stages of breast development, these observations support the exploration of Bcl-2 family proteins in lobular involution.

Bcl-2 family in breast cancer formation and progression

Anti-apoptotic Bcl-2 proteins were first acknowledged as oncogenes in 1984 upon discovery of the Bcl-2 chromosomal translocation t(14;18), which was a driving factor in B-cell leukemia (34) and changed the oncogene paradigm to include anti-apoptotic proteins. Evasion of cell death is now acknowledged as a hallmark of cancer, required to overcome the counterbalancing effects of cell death on enhanced cell proliferation (35). As an illustration of this point, studies performed in three-dimensional acinar cultures of untransformed MECs demonstrated that oncogene-induced proliferation was insufficient to support lumen filling due to equally increased cell death (36, 37). However, Bcl-2 and Bcl-xL overexpression in combination with oncogene-induced proliferation resulted in lumen filling, a morphological characteristic of ductal carcinoma in situ (DCIS), an early pre-malignant state (6, 38). Thus, pro-proliferative and anti-apoptotic signals, coordinated by anti-apoptotic Bcl-2 proteins, cooperate in early MEC transformation (**Figure 4**).

Consistent with these findings, although transgenic overexpression of BCL-2 alone did not generate mammary tumors, c-*Myc* induced (29) and SV40 Large T-antigen (TAg)-induced (31) mammary tumors developed at a remarkably faster rate in the context of BCL-2 overexpression. Similarly, genetic BAX ablation accelerated TAg-induced mammary tumor progression (28). In human breast cancers, up to 80% of *MYC*-amplified triple negative breast cancer (TNBC) cases harbor *MCL1* co-amplification (39). Further, Bcl-2 expression frequently correlates with Estrogen Receptor-α (ERα) expression levels in ERα+ breast cancers (40). Taken together, these studies suggest that anti-apoptotic Bcl-2 proteins may cooperate with pro-proliferative signals to support breast cancer initiation and progression.

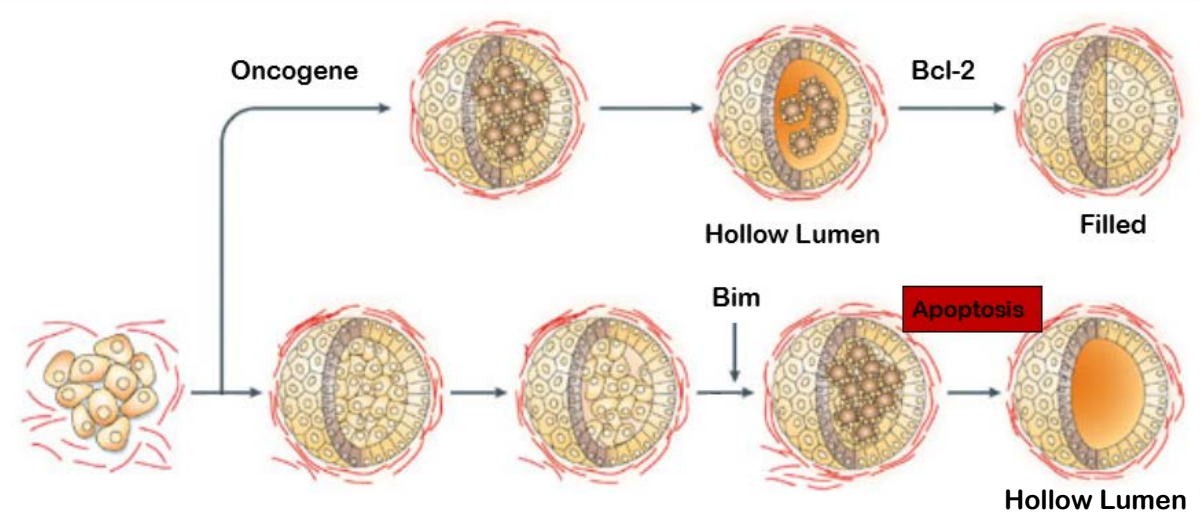


Figure 4. Evasion of cell death is essential for lumen filling and transformation.

Schematic of normal mammary epithelial cells (MECs) forming acinar structures when cultured in a 3D-matrix. Apoptosis, organized by the intrinsic apoptotic pathway, is essential for formation of normal acinar structures with a hollow lumen. However, in the presence of an oncogenic driver that promotes aberrant proliferation, anti-apoptotic Bcl-2 family proteins can halt the induction of cell death needed to clear the lumen. A filled lumen is a characteristic of early pre-malignant changes. Adapted from (6).

Bcl-2 family in therapeutic resistance

The goal of chemotherapies, therapeutic radiation, and molecularly targeted drugs is effective tumor cell killing. However, this goal is often thwarted by increased anti-apoptotic Bcl-2 family protein activity or expression (**Figure 5**). In the context of chemotherapy, clinical breast cancer specimens biopsied before and after a course of pre-operative neoadjuvant chemotherapy displayed increased Bcl-2 in residual post-treatment tumor cells (41). Furthermore, metastatic human breast cancer cell lines with high Bcl-2 levels were less sensitive to chemotherapies, including taxanes (42) and doxorubicin/Adriamycin (43). *MCL1* amplification was more frequently observed in chemo-refractory TNBC samples as compared to the total TNBC sample population (39).

Bcl-2 family proteins also mediate resistance to targeted breast cancer therapies. The anti-HER2 antibody, trastuzumab (trade name, Herceptin^{R}), is clinically approved for use in breast cancers that exhibit *HER2* gene amplification, accounting for ~20% of all breast cancers equating ~60,000 new cases each year in the U.S. However, many *HER2*-amplified tumors display innate trastuzumab resistance, while others rapidly acquire trastuzumab resistance. Trastuzumab-resistant HER2+ breast cancer cell lines frequently upregulate Bcl-2 and decrease Bax as a means of enhancing tumor cell survival (44).

55-65% of breast cancers are ERα+ (nearly 180,000 new cases each year in the U.S.) and are thus treated with drugs that interfere with ERα signaling, such as selective ERα modulators (SERMS, e.g. tamoxifen), selective ERα downregulators (SERDS, e.g., fulvestrant: trade name Faslodex^{R}), or drugs that decrease circulating estrogen levels, like aromatase inhibitors (AIs, e.g., anastrozole: trade name arimidex^{R}).

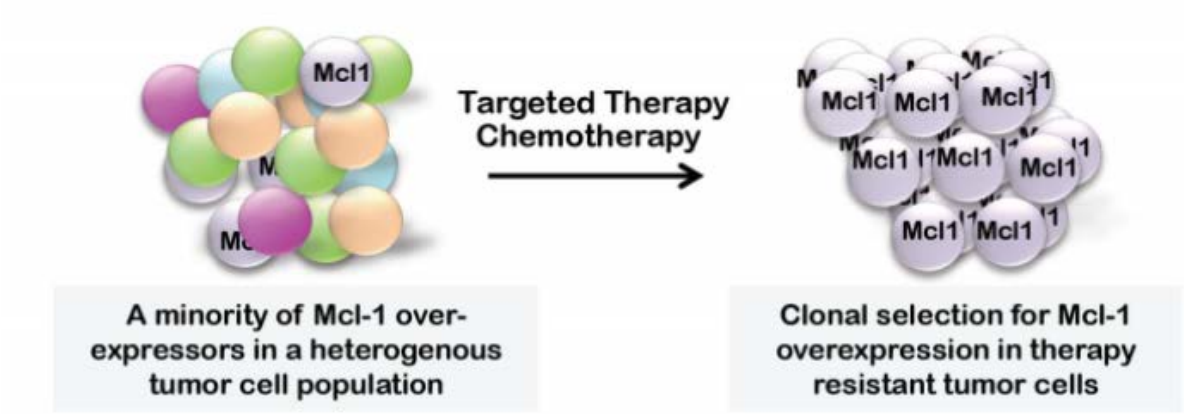


Figure 5. Standard of care breast cancer therapies increase Bcl-2 family member expression.

Chemotherapies or targeted therapies select for tumors that express high levels of anti-apoptotic Bcl-2 family members. Mcl-1 is used as an example.

Although these targeted therapies improve outcome for many patients with ERα+ breast cancers, 15-20% relapse within 5 years of treatment withdrawal (45). Tamoxifen treated ERα+ breast cancers often increase Bcl-2, Bcl-xL, or Mcl-1 levels, decreasing acute tumor response to tamoxifen and increasing long-term tamoxifen resistance (46-48). Similarly, Bcl-2, Bcl-w, and Mcl-1 expression is increased in models of fulvestrant resistance (47, 49). Using estrogen deprivation of ERα+ human breast cancer cell lines as a model of AI treatment, one study showed that cell survival under these conditions is supported by increased activity of Bcl-2 at the mitochondria (50). Thus, Bcl-2 protein dysregulation favoring expression or activity of anti-apoptotic family members is a driver of resistance to standard of care breast cancer therapies, including chemotherapies and targeted therapies (**Figure 5**). In fact, genetic silencing of Bcl-2 and Mcl-1 restored sensitivity to chemotherapies and targeted therapies in certain breast cancer models (39, 47, 49).

Targeting Bcl-2/Bcl-xL to enhance breast tumor cell killing

Based on observations that tumors require evasion of cell death, and that anti-apoptotic Bcl-2 family proteins are frequently overexpressed or hyper-activated in cancers, targeted inhibition of anti-apoptotic Bcl-2 proteins may be an effective therapeutic avenue for some tumors. This hypothesis is the basis for the relatively recent development of BH3-mimetics, a class of small molecular weight Bcl-2 family inhibitors that tightly bind the hydrophobic BH3 binding motif within anti-apoptotic Bcl-2 proteins. In the presence of drug, Bcl-2 activators (Bim, Puma, and Noxa) are released from sequestration by Bcl-2 inhibitors, resulting in MOMP and caspase-dependent cell death (51). Several BH3-mimetics have been developed (**Table 2**), including the Bcl-2/Bcl-xL inhibitor ABT-263 (52), which binds to Bcl-2 and Bcl-xL in a manner similar to the BH3 domain of the sensitizer Bad.

Target	*Compound: Company*	*Tested Pre-Clinically?*	*Subtype*	*Combination Therapy*	*Clinical Trial #: Combo. Therapy*	*Ref.*
Bcl-2	**ABT-199: AbbVie**	Yes	ERα+	Tamoxifen	ACTRN12615000702516: Tamoxifen	(48, 53)
			All	na	NCT02391480: Tested as single agent	(54)
Bcl-xL	**A-1155463 (A-1331852*): AbbVie**	Yes	TNBC	Docetaxel	na	(55)
	WEHI-539: Genentech	Yes	TNBC	PI3K Inhibitors, A-1210477	na	(56)
Bcl-2 + Bcl-xL	**ABT-263 (ABT-737*): AbbVie**	Yes	ERα+	Tamoxifen, PI3K Inhibitors	na	(48, 56, 57)
			TNBC	Docetaxel, PI3K Inhibitors	na	(56, 58)
Mcl-1	**AMG-176: Amgen**	No	na	na	na	(59)
	A-1210477: AbbVie	Yes	TNBC	ABT-263	na	(60)
	S63845: Servier	Yes	HER2+	Lapatinib	na	(61)
	VU-159: Vanderbilt University	No	na	na	na	(62)
Pan-Bcl2	**Gx15-070: Teva Pharm.**	Yes	HER2+	Lapatinib, Docetaxel	na	(63)

Table 2. Anti-apoptotic Bcl-2 family member inhibitors and their use in breast cancer models. List of current anti-apoptotic Bcl-2 family member inhibitors. All inhibitors are from the BH3-mimetic drug class. Also detailed is the use of these inhibitors in breast cancer models and effective drug combinations. * denotes orally bioavailable version of inhibitor, na = not available.

Although ABT-263 has shown promising anti-cancer activity in chronic lymphocytic leukemia (CLL) as a single agent (64), ABT-263 caused on-target induction of apoptosis in platelets due to Bcl-xL inhibition (65), limiting its clinical utility. However, development of Bcl-xL specific BH3-mimetics, like A-1155463 or the orally bioavailable A-1331852, has continued in hopes of teasing out the relative contribution of anti-apoptotic Bcl-2 family members to tumor cell survival (55, 66). Alternatively, the Bcl-2 selective inhibitor ABT-199 spares platelets due to a lack of Bcl-xL inhibition (67, 68). Although initial clinical trials using ABT-199 in CLL patients were suspended due to fatal levels of tumor lysis, ABT-199 has gained clinical approval in CLL, and is entering trials in other cancers, including breast cancers (53).

Although BH3-mimetics are just entering the clinic for breast cancer treatment, pre-clinical models support the use of BH3-mimetics in combination with existing breast cancer treatment strategies. For example, Bcl-2 is overexpressed in about 85% of ERα+ breast cancers, perhaps due to the fact that its promoter is directly bound and transactivated by ERα (69). Additionally ERα+ breast tumors have elevated levels of Bcl-xL (58), suggesting that ERα+ breast tumors may benefit from the targeting of multiple anti-apoptotic Bcl-2 family proteins. Patient-derived ERα+ breast cancer xenografts treated with tamoxifen in combination with the Bcl-2/Bcl-xL inhibitor ABT-737 (51), an analogue of ABT-263, displayed decreased tumor growth and increased tumor cell death (48). Similar results were obtained using tamoxifen in combination with ABT-199, the Bcl-2 selective inhibitor. However, BH3-mimetics alone failed to decrease tumor growth or induce apoptosis in this model. In models of TNBC, ABT-737 or A-1331852 sensitized patient-derived basal-like breast cancer xenografts to the chemotherapeutic agent docetaxel (58), and increased cell death in irradiated TNBCs in cell culture and *in vivo* (70). GX15-070 (obatoclax), a pan Bcl-2 family inhibitor, cooperated with lapatinib (an EGFR/HER2 inhibitor) to induce cell death in HER2+ breast cancer cells treated with radiotherapies (71). These studies suggest that targeting Bcl-2 family proteins in combination with current chemotherapies and/or targeted therapies may

increase tumor cell killing and therefore improve breast cancer patient outcome. These success of these preclinical findings has instigated Phase I studies to determine the efficacy of ABT-263 with paclitaxel, gemcitabine and sorafenib in solid tumors, which could include breast cancers (72-74). Additionally, the clinical combination of ABT-199 with tamoxifen in ERα+ breast tumors is currently being explored (53).

Evidence for targeting Mcl-1 in breast cancer

Although there is strong preliminary evidence supporting the roles of Bcl-2 and Bcl-xL in breast tumor formation and therapeutic resistance, high Bcl-2 protein levels in human breast cancer samples often correlate with a favorable prognosis (75), possibly because Bcl-2 is transcriptionally regulated by ERα, which can be successfully targeted in ~70% of ERα+ breast cancer cases. Furthermore, according to The Cancer Genome Atlas (TCGA), genetic amplification of *BCL2* and *BCL2L1* (encoding Bcl-xL) occurs in less than 3% of ERα+ breast cancer and TNBC cases (76), implying that BH3-mimetic targeting of Bcl-2 and Bcl-xL may not be beneficial in many breast cancer subtypes. However, targeting of other anti-apoptotic Bcl-2 family members, like Mcl-1, may provide an alternative approach.

At the initiation of this thesis research, little was known regarding the contribution of Mcl-1 to breast cancer formation and therapeutic response. However, unlike other Bcl-2 family members, Mcl-1 has a short protein half-life and is highly regulated at the transcriptional, post-transcriptional, translational, and post-translational levels (3, 77) by various oncogenic signaling pathways (**Figure 6**), including the mitogen activated protein kinase (MAPK) pathway (78), the mechanistic Target of the Rapamycin (mTOR) pathway (79), and the phosphatidylinositol-3 kinase (PI3K) pathway (80).

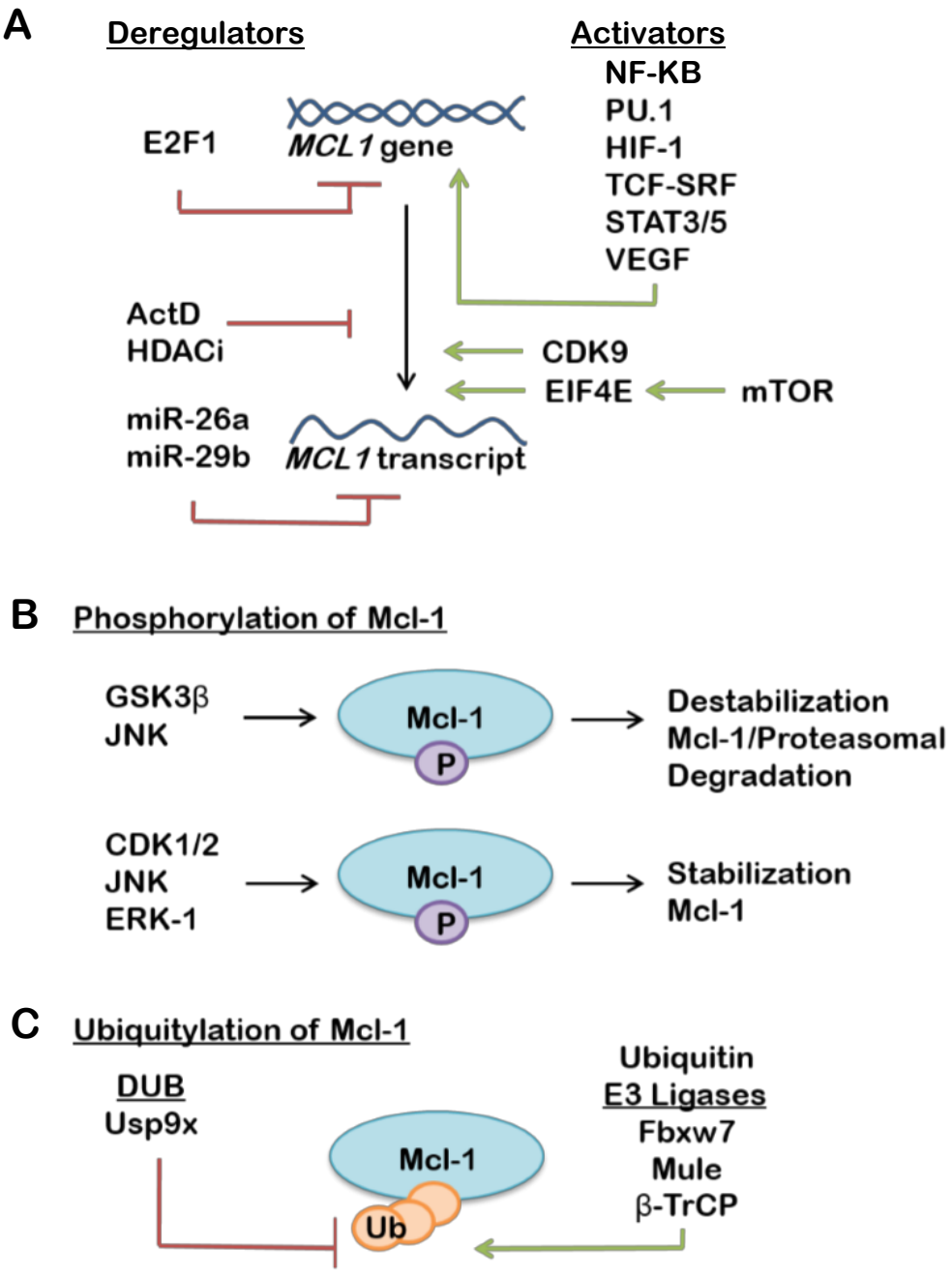


Figure 6. Regulation of Mcl-1 expression.

A. Transcriptional, translational and post translational mechanisms of Mcl-1 regulation (activators support Mcl-1 expression, while deregulators decrease Mcl-1 expression). **B-C.** A complex interplay of phosphatases (B), ubiquitinases and deubiquitinases (DUBs, C) regulate Mcl-1 stability through activation or suppression of ubiquitin-mediated proteasomal degradation of Mcl-1 [further reviewed in (2, 3)].

These oncogenic signaling pathways rapidly increase Mcl-1 to promote cell survival, suggesting that Mcl-1 might mediate apoptotic escape and therapeutic resistance in response to PI3K/mTOR signaling. This hypothesis is supported observations that the dual PI3K/mTOR inhibitor NVP-BEZ235 decreased Mcl-1 levels in ovarian carcinoma cells (81), as well as in ERα+ and ER-negative breast cancer cells (56). Additionally, Mcl-1 expression was inhibited by targeted mTOR inhibition using rapamycin, rapaloges (such as RAD001/everolimus), or ATP-competitive mTOR inhibitors (such as AZD8055), promoting increased tumor cell killing in culture and *in vivo* models of colon cancer (82), rhabdomyosarcomas (83), lung cancers (84, 85), and glioblastomas (86).

Specifically in the context of breast cancer, *MCL1* has genetic amplifications or mRNA upregulation in 16/58 (28%) of human breast cancer cell lines as curated by the CCLE (**Figure 7**), while *BCL2* is aberrantly upregulated in only 2/58 cases (3%) (76). Expression of Mcl-1 in human breast cancer samples correlates with high tumor grade and decreased patient survival, regardless of subtype (87). *MCL1* is genetically amplified in 9% of luminal B breast cancers (76), and in 54% of TNBCs resistant to neoadjuvant chemotherapies (39), suggesting that Mcl-1 targeting may be particularly favorable in these breast cancer subtypes.

Furthermore, targeting of Mcl-1, through use of a pan-anti-apoptotic Bcl-2 family member inhibitor in combination with lapatinib, sensitized breast cancer cells to radiotherapies, demonstrating that increased Mcl-1 levels may render HER2+ breast cancer cells less sensitive to radiation (88). Additionally, human ERα+ breast cancers saw increased Mcl-1 expression upon tamoxifen- and fulvestrant-resistance, resulting in exquisite sensitivity to Mcl-1 ablation (47). These preliminary studies suggest that Mcl-1 may support escape of breast cancer cells from therapy induced cell death (**Figure 5**). Thus, targeting Mcl-1 may be beneficial to improving the outcome of breast cancer patients, particularly those primed for high Mcl-1 expression by *MCL1*-amplification.

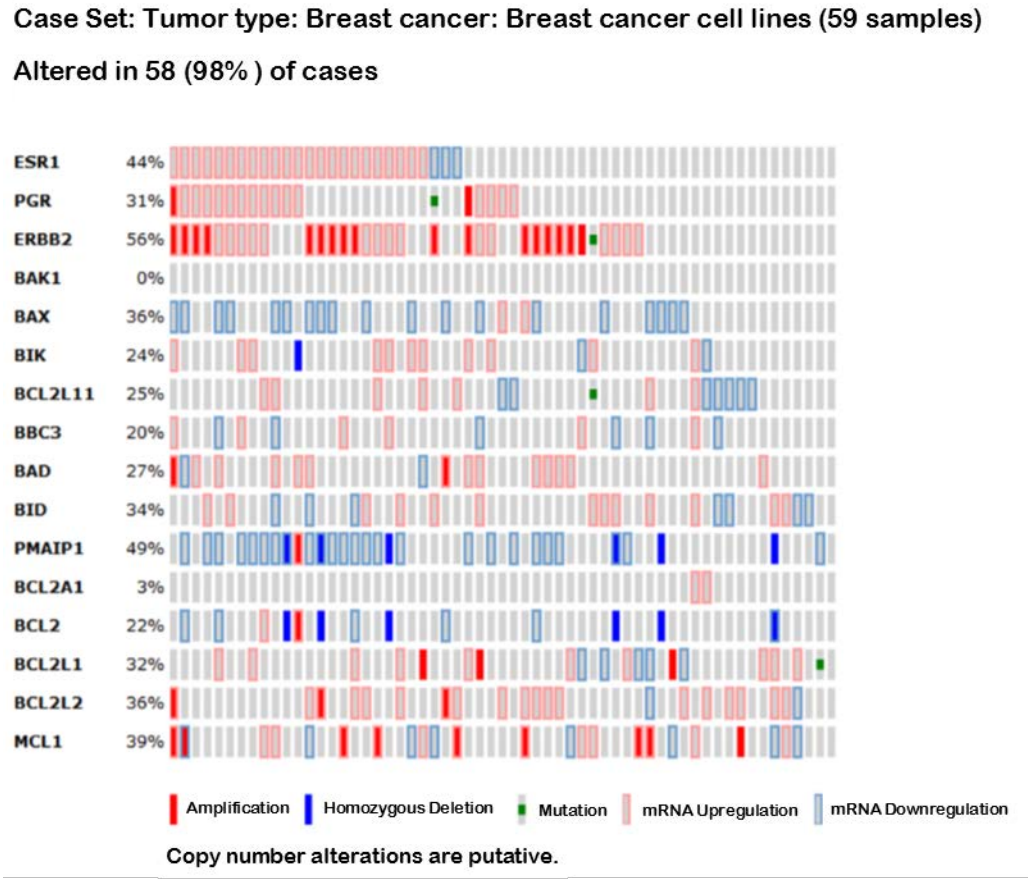


Figure 7. Expression signature of Bcl-2 family proteins in human breast cancer cell lines according to the Cancer Cell Line Encyclopedia (CCLE) as curated by the cancer genome atlas.

BCL2L11 = Bim, *BBC3* = Puma, *PMAIP1* = Noxa, *BCL2L1* = Bcl-xL, *BCL2L2* = Bcl-w.

The importance of targeting Mcl-1 in breast cancers is further supported by extensive studies conducted on anti-apoptotic Bcl-2 family proteins in other cancers. These studies suggest that high Mcl-1 expression or activity (the ability of Mcl-1 to interact with and sequester pro-apoptotic Bcl-2 family proteins) confer resistance to the Bcl-2/Bcl-xL inhibitor ABT-263 in leukemias, lymphomas, melanomas, and lung cancers, as well as certain breast cancer subtypes (56, 89-94). Specifically in triple negative breast cancers, preliminary studies show that indirect or direct Mcl-1 blockade restored sensitivity to Bcl-xL inhibitors or dual Bcl-2/Bcl-xL inhibitors (56, 60). These findings highlight the potential compensatory nature of anti-apoptotic Bcl-2 family members and suggest that maximum tumor cell killing may require Bcl-2, Bcl-xL, and Mcl-1 combined inhibition (**Figure 8**). For these reasons, great excitement has surrounded the recent development of Mcl-1 specific BH3-mimetics (61, 62, 95), many of which are in pre-clinical development, while some have recently entered clinical trials (59, 96). However, the use of these inhibitors in breast cancer models, particularly ERα+ breast cancers, remains understudied, while their clinical use in breast cancers remains on the horizon.

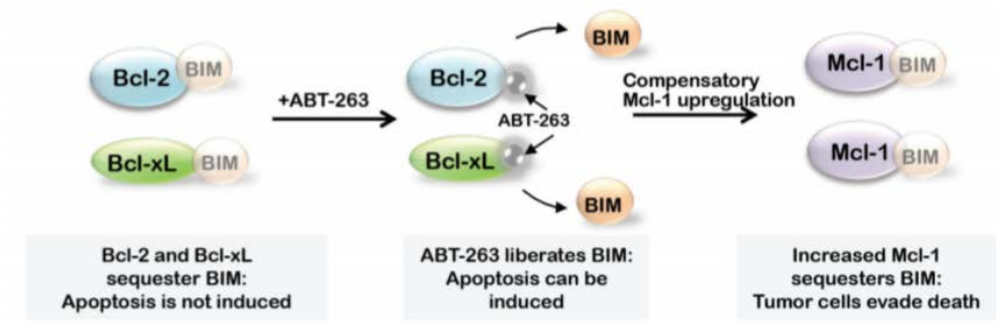


Figure 8. Proposed model for Mcl-1-mediated resistance to ABT-263.

The Bcl-2/Bcl-xL inhibitor ABT-263 blocks interactions between Bcl-2/Bcl-xL and pro-apoptotic Bcl-2 family members like Bim. Compensatory upregulation of Mcl-1 may however result in re-sequestration of Bim, and limited tumor cell death.

Summary and study aims

Cell death orchestrated by anti-apoptotic Bcl-2 family proteins is essential to normal mammary gland development. However, when aberrant overexpression of anti-apoptotic Bcl-2 family members occurs in the context of an oncogenic driver, evasion of cell death can promote breast tumor initiation, progression, and therapeutic resistance. Although preliminary studies in breast cancers demonstrate that inhibiting Bcl-2 or Bcl-2/Bcl-xL in combination with targeted therapies is sufficient to increase tumor cell killing and decrease tumor survival, this might not be the case for all breast cancers, particularly those with elevated Mcl-1 expression or activity. For example, ERα+ breast cancers express high levels of Bcl-2, Bcl-xL, and Mcl-1 (58), but more frequently amplify Mcl-1 over other anti-apoptotic Bcl-2 family members (**Figure 7**). Therefore, targeting Bcl-2 or Bcl-2/Bcl-xL may be ineffective in this breast cancer subset due to Mcl-1 compensation, a well-established resistance factor to ABT-263 (a Bcl-2/Bcl-xL BH3-mimetic) in other cancers. Instead, Mcl-1 has the potential to be an essential tumor cell survival factor supporting ERα+ breast tumor progression and therapeutic resistance.

Addressing this hypothesis, experimental results described in **Chapter 2** will detail the outcome of Mcl-1 specific inhibition in ERα+ breast cancers, and will compare Mcl-1 single blockade via genetic targeting (shRNA) to Bcl-2/Bcl-xL targeting with ABT-263. The role of Mcl-1 as a resistance factor to Bcl-2/Bcl-xL inhibition will be explored in **Chapter 3,** where experimental results will reveal that Mcl-1 ablation can sensitize ERα+ breast cancers to ABT-263. **Chapter 3** will also discuss results of experiments assessing the molecular mechanisms that support Mcl-1 upregulation upon Bcl-2/Bcl-xL blockade. A better understanding of Mcl-1 biology in ERα+ breast cancers will educate future clinical decisions and could aid in the development of novel methods to target Mcl-1.

In addition to promoting resistance to Bcl-2/Bcl-xL inhibition, Mcl-1 may promote resistance to various standard of care breast cancer therapies, including chemotherapies, HER2

targeted therapies, and anti-estrogens. However, current studies addressing the role of Mcl-1 as a tumor cell survival factor upon treatment with or acquired resistance to anti-estrogens are preliminary and do not compare Mcl-1 inhibition to targeting of other anti-apoptotic family members. Thus, **Chapter 4** will aim to determine whether Mcl-1 is the dominant anti-apoptotic Bcl-2 protein driving tumor cell survival during anti-estrogen treatment, and the results and interpretation of these studies.

A final theme of this thesis was to develop alternative, clinically relevant methods to target Mcl-1. Although the recent development of Mcl-1 specific BH3-mimetics may allow for Mcl-1 specific targeting in the clinic, these inhibitors were not available at the initiation of these studies. Instead, we explored Mcl-1 targeting through the use of indirect inhibitors that target Mcl-1 regulatory pathways (**Chapter 3**) and through use of polymeric nanoparticles encapsulating Mcl-1 siRNA (**Chapter 4**). These therapies were selected with the hope of developing a method to rapidly target Mcl-1 in the clinic. Overall, a better understanding of Mcl-1 in ERα+ breast cancers, as well as development of clinically relevant means to target Mcl-1, could greatly improve tumor cell killing in this breast cancer subtype, leading to increased patient survival.

CHAPTER II

Key Survival Factor, Mcl-1, Correlates with Sensitivity to Combined Bcl-2/Bcl-xL Blockade

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Abstract

An estimated 40,000 deaths will be attributed to breast cancer in 2016, underscoring the need for improved therapies. Evading cell death is a major hallmark of cancer, driving tumor progression and therapeutic resistance. To evade apoptosis, cancers use anti-apoptotic Bcl-2 proteins to bind to and neutralize apoptotic activators, such as Bim. Investigation of anti-apoptotic Bcl-2 family members in clinical breast cancer datasets, revealed greater expression and more frequent gene amplification of *MCL1* as compared to *BCL2* or *BCL2L1* (Bcl-xL) across three major molecular breast cancer subtypes, Luminal (A and B), HER2-enriched, and Basal-like. While Mcl-1 protein expression was elevated in Estrogen Receptor-α (ERα)-positive and ERα-negative tumors as compared to normal breast, Mcl-1 staining was higher in ERα+ tumors. Targeted Mcl-1 blockade using RNAi increased caspase-mediated cell death in ERα+ breast cancer cells, resulting in sustained growth inhibition. In contrast, combined blockade of Bcl-2 and Bcl-xL only transiently induced apoptosis, as cells rapidly acclimated through Mcl-1 upregulation and enhanced Mcl-1 activity, as measured *in situ* using Mcl-1/Bim proximity ligation assay. Importantly, *MCL1* gene expression levels correlated inversely with sensitivity to pharmacological Bcl-2/Bcl-xL inhibition in ERα+ breast cancer cells, whereas no relationship was seen between gene expression of *BCL2* or *BCL2L1* and sensitivity to Bcl-2/Bcl-xL inhibition. These results demonstrate that breast cancers rapidly deploy Mcl-1 to promote cell survival, particularly when challenged with blockade of other Bcl-2 family members, warranting the continued development of Mcl-1 selective inhibitors for targeted tumor cell killing.

Implications: Mcl-1 levels predict breast cancer response to inhibitors targeting other Bcl-2 family members, and demonstrate the key role played by Mcl-1 in resistance to this drug class.

Introduction

The intrinsic apoptotic pathway is tightly regulated by Bcl-2 family members to support developmental processes and proper physiological function (97). Apoptosis dysregulation often produces pathological consequences, including cancer formation, progression, and therapeutic resistance (98). At the center of the intrinsic apoptotic pathway are the 'effectors' Bax and Bak, which oligomerize at the outer mitochondrial membrane (OMM) by binding to 'activators' (Bim, Bid, and Puma) (99). Bak/Bax oligomerization promotes pore formation in the OMM, resulting in mitochondrial depolarization, disruption of oxidative phosphorylation, mitochondrial cytochrome-c release into the cytoplasm, and apoptosome activation, thus initiating caspase-dependent apoptosis (8-10). Anti-apoptotic Bcl-2 family proteins (Bcl2-A1, Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) restrain the intrinsic apoptotic pathway by binding and sequestering effectors (14) and/or activators (12), thus favoring cell survival. Pro-apoptotic 'sensitizers' (Bad, Hrk, and Noxa) bind and saturate anti-apoptotic Bcl-2 proteins, thus favoring apoptosis (18, 19, 100). A delicate balance in the relative ratio of anti-apoptotic Bcl-2 proteins to apoptotic activators or sensitizers is necessary for cell survival regulation. Cancers can exploit this pathway to evade apoptosis, often through increased levels of anti-apoptotic Bcl-2 factors (101-103).

Nearly 250,000 new breast cancer cases will be diagnosed in the U.S. in 2016 (104). Despite advances in detection and treatment of breast cancers, it is estimated that up to 40,000 patients will die from breast cancer in the U.S. each year, often due to recurrent metastatic disease, highlighting the need for improved treatments that promote tumor cell killing. Several studies suggest that anti-apoptotic Bcl-2 family proteins may be particularly attractive therapeutic targets in breast cancers. For example, Bcl-2 expression was observed in up to 70% of ERα+

breast cancers (40). While Bcl-xL remains less studied in primary breast tumors, Bcl-xL expression was increased in ductal carcinoma *in situ* (DCIS), an early pre-malignant state, when compared to normal breast (105). Interestingly, many breast cancers increase levels of Bcl-2 family proteins following treatment with tamoxifen (106), fulvestrant (107), and neoadjuvant chemotherapy (NAC) (39). Bcl-2 and Bcl-xL levels reportedly predict poor response to taxanes (42), Adriamycin (43), and the HER2-monocolonal antibody Herceptin (44). Additionally, Bcl-2 family proteins are often upregulated in endocrine-resistant cancers (46, 50). These findings support an intense interest in research strategies to block activity of Bcl-2 family proteins as a means to enhance tumor cell killing.

Pharmacological inhibition of Bcl-2 family proteins has been achieved using compounds that bind to the BH3 domain binding pocket of Bcl-2 family members. These 'BH3-mimetics' block the interaction of Bcl-2 family proteins with BH3 domain containing pro-apoptotic factors (i.e. Bcl-2 activators or sensitizers), including Bim (51). ABT-199/venetoclax, which specifically blocks Bcl-2 from interacting with BH3 domain containing pro-apoptotic factors, is currently approved for clinical use in chronic lymphocytic leukemia (CLL) (108), and is in clinical trial in several additional cancers, including breast cancers (53). ABT-263/navitoclax binds to and blocks activity of Bcl-2 and Bcl-xL and is showing efficacy in early-phase clinical trials in hematological malignancies (64, 65, 109). Pre-clinical studies in ERα+ breast cancers show that ABT-263 (targeting Bcl-2 and Bcl-xL) or ABT-199 (targeting Bcl-2) may effectively increase tumor killing when cells were first 'primed' with tamoxifen (48, 58, 110).

These advances in Bcl-2 family targeting warrant a greater understanding of the molecular characteristics of breast cancers that might benefit from this treatment strategy. Examination of large clinical datasets identified expression and gene amplification of *BCL2* and *BCL2L1* (Bcl-xL) in human breast cancers. However, we found that *MCL1* gene expression occurred more frequently in breast cancers than other Bcl-2 family members. Disruption of Mcl-1 activity

increased caspase-activated apoptosis and impaired cell growth to a greater extent than combined disruption of Bcl-2 and Bcl-xL. Importantly, expression levels of *MCL1* predicted sensitivity to ABT-263 in a panel of breast cancer cell lines, which may inform results in ongoing clinical trials, or guide patient selection for future trials.

Materials and Methods

Expression analysis of publically available cancer cell line and breast cancer datasets: mRNA expression of *MCL1*, *BCL2*, and *BCL2L1* (Bcl-xL) were curate using cBio Portal (www.cbio.org) for cancer cell lines (CCLE) and breast tumor specimens (TCGA). Breast cancer specimens were stratified based on PAM50 molecular markers (TCGA), and CGH analysis was used to evaluate observed alterations at the genetic level (amplifications). mRNA expression of *MCL1*, *BCL2*, and *BCL2L1* in breast cancer cell liens (CCLE*)* were correlated to the IC50 of ABT-263 as determined by the Sanger Institute (http://www.cancerrxgene.org/), data was fit to a linear regression.

Western blotting: Cells and tumor tissue were homogenized in ice-cold lysis buffer [50mM Tris pH 7.4, 100mM NaF, 120mM NaCl, 0.5% NP-40, 100 µM Na_3VO_4, 1X protease inhibitor cocktail (Roche), 0.5µM proteasome inhibitor (Santa Cruz Technologies)]. Proteins were resolved on 4-12% SDS-PAGE gels and transferred to nitrocellulose membranes, which were blocked in 3% gelatin in TBS-T [Tris-buffered saline, 0.1% Tween-20], incubated in primary antibody [Mcl-1 S19, Bim, Bcl-2, Bcl-xL (Santa Cruz 1:500); β-Actin, E-Cadherin (Cell Signaling, 1:10,000)], secondary antibody [Rabbit, Goat, Mouse (Santa Cruz, 1:5,000-10,000)], and developed with ECL substrate (Thermo Scientific).

Proximity ligation assay (PLA): Cells cultured in 96-well plates were fixed with methanol, stained with the Duolink (Sigma) PLA protocol according to manufacturer's directions using Mcl-1 (Santa Cruz, 1:25) and Bim (Santa Cruz, 1:25) antibodies, counterstained with Hoescht and

scanned by ImageXpress Micro XL Automated Microscope. PLA fluorescent puncta and Hoescht-stained cells were enumerated using ImageJ software.

Caspase-3/7 activity: 5,000 cells/well or 10,000 cells/well were seeded in 96-well plates in Growth Media and were treated with ABT-263 or DMSO for 4-48 hours. Caspase-3/7-Glo Assay (Promega) was used according to manufacturer's directions. Luminescence was measured on a Glomax Mutli+ Detection System (Promega) luminometer and was standardized to protein values.

Cell culture: Cell lines were purchased directly from American Tissue Type Collection (*Homo sapiens* ATCC CRL 2327; HTB-22; HTB-133; CRL-1500), and cultured in Growth Media (DMEM, 10% fetal bovine serum, 1x antibiotics/anti-mycotics). Cells were transduced with lentiviral particles expressing three distinct shControl or shMCL1 sequences (Santa Cruz Biotechnologies) and kept under constant Puromycin selection (1μg/mL, Life Technologies). For cell growth analyses, 2,500 cells/well [growth 3D-Matrigel (BD Bioscience)] or 5,000 cells/well [growth monolayer] were seeded in a 96-well or 12-well plate, respectively. Media, antibiotic and/or drug were changed every 3 days. For analysis, 3D colonies were imaged after 14 days (Motic AE3, ProRes CapturePro v2.8.0) and enumerated using ImageJ software. Colonies in monolayer were stained with 0.01% w/v crystal violet (Sigma Life Sciences) and measured using ImageJ. Trypan blue-excluding cells were counted after seeding 50,000 cells/well in 12-well plates and treating with drug for 48 hours.

Statistical analysis: Statistical significance ($P<0.05$) was determined using Student's unpaired 2-tailed T-Test or ANOVA with Bonferroni *post hoc* tests followed by Student's unpaired 2-tailed T-test using Graphpad Prism5 software.

Results

Mcl-1 is highly expressed in breast cancers.

Anti-apoptotic Bcl-2 family member transcripts were assessed in Cancer Cell Line Encyclopedia (CCLE) tumor cell line expression datasets (111). *BCL2* transcripts were high in tumors of hematological origin, but were relatively low in epithelial tumor cells, including breast, while *BCL2L1* (Bcl-xL) transcripts were higher in tumors of epithelial origin (**Figure 9A-B - red**). *MCL1* levels were relatively high across several cancers of epithelial (lung, breast, ovary, pancreas, prostate, and stomach) and hematological (B-cell lymphomas, myelomas) origin, and in melanomas (**Figure 10A - red**). Focusing specifically on breast cancer, we assessed *MCL1*, *BCL2*, and *BCL2L1* in cell lines stratified by PAM50 molecular subtypes (112-114). *MCL1* was detected at higher levels than *BCL2* and *BCL2L1* across all breast cancer molecular subtypes (**Figure 10B - red**). This observation was confirmed by western analysis in a smaller panel of breast cancer lines, showing abundant Mcl-1 expression across most lines, and variable levels of Bcl-2 and Bcl-xL (**Figure 9C**).

Next, we queried RNA-Seq data from The Cancer Genome Atlas (TCGA)-curated clinical breast cancer datasets for total *MCL1*, *BCL2*, and *BCL2L1* transcript counts, finding more *MCL1* in Luminal A (N = 333), Luminal B (N = 325), HER2-enriched (N = 150), and Basal-like (N = 211) samples than *BCL2* and *BCL2L1* (**Figure 10C - red**). Basal-like tumors harbored highest *MCL1* transcripts, followed by Luminal A, *HER2*-enriched, and finally Luminal B (**Figure 10D – luminal in red**).

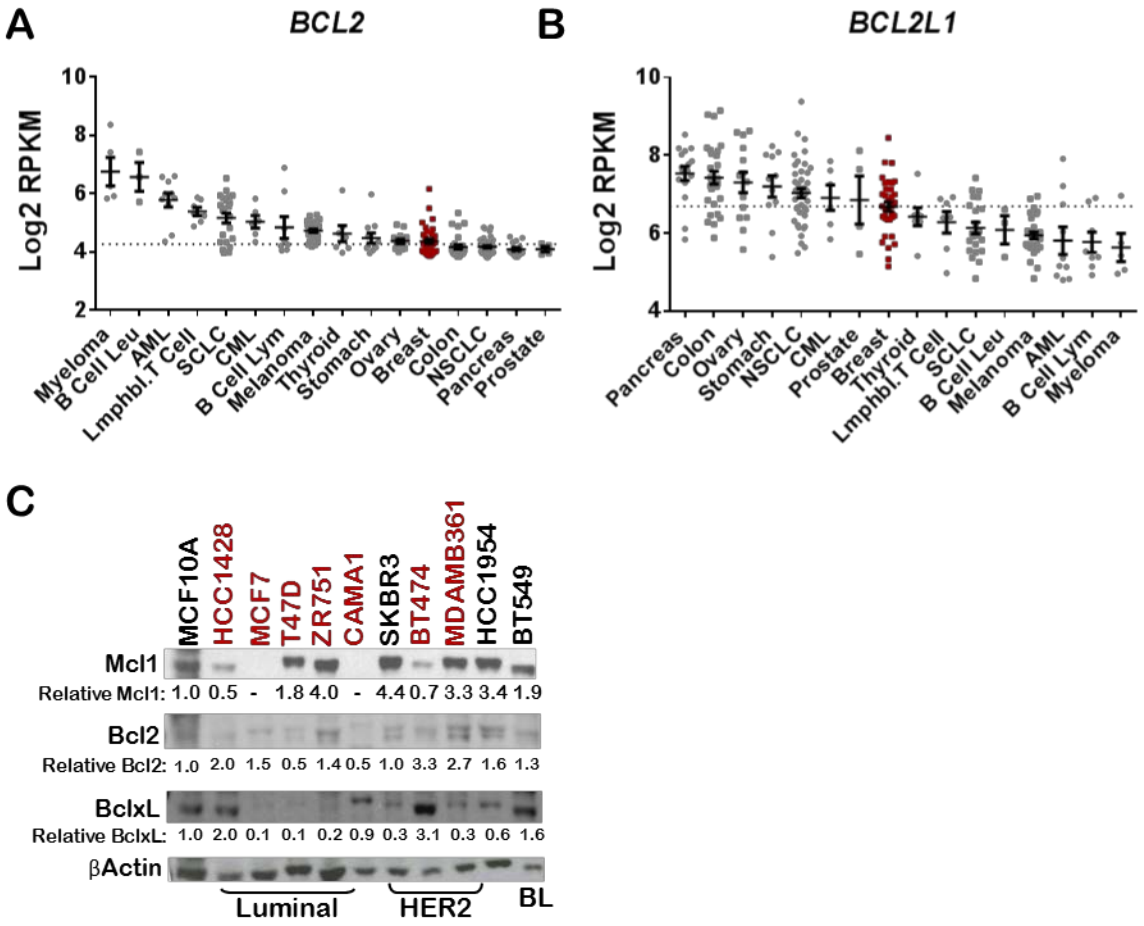


Figure 9. Breast cancers do not elevate Bcl-2 or Bcl-xL levels upon tumorigenesis.

A-B. CCLE-curated cancer cells were grouped according to tumor type and assessed for mRNA expression of *BCL2* (A) or *BCL2L1* (B) using cBio Portal (www.cbio.org). **B.** Whole cell lysates harvested from a panel of breast cancer cell lines observed with antibodies denoted on the left. Red text marks cell lines that are ERα+.

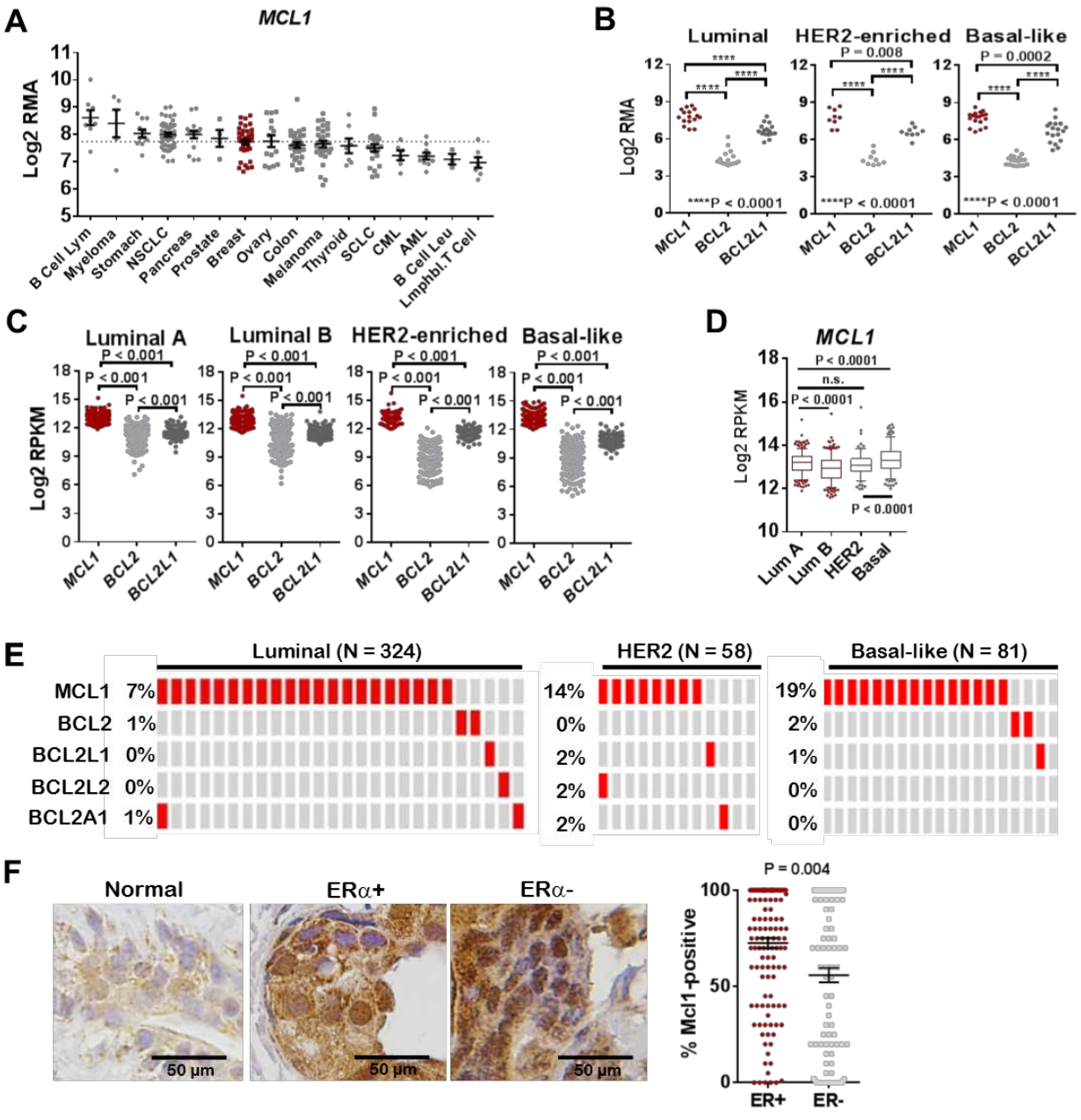

A
MCL1
Log2 RMA
B Cell Lym
Myeloma
Stomach
NSCLC
Pancreas
Prostate
Breast
Ovary
Colon
Melanoma
Thyroid
SCLC
CML
AML
B Cell Leu
Lmphbl.T Cell
B
Luminal
HER2-enriched
Basal-like
P = 0.008
P = 0.0002
****
****P < 0.0001
MCL1
BCL2
BCL2L1
C
Luminal A
Luminal B
HER2-enriched
Basal-like
Log2 RPKM
P < 0.001
D
MCL1
Log2 RPKM
P < 0.0001
n.s.
Lum A
Lum B
HER2
Basal
E
Luminal (N = 324)
HER2 (N = 58)
Basal-like (N = 81)
MCL1 7%
BCL2 1%
BCL2L1 0%
BCL2L2 0%
BCL2A1 1%
14%
0%
2%
2%
2%
19%
2%
1%
0%
0%
F
Normal
ERα+
ERα-
50 µm
P = 0.004
% Mcl1-positive
ER+
ER-

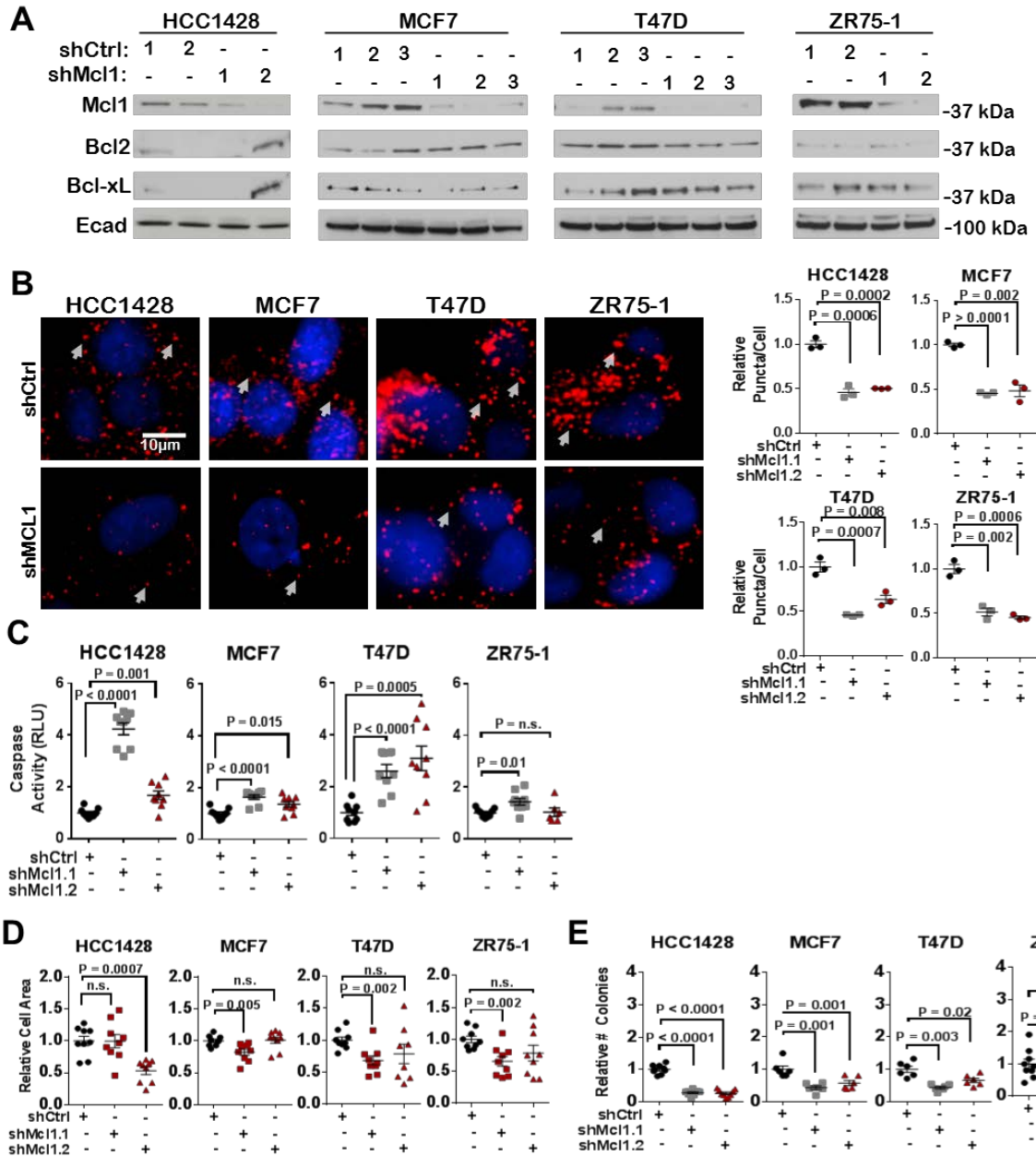
Figure 10. Mcl-1 expression is highest in ERα+ breast cancers.

A. CCLE-curated cancer cells were grouped according to tumor type and assessed for mRNA expression of *MCL1* using cBio Portal (www.cbio.org). **B.** CCLE-curated breast cancer cells were grouped according to PAM50 molecular subtype and assessed for mRNA expression of *MCL1*, *BCL2*, and *BCL2L1* (Bcl-xL) using cBio Portal (www.cbio.org). **C-D.** Clinical breast tumor specimens curated by TCGA and subjected to RNA-Seq analysis were grouped according to PAM50 molecular subtype, and then assessed for absolute read for transcripts encoding *MCL1*, *BCL2*, and *BCL2L1* using cBio Portal (www.cbio.org). Student's unpaired two-tailed t-test. **E.** Clinical breast tumor specimens curated by TCGA and subjected to CGH analysis were grouped according to PAM50 molecular subtype, and then assessed for amplifications of genes encoding anti-apoptotic Bcl-2 family members. **F.** Breast tumor tissue microarray (N = 266) was stained for Mcl-1 and scored for percent of tumor cells that are Mcl-1-positive by a breast pathologist. Samples were stratified by ER status, ERα+, N = 132; ERα-, N = 134. The average % Mcl-1–positive cells (SE) is shown as the midline. Individual samples are shown as data points (Student unpaired two-tailed t test).

Comparative genomic hybridization (CGH) analysis of TCGA Luminal (A and B) breast cancers (N = 324) demonstrated that *MCL1* gene amplifications were found in 21/324 tumor samples (7%), which was more frequent than amplifications in *BCL2* (2/324) and *BCL2L1* (1/324) (**Figure 10E**). Gene amplifications in *MCL1* also occurred in *HER2*-enriched and Basal-like breast cancer specimens at rates higher than what was seen for other Bcl-2 family-encoding genes. Immunohistochemical analysis of clinical breast tumor specimens (N = 266) showed little Mcl-1 staining in normal breast epithelium (**Figure 10F - right panel**). However, a substantial number of cells stained positive for Mcl-1 in breast tumor specimens, with the highest number of Mcl-1-positive cells seen in ERα+ tumors (**Figure 10F - left panel**). These results suggest a role for Mcl-1 in breast cancer biology, motivating our continued investigation of Mcl-1 in ERα+ breast cancers.

Mcl-1 inhibition decreases ERα+ breast cancer tumor cell growth.

We used *MCL1* shRNA sequences (shMCL1) (2-3 sequences per cell line) in the ERα+ breast cancer cell lines HCC1428, MCF7, T47D, and ZR75-1 to knock down Mcl-1 expression. Western analysis of polyclonal puromycin-selected cells demonstrated decreased Mcl-1 protein expression in each cell line, with no change in Bcl-2 and Bcl-xL expression (**Figure 11A**). Proximity ligation assay [PLA,(115)] showed a decreased association between Mcl-1 and Bim in cells expressing shMCL1 as compared to shControl non-targeting sequences (**Figure 11B - arrows**). Mcl-1 knockdown increased caspase-3/7 activity in three of four cells lines (**Figure 11C**), and decreased cell growth in three of four lines grown in monolayer (**Figure 11D**) or in 3D-Matrigel (**Figure 11E**). These results confirm that specific Mcl-1 inhibition increases cell death in some, but not all ERα+ breast cancer cells.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MCF7
T47D
ZR75-1
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shMcl1:
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-37 kDa
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HCC1428
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ZR75-1
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shMCL1
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P = 0.0002
P = 0.0006
P = 0.002
P > 0.0001
P = 0.008
P = 0.0007
P = 0.0006
P = 0.002
shCtrl
shMcl1.1
shMcl1.2
C
Caspase Activity (RLU)
P = 0.001
P < 0.0001
P = 0.015
P < 0.0001
P = 0.0005
P < 0.0001
P = n.s.
P = 0.01
D
Relative Cell Area
P = 0.0007
n.s.
n.s.
P = 0.005
n.s.
P = 0.002
n.s.
P = 0.002
E
Relative # Colonies
P < 0.0001
P < 0.0001
P = 0.001
P = 0.001
P = 0.02
P = 0.003
n.s.
P = 0.028

Figure 11. Decreased activity and expression of Mcl-1 induces tumor cell killing.

A–E. Polyclonal pools of cells stably expressing shCtrl (shControl) or shMcl1 sequences were assessed. A. Western blot analysis of whole-cell lysates. B. Cells were assessed by PLA to detect Mcl-1/Bim interactions. Right, representative images: red, Mcl-1/Bim proximity; blue, Hoechst. Left, average cytosolic puncta/cell (SE) was quantitated for 3 cells/sample, N = 3 (assessed in triplicate), Student unpaired two-tailed t-test. **C.** Cells were assessed for caspase activity by Caspase-3/7-Glo assay. Average RLU (SE) is shown, N = 6–9, Student unpaired two-tailed t-test. **D.** Cells were grown for 7 d in monolayer. Average cell area (SE) was determined using crystal violet, setting the number of shCtrl colonies equal to 1 as a common reference of comparison for each cell line (N = 6–9 per group). Student unpaired two-tailed t-test. **E.** Cells embedded in Matrigel were cultured for 14 d. Number of colonies/well was measured, setting the number of shCtrl colonies equal to 1 as a common reference of comparison for each cell line (N = 6–9 per group). Student unpaired two-tailed t-test.

ERα+ breast cancer cell lines have limited sensitivity to ABT-263.

To determine how blockade of other Bcl-2 family members impacts the growth and survival of ERα+ breast cancer cells, we treated HCC1428, MCF7, T47D, and ZR75-1 cells with ABT-263, a compound that inhibits two Bcl-2 family members, Bcl-2 and Bcl-xL. Although each cell line showed increased caspase-3/7 activity at 4 hours exposure to ABT-263 (1.0 μM), caspase activation was not sustained through 48 hours in three of the four cell lines (**Figure 12A**), suggesting a rapid loss of sensitivity to ABT-263. Consistent with these findings, ABT-263 did not affect the total number of MCF7, T47D, or ZR75-1 cells grown in monolayer for 48 hours (**Figure 12B**), or in three-dimensional (3D)-Matrigel for 14 days (**Figure 12C**). PLA (115) confirmed that ABT-263 disrupted interaction of its target protein Bcl-2 with Bim (**Figure 12D-E - arrows**), confirming on-target activity of ABT-263 at 4 and 24 hours treatment, despite the lack of caspase activation at distal time points in three of the four cell lines tested.

ABT-263 induces Mcl-1 expression and activity.

Cells were treated with a time course of ABT-263 to determine whether rapid upregulation of Bcl-2 family proteins could desensitize cells to ABT-263. Although Bcl-xL expression remained relatively unchanged after 24 hours treatment with ABT-263 (**Figure 13A**), Bcl-2 expression was elevated at this time point. Despite an increase in Bcl-2 levels, Bcl-2/Bim interactions remain inhibited at 24 hours treatment (**Figure 12D-E**), suggesting that elevated Bcl-2 expression does not enhance the ability for Bcl-2 to sequester Bim in the presence of ABT-263. Alternatively, Mcl-1 expression was promptly increased within 4-8 hours treatment with ABT-263 (**Figure 14A**).

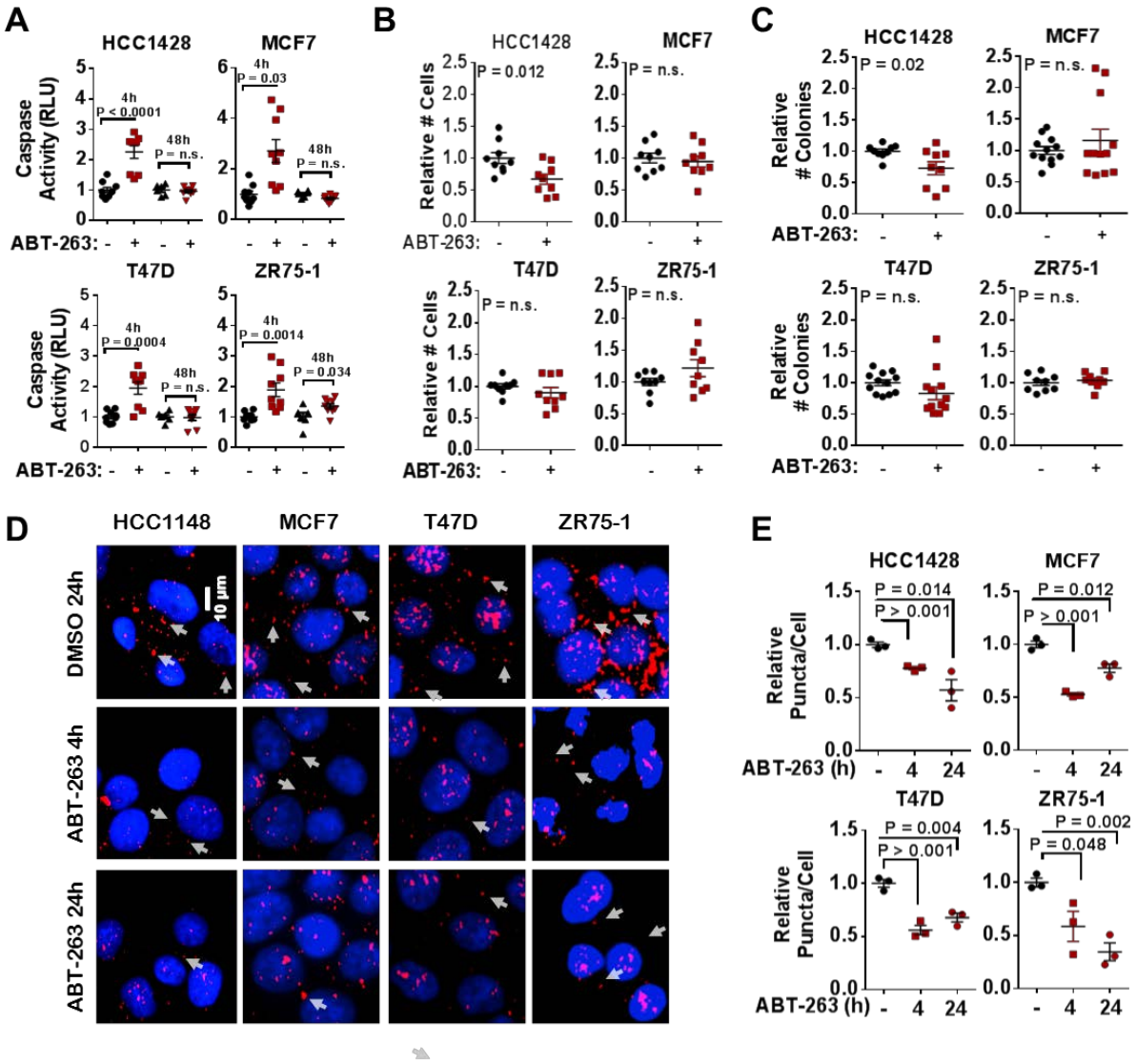

Figure 12. ERα+ breast cancer cell lines have limited sensitivity to ABT-263.

A. Caspase activity was measured in cells treated for 4 or 48 hrs with 1.0 µmol/L ABT-263 using a luminescent Cleaved Caspase-3/7-Glo assay. Average (SE) relative luminescence is shown, Student unpaired two-tailed t-test, N = 3. **B.** Cells were treated 48 hrs with 1.0 µmol/L ABT-263, and live cells (Trypan blue–negative) were counted. Values shown are average cell number (SE), Student's unpaired two-tailed t-test, N = 3. **C.** Cells were Matrigel embedded and cultured for 14 d. Average number of colonies/well (SE) is shown. T-test, N = 3. **D-E.** PLA of cells treated 4 or 24 hrs with 1.0 µmol/L ABT-263. Representative images are shown. Red, Bcl-2/Bim proximity; blue, Hoechst (D). Values shown are average cytosolic puncta/nuclei (SE), N = 3 (E).

This rapid upregulation was also sustained, as Mcl-1 levels were increased at 7 days treatment with ABT-263 in three of four cell lines (**Figure 14A-B**). Increased Mcl-1/Bim interactions were observed in each cell line upon treatment with ABT-263 (**Figure 14C-D - red puncta**). While Mcl-1/Bim interaction increased in three of four cell lines at 4 hours treatment, these interactions decreased or returned to baseline at 24 hours in MCF7 and ZR75-1 cells, possibly due to decreased Bim expression at this time point (**Figure 13B**). Additionally, the delayed upregulation of Mcl-1/Bim interactions in HCC1428 cells (24 hours) relative to the other three cell lines (4 hours) may explain the relatively increased sensitivity of HCC1428 cells to ABT-263 (as shown in **Figure 12**), suggesting that complex interactions between Bcl-2 family proteins may determine sensitivity to ABT-263.

We assessed MCF7 xenografts treated *in vivo* with ABT-263 (20 mg/kg) for 16 days. Co-precipitation of Bim with Bcl-2 was seen in control-treated tumors, but was absent in MCF7 xenografts treated with ABT-263 (**Figure 14E**), confirming on-target activity of ABT-263 within tumors. Increased Mcl-1 was seen in ABT-263-treated MCF7 whole tumor lysates (**Figure 14F**), and PLA measured increased Mcl-1/Bim interactions in tumors treated with ABT-263 as compared to vehicle-treated tumors (**Figure 14G - red puncta**). Although decreased recovery of signal was achieved in FFPE as compared to what was seen in methanol fixed MCF7 cell cultures (comparing Figure 12C to Figure 12G), these results are consistent with the idea that tumors *in vivo* upregulate Mcl-1 activity in response to blockade of other Bcl-2 family members similar to what was seen in cell culture.

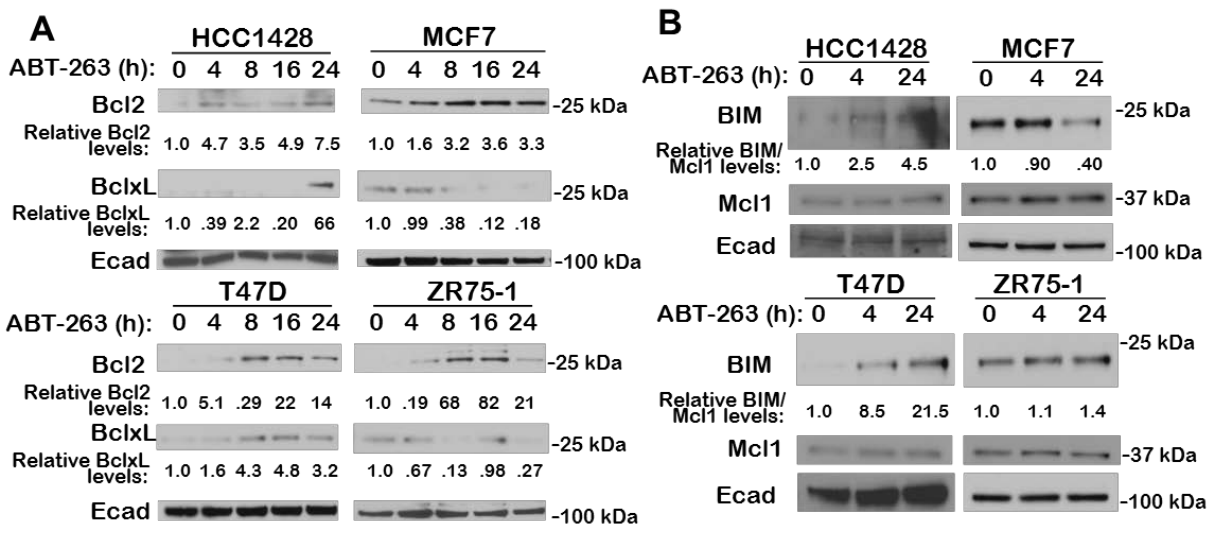

Figure 13. Expression levels of Bcl-2 and Bim, but not Bcl-xL, increase after ABT-263 treatment.

A-B. Whole cell lysates from cells treated with ABT-263 (1.0 μmol/L) for 0-24 h.

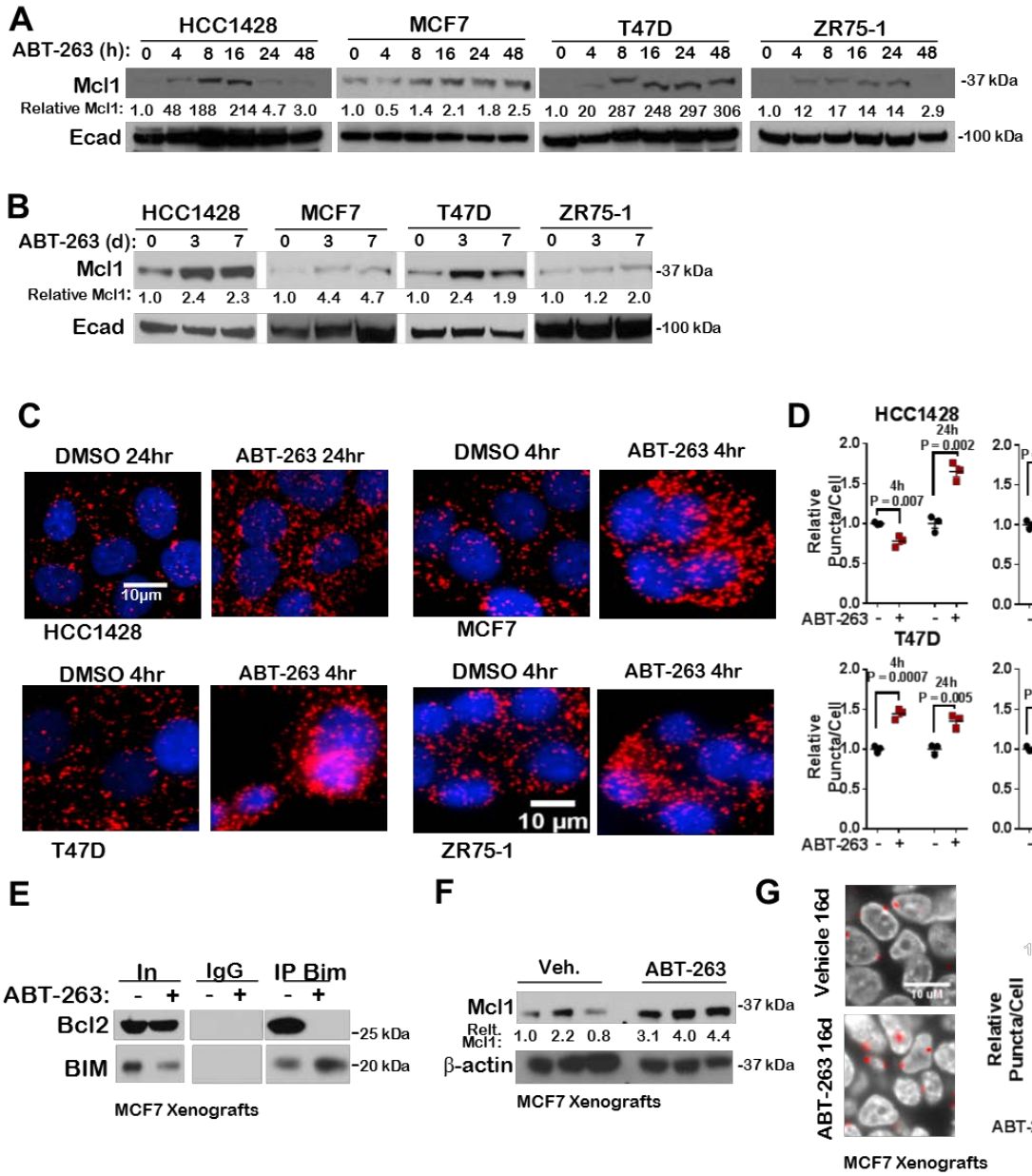
A
HCC1428
MCF7
T47D
ZR75-1
ABT-263 (h): 0 4 8 16 24 48
Mcl1
-37 kDa
Relative Mcl1: 1.0 48 188 214 4.7 3.0
1.0 0.5 1.4 2.1 1.8 2.5
1.0 20 287 248 297 306
1.0 12 17 14 14 2.9
Ecad
-100 kDa
B
HCC1428
MCF7
T47D
ZR75-1
ABT-263 (d): 0 3 7
Mcl1
-37 kDa
Relative Mcl1: 1.0 2.4 2.3
1.0 4.4 4.7
1.0 2.4 1.9
1.0 1.2 2.0
Ecad
-100 kDa
C
DMSO 24hr
ABT-263 24hr
DMSO 4hr
ABT-263 4hr
10μm
HCC1428
MCF7
T47D
ZR75-1
10 μm
D
HCC1428
MCF7
T47D
ZR75-1
Relative Puncta/Cell
ABT-263
4h
24h
P = 0.007
P = 0.002
P = 0.014
P = 0.005
P = 0.0007
P = 0.03
n.s.
E
In
IgG
IP Bim
ABT-263:
Bcl2
BIM
-25 kDa
-20 kDa
MCF7 Xenografts
F
Veh.
ABT-263
Mcl1
-37 kDa
Relt. Mcl1:
1.0 2.2 0.8
3.1 4.0 4.4
β-actin
-37 kDa
MCF7 Xenografts
G
Vehicle 16d
ABT-263 16d
10 uM
MCF7 Xenografts
P = 0.004
Relative Puncta/Cell
ABT-263
MCF7 Xenografts

Figure 14. Increased Mcl-1 expression and activity in ERα+ breast cancer cells treated with ABT-263.

A-B. Western blot analysis of whole-cell lysates after treatment with 1.0 µmol/L ABT-263 or DMSO for 0–48 hrs (A) or 0, 3, or 7 d (B). **C-D.** PLA of cells treated 4 or 24 hrs with 1.0 µmol/L ABT-263. Representative images are shown. Red, Bim/Mcl-1 proximity; blue, Hoechst (C). Average cytosolic puncta/cell (SE) was quantitated for 3 cells/sample, N = 3 (assessed in triplicate), Student unpaired two-tailed t-test (D). **E–G.** MCF7 xenografts were treated with daily ABT-263 (20 mg/kg) for 16 d by oral gavage. E. Immunoprecipitation (IP) of Bim or IgG control was conducted on whole tumor lysates. F. Whole tumor lysates were assessed by Western blot analysis. G. PLA of MCF7 xenografts treated daily with ABT-263 for 16 d. Average puncta/cell was quantitated for three fields/tumor, N = 4.

MCL1 *expression correlates inversely with sensitivity to Bcl-2/Bcl-xL inhibition.*

Bcl-2 and/or Bcl-xL inhibition is currently under clinical investigation for treatment of breast cancers. Given the high Mcl-1 expression levels in breast cancers, and the capacity for rapid Mcl-1 upregulation following Bcl-2/Bcl-xL using ABT-263, we assessed the relationship between *MCL1* and sensitivity to ABT-263 across a panel of CCLE-curated ERα+ (N = 16), *HER2*-amplified (N = 9), and triple negative (N = 18) breast cancer cell lines [using datasets published in (111) and (116)]. *MCL1* transcript levels correlated with ABT-263 IC50 in ERα+ and *HER2*-amplified cells, but not in triple negative breast cancer (TNBC) cells (**Figure 15A**). Interestingly, *BCL2* and *BCL2L1* transcript levels did not correlate directly or inversely with ABT-263 IC50 in any cell type. These findings support the notion that Mcl-1 may be for a marker for *de novo* resistance to ABT-263 in breast cancers. To test this idea directly, we overexpressed Mcl-1 in HCC1428, MCF7, and T47D cells (**Figure 15B**). Although Bim interactions with Mcl-1 were upregulated by 20-45% in cells transduced with the his-Mcl-1 adenovirus, we found ABT-263 more potently upregulated Mcl-1/Bim interactions in transduced cells (**Figure 15C - red puncta**), demonstrating that Mcl-1 acts as a sink for Bim upon ABT-263 treatment and that ABT-263 promotes maximal Mcl-1/Bim interactions. Consistent with the idea that Mcl-1 levels contribute to *de novo* ABT-263 resistance, we found that Mcl-1 overexpression substantially decreased caspase-3/7 activation in ABT-263-treated cells (**Figure 15D**), further suggesting that ERα+ breast cancer cells use upregulation of Mcl-1 to evade cell death when challenged with ABT-263 (**Figure 15E**).

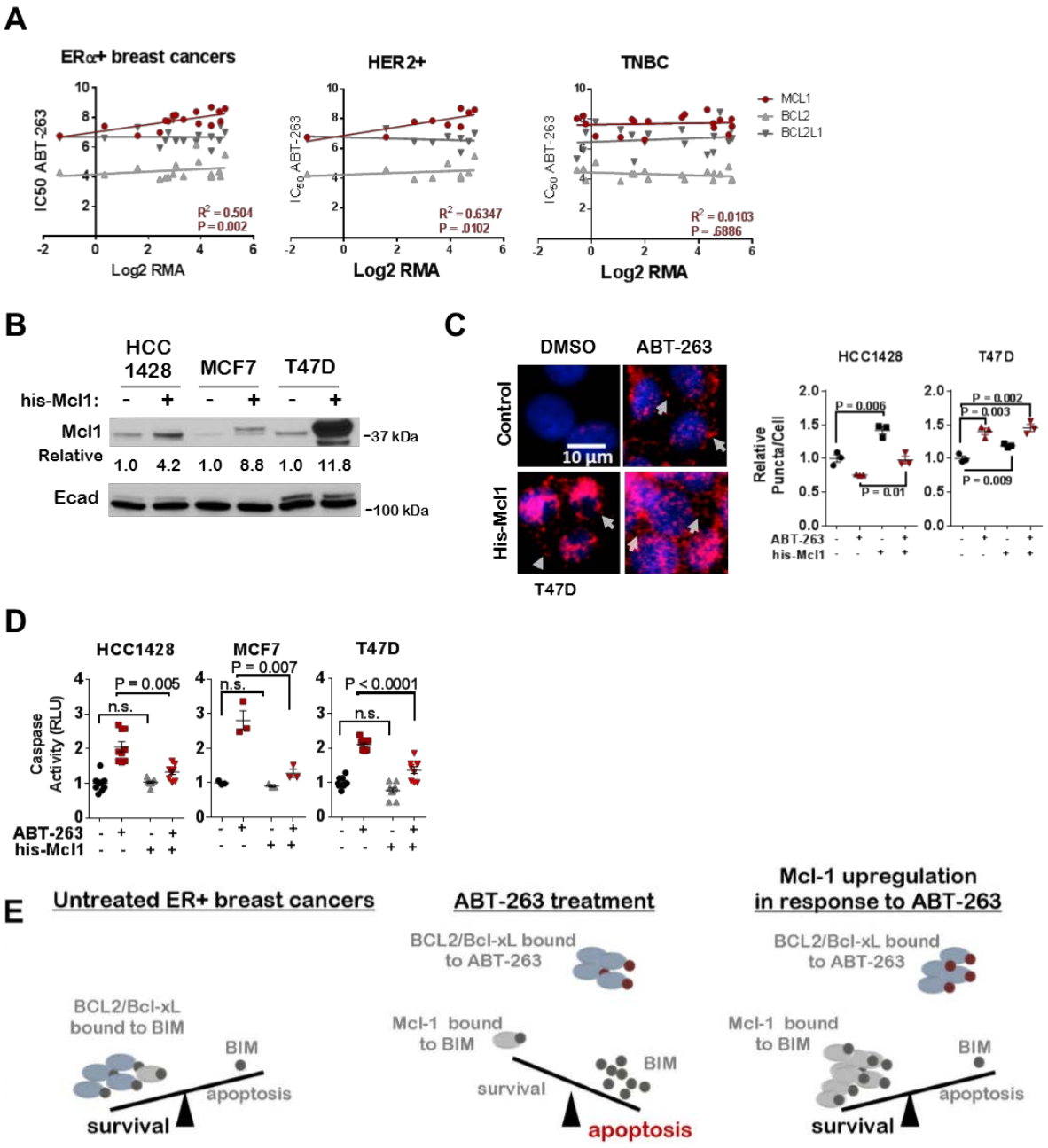
A
ERα+ breast cancers
HER2+
TNBC
IC50 ABT-263
Log2 RMA
R² = 0.504
P = 0.002
R² = 0.6347
P = .0102
R² = 0.0103
P = .6886
MCL1
BCL2
BCL2L1
B
HCC 1428
MCF7
T47D
his-Mcl1:
Mcl1
Relative
1.0 4.2 1.0 8.8 1.0 11.8
Ecad
~37 kDa
~100 kDa
C
DMSO
ABT-263
Control
His-Mcl1
10 µm
T47D
HCC1428
T47D
Relative Puncta/Cell
P = 0.006
P = 0.01
P = 0.002
P = 0.003
P = 0.009
ABT-263
his-Mcl1
D
HCC1428
MCF7
T47D
Caspase Activity (RLU)
P = 0.005
n.s.
P = 0.007
n.s.
P < 0.0001
n.s.
ABT-263
his-Mcl1
E
Untreated ER+ breast cancers
ABT-263 treatment
Mcl-1 upregulation in response to ABT-263
BCL2/Bcl-xL bound to BIM
BIM
apoptosis
survival
BCL2/Bcl-xL bound to ABT-263
Mcl-1 bound to BIM
BIM
survival
apoptosis
BCL2/Bcl-xL bound to ABT-263
Mcl-1 bound to BIM
BIM
apoptosis
survival

Figure 15. **Mcl-1 expression correlates with reduced sensitivity to ABT-263.**

A. *MCL1*, *BCL2*, and *BCL2L1* (Bcl-xL) mRNA expression in ERα+ breast cancer cell lines (CCLE) was compared with the ABT-263 IC50 (Sanger Institute). Data were fit to a linear regression. R^2 and P values shown for *MCL1*. R^2 and P values for *BCL2* are 0.05, 0.37 ($ERα^+$), 0.05, 0.55 (HER2), and 0.07, 0.28 (TNBC), respectively; and for *BCL2L1* are 0.00, 0.97 ($ERα^+$), 0.03, 0.52 (HER2), and 0.05, 0.58 (TNBC), respectively. **B–D.** Human Mcl-1 was overexpressed in cells by adenoviral expression. **B.** Whole-cell lysates after 48 hr treatment with adenovirus. **C.** PLA in HCC1428 and T47D cells treated with an his-Mcl-1 adenovirus for 48 hrs, then treated with ABT-263 (1.0 µmol/L) for 4 hrs, N = 3 (assessed in triplicate), Student unpaired two-tailed t-test. **D.** Relative caspase activity of cells treated ABT-263 (1.0 µmol/L) for 4 hrs. Average (SE) relative luminescence is shown, N = 3, Bonferroni *post hoc* test followed by Student unpaired two-tailed t-test. **E.** Model for Mcl-1–mediated resistance to ABT-263.

Discussion

Nearly 40,000 breast cancer deaths occur annually in the United States, often in the context of recurrent and/or therapeutically resistant disease (104). Additional molecularly targeted treatment strategies that effectively induce tumor cell killing could potentially decrease tumor recurrences, and would increase therapeutic options for patients with resistant disease. Tumors often rely on anti-apoptotic Bcl-2 family proteins to evade tumor cell death. Given the clinical success of ABT-199 in CLL (108), and ongoing clinical investigations of ABT-263 and ABT-199 in breast cancers (117), we were motivated to understand which breast cancers express high levels of Bcl-2 family members, as this might indicate which breast cancers would benefit from Bcl-2 and/or Bcl-xL inhibition. We also investigated the Bcl-2 family member Mcl-1, given recently developed compounds that target Mcl-1 activity that have transitioned into early phase clinical trials for hematological malignancies (59). Data shown herein suggest that Mcl-1 may be a key survival factor across all breast cancer subtypes.

In previous studies, a dependency screening approach identified Mcl-1 as a key survival factor in TNBCs (118), consistent with our observations presented herein that among Bcl-2 family members, *MCL1* mRNA expression was frequently higher than *BCL2* and *BCL2L1* mRNA in breast cancers, including TNBCs (**Figure 10C**). At the protein level, immunohistochemical Mcl-1 staining of breast cancer tissue microarrays demonstrated profoundly elevated Mcl-1 in breast cancers as compared to normal breast epithelium (**Figure 10F**). Further, Mcl-1 levels were elevated in breast tissue containing ERα expression, suggesting that Mcl-1 may confer a selective survival advantage to breast cancer cells, particularly luminal breast cancer cells, but may also represent a vulnerability that can be exploited therapeutically. Mcl-1 knockdown in ERα+ breast cancer cells decreased Mcl-1 activity, as measured *in situ* by Mcl-1/Bim interactions (**Figure 11B**). Importantly, Mcl-1 knockdown decreased tumor cell survival and tumor cell growth (**Figure 11C-E**). These studies complement earlier studies from other groups indicating that Mcl-1 knockdown

in some triple negative (39, 119) and/or *HER2*-amplified breast cancer cells (60, 120) increased caspase activity. In contrast, combined Bcl-2 and Bcl-xL inhibition using ABT-263 had only an acute impact on caspase activation (**Figure 12A**) and did not affect tumor cell growth (**Figure 12B-C**). These findings are in agreement with studies from other groups demonstrating that ABT-263 was insufficient as a single agent to induce tumor cell killing in ERα+ breast cancer cells, but only once cells were primed with tamoxifen did Bcl-2/Bcl-xL or selective Bcl-2 inhibition with ABT-199 induce cell death (48).

Several studies show Mcl-1 production and stability is responsive to cellular cues, including inhibition of other Bcl-2 family members (82, 85, 89, 95, 121), making Mcl-1 ideally suited for rapidly evading therapeutically-induced tumor cell death [reviewed in (98)], and emphasizing the value of Mcl-1 as a therapeutic target. Interestingly, we found that rapid and sustained Mcl-1 induction occurred in response to ABT-263 in ERα+ breast cancer cells (**Figure 14**), although Mcl-1 depletion did not result uniformly in Bcl-2 or Bcl-xL upregulation in ERα+ breast cancer cells (**Figure 11A**). This suggests that Mcl-1 may be a dominant anti-apoptotic signal in ERα+ breast cancers. Previous studies in hematological malignancies, colorectal cancers, and small cell lung cancers show that Mcl-1 drives innate resistance to ABT-263, while Mcl-1 suppression could restore sensitivity to ABT-263 (82, 89, 90). Although this idea needs to be tested further in ERα+ breast cancers, it is possible that maximal tumor cell killing may only be achieved when Bcl-2, Bcl-xL, and Mcl-1 are inhibited, suggesting that the toxicities associated with targeting all three family members need to be explored. Additionally, we show herein that *MCL1* gene expression levels correlated inversely with sensitivity to ABT-263 (**Figure 15A**), suggesting that *MCL1* might be used as predictor of patient response to ABT-263 or ABT-199, a hypothesis that could be tested in ongoing and future clinical trials.

In summary, we find that *MCL1* gene expression and amplification are frequent occurrences in breast cancers, and that genetic Mcl-1 inhibition increased apoptosis in ERα+

breast cancer cells, resulting in increased growth inhibition. In contrast, Bcl-2/Bcl-xL inhibition using ABT-263 did not sustain tumor cell killing or growth inhibition. Cells rapidly responded to ABT-263 by increasing Mcl-1 expression, but Mcl-1 depletion did not induce Bcl-2 or Bcl-xL upregulation. Together, these findings support a role for Mcl-1 in survival of breast cancer cells, warranting consideration of Mcl-1 in the design and interpretation of clinical trials investigating Bcl-2 family inhibitors.

CHAPTER III

Bcl-2/Bcl-xL Inhibition Promotes Rapid mTORC1-Dependent *MCL1* Translation and Treatment Resistance

This work is in preparation for submission to *Molecular Cancer Therapeutics*.

Abstract

Anti-apoptotic Bcl-2 proteins are pro-survival factors that act as brakes for the intrinsic apoptotic pathway, a molecular switch at the mitochondrial membrane that activates caspase-mediated cell death. Many tumors overexpress anti-apoptotic Bcl-2 proteins to evade tumor cell death, a known hallmark of cancer progression. For instance, Estrogen Receptor-alpha positive (ERα+) breast cancers express high levels of three anti-apoptotic Bcl-2 family members, Bcl-2, Bcl-xL, and Mcl-1, making them unique among breast cancer subtypes. However, pharmacological inhibition of Bcl-2/Bcl-xL failed to induce cell death in ERα+ breast cancer cell lines, a result which is attributed to rapid and robust Mcl-1 upregulation, albeit through mechanisms that remained undefined in this cancer subtype. We report here that cap-dependent protein translation of *MCL1* transcripts, driven in large part through increased mTOR signaling, was responsible for Mcl-1 upregulation in ERα+ breast cancer cells treated with the Bcl-2/Bcl-xL inhibitor ABT-263. Cells treated with a pharmacological inhibitor of cap-dependent translation or with the mTORC1 inhibitor RAD001/everolimus, displayed reduced protein levels of Mcl-1 under basal conditions, and failed to upregulate Mcl-1 protein expression following treatment with ABT-263. mTORC1 inhibition increased tumor cell killing in ERα+ cells and tumor models when used as a single agent, and further increased tumor cell death when used in combination with ABT-263, arguing for combined Bcl-2/Bcl-xL/Mcl-1 blockade. Thus, RAD001, which has achieved clinical approval in ERα+ breast cancers, may be a rapidly translatable means to target Mcl-1 clinically, and could be particularly useful after priming for Mcl-1 inhibition by Bcl-2/Bcl-xL blockade.

Introduction

The breast epithelium undergoes many dynamic changes throughout a woman's lifetime. The intrinsic apoptotic pathway, a master regulator of caspase-dependent cell death, is essential to maintain proper development throughout each of these processes, including puberty, pregnancy, lactation, and post-lactational involution (5). Not surprisingly, breast tumors frequently dysregulate this pathway to favor tumor cell survival, often through upregulation of anti-apoptotic Bcl-2 family proteins (Bcl2-A1, Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) that sequester the activity of pro-apoptotic family members [as reviewed in (122)]. Specifically, anti-apoptotic Bcl-2 proteins either **1)** bind to Bcl-2 effector proteins Bak and Bax to block Bak-Bax oligomerization and pore formation in the outer mitochondrial membrane (14), or **2)** sequester Bcl-2 activators (Bim, Bid, and Puma), which facilitate Bak/Bax oligomerization (12). Thus, a dynamic set of interactions between anti-apoptotic and pro-apoptotic Bcl-2 family proteins determines whether cell survival is favored over cell death.

Up to 70% of Estrogen Receptor (ER)-α positive breast cancers express cytoplasmic Bcl-2 (123), however other breast cancer subtypes have relatively decreased expression of this anti-apoptotic family member (58, 124). Bcl-xL is widely expressed across breast tumor subtypes (58) in both pre-malignant (105) and high grade tumors (124). Like Bcl-xL, Mcl-1 is overexpressed in ERα-positive and ERα-negative tumors, although Mcl-1 protein expression is higher in ERα-positive specimens (57, 58). At the genetic level, *MCL1* is the most frequently amplified anti-apoptotic Bcl-2 family member in breast cancers (125), while Mcl-1 protein expression correlates with poor patient survival in breast cancers regardless of subtype (126). These observations support intense research efforts into therapeutic targeting of anti-apoptotic Bcl-2 proteins in breast cancers, and emerging efforts specifically aimed at Mcl-1 blockade.

Because anti-apoptotic Bcl-2 family proteins bind to effectors (Bak and Bax) and activators (Bim, Bid, and Puma) specifically through their BH3 domain binding motif, anti-apoptotic Bcl-2

proteins can be inhibited using 'BH3-mimetics', compounds that specifically fit within the BH3 domain binding pocket (127). This liberates BH3 motif-containing proteins (Bim, Bax, Bak, etc.), allowing these apoptotic effectors and activators to engage the intrinsic apoptotic pathway. BH3-mimetics targeting Bcl-2 and/or Bcl-xL have been successful as single agents in clinical studies of hematological malignancies (64, 65, 68). However, single agent inhibition of Bcl-2 (using ABT-199) or dual inhibition of Bcl-2/Bcl-xL (using ABT-737 or ABT-263) was ineffective in human triple negative breast cancer (58) and ERα+ breast cancer models (48, 57). In ERα+ breast cancers, rapid Mcl-1 protein upregulation is reported after ABT-263 treatment (56, 57), suggesting that Mcl-1 compensation promotes cell death evasion upon Bcl-2/Bcl-xL inhibition. However, Mcl-1 biology requires further exploration, specifically to elucidate the molecular mechanism supporting Mcl-1 upregulation in response to Bcl-2/Bcl-xL inhibition.

Mcl-1 upregulation following Bcl-2/Bcl-xL blockade may prime breast cancer cells for Mcl-1 inhibitor-mediated cell death. This idea is supported by studies showing the converse. Genetic Mcl-1 inhibition primed breast cancer cells for cell death in response to Bcl-xL and/or Bcl-2 inhibition (56, 119). However, Mcl-1 specific inhibition is not clinically achievable, as Mcl-1 specific BH3-mimetics are just now progressing to Phase I/II clinical trials in hematological malignancies, but not in breast or other solid cancers (59). As an alternative, identification of the molecular mechanisms that support Mcl-1 upregulation may reveal therapeutically tractable alternatives for indirect Mcl-1 targeting. Herein we show that increased Mcl-1 translation upon ABT-263 treatment drives survival of ERα+ breast cancer cells. The combination of a translation inhibitor, or the mTORC1 inhibitor RAD001/everolimus with ABT-263 blocked Mcl-1 upregulation and produced robust killing of ERα+ breast tumor cells in culture and *in vivo*. These findings suggest that mTORC1 inhibitors already approved for use in ERα+ breast cancers, may be clinically efficacious in ERα+ breast tumors to block Mcl-1 upregulation in response to ABT-263.

Materials and Methods

RT-qPCR analysis: RT-qPCR was completed as previously described (128), Human *MCL1* primers [Integrated DNA Technologies, Forward (5'-CCTTCCAAGGATGGGTTTGTGGA) and Reverse (5'-TGCCACTTGCTT TTCTGGCT)], a 36B4 primer control (Integrated DNA Technologies). $\Delta\Delta C_t$ values were standardized to the 36B4 control and the average standardized $\Delta\Delta C_t$ values are presented.

Western analysis and immunoprecipitation: For immunoprecipitation, whole cell lysates were harvested after 2 hours treatment with 1.0 μM ABT-263 in ice-cold lysis buffer [50 mM Tris pH 7.4, 100 mM NaF, 12 0mM NaCl, 0.5% NP-40, 100 μM Na_3VO_4, 1X protease inhibitor cocktail (Roche), 0.5 μM proteasome inhibitor (Santa Cruz Technologies)]. 1000 μg lysate was rotated with 10 μL antibody cross-linked beads overnight at 4°C [50 μL protein A/G agarose beads (Santa Cruz Biotechnology) were cross-linked with 10 μg antibody using 5 mM BS3 (Sigma-Aldrich) by rotating at room temperature for 30 minutes. BS3 was quenched using 15 μL 1.0 M Tris (pH 7.4), and beads were washed 3x with ¼ NLB (see above) diluted in 1X PBS]. Cross-linked beads were washed the next day 6x with ¼ NLB diluted in 1x PBS and boiled in 1x reducing sample buffer (NuPAGE, Invitrogen), then run on a 4-12% SDS-PAGE gel as detailed in (125). Proteins were transferred to nitrocellulose (iBlot, Invitrogen), blocked in 3% gelatin (Sigma-Aldrich) in Tris buffered saline (TBS), 0.1% Tween-20 (Sigma-Aldrich), then probed with antibodies against Bim (Cell Signaling Technology, 1:500), Mcl-1 (Santa Cruz Biotechnology, 1:500), Bcl-xL (Cell Signaling Technology, 1:500), Actin (Cell Signaling Technology, 1:10,000) and Bcl-2 (DAKO, 1:1000). Western analysis was conducted after harvesting samples in ice-cold lysis buffer (as described above) and resolving on 4-12% SDS-PAGE gels (as described above).

Cell culture: All cell lines used in these study were purchased directly from American Tissue Type Collection (*Homo sapiens* ATCC CRL 2327; HTB-22; HTB-133), and cultured in Growth

Media [DMEM (Corning Cellgro), 10% fetal bovine serum (FBS, Life Technologies), 1x antibiotics/anti-mycotics (Life Technologies)]. Mcl-1 expression was stably ablated used lentiviral particles containing two separate sequences against Mcl-1 (shMCL1), and a scramble control (shControl), according to the manufactures protocol (Santa Cruz Biotechnology, SC-35878-V). Cells were grown constantly in the presence of puromycin (Life Technologies). Growth in 3D-Matrix (Cultrex) was determined by embedding 2,500 cells/well of a 96-well dish in 100 uL Matrigel, overlain with media and drugs (1.0 μM ABT-263 ± 200 nM RAD001), which were replenished on days 4, 7, and 10. Caspase-3/7 activity was measured using Caspase-3/7 Glo (Promega). 10,000 cells/well plated in a 96-well dish were treated the following day ± 1.0 μM ABT-263, 200 nM RAD001 for four hours. Mcl-1 protein stability was determined after treatment with 1.0 μM ABT-263 for 24 hours and treatment with cycloheximide (100 μg/mL, Sigma-Aldrich) for the final 0, 15, and 30 minutes. Whole cell lysates were harvested and analyzed by western analysis according to the above protocol.

Murine models: Mice were housed under pathogen-free conditions and all experiments were in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. MCF7 xenografts were generated as described previously (128). Spontaneous mammary tumors from FVB female *Wap-myc* transgenic mice (129) were homogenized to a single cell suspension, and tumor cells were injected into the inguinal mammary fat pads of 6 week old wild type mice (FVB, Jackson Laboratory). Tumor volume was measured every other day once tumors became palpable. Treatment of mice began when tumors reached 200 mm^3. Mice were randomized and treated with 50 μL vehicle control [0.1% Tween 80 and 0.5% methylcellulose] or RAD001 [1.0 mg/kg, suspended in vehicle control] daily by daily gavage for 28 days or until tumor volume reached 1000 mm^3. Tissues were collected 1 hour after final treatment.

Histological analysis: All samples were fixed in 10% formalin, paraffin-embedded and 5-μm sections were stained with TUNEL using the ApopTag kit (Calbiochem). Phospho-S6 staining (Cell Signaling Technologies) and Phospho-histone H3 staining (Santa Cruz Biotechnology) were performed on sections as described by (128, 130) respectively. All histological analyses were photographed using Olympus DP2 software (200x).

Proximity ligation assay: Cells cultured in 96-well plates were fixed with methanol and Duolink (Sigma) proximity ligation protocol was used according to manufacturer's directions using Mcl-1 (Santa Cruz Biotechnology, 1:25) and Bim (Santa Cruz Biotechnology, 1:25) antibodies. Cells were counterstained with Hoescht. Plates were scanned by ImageXpress Micro XL Automated Microscope. PLA fluorescent puncta were enumerated using the automated Duolink ImageTool. Puncta/cell values were recorded, and averaged, for 20 cells per 400x field. For FFPE tissues, slides were re-hydrated in ethanol, and then antigens were retrieved using citric acid. The above protocol was then followed using the same antibodies and DAPI as a counter stain.

Statistical analysis: Statistical significance ($P<0.05$) was determined using Student's unpaired two-tailed T-test or ANOVA with Bonferroni *post hoc* tests followed by Student's unpaired two-tailed T-test using the Graphpad Prism 6 software. For animal studies significance was determined by area under the cure. When appropriate outliers were excluded from data presentation, and were defined as any value ± two standard deviations of the average.

Results

Increased MCL1 *translation in ERα+ breast cancer cells treated with ABT-263.*

Previous studies show that Mcl-1 expression and activity increased upon inhibition of Bcl-xL (WEHI-539) or Bcl-2/Bcl-xL [ABT-263, (56, 57)], suggesting that tumor cells use Mcl-1 to evade cell death upon Bcl-2/Bcl-xL inhibition. We explored the molecular mechanism driving this Mcl-1 upregulation in ERα+ breast cancer cells treated with ABT-263, given that this molecular mechanism may **1)** be a dominant tumor cell survival pathway essential to ERα+ breast tumorigenesis, and **2)** could predict rational therapeutic combinations to limit or block Mcl-1 upregulation in response to ABT-263 or other factors. Towards this goal, *MCL1* transcript levels were measured in cells treated with ABT-263 for 24 hours, a time point when ABT-263 was previously shown to blocked Bcl-2/Bim interactions and induced Mcl-1 protein upregulation in ERα+ breast cancer cells lines (125). We found that *MCL1* transcripts, measured by RT-qPCR of reverse transcribed whole cell RNA, were unchanged in HCC1428 and MCF7 cells, and decreased in T47D cells after treatment with ABT-263 (**Figure 16A**). These findings suggest that changes in Mcl-1 transcript levels, either through transcription *per se*, or transcript stability, may not contribute significantly to Mcl-1 protein upregulation in response to ABT-263.

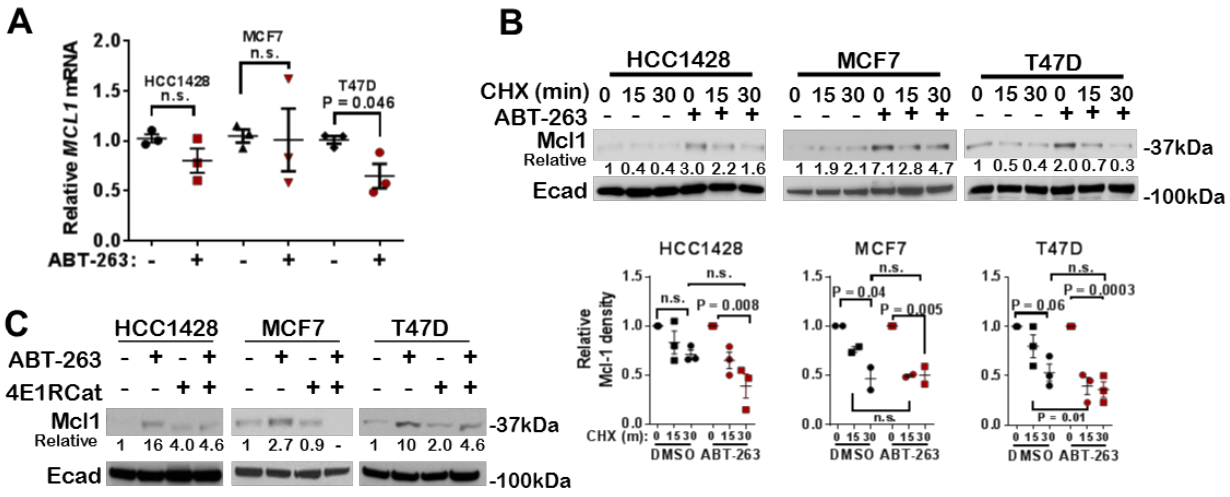

Figure 16. Inhibition of Bcl-2/Bcl-xL by ABT-263 only increases Mcl-1 expression through cap-dependent translation.

A. Relative transcript levels were determined by RT-qPCR after treatment with 1.0 µM ABT-263 for 24 hrs. Values were standardized to DMSO control for each cell line and represent the average of three separate experiments, Student's unpaired two-tailed t-test. **B.** Whole cell lysates from cells treated with 1.0 µM ABT-263 for 24 hrs. Cycloheximide (CHX) was used to block new protein synthesis in the final 0-30 min of ABT-263 treatment. A representative image is shown and quantitative analysis for three separate experiments depicts average density of Mcl-1/Ecad standardized to 0 min treatment with CHX, *Bonferroni* post hoc test followed by Student's unpaired two-tailed t-test. **C.** Whole cell lysates were harvested from cell treated with 1.0 µM ABT-263 for 24 hrs and 5.0 µM 4E1R-cat for the final 6 hrs.

Given that Mcl-1 protein is targeted for degradation by the ubiquitin-proteasome pathway (2), we tested the hypothesis that decreased Mcl-1 degradation following ABT-263 treatment causes Mcl-1 upregulation. Cycloheximide (CHX) was used to block new protein synthesis in cells treated 24 hours with ABT-263 or with DMSO control. After collecting cells at time points following CHX addition, Mcl-1 protein levels were assessed by western analysis of whole cell lysates. These studies revealed that Mcl-1 levels were upregulated by ABT-263, as expected (**Figure 16B**). Despite increased Mcl-1 protein expression, Mcl-1 diminution following CHX addition occurred in ABT-263 treated cells at a rate equal to or greater than what was seen in control treated cells. These findings rule out Mcl-1 protein stabilization as a major driver of Mcl-1 upregulation in response to ABT-263 in ERα+ breast cancer cells.

Increased cap-dependent translation of *MCL1* transcripts has been reported, particularly under conditions of cellular stress (79, 131). This possibility was tested in ERα+ cells treated with ABT-263, using a pharmacological inhibitor of cap-dependent translation, 4E1RCat (132). While Mcl-1 protein levels were increased in cells treated 24 hours with ABT-263, inhibition of cap-dependent translation with 4E1RCat blocked Mcl-1 upregulation following ABT-263 treatment in all three cell lines (**Figure 16C**). Interestingly, basal Mcl-1 levels were not downregulated by 4E1RCat, suggesting that *MCL1* translation occurs through cap-independent and cap-dependent mechanisms, but that in response to ABT-263, ERα+ breast cancer cells may rely heavily on cap-dependent translation of *MCL1* transcripts to drive rapid and potent Mcl-1 protein upregulation.

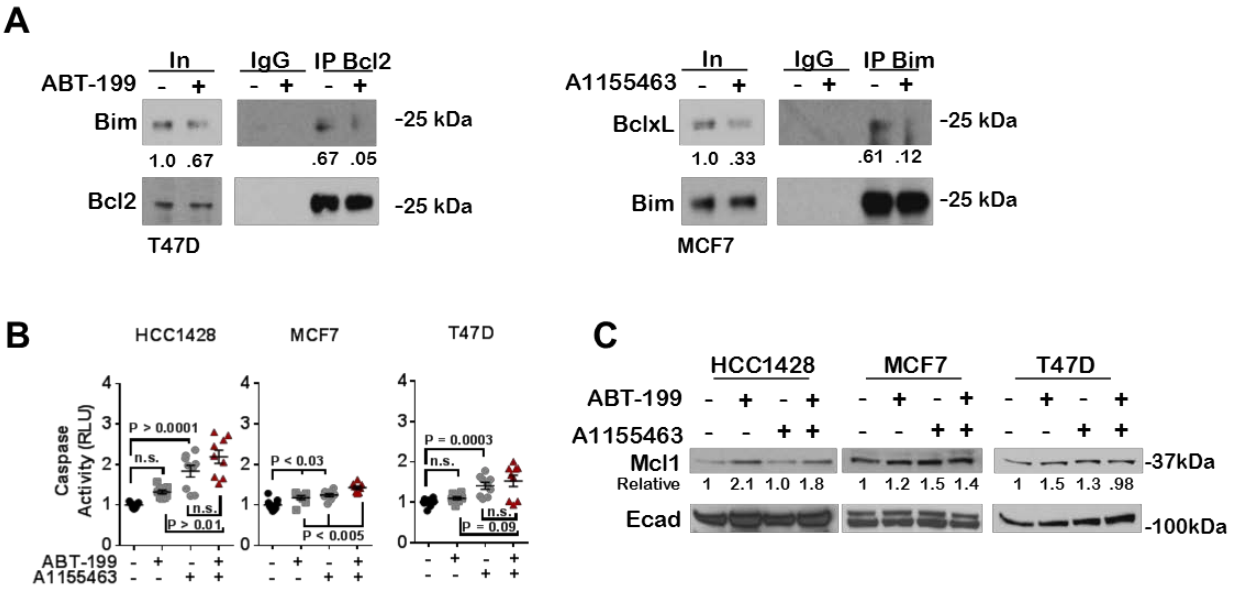

Figure 17. ERα+ breast cancers have varying sensitivities to Bcl-2 and Bcl-xL single inhibition.

A. Immunoprecipitation of Bcl-2 or Bim in whole cell lysates harvested after treatment with ABT-199 (1.0 μM) or A1155463 (1.0 μM) for 2 hrs. **B.** Caspase activity was determined after treatment with 1.0 μM ABT-199, 1.0 μM A1155463, or ABT-199 + A1155463 for 4 hrs. Average luminescence is depicted, N = 6-9, Bonferroni *post hoc* test followed by Student's unpaired two-tailed t-test. **C.** Whole cell lysates harvested from cells treated with ABT-199 (1.0 μM), A1155463 (1.0 μM), or ABT-199 + A1155463 for 24 hrs.

ERα+ breast tumors have varying response to Bcl-2 and Bcl-xL single inhibition.

To better understand if Mcl-1 upregulation is supported by Bcl-2 inhibition independently of Bcl-xL inhibition or vice versa, we treated cells with the Bcl-2 specific inhibitor ABT-199 (68) and the Bcl-xL specific inhibitor A-1155463 (55). ABT-199 blocked Bcl-2/Bim interactions after 2 hours treatment, while A-1155463 blocked Bcl-xL/Bim interactions, demonstrating on target inhibition by each compound (**Figure 17A**). Activity of caspases 3 and 7, irreversible molecular mediators of the intrinsic apoptotic pathway, was used to assess the impact of Bcl-2 and Bcl-xL single inhibition on apoptosis induction. ABT-199 induced marginal caspase-3/7 activity in MCF7 cells, but not HCC1428 or T47D cells after 4 hours treatment (**Figure 17B**). In contrast, A-1155463 increased caspase-3/7 activity in all three cell lines. ABT-199 in combination with A-1155463 increased caspase-3/7 activity beyond either agent alone in only MCF7 cells. HCC1428 and T47D cells did not benefit from the combination of ABT-199 and A-1155463 beyond what was seen with Bcl-xL blockade alone. These findings suggest that Bcl-xL is a key cell survival factor in many ERα+ breast cancers, while others depend on both Bcl-2 and Bcl-xL. Interestingly, Mcl-1 upregulation was seen in all cells treated with ABT-199, albeit only modestly in MCF7 cells (**Figure 17C**), perhaps explaining the reduced responsiveness of ERα+ breast cancers cells to ABT-199. Mcl-1 upregulation in response to A-1155463 was seen in MCF7 and T47D cells, but not HCC1428 cells, potentially explaining the increased sensitivity of HCC1428 cells to A-1155463 as compared to MCF7 and T47D cells. These results suggest that Bcl-2 and Bcl-xL inhibition are not equivalent across all ERα+ breast cancer cell lines, possibly due to varying Mcl-1 upregulation.

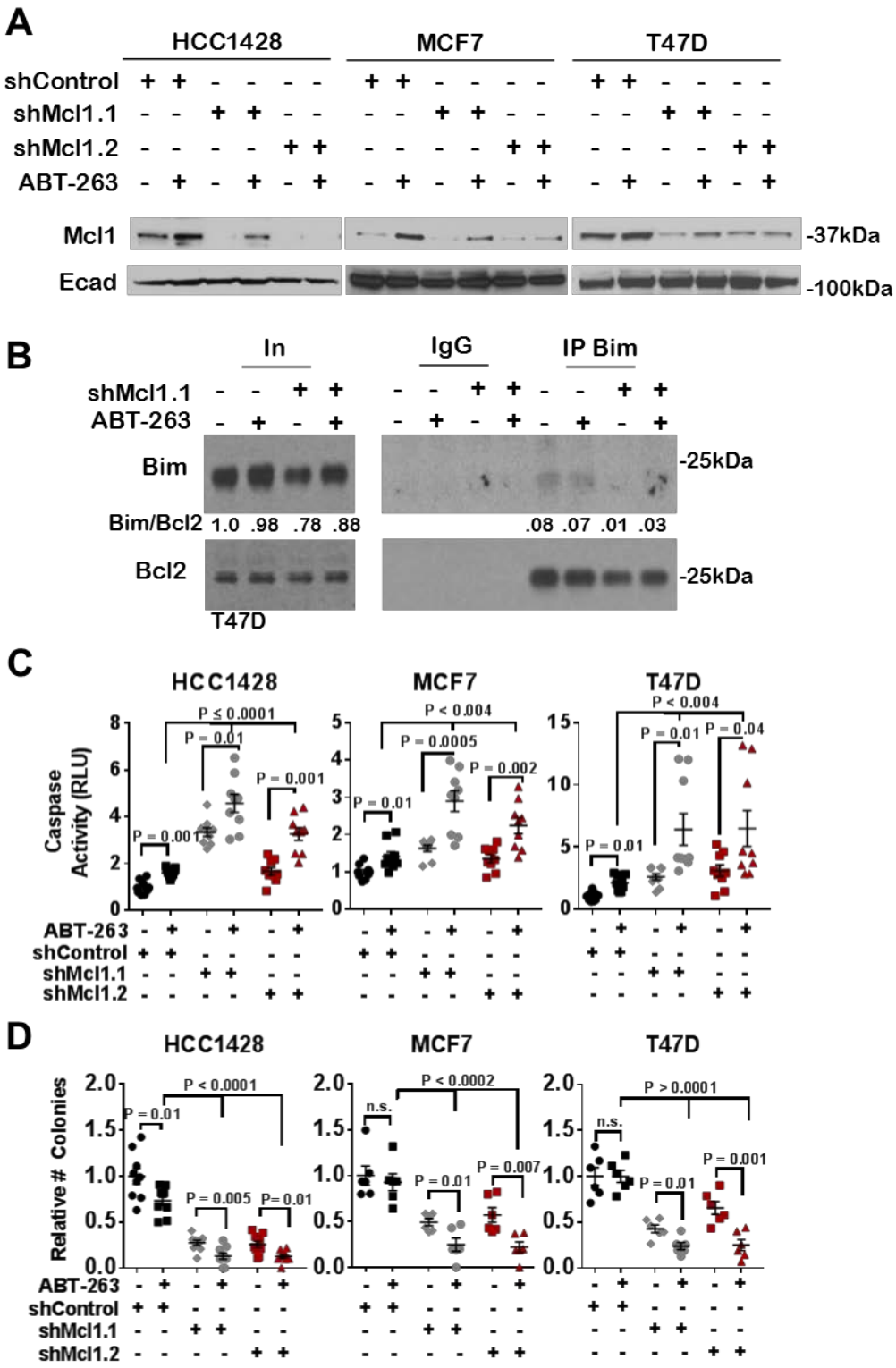
A
HCC1428
MCF7
T47D
shControl
shMcl1.1
shMcl1.2
ABT-263
Mcl1
-37kDa
Ecad
-100kDa
B
In
IgG
IP Bim
shMcl1.1
ABT-263
Bim
-25kDa
Bim/Bcl2 1.0 .98 .78 .88
.08 .07 .01 .03
Bcl2
-25kDa
T47D
C
HCC1428
MCF7
T47D
Caspase Activity (RLU)
P ≤ 0.0001
P = 0.01
P = 0.001
P = 0.001
P < 0.004
P = 0.0005
P = 0.002
P = 0.01
P < 0.004
P = 0.01
P = 0.04
P = 0.01
ABT-263
shControl
shMcl1.1
shMcl1.2
D
HCC1428
MCF7
T47D
Relative # Colonies
P < 0.0001
P = 0.01
P = 0.005
P= 0.01
P < 0.0002
n.s.
P = 0.01
P = 0.007
P > 0.0001
n.s.
P = 0.01
P = 0.001
ABT-263
shControl
shMcl1.1
shMcl1.2

Figure 18. Mcl-1 ablation increases sensitivity to ABT-263.

A. Whole cell lysates were prepared from cells treated for 24 hrs with 1.0 μM ABT-263 or DMSO control. **B.** Immunoprecipitation of Bim completed on MCF7 cells treated for 2 hrs with 1.0 μM ABT-263 or DMSO control. **C.** Average luminescence was measured in cells treated for 4 hrs with 1.0 μM ABT-263 or DMSO control after addition of Cleaved Caspase-3/7-Glo substrate. N = 6-9, Bonferroni *post hoc* test followed by Student's unpaired two-tailed t-test. **D.** Cells were grown in 3D-martigel and treated with 1.0 μM ABT-263 or control for 14 d. Data represents average number of colonies/20x-magnification, N = 6-9, Bonferroni *post hoc* test followed by Student's unpaired two-tailed t-test.

Combined Mcl-1 ablation and ABT-263 increase cell death in ERα+ breast cancers.

To determine if Mcl-1 inhibition sensitizes cells to ABT-263, we generated a panel of ERα-positive breast cancer cell lines (HCC1428, MCF7, and T47D) that stably express two independent shRNA sequences directed against *MCL1* (shMCL1) (**Figure 18A**). Western analysis confirmed knockdown of Mcl-1 protein expression in cells expressing shMCL1 as compared to cells expressing control shRNA sequences (shControl). Cells expressing shControl upregulated Mcl-1 upon treatment with ABT-263 (1.0 μM) for 24 hours. However, Mcl-1 induction in response to ABT-263 was reduced in cells expressing shMCL1. Further, despite Mcl-1 ablation ABT-263 inhibited Bcl-2/Bim interactions similarly in shMCL1 and shControl expressing cells (**Figure 18B**).

Cells were treated 4 hours with ABT-263 and apoptosis was measured by caspase-3/7 activity, showing a 1-2-fold increase (**Figure 18C**). However, Mcl-1 knockdown increased tumor cell death 1.5- to 3.5-fold above that seen in shControl cells. These findings are consistent with previous reports describing Mcl-1 inhibition in ERα+ breast cancer cells (57). The combination of Mcl-1 knockdown with ABT-263 markedly increased caspase-3/7 activity in each cell line. Further, the number of colonies that outgrew after single cell suspension in 3D-Matrix and treatment for 14 days with ABT-263 were marginally decreased in only HCC1428 shControl cells (**Figure 18D**), while Mcl-1 ablation (shMcl1) decreased colony number by ≥2-fold in each cell line. However, shMCL1-expressing cells treated with ABT-263 generated nearly 10-fold fewer colonies as compared to shControl-expressing cells. These data suggest that Mcl-1 primes ERα+ breast cancer cells for cell death in response to ABT-263, resulting in potent inhibition of tumor cell growth.

Mcl-1 expression is depleted by mTORC1 inhibition.

To pharmacologically deplete Mcl-1 expression, we explored the impact of mTORC1 blockade. Because mTORC1 is a dominant activator of cap-dependent translation, mTORC1 inhibition may restrain ABT-263-mediated Mcl-1 upregulation by limiting *MCL1* cap-dependent translation. This would be advantageous clinically, given that mTORC1 inhibitors may induce dramatic tumor cell killing in combination with ABT-263, and because mTORC1 inhibitors (e.g., RAD001/everolimus) are clinically approved for treatment of ERα+ breast cancers.

We assessed the impact of RAD001 (200 nM) on Mcl-1 by western analysis. Cells treated with RAD001 (200 nM) for 4 hours displayed decreased phosphorylation of 70^{S6K} (S6K) and 4EBP1, and decreased Mcl-1 protein expression (**Figure 19A**). These results were confirmed in a spontaneous mouse model of luminal-like breast cancer, the *Wap-myc* mammary tumor model, which expresses *c-myc* in the alveolar mammary epithelium under the control of the whey acidic protein (WAP) gene promoter (133, 134). Previous reports demonstrate that Myc-driven tumors often express high levels of Mcl-1, and in many cases, rely on Mcl-1 for tumor cell survival (135-137). We confirmed that *Wap-myc* tumors express high levels of MCL-1, comparing normal mammary gland tissues to tumor tissues taken from the same animal (**Figure 20A**). In *Wap-myc* tumors, RAD001 treatment decreased phosphorylation of downstream mTORC1 targets, and decreased MCL-1 protein levels on average 2.5-fold (**Figure 19B**). These data suggest that Mcl-1 may be a key mTORC1 effector driving tumor cell survival. To test this, we treated ERα+ breast cancer cell lines with RAD001 to block mTORC1, finding that RAD001 induced caspase-3/7 activity after 4 hours treatment in all cell lines tested (**Figure 19C**). Increased caspase-3/7 activity was sustained up to 48 hours in RAD001-treated MCF7 and T47D cells. Similarly, RAD001 treatment of *Wap-myc* transgenic mouse mammary tumors induced high levels of tumor cell death, as measured by *in situ* TUNEL analysis of tumor sections (**Figure 19D**). However, RAD001 had no effect on tumor cell proliferation (**Figure 20B**).

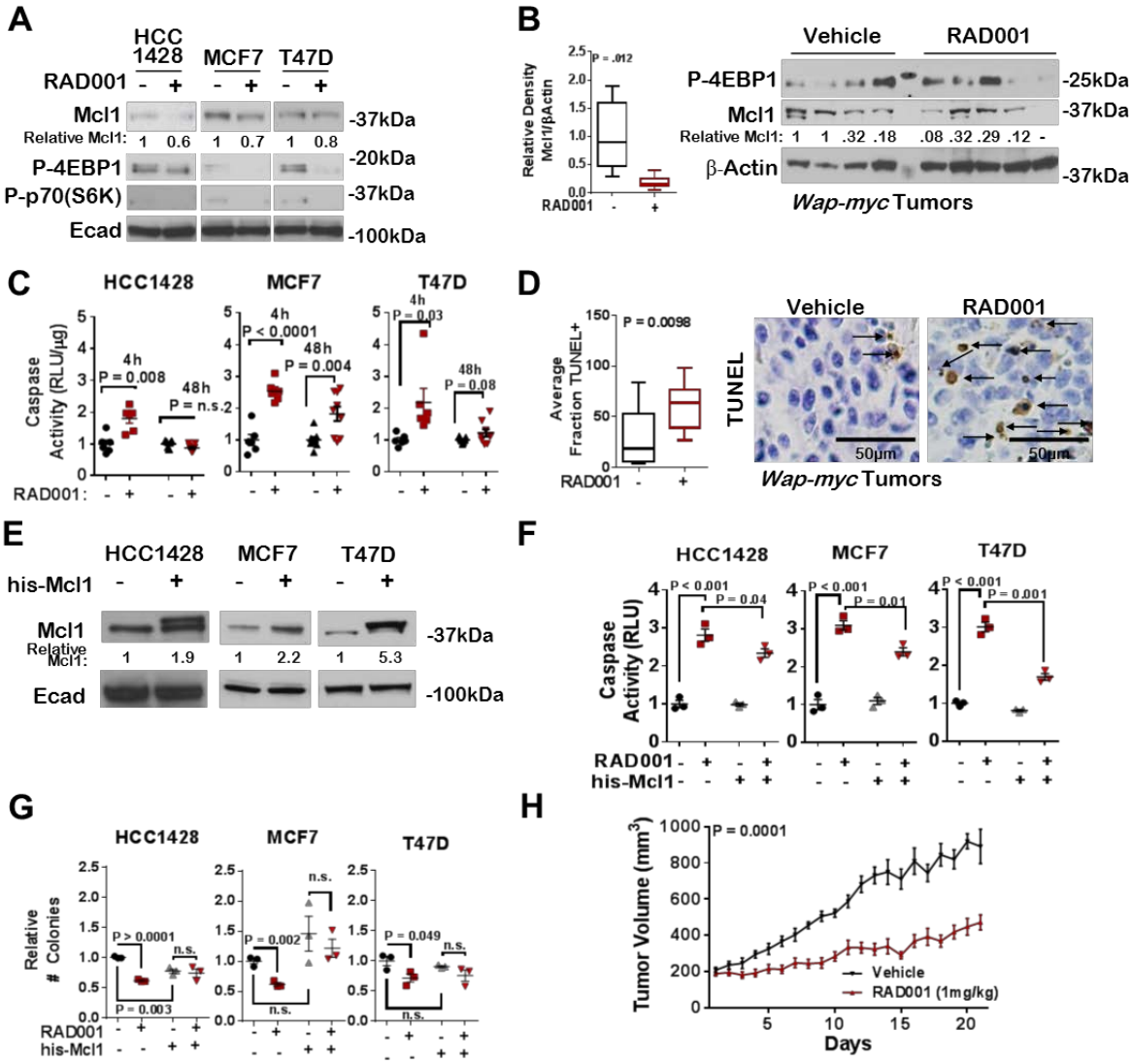
A
HCC 1428
MCF7
T47D
RAD001 - + - + - +
Mcl1
Relative Mcl1: 1 0.6 1 0.7 1 0.8
-37kDa
P-4EBP1
-20kDa
P-p70(S6K)
-37kDa
Ecad
-100kDa
B
Relative Density Mcl1/βActin
P = .012
RAD001 - +
Vehicle
RAD001
P-4EBP1
-25kDa
Mcl1
-37kDa
Relative Mcl1: 1 1 .32 .18 .08 .32 .29 .12 -
β-Actin
-37kDa
Wap-myc Tumors
C
HCC1428
MCF7
T47D
Caspase Activity (RLU/μg)
4h
P = 0.008
48h
P = n.s.
4h
P < 0.0001
48h
P = 0.004
4h
P = 0.03
48h
P = 0.08
RAD001: - + - + - + - + - + - +
D
Average Fraction TUNEL+
P = 0.0098
RAD001 - +
Vehicle
RAD001
TUNEL
50μm
50μm
Wap-myc Tumors
E
HCC1428
MCF7
T47D
his-Mcl1 - + - + - +
Mcl1
Relative Mcl1: 1 1.9 1 2.2 1 5.3
-37kDa
Ecad
-100kDa
F
HCC1428
MCF7
T47D
Caspase Activity (RLU)
P < 0.001
P = 0.04
P < 0.001
P = 0.01
P < 0.001
P = 0.001
RAD001 - + - + - + - + - + - +
his-Mcl1 - - + + - - + + - - + +
G
HCC1428
MCF7
T47D
Relative # Colonies
P > 0.0001
n.s.
P = 0.003
P = 0.002
n.s.
n.s.
n.s.
P = 0.049
n.s.
n.s.
RAD001 - + - + - + - + - + - +
his-Mcl1 - - + + - - + + - - + +
H
Tumor Volume (mm3)
P = 0.0001
Vehicle
RAD001 (1mg/kg)
Days

Figure 19. Inhibition of mTORC1 with RAD001 induces cell death through depletion of Mcl-1.

A. Whole cell lysates harvested from cells treated with 200 nM RAD001 for 4 hrs. **B.** Wap-myc mammary tumors treated with 1.0 mg/kg RAD001 or control by oral gavage daily for 21 d. Whole tumor lysates. Left, quantitation shows the average of three blots, Student's unpaired two-tailed t-test. Right, representative western analysis. **C.** Caspase activity was determined using the Cleaved Caspase-3/7-Glo assay after treatment with 200 nM RAD001 for 4 or 48 hrs. N = 6-9, Student's unpaired two-tailed t-test. **D.** TUNEL analysis was completed on FFPE tumor tissue from experiment detailed in B. Left, quantitation represents average TUNEL+ cells/field of view for 3 fields/tumor, N = 15, Student's unpaired two-tailed t-test. Right, representative images at 400x: arrows denote TUNEL+ cells. **E-G.** Adenovirus (his-Mcl1) was used to overexpress Mcl-1. All experiments were compared to control adenoviral transduced cells (Ad.GFP). E depicts whole cell lysates harvested from cells treated with adenovirus for 72 hrs. Cleaved caspase activity was determined in cells treated with adenovirus for 72 hrs and 200 nM RAD001 for the final 4 hrs, N = 3-9, Bonferroni *post hoc* test followed by Student's unpaired two-tailed t-test (F). G depicts single cells seeded in 3D-Matrigel 24 hrs post-transduction. Cells treated with 200 nM RAD001 for 7 d and average number of colonies/20x-field is shown for 2 experiments, N = 6, Student's unpaired two-tailed t-test. **H.** Average tumor volume, N = 15, area under the curve for experiment detailed in B.

Notably *MCL1* transcript levels in MCF7 and T47D cells were unchanged after 24 hours RAD001 treatment, suggesting that mTORC1 inhibitors must downregulate Mcl-1 expression downstream of RNA levels in these cells (**Figure 20C**). Despite decreased Mcl-1 levels in RAD001-treated HCC1428 cells, Mcl-1 mRNA levels were increased >150-fold, highlighting the complexity of the molecular signals that integrate to regulate Mcl-1 expression.

To further explore these mechanisms, we used adenoviral transduction to uncouple Mcl-1 expression from the cap-dependent translation machinery. Adenoviral *MCL1* (his-MCL1) increased Mcl-1 expression over what was seen in Ad.GFP control transduced cells (**Figure 19E**). RAD001 increased caspase-3/7 levels in cells expressing Ad.GFP (**Figure 19F**). However, his-MCL1 decreased RAD001-induced caspase-3/7 activity. Further, growth of Ad.GFP-expressing cells was impaired by RAD001, while his-MCL1 expressing cells were resistant to RAD001-mediated growth inhibition (**Figure 19G**). These results were confirmed in the transgenic *Wap-myc* mammary tumor model, revealing 60% decreased tumor volume in response to daily treatment with RAD001 (1.0 mg/kg) as compared to vehicle treated controls (**Figure 19H**). These results suggest that mTORC1 inhibition blocks Mcl-1 expression, resulting in increased tumor cell death and tumor growth inhibition.

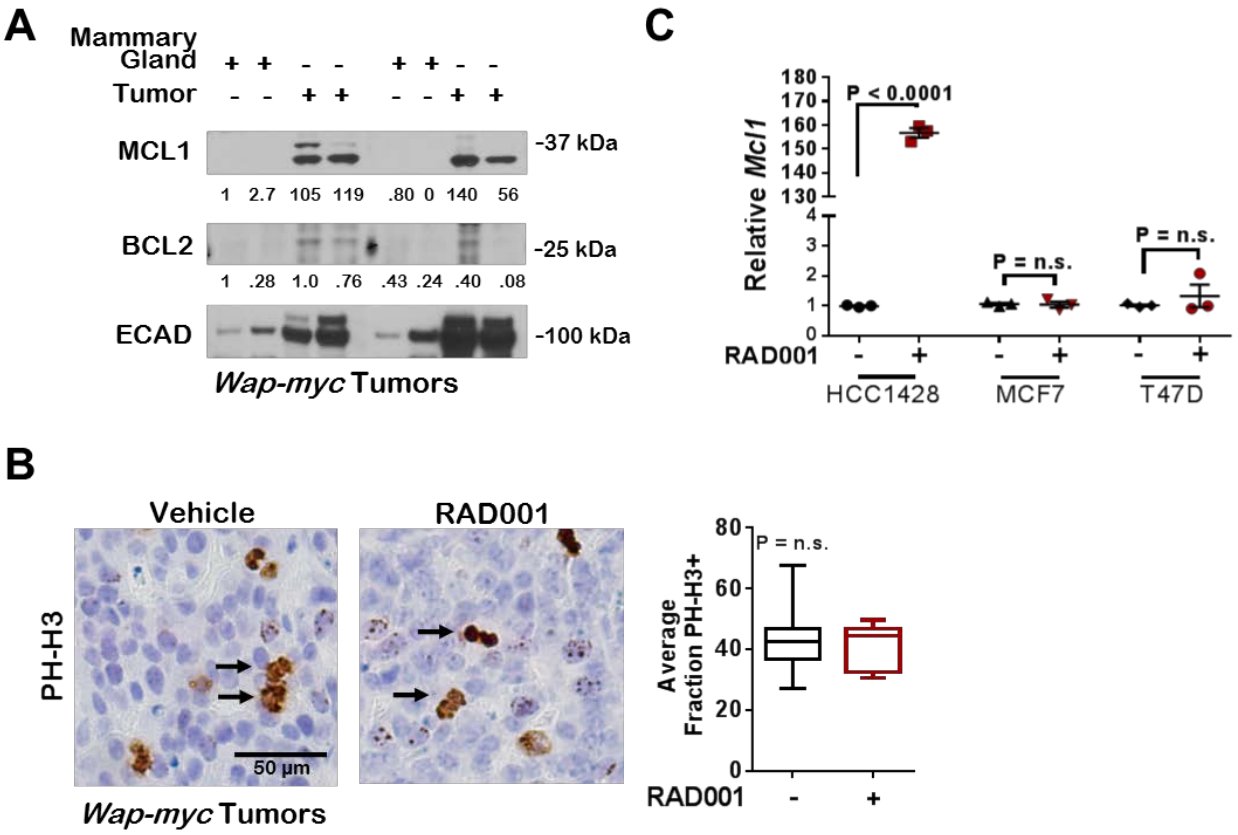

Figure 20. MCL1 expression is elevated in *Wap-myc* mammary tumors.

A. Whole tumor lysates harvested from *Wap-myc* mammary tumors and adjacent normal mammary glands, antibodies show on the left. **B.** Phospho-histone H3 staining in *Wap-myc* mammary tumors after treatment with RAD001 (1.0 mg/kg) or vehicle control for 21 d. Left, representative images taken at 400x: arrows denote positive cells. Right, quantitation of average PH-H3 positive cells/total cells for 3 fields per tumor is show. N = 15, Student's unpaired two-tailed t-test. **C.** RT-qPCR completed on reverse transcribed whole cell mRNA after treatment with RAD001 (200 nM) for 4 hrs, values were standardized to 36B4 control and averaged for three separate experiments, N = 9, Student's unpaired two-tailed t-test.

mTORC1 inhibition depletes Mcl-1 expression and activity induced by ABT-263.

To explore the utility of mTORC1 inhibition in combination with Bcl-2/Bcl-xL inhibition, we treated cells with ABT-263 in the presence or absence of RAD001 for 24 hours. While ABT-263 induced Mcl-1 protein upregulation, RAD001 abrogated Mcl-1 induction by ABT-263 (**Figure 21A**). These results are consistent with our data using 4E1RCat to block cap-dependent translation in these cell lines, and with the idea that mTORC1 is a dominant regulator of cap-dependent translation (138). A proximity ligation assay (PLA) was used to measure Mcl-1 activity (i.e., molecular interactions between Mcl-1 and Bim) in cells treated with ABT-263 and RAD001. Cells treated with ABT-263 showed potent upregulation of the number of fluorescent puncta generated by the detection of Mcl-1/Bim interactions (**Figure 21B-C – red puncta**). However, RAD001 abrogated the ABT-263-mediated increase in Mcl-1/Bim interactions. These data are consistent with previous reports that Mcl-1 upregulation occurs downstream of mTORC1 signaling, and support the notion that targeted inhibition of mTORC1 can block ABT-263-induced Mcl-1 expression and activity.

We tested the impact of combining ABT-263 with RAD001 on growth and survival of ERα+ breast cancer cell lines. Cells cultured 4 hours in the presence of ABT-263 plus RAD001 displayed greater than 5-fold increased caspase-3/7 activity as compared to cells cultured in either agent alone (**Figure 21D**). Single cell suspensions embedded in 3D-Matrigel and cultured 14 days in the presence of both ABT-263 and RAD001 generated fewer colonies than cells cultured in ABT-263 or RAD001 alone (**Figure 21E**), suggesting that the greatest growth inhibition is achieved upon combined Bcl-2/Bcl-xL/Mcl-1 inhibition.

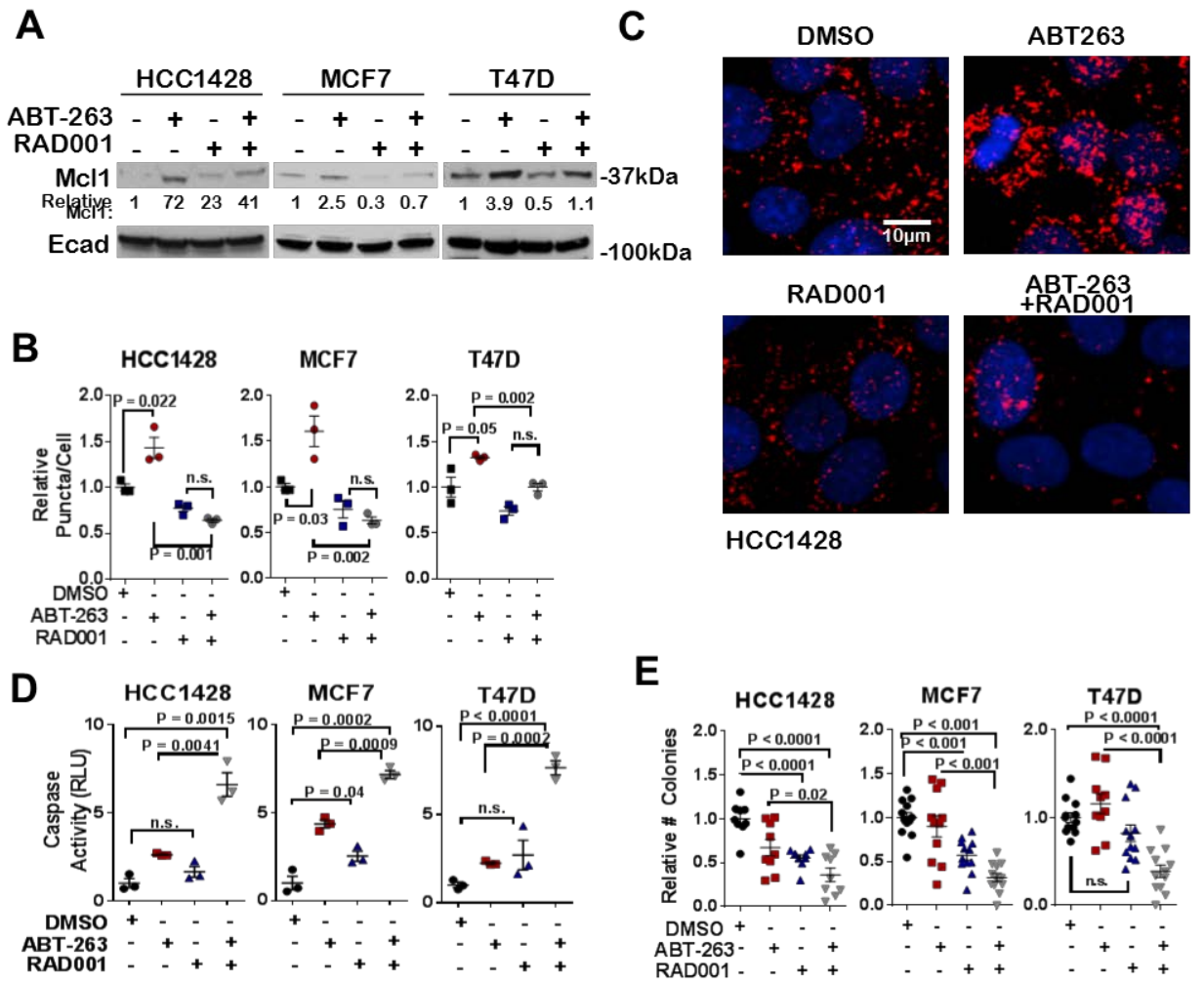

Figure 21. Figure 4. Inhibition of mTORC1 sensitizes ERα+ breast cancers to ABT-263.

A. Whole cell lysates harvested from cells treated with 1.0 μM ABT-263 ± 200 nM RAD001 for 24 hrs. **B-C.** Proximity ligation assay (PLA) was conducted on methanol fixed cells after treatment with 1.0 μM ABT-263 ± 200nM RAD001 for 4 hrs. **B.** Quantitation represents the average number of puncta/nuclei for 3 fields of view, N = 3. Bonferroni post hoc test followed by Student's unpaired two-tailed t-test. **C.** Representative images: red = Mcl-1/Bim proximity (arrows represent numerated puncta), blue = hoechst staining. **D.** Caspase activity was determined after treatment with 1.0 μM ABT-263 ± 200 nM RAD001 for 4 hrs. Average luminescence is depicted, N = 6-9, Bonferroni post hoc test followed by Student's unpaired two-tailed t-test. **E.** Cells grown in 3D-matrigel were treated with 1.0 μM ABT-263 ± 200 nM RAD001 for 14 d. Average number of colonies/20x field of view is shown, N = 6-9, Bonferroni post hoc test followed by Student's unpaired two-tailed t-test.

***Inhibition of Bcl-2/Bcl-xL combined with mTORC1 blockade decreases growth of ERα+ breast tumors* in vivo.**

We treated MCF7 xenografts with ABT-263 (20 mg/kg) in the presence or absence of RAD001 (1 mg/kg) for 16 days. Although ABT-263 alone (N = 10) did not significantly alter the growth of MCF7 tumors as compared to those treated with vehicle control (**Figure 22A**), RAD001 (N = 7) decreased MCF7 tumor growth by nearly 50% as compared to controls (N = 9). However, tumor growth was potently reduced upon combined inhibition of mTORC1 and Bcl-2/Bcl-xL (N = 7), causing complete abrogation of tumor growth throughout the 16-day treatment period. We used PLA to confirm the on-target activity of ABT-263, which disrupted interactions between Bcl-2 and Bim (**Figure 22B – red puncta**). Western analysis and immunohistochemistry demonstrated that canonical downstream mTORC1 targets, including p4EBP1 (**Figure 23A**) and pS6K (**Figure 23B**), were decreased after RAD001 treatment. Consistent with the idea that Mcl-1 levels are highest in tumors that have mTORC1 pathway activation, blockade of mTORC1 signaling by RAD001 resulted in tumors that express the lowest Mcl-1 levels (**Figure 22D**). These data further support the idea that ABT-263 treatment relies on mTORC1-mediated Mcl-1 upregulation, which can be blunted by mTORC1 inhibitors.

In addition to decreased tumor volume, total cellularity was decreased by RAD001 + ABT-263 treatment (**Figure 22C**), suggesting that combined mTORC1 and Bcl-2/Bcl-xL inhibition may induce dynamic tumor cell killing. To test this idea, TUNEL analysis was used to measure tumor cell death, revealing an upregulation of tumor cell killing in tumors treated with RAD001 as a single agent (**Figure 22E**). However, the combination of ABT-263 + RAD001 induced the greatest tumor cell killing. Tumor cell proliferation was unaffected by treatment with RAD001 or ABT-263 (**Figure 23C**). These data suggest that mTORC1 inhibition culled Mcl-1 upregulation following Bcl-2/Bcl-xL inhibition, increasing the net therapeutic value of ABT-263.

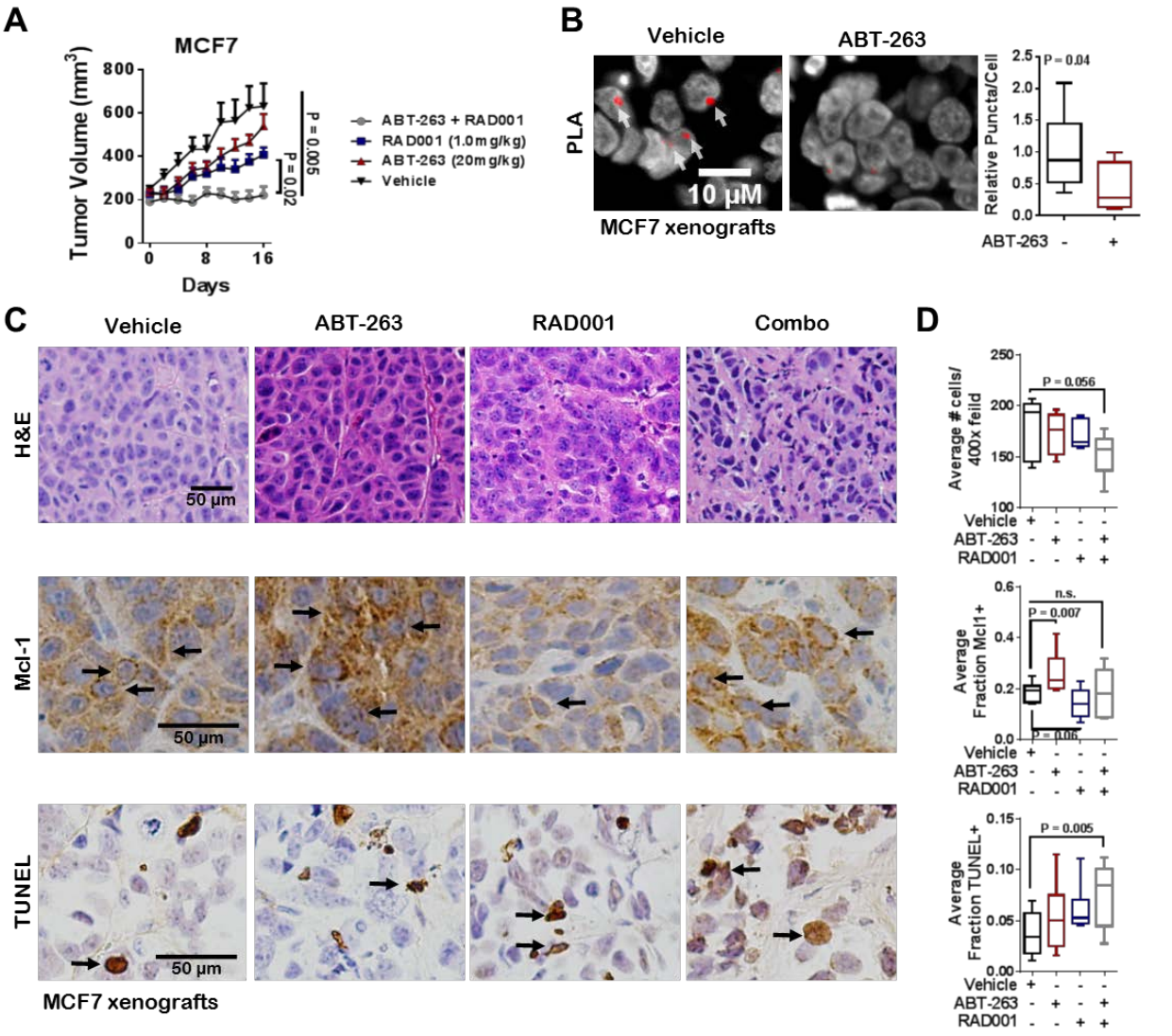

Figure 22. Inhibition of mTORC1 in combination with ABT-263 increases tumor cell killing *in vivo*.

A-C. MCF7 xenografts were treated with ABT-263 (20 mg/kg), RAD001 (1.0 mg/kg) or both by oral gavage daily for 16 d. A depicts average tumor volume N = 6-10, area under the curve. Proximity ligation assay conducted on FFPE tissues harvested 1 hr after final treatment. Left, 630x representative images: grey, DAPI, red, Mcl-1/Bim proximity denoted by arrows. Right, quantitation represents average puncta/cell for 6 photos/tumor, N = 6-10. Student's unpaired two-tailed t-test. Error bars represent min to max (B). **C-D.** Hematoxylin and eosin (H&E) staining, Mcl-1 immunohistochemical staining, and TUNEL analysis of MCF7 xenografts. C, representative images at 200x or 400x: arrows denote enumerated cells. D, depicts total number of cells per field for H&E, or average Mcl-1+,TUNEL+ cells/total number of cells for three fields/tumor, N = 6-10, Student's unpaired two-tailed t-test.

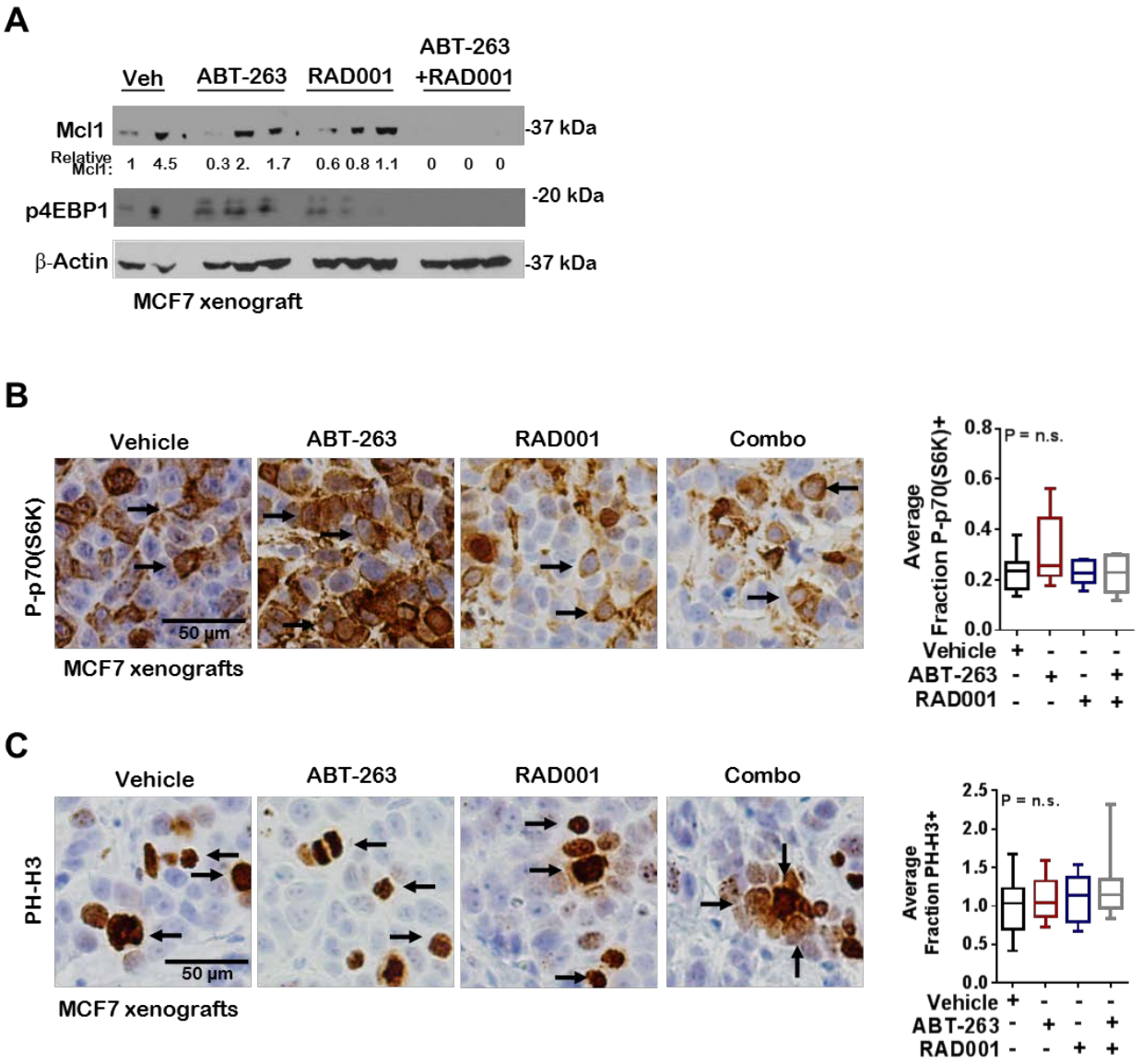

Figure 22. Expression of Mcl-1 correlates with mTORC1 pathway activation.

A-C. MCF7 xenografts were treated with ABT-263 (20 mg/kg), RAD001 (1.0 mg/kg) or both by oral gavage daily for 16 d. (A) depicts whole tumor lysates probed for antibodies shown on the left. In (B) FFPE tissues harvested 1 hr after final treatment were analyzed for P-p70(S6K) expression. Left: 400x representative images. Right: quantitation represents average P-p70(S6K) positive cells/total cells for 3 photos per tumor, N = 6-10. Student's unpaired two-tailed t-test. Error bars represent min to max. (D) represents Phospho-histone H3 staining. Left: 400x representative images. Right: quantitation represents average P-p70(S6K) positive cells/total cells for 3 photos per tumor, N = 6-10. Student's unpaired two-tailed t-test. Error bars represent min to max.

Discussion

The anti-apoptotic Bcl-2 family member Mcl-1 is highly expressed in all breast cancer subtypes, however compared to other cancers relatively little is known about Mcl-1 in breast tumor biology. With the ongoing development of Mcl-1 specific BH3-mimetics (61, 62), a better understanding of the role of Mcl-1 in breast tumorigenesis is imperative. Previous studies by our laboratory and others are addressing this need in ERα+ breast cancers. These studies show that Mcl-1 depletion induces cell death in ERα+ breast cancer cell lines (56, 57). Additionally, Mcl-1 is a primary tumor cell survival factor upon pharmacological inhibition of other anti-apoptotic Bcl-2 family proteins using ABT-263 or ABT-737 (Bcl-2/Bcl-xL specific inhibitors), and WEHI-539 (a Bcl-xL specific inhibitor) (60). For instance, triple negative breast cancers are more sensitive to Mcl-1 inhibition if Bcl-xL is also targeted (119). To better understand mechanistically why Bcl-2 and/or Bcl-xL inhibition may prime cells for Mcl-1 blockade, we observed Mcl-1 biology upon ABT-263 treatment, finding that direct pharmacological inhibition of cap-dependent translation using 4ER1Cat depleted Mcl-1 levels stimulated by ABT-263 treatment (**Figure 16C**). These findings are consistent with previous reports in other cancers. However, we found that single agent Bcl-2 inhibition (ABT-199) increased Mcl-1 levels in more cell lines (3/3) than single agent Bcl-xL inhibition (2/3 cell lines, A-1155463), perhaps explaining the innate resistance of ERα+ breast cancer cells to Bcl-2 inhibition as a single treatment (**Figure 17C**). Therefore, Mcl-1 upregulation by ABT-263 may be due to inhibition of Bcl-2 in some ERα+ breast tumors, but these results do not rule out the impact of Bcl-2/Bcl-xL blockade on Mcl-1 expression levels. The idea that ERα+ breast cancers are heterogeneously dependent on anti-apoptotic Bcl-2 family members will require further exploration.

Increased Mcl-1 expression in response to ABT-263 should sensitize ERα+ breast cancer cells to Mcl-1 ablation. Indeed, Mcl-1 knockdown conferred sensitivity to ABT-263 in otherwise resistant ERα+ breast cancer cells (**Figure 18C-D**), demonstrating that targeting multiple Bcl-2

family proteins may be necessary to enable therapeutic tumor cell killing in some ERα+ breast cancers. We hypothesized that pharmacological inhibition of mTOR, a serine-threonine kinase that regulates cap-dependent translation [as reviewed in (138)], would depleted Mcl-1 levels and similarly induce tumor cell killing in ERα+ breast cancer cells alone and in combination with ABT-263. The idea that Mcl-1 is regulated by mTOR in ERα+ breast cancers is consistent with the well-established oncogenic role of mTOR in this cancer type [as reviewed in (139)]. Further, previous work demonstrated that under conditions of cellular duress, including ABT-263 treatment, Mcl-1 protein levels are regulated by mTORC1 signaling in colon cancers (82), rhabdomyosarcomas (140), lung cancers (85, 141), glioblastomas (86), and ovarian cancers (81). In fact, one study showed that PI3K/mTOR inhibitors effectively decreased Mcl-1 levels in *PIK3CA*-mutant breast cancer cell lines, sensitizing mutant cells to ABT-263 (56). Although the data herein support these findings, our studies suggest a wider impact of mTORC1 inhibition. For example, RAD001 decreased Mcl-1 levels and increased apoptosis even in the absence of ABT-263 (**Figure 19A-C**). Further, Mcl-1 downregulation using mTORC1 inhibition induced cell death in *PIK3CA*-WT cells (e.g. HCC1428), suggesting that Mcl-1 is a critical effector of mTORC1 signaling independent of *PIK3CA* status. More importantly, this study is the first to use the clinically approved mTORC1 inhibitor RAD001/everolimus as a tool to target Mcl-1 in both human breast cancer cell lines in culture and *in vivo*, and in transgenic mammary tumor models. Thus, RAD001 may be an ideal candidate to target Mcl-1 clinically. This notion is supported by a recently published case study showing pathological complete response in a patient with late stage *MCL1*-amplified ERα+ breast cancer after treatment with aromatase inhibition (anastrozole) and RAD001/everolimus (142).

Although excitement surrounds the development of Mcl-1 specific BH3-mimetics, the results shown herein suggest that Mcl-1 may be targeted in the clinic indirectly through the use of mTORC1 inhibitors. This approach is particularly appealing given the fact that a recent preclinical

test of Mcl-1 specific BH3-mimetics caused death in all mice receiving elevated doses (61), possibly due to on-target toxicities of systematic Mcl-1 inhibition. For instance, MCL-1 loss in mice is associated with cardiac failure (143), immune deficiency (144-146), and mitochondrial dysfunction (143). However, Mcl-1 diminution, as opposed to complete inhibition, by mTORC1 inhibitors maybe efficacious clinically, particularly when ABT-263 is used to prime ERα+ breast cancers for Mcl-1-dependence. These data support further clinical analysis of mTORC1 inhibitors as indirect Mcl-1 inhibitors, as well as combined anti-apoptotic Bcl-2 family member inhibition in ERα+ breast cancers and other tumor types.

CHAPTER IV

Intrinsic Apoptotic Pathway Activation Increases Response to Anti-Estrogens in ERα+ Breast Cancers

This work is under revision for acceptance at Cell Death and Disease under the same title.

Abstract

Estrogen receptor-α positive (ERα+) breast cancer accounts for approximately 55-65% of the nearly 250,000 new cases of breast cancer diagnosed in the U.S. each year. Endocrine-targeted therapies (those that block ERα activity) serve as the first line of treatment in most cases. Despite the proven benefit of endocrine therapies, ERα+ breast tumors often escape endocrine therapy, causing disease progression or recurrence, particularly in the metastatic setting. Anti-apoptotic Bcl-2 family proteins enhance breast tumor cell survival, often promoting resistance to targeted therapies, including endocrine therapies. Herein, we investigated whether blockade of anti-apoptotic Bcl-2 family proteins could sensitize ERα+ breast cancers to anti-estrogen treatment. We used long-term estrogen deprivation (LTED) of human ERα+ breast cancer cell lines, an established model of sustained treatment with, and acquired resistance to aromatase inhibitors (AIs), in combination with Bcl-2/Bcl-xL inhibition (ABT-263), finding that ABT-263 induced only limited tumor cell killing in LTED-selected cells in culture and *in vivo*. Interestingly, expression and activity of the Bcl-2-related factor Mcl-1 was increased in LTED cells. Genetic Mcl-1 ablation induced apoptosis in LTED-selected cells, and potently increased their sensitivity to ABT-263. Increased expression and activity of Mcl-1 similarly was seen in clinical breast tumor specimens treated with AI + the selective estrogen receptor downregulator fulvestrant. Fulvestrant caused upregulation of Mcl-1, but not Bcl-2 or Bcl-xL, in cultured breast cancer cell lines. Delivery of Mcl-1 siRNA-loaded into polymeric nanoparticles (MCL1si-NPs) decreased Mcl-1 expression in LTED-selected cells and in fulvestrant-treated cells, increasing tumor cell death and blocking tumor cell growth. These findings suggest that Mcl-1 upregulation in response to anti-estrogen

treatment enhances tumor cell survival, decreasing response to therapeutic treatments. Therefore, strategies blocking Mcl-1 expression or activity used in combination with endocrine therapies would enhance tumor cell death.

Introduction

The American Cancer Society estimated that approximately 250,000 women were diagnosed with breast cancer in 2016 in the United States alone (104). The most frequently diagnosed clinical breast cancers are those expressing Estrogen Receptor-α (ERα), an intracellular nuclear receptor that drives tumor cell cycle progression. ERα+ breast cancers are treated with targeted inhibitors that block ERα signaling, including selective ERα modulators (SERMS, *e.g.*, tamoxifen), selective ERα downregulators (SERDs, *e.g.*, fulvestrant), and aromatase inhibitors (AIs, *e.g.*, anastrozole), which decrease circulating estrogen levels in post-menopausal women. Although these treatments are successful for a large number of breast cancer patients, approximately 15-30% have *de novo* or acquired resistance to anti-estrogens [reviewed in (46, 147)]. Given the number of new diagnoses, and the number of breast cancer-related deaths caused by anti-estrogen resistance each year, there is a need to identify molecular vulnerabilities in ERα+ tumors that can be targeted to prevent or overcome anti-estrogen resistance.

Resistance to many cancer therapeutics relies on evasion of cell death, a hallmark of cancer (35). Tumor cells can evade apoptosis by increasing expression or activity of anti-apoptotic Bcl-2 family proteins (Bcl2-A1, Bcl-2, Bcl-xL, Bcl-w, and Mcl-1). These factors prevent Bak/Bax oligomerization and pore formation in the outer mitochondrial membrane [as reviewed in (8, 10)] by binding directly to Bak or Bax (148), or to Bim, an activator of Bak/Bax oligomerization (12). ERα+ breast cancers frequently overexpress anti-apoptotic Bcl-2, Bcl-xL, and Mcl-1 (58, 124-126). Bcl-2 and Bcl-xL are further elevated upon anti-estrogen treatments (47-49, 149),

suggesting that ERα+ breast cancers may use anti-apoptotic Bcl-2 family members to drive cell survival and treatment resistance (5, 150).

In further support of this idea, anti-estrogens are often cytostatic (151), halting cell proliferation without activating apoptosis. Survival of tumor cells during treatment would increase the likelihood of recurrence upon treatment withdraw, and may enforce treatment resistance, suggesting that blockade of anti-apoptotic Bcl-2 proteins in combination with anti-estrogens may decrease recurrence and/or resistance in ERα+ breast cancers. This idea has been tested using a class of small molecular weight compounds known as 'BH3-mimetics,' designed to bind anti-apoptotic Bcl-2 proteins with specificity to BH3 domain interaction motifs, preventing their interaction with pro-apoptotic factors like Bak/Bax and Bim (51). Although Bcl-2/Bcl-xL inhibition, using the BH3-mimetic ABT-737, or Bcl-2 specific inhibition, using the BH3-mimetic ABT-199, had little activity as single agents, their use in combination with tamoxifen resulted in tumor regression in some, but not all, patient-derived ERα+ breast cancer xenografts tested (48), supporting a role for Bcl-2 in endocrine resistance. In contrast, other studies show that *BCL2*, an ERα transcriptional target, is decreased in tamoxifen-treated and tamoxifen-resistant xenografts (152). These conflicting results require continued exploration of the Bcl-2 family in ERα+ breast cancers.

To investigate this, we used long term estrogen deprivation (LTED) to model treatment with and acquired resistance to AIs in human ERα+ breast cancer cell lines. We found that Bcl-2/Bcl-xL inhibition did not increase cell death in LTED-selected cells. However, Mcl-1 expression and activity were upregulated upon estrogen deprivation, as well as in response to fulvestrant. The recent development of Mcl-1 specific BH3-mimetics is allowing preclinical testing of Mcl-1 inhibition in some cancers (61, 62, 95), leading in some cases to clinical trials (59). However, preclinical and clinical testing of Mcl-1 blockade in combination with endocrine inhibition in ERα+ breast cancers is not fully explored. We show here that targeted inhibition of Mcl-1 in ERα+ breast cancers using Mcl-1 siRNA encapsulated in polymeric nanoparticles (si-NPs) increased tumor

cell killing in LTED-selected cells and in fulvestrant-treated cells, demonstrating that anti-estrogens prime ERα+ cells for cell death upon targeted Mcl-1 inhibition.

Materials and Methods

Cell culture: All cells lines were purchased from the American Tissue Type Collection (*Homo sapiens* ATCC CRL 2327; HTB-22; HTB-133). Cells were maintained in normal growth media (DMEM, 10% fetal bovine serum, 1x antibiotics/anti-mycotics). To generate LTED cells, each cell line was cultured in LTED growth media (phenol-red free Opti-Mem, 10% charcoal stripped fetal bovine serum, 1x antibiotics/anti-mycotics) until they grew at a similar rate to parental cells, 3-6 months. Growth in monolayer and cell count analyses were completed as in (125). In cases where parental cell lines were treated with or without estrogen, cells were cultured in LTED growth media supplemented with or without 2.0 nM 17β-estradiol (Sigma). Fulvestrant (Selleckchem) and ABT-263 (Selleckchem) were dissolved in DMSO and used at a final concentration of 1.0 μM.

Caspase-3/7 activity: 5,000 cells/well were plated in 96-well plates in growth media or LTED growth media, and treated with 1.0 μM ABT-263. For experiments with si-NPs, 5,000 cells/well (parental cells) or 10,000 cells/well (LTED-selected cells) were plated in 96-well plates and treated with si-NPs for 24 hours, and then replenished with growth media ± 1.0 μM fulvestrant. Caspase activity was determined using the Caspase-3/7-Glo assay (Promega) according to manufacturer's instructions at 4 hours after adding ABT-263 or 48 hours after adding si-NPs. Overall luminescence was read on a Glomax Mutli+ Detection System luminometer (Promega).

Western and immunoprecipitation: 10 μg Bcl-2 (DAKO) or IgG control (Santa Cruz Biotechnologies) antibodies were crosslinked to 50 μL protein A/G agarose beads (Santa Cruz Biotechnologies) using 5.0 mM BS3 (Sigma-Aldrich) by rotating at room temperature for 30 minutes. BS3 was quenched using 15 μL 1.0 M Tris (pH 7.4), and beads were washed 3x with ¼ NLB (see below) diluted in 1X PBS. 10 μL crosslinked beads (5.0 μg antibody) were rotated

overnight at 4°C with 1000 μg protein lysate, harvested [in 0.1% Nonident-P40 (NP-40) lysis buffer (NLB) supplemented with protease inhibitor cocktail (Roche), proteasome inhibitor MG-132 (Selleckchem), and sodium orthovanadate (Pierce)] as described in (125). Samples were washed with NLB, boiled in 1x reducing sample buffer (NuPAGE, Invitrogen) and run on a 4-12% SDS-PAGE gel as detailed in (125). Proteins were transferred to nitrocellulose (iBlot, Invitrogen), blocked [3% gelatin in Tris buffered saline (TBS), 0.1% Tween-20 (Sigma-Aldrich) and 3% cold fish gelatin (Sigma-Aldrich)], and then probed with antibodies against Bim (Cell Signaling Technology, 1:500), Mcl-1 (Santa Cruz Biotechnologies, 1:500), Bcl-xL (Santa Cruz Biotechnologies, 1:500), Actin (Cell Signaling, 1:10,000) and Bcl-2 (DAKO, 1:1000).

Murine models: Mice were housed under pathogen-free conditions and all experiments were in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. MCF7 and MCF7-LTED xenografts were generated as described previously (128). Tumor volume was measured every other day once tumors became palpable. Treatment of mice began when tumors reached 200 mm^3. Mice were randomized and treated with 50 μL vehicle control [0.1% Tween 80 and 0.5% methylcellulose] or ABT-263 [20mg/kg, dissolved in vehicle control] daily by gavage for 16 days or until tumor volume reached 1000 mm^3. Tissues were collected 1 hour after final treatment. MCF7 xenografts were similarly generated and treated with fulvestrant as described in (128).

Histological analyses: Tumors were fixed in 10% formalin. Formalin-fixed, paraffin-embedded (FFPE) tumor sections were assessed by TUNEL analysis using the *In Situ* TUNEL detection kit (Bio-Rad) and for Mcl-1 using methods described in (39) and anti-Mcl-1 antibody (Santa Cruz Biotechnologies).

Proximity ligation assay: Formalin fixed paraffin embedded (FFPE) sections of human breast tumor biopsies collected as part of a single-stage, single institution phase II neoadjuvant trial, in

which post-menopausal women with newly diagnosed breast cancer were treated with anastrozole (1 mg daily, Arimidex) and fulvestrant (250 mg, one per month, Faslodex). After a baseline tumor core biopsy on day 0, patients received treatment (Faslodex and Arimidex). In some cases, patients received the EGFR kinase inhibitor gefitinib (Iressa) (153, 154). Tissues were rehydrated using ethanol. Following antigen retrieval using citric acid, the Duolink (Sigma) proximity ligation assay kit was used according to manufacturer's directions using Mcl-1 (Santa Cruz Biotechnologies, 1:25) and Bim (Santa Cruz Biotechnologies, 1:25) antibodies. A Duolink DAPI mounting media was used to counter stain cells. Images were taken at 63x and total puncta/nuclei were counted per field using the ImageJ (Windows 64) Software.

siRNA-loaded nanoparticles: All polymers were synthesized and characterized as described previously (155). Two polymers, (p(DMAEMA-co-BMA), DB) and (PEG-b-p(BMA-co-DMAEMA), PDB), were used to form si-NPs. Both polymers were dissolved in a 10 mM citric acid buffer (pH 4.0). The siRNA (50 μM in diH_2O) was initially complexed at 4:1 N:P ratio (ratio of polymer amines:siRNA backbone phosphates) with the DB polymer (0.5 mg/mL polymer concentration) for 15 minutes at room temperature. Then, PDB polymer (3.33 mg/mL polymer concentration) was added to the mixture to achieve a final N:P ratio of 12:1 and incubated for 30 minutes at room temperature. 5-fold excess of 10 mM phosphate buffer (pH 8.0) was added to the samples before diluting in 5-fold excess of respective growth media. A final siRNA concentration of 100 nM was used to treat all cell lines.

Statistics: Data presentation and statistics were generated using GraphPad Prism 6 software. Significance was determined by ANOVA with Bonferroni *post hoc* tests followed by Student's unpaired two-tailed t-test for experiments with more than two conditions, or a Student's unpaired two-tailed t-test for experiments with only two conditions ($P \geq 0.05$). For tumor growth curves, significance was determined by area under the curve.

Results

Long term estrogen deprivation does not sensitize cells to Bcl-2/Bcl-xL inhibition.

To model long term treatment with AIs, three human ERα+ breast cancer cell lines, HCC1428, MCF7, and T47D, were cultured under estrogen deprivation for 3-6 months as described in previous studies (156). While parental cells exhibited growth inhibition when cultured in the absence of estrogen, LTED-selected cells grew at a similar or accelerated rate in estrogen-depleted media as compared to their growth in estrogen-replete conditions (**Figure 24A-B**). Since previous studies show that anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL promote cell survival in tamoxifen-treated breast cancers (48), we measured expression of Bcl-2 and Bcl-xL in parental and LTED-selected breast cancer cells, finding similar levels of Bcl-2 and Bcl-xL in LTED-selected HCC1428 and T47D cells as compared to their parental counterpoints cultured in estrogen-replete media (**Figure 24C**). LTED-selected MCF7 cells expressed less Bcl-xL and Bcl-2 as compared to parental counterparts. Interestingly, parental MCF7 cells upregulated Bcl-xL, but not Bcl-2, upon acute estrogen deprivation (3 days and 7 days), while parental HCC1428 cells upregulated Bcl-2 and Bcl-xL upon acute estrogen deprivation (**Figure 25A**). Despite the heterogeneous impact of acute estrogen deprivation on Bcl-2 and Bcl-xL levels, LTED does not support upregulation of Bcl-2 or Bcl-xL in ERα+ breast cancers.

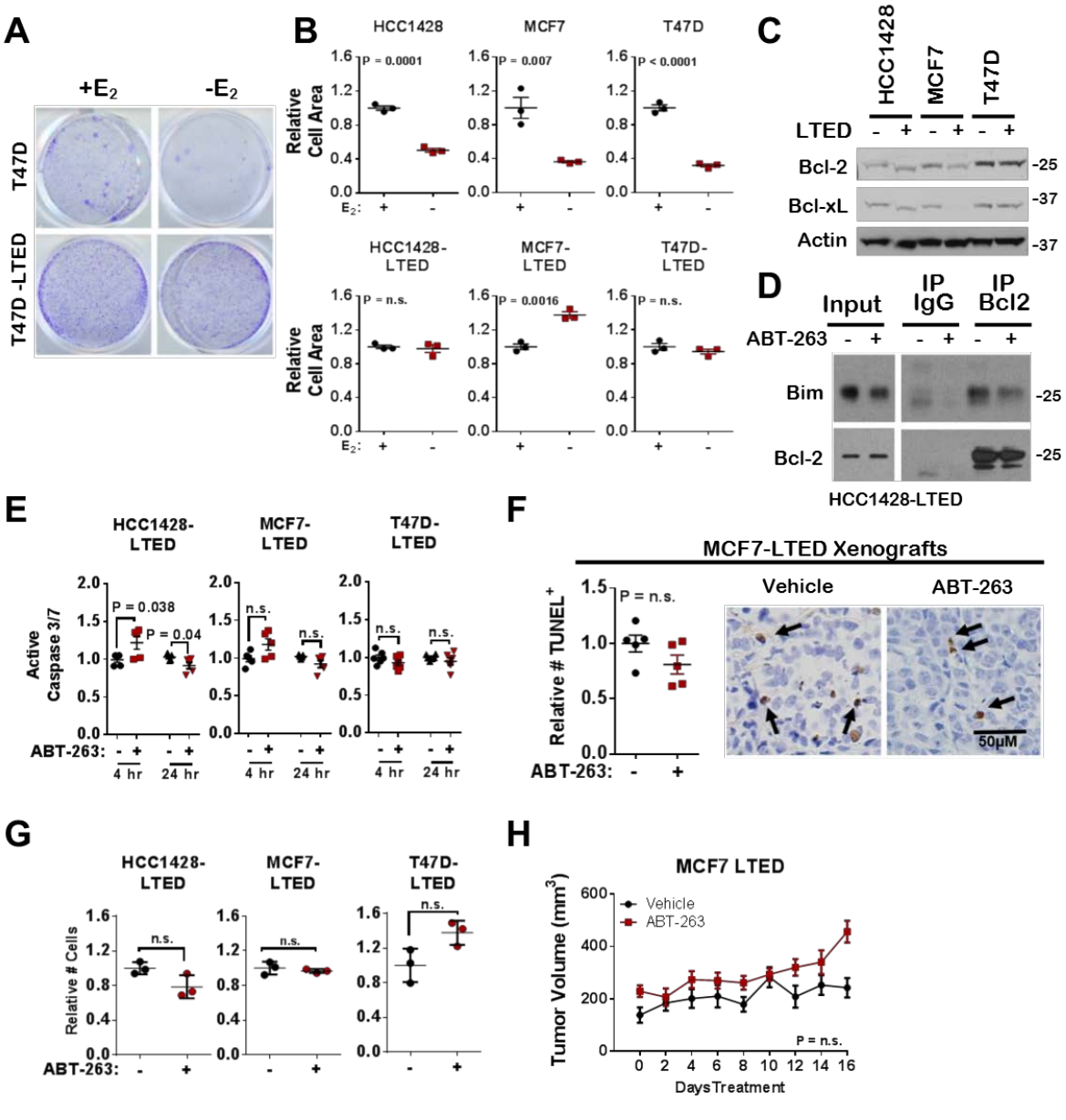
A
+E2
-E2
T47D
T47D -LTED
B
HCC1428
MCF7
T47D
Relative Cell Area
P = 0.0001
P = 0.007
P < 0.0001
HCC1428-LTED
MCF7-LTED
T47D-LTED
P = n.s.
P = 0.0016
P = n.s.
E2:
C
HCC1428
MCF7
T47D
LTED
Bcl-2
Bcl-xL
Actin
-25
-37
-37
D
Input
IP IgG
IP Bcl2
ABT-263
Bim
Bcl-2
-25
-25
HCC1428-LTED
E
HCC1428-LTED
MCF7-LTED
T47D-LTED
Active Caspase 3/7
P = 0.038
P = 0.04
n.s.
ABT-263:
4 hr
24 hr
F
MCF7-LTED Xenografts
Relative # TUNEL+
P = n.s.
Vehicle
ABT-263
50μM
ABT-263:
G
HCC1428-LTED
MCF7-LTED
T47D-LTED
Relative # Cells
n.s.
ABT-263:
H
MCF7 LTED
Tumor Volume (mm3)
Vehicle
ABT-263
P = n.s.
Days Treatment

Figure 24. LTED-selected cells remain insensitive to Bcl-2/Bcl-xL inhibition.

A-B. Crystal violet staining completed on parental and LTED-selected cells grown ± estrogen (E2) for 7 d. Representative images are shown (A). Stained cell area/well was quantitated using the ImageJ software then used to quantitate average cell area (B). N = 3, each measured in triplicate. Student's unpaired two-tailed t-test. **C**. Whole cell lysates harvested from parental or LTED-selected cells were assessed by western analysis using antibodies indicated at left, molecular weight in kiloDaltons. **D.** Western analysis of whole cell lysates (input) or Bcl-2 IPs from whole cell lysates harvested from HCC1428-LTED cells treated 2 hrs with ABT-263 (1.0 μM) was performed using antibodies shown at left, molecular weight in kiloDaltons. **E.** Caspase-3/7 activity was evaluated in cells treated 4 and 24 hrs with 1.0 μM ABT-263 (Caspase-3/7-Glo). Data points are shown relative to the average luminescence measured in DMSO-treated cells, set equal to a value of 1. Luminescence for the average of five separate experiments, N = 5, repeats of three experimental replicates each. Student's unpaired two-tailed t-test. **F.** TUNEL analysis of MCF7-LTED tumors grown in mice treated 16 d with daily ABT-263 (20 mg/kg) or vehicle control and harvested 1 hr after final treatment. Left, representative images (200X). Right, average TUNEL+ cells/field, N = 5 tumors, 6 fields per tumor; arrows denote TUNEL+ cells. **G.** Total number of cells/well were counted after treatment with DMSO or ABT-263 (1.0 μM) for 48 hrs. Data represents average of three separate experiments, N = 3, Student's unpaired two-tailed t-test. **H.** Average tumor volume of MCF7-LTED xenografts treated for 16 d with daily ABT-263 (20 mg/kg). For B, F-H error bars represent standard error.

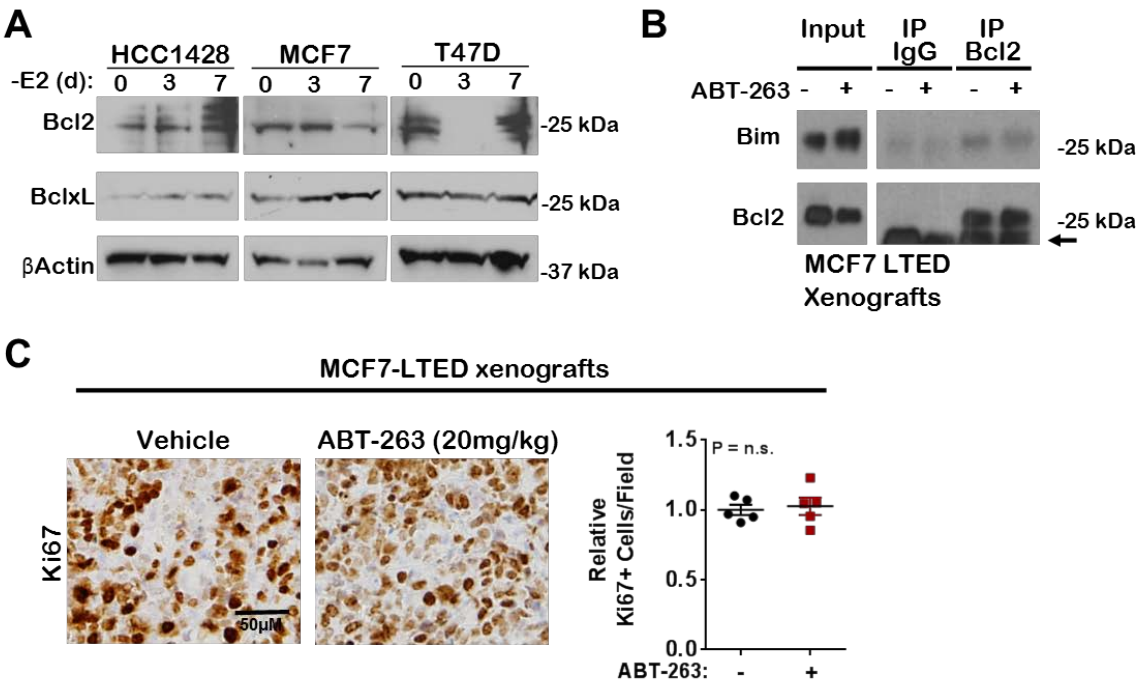

Figure 25. Bcl-2 and/or Bcl-xL expression is elevated in some, but not all, ERα+ breast cancers after short term estrogen deprivation.

A. Whole cell lysates from ERα+ breast cancer cell lines grown in estrogen containing conditions (0 d) and estrogen depleted media for 3 and 7 d. **B-C.** MCF7 LTED xenografts treated 16 d with ABT-263 (20 mg/kg) or vehicle control. Immunoprecipitation on whole tumor lysates. Arrow points to IgG light chain (~22 kDa, B). Ki67 staining to demark proliferating cells. Left = representative images, right = quantitation of average Ki67+ cells/field for 6 fields per tumor. N = 5. Student's unpaired two-tailed t-test, error bars represent standard error (C).

Despite no obvious Bcl-2 and/or Bcl-xL protein upregulation in LTED-selected cells, it is possible that the anti-apoptotic activity of Bcl-2 and/or Bcl-xL (i.e. sequestration of pro-apoptotic Bim or Bax) supports cell survival and therapeutic resistance in this setting. To test this, activity of Bcl-2 and Bcl-xL was blocked in LTED-selected cells using the BH3-mimetic ABT-263 (1.0 μM). Bcl-2 immuno-precipitates assessed by western analysis for Bim confirmed that ABT-263 reduced Bcl-2/Bim interactions (**Figure 24D**). Previous reports show that ABT-263 treatment of HCC1428, MCF7, and T47D cells induced caspase-3/7 activity after 4 hours (125), similar to observations in other cell lines (157). In contrast, ABT-263 did not increase caspase-3/7 activity in MCF7-LTED and T47D-LTED cells at 4 or 24 hours (**Figure 24E**), while HCC1428-LTED cells responded to ABT-263 with a subtle increase in caspase-3/7 activity at 4 hours, returning to baseline at 24 hours. These results were confirmed *in vivo* using MCF7-LTED xenografts treated daily with ABT-263 (20 mg/kg), which did not increase tumor cell death as assessed by terminal dUTP nick end labeling (TUNEL) analysis (**Figure 24F - arrows**). Consistent with these results, growth (total cell number) of LTED-selected cells was not affected by 48 hours treatment with ABT-263 (**Figure 24G**). Similarly, growth of MCF7-LTED xenografts (tumor volume) was not affected by daily treatment with ABT-263 (**Figure 24H**), even though Bim interactions with Bcl-2 were disrupted by ABT-263 (**Figure 25B**). Tumor cell proliferation was unaffected by ABT-263 (**Figure 25C**). These data suggest that sustained estrogen deprivation, such as that achieved by AIs, may not prime ERα+ breast cancer cells for Bcl-2/Bcl-xL targeting.

Mcl-1 expression increases under long term estrogen deprivation.

Previous studies demonstrated that Mcl-1, a related anti-apoptotic Bcl-2 protein, is upregulated upon Bcl-2/Bcl-xL inhibition, resulting in resistance to ABT-263 (56, 85, 95, 158-160). In fact, Mcl-1 expression levels correlate inversely with sensitivity to ABT-263 in ERα+ breast cancer cells (125). However, the impact of Mcl-1 sensitivity to LTED is currently unknown. We measured Mcl-1 expression levels in LTED-selected ERα+ breast cancer cells by western

analysis, finding increased Mcl-1 in all LTED cells as compared to parental counterparts (**Figure 26A**). Mcl-1 upregulation *in vivo* upon sustained estrogen deprivation was confirmed by immunohistochemical staining in MCF7 xenografts grown in ovariectomized hosts (thus, estrogen-deprived for 42 days), revealing increased Mcl-1 staining intensity, and increased number of Mcl-1-positive cells, as compared to MCF7 xenografts grown in ovariectomized hosts supplemented with 42-day slow release estrogen pellets (**Figure 26B**). We used Proximity Ligation Assay [PLA, (161)], a technique that measures protein-protein interactions *in situ*, to quantitate Mcl-1 activity (Mcl-1/Bim interaction) in MCF7 xenografts. Fluorescent puncta, indicating molecular interaction between Mcl-1 and Bim, were significantly increased in tumors grown in ovariectomized mice without estrogen supplementation (**Figure 26C – red puncta**), suggesting that Mcl-1 expression and activity increase upon sustained estrogen deprivation.

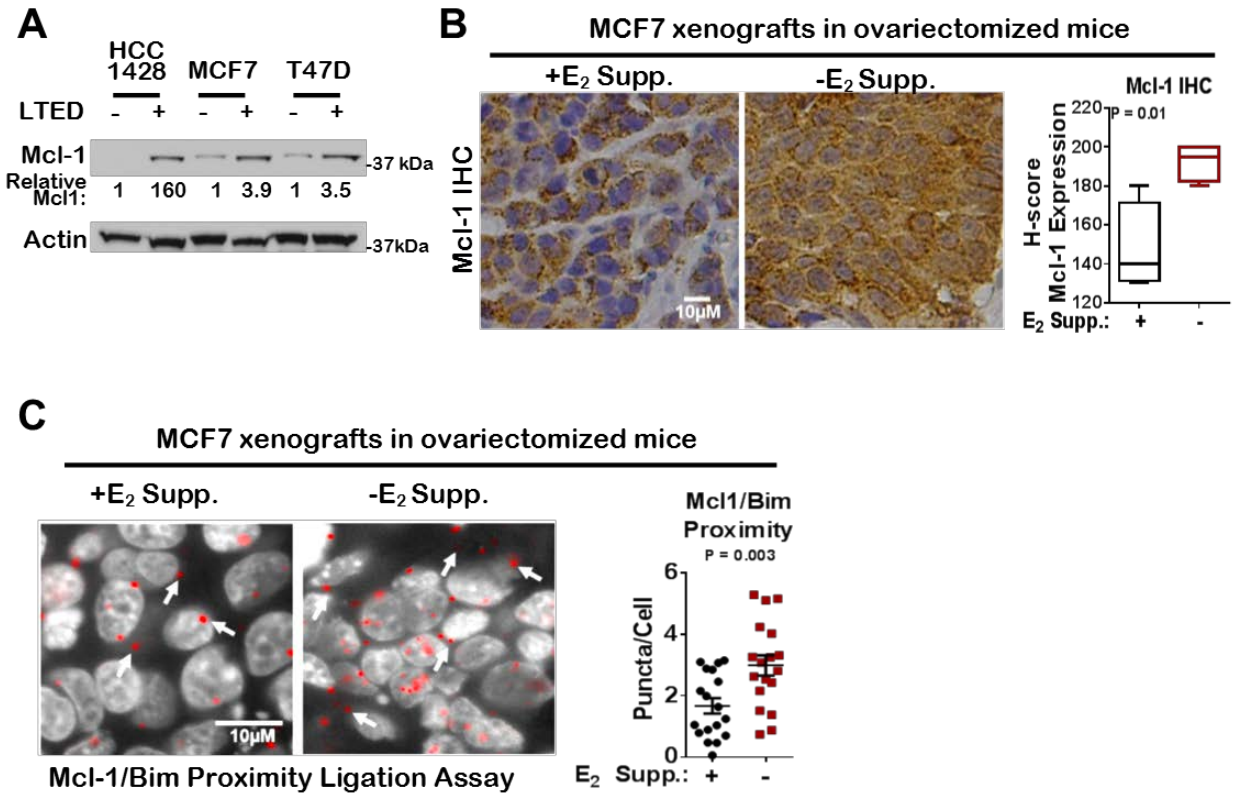

Figure 26. Mcl-1 upregulation in response to LTED.

A. Whole cell lysates were assessed by western analysis using antibodies shown at right. Image J was used for densitometry analysis to assess Mcl-1 levels (corrected for actin levels) shown relative to levels seen in the respective parental cell line set to a value of 1. **B-C**. MCF7 xenografts were grown for 42 days in ovariectomized mice with or without estrogen supplementation (E_2). Left, Mcl-1 immunohistochemistry with representative images at 400x. Right, quantitation of Mcl-1 IHC performed by a pathologist is represented as an H-score (a semi-quantitative scoring system that factors staining intensity and percentage of cells positive for staining). Student's unpaired two tailed t-test, error bars represent min to max (B). C depicts Mcl-1/Bim PLA. Left, representative images at 630x: grey, DAPI; red, Mcl-1/Bim proximity (denoted by arrows). Right, quantitation of Mcl-1/Bim proximity as puncta/cell for 6 fields per tumor. Student's unpaired two tailed t-test, error bars represent standard error.

Fulvestrant primes ERα+ breast cancers for Mcl-1 targeting.

To expand our findings to another mechanism of endocrine inhibition, we examined Bcl-2, Bcl-xL, and Mcl-1 expression changes in response to fulvestrant, a SERD that blocks estrogen binding to ERα and induces ERα degradation. Parental HCC1428, MCF7, and T47D cells treated 24 hours with fulvestrant (1.0 μM) showed decreased *BCL2* transcripts (**Figure 27A**), while *BCL2L1* (Bcl-xL) increased in HCC1428 and T47D cells. *MCL1* transcripts were increased in all three fulvestrant-treated cell lines. At the protein level, Mcl-1 expression in parental MCF7 and T47D cells increased upon fulvestrant treatment, while Bcl-2, detected at very low levels in MCF7 and T47D cells, diminished further in response to fulvestrant. Bcl-xL protein expression similarly decreased in response to fulvestrant (**Figure 27B**). These findings were verified *in vivo* using MCF7 and T47D xenografts treated with fulvestrant for 7 days, revealing fulvestrant-mediated upregulation of Mcl-1 in MCF7 and T47D tumors, while Bcl-2 levels increased in MCF7 (but not T47D) tumors and Bcl-xL showed relatively little change (**Figure 27C**). We confirmed fulvestrant-mediated down-regulation of ERα, suggesting on target inhibition by fulvestrant, using immunohistochemistry (**Figure 28**). Further, PLA for Mcl-1/Bim interactions demonstrated that fulvestrant increased Mcl-1 activity (**Figure 27D – red puncta**).

Next, we used PLA to measure Mcl-1/Bim interactions in matched pairs of clinical breast tumor specimens from patients with ERα+ breast cancers. Breast tumor biopsies were collected from post-menopausal women with a new diagnosis of ERα+ breast cancer as part of a single-stage, single institution phase II neoadjuvant trial (153, 154). Tumors were biopsied 24 hours prior to any treatment (day 0). Patients were treated with a single fulvestrant dose (Faslodex, 250 mg) followed by daily treatment with the AI anastrozole (Arimidex, 1.0 mg daily). In some cases, patients also received daily treatment with the EGFR inhibitor gefitinib. Tumor biopsies were collected again on treatment day 21.

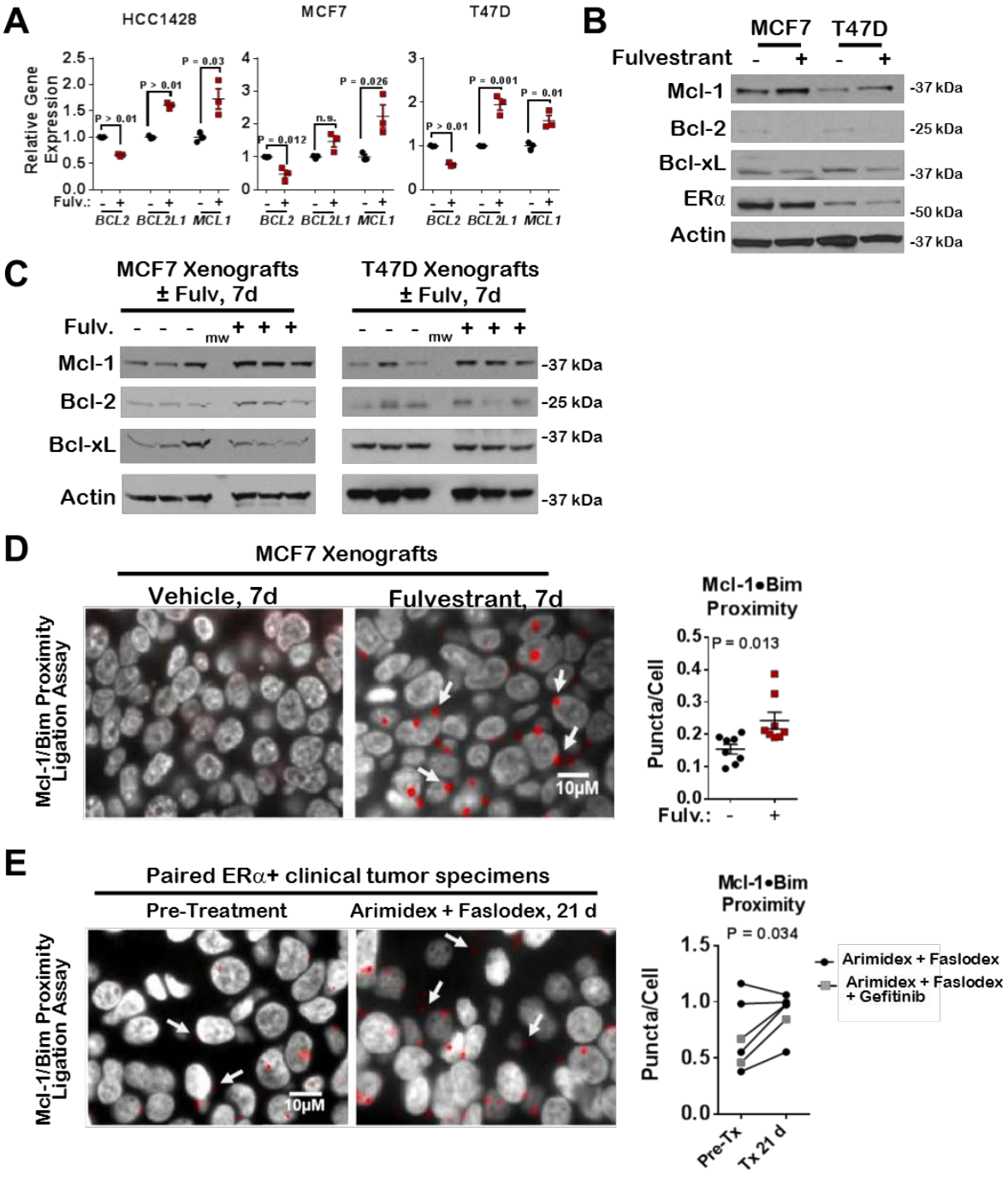
A
HCC1428
MCF7
T47D
Relative Gene Expression
P > 0.01
P > 0.01
P = 0.03
P = 0.012
n.s.
P = 0.026
P > 0.01
P = 0.001
P = 0.01
Fulv.:
BCL2
BCL2L1
MCL1
B
MCF7
T47D
Fulvestrant
Mcl-1
Bcl-2
Bcl-xL
ERα
Actin
-37 kDa
-25 kDa
-37 kDa
-50 kDa
-37 kDa
C
MCF7 Xenografts
± Fulv, 7d
T47D Xenografts
± Fulv, 7d
Fulv.
mw
Mcl-1
Bcl-2
Bcl-xL
Actin
-37 kDa
-25 kDa
-37 kDa
-37 kDa
D
MCF7 Xenografts
Vehicle, 7d
Fulvestrant, 7d
Mcl-1/Bim Proximity Ligation Assay
10μM
Mcl-1•Bim Proximity
P = 0.013
Puncta/Cell
Fulv.:
E
Paired ERα+ clinical tumor specimens
Pre-Treatment
Arimidex + Faslodex, 21 d
Mcl-1/Bim Proximity Ligation Assay
10μM
Mcl-1•Bim Proximity
P = 0.034
Puncta/Cell
Arimidex + Faslodex
Arimidex + Faslodex + Gefitinib
Pre-Tx
Tx 21 d

Figure 27. Selective estrogen receptor downregulators increase Mcl-1 expression and activity.

A-B. RNA (A) or whole cell lysates (B) were harvested from cells treated with 1.0 μM fulvestrant for 24 or 6 hrs, respectively. mRNA expression was analyzed by RT-qPCR and protein expression was analyzed using antibodies denoted on the left. **C-D.** MCF7 and T47D xenografts grown in mice with intact ovaries were treated with fulvestrant once by intraperitoneal injection and harvested after 7 d. Whole tumor lysates were assessed by western analysis using antibodies shown at left (C). D depicts PLA completed on FFPE tissues. Left, representative images (630x): grey, DAPI; red, Mcl-1/Bim proximity (arrows denote Mcl-1/Bim interactions). Right, quantitation represents the average number of puncta/cell for 6 images per tumor, N = 8. Student's unpaired two-tailed t-test, error bars represent standard error. **E.** PLA to detect Mcl-1/Bim interactions was completed on de-identified clinical breast tumor specimens collect pre-treatment (Pre-Tx) and 21 d post treatment (Tx 21 d) with Fulvestrant/Anastrozole ± gefitinib. Left, representative images (630x): grey, DAPI; red, Mcl-1/Bim proximity (arrows denote Mcl-1/Bim interactions). Right, quantitation represents the number of puncta/cell for each specimen.

Student's paired one-tailed t-test.

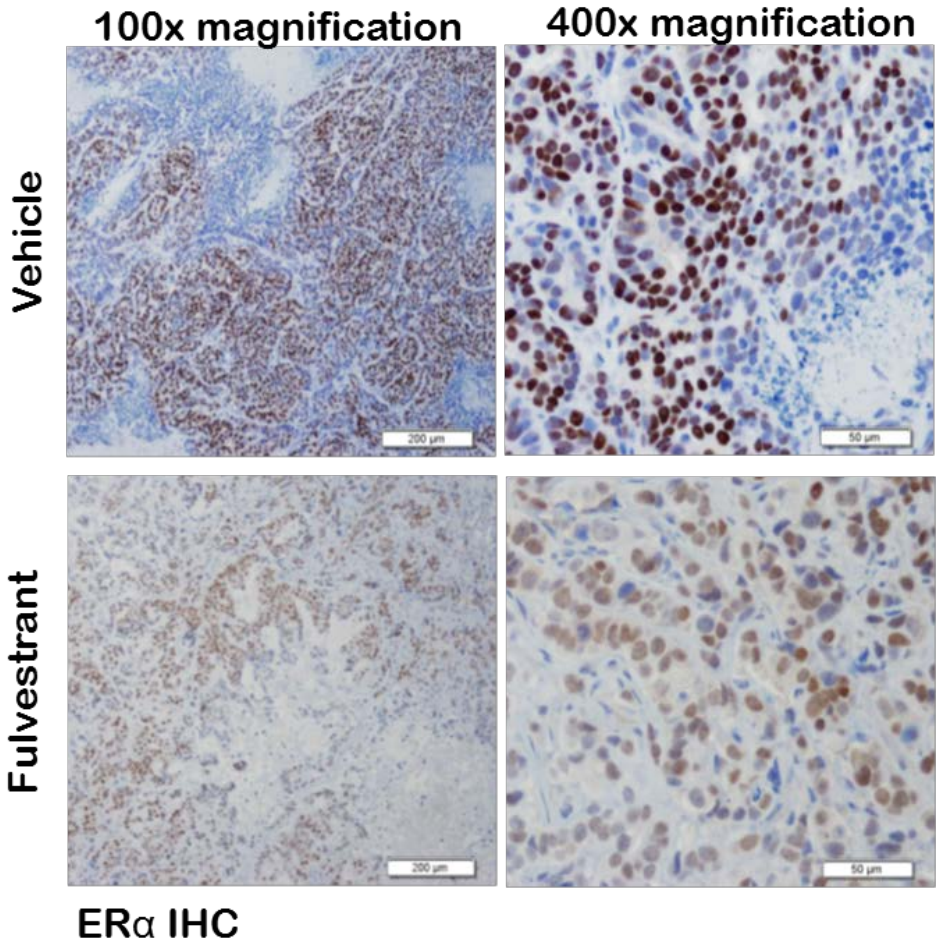

Figure 28. Fulvestrant decreases ERα expression *in vivo*.

ERα immunohistochemistry performed on sections of MCF7 xenografts treated for 7 d with vehicle or fulvestrant (once weekly by i.p. injection). Representative images taken at 100x and 400x are shown.

These studies revealed that Bim interactions with Mcl-1 were increased in post-treatment specimens (day 21) as compared to those collected prior to treatment (day 0, **Figure 27E – red puncta**), consistent with the results shown here in animal models. Although inclusion of the EGFR kinase inhibitor gefitinib is a caveat of the clinical data set that is inconsistent with the endocrine treatment modalities used herein, these results support the idea that increased Mcl-1 expression and activity may be clinically meaningful.

Genetic targeting of* MCL1 *increases apoptosis in LTED-selected breast cancer cells.

We delivered siRNA sequences directed against Mcl-1 to LTED-selected ERα+ breast cancer cells as an experimental approach at targeted and highly selective Mcl-1 inhibition. As a control, we used luciferase (LUC)-directed siRNA sequences. Delivery of siRNA sequences was achieved using polymeric nanoparticles termed si-NPs (MCL1si-NP and LUCsi-NP, respectively). These recently-developed nanocarriers enable effective siRNA delivery/activity in cell culture models due to high cell uptake and efficient endosomal escape capability (155). Also, unlike other off-the-shelf agents, these NPs can be directly translated into future *in vivo* studies due to sufficient serum stability that enables i.v. injection and biodistribution/bioactivity within breast tumors. Cells were assessed for Mcl-1 knockdown by western analysis 48 hours after treatment with si-NPs, demonstrating that MCL1si-NPs decreased Mcl-1 protein expression in all three LTED-selected cells (**Figure 29A**). Importantly, Bcl-2 and Bcl-xL levels remained unchanged despite Mcl-1 knockdown in MCL1si-NP treated cells, confirming sequence specificity to the target, and suggesting that acute compensatory upregulation of Bcl-2 and Bcl-xL is not induced by Mcl-1 gene targeting.

We tested the impact of MCL1si-NP on caspase-3/7 activity in LTED-selected ERα+ breast cancer cells cultured in estrogen-deprived media, revealing increased caspase-3/7 activity in all three cell lines as compared to LUCsi-NP controls (**Figure 29B**), suggesting that LTED induces Mcl-1 expression and activity, which may prime cells for Mcl-1 inhibition.

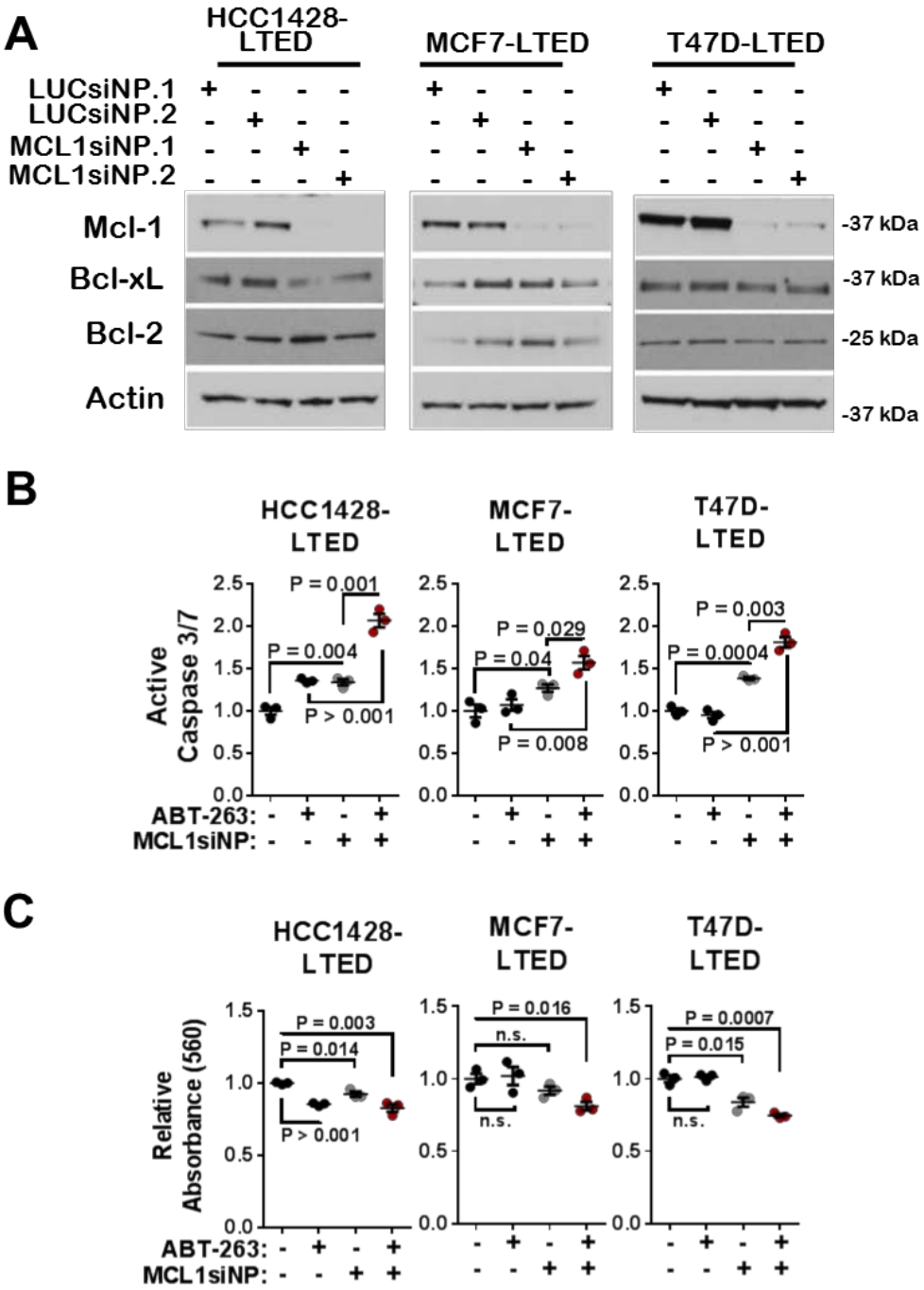
A
HCC1428-LTED
MCF7-LTED
T47D-LTED
LUCsiNP.1
LUCsiNP.2
MCL1siNP.1
MCL1siNP.2
Mcl-1
Bcl-xL
Bcl-2
Actin
-37 kDa
-37 kDa
-25 kDa
-37 kDa
B
HCC1428-LTED
MCF7-LTED
T47D-LTED
Active Caspase 3/7
P = 0.001
P = 0.004
P > 0.001
P = 0.029
P = 0.04
P = 0.008
P = 0.003
P = 0.0004
P > 0.001
ABT-263:
MCL1siNP:
C
HCC1428-LTED
MCF7-LTED
T47D-LTED
Relative Absorbance (560)
P = 0.003
P = 0.014
P > 0.001
P = 0.016
n.s.
n.s.
P = 0.0007
P = 0.015
n.s.
ABT-263:
MCL1siNP:

Figure 29. Mcl-1 inhibition restores sensitivity of LTED breast cancers to ABT-263.

A. Whole cell lysates from LTED-selected cells were assessed by western analysis for antibodies indicated at left after treatment with si-NPs loaded with 100 nM siControl or siMCL1 for 48 hrs. **B-C.** LTED-selected cells were treated for 48 hrs with 1000 nM si-NP. 1.0 μM ABT-263 was added for the final 4 hrs. Caspase-3/7 activity was measured (Caspase-3/7-Glo, B) and used to calculate average luminescence, relative to values obtained for LUCsi-NP-treated cells with DMSO treatment, set to a value of 1. N = 3, each assessed in duplicate. Student's unpaired two-tailed t-test, error bars represents standard error. Total protein was quantitated using an SRB assay (C), relative to values obtained for LUCsi-NP-treated cells with DMSO treatment, set to a value of 1. N = 3, each assessed in duplicate. Student's unpaired two-tailed t-test, error bars represents standard error.

Caspase-3/7 activity was increased further in LTED-selected cells when MCL1si-NPs were used in combination with ABT-263. MCL1si-NP also decreased growth of LTED-selected cells as compared to LUCsi-NP (**Figure 29C**), and increased growth inhibition in response to ABT-263.

Genetic targeting of* MCL1 *increases apoptosis in fulvestrant-treated breast cancer cells.

We next examined the impact of selective Mcl-1 gene targeting on response of parental ERα+ breast cancer cells to fulvestrant, first verifying Mcl-1 knockdown in parental HCC1428, MCF7, and T47D cells treated with MCL1si-NPs (**Figure 30A**). RNA collected 48 hours after treatment with MCL1si-NP harbored 70%-90% fewer *MCL1* transcripts as compared to cells treated with LUCsi-NPs. These results were confirmed by western analysis of lysates collected 48 hours after treatment with MCL1si-NPs (**Figure 30B**). We next measured caspase-3/7 activity in ERα+ breast cancer cells 48 hours after treatment with MCL1si-NP. Unlike what was seen in LTED-selected cells, which exhibited increased cell death upon knockdown of Mcl-1, two of three parental cell lines did not increase caspase-3/7 activity in response to MCL1si-NP (**Figure 30C**). Consistent with previous reports, fulvestrant as a single agent did not induce caspase-3/7 activity in any of the parental ERα+ breast cancer cell lines. However, the MCL1si-NPs combined with fulvestrant robustly increased caspase-3/7 activity in each of the parental ERα+ cell lines. Similarly, MCL1si-NPs alone decreased growth of parental MCF7 cells, but not HCC1428 or T47D cells (**Figure 30D**). While fulvestrant decreased growth of all three ERα+ cell lines, the combination of fulvestrant + MCL1si-NPs decreased growth to a greater extent than either agent alone. These findings support the feasibility of therapeutic Mcl-1 gene ablation in combination with anti-estrogens (**Figure 30E**).

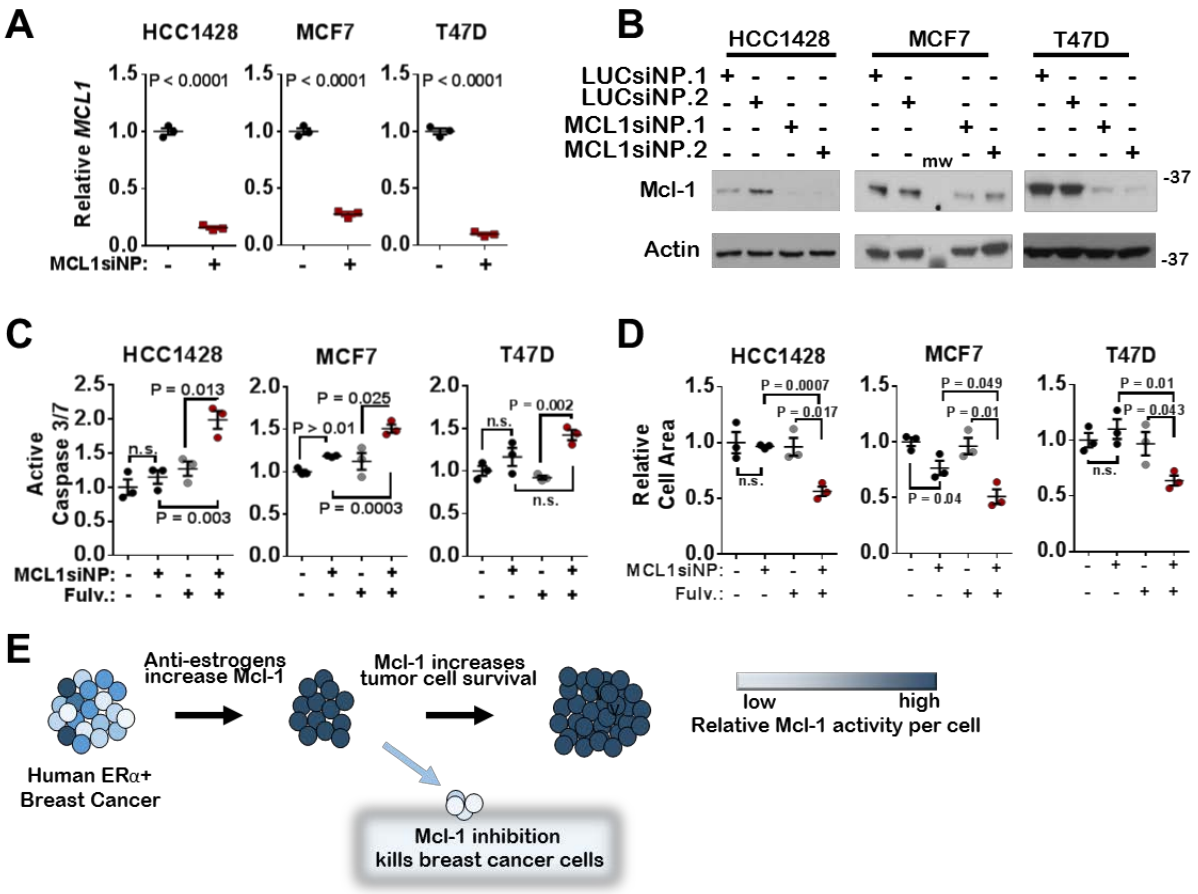

Figure 30. Mcl-1 inhibition sensitizes ERα+ breast cancers to fulvestrant.

A-C. Cells were treated with si-NPs loaded with 100 nM siControl or siMcl1 for 24 hrs, and cultured for an additional 24 hrs in growth media (A-B) or in growth media with and without 1.0 μM fulvestrant (C). *MCL1* expression was assessed in total RNA by RT-qPCR, N = 3, assessed in triplicate (A). Whole cell lysates were assessed by western analysis using antibodies shown at left, molecular weight kiloDaltons (B). Caspase-3/7 activity was measured (Caspase-3/7-Glo) in duplicate, N = 3 (C). **D.** Crystal violet staining of cells treated with si-NP (100 nM siControl or siMcl1) for 24 hrs then cultured 7 d in growth media supplemented with or without 1.0 μM fulvestrant. Quantitation represents average area stained with crystal violet per well, N = 3-4, each conducted in triplicate. Student's unpaired two-tailed t-test. For A, C, and D error bars represent standard error. **E.** Model of Mcl-1 upregulation and tumor cell survival promoted by anti-estrogen therapeutics.

Discussion

Preclinical studies suggest that anti-apoptotic Bcl-2 family proteins promote resistance to standard of care breast cancer therapies, including endocrine inhibitors (47-49, 149), HER2 inhibitors (63), and chemotherapies (39, 42, 162). An impactful study using preclinical models of ERα+ breast cancers showed extreme sensitive to the combination of tamoxifen and the Bcl-2/Bcl-xL inhibitor ABT-737 or the Bcl-2 specific inhibitor ABT-199, suggesting that Bcl-2 drives resistance of ERα+ breast cancers to tamoxifen (48). Based on these and other studies, clinical testing of ABT-199 in combination with tamoxifen has begun (53). However, in a separate study Bcl-2 expression was decreased in MCF7 xenografts upon tamoxifen treatment and decreased further upon acquisition of tamoxifen resistance (152), consistent with the clinical finding that Bcl-2 expression often predicts a favorable prognosis (75), and with the idea that *BCL2* is a transcriptional target of ERα. These conflicting reports, in addition to a lack of studies observing the role of other anti-apoptotic factors in ERα+ breast cancer resistance, led us to further explore the role of Bcl-2, as well as Bcl-xL and Mcl-1, in response of ERα+ breast cancers to endocrine inhibition.

We found that LTED cells were not sensitive to a physiologically relevant dose of ABT-263, a Bcl-2/Bcl-xL inhibitor that functions similarly to ABT-737. ABT-263 induced tumor cell killing transiently in one of the three LTED-selected ERα+ breast cancer cell lines tested, but this response was not sustained, and ABT-263 did not affect tumor cell growth in culture or *in vivo* (**Figure 24E-H**). Interestingly, short term estrogen deprivation (0-48 hours) increased Bcl-2 and/or Bcl-xL levels in some, but not all ERα+ cell lines (**Figure 25A**), although sustained estrogen deprivation caused Bcl-2 and Bcl-xL levels to decrease substantially (**Figure 24C**). It is possible that short term treatment (0 – 48 hours) with tamoxifen or AIs increases Bcl-2 and/or Bcl-xL levels, priming ERα+ breast tumor cells for Bcl-2/Bcl-xL inhibition, as was seen in ERα+ patient derived xenograft models (48). However, upon acquisition of resistance, Bcl-2/Bcl-xL levels become

depleted, and survival then depends on different anti-apoptotic factors. These ideas will require further exploration in future studies.

Transient sensitivity to ABT-263, as seen in HCC1428-LTED cells, was previously explored in ERα+ breast cancer cell lines grown in estrogen replete conditions (57). These studies showed that Mcl-1, a Bcl-2-related anti-apoptotic factor, is a key driver of ABT-263 resistance, supporting the concept of compensatory and overlapping functions across anti-apoptotic Bcl-2 family members. Therefore, we were motivated to explore the role of Mcl-1 in our LTED models that were not only resistant to estrogen deprivation, but also were unresponsive to ABT-263. We found abundant Mcl-1 expression (but not Bcl-2 or Bcl-xL) upon LTED in cell culture and *in vivo* (**Figure 26A-C**), suggesting that Mcl-1, rather than Bcl-2 or Bcl-xL, may be the primary survival factor in AI-resistant ERα+ breast cancers. We similarly found that Mcl-1 was a key survival factor in fulvestrant-treated ERα+ breast cancer cells (**Figure 27**), supporting previous reports that indirectly identified Mcl-1 as a survival factor in fulvestrant-resistant cell lines (47). Interestingly, we found increased Mcl-1/Bim interactions in fulvestrant-treated MCF7 and T47D xenografts (**Figure 27C-D**), and in clinical ERα+ tumor specimens treated with Faslodex/Arimidex (**Figure 27E**). Thus, multiple endocrine inhibitors increase Mcl-1 expression and activity in cell culture, *in vivo*, and in clinical samples, suggesting that endocrine inhibitors may prime ERα+ breast cancers for Mcl-1 inhibition.

Since high Mcl-1 activity promotes ABT-263 resistance in breast cancers (56, 60, 125), these findings suggest that LTED-mediated Mcl-1 induction may indirectly increase resistance to Bcl-2/Bcl-xL inhibition. Thus, it became imperative to inhibit Mcl-1 in combination with Bcl-2/Bcl-xL in LTED cell lines. Mcl-1 inhibition was achieved using nanoparticles loaded with Mcl-1 siRNA (MCL1si-NP). The combination of ABT-263 with MCL1si-NPs increased tumor cell killing and diminished tumor cell growth in each LTED cell line tested (**Figure 29B-D**). These findings support the idea that inhibition of multiple anti-apoptotic Bcl-2 family proteins may be required to maximize

tumor cell killing. However, a pan-Bcl-2 family inhibitor would have to overcome the challenges of clinical toxicities. For instance, ABT-263 was previously suspended from clinical trials due to extreme thrombocytopenia (64, 65). Further, it is possible that Mcl-1 targeting alone will be toxic, because Mcl-1 is essential to the survival of normal tissues, such the heart (143), brain (163, 164) and the immune compartment (144-146). For this reason, we explored Mcl-1 targeting using siRNA, which offers a genetically selective, and possibly less toxic, method to deplete Mcl-1. The nanoparticles into which siRNA sequences were loaded have been vetted for toxicity, and were optimized for superior systemic *in vivo* delivery over what can be achieved using commercially-available reagents. Further, si-NPs penetrate less into healthy tissues as compared to small molecule inhibitors, thus increasing tumor-specific delivery via the enhanced permeability and retention effect (61, 95, 165), a term describing the leaky vasculature of tumors caused by rapid and irregular tumor angiogenesis. Based on their relative size, si-NPs passively enter and accumulate in tumors (166), limiting extra-tumoral side effects. Due to these favorable pharmacokinetics, many nanoparticle-based drugs, including siRNA-loaded particles, are in clinical development [as reviewed in (167)].

Although si-NPs targeting Mcl-1 remain to be tested *in vivo*, Mcl-1 specific inhibition in cell culture based models dramatically reduced Mcl-1 expression in both LTED and parental breast cancer cells (**Figure 29A,30B**), increasing tumor cell killing in cells that have developed resistance to current standard of care therapies. Further, we tested the utility of MCL1si-NPs in combination with fulvestrant, because Mcl-1 expression was induced upon short term fulvestrant treatment in culture and *in vivo* (**Figure 27B-D**). Tumor cell death was induced upon combined treatment with fulvestrant and MCL1si-NPs (**Figure 30C**). Overall, these studies demonstrate that ERα+ breast cancers treated with standard of care endocrine inhibitors may induce Mcl-1 expression and/or activity, which could represent a potential dynamic biomarker of sensitivity to

Mcl-1 inhibition. Further, inhibition of Mcl-1 in tumors that are primed by endocrine inhibition may be viable strategy to increase tumor cell killing and breast cancer patient survival.

CHAPTER V

Conclusions and Future Directions

Conclusions

Anti-apoptotic Bcl-2 family proteins (Bcl2-A1, Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) are inhibitors of programmed cell death. In the breast, where apoptosis is essential to normal tissue development, aberrant expression of anti-apoptotic Bcl-2 family proteins disrupts almost every stage of normal mammary gland development. Not surprisingly, evasion of cell death, through overexpression of anti-apoptotic Bcl-2 family proteins, is a process often utilized by breast tumors to support tumor initiation, progression, and therapeutic resistance by favoring tumor cell survival over tumor cell death. For instance, overexpression of anti-apoptotic Bcl-2 is required for oncogenic drivers like Cyclin D1 or ErbB2 to promote lumen filling in cell culture models of breast epithelial cell transformation (37), while overexpression of BCL-2 accelerates tumor formation and tumor progression in multiple mammary tumor models (28, 29, 31). Additionally, anti-apoptotic Bcl-2 family proteins promote resistance to various standard of care breast cancer therapies, including chemotherapies, HER2 inhibitors, and anti-estrogens. Thus, targeting of anti-apoptotic Bcl-2 family proteins has been pursued in preclinical breast cancer models, and in some cases is entering clinical trials, as with the combination of the Bcl-2 specific inhibitor ABT-199 and tamoxifen in ERα+ breast cancers (53).

Although this combination is promising, one anti-apoptotic Bcl-2 family protein, Mcl-1, was understudied in breast cancers at the initiation of this thesis work. Because Mcl-1 is expressed highly in all breast cancer subtypes, we were interested in determining whether Mcl-1 targeting was effective in breast cancers, focusing on ERα+ breast cancers where Mcl-1 expression was the highest (**Figure 10**). Further, Mcl-1 is a primary resistance factor to Bcl-2/Bcl-xL targeting in

other cancers, suggesting that Bcl-2/Bcl-xL inhibitors may have limited clinical benefit in ERα+ breast cancers due to Mcl-1 compensation. Hence, the studies presented herein focused on Mcl-1 targeting in ERα+ breast cancers as a single agent and in combination with Bcl-2/Bcl-xL inhibitors, and were the first to demonstrate that Mcl-1 is a dominant tumor cell survival factor in ERα+ breast cancers. Additionally, Mcl-1 was the most effective anti-apoptotic Bcl-2 family target in endocrine resistant breast cancers, suggesting that Mcl-1 is a primary resistance factor to targeted therapies in ERα+ breast cancers.

Mcl-1 targeting is superior to Bcl-2/Bcl-xL targeting in ERα+ breast cancers.

First, the role of Mcl-1 as a tumor cell survival factor was explored in ERα+ breast cancers. Although previous studies show that human ERα+ breast cancer specimens maintain high Mcl-1 expression when compared to other breast cancer subtypes, no study to date explored direct Mcl-1 targeting in ERα+ breast cancers. Further, this study was the first to compare Mcl-1 targeting to blockade of other anti-apoptotic Bcl-2 family proteins in ERα+ breast cancers.

Clinical breast tumor datasets were first observed for *BCL2*, *BCL2L1* (Bcl-xL), and *MCL1* alterations at the genetic level and for expression at the transcript level. *MCL1* genetic amplifications were more frequent in each breast cancer subtype when compared to *BCL2* and *BCL2L1* amplifications, while *MCL1* transcripts were expressed at higher levels than *BCL2* and *BCL2L1* transcripts in each breast cancer subtype (**Figure 10**). At the protein level, Mcl-1 expression was elevated in breast tumor specimens when compared to normal tissues, but Mcl-1 expression was highest in ERα+ breast cancers. These data supported previous findings (58), and justified Mcl-1 targeting over Bcl-2/Bcl-xL inhibition in ERα+ breast cancers.

In further support of this idea, genetic Mcl-1 depletion increased tumor cell killing and decreased tumor cell growth in human ERα+ breast cancer cell lines (**Figure 11**). In contrast, targeting of Bcl-2/Bcl-xL with the BH3-mimetic ABT-263 did not decrease tumor cell growth in

culture or *in vivo* (**Figure 12, Figure 22**), because ABT-263 failed to induce sustained tumor cell killing. These findings suggest that ERα+ breast tumor cells rapidly acquire resistance to ABT-263, demonstrating that Bcl-2/Bcl-xL inhibition may not be an effective strategy to increase tumor cell killing clinically. Indeed, Mcl-1 may be the preferred anti-apoptotic target in ERα+ breast cancers.

Mcl-1 is a primary resistance factor to ABT-263.

To better understand why ABT-263 did not sustain tumor cell killing in ERα+ breast cancers, we observed the expression and activity of Mcl-1, a known resistance factor to Bcl-2/Bcl-xL blockade in other cancers (55, 82, 85, 89, 121). Mcl-1 expression and activity were increased upon ABT-263 treatment (**Figure 14**). In fact, *MCL1* transcript levels correlated positively with ABT-263 IC50 in human ERα+ breast cancer cell lines, demonstrating that high Mcl-1 expression can predict innate resistance to ABT-263 (**Figure 15**). Further, genetic ablation of Mcl-1 restored sensitivity to ABT-263, suggesting that Mcl-1 upregulation is a main resistance factor to Bcl-2/Bcl-xL blockade in ERα+ breast cancers (**Figure 18**). These findings are supported by previous works in chronic lymphocytic leukemia which demonstrate that clinical benefit from ABT-263 is only seen in tumors that maintain low Mcl-1 levels (168). Thus, Bcl-2/Bcl-xL/Mcl-1 inhibition, if clinically achievable, may induce greater tumor cell killing than inhibition of each factor alone.

mTORC1 inhibitors deplete ABT-263-mediated Mcl-1 upregulation.

Although the findings presented herein argue for combined Bcl-2/Bcl-xL/Mcl-1 targeting in breast cancers, at the initiation of these studies a Mcl-1 specific BH3-mimetic did not exist. Thus, to test combined Bcl-2/Bcl-xL/Mcl-1 inhibition we decided to identify an indirect Mcl-1 inhibitor, focusing our efforts on inhibitors of oncogenic signaling pathways that have achieved clinical approval. Not only was this approach feasible, because Mcl-1 is regulated by numerous oncogenic drivers that are targetable [as reviewed in (97)], but this approach may provide a rapidly

translatable means to target Mcl-1 clinically. Further, since Mcl-1 expression and activity increased upon treatment with ABT-263, human ERα+ breast cancer cell lines were studied after Bcl-2/Bcl-xL blockade to determine which mechanism supports elevated Mcl-1 expression under these conditions, reasoning that this mechanism must be a dominant regulator of Mcl-1 expression in ERα+ breast cancers. *MCL1* transcription and Mcl-1 protein stability remained the same or decreased after treatment with ABT-263 (**Figure 16**). However, blockade of cap-dependent translation blunted Mcl-1 levels induced by ABT-263, suggesting that cap-dependent translation, not transcription nor protein stability, governs Mcl-1 expression in response to stress upon the intrinsic apoptotic pathway.

Interestingly, mTORC1 is a dominant regulator of cap-dependent translation, and findings in other malignancies show that ABT-263 is more effective in combination with various PI3K/mTOR inhibitors (56, 81-86). These observations suggest that mTOR inhibition may limit cap-dependent translation of *MCL1* following ABT-263 treatment. To test this idea, we used the mTORC1 inhibitor RAD001/everolimus, having already achieved clinical approval in ERα+ breast cancers. RAD001 depleted Mcl-1 levels as a single agent, and induced sustained tumor cell killing in cell culture and *in vivo* (**Figure 19**). Importantly, mTORC1 inhibition blunted ABT-263-mediated Mcl-1 upregulation (**Figures 21, 22**), further supporting the idea that cap-dependent translation is an important mechanism supporting Mcl-1 expression during conditions of cellular stress. Hence, RAD001 may be a rapidly translatable means to target Mcl-1 in the clinic. Although the findings presented herein support combined use of ABT-263 + RAD001, Mcl-1 single inhibition by RAD001 could provide an immediate means to clinically study anti-apoptotic Bcl-2 protein inhibition, since BH3-mimetics targeting Bcl-2 are just now entering clinical trials in breast cancers and inhibitors of other anti-apoptotic Bcl-2 family proteins remain to be tested in breast cancers.

ERα+ breast cancers respond differently to Bcl-2 or Bcl-xL inhibition.

Although the Bcl-2/Bcl-xL inhibitor ABT-263, and its oral analogue ABT-737, have been extensively studied in hematological malignancies and solid tumors, very few studies observe whether tumor cell killing by ABT-263 is mediated by Bcl-2, Bcl-xL, or combined inhibition of both family members. To fill this knowledge gap in ERα+ breast cancers, the Bcl-2 specific inhibitor ABT-199 and the Bcl-xL specific inhibitor A-1155463 were used. Interestingly, Bcl-xL inhibition was superior to Bcl-2 inhibition in some cell lines tested, suggesting that transient tumor cell killing induced by ABT-263 is largely due to Bcl-xL inhibition (**Figure 17**). In another cell line, maximal tumor cell killing was achieved when both Bcl-2 + Bcl-xL were inhibited, demonstrating that ERα+ breast cancers are heterogeneous in their response to anti-apoptotic Bcl-2 family member blockade. However, all tested ERα+ breast cancer cell lines and *in vivo* models were sensitive to Mcl-1 inhibition, suggesting that while Bcl-2/Bcl-xL/Mcl-1 blockade may induce the largest tumor cell killing, Mcl-1 inhibition alone may be sufficient for clinical efficacy in a majority of ERα+ breast cancer patients.

Mcl-1 promotes resistance to anti-estrogens.

In addition to promoting resistance to BH3-mimetics targeting Bcl-2 and/or Bcl-xL, Mcl-1 may be a prominent resistance factor to other targeted therapies. For instance, a preliminary study showed that Mcl-1 targeting decreased tumor cell growth in fulvestrant- and tamoxifen-resistant ERα+ breast cancers (47). Thus, we were interested in further observing the role of Mcl-1 in response to anti-estrogens, which are the clinical standard of care for ERα+ breast cancers. Additionally, as in **Chapter 2**, we were interested in comparing Mcl-1 inhibition to blockade of other anti-apoptotic family members in order to determine which anti-apoptotic factor is most essential to tumor cell survival during anti-estrogen treatment. Long term estrogen deprived (LTED) ERα+ breast cancer cell lines were used to model sustained treatment with aromatase inhibitors. As seen in parental ERα+ breast cancer cells grown continuously in the presence of

estrogen, LTED cells were only transiently sensitive to ABT-263 (**Figure 24**). Further, some parental cell lines sensitive to ABT-263 lost sensitivity to Bcl-2/Bcl-xL inhibition after sustained estrogen deprivation, perhaps due to Mcl-1 upregulation after LTED driving resistance to Bcl-2/Bcl-xL targeting (**Figure 26**). In support of this idea, Mcl-1 targeting using polymeric nanoparticles containing Mcl-1 siRNA (MCL1si-NPs) increased tumor cell killing and decreased tumor cell growth in LTED cells when used in combination with ABT-263 (**Figure 29**). Importantly, while ABT-263 alone only induced transient tumor cell killing in one LTED cell line, MCL1si-NPs increased tumor cell killing in all LTED cell lines tested, supporting our previous findings that Mcl-1 is a dominant tumor cell survival factor in ERα+ breast cancers.

In addition to observing the role of Mcl-1 in response to AIs, we were interested in exploring the contribution of Mcl-1 to tumor cell survival after treatment with another class of anti-estrogen, selective estrogen receptor downregulators like fulvestrant. Fulvestrant's mechanism of action varies from aromatase inhibitors, and thus fulvestrant may depend on a different anti-apoptotic Bcl-2 family protein for tumor cell survival. However, we observed increased Mcl-1 expression and activity in ERα+ breast tumors from patients treated with fulvestrant, and in human ERα+ breast cancer cell lines and xenograft models (**Figure 27**). MCL1si-NPs were particularly effective when used in combination with fulvestrant in all cell lines tested (**Figure 30**), supporting the idea that Mcl-1 targeting can increase tumor cell killing in combination with multiple classes of anti-estrogens, a finding that could have immense clinical impact due to the large number of ERα+ breast cancers diagnosed each year.

Targeting Mcl-1 using alternative methods.

Mcl-1 specific BH3-mimetics were not available at the start of these studies. One focus of this thesis was to develop alternative methods to target Mcl-1 in ERα+ breast cancers. This was successfully accomplished by three means: 1) using genetic ablation via shRNA (shMCL1, **Chapter 2**), 2) targeting Mcl-1 through indirect, upstream regulatory mechanisms (**Chapter 3**),

and 3) targeting Mcl-1 via siRNA encapsulated in nanoparticle carriers (MCL1si-NPs, **Chapter 4**). Two strategies to ablate Mcl-1 levels, Mcl-1 genetic depletion and mTORC1 inhibitors, which block *MCL1* cap-dependent translation, decreased Mcl-1 levels and increased tumor cell killing in all cell lines tested (**Figures 11, 20**). However, MCL1si-NPs only increased tumor cell killing in one cell line tested (**Figure 30**). This discrepancy may be due to the potency of Mcl-1 inhibition by MCL1si-NPs leading to dramatic tumor cell killing at time points prior to those tested (48 hours). Despite this, shMCL1, mTORC1 inhibition, and MCL1si-NPs each increased tumor cell killing when used in combination with targeted therapies. Importantly these targeted therapies, ABT-263 and anti-estrogens, did not sustain tumor cell killing in ERα+ breast cancers when used as single agents, possibly due to elevated Mcl-1 expression and activity. These results suggest that multiple targeted therapies increase Mcl-1 expression and activity in ERα+ breast cancers, further supporting the idea that these malignancies rely heavily on Mcl-1 for tumor cell survival and that Mcl-1 targeting should be clinically tested in ERα+ breast cancers.

Although BH3-mimetics targeting Mcl-1 have recently become available, we believe that the Mcl-1 inhibitors outlined in this thesis may yet have clinical relevance. Preliminary studies with Mcl-1 specific BH3-mimetics have already been met with complications due to toxicities (61), possibly due to the important role of Mcl-1 in survival of immune cells (144-146), cardiac cells (143), and neurons (163, 164). Thus, Mcl-1 inhibitors could be toxic in patients. However, RAD001/everolimus is already approved for use in ERα+ breast cancers, and is well-tolerated. Further, nanoparticle technology provides increased tumor-specific targeting and could spare on-target toxicities of Mcl-1 inhibition at alternative sites. Thus, while Mcl-1 BH3-mimetics need to be tested for tolerance in patients, we believe that further exploration of alternative methods to target Mcl-1 need to continue in ERα+ breast cancers.

Future Directions

Heterogeneous response to anti-apoptotic Bcl-2 family member inhibition.

The data presented herein show that all tested ERα+ breast cancer cell lines and *in vivo* models were sensitive to Mcl-1 inhibition. However, only small panels of ERα+ breast cancer cell lines were studied, leaving the possibility that some ERα+ breast cancers will be more sensitive to Bcl-2 inhibition or Bcl-xL inhibition than Mcl-1 blockade. Further, ERα+ breast cancers had a heterogeneous response to Bcl-2 and Bcl-xL inhibition, where some cell lines were sensitive to Bcl-2 + Bcl-xL blockade, while others were only sensitive to Bcl-xL inhibition, not Bcl-2 inhibition (**Figure 18**). Thus, we believe that further exploration of the heterogeneous response of ERα+ breast tumors to Bcl-2 family member inhibition is needed. For instance, a large scale screen comparing Bcl-2, Bcl-xL, and Mcl-1 inhibition across ERα+ breast cancer cell lines would be very informative.

Mcl-1 targeting in Myc-driven breast tumors.

Previous studies in lung cancers (135), lymphomas (137), and HER2+ breast cancers (71) demonstrate that Myc-driven tumors rely on Mcl-1 for tumor cell survival, possibly because Mcl-1 evades the pro-apoptotic functions of Myc. The studies presented herein support these findings, showing that RAD001/everolimus targeting of Mcl-1 was extremely effective in *Wap-Myc* mammary tumors (**Figure 19**). However, the role of Mcl-1 in Myc-driven breast tumorigenesis is largely unexplored, despite the fact that over 15% of breast cancers contain a *MYC*-amplification (169). Further, *MCL1* was frequently co-amplified with *MYC* in triple negative breast cancer patients refractory to chemotherapy (39), suggesting that Myc and Mcl-1 cooperate to promote sustained tumor cell survival throughout breast tumorigenesis. Because Myc is a difficult transcription factor to target, Mcl-1 inhibition may provide an alternative means to treat Myc-driven

breast cancers throughout tumor progression, particularly after acquisition of therapeutic resistance. This idea needs to be further explored in future studies.

Develop a clinical assay to determine sensitivity to Bcl-2 family member inhibition.

The development of a clinically relevant method to determine sensitivity to anti-apoptotic Bcl-2 family protein inhibition is imperative. Data presented herein suggest that *MCL1* transcript levels may be a useful biomarker of sensitivity to Bcl-2/Bcl-xL inhibitors (**Figure 15**). Since the Bcl-2 specific inhibitor ABT-199 is currently being tested clinically in ERα+ breast cancers, studies should observe whether patients with high *MCL1* expression receive less clinical benefit from ABT-199 than patients with low *MCL1* expression.

Additionally, excitement surrounds the use of dynamic BH3-profiling to determine how 'primed' a tumor is for cell death [by quantitating outer mitochondrial membrane permeabilization upon stimulation with pro-apoptotic Bcl-2 family members (170)]. This assay could also be used to identify which anti-apoptotic Bcl-2 family protein is most 'primed' for inhibition (i.e. which anti-apoptotic factor this most essential for continued tumor cell survival). Importantly, BH3-profiling can predict sensitivity to BH3-mimetics, like ABT-737 (171). This assay is being developed in preclinical models of solid tumors (172), and could be an invaluable tool to aid in deciding which BH3-mimetic is used in patients, on a patient to patient basis.

Although BH3-profiling is very appealing, we believe that the use of proximity ligation assay (PLA), to observe anti-apoptotic Bcl-2 family member interactions with pro-apoptotic proteins, could be particularly useful in the clinic in breast cancers. Importantly, as shown in these studies, PLA can be completed on paraffin embedded-formalin fixed human tissues, a standard method of tissue collection for breast tumors. Further, PLA could be used to retrospectively observe anti-apoptotic Bcl-2 family member dependence, which could allow for rapid analysis of the importance of Bcl-2 family proteins to patient survival, a statistic that is often challenging to

analyze due to the length of time to tumor progression in ERα+ breast cancer patients. Conversely, BH3-profiling requires fresh or frozen tumor tissues, as well as a large number of viable cells, which is often a limiting factor in solid tumors. Hence, we believe that future preclinical studies should evaluate whether Bcl-2/Bim, Bcl-xL/Bim, and/or Mcl-1/Bim interactions determined by PLA can predict sensitivity to BH3-mimetics that target each anti-apoptotic family member alone (ABT-199 or A-1155463) or in combination (ABT-263 or obatoclax).

mTORC1 inhibition in combination with aromatase inhibitors.

The mTOR inhibitor RAD001/everolimus is approved in the clinic in combination with aromatase inhibitors (AIs) in ERα+ breast cancers. The rationale for this combination is the fact that mTORC1 signaling is aberrantly regulated in many tumors that have acquired resistance to AIs; however, the exact molecular mechanism driving the efficacy of RAD001 + AIs is not fully understood. Data presented herein suggest that Mcl-1 is a main mTORC1 target, demonstrating that Mcl-1 inhibition may be a primary contributor to the clinical efficacy of RAD001 in ERα+ breast cancers. This idea is supported by a case study that noted complete response in a patient with ERα+ breast cancer containing an *MCL1*-amplifcation after treatment with the AI anastrozole and RAD001 (142). Although we have observed that Mcl-1 expression is increased upon treatment with AIs, future studies should observe whether Mcl-1 inhibition via RAD001 blocks this compensatory function of Mcl-1. These studies could explain why RAD001 is clinically effective ERα+ breast cancers, and thus could have immense impact.

Alternative roles of Mcl-1

Although these studies focused on the anti-apoptotic role of Mcl-1 in the tumor epithelium, Mcl-1 targeting may also impact the tumor microenvironment. In normal biology, Mcl-1 is essential to development and maintenance of cells in the hematopoietic compartment, including plasma cells (144), T-lymphocytes (145), B-cells (173), and macrophages (146). Although this supports the idea that Mcl-1 targeting may be toxic in patients, many of these studies were

completed in transgenic models that had complete tissue specific ablation of *Mcl1*. It is possible that Mcl-1 inhibition by mTOR inhibitors or si-NPs could spare some of these off target effects, due to incomplete Mcl-1 ablation or to superior tumor specific targeting, respectively. For instance, BH3-mimetics that specifically target Mcl-1 were recently shown to have minimal effect on the survival of some leukocyte populations in preclinical immunocompetent murine models (61). However, since studies show that response of ERα+ breast cancers to conventional therapies relies on the number of infiltrating lymphocytes [as reviewed in (174)], we believe it is essential to better understand how Mcl-1 inhibition will effect survival and infiltration of immune cells into the ERα+ breast tumor microenvironment. The *Wap-myc* mammary tumor model may serve as an important model to study this concept in an immune competent host.

In addition to its role as an anti-apoptotic factor, Mcl-1 has alternative functions. For instance, a truncated version of Mcl-1 is involved in mitochondrial fission and fusion, mitochondrial respiration, and other essential mitochondrial functions (143, 175). Although this role of Mcl-1 has not been explored in tumor cells, cancers are defined by metabolic reprogramming, also known as the Warburg effect. Thus, it is possible that Mcl-1 regulates breast tumor metabolism. Future studies should address this idea to better comprehend the effects of Mcl-1 targeting on both tumor mitochondrion and normal mitochondrion, with the possibility that Mcl-1 inhibition could affect normal mitochondrial respiration as a potentially toxic on-target effect.

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