Modulation of Epigenetic Regulatory Pathways as a Therapeutic Strategy in Breast Cancer

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MODULATION OF EPIGENETIC REGULATORY PATHWAYS
AS A THERAPEUTIC STRATEGY IN BREAST CANCER

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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<thead>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AV</td>
<td>Annexin V</td>
</tr>
<tr>
<td>Baf</td>
<td>Bafilomycin A1</td>
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<td>Bak</td>
<td>Bcl-2 Homologous Killer</td>
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<td>Bax</td>
<td>Bcl-2-Associated X Protein</td>
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<td>BCL-2</td>
<td>B-Cell Lymphoma 2</td>
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<tr>
<td>BCL-W</td>
<td>Bcl-2-Like Protein 2</td>
</tr>
<tr>
<td>BCL-X_L</td>
<td>B-Cell Lymphoma-Extra Large</td>
</tr>
<tr>
<td>BET</td>
<td>Bromo- and Extra-Terminal Domain</td>
</tr>
<tr>
<td>BRD4</td>
<td>Bromodomain-Containing Protein 4</td>
</tr>
<tr>
<td>C12FDG</td>
<td>5-Dodecanoylamino-fluorescein Di-β-D-Galactopyranoside</td>
</tr>
<tr>
<td>cCasp3</td>
<td>Cleaved caspase 3</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent Kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin-dependent Kinase Inhibitor</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA Damage Response</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ETO</td>
<td>Etoposide</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
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<tr>
<td>HSP90</td>
<td>Heat Shock Protein 90</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing Radiation</td>
</tr>
<tr>
<td>KD</td>
<td>Knock Down</td>
</tr>
<tr>
<td>LC3B</td>
<td>Light Chain Microtubule-Associated Protein</td>
</tr>
<tr>
<td>MCL-1</td>
<td>Myeloid Cell Leukemia-1</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse Double Minute 2 Homolog</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-Light-Chain Enhancer of Activated B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>OIS</td>
<td>Oncogene-Induced Senescence</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Solution</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PROTAC</td>
<td>Proteolysis Targeting Chimera</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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Abstract

Anti-estrogens or aromatase inhibitors in combination with cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors are the current standard of care for estrogen receptor-positive (ER+) Her-2 negative metastatic breast cancer. In triple negative breast cancer, cytotoxic drugs and ionizing radiation are often used as standard of care. Although these therapies prolong progression-free survival, the growth-arrested state of residual tumor cells is clearly transient. Tumor cells that escape what might be considered a dormant state and regain proliferative capacity often acquire resistance to further therapies. Epigenetic regulation can modulate the effects of cancer therapeutics. Our studies are based upon
the observation that breast tumor cells arrested by Fulvestrant + Palbociclib enter into states of both autophagy and senescence from which a subpopulation ultimately escapes, potentially contributing to recurrent disease. Autophagy inhibition utilizing pharmacologic or genetic approaches only moderately enhanced the response to Fulvestrant + Palbociclib in ER+ MCF-7 breast tumor cells, slightly delaying proliferative recovery. In contrast, the epigenetic regulator and BET inhibitor/degrader, ARV-825, prolonged the growth-arrested state in both p53 wild-type MCF-7 cells and p53 mutant T-47 D cells and significantly delayed proliferative recovery. In addition, ARV-825 added after the Fulvestrant + Palbociclib combination promoted apoptosis. We further studied another epigenetic regulator, a bromodomain PHD finger transcription factor subunit (BPTF) of the nucleosome remodeling factor (NURF), which promotes resistance to chemotherapies in triple negative breast cancer. We observed sensitization to topoisomerase II (TOP2) inhibitors doxorubicin, etoposide, as well as microtubule poisons paclitaxel, vinorelbine, and vinblastine. The observed sensitization appeared to be specific to these mechanisms of action and indicates a potential role of autophagy in these treatments. These studies indicate that the administration of epigenetic regulators, such as BET and NURF inhibition, may potentially improve the standard of care therapy in metastatic ER+ breast cancer and triple negative breast cancer patients and may further prolong progression-free survival.
Disclosures

1. Parts of the writing and data presented in this thesis have previously been published in:


Chapter 1 : General Introduction

1.1. Breast Cancer

1.1.1. Breast Cancer: Overview

Breast cancer is the most common type of cancer diagnosed in women and the second most common cause of cancer-related death in the United States in 2022 (1). Breast cancer is generally divided into four major subtypes, which consist of hormone Receptor positive (ER or PR), which includes Luminal A (HER2+) and Luminal B (HER2-), triple negative (ER-/PR-/HER2-), and HER2 overexpressing (2). Among these, hormone receptor-positive is the most common subtype, constituting approximately 70% of breast cancer cases, while triple-negative is the least common subtype, which includes about 10% of cases (2).

Despite being the most common breast cancer subtype, HR+/HER2- breast cancer has the best 5-year survival rate, about 94% (3). In contrast, despite being the lowest occurring subtype, the 5-year survival of triple-negative breast cancer patients is around 76 percent, making it the most-deadly subtype (3). While the specific subtype may be used as an indicator of survival, the cancer stage at diagnosis may be one of the most influential factors when determining the survival outcomes.

1.1.2. Treatment options for Breast Cancer

The treatments used in HR+ HER2- breast cancer and triple-negative breast cancer vary depending on the cancer stage. In the case of HR+ HER2- breast cancer,
hormonal therapy is primarily considered the standard of care for initial treatment (4). In instances where the initial therapy is ineffective or not well tolerated, switching to another class of hormonal therapy is required (5). There are numerous clinically available endocrine therapies, including selective estrogen receptor modulators (SERMS) such as tamoxifen, selective estrogen receptor degraders (SERDs), such as Fulvestrant (6) and aromatase inhibitors (AIs). Tamoxifen, one of the oldest and most utilized SERMs, is now typically prescribed to treat hormone receptor-positive, early-stage breast cancer after surgery to reduce disease recurrence in post-menopausal women (7). AIs, which show increased efficacy compared to TAM therapy (8), are the preferred endocrine treatment for post-menopausal women with all stages of ER+ breast cancer. Unfortunately, while early ER-positive breast cancer treatment with SERMs, SERDs, and AIs can reduce recurrence for up to 5 years, resistance to hormone therapy is common, and most cases eventually result in metastatic disease progression (9,10). While endocrine therapies remain the standard of care for ER+ breast cancer, a significant hindrance to the success of these therapies is due to resistance.

Metastatic HR+ Her2- breast cancer standard of care usually involves either single hormonal therapy or a combination with targeted therapy such as CDK4/6 inhibitors (5,11). Palbociclib is the most utilized and commonly studied CDK4/6 inhibitor. Under normal conditions, CDK4/6 binds to cyclin D, phosphorylates and inactivates retinoblastoma (RB) protein, releases transcription factor E2F, resulting in the transcription of the genes necessary for progression from the G1 to S phase of the cell
cycle (Figure 1.1). Palbociclib works by inhibiting the binding of CDK4/6 with cyclin D, and thereby blocking cell cycle progression (12).

In extreme cases, chemotherapy is utilized when hormone therapies are no longer effective. Although chemotherapy is an option, there is no specific type of chemotherapy recommended for this subtype of breast cancer (5). In rare instances, when ER+ HER2- breast tumors have deficient mismatch repair or a high tumor mutation burden, immunotherapy may be used, although very rarely used in combination with chemotherapy (13). In triple-negative breast cancer, the standard of care currently utilized in early diagnosed cases is primarily neoadjuvant chemotherapy, followed by surgery (14). Common classes of chemotherapies utilized in TNBC are anthracyclines (such as doxorubicin), alkylating agents (such as cyclophosphamide), anti-microtubule agents (such as taxanes), and anti-metabolites (such as fluorouracil or 5-FU).
1.2. Apoptosis

One of the most desired outcomes for anti-tumor therapies is cell death. Apoptosis is programmed cell death defined by several hallmarks, such as irreversible chromatin condensation, cell shrinkage, membrane blebbing, and DNA fragmentation (15). Apoptosis comprises two major pathways: an extrinsic, death receptor-driven
pathway and an intrinsic, mitochondrial-driven pathway (15), and a third perforin/granzyme pathway. These pathways converge at caspase 3, known as the executioner caspase, which leads to the distinctive morphological changes and the formation of apoptotic bodies (15,16). The extrinsic pathway is initiated by an external stimulus or ligand molecule, which binds to a “death receptor,” which is a transmembrane receptor, such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and FAS (15,16). The binding of the death ligand with the death receptor activates the formation of the Death-inducing signaling complex (DISC), which results in the activation and dissociation of active caspase-8, which cleaves and activates caspase 3 (15).

The intrinsic pathway is triggered by external stimuli such as radiation, toxins, chemotherapies, or hypoxia, inducing mitochondrial changes and pore formation by BAX and BAK proteins, resulting in mitochondrial outer membrane permeabilization (15,16), allowing cytochrome C release leads to the formation of apoptosomes, leading to the cleavage and activation of caspase 9, which then cleaves and activates caspase 3, leading to the execution pathway (15,16).

In the last pathway mentioned, the perforin/granzyme pathway, cytotoxic T lymphocytes target cells through FasL and FasR interactions, leading to secretion of perforin from cytotoxic T lymphocytes, which creates pores in the membrane, followed by the release of cytoplasmic granules, composed of granzyme A and granzyme B into these pores (15,17). Granzyme can then directly activate caspase 3 or induce cytochrome C release, resulting in the execution pathway (15,17). Granzyme A can trigger cell death through the endoplasmic reticulum-associated SET complex, which translocates to the nucleus and causes DNA cleavage, inducing apoptosis independent of caspase 3.
1.3. Autophagy

1.3.1. Introduction and Types of Autophagy

Autophagy can be divided into three main classifications: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Each of these pathways, although morphologically distinct, ultimately involves the delivery of cargo to the lysosome. The lysosome provides the acidic environment and enzymes necessary for the degradation and recycling of cargo (18). Macroautophagy is the autophagy pathway most commonly observed in cells and tissues and has been implicated in cancer cell resistance to therapy, including hormonal therapy resistance (19). Macroautophagy is an evolutionarily conserved catabolic process through which cellular cargo is initially sequestered within a double membrane vesicle, prior to fusion with the lysosome (20). In this regard, macroautophagy is distinct from chaperone-mediated autophagy and microautophagy in that each of these types of autophagy does not rely on an autophagosome to bring cargo to the lysosome (21). To date, the components of autophagy and the required autophagic machinery are encoded by 31 autophagy-related genes (ATG). Many of these genes and the autophagy pathway itself have been shown to be necessary components of a number of cellular processes such as immune cell development, maintaining cell and tissue homeostasis, cellular metabolism, aging, and cancer (22). Thus, it is not surprising that the impairment of autophagy in normal cells has been associated with multiple disease processes (23,24).
1.3.2. Mechanism of Autophagy

The autophagy pathway is typically divided into separate stages: initiation of the double membrane phagophore, elongation and closure of the autophagosome membrane, fusion with the lysosome forming the autolysosome, and degradation of the intravesicular cargo. The initiation phase is regulated by the mammalian target of rapamycin, mTOR, which is a central component of two multiprotein complexes, designated mTORC1 and mTORC2. The mTORC1 complex is highly responsive to nutrient deprivation and limited amino acid availability, while mTORC2 responds to growth factor availability. When mTORC1 activity is low, ULK1/2 (Unc-51-like kinase 1/2) are activated via dephosphorylation. The ULK1/2 complex is comprised of ULK1/2, FIP200 and ATG13. This complex, once assembled, phosphorylates members of the class III PI3K complex, consisting of AMBRA1, Beclin1, VPS15/34, UVRAG and ATG14(25). Phosphorylation of PI3KC, Beclin-1, and VPS34 is required for initiation of phagophore nucleation, which is hypothesized to originate from multiple membrane sources, including the endoplasmic reticulum, mitochondria, Golgi apparatus and recycling endosomes (26–29). The phagophore is then elongated by the ATG5/12 complex, which is conjugated by ATG16L and by the conjugation of active cytosolic LC3-I (encoded by ATG8) to phosphatidylethanolamine (PE), generating LC3-II. Prior to LC3-I conjugation, cleavage of the C-terminal region of the inactive pro-form of LC3 is mediated by ATG4B. LC3-II is recruited to the phagophore membrane and is required for elongation of the inner and outer membrane of the autophagosome. Following phagophore maturation, the autophagosome fuses with the lysosome, forming an autolysosome and leading to the
degradation of the autophagic cargo, along with LC3-II. Thus, LC3-II turnover is often utilized as a marker for autophagosome formation and functional autophagic flux (30).

The protein Sequestosome 1 (p62/SQSTM1) is a ubiquitin and LC3 binding protein that is also degraded during autolysosomal turnover and can also be a marker of functional autophagy. Although p62/SQSTM1 plays a key role in clearing protein aggregates in cells (termed aggrephagy), it is also a selective autophagy receptor and facilitates mitochondrial and lipid droplet turnover specifically termed mitophagy and liophagy, and also has roles in the ubiquitin-proteasome system, cellular metabolism, signaling, and apoptosis (31).

Figure 1.2 Mechanism of Autophagy. Primary mechanism of autophagy. When growth factors or nutrients become scarce, AMPK or mTOR inhibition results in activation of the ULK complex, which leads to phagophore initiation through mediation by the Beclin1 complex. The phagophore elongates and matures with the recruitment of ATG proteins, which contribute to the formation of the phosphatidylethanolamine (PE)-Conjugated LC3-II, which incorporates into the autophagosome membrane. After fusion with the lysosome, the autophagic cargo, comprising nutrients and metabolites, is degraded in the autolysosome and recycled back into the cytoplasm.
1.3.3. Functional forms of Autophagy

There are a few distinct functional forms of autophagy, each of which influence the tumor cell response and effectiveness of the therapy being utilized. Protective autophagy refers to instances where the induction of autophagy promotes the survival of tumor cells. In these occurrences, genetic or pharmacological inhibition of autophagy may be utilized to enhance the tumor cell sensitivity to the therapy being employed and promote apoptosis (13,32). In the case of protective autophagy, many variables influence the survival of cells including the inhibition of apoptosis, diminished free radicals, and elimination of damaged proteins by autophagy (33).

The nature of autophagy is often protective in anti-estrogen therapies and has been associated with the development of resistance, which occurs in approximately 40% of patients (34,35). Several studies have evaluated the importance of ER-alpha in malignant ER+ breast tumor growth, given its close association with the autophagy gene Beclin-1 (36,37). In this case, genetic inhibition of ER-alpha, along with pharmacological inhibition, using Fulvestrant, have shown autophagy to be protective (38). Studies assessing tamoxifen treatment showed an upregulation of autophagy, where inhibiting autophagy through genetic and pharmacological inhibition revealed a cytoprotective role of autophagy (39). Along with tamoxifen, studies with other ER ligands, along with aromatase inhibitors, have displayed a protective role of autophagy in these therapies (40,41). These observations demonstrate that estrogen receptor modulation can often induce cytoprotective autophagy, which is pro-survival in nature.

Another functional form of autophagy is cytotoxic autophagy, where the induction of autophagy works synergistically with the applied therapy, resulting in increased tumor cell
death and anti-proliferative effects. This mode of autophagy may be a result of over-digestion of intracellular components by extensive or prolonged autophagy, although the literature distinguishing the protective from the toxic forms is scarce (42,43). While cytotoxic autophagy and cellular stress can induce apoptosis, situations can arise where apoptosis is blocked, in which case autophagic cell death can occur as an alternative cell death mechanism as well as in parallel with apoptosis (44).

As mentioned previously, Beclin-1 is an essential component of autophagy initiation and is involved in the class III PI3K complex phosphorylation. Beclin-1 has been shown to be unregulated when the anti-apoptotic protein, BCL-2, is silenced, leading to upregulated autophagy and subsequent autophagic cell death in MCF-7 tumor cells treated with doxorubicin (45). Furthermore, when autophagy is inhibited via silencing of ATG5 and BCL-2, the autophagic cell death induced by doxorubicin is ameliorated. In contrast to the classical autophagic cell death, non-canonical autophagy can also occur, inducing cell death without the involvement of Beclin-1. One study observed cytotoxic autophagy independent of Beclin-1 when MCF-7 cells were treated with resveratrol, a phenol produced by several types of plants (46).

While autophagy can be functionally protective or cytotoxic, in some cases autophagy plays no distinct role in promoting or suppressing the growth or sensitivity of the tumor cells in response to therapy (42,47). In these cases, autophagy is non-protective in nature. Chemotherapy and radiation, in addition to promoting cytoprotective autophagy, have also been shown to induce non-protective autophagy that may not change outcome of treatment (48,49).
Delineating the role of autophagy in each therapy and specific cancer type is advantageous in determining an effective route of modulation to improve the treatment. Another form of autophagy is the cytostatic form, where activation of autophagy results in prolonged growth arrest in order to protect the tumor cell from the therapy being utilized, with no effect on cell viability (50).

1.3.4. Autophagy and the Immune System

There is extensive interplay between autophagy and the immune system, resulting in conflicting information on the potential of autophagy inhibition in mice and patients. Often referred to as a “double-edged sword,” autophagy can have either pro-tumorigenic or anti-tumorigenic impact on the innate, adaptive immune system and immune tolerance (51). Autophagy can be upregulated by innate immune receptor activation, including toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (51,52). Additionally, autophagy activation can either promote or inhibit the development of tumors through modulation of the homeostasis, activation, and differentiation of immune cells (51). Autophagy facilitates T cells differentiation into cytotoxic T cells or Th cells (53), drives dendritic cell (DC) and B cell development (54–56), plasma cell differentiation as well as IgM and IgG production (57), enhancing antigen-presentation.

While autophagy promotes anti-tumor immunity, it also promotes tumor growth, invasion, angiogenesis, and metastasis. In the tumor microenvironment, autophagy is essential for Treg-mediated tolerance and survival, inducing polarization towards
immunosuppressive M2-like macrophages, while autophagy inhibition can result in M1-like TAM polarization (58,59). Furthermore, autophagy enables myeloid-derived suppressor cell growth, further promoting a pro-tumorigenic microenvironment. Immune checkpoint molecules such as IDO, CTLA-4 and PD1 can regulate the tumor immune tolerance through autophagy (51). Autophagy has also been shown to degrade NK-cell derived granzyme B.

1.3.5. Role of Autophagy in Immunogenic Cell Death

While apoptosis is the desired outcome of cancer therapy alone, it is primarily considered a sterile mode of cell death, having little effect on immune response since apoptosis plays such a vital role in normal body functions (60). Alternatively, when apoptosis is coupled with autophagy or senescence, this has been shown to elicit immune responses against tumor cells (61). Many chemotherapeutic therapies, such as anthracyclines, while inducing apoptosis, autophagy, and senescence, have also been linked to immunogenic cell death (ICD), making tumors more immunogenic (Figure 1.3) (62,63).

Damage-associated molecular patterns (DAMPs) are essential for attracting immune cells into the tumor microenvironment and are a necessary factor in ICD (60). Autophagy plays a vital role in adenosine triphosphate (ATP) release, which leads to the induction of ICD (60). Studies in autophagy-deficient tumor cells have shown that they secrete less ATP than their autophagy-competent counterparts (64). ATP is a DAMP released from dying tumor cells to attract and activate immature macrophages and DCs.
Another major influencer of ICD induction is stress in the Endoplasmic reticulum and increased levels of reactive oxygen species (ROS) due to the aggregation of misfolded and unfolded proteins, leading to the release of DAMPs (66). Endoplasmic reticulum stress-related signaling targets calreticulin, which can draw immune cells to the tumor microenvironment (67). Modulation of ICD in cancer treatment has been previously studied through modulation of ER stress (68), calreticulin (69), heat shock protein (70), ATP secretion (71) and caspase-3 activation (72,73).
Figure 1.3 Immunogenic Cell Death and Autophagy. ICD inducers, such as radiation and chemotherapies triggering induction of apoptosis, autophagy, and necrosis in dying tumor cells, resulting in secretion of DAMPS. These DAMPs cause maturation of DCs, releasing proinflammatory cytokines, leading to downstream effector immune cell responses targeting the tumor cells (adapted from Tang, 2013).
1.3.6. Autophagic cell death

While autophagy can induce apoptosis and ICD, it can also lead to its own form of cell death, autophagic cell death (ACD). ACD is considered type 2 programmed cell death and is defined as cell death occurring without the involvement of other forms of programmed cell death, elevated autophagic flux, and when inhibition of autophagy through pharmacological or genetic methods, prevents cell death from occurring (Figure 1.3) (74). Morphologically, ACD can be observed by the vacuolization of the cytoplasm without chromatin condensation, observed in apoptosis, or lack of organelle swelling, which occurs during necrosis (75). ACD is referred to as a controversial term as Kroemer and Levine suggested that “autophagic cell death” is a misnomer and should be referred to as “cell death with autophagy” or “cell death by autophagy” (Figure 1.4) (75). Despite this controversy, there is an increasing body of evidence that, in many cases, autophagy and autphagic machinery is essential for cell death (76–78).
Figure 1.4 Autophagic cell death. Autophagic cell death may refer to cell death which occurs concurrently with autophagy, or cell death which occurs as a result of autophagy. **A.** In cell death with autophagy, inhibition of autophagy following a death stimulus may have no impact on survival but may impact the rate of degradation of the cell. If autophagy is cytoprotective in this case, inhibition of autophagy may accelerate cell death. **B.** If cell death occurs by autophagy, inhibition of autophagy would result in cell survival (adapted from Kroemer, 2008).
1.4 Senescence

1.4.1. Introduction to Senescence

Senescence was originally studied in the context of aging, where it was defined as the limit to replicative capacity, where cells enter a growth arrested state and are unable to divide (79). This form of senescence was related to shortening of telomeres during cell division where the term “Hayflick limit” referred to the limited replicative capacity of these cells (80–82). Senescent cells’ inability to replicate is a result of cell cycle arrest in the G1/S phase, resulting in halted DNA synthesis (82). Traditionally, senescence was thought to represent solely a permanent form of growth arrest but has been gradually accepted as a transient arrest in tumor cells and a form of tumor dormancy (83,84). While replicative capacity can drive senescence induction in normal cells, there are other conditions which induce senescence. Oncogene-induced senescence is one condition where growth arrest is utilized by the cell to delay or circumvent oncogenic transformation (85). Environmental stressors, exposure to UV or radiation, and an accumulation of ROS can also lead to stress-induced senescence (86,87). Cancer cells can also enter senescence as a response to various treatments such as cytotoxic therapies, CDK4/6 inhibition, or ionizing radiation (88–90). The literature is quite divided on whether or not senescence is a beneficial outcome, but the majority of studies in the field agree that while cell death is the preferred outcome, induction of growth arrest through senescence is a beneficial alternative [13].
1.4.2. Features of Senescent Cells

Typical features of senescent cells include increased size, expression of BCL-2 protein, CDK inhibitors p16\textsuperscript{INK4a} and p21\textsuperscript{WAF1}, and reduced proapoptotic caspase activity [15,16]. Another feature, and the most common indication of senescence, is the senescence-associated beta-galactosidase (SA beta-gal) assay, which involves detection of the ubiquitous lysosomal hydrolase, beta-galactosidase, which is active at pH 6. While SA-\beta-gal is an accepted measure of senescence in the field, quantification of other hallmarks is often used for confirmation of senescence induction (95,96).

Another hallmark of senescent cells is the release of inflammatory cytokines, chemokines, growth factors and matrix metalloproteinases (97). These secreted factors comprise the senescence-associated secretory phenotype (SASP,) which are thought to function as messengers between senescent cells and cells in their surrounding cells such as stromal, immune or other cancer cells (92). Similar to autophagy, DNA damage response (DDR) is also an inducer of the senescence response, triggered by DNA damage. DNA damage causes ATM kinase recruitment to the site of damage, driving the phosphorylation of histone H2AX, and directing the necessary DNA repair complexes (92).

1.4.3. Recovery and Senescence-targeted Therapies

Our laboratory, along with others, has reported the promotion of senescence by cancer therapies as well as clearance of senescent tumor cell populations by senolytic agents (84,89,98–100). Several senolytic agents such as BCL-2/BCL-xL inhibitors Navitoclax (ABT-263), Venatoclax (ABT-199), Panobinostat, and Fisetin are currently in preclinical
studies and/or in clinical trials in combination with conventional therapies (101–104). ABT-263 is effective at the elimination of tumor cells induced into senescence by various chemotherapeutic strategies in triple-negative breast, lung, head and neck, and prostate tumor cells (89, 99, 100). Unfortunately, one fundamental limitation in the use of ABT-263 is thrombocytopenia and neutropenia, given that the target of ABT-263 is the Bcl-xL protein, upon which platelets and neutrophils are dependent for survival (105, 106).

While some senescence-targeted therapies are aimed at the elimination of senescent cells, others take different approaches, such as inhibiting specific SASP components, improving senescent cell clearance by the immune system, and further inducing growth arrest of the tumors, causing them to remain in a prolonged dormant state (107).

**Chapter 2 : Materials and Methods**

**2.1. Antibodies and reagents**

The following primary antibodies were used: SQSTM1/p62 (Cell Signaling Technology, 5114T); c-Myc (Cell Signaling Technology, 5605); ATG5 (Cell Signaling Technology, 2630); LC3B (Cell Signaling Technology, 3868); BRD4 (Cell Signaling Technology, 13440S); β-actin (Cell Signaling Technology, 4970); and GAPDH (Cell Signaling Technology, 2118). Secondary antibodies: Horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling, anti-mouse, 7076S; anti-rabbit, 7074S).
2.2. Cell Lines

MCF7 cells were generously gifted by Dr. Clevenger, at Virginia Commonwealth University. MCF7 and T47D cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (Thermo Scientific, SH30066.03), 100 U/mL penicillin G sodium (Invitrogen, 15140–122), and 100 μg/mL streptomycin sulfate (Invitrogen, 15140–122).

The ATG5-knockdown was generated as follows: Mission shRNA bacterial stocks of ATG5 were purchased from Sigma Aldrich. Lentivirus was produced in HEK 293T cells, which were co-transfected using EndoFectinTM Lenti Transfection Reagent (GeneCopoeia, 1001-01) with a packaging mixture of psPAX2 and pMD2.G constructs (Addgene). Media containing the virus was used to infect the MCF7 cells. Puromycin (1 μg/mL) was used as a selection marker to enrich for the infected cells.

Lentiviral packaging of H2B-GFP vector, pLenti0.3UbCGWH2BC1-PatGFP was carried out in 293FT cells. Exponentially growing MCF7 and T47D cells were infected with the H2B-GFP lentivirus in the presence of polybrene (4μg/mL). GFP positive cells were selected using FACS Aria cell sorter.

MCF7 RB-del and T47D-RB-del were gifted from Dr. Knudsen, at Roswell Park Comprehensive Cancer Center, New York. Isogenic MCF7-RB-del and T47D-RB-del cell lines were developed by CRISPR mediated deletion using guide sequences designed to target exon 2 of RB1 as previously described (108).
2.3. Drug Treatments

Fulvestrant (Millipore Sigma, I4409), Palbociclib (LC Laboratories, P-7788), Bafilomycin A1 (Millipore Sigma, 196000), and ARV-825 (MedChemExpress, HY-16954) were dissolved in DMSO. Chloroquine (Millipore Sigma, C6628) was dissolved in sterile 1X PBS (Thermo Fisher Scientific, 10010).

For sensitization studies, cells were exposed to Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days.

For early autophagy inhibition studies, cells were pre-treated with Chloroquine (10 µM) or Bafilomycin (2.5 nM) for 2 h prior to exposure with Fulvestrant, Palbociclib or the combination. CQ or Baf were given in combination with the respective conditions for an addition 48 h, CQ or Baf was removed and Fulvestrant, Palbociclib or the combination were given for an additional 4 days post-CQ or Baf removal to meet the 6-day drug treatment regimen.

For late autophagy inhibition studies, cells were treated with Fulvestrant, Palbociclib or the combination for 6 days and CQ (10 µM) or Baf (2.5 nM) was given for 48 h post-anti-estrogen and CDK 4/6 inhibition therapy.

For senolytic exposure, cells were treated with Fulvestrant, Palbociclib or the combination for 6 days and the respective senolytic was given for 48 h post-anti-estrogen and CDK 4/6 inhibition therapy. ARV-825 was administered for 96 h post-anti-estrogen and CDK 4/6 inhibition therapy.
2.4. Cell Viability

Trypan blue exclusion was utilized to assess cell viability. Cells were plated at 20,000 cells per well in a 6-well plate and treated with the respective conditions. On the indicated days, cells were trypsinized, stained with 0.4% trypan blue (Sigma, T01282), and counted on the indicated days using a hemocytometer. Growth curves were generated from the collected data.

2.5. Clonogenic Survival Assay

Cells were plated at a density of 200 cells per well in 6-well plates and treated with the respective conditions. Media was replenished every other day until colonies formed. Colonies were washed with 1X phosphate-saline buffer (PBS, Life Technologies), fixed with 100% methanol, and stained with 0.1% crystal violet (Sigma). The number of colonies formed was counted. The fraction viable after treatment was calculated by the ratio # colonies treated / # colonies untreated.

2.6. Promotion of Apoptosis

The extent of apoptotic cell death was measured using Annexin V-FITC/Propidium iodide staining. On the indicated day, cells were trypsinized, washed with 1X PBS and stained according to manufacturer protocol (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences, 556547). Fluorescence was quantified by flow cytometry using BD FACSCanto II and BD FACSDiva software at the Flow Cytometry Core Facility at Virginia Commonwealth University. For all flow cytometry experiments, 10,000 cells per replicate were analyzed and three replicates for each condition were analyzed per
independent experiment unless otherwise stated. All experimental protocols were performed with cells protected from light.

2.7. Acridine Orange

On the indicated days, cells were stained with 1 μg/ml acridine orange at 37°C for 20 min and then washed with 1X PBS. Cells were imaged using an inverted fluorescence microscope (Olympus, Tokyo, Japan) at 20X magnification. For quantification of autophagic vesicles (AVOs), on the indicated days, cells were trypsinized, harvested and washed with 1X PBS. Pellet fractions were resuspended in 1X PBS and analyzed by BD FACSCanto II and BD FACSDiva software. For all flow cytometry experiments, 10,000 cells per replicate were analyzed and three replicates for each condition were analyzed per independent experiment unless otherwise stated. All experimental protocols were performed with cells protected from light.

2.8. SA-β-gal staining

On the indicated days, cells were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining as previously described by Dimri et al. (109). Cells were washed with 1X PBS and phase contrast images were taken using an inverted microscope (Olympus, Tokyo, Japan).

T47D-WT cells were treated with Palbociclib (1 μM) in combination with Fulvestrant (100 nM) for 6 days. The cells were stained for β-galactosidase to determine the senescence phenotype by using the commercially available kit (Cell Signaling; 9860)
according to the manufacturer's protocol. Cell images were taken using phase-contrast microscope at 20X magnification.

To quantify β-gal positive senescent cells, after treatment, cells were treated with Bafilomycin A1 (100 nM) for 1 h to achieve lysosomal alkalinization, followed by staining with C₁₂FDG (10 μM) for 2 h at 37 °C. After incubation, cells were collected and analyzed by BD FACSCanto II and BD FACSDiva software. For all flow cytometry experiments, 10,000 cells per replicate were analyzed and three replicates for each condition were analyzed per independent experiment unless otherwise stated. All experimental protocols were performed with cells protected from light.

2.9. Western blot analysis

Western blotting was performed as previously described (110). In brief, after indicated treatments, cells were trypsinized, harvested, and washed with 1X PBS. Pellets were lysed and protein concentrations were determined by the Bradford Assay (Bio-Rad Laboratories, 5000205). Protein samples were loaded and subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and blocked with 5% milk in 1X PBS with 0.1% Tween 20 (Fisher, BP337). The membrane was incubated overnight at 4°C with the indicated primary antibodies at a dilution of 1:1000 in 5% BSA. The membrane was then washed, secondary antibody was added at a dilution of 1:2000 in 5% BSA for 2 h at room, and the membrane was washed again with 1X PBS with 0.1% Tween 20 three times. Blots were developed using Pierce enhanced chemiluminescence reagents (Thermo Scientific, 32132) on BioRad ChemiDoc System.
2.10. qRT-PCR

Fulvestrant + Palbociclib treated cells were harvested at Day 6 after drug treatment, and total RNA was extracted using RNeasy kit (QIAGEN, Germany) following the manufacturer’s instructions. cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad, USA) based on the protocol that manufacture provided. cDNAs from different samples were amplified in technical triplicates using iTaq Universal SYBR® Green Supermix from BioRad in QuantStudio™ 3 Real-Time PCR System (Thermofisher, USA). QuantiTect primers were purchased from Qiagen: CXCL8: QT0000322; IL-6: QT00083720; IL-1β: QT00021385; MMP3: QT00060025; GAPDH: QT00079247. Relative mRNA expression was determined using the ∆∆Ct method.

2.11. Cell proliferation assay

To determine the proliferation of T47D cells in real time, live cell imaging using IncuCyte S3 was performed. Cells were seeded in 96 well dish (1000 cells/well) and allowed to adhere overnight. The cells were exposed to Palbociclib (1 µM) in combination with Fulvestrant (100 nM) for 6 days and the cell division as monitored using IncuCyte that performs nuclei count. Following 6 days, the cells were released from the Palbociclib/Fulvestrant combination treatment and allowed to grow in the absence and presence of ARV825 (50 nM) for 96 H. Following 96H exposure, the cells were released from ARV825, and the cellular outgrowth was monitored. Based on the nuclei counts, the relative proliferation rate was determined. Growth curves were generated using GraphPad Prism.
2.12. DNA Damage

Cells were treated with Doxorubicin (50 nM), Etoposide (300 nM) and Paclitaxel (50 nM) for 24 hours. After drug removal cells were collected, washed with PBS and centrifuged. Pellets were resuspended in 70% ice cold Ethanol fixed and permeabilized for 30 min at RT. Then cells were washed in PBS again, resuspended in 5% BSA and blocked for 30 min on shaker. αH2AX conjugated antibody (BD Pharmigen, Cat#560445) was added 1:100 and cells were stained for 1.5 hours on shaker protected from light. Cells were washed and analyzed by flow cytometry.

2.13. Tumor Studies

BALB/c female mice 6-8 weeks of age weighting ~ 20 g (Jackson Laboratory) were used in animal studies. 5x10⁴ 4T1 cells were surgically transplanted into the fourth mammary fat pad. Tumors were measured by caliper measurements 3 days/week starting from day 7 post-transplantation. Doxorubicin (5mg/kg) doses were administered via i.p. once a week for three weeks and weight was monitored. For AU1 studies, AU1 was administered 2 days before doxorubicin treatment, and concurrently with the AU1+/−-doxorubicin groups. Tumors were harvested at week three for staining and analysis.
2.14. Statistics

Unless otherwise indicated, all quantitative data is shown as mean ± SEM from at least three independent experiments, all of which were conducted in triplicates or duplicates. GraphPad Prism 9.0 software was used for statistical analysis. All data was analyzed using either a one- or two-way ANOVA, as appropriate, with Tukey or Sidak post hoc.
Chapter 3: Evaluation of autophagy inhibition and BET inhibition as strategies to extend the response to Fulvestrant + Palbociclib in ER-positive breast cancer

3.1. Introduction

The number of new cases of invasive breast cancer in women is projected to be over 287,000 in 2022, resulting in over 43,000 deaths; in fact, rates of breast cancer have been gradually increasing by 0.5% per year since the mid-2000s (1). Amongst these cases, approximately 73% are comprised of hormone-receptor-positive breast cancer (2). The first-line hormonal therapy for this subtype of breast cancer utilizes selective estrogen receptor modulators (SERMs) such as tamoxifen, aromatase inhibitors (AIs), and selective estrogen receptor degraders (SERDs), such as Fulvestrant (6). Tamoxifen, a SERM that has been utilized in the clinic for decades, is usually prescribed to treat hormone receptor-positive, early-stage breast cancer after surgery to reduce disease recurrence. Despite reducing recurrence for up to five years, resistance to hormonal therapy is a major drawback to the success of these therapies and most cases eventually result in metastatic disease progression (9,10).
3.1.1. Standard of Care for ER-positive Her2-negative Breast Cancer

The current standard of care for metastatic ER-positive/Her2 negative breast cancer utilizes the combination of either the estrogen receptor degrader Fulvestrant or aromatase inhibitors such as letrozole with CDK4/6 inhibitors such as Palbociclib (5,11). Fulvestrant binds and destabilizes the estrogen receptor, resulting in destruction of the receptor by the cells' normal degradation pathways (111). CDK 4/6 inhibitors, such as Palbociclib, interfere with cell cycle progression by suppressing the CDK-cyclin complex mediated phosphorylation of Rb, thereby allowing the dephosphorylated form of Rb to bind to the transcriptional regulator, E2F, blocking G1/S cell cycle transition (11,112). In patients whose disease has progressed on prior endocrine therapy, the combination of Fulvestrant with Palbociclib had extended progression-free survival in breast cancer patients from 4.6 to 11.2 months (113). While these treatments represent remarkable improvements, escape from the tumor suppressive effects of even the combination therapies occurs frequently, with the consequence that the patients unfortunately succumb to this disease.

3.1.2. Autophagy inhibition and ER-positive Her2-negative Breast Cancer

Cancer

Almost invariably, autophagy also accompanies the induction of senescence by chemotherapy and radiation (114). Autophagy or “self-eating” is an evolutionarily conserved catabolic process through which cellular cargo is sequestered within a double membrane vesicle and ultimately undergoes lysosomal degradation. Many antitumor therapies, such as chemotherapy and radiation, have been shown to promote the cytoprotective form of autophagy, whereby autophagy inhibition sensitizes the tumors to
the therapy (115,116). It is well established in the literature that endocrine therapies display protective autophagy and consequently the autophagy inhibitors chloroquine and hydroxychloroquine show positive outcomes in combination with endocrine therapies, such as fulvestrant or tamoxifen, in preclinical studies (117). Additional studies have investigated the induction of autophagy by palbociclib and the impact of autophagy inhibition on drug sensitivity in breast tumor cells (118). These studies, however, did not involve the current standard of care combination of fulvestrant + Palbociclib.

3.1.3. BET inhibition/degradation and ER-positive Her2-negative Breast Cancer

The Bromodomain and extra terminal (BET) protein family comprises four proteins (BRD2, BRD3, BRD4 AND BRDT) which are epigenetic readers that bind to acetylated histones and synchronize gene transcription, by binding to different DNA or histone proteins laid down by writers, resulting in modifying cellular and physiological outcomes (119). BET proteins are fundamental for cell cycle progression, neurogenesis, maturation of erythroids, and other normal cellular processes (119). Research on BET proteins, specifically BRD4, indicate an association with numerous pathological conditions, such as cancer, inflammation, infection, renal and cardiac diseases (119,120). Additionally, inhibition of BRD4 has demonstrated senolysis which has been proposed to be through interference with non-homologous end joining (NHEJ) repair, intensifying DNA double-strand breaks, and the activation of autophagy in senescent cells (120). BRD4 also localizes to super enhancers, upstream of oncogenes such as c-Myc and BCL-XL (121). BRD4 amplification transcriptionally activates c-Myc (122), leading to promotion of
growth, invasion, and metastasis of cancer (123,124). c-Myc has been shown to be upregulated by estrogen in ER positive breast cancer cells, playing a crucial role in ER+ breast cell proliferation (125).

BET inhibitors, such as JQ1, have been studied for years as potential anti-tumor drugs. JQ1 has been used in combination with Fulvestrant showing increased survival in mice with tamoxifen- resistant breast cancer, ultimately through degradation of the estrogen receptor (126). In another study performed in human dermal fibroblasts treated with bleomycin, JQ1 was shown to have senolytic properties (126). Despite the toxicity of JQ1 being too high to be utilized in the clinic, the extensive studies with this compound provide proof of concept to drive the development of additional BET inhibitors or degraders with less toxicity. BET inhibitor PROTACS, and specifically ARV-825, have demonstrated activity against triple negative breast cancer (127), gastric cancer (128), neuroblastoma (129), thyroid cancer (130), and lymphoblastic leukemia (131).
3.1.4 Overarching hypotheses

It is well established that most conventional antitumor drugs and radiation promote autophagy and senescence. Since autophagy and senescence can contribute to survival of tumors, we hypothesize that following Fulvestrant+ Palbociclib therapy, the population of tumor cells undergo both autophagy and senescence; consequently, either autophagy inhibition or senolytic agents can prevent recovery of these senescent cells in ER+ breast cancer.

3.2. Results

3.2.1. The growth arrest response to Fulvestrant + Palbociclib in MCF7 breast tumor cells

While anti-estrogen therapy combined with CDK 4/6 inhibition is the standard of care for HR+/HER2- breast cancer patients diagnosed with locally advanced or metastatic disease progression following endocrine therapy, this combination therapy only modestly prolongs patient survival (132). In an effort to simulate the clinical treatment regimen in an in vitro environment, MCF7 cells were exposed to Fulvestrant and Palbociclib for 6 days and fresh media was replenished after drug removal on day 6. Cell viability was
assessed utilizing trypan blue exclusion on the indicated days (Figure 3.1A). Fulvestrant initially delayed tumor cell growth and, after a delay, arrested the cells. Palbociclib, alone, and in combination with Fulvestrant, completely halted the growth of the MCF7 cells; however, the cells generally began to recover from treatment after a period of approximately 8-12 days (see Figures 3.3, 3.5, 3.7, 3.10), which is consistent with earlier work from this and other laboratories (83,84,98).

The effectiveness of Palbociclib alone as well as the combination treatment in suppressing cell growth was confirmed by clonogenic survival studies (Figures 3.1B and C). The growth inhibitory effect of fulvestrant alone did not achieve significance, in contrast to the moderate effects in the temporal response assay; consequently, the observed effects are largely palbociclib driven. While therapy-induced tumor cell death is the desired outcome of anti-cancer treatment, there was a relatively low degree of apoptosis in MCF7 cells treated with Fulvestrant, Palbociclib or the combination (Figure 3.1D), which may be permissive for proliferative recovery. Consequently, one of the primary goals of the present work was to identify a strategy that might convert the growth arrest response to one of cell death, initially through efforts to block autophagy.
Figure 3.1 Fulvestrant in combination with Palbociclib sensitizes MCF7 cells. Cells were treated with Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days (A-C). (A) Viability of MCF7 cells was monitored based on trypan blue exclusion at indicated days following drug exposure (n=3). *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to Fulvestrant alone. (B) After exposure for 6 days, cells were incubated in fresh medium for 7 days. Quantification of colonies expressed as relative percentage compared to controls (n=3). (C) Representative images of colony formation with each treatment. (D) Apoptosis was measured using annexin V/PI staining at the end of the 6-day treatment and fluorescence was measured using flow cytometry (n=3). Unless stated otherwise, data were from three independent experiments. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak’s post hoc test.
### 3.2.2. Fulvestrant and Palbociclib-induced Autophagy

In response to therapy, cancer cells upregulate multiple mechanisms in attempts to evade cell death, one of which is autophagy (50,133). Autophagy is conventionally considered to be a cytoprotective process that allows cells to combat either intrinsic or extrinsic forms of injury; however, other functions of autophagy have been identified, specifically a cytotoxic form (43) and what has been termed as a nonprotective form (50,64,134). Consequently, we examined whether autophagy was induced in response to the anti-estrogen, Fulvestrant, and CDK4/6 inhibition therapy in MCF7 cells. Initially, acridine orange was utilized at day 4 to assess acidic vesicle formation. **Figure 3.2A** demonstrates basal autophagy in these cells as well as increased acidic vesicle generation in response to Fulvestrant or/and Palbociclib. **Figure 3.2B** further provides quantification of fluorescence via flow cytometry and indicates that autophagy is marginally higher for the combination treatment at day 3 and significantly higher at day 6. To verify autophagy induction, western blot analysis in **Figure 3.2C** revealed a temporal decline in p62/SQSTM1 levels in MCF7 cells treated with Fulvestrant, Palbociclib and the drug combination.
Figure 3.2 Fulvestrant in combination with Palbociclib induces autophagy. Cells were treated with Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days (A-C). Lysates were collected at specified days (A) Cells were stained with acridine orange on day 4 and imaged using a fluorescent microscope. All images were taken at the same magnification (scale bar= 200 µm, n=3). (B) Cells were stained with acridine orange and fluorescence was quantified using flow cytometry. (C) Autophagy induction over time was assessed by degradation of p62/SQSTM1 protein levels. All images are representative fields or blots from at least three independent experiments. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to Fulvestrant as determined using two-way ANOVA with Sidak's post hoc test.
3.2.3. Efforts to sensitize MCF-7 breast tumor cells via early autophagy inhibition

In an effort to sensitize MCF7 cells to the Fulvestrant, Palbociclib, and the combination therapy, the lysosomotropic agents, chloroquine (CQ) and bafilomycin A1 (Baf), were utilized as pharmacological inhibitors of autophagy, based on their ability to interfere with autophagosome-lysosome fusion (135). Chloroquine and bafilomycin were added concurrently with the Fulvestrant + Palbociclib for 48 hrs; CQ and Baf were removed, and the Fulvestrant + Palbociclib treatment restored for an additional 4 days (Figure 3.3A). In order to visualize autophagy, acridine orange staining was utilized as it fluoresces bright orange in acidic environments, such as the lysosomes, and shifts to an orange/yellow color when pH becomes less acidic (136,137). We also observed an increased number of autophagic vacuoles upon addition of the CQ and Baf, which is indicative of autophagic vacuole accumulation when the autophagic process is prevented from going to completion (Figure 3.3B) (138). The inhibition of early autophagy in cells exposed to CQ and Baf was further confirmed by western blot analysis of LC3-II and p62/SQSTM1. We observed an increase in accumulation of LC3-II in the presence of CQ and Baf (due to inhibition of autolysosome formation as well as accumulation of autophagosomes) in both control and drug treated groups, indicating that CQ and Baf inhibited both basal and treatment-induced autophagy (Figure 3.3C). Accumulation of p62/SQSTM1 indicative of interference with p62/SQSTM1 degradation further confirmed that CQ and Baf inhibited treatment-induced autophagy. However, autophagy inhibition failed to sensitize the MCF-7 cells to Fulvestrant, Palbociclib or the combination therapy
(Figure 3.3D-F), indicating that the autophagy was functionally nonprotective, as we have observed previously in other experimental systems (47,139,140). Of note is the recovery of proliferative capacity that occurs between days 8-12, which is indicative of escape from senescence (see below). Consistent with the lack of sensitization, pharmacological autophagy inhibition did not significantly promote apoptotic cell death by the combination of the anti-estrogen and CDK 4/6 inhibitor (Figure 3G-I).
Figure 3.3 Early autophagy inhibition does not alter sensitivity to Fulvestrant in combination with Palbociclib in MCF7 cells. Cells were pre-treated for 3 h with either CQ (10 μM) or Baf A1 (2.5 nM). CQ and Baf A1 were given for an additional 48 h, alongside the treatment with Fulvestrant (100 nM), Palbociclib (1 μM) or the combination for 6 days. (A) Schematic of in vitro treatment. (B) Cells were stained with acridine orange on day 3 and imaged using a fluorescent microscope. All images were taken at the same magnification (scale bar=200 μm, n=3). (C) Western blot analysis at day 3 assessing accumulation of LC3 I-II and p62/SQSTM1 protein levels. (D-F) Viable cell number was counted via trypan blue exclusion on the indicated days. (E) Apoptosis was measured by annexin V/PI staining. Staining was performed on day 6 and fluorescence was measured using flow cytometry. All images are representative fields, blots, or data from at least three independent experiments. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to Fulvestrant, Palbociclib or the combination of Fulvestrant and Palbociclib as determined using two-way ANOVA with Sidak’s post hoc test.
3.2.4. Efforts to sensitize T47D breast tumor cells via early autophagy inhibition

Approximately 20% of ER positive breast cancers present with p53 mutations (141). In order to evaluate whether autophagy inhibition would also be effective against p53 mutant ER+ breast tumors treated with the Fulvestrant + Palbociclib combination, we assessed the number of viable cells at day 6 using MTS cell viability assay. Looking at percent viability, autophagy inhibition appeared to not sensitize T47D cells to the Fulvestrant + Palbociclib combination treatment (Figure 3.4B), indicating that the autophagy was functionally nonprotective. We observed an increase in accumulation of LC3-II in the presence of CQ and Baf in both control and drug treated groups, indicating that CQ and Baf inhibited both basal and treatment-induced autophagy in T47D cells (Figure 3.4C).
Figure 3.4 Early Autophagy inhibition does not alter sensitivity to Fulvestrant in combination with Palbociclib in T47D cells. Cells were pre-treated for 3 h with either CQ (10 uM) or Baf A1 (2.5 nM). CQ and Baf A1 were given for an additional 48 h, alongside the treatment with Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days. (A) Schematic of in vitro treatment of early addition of CQ and Baf. (B) Percent cell viability was measured using MTS viability assay. (C) Western blot analysis at day 6 assessing accumulation of LC3 I-II. (D) Schematic of in vitro treatment of late addition of CQ and Baf. (E) Percent cell viability was measured using MTS viability assay. (F) Western blot analysis at day 6 assessing accumulation of LC3 I-II.
3.2.5. Efforts to sensitize MCF-7 breast tumor cells via late autophagy inhibition

Conventionally, most preclinical, and clinical studies designed to evaluate the effect of autophagy inhibition involve the addition of the pharmacological autophagy inhibitors concurrently or as a pre-treatment to therapy (40,139,142). Preliminary experiments (not shown) suggested the possibility that a more delayed approach to autophagy inhibition might prove to be a more effective sensitization strategy. Consequently, MCF7 cells were treated with Fulvestrant, Palbociclib or the combination for 6 days, and then exposed to CQ or Baf for 48 hrs post treatment (Figure 3.5A). Similar to the experiments in Figure 3.3, Figure 3.5B demonstrates autophagy inhibition based on interference with lysosomal acidification by acridine orange staining. Autophagy inhibition by Baf and CQ was additionally confirmed by western blot analysis of LC3-II and p62/SQSTM1. Similar to the data generated in Figure 3.3, we observed an increase in LC3-II accumulation (Figure 3.5C). and accumulation of p62/SQTM1 degradation with CQ and Baf. Figures 3.5D-F indicate that while addition of CQ or Baf provided a modest degree of sensitization to Fulvestrant alone, in terms of delaying proliferative recovery, sensitization of the MCF7 cells to Palbociclib or the combination treatment was barely significant. Additionally, there was minimal promotion of apoptosis with autophagy inhibition, (Figure 3.5G-H), again consistent with the autophagy being largely nonprotective in function.
Figure 3.5 Late autophagy inhibition only moderately increases sensitivity to Fulvestrant in combination with Palbociclib in MCF7 cells. Cells were treated with Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days, drugs were removed, and cells were given an additional 48 h CQ (10 µM) or Baf A1 (2.5 nM). (A) Schematic of in vitro treatment. (B) Cells were stained with acridine orange on day 8 and imaged using a fluorescent microscope. All images were taken at the same magnification (scale bar= 200 µm, n=3). (C) Autophagy inhibition was confirmed by western blot analysis assessing accumulation of LC3 I-II and p62/SQSTM1 protein levels. (D-F) Viable cell number was counted via trypan blue exclusion on the indicated days. (G-I) Apoptosis was measured using annexin V/PI staining. Staining was performed on day 8 and fluorescence was measured using flow cytometry. All images are representative fields, blots or data from at least three independent experiments. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to Control as determined using two-way ANOVA with Sidak's post hoc test.
3.2.6. Efforts to sensitize T47D breast tumor cells via late autophagy inhibition

In order to evaluate whether late autophagy inhibition would be effective against p53 mutant ER+ breast tumors treated with the Fulvestrant + Palbociclib combination, we assessed the number of viable cells at day 8 using the MTS cell viability assay. T47D cells were treated with Fulvestrant + Palbociclib for 6 days followed by 48-hour exposure to CQ or BAF A1 (Figure 3.6A). Looking at percent viability, autophagy inhibition failed to sensitize T47D cells after exposure to the Fulvestrant + Palbociclib combination treatment (Figure 3.6B), indicating that the autophagy was, again, functionally nonprotective. We observed an increase in accumulation of LC3-II in the presence of CQ and Baf in both control and drug treated groups, indicating that CQ and Baf inhibited both basal and treatment-induced autophagy in T47D cells (Figure 3.6C).
Figure 3.6 Late Autophagy inhibition does not alter sensitivity to Fulvestrant in combination with Palbociclib in T47D cells. T47D cells were treated with Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days, drugs were removed, and cells were given an additional 48 h CQ (10 µM) or Baf A1 (2.5 nM). (A) Schematic of in vitro treatment of late addition of CQ and Baf. (B) Percent cell viability was measured at day 8 using MTS viability assay. (C) Western blot analysis at day 8 assessing accumulation of LC3 I-II.
3.2.7. Efforts to sensitize MCF-7 breast tumor cells via autophagy inhibition via genetic silencing

To further confirm the absence of a pronounced sensitization to Fulvestrant, Palbociclib and the combination treatment via autophagy inhibition, the MCF-7 cells were stably transfected using short hairpin RNA for ATG5 (shATG5) or scrambled control (shControl). Knockdown of ATG5 and impairment of autophagy was confirmed by western blot analysis indicating reduced levels of ATG5 and accumulation of p62/SQSTM1 (Figure 3.7A). Temporal analysis of cell viability showed that autophagy deficient cells were significantly more sensitive to Fulvestrant, as was the case with pharmacologic autophagy inhibition; however, the MCF7 shATG5 cells were only slightly more sensitive to Palbociclib and the Palbociclib + Fulvestrant combination therapy when compared to shControl cells (Figure 3.7B-D). These data with genetic knockdown of ATG5 are consistent with the pharmacological inhibition of autophagy with CQ and Baf (Figures 3.3, 3.5). Genetic silencing of autophagy also did not promote apoptosis in MCF-7 cells when exposed to Ful or Pablo, but exhibited significant, albeit minimal apoptotic cell death with the combination treatment compared to shControl MCF7 cells (Figure 3.7E). Taken together, these studies indicate that the autophagy induced by the Fulvestrant, Palbociclib, and the combination treatment is largely nonprotective, and consequently that autophagy inhibition is not likely to be of significant biological or clinical relevance to disease treatment (47,140).
Figure 3.7 Genetic knockdown of autophagy only moderately increases sensitivity to Fulvestrant in combination with Palbociclib in MCF7 cells. Cells were treated with Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days (A-D). Short hairpin RNA was used to knockdown ATG5. (A) Western blot analysis was used to show reduced ATG5 protein levels. Autophagy inhibition was confirmed by assessing accumulation of p62/SQSTM1 protein levels. (B-D) Viable cell number was counted via trypan blue exclusion on the indicated days. (E) Apoptosis was measured using annexin V/PI staining. Staining was performed on day 8 and fluorescence was measured using flow cytometry. Unless stated, otherwise data were from three independent experiments. #p < 0.05 compared to shControl cells treated with Fulvestrant, Palbociclib or the combination of Fulvestrant and Palbociclib.
3.2.8. Fulvestrant + Palbociclib induce senescence in MCF-7 breast tumor cells

Given that administration of Fulvestrant + Palbociclib either alone or in combination, induces a transient growth arrest, and that autophagy and senescence tend to occur in parallel, we examined senescence induction, a durable growth arrest induced by therapy (110,114,143–145). Previous work from our group and others has consistently shown proliferative recovery from various models of therapy induced senescence (83,84,89,98–100). Figure 3.8A demonstrates the promotion of senescence by β-gal staining with exposure to Fulvestrant alone, Palbociclib alone and the Fulvestrant and Palbociclib combination in MCF7 cells as well as morphological changes (cell enlargement and flattening) associated with senescence (143). Senescence was further confirmed using flow cytometry quantification of C12FDG fluorescence, a metabolite for SA-β-gal. Figure 3.8B demonstrates increased SA-β-gal activity in Fulvestrant, Palbociclib and combination treated MCF7 cells when compared to untreated controls. The senescence in the combination therapy appears to be largely due to the Palbociclib (144,146). Figure 3.8C presents qRTPCR data indicating significant increases of the expression of IL-6, IL-8, and MMP3, components of the senescence-associated secretory phenotype (143,147), at Day 6 post-combination treatment with Fulvestrant and Palbociclib.
Figure 3.8 Fulvestrant in combination with Palbociclib induced senescence in MCF-7 cells. Cells were treated with Fulvestrant (100nM), Palbociclib (1uM) or the combination for 6 days. (A) Cells were fixed on Day 6, stained with x-gal staining solution, and imaged using brightfield microscope. All images were taken with the same magnification. (B) Quantification of SA-βgal using C12FDG at indicated timepoints. (C) qRT-PCR examining SASP mRNA expression of IL-6, IL-8, and MMP3 on D6 post-combination treatment. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak’s post hoc test. All images are representative fields, blots, or data from three independent experiments (n = 3).
3.2.9. Lack of effect of conventional senolytics

Given the extensive literature demonstrating that targeting Bcl-XL is an effective senolytic strategy, we tested the Bcl-XL inhibitor, ABT-263, along with other therapies which have recently been studied in the literature as senolytics. ABT-263 is a BH3 mimetic compound that binds to pro-survival BCL-2 family member proteins, preventing their association with pro-death BCL-2 family members, resulting in cell death (148). ABT-263 inhibits the functional activities of BCL-2, BCL-XL, BCL-W, but not MCL-1 (148). The effectiveness of the senolytic actions of ABT-263 has been observed to be dependent on BCL-XL and BCL-W, while BCL-2 inhibition is not required (149,150). We additionally evaluated an MCL-1 inhibitor, S63845, and the HSP-90 inhibitor, 17DMAG, against MCF-7 cells induced into senescence by fulvestrant + palbociclib. However, none of these suppressed the growth/survival of these cells (Fig 3.9 A). Another resistance mechanism to ABT263 could be through low levels of NOXA, a BH3-only protein that can neutralize the anti-apoptotic protein MCL-1 (153).

To evaluate the efficacy of ABT-263 and BCL-2 inhibitor ABT-199 long-term, cell viability was assessed utilizing trypan blue exclusion up to 12 days (Fig. 3.9 B,D). There was no significant reduction in the number of viable cells in treated groups for over 12 days, and the cells appeared to recover quickly independent of ABT-199 and ABT-263. Consistent with these outcomes, we confirmed there was no change in BCL-XL protein expression at days 2,4 and 6 of the treatment with Fulvestrant +Palbociclib, which could explain why BCL inhibition is not effective (Fig. 3.9 C).
Figure 3.9 Conventional senolytics have no impact on cell viability after Fulvestrant + Palbociclib treatment in MCF7 cells. Cells were treated with the Fulvestrant (100 nM) + Palbociclib (1 µM) combination for 6 days, followed by drug removal and the addition of senolytic drugs for 48 hours. (A) Cell viability on day 8, following addition of conventional senolytics MCL1, ABT-263, 17DG. (B,D) Viable cell number was counted via trypan blue exclusion on the indicated days. (C) Western blot analysis of BCL-XL protein levels.
3.2.10. Fulvestrant + Palbociclib induce senescence in MCF-7 breast tumor cells and ARV-825 extends growth delay and suppresses proliferative recovery

Inhibitors of bromodomain-containing protein 4 (BRD4), particularly ARV-825, have demonstrated antitumor activity in multiple preclinical models, and have recently been considered as potential senolytics (120). To investigate whether ARV-825 might act as a senolytic in combination with the senescence induced by the Fulvestrant + Palbociclib combination, cells were treated for 6 days with Fulvestrant (100 nM) and Palbociclib (1 μM), followed by ARV-825 (50 nM) for 96 h post-treatment. Temporal analysis of cell viability demonstrated that ARV-825, alone, moderately suppressed growth of the MCF-7 cells (Figure 3.10A); this is consistent with prior literature studies of the action of ARV-825 (128,154), and with the degradation of BRD4 and the suppression of downstream c-Myc shown in Fig. 3.10C. Our laboratory as well as others have shown that c-Myc is upregulated in breast cancer and involved in breast cancer proliferation (128,155,156).

The most critical observation in this work is that ARV-825 treatment sequentially after the Fulvestrant + Palbociclib combination resulted in a prolonged growth arrest with suppression of proliferative recovery (Figure 3.10A). This finding is consistent with the pronounced suppression of both BRD4 and c-Myc for the combination of Fulvestrant + Palbociclib and ARV-825 in Figure 3.10C. Although the addition of ARV-825 to the Fulvestrant + Palbociclib combination appeared to result in prolongation of growth arrest
rather than cell killing, this therapeutic strategy nevertheless demonstrated some characteristics of senolysis, specifically the promotion of apoptosis. As shown in Figure 3.10B, a significant increase in apoptosis was evident at Day 10 with ARV-825 treatment compared to the combination treatment alone; the percentage of apoptosis stayed relatively constant through day 14 (data not shown).
Figure 3.10 ARV prolongs growth arrest induced by Fulvestrant in combination with Palbociclib in MCF-10A cells. Cells were treated with Fulvestrant (100nM), Palbociclib (1µM) or the combination for 6 days. (A) Cell viability was monitored over a period of 14 days by trypan blue exclusion. (B) Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit. (C) Western blotting for BRD4, c-Myc at day 4 of ARV treatment. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak’s post hoc test. All images are representative fields, blots, or data from three independent experiments (n = 3).
3.2.11. ARV-825 induces increased apoptosis in sorted senescent MCF-7 cells

Although the combination treatment promotes substantial senescence in the MCF-7 cells (Figure 3.8A,B), the entire cell population is not senescent, and consequently it was necessary to address whether the ARV-825 was functioning as a senolytic and that the senescent cell population might be particularly vulnerable to the ARV-825. To address this question, cells were sorted by flow cytometry to distinguish the SA-β-Gal highly positive and low positive populations (high 30% and low 20% SA-β-Gal positive). Control cells were also sorted for SA-β-Gal positive cells (Figure 3.11A). Flow cytometry was performed post-sorting for C12FDG staining to confirm the senescent population (Figure 3.11B). Figure 3.11C indicates that the senescent high cell population underwent significantly more apoptosis compared to the non-senescent cells after treatment with Fulvestrant/Palbociclib + ARV-825. These data indicate that the senescence induced by the combination of an anti-estrogen and CDK4/6 inhibitor increases susceptibility to ARV-825 induced apoptotic cell death.
Figure 3.11 ARV prolongs growth arrest induced by Fulvestrant in combination with Palbociclib in MCF- cells. Cells were treated with Fulvestrant + Palbociclib combination for 6 days prior to cell sorting. (A) Sorted cells was analyzed by flow cytometry to verify purity of the SA-β-Gal positive population (high 30% and low 20% SA-β-Gal positive). Control cells were sorted for high 80% SA-β-Gal positive. (B) Flow cytometry was performed post-sort for C12FDG staining to confirm senescence induction. (C) Sorted senescent cells were plated for 24-hours and treated with ARV-825 for 4 days followed by annexin V/PI apoptosis staining. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak’s post hoc test. All images are representative fields, blots, or data from three independent experiments (n = 3).

Approximately 20% of ER positive breast cancers present with p53 mutations (141). To evaluate whether ARV-825 would also be effective against p53 mutant ER+ breast tumors treated with the Fulvestrant + Palbociclib combination, we assessed the temporal response by real-time, live cell imaging using IncuCyte S3 in T47D breast tumor cells. Analogous to the outcomes in MCF-7 cells (Figure 3.10A,B), we observed that the combination of Fulvestrant + Palbociclib induced senescence based on senescence associated β-galactosidase staining (Figure 3.12A,B) that was followed by proliferative recovery (Figure 3.12C); as in the studies with the p53 wild-type MCF-7 cells, the addition of ARV-825 resulted in prolonged growth arrest without recovery, at least over the ~13-day time course of this study (Figure 3.12C). Quantifying the extent of senescence using C12FDG staining indicated that approximately 40% of the population represented senescent cells at both day 6 and day 8 (Figure 3.12B). Analysis of apoptosis indicated that there was no significant difference with the combination + ARV825, compared to the combination alone (Figure 3.12D), despite some evidence of a decline in cell number in the temporal response study. Additionally, western blot analysis confirmed a reduction in target protein, BRD4, by ARV-825 in both control and Fulvestrant + Palbociclib (Figure 3.12E). Similarly, a profound suppression of downstream c-Myc by ARV-825 is evident in control cells, the combination alone and the combination + ARV825 (Figure 3.12E).
Figure 3.12 ARV prolongs growth arrest induced by Fulvestrant in combination with Palbociclib in p53 mutant T-47D cells. T47D-WT cells that were treated with palbociclib (1 µM) in combination with Fulvestrant (100 nM) for 6 days. (A) Cells were fixed on Day 6, stained with x-gal staining solution, and imaged using brightfield microscope. All images were taken with the same magnification. (B) Quantification of SA-βgal using C12FDG at indicated timepoints. (C) Live cell viability was monitored via IncuCyte over a period of 14 days and normalized to GFP count. (D) Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit. (E) Western blotting for BRD4, c-Myc at day 4 of ARV treatment. Mean and SD were determined based on triplicates from 3 independent experiments. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak’s post hoc test.
3.2.13. Sensitization by ARV-825 in resistant MCF-7 cells deficient in Rb and treated with the fulvestrant + palbociclib combination.

One common mechanism of resistance that develops to palbociclib therapy in ER positive breast tumor cells is the loss of Rb, which prevents the cells from entering into a state of senescence (157). However, it is possible that the ARV-825 is not acting as a classical senolytic, which might make cells treated with fulvestrant + palbociclib susceptible to cell killing even where senescence is not the primary response. To address this possibility, MCF-7 and T47D cells, where Rb function was genetically deleted, were exposed to the fulvestrant + palbociclib combination, followed by ARV-825. As shown in Figure 3.13A, in MCF-7 Rb deleted cells, the combination treatment promotes delayed growth compared to control, followed by proliferative recovery, similar to what we observe with Rb competent MCF-7 cells. Figure 3.13B demonstrates the lack of senescence induction with MCF-7 Rb deleted cells, which was confirmed with quantification of senescence using C12FDG where we observe a relatively low percentage of senescent cells (Figure 3.13C). The addition of ARV-825 to the combination treatment of fulvestrant + palbociclib clearly promotes an increase in apoptosis over the low level of apoptosis induced by fulvestrant + palbociclib (Figure 3.13D). Figure 3.13E indicates that ARV-825 suppressed both the target protein, BRD4, as well c-Myc, which is presumably downstream of BRD4, in both control and fulvestrant + palbociclib treated cells.
Figure 3.13 ARV-825 prolongs growth arrest induced by the combination of Fulvestrant and Palbociclib in resistant MCF-7 cells. Live cell imaging in MCF-7 Rb-del cells that were treated with palbociclib (1 µM) in combination with fulvestrant (100 nM) for 6 days. (A) Live cell viability of Rb-deficient MCF-7 cells was monitored via IncuCyte over a period of 14 days and normalized to GFP count. (B) MCF-7 Rb deleted cells were fixed on Day 6, stained with x-gal staining solution, and imaged using brightfield microscope. All images were taken with the same magnification. (C) Quantification of SA-Beta gal using C12FDG at indicated timepoints. (D) Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit. (E) Western blotting for BRD4, c-Myc at day 4 of ARV treatment. Mean and SEM were calculated based on triplicates from 2 independent experiments. (**P≤ 0.001 as determined by 2-way ANOVA).
3.2.14. Sensitization by ARV-825 in resistant T47D cells deficient in Rb and treated with the fulvestrant + palbociclib combination.

In contrast to the MCF-7/Rb deleted cells, growth arrest by the treatment with the fulvestrant + palbociclib combination is markedly delayed in the T47D Rb deleted cells; nevertheless, the addition of ARV-825 significantly suppresses growth (Figure 3.14A). As was largely the case with the MCF-7/Rb deleted cells, the combination treatment fails to promote senescence (Figure 3.14B), as further confirmed by C₁₂FDG quantification (Figure 3.14C). Furthermore, while MCF-7 Rb deficient cells demonstrated enhanced apoptosis with the combination treatment followed by ARV-825 (Figure 3.13D), there was no increase in apoptosis in the T47D Rb deficient cells (Figure 3.14D). This is likely related to the absence of functional p53, as there was also no increase in apoptosis in the Rb proficient T-47D cells (Figure 3.12D). Finally, the targeting of BRD4 and c-Myc by the ARV-825 was confirmed by the Western blot presented in Figure 3.14E.
Figure 3.14 ARV-825 prolongs growth arrest induced by the combination of Fulvestrant and Palbociclib in resistant T47D cells. (A) Live cell viability of Rb-deficient T47D cells was monitored via IncuCyte over a period of 14 days and normalized to GFP count. (B) Cells were fixed on Day 6, stained with x-gal staining solution, and imaged using brightfield microscope. All images were taken with the same magnification. (C) Quantification of SA-Beta gal using C12FDG at indicated timepoints. (D) Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit. (E) Western blotting for BRD4, c-Myc at day 4 of ARV treatment. Mean and SEM were calculated based on triplicates from 2 independent experiments. (**P ≤ 0.001 as determined by 2-way ANOVA).
3.3. Discussion

Disease recurrence, both local and distal, is an ongoing issue contributing to the majority of hormone receptor-positive breast cancer deaths and is observed in many other types of cancers, such as triple-negative breast cancer, lung, and prostate cancer. Often this recurrence can be associated with therapy-induced residual dormant tumor cell populations, that can escape and often become more aggressive in nature (158–160). While therapy-induced senescence has been studied for decades, successful utilization of senolytics in cancer treatment has not yet been implemented. Despite this, several senolytic agents have been considered and studied to modulate and eliminate senescent tumor cells.

Another potential route for modulation of drug sensitivity in cancer is autophagy inhibition, (42,116). Estrogen receptor-targeted therapies are generally the first-line treatment for hormone receptor-positive breast cancer and autophagy in response to tamoxifen has been shown quite convincingly to be cytoprotective (161). In fact, this form of autophagy has been shown to lead to the development of resistance to anti-estrogen therapies (35,40,162). In cases where autophagy is cytoprotective, pharmacological inhibition of autophagy may be utilized to enhance the tumor cell sensitivity to treatment. In addition to cytoprotective autophagy, we have identified a non-protective form of autophagy, which apparently plays no distinct role in promoting or suppressing the growth or sensitivity of the tumor cells in response to therapy (42,47,138). Clinical trials are currently underway evaluating HCQ as a pre-treatment with the combination of letrozole + Palbociclib, based on preclinical studies showing efficacy in autophagy inhibition as a pretreatment with this combination (118).
We and others have demonstrated that the senolytic ABT-263 (navitoclax), is effective at the elimination of tumor cells induced into senescence by various chemotherapeutic strategies in breast, lung, head and neck and prostate tumor cells (89,99,100). However, one fundamental limitation in the use of ABT-263 is thrombocytopenia, given that the target of ABT-263 is the Bcl-xL protein, upon which platelets depend for survival (105). While ABT-263 was effective in many cancer types, we did not observe any effect in MCF-7 breast cancer cells.

The current work evaluated the potential of utilizing autophagy inhibition, before and after treatment, to sensitize ER positive MCF-7 breast tumor cells to the combination of Fulvestrant + Palbociclib. This treatment promotes significant growth arrest and both autophagy and senescence induction after 6 days of treatment with Fulvestrant and Palbociclib. Early administration of pharmacological autophagy inhibitors did not improve tumor cell responsiveness to the combination treatment, leading to the conclusion that the autophagy was non-protective. Slight sensitization was evident with late addition of pharmacological autophagy inhibitors as well as with genetic knockdown. The latter was most pronounced with Fulvestrant, which may relate to previous studies demonstrating that ER targeted therapies are generally cytoprotective (38,39). However, the autophagy for the combination treatment appears to be largely nonprotective, indicating that autophagy inhibition is unlikely to become a clinically useful therapeutic strategy (42,163). This does not rule out the possibility that the autophagy induced by aromatase inhibitors in combination with CDK4/6 inhibitors could be cytoprotective and amenable to autophagy inhibition in the clinic.
Our studies further examined the incorporation of the BET degrader, ARV-825, into the combination treatment. ARV-825 has been used in pre-clinical studies with different cancer types, and we hypothesized that ARV-825 could potentially improve ER+ breast cancer tumor response following Fulvestrant + Palbociclib treatment (59–62). ARV-825 suppressed tumor growth for both the ER positive p53 wt MCF-7 cells and the ER positive p53 mutant T-47D cells, significantly delaying proliferative recovery. We also observed significant induction of apoptosis in the MCF-7 cells treated with Fulvestrant + Palbociclib followed by ARV-825, but not in T47D cells, which may be due to T-47D p53 mutational status. The senescent MCF-7 cell population appears to be more susceptible to ARV-825 induced apoptosis, although a low degree of apoptosis is also observed in non-senescent cells exposed to the ARV-825. While the combination of Fulvestrant + Palbociclib rapidly induced growth arrest in MCF-7 Rb deficient cells, the growth was delayed in the T47D Rb deficient cells. The observed growth arrest was confirmed to be independent of senescence, as Rb loss is a well-known factor of resistance to CDK 4/6 inhibitors (167,168).

The observed growth arrest is consistent with the degradation of BRD4 and the suppression of downstream c-Myc as well as with previous studies by our laboratory and others demonstrating c-Myc to be upregulated in ER+ breast cancer and involved in breast cancer proliferation (128,155,156). These findings are supported by an analysis of three patient database sets, demonstrating that higher expression of BRD4 in breast cancer patients correlated with overall lower recurrence-free survival when compared to patients with lower BRD4 expression levels (122).
Taken together, the current studies indicate that administration of BET inhibitors/degraders may potentially improve the standard of care therapy in metastatic ER+ breast cancer patients and may further prolong progression-free survival. The BET inhibitors, ABBV-075 and ABBV-744, have shown promise in preclinical studies and will be tested in the future both in cell culture and in tumor-bearing animal models (169–171).
Chapter 4: Suppression of Epigenetic Regulator

NURF leads to autophagy mediated sensitization of triple negative breast cancer

4.1. Introduction

Triple-negative breast cancer (TNBC) comprises approximately 10% of breast cancer cases and is generally more aggressive than other types of breast cancer (14). In contrast, despite being the lowest occurring subtype, the 5-year survival of TNBC patients is around 76 percent, making it the most-deadly subtype (3). TNBC is chemotherapy sensitive, making it the standard of care. In the case of early diagnosis, neoadjuvant chemotherapy is normally utilized followed by surgery (14). The most common chemotherapies used include topoisomerase II inhibitors (doxorubicin), alkylating agents (cyclophosphomide), anti-microtubule agents (Paclitaxel), and anti-metabolites fluorouracil (5-FU) (14).

4.1.1. Involvement of NURF in Cancer

Cancers often undergo genetic and epigenetic changes which lead to resistance to both chemotherapy and immune surveillance (172). Epigenetic regulators play many important roles in cancer biology, making them a potential target for cancer therapies (172). The
Nucleosome Remodeling Factor (NURF) is an ATP-dependent chromatin remodeling enzyme that regulates gene expression by sliding the nucleosome along the DNA. NURF contains three subunits: pRBAP46/48, SNF2L, and the bromodomain PHD-finger containing transcriptional factor (BPTF) (173). BPTF is overexpressed in many cancer types, including TNBC, and is linked in resistance to therapy by promoting the expression of the anti-apoptotic protein, BCL-2, and the oncogenic protein, c-Myc (174). High expression of BPTF correlates with decreased patient survival (Figure 4.1.1). BPTF has also been associated with suppression of CD4+, CD8+ T cell and NK cell mediated antitumor immunity through suppression of antigen presentation and ligand expression (175). AU1 is a selective pharmacological inhibitor BPTF and one of the first designed to target BPTF.

![Figure 4 Clinical Significance of BPTF in BRCA-basal-like (TNBC) breast cancer. Survival analysis of BPTF high and low expression in BRCA patient samples using TIMER database.](image-url)
4.1.2 Overarching hypotheses

We hypothesize that BPTF/NURF inhibition could be utilized to enhance the response to conventional drugs utilized against TNBC as well as to overcome intrinsic and acquired drug resistance.

4.2. Results

4.2.1. Inhibition of NURF sensitizes breast tumor cells to specific chemotherapeutic agents

In order to determine the effect of NURF inhibition on sensitivity of TNBC to multiple chemotherapeutic drugs, members of the Landry lab initially assayed sensitivity to doxorubicin, etoposide, paclitaxel, and cisplatin as well as ionizing radiation utilizing clonogenic survival assays. These studies demonstrated that the genetic inhibition of BPTF sensitized 4T1 cells to the topoisomerase 2 inhibitors doxorubicin and etoposide and the microtubule inhibitor paclitaxel, but did not sensitize to radiation, the DNA cross-linking agent cisplatin, or the antimetabolite, 5-flourouracil (5-FU) (Figure 4.1). These data suggest that the BPTF KD can sensitize tumor cells to therapies with different mechanisms of action.
Figure 4.1 Inhibition of NURF sensitizes breast tumor cells to specific chemotherapeutic agents. Fraction of viable 4T1 cells after 24-hour treatment with doxorubicin (50nM), Etoposide (500nM), radiation (5Gy), paclitaxel (50nM), cisplatin (500nM), and 5-fluorouracil (5µM). Mean and SEM were calculated based on triplicates from 3 independent experiments. (**P≤ 0.001 as determined by 2-way ANOVA).
4.2.2. BPTF sensitization to doxorubicin independent of senescence and apoptosis

Chemotherapies, such as doxorubicin, etoposide, and paclitaxel have been shown to induce apoptosis, growth arrest, autophagy, and/or DNA damage. All these factors may contribute to cell death. To elucidate the mechanism of sensitization observed previously in Figure 4.1, these potential endpoints were assessed by flow cytometry. An analysis of apoptosis in response to etoposide, and paclitaxel indicated that the BPTF KD moderately increased apoptosis with paclitaxel and etoposide (Figure 4.2A); however, there was no significant alteration in apoptosis with doxorubicin with the BPTF KDs. Senescence was quantified by measuring SA-β-galactosidase activity using C12FDG. While doxorubicin, etoposide, and paclitaxel all induced cellular senescence, the BPTF KD had no impact on the degree of senescence (Figure 4.2B). AO staining shows an extensive increase in autophagic vesicles with doxorubicin and etoposide in the BPTF KD cells, while there is no difference in autophagy with paclitaxel in the BPTF KD cells (Figure 4.2C). Therefore, the observed sensitization could be driven by BPTFs regulation of autophagy, but not through regulation of apoptosis or senescence with doxorubicin and etoposide treatment.

Another potential mechanism contributing to the observed sensitization with doxorubicin and etoposide could be through the regulation of TOP2 crosslinking, resulting in enhanced DNA double strand breaks. To explore this possibility, analysis of the DNA-damage marker, γ-H2AX, revealed a significant increase in DNA damage with doxorubicin and etoposide, but not paclitaxel, in the BPTF KDs. This might be expected since paclitaxel is not considered to act through the promotion of DNA damage. These
data suggest a potential protective role of BPTF and NURF in autophagy and DNA damage repair for doxorubicin and etoposide’s mechanism of action.
Figure 4.2 Inhibition of NURF sensitzes breast tumor cells to specific chemotherapy. 4T1 cells were treated for 48 hours with doxorubicin (50nM), Etoposide (500nM), and Paclitaxel (50nM), followed by 48 hours of recovery. A. Apoptosis was measured using annexin V/PI staining. B. SA-βgal was measured using C12FDG. C. Autophagy was measured using AO staining. D. DNA damage was measured using γ-H2AX staining. Mean and SEM were calculated based on triplicates from 3 independent experiments. (***P ≤ 0.001 as determined by 2-way ANOVA).
4.2.3. Autophagy modulates sensitivity to doxorubicin

In order to elucidate whether the sensitization observed in Figure 4.1 was conserved in vivo, the Landry lab surgically implanted 4T1 cells, WT and BPTF KD, into the T4 mammary fat pad of syngeneic BALB/c mice. Doxorubicin was administered at 5mg/kg once a week for three weeks. While there was a slight reduction in tumor volume with Doxorubicin, compared to WT alone, the BPTF knockdown tumor volume was significantly reduced with Doxorubicin compared to BPTF KD alone (Figure 4.3 A, B). Furthermore, WT tumors with ATG5 KD demonstrated an increased reduction in tumor volume compared to WT alone, demonstrating a protective role of autophagy also in-vivo (Figure 4.3C). This was further confirmed in the 4T1 tumors with both BPTF and ATG5 knocked down, where there was no tumor sensitization to doxorubicin observed (Figure 4.3D). These data suggest that functional autophagy is required for sensitization (at least in the case of doxorubicin) by genetic NURF inhibition.
Figure 4.3 Autophagy modulates sensitivity to doxorubicin. Mice were challenged with BPTF WT and BPTF KD tumor cells (50X10⁴). When palpable tumors appeared, animals received doxorubicin ones a week for 3 weeks at concentration of 5 mg/kg via i.p. injections. 

A-B. In-vivo growth of BPTF WT 4T1 cells, and BPTF KD 4T1 with and without doxorubicin treatment. 

C-D. In-vivo growth of ATG5 KD or ATG5KD + BPTF KD 4T1 cells. Mean and SEM were calculated based on triplicates from 3 independent experiments. (**P ≤ 0.001 as determined by 2-way ANOVA).
4.2.4. NURF inhibition enhances doxorubicin and etoposide induced autophagic flux

Given our previous findings indicating that NURF may regulates protection to doxorubicin through regulation of autophagy (Figure 4.3), an LC3 reporter cell line was generated from a GFP-LC3-RFP-LC3ΔG reporter construct. GFP-LC3 binds to the inner membrane of the autophagosome and is degraded once fusion with the lysosome occurs. RFP-LC3ΔG, on the other hand, is unable to undergo lipidation and therefore remains in the cytoplasm serving as an internal control (176). Autophagic flux can be estimated by quantifying the GFP/RFP signal ratio, where an increase in autophagy results in a degradation of the GFP signal, but not the RFP control, resulting in a decreased GFP/RFP signal ratio (Figure 4.4A). Initially an increase in autophagic flux was observed with Doxorubicin alone, compared to control, while pharmacological NURF inhibitor AU1 + dox significantly increased autophagic flux (Figure 4.4B). Similar to doxorubicin, etoposide alone demonstrated increased autophagic flux and further enhanced autophagic flux with the AU1 + etoposide combination (Figure 4.4C). Additionally, paclitaxel alone had minimal impact on autophagic flux, while we observe an increase in autophagic flux with AU1 + Paclitaxel, compared to paclitaxel alone (Figure 4.4D). However, this effect is primarily driven by AU1 in these cells as there is no difference in autophagic flux compared to AU1 alone.
Figure 4.4 NURF inhibition enhances doxorubicin induced autophagic flux. 4T1 cells were transduced with a LC3B reporter plasmid. (A) General graph demonstrating the trend of GFP/RFP ratio during high vs low autophagic flux. Reporter 4T1 cells were treated with (B) doxorubicin (50nM), (C) etoposide (500nM), and (D) paclitaxel (50nM) for 48 hours with or without AU1(10µM) followed by flow cytometry. Mean and SEM were calculated based on triplicates from 3 independent experiments. (**P≤ 0.001 as determined by 2-way ANOVA).
4.2.5. NURF inhibition enhances doxorubicin induced autophagy in multiple cell lines

Given the importance of autophagy in the mechanism of NURF protection from chemotherapy in 4T1 breast tumor cells, additional studies were performed to determine whether the upregulation of autophagy is conserved among multiple breast cancer cell lines. In order to quickly assess autophagy in these cell lines, acridine orange (AO) staining was utilized. AO quantification confirmed a similar trend; while doxorubicin alone enhanced autophagy significantly in 436, MCF-7, 67NR and 4T1 cells, there is a further enhancement of autophagy in all cell lines with the AU1 + doxorubicin combination (Figure 4.5). However, in the case of 436 cells, there is no increase in AO staining between pharmacological inhibition of NURF with doxorubicin, compared to doxorubicin alone. It is unknown why epigenetic regulator NURF has no enhancement in this cell line and would require further investigation.
Figure 4.5 NURF inhibition enhances doxorubicin induced autophagy in multiple BC cell lines. Multiple breast cancer cell lines as indicated were treated with doxorubicin (50nM) for 48 hours, followed by recovery for 48 hours. Autophagy was quantified using AO staining. Mean and SEM were calculated based on 3 independent experiments. (**P ≤ 0.001 as determined by 2-way ANOVA).
4.2.6. NURF inhibition enhances doxorubicin and etoposide induced mitophagy

Autophagy occurs in many forms including macroautophagy, microautophagy, chaperone-mediated autophagy and mitophagy. These processes differ in the type of cargo targeted for degradation by fusion with the lysosome. In the case of mitophagy, defective mitochondria are targeted for degradation, improving cell homeostasis in times of metabolic stress (177). The co-localization of mitochondria with lysosomes is characteristic of mitophagy, an autophagy promoted process which selectively targets defective mitochondria (178). A significant increase was evident in the percentage of Lysotracker positive organelles that overlap with Mitotracker positive organelles in the BPTF KDs with both doxorubicin and etoposide treatment, but not with paclitaxel, as determined by fluorescence microscopy (Figure 4.6A,B). Measurement of mitochondrial/lysosomal overlap with AU1 + chemotherapy treatment, indicated a significant increase in lysosome/mitochondrial overlap with AU1 + Doxorubicin, compared to AU1 alone in both 4T1 and MDA-MB-231 cells (Figure 4.6C,D). These results are consistent with an overall increase in autophagy in Figures 4.2, 4.4 and 4.5, where BPTF inhibition demonstrates an overall increase in autophagy in doxorubicin and etoposide treated cells, which includes the selective process of mitophagy.
Figure 4.6 NURF inhibition enhances doxorubicin and etoposide induced mitophagy. 4T1 WT cells and BPTF KD cells were treated with doxorubicin (50nM), Etoposide (500nM), and paclitaxel (50nM) for 48 hours, followed by recovery for 48 hours, followed by staining with Lysotracker and Mitotracker and imaged on a fluorescent microscope. A. Representative images of Lysotracker positive organelles that overlap with Mitotracker positive organelles in WT and KD cells. B. Quantification of percentage of Lysotracker positive organelles that overlap with Mitotracker positive organelles in WT and KD cells. C. Quantification of percent lysosomes overlapping with mitochondria in AU1 treated 4T1 cells. D. MDA-MB-231s cells. Mean and SEM were calculated based on triplicates from 3 independent experiments. (**P≤ 0.001 as determined by 2-way ANOVA).
4.2.7. **NURF inhibition enhances doxorubicin induced DNA damage in multiple cell lines**

Cytotoxic therapies often cause DNA damage, contributing to cell death, senescence, and/or autophagy [1,2]. Similar to autophagy, our earlier studies demonstrated a potential protective role of BPTF and NURF in the treatment of doxorubicin and etoposide, where we observed an increase in DNA damage when NURF is inhibited in 4T1 breast tumor cells (Figure 4.2). In order to see if this effect is consistently conserved in breast cancer, we evaluated if there was a similar upregulation of DNA damage in a few breast cancer cell lines. Analysis of DNA-damage marker y-H2AX revealed pharmacological NURF inhibition similarly enhanced DNA induced by doxorubicin in all 4 cell lines (Figure 4.7).
Figure 4.7 NURF inhibition enhances doxorubicin induced DNA damage in multiple BC cell lines. Multiple breast cancer cell lines as indicated were treated with doxorubicin (50nM) for 48 hours, followed by recovery for 48 hours. DNA damage was quantified using γ-H2AX staining. Mean and SEM were calculated based on 3 independent experiments. (***P≤ 0.001 as determined by 2-way ANOVA).
4.2.8. NURF inhibition enhances breast tumor cell sensitivity to doxorubicin in vivo

In order to demonstrate the clinical significance of AU1, the next step would be to confirm if BPTF is a druggable target in mice. In tumors treated with vehicle, the pharmacological BPTF/NURF inhibitor, AU1, and/or doxorubicin, an immunohistochemistry assessment for autophagy, apoptosis, and DNA damage was performed. In figure 4.8A, LC3B staining largely shows no alterations in the extent of autophagy between vehicle, AU1, Dox, while AU1+Dox treated tumors show significantly enhanced staining. These studies in tumor bearing animals support the previous data demonstrating potentially direct involvement of autophagy in sensitization by the BPTF inhibitor (Figures 4.2, 4.3). Further histological analysis of cleaved caspase 3 shows increased apoptosis, as observed by darker staining upon treatment with Dox and AU1 + Dox, compared to the vector controls as well as with AU1 alone (Figure 4.8B). There appears to be no increase in cleaved caspase-3 in AU1 + Dox treated tumors, compared to Dox alone, which is consistent with our in vitro data, demonstrating that BPTF/NURF inhibition does not significantly increase apoptosis, compared to doxorubicin treatment alone (Figure 4.2A). These observations, along with those relating to autophagy, suggest that sensitization in vivo may occur via a similar pathway as in cell culture. Tissues were further analyzed for Gamma-H2AX staining, showing
increased DNA damage in AU1+ doxorubicin treated cells compared to doxorubicin alone (Figure 4.8C). Again, these data are consistent with the findings in vitro.

**Figure 4.8 NURF inhibition enhances breast tumor cell sensitivity to doxorubicin in vivo.** BALB/c mice were transplanted with 4T1 tumor cells and tumors were treated with AU1 intraperitoneally (10µL at 10mM in DMSO and/or 5mg/kg doxorubicin intraperitoneally at days 2, 4, 7 and harvested after 30 days. Tumors were fixed in 10% NBF and stained for LC3B, Cleaved Caspase-3, and γ-H2AX. Representative images taken from 5-7 fields of 3 biological replicates of each group (20x).
4.2.9. NURF inhibition enhances cell sensitivity to microtubule inhibitors

To extend these findings to additional antitumor drugs, an initial screening of FDA compounds, performed in collaboration with the Landry lab, identified other potential agents from a broad range of different classes, including Topoisomerase II inhibitors, platelet inhibitors used for blood clot prevention, proteasome inhibitors, insecticides/macrolides, and microtubule inhibitors. These initial BPTF knockdown studies suggested that these compounds might be susceptible to sensitization through BPTF/NURF inhibition. We performed MTS and clonogenic studies involving pharmacological inhibition experiments using AU1 with both 4T1 murine and MDA-MB231 human breast tumor cell lines. Although our studies with the pharmacological BPTF inhibitor failed to confirm many of the observations with the BPTF knockdown, in quite dramatic contrast, we observed marked sensitization to the microtubule poison, vinorelbine in 4T1 cells (Figure 4.9). Doxorubicin was used as a positive control.

These findings were confirmed in a clonogenic survival assay, where each agent alone had minimal effects on the survival of the TNBC 4T1 mouse breast tumor cell line. In contrast, the combination treatment of AU1 + vinorelbine essentially eliminated the entire tumor cell population (Figure 4.9A,B). Additionally, we ran a time course viability study utilizing 2.5 µM AU1 and 50 nM vinorelbine where neither vinorelbine nor AU1 alone inhibit tumor cell growth in culture in the 4T1 cell line (Figure 4.9C). In dramatic contrast, the combination treatment results in complete suppression of tumor growth where the cells do not begin to recover until as late as day 16 (Figure 4.9C).
Figure 4.9 NURF inhibition sensitizes breast tumor cells to vinorelbine, delaying recovery. 4T1 cells were pre-treated with AU1 overnight, followed by combination treatment with vinorelbine (50nM). Treatments were replenished after 48 hours and removed after 96 hours. AU1 was thereafter replenished every two days. Viable cell number was monitored by trypan blue exclusion. A. Quantification of colony formation in clonogenic survival assay. B. Representative images of colony formation with each treatment. C. Viable cell number for each treatment was quantified by trypan blue exclusion. Mean and SEM were calculated based on triplicates from 3 independent experiments. (**P ≤ 0.001 as determined by 2-way ANOVA).
To explore potential mechanisms that may be responsible for the observed sensitization, we next performed assays probing for apoptosis and senescence using flow cytometry. The control, AU1 alone, and vinorelbine alone groups all showed minimal levels of apoptosis (Figure 4.10A). In contrast, the combination treatment promoted about 70 percent apoptosis (Figure 4.10A). STS (staurosporine) is utilized as a positive control. Clearly, apoptosis plays a role in the sensitization of 4T1 cells with the combination treatment. In order to evaluate if senescence is playing a role in sensitization, we measured senescence induction through C₁₂FDG labeling of cells at days 2, 3 and 4 of treatment (Figure 4.10B). Days 3 and 4 show 10 percent and 30 percent C₁₂FDG positive cells with the AU1 + Vinorelbine combination respectively. These data indicate that senescence also appears to play a role in and is a consequence of combination treatment sensitization. However, clearly apoptosis is predominant.
Figure 4.10 NURF inhibition by AU1 enhances apoptosis in combination with Vinorelbine. 4T1 cells were pre-treated with AU1 overnight prior to exposure to vinorelbine (50 nM). A. Apoptosis was measured by FITC Annexin/PI staining at day 4. Staurosporine (STS) was used as a positive control for apoptosis. B. Senescence was measured by C12FDG staining at indicated days. Mean and SEM were calculated based on triplicates from 3 independent experiments. (***P≤ 0.001 as determined by 2-way ANOVA).
In order to build upon these findings that AU1 sensitizes breast tumor cells in combination with microtubule poison, vinorelbine, we determined the IC50 values for Vinorelbine, Vinblastine, Doxorubicin, Paclitaxel, 5-FU, and Cisplatin using an MTS viability assay. We observe the IC50 value in 4T1 breast tumor cells for vinorelbine is 221 nM and shifts to 26.5 in combination with AU1, which is approximately an 8-fold increase in sensitivity in the 4T1 cell line when vinorelbine is combined with AU1 (Table 4.1). In order to confirm the previous findings, we utilized doxorubicin, where combination with AU1 resulted in approximately a 2-fold increase in sensitivity, while vinblastine and paclitaxel in combination with AU1 resulted in approximately 3-fold increases in sensitivity. AU1 had no effect on sensitization of 5-FU and cisplatin, which is consistent with our previous work, which indicated that genetic inhibition of BPTF/NURF did not result in sensitization to cisplatin or 5-FU (Figure 4.1).
Table 4.1 Shift in IC50 values for antitumor drugs induced by AU1. 4T1 cells were treated with AU1, followed by exposure to indicated chemotherapeutic agents with or without AU1 and incubated for an additional 96 hr prior to evaluation via the MTS viability assay.

<table>
<thead>
<tr>
<th>IC50 values (nM)</th>
<th>Control</th>
<th>With AU1</th>
<th>IC50 shift</th>
<th>Control/AU1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinorelbine</td>
<td>221</td>
<td>26.5</td>
<td>194</td>
<td>8.31</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>1.97</td>
<td>0.701</td>
<td>1.27</td>
<td>2.81</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>28.0</td>
<td>15.4</td>
<td>12.6</td>
<td>1.81</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>43.0</td>
<td>15.2</td>
<td>27.8</td>
<td>2.83</td>
</tr>
<tr>
<td>5-FU</td>
<td>366</td>
<td>408</td>
<td>42.1</td>
<td>0.897</td>
</tr>
<tr>
<td>Cisplatin (μM)</td>
<td>5.73</td>
<td>5.91</td>
<td>0.175</td>
<td>0.970</td>
</tr>
</tbody>
</table>
4.3. Discussion

The focus of this component of our research was to evaluate the therapeutic potential of NURF suppression to enhance sensitivity of triple negative breast cancer cells to chemotherapy. NURF suppression appeared to be effective in tumor cell sensitization to some drugs, specifically doxorubicin, etoposide, paclitaxel, and vinorelbine; however, this did not appear to be the case for platinum compounds, ionizing radiation, antimetabolites, or kinase inhibitors. The observed sensitization to doxorubicin was dependent on autophagy, where autophagy inhibition ameliorated the observed sensitization to doxorubicin. Given this dependence on autophagy, it would appear that autophagy may be killing the cells or contributing to cell death.

It was additionally confirmed that autophagy is promoted in breast cancer cells by doxorubicin and etoposide, and further enhanced in instances where BPTF is knocked down, but no difference in autophagy was observed with Paclitaxel treatment. We further observed an increase in mitophagy, or the selective targeting of mitochondria through autophagy, when NURF inhibition was combined with doxorubicin. Taken together, these studies reveal a potential protective role of BPTF and NURF against cancer chemotherapeutic drugs which may require autophagy. While induction of senescence and apoptosis were evident in some of these treatments, these mechanisms did not appear to be responsible for the observed sensitization of doxorubicin, etoposide, and paclitaxel, although we cannot rule out a potential role for apoptosis in other drug treatments.
In addition to autophagy playing a role, an increase in DNA damage was observed when NURF inhibition was combined with doxorubicin and etoposide, but interestingly not in the case of paclitaxel. Given that these drugs interact with topoisomerase II, resulting in DNA damage, it may be possible that BPTF is interfering with this interaction, and consequently inhibiting BPTF results in increased sensitivity to topoisomerase II inhibitors.

In vivo tissue analysis confirmed sensitization to doxorubicin through NURF inhibition resulted in increased autophagy and DNA damage, which confirmed the in vitro findings. The fact that these observations are conserved in both cell culture and in tumor bearing mice indicate that these findings may have translational potential. Additionally, tissue analysis confirmed the in vitro findings that BPTF inhibition had no impact on apoptosis, indicating that the sensitization occurring may be independent of apoptosis. Topoisomerase II inhibitors, among other chemotherapeutic therapies, while inducing apoptosis, autophagy, and senescence, have also been shown to induce immunogenic cell death (ICD), making tumors more immunogenic (62,63). Autophagy plays a vital role in ATP release, which leads to the induction of ICD (60). So, despite apoptosis not playing a role in sensitization, one potential mechanism could be through the induction of ICD. Alternatively, the increased autophagy could be directly inducing ACD, cell death dependent on autophagy (75).

Our studies further examined the combination of the pharmacological BPTF inhibitor, AU1, with a broad range of chemotherapeutic agents in triple negative breast tumor cells. These agents, although not currently used as drugs in the treatment of breast cancer, may prove to be effective in the treatment in combination with NURF inhibition in
the future. Pharmacological NURF inhibition confirmed sensitization to vinorelbine, an effect that was largely driven by the induction of apoptosis.

Chapter 5  : Future Directions

5.1. The Future of Epigenetic Regulators and Breast Cancer

This dissertation describes studies which investigate the efficacy of modulating epigenetic regulators in order to sensitize breast cancer cells to therapy. Project 1 evaluates autophagy and BET inhibition in response to the combination treatment of Fulvestrant and Palbociclib in ER positive breast cancer. Project 2 evaluates the effectiveness of suppression of the nucleosome remodeling factor NURF in sensitizing TNBC cells to chemotherapies. Results from these studies further our understanding of how inhibition of epigenetic regulators alters the response of these tumor cells to select therapies in breast cancer.

For project 1, we initiated our studies evaluating autophagy inhibition as a potential route for modulation of the breast tumor cells to Fulvestrant + Palbociclib. This direction was based on the extensive data in the literature that many antitumor therapies, including chemotherapy, radiation, and endocrine treatments, promote the cytoprotective form of autophagy, whereby autophagy inhibition sensitizes the tumors to the therapy (25,115,116). Additional studies have investigated the induction of autophagy by palbociclib and the impact of autophagy inhibition on drug sensitivity in breast tumor cells (118). We observed that early administration of pharmacological autophagy inhibitors, prior to the induction of autophagy, did not improve tumor cell responsiveness to the
combination treatment, leading to the conclusion that the autophagy was non-protective. Similarly, with late inhibition of autophagy, the combination treatment appears to be largely nonprotective. These data were confirmed by genetic inhibition of autophagy. Given these data, we concluded that autophagy inhibition is unlikely to become a clinically useful therapeutic strategy (42,163). Despite this, future studies in this area may be targeted at the possibility that autophagy induced by aromatase inhibitors in combination with CDK4/6 inhibitors could be cytoprotective and amenable to autophagy inhibition in the clinic.

Following autophagy inhibition, we evaluated another potential mechanism of sensitization through modulation of senescence. Our data confirms that similar to autophagy, the combination of Fulvestrant and Palbociclib induce significant senescence. Our studies further examined the incorporation of the BET degrader, ARV-825, into the combination treatment. We observed suppressed tumor growth for both the ER positive p53 wildtype MCF-7 cells and the ER positive p53 mutant T-47D cells. Additionally, we confirmed the senescent MCF-7 cell population appears to be more susceptible to ARV-825 induced apoptosis, although a low degree of apoptosis is also observed in non-senescent cells exposed to the ARV-825. Extending these studies into RB deficient cells, we demonstrated efficacy in cells resistant to Fulvestrant and Palbociclib, which are unable to enter senescence. These findings provide a clear indication that the p53 status of the cells as well as the development of resistance, may not be a factor in their susceptibility to the proposed therapeutic approach, broadening its potential utility in breast cancer patients.
ARV-825 has been demonstrated to activate autophagy as one possible component involved in senolysis (120); therefore, it would be beneficial to study the mode of cell death, especially in the case of T47D cells, where apoptosis is not occurring. Understanding the mechanism of cell death, whether it is through ACD or another signaling pathway, could help to determine how this treatment might be manipulated to achieve the best therapeutic outcome. To verify BRD4 is regulating myc expression, overexpressing BRD4 would be one method of confirming these findings. Further studies could be developed utilizing ABBV-075 and ABBV-744, recently developed BET inhibitors that have shown promise in preclinical studies (169–171). It would be beneficial in testing these agents with Palbociclib, in combination with other estrogen therapies, such as Fulvestrant and aromatase inhibitors in cell culture and in tumor-bearing animal models.

For project 2, the suppression of the epigenetic regulator, NURF, was evaluated as a strategy to enhance sensitivity to chemotherapy. Both genetic silencing approaches, and, in some cases, the pharmacological BPTF/NURF inhibitor, AU1, were utilized both in cell culture and in tumor-bearing animals. These studies demonstrated sensitization to the cancer chemotherapeutic drugs doxorubicin, etoposide, and paclitaxel, but not to cisplatin or 5-fluorouracil, identifying a protective function of NURF, which was regulated through autophagy.

Autophagy plays a distinct immunomodulatory role through the release of ATP, a DAMP that activates many components of the immune response, but that is not completely understood. Potential future studies would be to investigate the role that cytokines and chemokines play to enhance the immune system, and whether immune activation mediates sensitization *in vivo*. While 4T1 cells are a murine model
representative of triple-negative breast cancer, investigating how NURF suppression modulates metastasis would be an important area to study, utilizing E0771-LMB cells, which metastasize spontaneously to the lungs in mice.

We recently discovered that pharmacological inhibition of NURF sensitizes TNBC cells to Vinorelbine, a drug from the class vinca alkaloids. Future studies will extend the approaches to vinblastine and vincristine, in combination with AU1 as well as determine if the promotion of autophagy is involved in sensitization for all viable chemotherapeutic and NURF inhibitor combinations. Additional studies have implicated the potential involvement of AU1 in drug efflux interference through one or more multidrug efflux pumps. An off-target effect of AU1 could be acting through inhibition of these pumps, which could be enhancing sensitization and apoptosis in these TNBC tumor cells. This premise is supported by a recent study evaluating that targeting BPTF sensitized pancreatic tumor cells to chemotherapy through inhibition of ABC-transporters, impairing multidrug resistance (179). Further directions will aim to determine whether multidrug resistance pumps are a target, potentially through utilization of calcium channel antagonists to assess sensitization to vinorelbine, vincristine, paclitaxel, and doxorubicin in TNBC lines.

While pharmacological BPTF inhibition is effective in these studies, there is some toxicity when administered systemically. New, more specific BPTF or chromatin remodeling protein inhibitors are needed to overcome toxicity and potential off-target effects.
References


54. Liu E, Van Grol J, Subauste CS. Atg5 but not Atg7 in dendritic cells enhances IL-2 and IFN-γ production by Toxoplasma gondii-reactive CD4+ T cells. Microbes Infect. 2015 Apr;17(4):275–84.


152. Siddiqa A, Long LM, Li L, Marciniak RA, Kazhdan I. Expression of HER-2 in MCF-7 breast cancer cells modulates anti-apoptotic proteins Survivin and Bcl-2 via the extracellular signal-related kinase (ERK) and phosphoinositide-3 kinase (PI3K) signalling pathways. BMC Cancer. 2008 May 2;8:129.


170. Sheppard GS, Wang L, Fidanze SD, Hasvold LA, Liu D, Pratt JK, et al. Discovery of N-Ethyl-4-[2-(4-fluoro-2,6-dimethyl-phenoxy)-5-(1-hydroxy-1-methyl-


