Dual Inhibition of the PI3K/Akt and MEK5/ERK5 Pathways for the Treatment of Breast Cancer

Thomas Douglas Wright
Duquesne University

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DUAL INHIBITION OF THE PI3K/AKT AND MEK5/ERK5 PATHWAYS FOR THE TREATMENT OF BREAST CANCER

A Dissertation
Submitted to the School of Pharmaceutical Sciences

Duquesne University

In partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By
Thomas Douglas Wright

August 2019
DUAL INHIBITION OF THE PI3K/AKT AND MEK5/ERK5 PATHWAYS FOR THE TREATMENT OF BREAST CANCER

By

Thomas Douglas Wright

Approved April 23rd, 2019

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ABSTRACT

DUAL INHIBITION OF THE PI3K/AKT AND MEK5/ERK5 PATHWAYS FOR THE TREATMENT OF BREAST CANCER

By

Thomas Douglas Wright

August 2019

Dissertation supervised by Dr. Jane Cavanaugh

Breast cancer is a heterogeneous disease state with several challenging frontiers. In particular, aberrations in the Phosphoinositide-3-kinase (PI3K) and Mitogen Activated Protein Kinase (MAPK) pathways have been linked to increased breast cancer proliferation and survival. It has been proposed that these survival characteristics are enhanced through compensatory signaling and crosstalk mechanisms. The crosstalk between PI3K/Akt and MEK1/2/ERK1/2 has been characterized in several systems. However, new evidence suggests that MEK5/ERK5, a member of the MAPK family, is a crucial component in the proliferation and survival of several aggressive cancers. We hypothesize that inhibiting both PI3K/Akt and MEK5/ERK5 pathways will decrease cell viability while maintaining limited collateral toxicity. In this study, we examined the effects of dual inhibition of PI3K/Akt and MEK5/ERK5 in a panel of hormonally diverse
cell lines. Additionally, we investigated dual inhibition in triple-negative breast cancer (TNBC) and tamoxifen resistant models. Both of which do not currently have targeted therapy available. Our results (in TNBC cells) indicate that the dual inhibition strategy was more effective than single inhibition due to the loss of crosstalk between the two pathways. In particular, a loss of Bad phosphorylation at two distinct sites was observed with dual inhibition. Interestingly, this signaling pattern was observed without disturbing the ERK1/2 pathway. Furthermore, the inhibition of both pathways led to p21 restoration, decreased cell proliferation, and induced apoptosis. Also, the dual inhibition strategy was determined to be synergistic in TNBCs and was nontoxic in the non-neoplastic MCF-10A cell line. In summary, the results from this study provide a unique perspective into the utility of a novel dual inhibition strategy for targeting TNBCs.
DEDICATION

To my parents, David and Diane, my sister, Katy, my brother, John, and especially my wife, Jenny. All of your encouragement, sacrifice, and love has made all of the difference.
ACKNOWLEDGEMENT

I want to thank faculty, staff, and students who have aided me throughout my graduate education. Dr. Jane Cavanaugh has been an amazing support to me both professionally and personally. I have had many trials over the last 4.5 years and Jane’s support has been unwavering. I would also like to thank my committee members: Dr. Paula Witt-Enderby, Dr. Patrick Flaherty, Dr. Wilson Meng, and Dr. Lauren O’Donnell. Dr. Witt-Enderby has provided me valuable insight and mentorship through courses taught and collaborative experiences. Dr. Flaherty has been a crucial collaborator over the years and his expert knowledge has broadened my understanding of medicinal chemistry. Dr. Meng, has inspired me through various courses and taught me concepts that are fundamental to my growth as a scientist. Dr. O’Donnell has been a valuable member of my committee and her unique insight has aided my project immensely. I would also like to thank other faculty members who have taught and supported me over the years. Dr. Rehana Leak has been a wonderful teacher and mentor to me throughout my graduate education. Dr. James Drennen has been an amazing mentor for my time as a tutor for the school of Pharmacy. Additionally, Drs. Peter Wildfong and Janet Astle have aided in my growth as a teacher with their insightful comments and feedback.

There are many students who have helped me throughout my time at Duquesne. Dr. Sneha Potdar helped me get acquainted when arrived at Duquesne and I sincerely appreciate that. Akshita Bhatt has been a wonderful lab mate and a crucial support for me over the past few years. I want to acknowledge my classmates: Mike Wasko, Mamud Hassan, Tarun Bhatia, Mohit Gupta and the rest of the graduate department. I also would like to extend my gratitude to members of the Cavanaugh lab who have helped with my
experiments: Chris Raybuck, Kate Wendiker, Nathan Gartland, Alexis Puk, Katie Anna, Victoria Nesbit, and Seraina Schottland.

Special thanks to the Cavanagh and Flathery labs for donating a magnolia tree in honor of my late brother, John.
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Ipat+XMD decreases proliferation marker Ki67 and induces p21 expression in BT-549 cells

Both Ipatasertib and XMD8-92 decreased pBad S112 while Ipat increased Cleaved caspase-3 in BT-549 cells

Ipatasertib plus XMD8-92 combination inhibits respective kinases while sparing ERK1/2 activity in BT549 cells

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Ipatasertib plus XMD8-92 decreases Ki67 expression in MDA-MB-468 cells

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<tr>
<td>AI</td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2 associated death promoter</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer susceptibility gene</td>
</tr>
<tr>
<td>BRD</td>
<td>Bromodomain</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster differentiation</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CI</td>
<td>Combination index</td>
</tr>
<tr>
<td>cMET</td>
<td>Receptor for hepatocyte growth factor</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>Era</td>
<td>Estrogen receptor alpha</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related protein kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumor</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HIF1a</td>
<td>Hypoxia inducible factor 1 alpha</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive ductal carcinoma</td>
</tr>
<tr>
<td>IGF1R</td>
<td>Insulin like growth factor-1 receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2</td>
</tr>
<tr>
<td>RPS6</td>
<td>Ribosomal protein S6</td>
</tr>
<tr>
<td>RSK</td>
<td>Ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>TamR</td>
<td>Tamoxifen resistant</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
</tr>
<tr>
<td>V600E</td>
<td>BRAF codon mutation</td>
</tr>
<tr>
<td>4-OH</td>
<td>4-OH Hydroxy-Tamoxifen</td>
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Chapter 1: Literature Review

1.1 Introduction

1.1.1 Cancer Epidemiology and Etiology

Cancer is the second leading cause of death in the United States with an estimated 1.7 million new cases and 600,000 deaths annually. Of the various cancer types, breast and prostate cancer are the most common for women and men, respectively (Figure 1-1A). Recent advances in early detection and treatment have led to approximately 2.6 million averted cancer deaths since 1990 (Siegel et al. 2019). However, many challenges remain in treating cancer because cancer is a diverse disease of 200 distinct types and is considered to be a “moving target” due to the vast genetic and epigenetic alterations (Komarova, 2015).

According to the American Cancer Society, 250,000 new cases of breast cancer will develop each year resulting in 40,000 deaths (American Cancer Society, Breast cancer Facts and Figures 2017-2018). Breast cancer remains the second leading cause of cancer related deaths in women and 1 in 8 women will develop the disease in their life-time. Almost all new cases (95%) occur in women over 40 years of age (DeSantis, 2017). Breast cancer deaths have decreased steadily since 1990 due to improvements in detection, treatment, surgery and molecular profiling (Figure 1-1B). Exposure to ionizing radiation and estrogens are risk factors for developing breast cancer. In addition to external factors, breast cancer arises from a series of genetic and epigenetic events that result the inhibition of apoptosis, increased invasiveness, immune suppression, and deregulation of cell growth.
1.1.2 Breast Cancer Anatomy and Pathophysiology

The female breast contains two main anatomical features: lobules and ducts. Breast ducts connect the lobules via a converging nipple-areola complex (Figure 1-2A). The ducts are comprised of three types of cells: luminal, basoluminal, and basal (Figure 1-2B). The basal cells are responsible for contraction of the ducts while the luminal cells line the ducts. Duct cells are
epithelial in origin and cancers that arise from the ducts are called Ductal Carcinoma In Situ (DCIS) and Invasive Ductal Carcinoma (IDC). DCIS means that the cancer is localized in the duct and has not disseminated to lymph nodes or formed distant metastases. The incidence of both DCIS and IDC increases after the age of 50 due to fluctuations in estrogen concentration and accumulation of somatic mutations (Figure 1-3). DCIS represents 83% of in situ cases while IDC represents 80% of invasive cases. Malignancies can also form in the lobules to become Lobular Carcinoma In Situ (LCIS) and Invasive Lobular Carcinoma (ILC); albeit at lower rates than their respective ductal counterparts (Collins et al. 2005; Sanders et al. 2005; Erbas et al. 2006).

Figure 1-2. Anatomical and cellular structure of the female breast. Anatomy of the female breast (A). Cross-section of breast duct (left) and cellular subtypes (right) (B). [Link](http://www.cancer.gov/cancertopics/pdq/treatment/breast/Patient/page2).
Breast cancer is staged according to the size and characteristics of the primary tumor (T), involvement of regional lymph nodes (N), and presence of metastatic lesions (M). TNM stage ranges from 0 to IV (Edge et al. 2010). DCIS is commonly referred to as TNM stage 0 and considered to be “in place” or pre-invasive. Whereas, metastatic IDC are considered to be TNM stage IV. Intermediate stages are determined by tumor size and degree of lymphatic involvement (Shahar 2005). Sentinel lymph node biopsy in the axillary region is a commonly performed procedure used to determine lymphatic involvement in invasive breast cancer (Strom 2005). In general, low grade and low TNM score (Stage 0-1) tumors are characterized as “local” and therefore DCIS. The treatment regimen for DCIS is radiation followed by surgery and postoperative radiation. Surgical technique has drastically improved since early breast surgeries.
in the 1950s. Instead of radical mastectomies, patients today have tumors removed and immediately undergo reconstructive surgery after the pathology is determined to be clear (Cotlar et al. 2003). Due to advances in surgery, the prognosis for localized DCIS is favorable with over 93% 5-year survival rates across all ethnicities of US women (Figure 1-4A). Mid-grade and mid TNM score (Stage II-IIIa) are more difficult to characterize as DCIS or IDC. However, their prognosis is relatively favorable with approximately 80% 5-year survival across all ethnicities (Figure 1-4A). Lastly, patients with “distant” pathology are considered high grade and high TNM score (Stage IV) have a worse prognosis than localized or regional disease with 5-year survival rates ranging from 26-39% (Figure 1-4A).

In addition to tumor staging, biopsies are examined by pathologists to determine the morphological appearance, molecular, and genetic characteristics of the tumor. Tumor grade refers to several factors such as the rate of growth, the degree of differentiation, mitosis, and metastatic spread. Tumors with poor differentiation, multiple mitoses, and high Ki67 staining (a proliferative marker) are said to be high grade. Similar to Stage IV tumors, high-grade tumors have a worse prognosis (Centers for Disease Control and Prevention, 2017). In breast cancer, patients with invasive disease (IDC) and distant metastases are considered stage IV and high grade.
Figure 1-4. Breast cancer 5-year survival based on site at detection and race. Patients were diagnosed from 2007 to 2013 and were followed through 2014 (A). NHW= non-hispanic white, NHB= non-hispanic black, API= Asian/Pacific islander, and AI/AN= American Indian/Alaska Native. Adapted from National Center for Health Statistics, Centers for Disease Control and Prevention, 2017. Reproduced with permission; License number: 4565100729408.

The metastatic process is a primary driver of IDC mortality and presents a significant challenge for treatment (Figure 1-4A). Metastasis begins with changes in the primary tumor microenvironment. First, tumor cells secrete vascular endothelial growth factor (VEGF) in response to the hypoxia of the tumor microenvironment. VEGF expression is increased under hypoxia due to the stabilization of hypoxia inducible factor 1 alpha (HIF1α) (Galanis et al. 2008). Neovascularization (angiogenesis) occurs in response to the expression of VEGF. However, the newly formed vessels are leaky and offer less of a barrier to cell invasion than compared to normal vasculature. Local invasion begins with the cell transformation process called epithelial to mesenchymal transition (EMT). EMT is characterized by a loss of cell-cell interactions and cytoskeletal rearrangements. E-cadherin and claudins are key proteins that establish cell-cell interactions (Hugo et al. 2007). Loss of E-cadherin promotes cell-matrix interactions rather than cell-cell interactions. Unlike normal cells, cancer cells do not undergo apoptosis (programmed...
cell death) when they detach from neighboring cells. Therefore, cancer cells are not bound to their tissue of origin and can migrate to more nutrient rich microenvironments.

Regulation of E-cadherin is both reversible and irreversible. Irreversible mechanisms such as mutations primarily occur in lobular carcinoma and are rare in IDC (Cowin et al. 2005). Epigenetic suppression of E-cadherin through promoter methylation is common in IDC and is often driven in response to a change in metabolic demands (Graff et al., 2000). In mesenchymal cells, loss of E-cadherin frequently accompanies the expression of N-cadherin (Cowin et al. 2005). Clinically, loss of E-cadherin is breast cancer is correlated with increased invasiveness and a worse prognosis (Paredes et al., 2007). Down regulation of E-cadherin promotes Wnt signaling by releasing beta catenin from the membrane (Nelson & Nusse, 2004). Once activated, beta catenin promotes an invasive phenotype by activating EMT related genes: TGFbeta, and snail (Guarino et al., 2007, Katoh and Katoh, 2008). Additionally, activation of survival pathways such as, PI3K, MAPK, and Smad also promote EMT (Peinado et al., 2003). Another feature of EMT transformed cells is that they secrete matrix metalloproteinases (MMPs). MMPs are enzymes that cleave fibrin, plasminogen, and type IV collagen (Weber 2007). Therefore, MPP secreting EMT transformed cells have increased motility. Additionally, the mechanical pressure that arises from excessive proliferation in the primary tumor also contributes to basement membrane penetration. Once cells have escaped the primary site, with the help of MMPs, they must survive in the vascular system until they reach a metastatic site. Detached cancer cells are anoikis resistant, meaning they are anchorage independent. Although cancer cells have numerous survival advantages, metastasis is an inefficient process and only 1 in 10,000 cells (0.1%) survive the journey through circulation (Anders and Carey, 2009). The cells that arrive at the metastatic site (lung, bone, and liver are most common) undergo another
transformative change from mesenchymal to epithelial and begin to grow and secrete growth factors and hormones that act to attract cells in blood stream to the metastatic site (Figure 1-5). Once a metastatic colony is established, the affected is a risk for tissue destruction and dysfunction. In particular, metastatic colonies in the lungs, brain, or bone are devastating due to their disruption of normal tissue functions (Chen et al., 2018).
Figure 1-5. **Metastatic process in breast cancer.** Anatomical representation of metastasis (A). Invasion and Metastatic process (B). [http://clinchem.aaccjnls.org/content/60/1/197](http://clinchem.aaccjnls.org/content/60/1/197) Reproduced with permission; License number: 11807713.

### 1.1.3 Molecular profile of Invasive Ductal Carcinoma and current treatments

Under normal conditions, hormones such as estrogen and progesterone maintain the female breast. In cancer cells, these hormones are mitogenic and stimulate excessive growth. As mentioned earlier, the ducts of the breast have three main types of cells: basal, basoluminal, and luminal. In general, the hormone receptor positive breast cancers resemble the luminal cells of the ducts. Luminal cells tend to express estrogen receptors (ER) on their cell surface and make up the majority of IDCs (Figure 1-6). Luminal cells are further subdivided into luminal A and B (Perou et al., 2000, Sorlie et al., 2001). Luminal A cells express ER and the progesterone receptor (PR). Luminal B cells also express ER and PR. However, Luminal B cells also express human epidermal growth factor receptor 2 (HER2). Since Luminal B cell express three hormone receptors they are considered to be “triple-positive.” Conversely, triple-negative breast cancer cells (TNBCs) lack the ER, PR, and HER2 receptors. TNBC cells resemble the basal cells of the ducts. Heterogeneity is also observed in basal like TNBCs with the cells divided into Basal A and Basal B. A study found that 123 out of 172 TNBC samples (71%) were Basal A (Bertucci et al. 2008). Basal A cells have epithelial character and express Keratin 5 and 14. Whereas, Basal B cells exhibit mesenchymal character and express vimentin (Neve et al., 2006).

Endocrine therapy is the primary treatment for ER+ and HER2+ (luminal A and B) breast cancer. Prognosis for luminal IDC is favorable compared to the other subtypes. However, resistance to endocrine therapy is a major mechanism of relapse and mortality (Creighton et al., 2009). Basal cell type is referred to as triple-negative breast cancer (TNBC). These cancers have the least favorable prognosis and do not respond to endocrine therapeutic intervention since they
lack all three hormone receptors: ER, PR, and HER2. As such, TNBCs have a worse prognosis compared to hormone positive IDC and chemotherapy is the primary treatment option (Figure 1-6).

Figure 1-6. Molecular subtypes of invasive breast cancer and incidence by race. Molecular spectrum of IDC with histological and clinical correlates (A). Data represented as new cases per 100,000 of US population in 2017. Cases further divided by race and hormonal profile (B). NHW= non-hispanic white, NHB= non-hispanic black, API= Asian/Pacific islander, and AI/AN= American Indian/Alaska Native. Adapted from National Center for Health Statistics,
Figure 1-7. Treatment paradigm for Metastatic Breast Cancer.
Adapted from MD Anderson Manual of Medical Oncology, Third edition (2016).

<table>
<thead>
<tr>
<th>Agent</th>
<th>Response rate</th>
<th>Response duration</th>
<th>Citation(s)</th>
</tr>
</thead>
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<tr>
<td>Doxorubicin</td>
<td>25-60%</td>
<td>7.8 months</td>
<td>Oosterom et al., 1986</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>21-62%</td>
<td>7.5 months</td>
<td>Sledge et al., 2003</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>37-57%</td>
<td>8.4 months</td>
<td>Harvey et al., 2006</td>
</tr>
<tr>
<td>Capecitabine</td>
<td>30%</td>
<td>8.1 months</td>
<td>Blum et al., 1999</td>
</tr>
<tr>
<td>Ixabepilone</td>
<td>11.5%</td>
<td>5.7 months</td>
<td>Perez et al., 2007</td>
</tr>
<tr>
<td>Eribulin</td>
<td>29%</td>
<td>5.8 months</td>
<td>McIntyre et al., 2014</td>
</tr>
<tr>
<td>Docetaxel/Capecitabine</td>
<td>42%</td>
<td>6.1 months</td>
<td>O’Shaughnessy et al., 2002</td>
</tr>
</tbody>
</table>
Table 1-1. Clinical outcomes for Metastatic Breast Cancer patients treated with standard chemotherapy.

Figure 1-8. Gompertzian kinetics of tumor growth and treatment. Adapted from Katzung and Trevor 2015.

Systemic chemotherapy is the current standard of care for metastatic breast cancer (MBC). Single-agent chemotherapy is administered IV every three weeks with one off week. The dose depends on the agent however dose-dense therapeutic regiments as described above are more effective than two weeks on and one week off. This is because infrequent scheduling of treatment courses allows the tumor to grow faster than the kill rate (dark blue line), whereas more intensive and frequent treatment (teal line) is more effective. Similarly, frequent and intense dosing given after surgery is also effective (green line) (Figure 1-8). Concurrent therapy is more effective than single therapy although toxicities are more common. (Norton 2005).

Doxorubicin was one of the first agents used to treat MBC. Doxorubicin is anthracycline-based chemotherapy that disrupts replication of DNA. More specifically, the planer structure of
Doxorubicin allows it to intercalate between two base pairs of DNA and stabilize the topoisomerase II complex, thus halting replication. Doxorubicin is administered IV every three weeks with one off week. Phase II and III trial data reported a 25-60% response rate for patients with MBC. Note that “response” refers to partial or complete response. Additionally, the duration of the response was 7.8 months (Van Oosterom 1986; Table 1-1). Taxanes are another class of agents used for MBC. Taxane-based therapies function by inhibiting the formation of the mitotic spindle. More specifically, Paclitaxel targets tubulin and stabilizes it polymerization, which inhibits mitotic spindle formation. Thus, Paclitaxel causes G2/M cell cycle arrest and induces apoptosis. Paclitaxel is administered via IV every three weeks with one week off. Clinical trial data report a 21-62% response rate for patients with MBC. The duration of response was 7.5 months (Sledge et al., 2003; Table 1-1). Ixabepilone is a microtubule stabilizer that used in taxane-resistant tumor cells. However, the efficacy is limited with only 11.5% of patients responding in trials (Perez et al., 2007; Table1-1). Capecitabine is an antimetabolite therapy that is converted to 5-FU in vivo. Once converted, 5-FU inhibits thymidylate synthase and ultimately DNA synthesis. In a Phase II trial, 30% of MBC patients responded to Capecitabine, and the response duration was 8.1 months (Blum et al., 1999; Table 1-1). Lastly, combinations of Docetaxel/Capecitabine have been tried in MBC but had similar response rates to monotherapy (42%) (O’Shaughnessy et al., 2002; Table 1-1).

The lack of sustained response and high collateral toxicity associated with chemotherapy have underscored the need for targeted therapy for MBC. New advances in genomic screening, such as next generation sequencing (NGS), have revealed promising leads for targeted therapy. More specifically, driver mutations in the MAPK and PI3K/Akt survival pathways have become
a central focus for developing targeted agents (Bailey et al. 2018). Successful translation of these agents to the clinic and implementation as the standard of care is the goal of precision oncology.

1.1.4 Genetics and Driver Mutations in Breast Cancer

Genetic alterations in breast cancer are familial associated 20-25% of the time but the vast majority of events are somatic or sporadic mutations in tumor suppressor genes or oncogene activation (Buchholz 1999). The BRCA1 and BRCA2 genes are strongly associated with familial inheritance (germ line) and patients with this mutation tend to develop ER+ cancer and are at an increased risk for ovarian cancer (Petrucelli 2010). BRCA mutations occur through point mutations and complex rearrangements less frequently occur (<1%). BRCA mutations affect the ability of a cell to repair double strand breaks. As a result, additional mutations, amplifications, and gene fusions result in tumor formation. BRCA mutant breast and ovarian cancer patients are responsive to PARP inhibition due to synthetic lethality (Farmer et al., 2005). Synthetic lethality occurs when two simultaneous gene ablations results in a loss of viability. In the case of BRCA and PARP, the loss of both double strand break repair mechanism results in catastrophic DNA damage and eventually apoptosis (Farmer et al., 2005). Preemptive removal (mastectomy) is sometimes indicated for patients harboring BRCA mutations (Hartman 1999, Rebbeck 2004). In addition to familial inheritance, genetic screening for mutations of tumor suppressors: PTEN (proliferation), TP53 (cell cycle), and CDH1 (E-cadherin expression) are also an effective strategy to assess risk. Since, the loss of function of PTEN, TP53, or CDH1 increases the risk of cancer formation due to unregulated growth and proliferation.

Although hormone therapy and surgery are the mainstays in breast cancer therapy for localized disease there are few options for invasive cases. Therefore, attention has shifted to the
genomic profile of many cancers, including invasive breast cancer, in hope of discovering “actionable” driver mutations that can be exploited for therapeutic benefit.

Bailey et al., 2018 analyzed the genomics of 9423 tumors that represented 33 different tumor types (including breast). 299 driver genes and 579 consensus mutations (out of 3437 mutations) were identified. The most common driver mutations were TP53 (27 out of 33 tumor types) followed by PIK3CA, KRAS, and PTEN (15 or more cancer types). The main mechanisms of mutation were truncation and missense for TP53 and missense for KRAS and PI3KCA. Interestingly, oncogenic driver mutations in the survival pathways MAPK and PI3K were found in 24 and 22 distinct cancer types, respectively. Discovery of the V600E BRAF mutation in melanoma has directly led to the development of FDA approved inhibitors Dabrafenib (BRAFi) and Trametinib (MEKi). This is one of the earliest examples of mutation driven precision oncology (Figure 1-8). Thyroid cancer is another example of using mutations to drive clinical innovation. Receptor tyrosine kinase inhibitors such as, Vandetanib, Cabozantinib, and Sorafenib are standards of care for medullary, papillary, and follicular thyroid cancers. Additionally, BRAF mutant anaplastic thyroid cancer is targeted with Dabrafenib (BRAFi) and Trametinib (MEKi). Gastrointestinal stromal tumor (GIST) is another example of successful targeted therapy. GIST patients with the BCR-Abl gene fusion are treated with Imatinib, a drug originally used for chronic myeloid leukemia (CML). As more of the genome is uncovered for each cancer, the number of successful FDA approvals is likely to increase. However, some cancers such as breast cancer are lagging despite the large number of “actionable” mutations across the disease. Therefore, in our study we will examine the genetic prolife of cell lines used but recognize that these mutations may not be “oncogenic addictions” that are susceptible to single pathway targeting.
Figure 1-9. Summary of mutation driven precision oncology by cancer type.
From the figure above, shows the tumors types that have had successful therapies developed from actionable mutations. For SKCM, melanoma, in particular V600E BRAF mutant melanoma have used Dabrafenib (BRAFi) and Trametinib (MEKi) to successfully treat patients with precision oncology. Adapted from Bailey et al. 2018.

1.1.5 MAPK and MEK5/ERK5 pathways

The mitogen-activated protein kinase (MAPK) pathway is a family of kinases that transduce extracellular signals intracellularly and influence cellular processes such as proliferation, differentiation, motility, metabolism, development, survival, and gene expression. There are six members of the MAPK family in mammals, 4 are extracellular signal related kinases (ERK) ERK1/2, ERK3/4, ERK5, and ERK7/8. The other two members are c-Jun N-terminal kinase (JNK1/2/3) and p38 (a/b/c/d) (Krishna and Narang 2008). Each MAPK contains a three tiered kinase signaling cascade that are initiated by a multitude of extracellular signals such as, growth factors, cytokines, and stress. At the receptor level, MAPKs are activated by Receptor Tyrosine Kinases (RTK), G-protein coupled receptors (GPCR), and hormone receptors (Flaherty et al., 2010). Upon activation, the receptor undergoes a conformational change that
facilitates the activation of the first tier of the MAPK pathway: Mitogen-activated protein kinase kinase kinase (MAPKKK). MAPKKK then phosphorylates Mitogen-activated protein kinase (MAPK), which subsequently phosphorylates Mitogen-activated protein kinase (MAPK). MAPK then phosphorylates cytosolic and nuclear substrates. The specific substrates that are modified depend on the initial signal, the MAPK activated, and how long the signal is maintained (Marshall 1994).

The Ras/Raf/MEK1/2/ERK1/2 pathway plays a key role in various cellular processes such as proliferation, survival, motility, and development. In particular, ERK1/2 is ubiquitously expressed in all tissue types and is key for embryonic angiogenesis and proliferation. Knockout of the ERK2 gene in mice is embryonically lethal, thus underscoring its importance to development and survival (Saba-El-Leil et al., 2003). Signaling is initiated by an EGF binding to EGFR (RTK) on the surface of the cells which stimulates the conversion of GDP bound Ras to the active GTP bound Ras. Next, Raf family kinases (Raf-1, A/B/C Raf) phosphorylate MEK1/2 at two sites (Fremin and Meloche, 2010). MEK1/2 then phosphorylates ERK1/2 at two sites via TEY motif. ERK1/2 then phosphorylates cytosolic substrates p90RSK, Elk-1, and Sapla. ERK1/2 also translocates to the nucleus and activate transcription factors related to proliferation, angiogenesis, and EMT (Lenormand et al., 1993, Yoon and Seger, 2006, Pecorino, 2016). Approximately 50% of ERK1/2 is found in the nucleus depending on the time after growth factor activation (Chen et al., 1992). In some cases the activation of ERK1/2 is transient (less than 20 minutes) while in other scenarios its activation is sustained (greater than 3 hours) (Wortzel et al., 2011).

Activating mutations KRAS and BRAF are observed in 30% and 20% of all cancers, respectively (Downward, 2003, Fremin and Meloche, 2010). More specifically, KRAS mutations
are observed in bladder, breast, colon, kidney, lung, hematopoietic malignancies, ovarian, and thyroid cancers. KRAS activates growth factors such as EGFR and ERBB2 (Downward 2003). Clinically, many approaches using Receptor Tyrosine Kinase inhibitors (RTKis) have been used in KRAS mutant cancers. BRAF on the other hand has been successfully targeted in V600E mutant melanoma. Downstream of Raf, MEK1/2 or ERK1/2 overexpression in mouse models led to aggressive tumors and increased metastasis (Fremin and Meloche 2010). Preclinical tumor models with BRAF mutations revealed that MEK1/2 inhibition reduced tumor volume and metastasis. Furthermore, the MEK1/2, Trametinib, is FDA approved to treat BRAF mutant melanoma and other cancers (FDA reference ID: 4255758)

Figure 1-10. BRAF mutation frequency in cancer. Adapted from Bailey et al., 2018.

In breast cancer estrogen has been shown to modulate ERK1/2 via cSrc (Yi et al., 2013). Additionally, ERK1/2 was observed to activate the estrogen receptor via p90RSK, which resulted in increased transcriptional efficiency of ER, related genes (Santen et al. 2002, Neuzillet et al., 2014). Although KRAS and BRAF are common mutations in cancer, they are relatively rare in breast cancer with 1.5% and 3% incidence, respectively (Saini et al., 2013). Although
MEK1/2 and ERK1/2 are not mutated in breast cancer they still play an important role. For example, ERK1/2 overexpression in TNBC patients was correlated with a worse survival rate (Bartholomeusz et al., 2012). In IDCs, both *in vitro* and *in vivo* ERK1/2 activation increased metastatic potential (Ma et al., 2012).

![Diagram of MAPK Signaling](image)

**Figure 1-11. Summary of MAPK Signaling.** Adapted from Drew et al., 2012. Reproduced with permission; License number: 4570510965494.

The MEK5/ERK5 pathway is a relatively recent discovery in the MAPK family and has several distinct features. In particular, ERK5 is structurally similar to ERK1/2 (50% sequence homology) but differs in size: 44/42 kD for ERK1/2 and 110 kD for ERK5. Hence ERK5 was aptly named “Big MAPK” or BMK-1 by Lee et al., 1995. ERK5 also has many similar functions
as ERK1/2 such as promoting survival, differentiation, proliferation, and development (Kato et al., 1997, Suzuki et al., 2002, Nishimoto et al., 2006, Li et al., 2008). In neurons ERK1/2 was observed to be active within minutes of BDNF stimulation while ERK5 was activated later and sustained activation for 24 hours (Cavanugh et al., 2001). ERK5 is ubiquitously expressed in cardiac myocytes, lungs, brain, and kidney (Lee et al., 1995, Zhou et al., 1995). ERK5 knockout in mice was embryonically lethal at day 10 and the mice were unable to form the neural crest (Hayashi and Lee, 2004, Drew et al., 2012). Ablation of the ERK5 gene also adversely affects the stability of the cardiovascular system due to compromised vascular integrity (Hayashi and Lee, 2004).

Like the other MAPKs, MEK5/ERK5 is a three tiered kinase cascade and is activated by extracellular signals such as epidermal growth factor (EGF), neural derived growth factor (NDGF), cytokines, and oxidative stress. Upon activation, MEKK2 or MEKK3 phosphorylates MEK5 at two separate sites: Ser311 and Thr315 (Chao et al., 1999). MEK5 then phosphorylates ERK5 at the Thr218 and Tyr220 residues in the kinase domain. Once active by MEK5, ERK5 undergoes a conformational change that exposes the nuclear localization domain (NLS) (Kondoh et al., 2006). ERK5 then translocates to the nucleus where it phosphorylates distinct targets (from ERK1/2): myocyte enhancer factor (MEF): MEF2A, MEF2C, and MEF2D (Kato et al., 1997). Activation of MEF genes are crucial for angiogenesis, endothelial cell survival, and heart development (Hayashi and Lee, 2004). The transcriptional activation domain, towards the C-terminus, of ERK5 is unique and is required for phosphorylation of cFos and fos-related antigen-1 (Fra-1) (Terasawa et al., 2003, Buschbeck and Ullrich, 2005). In contrast, ERK1/2 does not contain a NLS and enters the nucleus through three mechanisms: passive diffusion, active transport, or interaction with the nuclear pore complex (Knokhlatchet et al., 1998, Adachi et al.,
1999, Matsubayashi et al., 2001). Also, ERK1/2 does not have a designated transcriptional activation domain and directly phosphorylates from the kinase domain (Nishimoto and Nishida, 2006). Lastly, ERK5 phosphorylates many distinct cytosolic targets such as Bad (survival, serum regulated kinase (SGK, metabolism), and cMYC (proliferation) (Drew et al., 2012).

MEK5/ERK5 signaling is altered in many cancer types and is considered an important factor in oncogenic signaling (Hoang et al., 2017). Overactive ERK5 signaling has been observed in cancers that overexpress receptor tyrosine kinases (RTKs) and is associated with a worse prognosis (Esparis-Ogando et al., 2002). The role of ERK5 in cancer has also been shown to be distinct from ERK1/2. For example, ERK5 expression was found to correlate with advanced tumor stage and lymphatic involvement in oral squamous cell carcinoma and not ERK1 (Sticht et al., 2008). Prostate cancer bone metastases with overexpression of MEK5 were associated with increased MMP-9 expression and a poorer prognosis (Mehta et al., 2003). Association of ERK5 and Focal adhesion kinase (FAK) has been correlated with increased cellular motility and metastasis (Sawhney and Brattian, 2009). In NIH3T3 fibroblast cells, ERK5 activation mediated by Src overexpression led to increased invasion (Schramp et al., 2008).

MEK5/ERK5 signaling also has many implications in breast cancer. High ERK5 (20%) expression was observed in high-grade ER- breast tumors and correlated with decreased disease free survival (Hsieh et al., 2005). ERK5 has also been linked to Breast tumor kinase (Brk) which is overexpressed in 86% of IDCs (Drew et al., 2012). In MDA-MB-231 (TNBC) cells ERK5 mediated proliferation via cyclin dependent kinase 1 (CDK1) and p21 expression (Perez-Madrigal et al., 2012).

The tools for studying MEK5/ERK5 are currently limited to small molecule inhibitors and genetic ablation with shRNA or Crisper/Cas-9 knock out (KO). The inhibitors of MEK5,
BIX02188 and BIX02189, are ATP competitive inhibitors with cell free IC\textsubscript{50} values of 4.3 nM and 1.5 nM, respectively (Tatake RJ, et al., 2008). XMD8-92 has been show to inhibit ERK5 (downstream of MEK5) with a cell free IC\textsubscript{50} of 80nM (Yang et al., 2010). However, recent studies have demonstrated XMD8-92 is also an inhibitor of BRD4 (an epigenetic regulator) with an IC\textsubscript{50} of 190 nM. Therefore, AX15836 was developed as a selective, ATP competitive ERK5 inhibitor with a cell free IC\textsubscript{50} of 8 nM vs ERK5 and 3,600 nM vs BRD4 (Lin EC et al., 2016). siRNA for ERK5 decreased proliferation and mitotic entry in SNU449 hepatocellular carcinoma cells (HCC) (Zen et al., 2009, Lochhead et al., 2016). However BIX02189 was not able to recapitulate the siRNA effects in the same HCC cells (Lochhead et al., 2016). ERK5 knock down alone was not sufficient to decrease proliferation of lung, multiple myeloma (MML), and chromic myeloid leukemia (CML). However, combinations of ERK5 knock down with conventional therapy were effective (Wang et al., 2014). ERK5 knock down or inhibition combined with Doxorubicin for enhanced efficacy in lung cancer (Shukla et al., 2013). Additionally, dominant negative forms of ERK5 in MML and CML combined with the proteasome inhibitor Bortezombi and BCR-Abl tyrosine kinase inhibitor Imatinib for increased \textit{in vitro} efficacy (Buschbeck et al. 2005, Carvajal-Vergara et al. 2005). Taken together, these studies implicate MEK5/ERK5 as an important mediator of various oncogenic processes such as proliferation and survival. Additionally, the MEK5/ERK5 pathway also has a key role in tumorgenesis and drug resistance. Although initial studies with MEK5/ERK5 inhibition or genetic ablation have shown promise, there is a need for new tools to study this pathway.

1.1.6 PI3K/Akt/mTOR pathway
The PI3K/Akt/mTOR pathway is an evolutionarily conserved signal transduction pathway that is responsible for many crucial cell functions, such as metabolism, motility, survival, and proliferation (Cantley 2002, Goncalves et al. 2019). Activation of the pathway is initiated by extracellular growth factors and cytokines that bind to RTKs and GPCRs. Signal is transduced intracellularly beginning with Phosphoinositide 3-Kinase (PI3K) which converts Phosphatidylinositol (4,5)-bisphosphate (PIP2) to Phosphatidylinositol (3,4,5)-triphosphate PIP3. The conversion of PIP2 to PIP3 is facilitated by the catalytic subunit of PI3K p110 (Gomparts et al. 2009). There are four isoforms of p110 and many are investigated for therapeutic intervention. Once PIP3 is formed, it phosphorylates PDK1, which in turn phosphorylates Akt (also known a protein kinase B; PKB) at the threonine 308 residue (Cantley 2002). Akt then phosphorylates mTOR, which subsequently phosphorylates Akt at the serine 473 residue (Cidado and Park 2012). Activated Akt then phosphorylates a multitude of targets that influence cellular functions: p27 (cell cycle), glucose transporters and GSK3B (metabolism), Forkhead box (FOXO) and Bad proteins (survival and apoptosis) (Hemming and Restuccia 2012, Costa et al. 2018). The PI3K/Akt/mTOR pathway is mainly regulated by two phosphatases: PP2A and PTEN. PP2A dephosphorylates Akt at both T308 and S473 residues (Hemming and Restuccia 2012). PTEN inhibits the conversion of PIP2 to PIP3 (Cantley 2002). Therefore, a loss or downregulation of PTEN frequently leads to hyperactivated Akt signaling due to increased PIP3 and subsequent PDK1 phosphorylation of Akt.
Figure 1-12. Summary of PI3K/Akt/mTOR signaling. Adapted from Carnero and Paramino 2014.

The PI3K/Akt pathway is frequently altered in cancer and mutations in this pathway are considered oncogenic driver mutations due to their crucial role in cellular functions. PIK3CA (encodes for the p110 alpha isoform of PI3K) mutations occur on the catalytic subunit of p110. More specifically these mutations predominantly affect the alpha isoform (Figure 1-13). Mutations in the catalytic domain confer constitutive activity of the PI3K pathway and have been observed in brain, breast, colon, liver, lung, and ovarian cancers. Additionally, mutations of the downstream kinase Akt (E17K) have also been observed in breast, brain, lung, and bladder cancers (Hyman et al. 2017). Lastly, alterations in the tumor suppressor PTEN appears in about 15% of all cancers (Baliey et al. 2018). PTEN alterations are variable in mechanism: loss of function point mutation, hetero or homozygous deletion, degradation, miRNA, or epigenetic suppression (McCubrey et al. 2012). In breast cancer, PTEN alterations are as a whole (3% of
cases) but in TNBC 35-50% of patients harbor PTEN alterations (Saini et al. 2013). Several PI3K/Akt/mTOR inhibitors are currently under clinical evaluation in TNBC (Table 1-2).

![Figure 1-13. PIK3CA mutation frequency in cancer. Adapted from Bailey et al. 2018.](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target(s)</th>
<th>Study Phase</th>
<th>Trial ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpelisib</td>
<td>PI3Kα</td>
<td>2</td>
<td>NCT02506556</td>
</tr>
<tr>
<td>Taselisib</td>
<td>PI3K (β sparing)</td>
<td>Ib/2</td>
<td>NCT02457910</td>
</tr>
<tr>
<td>MK2206</td>
<td>Pan Akt (allosteric)</td>
<td>2</td>
<td>NCT01277757</td>
</tr>
<tr>
<td>Ipatasertib</td>
<td>Pan Akt (ATP-comp)</td>
<td>2</td>
<td>NCT02301988</td>
</tr>
<tr>
<td>Temsirolimus</td>
<td>mTOR ATP-comp</td>
<td>1</td>
<td>NCT01111825</td>
</tr>
</tbody>
</table>

Table 1-2. PI3K/Akt/mTOR inhibitors in clinical trials for TNBC.
1.1.7 Crosstalk between MEK5/ERK5 and PI3K/Akt pathways

Crosstalk is defined, as the combined signal from two pathways is different than each pathway alone. The two pathways can be directly or indirectly related. For example if one pathway is modified directly by another pathway (e.g. phosphorylation) then the pathways are directly related. Examples of direct crosstalk are crossactivation via phosphorylation and convergence on a single target at separate phosphorylation sites. Additionally, a pathway may indirectly influence another by altering gene expression (Vert and Chory 2011).

MEK5/ERK5 and PI3K/Akt crosstalk is less studied than MEK/Akt crosstalk. However reports of crossactivation and points of signaling convergence have underscored its importance in oncogenic signaling. For example, genetic ablation of Akt in neuroblastoma cells led to a decrease in ERK5 phosphorylation. Also, Akt was shown to phosphorylate MEKK3, the upstream kinase of MEK5 (Umapathy et al. 2014). In our preliminary studies, we observed similar results: the Akt inhibitor Ipatasertib decreased phosphorylated ERK5 in U87 glioblastoma cells (Figure 1-14). U87 cells are PTEN mutant and have hyperactive Akt. Therefore, the crossactivation of MEK5/ERK5 via Akt is likely to be present in cancers with similar PTEN alterations. In addition to crossactivation, both MEK5/ERK5 and PI3K/Akt have been shown to converge on the survival proteins Bad and FOXO3a (Pi et al. 2004, Drew et al. 2012). In particular Akt has been shown to phosphorylate Bad at Ser136 while ERK5 phosphorylates Bad at Ser112 (Datta et al. 1997, Pi et al. 2004). Interestingly, both pBad S112 and S136 expression was shown to be higher in the TNBC cell line MDA-MB-231 vs the normal tissue cell line MCF-10A (Sickels et al. 2015). Therefore, targeting both MEK/ERK5 and PI3K/Akt in a serial rather than parallel manner will perhaps yield effective and less-toxic results
(Yin et al., 2014). Figure 1-15 summarizes the known crosstalk between the MEK5/ERK5 and PI3K/Akt pathways.

![Figure 1-14. Akt inhibitor Ipatasertib decreases pERK5 in PTEN mutant GBM cells.](image1)

U87 glioblastoma cells were treated with Akt inhibitor Ipatasertib for 1 hour prior to 5% FBS stimulation for 24 hours. DMSO has used as a vehicle control. Cellular lysates were collected and proteins were visualized via western blot. (Seraina Schottland, Unpublished work)

![Figure 1-15. Akt crossactivation of MEK5/ERK5 pathway.](image2)

Summary of MEK5/ERK5 and Akt crosstalk: (1) Akt crossactivation of MEKK3 (2) convergence of Akt and ERK5 on survival proteins.
1.2 Statement of the problem

Breast cancer is a heterogeneous disease and according to the American Cancer Society, 250,000 new cases are reported annually. Breast cancer is the second leading cause of cancer death in women with 40,000 deaths annually. The majority of breast cancer mortality is attributed to invasive disease. In particular, Invasive Ductal Carcinomas (IDC) make up over 80% of all invasive cases (Allred 2010). IDCs are primarily categorized by hormonal profile. IDCs that express ER+ and HER2+ are responsive to endocrine therapy and have a favorable prognosis. However, endocrine resistance remains a major challenge to treating ER+ and HER2+. Triple-negative breast cancers (TNBCs) are a type of IDC that lack hormone receptors. TNBCs are not responsive to chemotherapy and have the poorest prognosis of IDCs. TNBCs are primarily treated with chemotherapy and no targeted treatment is available.

Recent advances in genomic screening have yielded promising leads for targeted therapy in cancer. For example, the discovery of the V600E BRAF mutation led to the development of agents that target the Raf/MEK/ERK axis in BRAF mutant cancers. However, not all cancers have been successfully treated with targeted agents and there is a need to investigate unique signaling pathways that contribute to oncogenic phenotypes. The MEK5/ERK5 pathway is a less studied member of the MAPK family and has a role in the oncogenic signaling of many cancer cell types (Hoang et al. 2017). Additionally, MEK5/ERK5 has been shown to interact with other “driver” cancer pathways such as PI3K/Akt (Umapathy et al. 2014). Therefore, we propose the study of MEK5/ERK5 signaling in a variety of breast cancer cell lines: both hormonally diverse and mutationally distinct.
1.2.1 Research Objectives

1.2.2 Hypotheses and Research Aims

1a). Combination of Akt and ERK5 inhibition will more effectively decrease MDA-MB-231 TNBC viability, proliferation, and migration than either inhibitor alone.

1b). Ipatasertib plus XMD8-92 will decrease viability proliferation, and migration in PTEN mutant TNBC cell lines MDA-MB-468 and BT549 than either drug alone.

2). Novel diphenylamine derivatives will provide selective inhibition of the MEK5/ERK5 pathway in MDA-MB-231 TNBC cells.

3). The combination of Akt and ERK5 inhibition will reduce proliferation in an acquired tamoxifen resistant MCF-7 cell line.
Chapter 2: Materials and Methods

2.1 Cell culture

Human breast cancer cell lines were obtained from ATCC (Manassas, VA) and maintained in accordance with the manufactures’ instructions (Table 2-1). Cell lines were selected based on hormone and genetic profiles (Table 2-1). The human triple-negative breast cancer cell line MDA-MB-231 was obtained from ATCC and was maintained in DMEM:F-12 (1:1) (Life Technologies), 10% Fetal Bovine Serum (Atlanta Biologicals), and 1% Penicillin/Streptomycin (Sigma). The MCF-10A cell line was also obtained from ATCC and was used as a normal tissue control. Each cell line was utilized for fewer than 15 passages after thawing except for the MCF-7 and TamR cell lines which were both passaged approximately 50 times over a 6 month period.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Disease</th>
<th>Hormone profile</th>
<th>Culture Conditions</th>
<th>Source</th>
<th>Key Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>Triple negative</td>
<td>DMEM/F-12, 10% FBS, and 5% CO₂</td>
<td>Metastasis, Pleural effusion</td>
<td>BRAF, CDKN2A, KRAS, NF2, and TP53</td>
</tr>
<tr>
<td>MDA-MB-231 ERK5 KO</td>
<td>Breast</td>
<td>Triple negative</td>
<td>DMEM/F-12, 10% FBS, and 5% CO₂</td>
<td>Dr. Burow’s lab</td>
<td>BRAF, CDKN2A, KRAS, NF2, and TP53</td>
</tr>
<tr>
<td>BT549</td>
<td>Breast</td>
<td>Triple negative</td>
<td>1640 RPMI, 10% FBS, and 5% CO₂</td>
<td>Primary tumor</td>
<td>PTEN, RB1, and TP53</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Breast</td>
<td>Triple negative</td>
<td>L-15, 10% FBS, and 0% CO₂</td>
<td>Metastasis, Pleural effusion</td>
<td>PTEN, RB1, SMAD4, and TP53</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>ER+</td>
<td>1640 RPMI, 10% FBS, and 5% CO₂</td>
<td>Metastasis, pleural effusion</td>
<td>CDKN2A and PIK3CA</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>ER+</td>
<td>1640 RPMI (-)</td>
<td>Metastasis</td>
<td>CDKN2A and</td>
</tr>
<tr>
<td>TamR</td>
<td>Phenol Red), 10% Charcoal Stripped FBS, 100nM 4-OH Tamoxifen, and 5% CO₂</td>
<td>pleural effusion</td>
<td>PIK3CA</td>
<td></td>
<td></td>
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<td>----------</td>
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<td>-----------------</td>
<td>--------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT474</td>
<td>Breast, Triple positive, Hybricare, 10% FBS, and 5% CO₂</td>
<td>Ductal carcinoma</td>
<td>PI3KCA, P53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-10</td>
<td>Normal Fibroblast, N/A, MEM Bullet Kit, 2.5% FBS, and 5% CO₂</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87-MG</td>
<td>Glioblastoma, N/A, MEM, 10% FBS, and 5% CO₂</td>
<td>Primary tumor</td>
<td>CDKN2A and PTEN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 2.1.1 Generation of MDA-MB-231 ERK5 KO cells

MDA-MB-231 ERK5 KO cells were generously gifted by Dr. Matthew Burow’s laboratory (Tulane University, New Orleans, LA). The Burow lab employed a pU6 driven guide strand with dual expression cassettes for Cas9/EGFP plasmids based approach (Horizon, Cambridge, UK). Using 5 individual guide strands targeting exons 3 and 5 of the MAPK7 gene they transfected TNBC (MDA-MB-231) cells. After 24 hours, cells were FACS for EGFP expression and plated for stable colony formation. The purity of ERK5 KO was determined by western blot analysis of ERK5 expression.

### 2.1.2 Generation of tamoxifen resistant MCF-7 cells

TamR cells were established as described in Rabenoelina et al. 2002. In brief, MCF-7 cells were treated with 100 nM 4-OH Tamoxifen (Sigma) in phenol red free medium with charcoal stripped FBS for 6 months. Resistance was established by assessing sensitivity to 4-OH Tamoxifen (MTT assay), proliferation (Ki67 staining), radioligand binding of Estrogen to ERalpha, and ER-ERE complex formation.
2.2 Cell viability

Cell viability was determined using the MTT assay. Breast cancer cells were plated at a density of 5x10^3 cells per well (96 well plate). Cells were allowed to attach overnight and were treated with inhibitors alone or in combination with 5% FBS stimulation for 72 hours. DMSO was used as a vehicle control. After treatment, 10µL MTT (Sigma) was added to each well (0.5 mg/mL final concentration) and the plates were incubated for 3 hours (5% CO₂ and 37°C). The medium was removed and the MTT-formazan crystals were dissolved with 100µL DMSO per well. Absorbance was measured at 570 nm with a VICTOR³ 1420 multilabel counter (Perkin Elmer). Three wells were analyzed for each condition, and wells containing medium-MTT only (no cells) and vehicle were used as controls. IC₅₀ values from the MTT experiments were calculated with Microsoft Excel.

2.3 Western blot analysis

Protein levels of ERK1/2, ERK5, Akt and apoptotic markers were measured using western blot. Cells were plated at a density of 5x10^5 per well (6 well plate). Cells were allowed to attach overnight and then serum starved for 24 hours before treatment. Cells were treated with vehicle or inhibitors one hour prior to 5% FBS stimulation for 24 hours. For Bad signaling, 50ng/mL EGF for 4 hours was used as a stimulus. Following treatment, cells were lysed and protein content was determined by a Bradford assay (Bio-Rad). 30µg of protein was loaded on a 4-15% SDS-PAGE gradient gel (Bio-Rad). The contents of the gel were transferred to a membrane and then probed with various antibodies: anti-phospho-ERK1/2, anti-total ERK1/2, anti-total ERK5, anti-phospho-ERK5, anti-phospho-Akt (Ser 473), anti-Akt, anti-pS6 (Ser 240/244), anti-p21, anti-cMYC, anti-Bad, anti-phospho-Bad (Ser112, Ser136), and anti-Cleaved PARP (1:1,000, Cell Signaling). Anti-GAPDH (1:10,000; Millipore) was used as a loading
control. The binding of antibody to antigen was detected by incubating membranes with secondary antibodies and scanning on an Odyssey Infrared Imager (LICOR biosciences). Blots were analyzed using ImageStudioLite by a blinded observer (LICOR biosciences). Phospho-Bad Ser112 was quantified with PathScan ELISA (Cell Signaling; 7182C) with the same lysates used in western blot analysis.

2.4 Invasion/ Migration

2.4.1 Scratch Assay

Cell migration was measured using the “scratch” wound healing assay. Cells were seeded at a density of 200,000 cells/well in a 12 well plate. Cells were allowed to attach and grow to 70% confluence. Prior to treatment, the medium was removed and a “scratch” was made with a 200 μL pipet tip. The underside of the plate was marked to denote the location of the initial wound. Detached cells and debris were washed off and removed with 1x PBS. After the wash, treatments were added to each well and initial images were obtained with an EVOS-fl inverted microscope under 10x magnification. The assay was ended with images were obtained after 24 hours. Wound closure was calculated by the formula: \((\text{border at 24 hr} – \text{border at 0 hr})/(\text{border at 0 hr}) \times 100\).
2.4.2 Invasion assay

Cell invasion was assessed using a basement membrane invasion assay. A Boyden chamber with 12 basement membrane inserts were brought to room temperature for 10 minutes and were rehydrated with 300 μL base medium with no FBS for 1 hr. The media was removed prior to adding 270 μL of the cell suspension in base medium with no FBS (125,000 cells/well) and 30 μL of the 10x inhibitor solution (1:10 dilution will make 1x final concentration) to the basement membrane insert. 500 μL of the base medium plus 10% FBS was added to the bottom layer. The plate was incubated for 48 hours. The medium was aspirated from the inner layer and the insert was transferred to a well with 225 μL of cell detachment solution for 30 minutes at 37°C. 75 μL of the Cytoquant/4x Lysis buffer solution was added to each well and incubated for 20 minutes at room temperature 200 μL of the resulting mixture was transferred to a blacked out Fluorescence plate and measured at 485 nm using a micro plate reader. Three wells were analyzed for each condition (n=3).

2.5 ELISAs

ELISA assays were conducted for Cleaved caspase-3, pBad S112, PTEN, and ER-ERE complexes. Breast cancer cells were treated with inhibitors for 48 hours then lysed in accordance with the PathScan Sandwich ELISA (Asp 175) protocol (Cell Signaling; 7190C). The absorbance was measured at 450 nm with a VICTOR\(^3\) 1420 multilabel counter (Perkin Elmer). Three wells were analyzed for each condition and the results were normalized to lysate protein concentration.

2.5.1 Cleaved caspase 3 Cell Signaling #7190C
2.5.2 ERE Active Motif
2.5.3 PBad S112 Cell Signaling #7182C
2.5.4 PTEN Cell Signaling #7882C

2.6 Immunofluorescence

Cellular proliferation and morphology were visualized with immunofluorescence. Cells were plated and treated as described in the MTT assay. After treatment, cells were fixed in 4% paraformaldehyde for 15 minutes. Cells were then permeabilized with 0.3% Triton-X followed by the addition of rabbit Ki67 and mouse α-Tubulin primary antibodies (1:1000, Cell Signaling). Goat anti-mouse Alexa Flour 488 nm and goat anti-Rabbit Alexa Flour 555 nm (1:1000, Invitrogen) were used as secondary antibodies. A Hoechst (Fisher) stain was used to visualize the nucleus. Images were obtained with an EVOS fl inverted microscope (Life Technologies) under 10x objective. A blinded observer counted Hoechst+ and Ki67+ cells using Image J software.

2.7 Inhibitors

Various inhibitors of the MEK1/2/ERK1/2, MEK5/ERK5, and PI3K/Akt pathways were selected due to their potencies (Table 2-2). Additionally, the microtubule inhibitor paclitaxel and ER antagonist tamoxifen were used for select experiments (Table 2-2). All inhibitors were dissolved in DMSO (Fisher) and used in the concentrations noted in the text and figures. Control groups were treated with a percentage of DMSO (0.2% maximum in combination groups) equal to the treatment groups. Structures are listed in Appendix A.
Table 2-2. List of known compound target(s) and potencies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target(s)</th>
<th>Potency (IC_{50})</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMD8-92</td>
<td>ERK5 and BRD4</td>
<td>80, 190 nM</td>
<td>Yang et al. 2010; Lin EC et al. 2016</td>
</tr>
<tr>
<td>AX15836</td>
<td>ERK5</td>
<td>8 nM</td>
<td>Lin EC et al. 2016</td>
</tr>
<tr>
<td>U0126</td>
<td>MEK1/2</td>
<td>60, 70 nM</td>
<td>Duncia et al. 1998</td>
</tr>
<tr>
<td>Trametinib</td>
<td>MEK1/2</td>
<td>0.92, 1.8 nM</td>
<td>Yamaguchi et al. 2011</td>
</tr>
<tr>
<td>CPI-203</td>
<td>BRD4</td>
<td>37 nM</td>
<td>Ballachanda et al. 2012</td>
</tr>
<tr>
<td>LY294002</td>
<td>Pan PI3K</td>
<td>500-570 nM</td>
<td>Chau ssade et al. 2007</td>
</tr>
<tr>
<td>Ipatasertib</td>
<td>Akt 1/2/3</td>
<td>5, 18, 8 nM</td>
<td>Kui et al. 2012</td>
</tr>
<tr>
<td>4-OH Tamoxifen</td>
<td>ER alpha</td>
<td>2-10 nM</td>
<td>Horwitz et al. 1978</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Microtubule</td>
<td>0.1 pM</td>
<td>Wang et al. 2003</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>Alkylating agent</td>
<td>Variable</td>
<td>Lee 2016</td>
</tr>
<tr>
<td>VO-Ohpic trihydrate</td>
<td>PTEN</td>
<td>35 nM</td>
<td>Rosivatz et al. 2006</td>
</tr>
</tbody>
</table>

2.8 Colony formation

Three dimensional cell growth was assessed using a Soft Agar Colony Formation assay (Cell Biolabs: CBA-130). 1×10^4 MDA-MB-231 cells were mixed with a 1.2% agar solution in growth medium (5% FBS) for a final concentration of 0.4% agar. The cell mixture was plated on top of a solidified base agar layer. Inhibitor solutions were added on top of the cell layer and colonies were allowed to grow for 7 days. The contents from each well were solubilized and quantified according to the manufacturer’s instructions. Fluorescence was measured at 485 nm with a VICTOR³ 1420 multilabel counter (Perkin Elmer). Three wells were analyzed for each condition (n=3).

2.9 Synergy calculations

Cell viability data from the MTT assay were analyzed for synergy using the Chou Talalay method (1984). Individual concentration response curves were compared to 1:1 and 1:3 combinations of Ipatasertib:XMD8-92. Combination indexes (CI) were obtained and CI<1 were considered to be synergistic. Synergy data were analyzed with CompuSyn (v 1.4) software.
2.10 Statistical inference

The data are presented as the mean +/- SEM of at least three independent experiments run in triplicate (n=3). Results were analyzed using Graph Pad Prism software (La Jolla, CA). Data were compared to respective controls with a One-Way ANOVA (Boferroni post hoc analysis), Two-Way ANOVA, or t-test (two tailed) where appropriate. A p value <0.05 was considered significant.
Chapter 3: Evaluation of PI3K and ERK5 inhibition in hormonally diverse breast cancer

3.1 Introduction

Breast cancer is a heterogeneous disease state that is primarily characterized by hormonal profile. The majority of breast cancers are estrogen receptor (ER) positive (30-40%) and, HER2 positive (25%) (The cancer genome atlas 2012). In some cases, breast cancer is characterized as triple positive meaning it has receptors for ER, PR and HER2. The standard of care for hormone positive breast cancers is endocrine treatment via hormone receptor antagonists or antibodies. Even though response rates to endocrine therapy are high, relapse and resistance remain a problem and drive mortality (Ali et al. 2016). Resistance is in many cases is attributed to genetic aberrations in survival pathways that confer an advantage for survival. Breast cancers that lack hormone receptors are called triple-negative breast cancer (TNBC). TNBCs constitute 10-15% of all invasive breast cancers and are associated with a poorer prognosis (Chavez et al. 2010). The mainstay of TNBC is chemotherapy and radiation (MD Anderson Manual of Medical Oncology, 2016). With no targeted therapy for TNBC, there is an opportunity to explore MAPK and Akt as possible candidates for targeted treatment. In summary, targeting MAPK and Akt aberrations in hormonally distinct breast cancer could have applications for treatment-resistant ER+ breast cancer and TNBC.

The prevalence of genomic aberrations in the PI3K/Akt/mTOR and Ras/Raf/MEK/ERK pathways have been demonstrated to be key drivers of cancer cell signal transduction (Bailey et al. 2018). With respect to breast cancer, PI3K/Akt mutations are prevalent in certain subtypes: PIK3CA 40% of luminal A and B (ER+), 7% Basal Triple-negative, and 42% HER2 triple positive. PTEN 19% in luminal A and B (ER+), 35% Basal Triple-negative, 19% HER2 triple
positive (The cancer genome atlas 2012). Ras/Raf/MEK/ERK mutations: MAP3K1 14% and 5% of luminal A and B, KRas 1.5% of all breast cancer, BRaf 3% of all breast cancer (Santarpia et al. 2012). Although rarer, the MEK/ERK aberrations are more common in triple-negative and basal subtypes.

Monotherapy against either MAPK or PI3K/Akt has been extensively studied in breast cancer with limited success (Saini et al. 2013). Therefore, combinations of PI3K or Akt and MEK or ERK inhibition were tried in hopes of improved efficacy. However, these pre-clinical studies and clinical trials yielded results less efficacious than expected (Saini et al. 2013). Many combination trials were terminated due to high collateral toxicity. For example, GDC-0941 (PI3K) plus GDC-0973 (MEK1/2) NCT00996892 Phase I study was terminated early due to high grade (3 and 4) toxicities of diarrhea and ocular toxicity. Hyperglycemia is a “class effect” of PI3K inhibition since it decouples PI3K from the insulin receptor and does not allow for glucose to be uptaken into the cells (Moreno et al. 2011). Lastly, pan-isoform PI3K inhibition can anemia due to the inhibition of the delta isoform with is a key factor in blood cells (neutropenia). In response, isoform specific Inhibitors of PI3K such as Taselisib (PI3K, β sparing) and Idelalisib (PI3K delta) were developed. Taselisib is currently in phase III trials for breast cancer. Idelalisib is FDA approved for treating hematopoietic malignancies such as Chronic Lymphoblastic Leukemia (CLL) (Godbersen et al. 2014). The MEK induced retinopathy is a particular “class effect” of MEK1/2 inhibition (Houede et al. 2011, Stjepanovic et al. 2016). MEK signaling maintains the retinal pigment epithelium (RPE) integrity and protects the RPE from oxidative stress, light induced damage and inflammation (Jiang et al. 2009). Clinically, MEK inhibition manifests as retinal detachment and periorbital edema (Stjepanovic et al. 2016). In a phase 1b trial of selumetinib (MEKi) and MK2206 (Akti) the combination group reported
multiple cases of serous retinal detachment (Tolcher et al. 2011). Therefore there is a need to utilize other inhibitory patterns in hopes of better efficacy and lowered toxicity.

As mentioned above, the combination of inhibitors for MEK1/2 and PI3K have been studied extensively in various cancers; however, PI3K inhibitors combined with MEK5/ERK5 inhibition has not been extensively explored in breast cancer. The MEK5/ERK5 pathway is a novel, interesting target because of its diverse roles in the cell. For example, in estrogen positive cancer ERK5 localizes to the nucleus and aids in gene transcription. Whereas, in hormone-independent cancer, ERK5 localizes to the cytosol and is involved in epithelial to mesenchymal transition (EMT) and metastasis (Madak-Erdogan et al. 2014). Moreover, investigations of the role of the MEK5/ERK5 pathways in breast cancer, and cancer in general are limited. One inhibitor for ERK5, XMD8-92, was well tolerated in vivo (Yang et al. 2010) and will be used through out this study. The results of the current study will add to our understanding of the role of this novel pathway in cancers and provide a foundation for the development of novel therapies.

3.2 Hypothesis
Dual inhibition of the PI3K/Akt and MEK5/ERK5 pathways will decrease viability and migration in a panel of hormone receptor diverse breast cancers.

3.3 Results
3.3.1 MDA-MB-231 (TNBC cell line)

First, the MDA-MB-231 TNBC cell line was selected due to its prominence in the literature and unique mutational profile (Chavez et al. 2010). The MDA-MB-231 cell line has a BRAF and KRas “driver mutations” which is crucial for ERK1/2 signal activation. Interestingly,
the MDA-MB-231 cell line is relatively insensitive to MEK1/2 inhibition, in cell culture, despite its BRAF and KRas driver mutations (Hollestelle et al. 2009). Therefore, we propose that sparing ERK1/2 signaling in MDA-MB-231 cells will not affect the efficacy of MEK5/ERK5 and PI3K/Akt dual inhibition.

The PI3K inhibitor LY294002 significantly decreased MDA-MB-231 cell viability in a concentration dependent manner beginning at 1 μM (75% viability) to 100 μM (6% viability) (Figure 3-1A). The MTT IC\textsubscript{50} was determined to be 13.8 μM for LY294002 (Figure 3-2A). The MEK1/2 inhibitor U0126 significantly decreased cell viability beginning at 3 μM (75% viability) however was unable to further reduce viability even at 100 μM (72% viability) (Figure 3-1B). The lack of MEK1/2 inhibitor efficacy was interesting since MDA-MB-231 cells harbor a BRAF driver mutation. However, these results are consistent with previous reports that MDA-MB-231 cells are relatively resistant to MEK1/2 inhibition in 2D culture (Mirzoeva et al. 2009). Lastly, the ERK5 inhibitor XMD8-92 significantly reduced viability in concentration dependent manner from 3 μM (70% viable) to 100 μM (11% viable) (Figure 3-1C). The MTT IC\textsubscript{50} was determined to be 31.3 μM for XMD8-92 (Figure 3-2A). Both the IC\textsubscript{50}s, from the MTT assay, of LY294002 and XMD8-92 were confirmed with crystal violet staining followed by microscopy (Figure 3-2B).

Once LY294002 and XMD8-92 potencies were established, we sought to investigate combinations with the aim of enhanced efficacy. We examined various inhibitor stoichiometries such as 1:1 and 1:3 ratios of LY:XMD. In our study, the 1:1 and 1:3 combinations of LY:XMD decreased viability starting at 3,3 (75% viable) and 5,5 (50%) μM for 1:1 and 1,3 (58% viable) for 1:3 ratio (Figure 3-3A). Additional analysis of the 5,5 μM combination revealed that the combination reduced viability more than each drug alone (Figure 3-3B). Next, the migratory capacity of MDA-MB-231 cells was analyzed with the scratch/wound healing assay (Figure 3-
LY294002 alone decreased wound closure 47% while XMD8-92 decreased wound closure 22% versus control. The 5,5 μM LY+XMD combination decreased wound closure 36% versus control and was not significantly different than each drug alone. Lastly, kinase signaling was analyzed for each inhibitor condition. The ERK5 inhibitor, XMD8-92, decreased the ratio of phosphorylated to total (p/t) ERK5 alone (57% inhibition) and in combination with LY (79% inhibition) (Figure 3-4A). Neither LY294002 nor XMD8-92 alone or in combination inhibited ERK1/2 (Figure 3-4B). PI3K inhibitor, LY294002, decreased the ratio of p/t Akt alone (59% inhibition) and in combination with XMD8-92 (71% inhibition). The inhibitory actions of LY294002 and XMD8-92 were as expected.
Figure 3-1. Evaluation of PI3K, ERK5, and MEK1/2 inhibitors in MDA-MB-231 triple-negative breast cancer cells. MDA-MB-231 TNBC cells were treated with PI3K inhibitor LY294002 (A.), MEK1/2 inhibitor U0126 (B.), and ERK5 inhibitor XMD8-92(C.) for 72 hours under 5% FBS stimulation. Cell viability was determined using the MTT assay. DMSO was used as a vehicle control. Data represent Mean +/- SEM of three independent experiments run in triplicate (n=3). One-Way ANOVA analysis performed with GraphPad prism software. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 vs DMSO+FBS vehicle control.
Figure 3-2. Determination of IC$_{50}$ of LY294002 and XMD8-92 in MDA-MB-231 cells. MTT results from figure 1 were analyzed with Microsoft excel to determine IC$_{50}$ values (A). MDA-MB-231 cells were treated with IC$_{50}$ concentrations obtained from MTT assay for 72 hours under 5% FBS stimulation. Cells were fixed with 4% paraformaldehyde, stained with 0.1 % crystal violet, and imaged under 10x magnification with an EVOS fl microscope (B).
Figure 3-3. Simultaneous ERK5 and PI3K inhibition decreased viability and wound closure in MDA-MB-231 cells. MDA-MB-231 were treated with various 1:1 and 1:3 combinations of LY:XMD for 72 hours under 5% FBS stimulation (A and B). Cell viability was determined using the MTT assay. MDA-MB-231 cells were treated with LY294002 and XMD8-92 alone or in combination for 24 hours under 5% FBS stimulation (C). DMSO was used as a vehicle control. Wound closure measured using the “scratch” assay. Data represent Mean +/- SEM of three independent experiments run in triplicate (n=3). One-Way ANOVA analysis performed with GraphPad prism software. *P<0.05, **P<0.01, and ***P<0.001 vs DMSO+FBS vehicle control. #P<0.05 vs each drug alone.
Figure 3-4. Combination of ERK5 and PI3K blockade inhibited respective targets while sparing ERK1/2 activity in MDA-MB-231 cells. Kinase analysis of LY+XMD 5 μM each combination in MDA-MB-231 cells. MDA-MB-231 cells were treated with 5 μM LY294002 and XMD8-92 alone or in combination for 1 hour prior to 50ng/mL EGF stimulation for 24 hours. Western blot analysis was performed on cellular lysates. GAPDH was used as a loading control. ERK5 (A), ERK1/2 (B), and Akt (C) were quantified using Image Studio Lite. *P<0.05 and **P<0.01 vs DMSO+EGF control.
3.3.2 MCF-7 (ER+ cell line)

Next, the MCF-7 cell line was selected because of its ER+ status, luminal A subtype, and PI3K mutational status (Perou et al. 2000, Sorlie et al. 2001). As mentioned in the introduction, Akt can crosstalkactivate the MEK5/ERK5 via MEKK3 phosphorylation (Umapathy et al. 2014). Therefore, we propose that cancers harboring PI3K and PTEN alterations perhaps activate the MEK5/ERK5 pathway and necessitate the inhibition of both pathways. Additionally, we also propose that dual MEK5/ERK5 and PI3K/Akt inhibition can be achieved without disrupting MEK1/2/ERK1/2 signaling.

In the MCF-7 cell line, the PI3K inhibitor LY294002 significantly decreased viability at 5 μM (58% viability; Figure 3-5A). The ERK5 inhibitor XMD8-92 also significantly decreased viability at 5 μM although to a lesser degree than LY294002 (79% viability). In combination the viability was 45%, which was significantly less than both drugs alone (Figure 3-5A). With respect to migration, LY294002 and XMD8-92 decreased wound closure 60% and 45%, respectively. In combination the drugs decreased wound closure 80%, which was significantly greater than both drugs alone (Figure 3-5B). XMD8-92 decreased the ratio of p/t ERK5 alone (74% inhibition) and in combination (59% inhibition) (Figure 3-6A). Neither LY294002 nor XMD8-92 decreased the p/t ratio of ERK1/2 (Figure 3-6B). Both LY294002 alone or in combination inhibited the p/t ratio of Akt by 97% (Figure 3-6C). XMD8-92 increased the p/t ratio of Akt by two fold of control.
Figure 3-5. ERK5 and PI3K combination decreases viability and wound closure in Estrogen positive MCF-7 cells. MCF-7 cells were treated with 5 μM LY294002 and XMD8-92 alone or in combination for 72 hours under 5% FBS stimulation (A). DMSO was used a vehicle control. Cell viability was determined using the MTT assay. MCF-7 cells were treated with LY294002 and XMD8-92 alone or in combination for 24 hours under 5% FBS stimulation (B). Wound closure measured using the “scratch” assay. *P<0.05, **P<0.01, and ***P<0.001 vs DMSO+FBS control. #P<0.05 vs drugs alone.
Figure 3-6. Dual ERK5 and PI3K combination inhibits respective targets while sparing ERK1/2 activity in MCF-7 cells. MCF-7 cells were treated with 5 μM LY294002 and XMD8-92 alone or in combination for 1 hour prior to 50ng/mL EGF stimulation for 24 hours. Western blot analysis was performed on cellular lysates. GAPDH was used as a loading control. ERK1/2 (A), Akt (B), and ERK5 (C) were quantified using Image Studio Lite. ***P<0.001 vs DMSO+EGF control.
3.3.3 BT474 (Triple positive cell line)

Lastly, the cell line BT474 was evaluated due to its triple positive hormone profile, luminal B status and PI3K mutational status (Perou et al. 2000, Sorlie et al. 2001). In the viability assay, only the combination of LY+XMD decreased viability 51% (Figure 3-7A). Additionally, the scratch assay revealed interesting results with each of the compounds alone or in combination “widening” the initial wound (Figure 3-7B). Initial interpretations would be to assume that this was cell loss, however, these results appear to contradict the viability results where cell loss was only observed in the combination group. This could be due to an early loss of cells at the 24 hour time point as seen in the scratch assay and then the cells grow back in the single drug groups by the 72 hour mark and therefore the drugs appear to be ineffective. Alternatively, this could all be an artifact due to a surfactant effect. To account for this, we centrifuged our plates to pull down any floating cells that may still be viable. Concerning kinase activity, Only the combination of LY+XMD significantly the pERK5/tERK5 ratios compared to control (46% decrease Figure 3-8). pERK1/2/tERK1/2 ratios were unaffected in all three groups (Figure 3-8). The PI3K inhibitor LY294002 worked as expected and decreased pAkt/tAkt ratios 84% alone and 40% in combination (Figure 3-8).
Figure 3-7. ERK5 and PI3K combination decreases viability in triple-positive BT-474 cells. BT-474 cells were treated with 5 μM LY294002 and XMD8-92 alone or in combination for 72 hours under 5% FBS stimulation (A). DMSO was used as a vehicle control. Cell viability determined using the MTT assay. BT-474 cells were treated with LY and XMD alone or in combination for 24 hours under 5% FBS stimulation (B). Wound closure measured using the “scratch” wound healing assay. *P<0.05 vs DMSO+FBS control.
Figure 3-8. **BT-474 cells western blot analysis.** BT-474 cells were treated with 5 μM LY294002 and XMD8-92 alone or in combination for 1 hour prior to 50ng/mL EGF stimulation for 24 hours. Western blot analysis was performed on cellular lysates. GAPDH was used as a loading control. ERK5 (A), ERK1/2 (B), and Akt (C) were quantified using Image Studio Lite. **P<0.01 vs DMSO+EGF control.**
3.4 Discussion

The 5.5 μM combination of LY+XMD reduced viability and migration in all three cell lines regardless of hormonal profile. In some cases the drugs were more potent alone such as LY294002 in the MCF7 cell line. This is logical because the MCF7 cell line has an E545K PI3K mutation that leads to constitutive activity and increased pAkt signaling (Wu et al. 2005). Clinically evaluated compounds such as the Pan PI3K inhibitor Pictisilib have shown enhanced efficacy in the MCF7 cell line (Liu S et al. 2018). The 5.5 μM combination also spared ERK1/2 signaling in all three cell lines. These results were consistent with Yang et al. 2010 who found that concentrations of 5 μM or less XMD8-92 did not inhibit ERK1/2 kinase activity. However, only one time point was tested, so it is possible there was transient inhibition or activation of ERK1/2 that was not detected. Therefore, in chapter 4 we will use additional time points to fully characterize the ERK1/2 phosphorylation state over time. Interestingly, the signaling produced in MDA-MB-231 and MCF-7 cells was similar: both phosphorylated and total Akt levels increased under XMD8-92 inhibition. Perhaps this is a compensatory pathway as described in (Umapathy et al. 2014) where the inhibited ERK5 pathway signals to Akt to upregulate and then reactivate MEK5/ERK5 via MEKK2 phosphorylation. This is also evidenced by the MCF-7 viability data where XMD only reduced viability by 21% alone, perhaps the increase in Akt reduced the effects of XMD. Therefore both PI3K and ERK5 need to be inhibited for efficacy.

Further characterization is needed to determine if the PI3K and ERK5 inhibitor combination is synergistic. Similar studies of dual Akt and MEK inhibition demonstrated 1:2, and 1:4, and 1:8 ratios of Akt:MEK inhibition were effective in a panel of Non-small cell lung cancer (NSCLC) cell lines (Meng et al. 2010). Therefore, we will expand our study in the next chapter to encompass staggered ratios of PI3K/Akt to MEK5/ERK5 inhibition. Also, we will
continue to monitor the role of ERK1/2 because sparing its activity may be key to limiting toxicity. This is particularly important since many combination trials with MEK inhibition have been halted due lack of efficacy, and high incidence of adverse events (NCT01907815, NCT00996892). However, it is possible that sparing ERK1/2 activity may enhance invasion, which has been reported in breast cancer both cell lines and in patients (Barholomeusz et al. 2012, Ma et al. 2012). Therefore, we will monitor the invasive potential of combination treated cells in chapter 4 to ensure the combination does not enhance invasion by sparing ERK1/2 activity.
Chapter 4: Evaluation of clinically relevant Akt inhibitor Ipatasertib in combination with ERK5 inhibitor XMD8-92 in TNBC

4.1 Introduction

To determine if the LY+XMD combination synergistically inhibits TNBC cell viability, a therapeutically relevant Akt inhibitor, Ipatasertib (GDC-0068; Ipa), was investigated. Ipatasertib is currently in a Phase III trial for patients with PI3K/ATK1/PTEN altered TNBC (NCT03337724), thus a primary reason why it was included in our study. Ipatasertib is more effective in pAkt high (low PTEN or Akt mutant) environments because it binds to Akt in the ATP site in the active form (Kui et al. 2012). The resulting Akt inhibition decreases phosphorylation of downstream targets: S6 ribosomal protein, Bad, and FOXO3a (Kui et al. 2012, Lin J et al. 2013). In addition to promising pre-clinical results, Ipatasertib was well tolerated in combination with the MEK inhibitor GDC-0973 in a phase I clinical trial (NCT01562275). The trial yielded less severe toxicities than previous MEK+PI3K/Akt combinations: GDC-0973 (MEK) combined with GDC-0941 (PI3K) (NCT00996892). For example, Ipatasertib treated patients did experience the “class effects” of PI3K/Akt inhibition which included transient hyperglycemia and low grade (1 and 2) GI upset. This was in contrast to PI3K inhibition that produced sustained hyperglycemia, increased insulin levels, and severe colitis. Therefore, Ipatasertib appears to display an improved “class effect” profile compared to clinically investigated PI3K inhibitors. This is a key factor in ensuring the tolerability of Akt inhibition in a patent population. Even though Ipatasertib had fewer side effects than its PI3K predecessors, the combination trial phase 1b with MEK1/2 inhibition was not progressed to phase 2. This was primarily due to lack of efficacy and “class effect” ocular toxicity from MEK1/2 inhibition persisted in several patients: 14% on 300mg GDC-0973 and 33% on 400mg
GDC-0973 (NCT01562275). Therefore, we propose our strategy of sparing MEK1/2 will help reduce toxicity.

4.2 Hypothesis

Combination of ERK5 inhibitor XMD8-92 with clinically relevant Akt inhibitor Ipatasertib will decrease viability, colony formation, and migration in MDA-MB-231 TNBC cells.

4.3 Results

4.3.1 Ipatasertib in MDA-MB-231 WT

In chapter 3, the PI3K inhibitor LY294002 in combination with the ERK5 XMD8-92 significantly reduced viability in MDA-MB-231 cells. However, issues with PI3K inhibitor toxicity at the clinical level prompted us to use a more relevant and translatable inhibitor. As previously stated, Ipatasertib was tolerated better than PI3K inhibitors and is currently under investigation in TNBC. Therefore, we investigated Ipatasertib in combination with XMD8-92 in MDA-MB-231 TNBC cells.

Ipatasertib, binds to Akt in the active conformation. This conformation protects the Thr308 and Ser473 phosphorylation sites from becoming dephosphorylated by the phosphatase PP2A. Therefore, pAkt is observed in the presence of Ipatasertib (Kui et al. 2012). In our study, the efficacy of Ipatasertib was determined by measuring phosphorylation of the ribosomal protein S6 (pS6; Ser 240/244), a downstream target of Akt. As expected, Ipatasertib significantly increased pAkt vs control and a significantly decreased of pS6 vs control (Figure 4-1). The PI3K inhibitor LY294002 worked as expected by inhibiting pAkt 79% and its downstream target pS6 89% (Figure 4-1). Ipatasertib also worked as expected by increasing pAkt 26% over control while inhibiting pS6 40% (Figure 4-1). Interestingly, adding LY294002 first prevented the
increased of pAkt by Ipatasertib. These results were consistent with previous findings (Kui et al. 2012).

Next, we assessed the effect on viability, proliferation, invasion, and colony formation in MDA-MB-231 TNBC cells. Ipatasertib reduced viability in a concentration dependent manner from 1 \(\mu\)M (62% viable) to 300 \(\mu\)M (4.5% viable) with an IC\(_{50}\) value of 10.4 \(\mu\)M (Figure 4-2 A). Equal ratios (1:1) of Ipatasertib to XMD8-92 reduced viability in a concentration-dependent manner (Figure 4-2 B). In Wright et al. 2018 the 1:1 combinations were determined to be synergistic (CI<1). Additionally, staggered ratios of 1:3 of Ipatasertib to XMD8-92 exhibited the strongest synergy with CIs of 0.11 and 0.25, respectively (figure 4-2 C). Conversely 3:1 ratios of Ipatasertib to XMD8-92 resulted in antagonism with CIs of 1.24 and 1.09, respectively (Figure 4-2 C). Additional analysis of the 1:3 Ipat:XMD combo revealed the sparing of ERK1/2 kinase activity and low toxicity in MCF10A cells (Wright et al. 2018). The MCF-10A cell line was selected as a normal tissue control because it is considered to be basal B but it does not contain driver mutations such as BRAF, KRas, or TP53 (MDA-MB-231 is also basal B; Kao et al. 2004).

The Ipatasertib plus XMD8-92 combination also decreased proliferation in MDA-MB-231 cells (Figure 4-3). This was evidenced by the decrease in Ki67 expression in both the XMD8-92 and combination groups. These results were similar to other reports of p21 induced cell cycle arrest in TP53 mutant TNBC cells (He et al. 2019). The morphology of the cells in the combination group appeared mesenchymal and ERK1/2 activity was spared; which prompted concerns of cell invasion (Liu Y et al. 2015). Therefore, we examined invasion in a basement membrane trans-well assay. Each drug alone decreased invasion by 58% (Ipatasertib) and 48%
(XMD8-92) (Figure 4-4 A). The combination decreased invasion by 50% and was not significantly different from the drugs alone.

As mentioned in chapter 3, MDA-MB-231 cells are resistant to MEK inhibition in 2 D culture but are sensitive in 3 D culture (Li Q et al. 2010). Therefore, we determined to effect of dual Akt and ERK5 inhibition on colony formation in a 3 D culture system. MDA-MB-231 cells were grown in soft agar for 7 days and treated with Ipatasertib and XMD8-92 alone or in combination. Ipatasertib did not decrease colony formation 103% vs control while XMD8-92 did significantly decrease colony formation alone (44%) and in combination with Ipatasertib (73%) (Figure 4-4 B). The latter was significantly different than both drugs alone.

Figure 4-1 Evaluation of clinical Akt inhibitor Ipatasertib in MDA-MB-231 cells. MDA-MB-231 cells were treated with 1 μM Ipatasertib and LY294002 for 1 hour prior to 50ng/mL EGF stimulation for 15 minutes. DMSO was used as a vehicle control. Western blot analysis was performed on cellular lysates. Phosphorylated Akt (Serine 473) (A) and Phosphorylated S6 (Serine 240/244) (B) levels were quantified using image studio lite. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 vs DMSO+EGF control.
Figure 4-2. Ipatasertib combined with XMD8-92 synergistically reduces viability in MDA-MB-231 cells. MDA-MB-231 cells were treated with Ipatasertib alone (A) or in 1:1 (B) and 1:3 or 3:1 (C) combinations with XMD8-92 for 72 hours. Cell viability was determined using the MTT assay. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 vs control.
Figure 4-3. Ipatasertib combined with XMD8-92 decreases proliferation marker Ki67 in MDA-MB-231 cells. MDA-MB-231 cells were treated with Ipatasertib and XMD8-92 alone or in combination for 72 hours under 5% FBS stimulation. DMSO was used as a vehicle control. Cells were fixed and probed with fluorescent antibodies for Tubulin and Ki67 and imaged with an EVOS-fl inverted microscope under 20x magnification. The ratio of Ki67 to Hoechst positive cells was used to determine the proliferative fraction.
Figure 4-4. Ipatasertib plus XMD8-92 decreases colony formation and invasion in MDA-MB-231 cells. MDA-MB-231 were seeded in the top chamber of a matrigel transwell system and were allowed 24 hours to invade to the bottom chamber containing 10% FBS (A). Invasive cells were analyzed fluorescently as per the kit instructions. *P<0.01 vs FBS control. MDA-MB-231 cells were grown in soft agar and treated with Ipat and XMD alone or in combination for 7 days (B). Colony formation was determined as per the kit instructions. **P<0.01 and ****P<0.0001 vs control. #P<0.05 vs each drug alone.
4.3.2 Ipatasertib in MDA-MB-231 ERK5 KO

Next, we sought to elucidate the mechanism of synergy the Ipatasertib plus XMD8-92 combination. Since the molecular tools for investigating ERK5 are limited, we used MDA-MB-231 ERK5 KO cells obtained from Matthew Burow’s Lab at Tulane University, New Orleans. The ERK5 KO cells were first characterized for ERK5 expression (Figure 4-5A). The MDA-MB-231 ERK5 KO cells were then treated with increasing concentrations of the Akt inhibitor Ipatasertib. In particular, 100 nM Ipatasertib significantly decreased viability by 28% (compared to vehicle control) in the ERK5 KO cell line compared to 12% (compared to vehicle control) for the same concentration in the wild type cell line (Figure 4-5B). Additionally, a modest difference in IC_{50} was observed between the wild type (10.4 μM) and ERK5 KO (6.6 μM) cell lines. These results were less encouraging than the Ipatasetib plus XMD8-92 combination (Chapter 4).

Therefore, we investigated an off target effect of XMD8-92: BRD4 inhibition (Lin EC et al. 2016). BRD4 a member of the bromodomain and extra terminal domain family (BET) and is an epigenetic regulator involved in expression of the oncogene cMYC in several cancers (Da Costa et al. 2013). The BRD4 inhibitor CPI203 (Wong et al. 2014) was selected and administered in combination with Ipatasertib in the MDA-MB-231 ERK5 KO cells. Both CPI and Ipatasertib alone decreased viability 28% (compared to vehicle control) at a concentration of 100 nM in the ERK5 KO cell line (Figure 4-6A). The 1:1 combination of Ipatasertib to CPI (100 nM each) decreased MDA-MB-231 ERK5 KO cell viability by 63% (compared to vehicle control) and was significantly different than either drug alone (Figure 4-6A). Additionally, the 1:1 combination results approximately recapitulated the Ipat+XMD combo from Figure 4-2B.

With BRD4 looking like a major factor in the proliferative component, we sought to explore the role of ERK5 in apoptosis. Previous studies have shown that ERK5 regulates the
proapoptotic protein Bad through phosphorylation of serine 112 (Pi et al. 2004, Wright et al. 2019). The regulation of Bad by ERK5 was shown to be independent of ERK1/2 and p90RSK. To address this we performed an ELISA for pBad S112 in MDA-MB-231 WT and MDA-MB-231 ERK5 KO cell types (Figure 4-7). Interestingly, the pBad 112 activity was abolished in the 231 ERK5 KO cell line since the cells were not responsive to EGF (Figure 4-7A). To ensure this result was not due to assay error, control proteins for Bad and pBad were incubated with spare wells from the ELISA plate. The results indicated that the kit was working properly (Figure 4-7C). We considered the possibility that the pBad s112 is transiently inhibited or activated by EGF. MDA-MB-231 WT and MDA-MB-231 ERK5 KO cells were then treated with EGF for 15 minutes, 1 hour 4 hours and 24 hours. In the MDA-MB-231 WT cell line pBad S112 phosphorylation was sustained for 24 hours while the MDA-MB-231 ERK5 KO cell line did not show pBad S112 levels above basal activation (Figure 4-8A). Next, we considered that the pBad S112 downregulation in MDA-MB-231 ERK5 KO cells could be due to downregulation of ERK1/2 and p90RSK. However, pERK1/2 and p90RSK levels were not decreased by Ipatasertib or CPI-203 treatment across all time points tested (Figure 4-8C). Therefore, the phosphorylation of pBad S112 appears to be independent of pERK1/2 and p90RSK.
Figure 4-5. ERK5 KO in MDA-MB-231 cells modestly enhanced sensitivity to Ipatasertib. ERK5 levels were determined via western blot analysis (A). 231 WT and 231 ERK5 KO cells were treated with various concentrations of Ipatasertib for 72 hours under 5% FBS stimulation (B). Cell viability was determined using the MTT assay. IC\textsubscript{50} values were obtained using Microsoft excel. *P<0.05 vs 231 WT cell line.
Figure 4-6. BRD4 inhibition enhances the effect of Ipatasertib in MDA-MB-231 ERK5 KO cells. MDA-MB-231 ERK5 KO cells were treated with Ipatasertib alone or in combination with BRD4 inhibitor CPI203 for 72 hours under 5% FBS stimulation (A). Cell viability was determined using the MTT assay. Cells were fixed and probed with fluorescent antibodies for
Tubulin and Ki67 (B). The ratio of Ki67 (D) to Hoechst (C) positive cells was used to determine the proliferative fraction. *P<0.05 vs drugs alone.

Figure 4-7. ERK5 KO abolishes pBad 112 in EGF stimulated MDA-MB-231 cells. MDA-MB-231 ERK5 KO cells were treated with Ipatasertib and CPI203 alone or in combination for 1 hour prior to 50ng/mL EGF stimulation for 4 hours (A). pBad s112 levels were determined via ELISA. Basal and stimulated pBad 112 levels were compared between 231 WT and 231 ERK5 KO (B). ELISA sensitivity for controls: Bad, pBad 112 and secondary antibody (C). P<0.001 vs No Tx 231 WT.
Figure 4-8. **pBad 112 decrease in 231 ERK5 KO cells was independent of pERK1/2 and p90RSK signaling.** MDA-MB-231 ERK5 KO cells were treated with Ipatasertib and CPI203 for 1 hour prior to 50ng/mL EGF stimulation for 15 min, 1hr, 4hr, and 24hr. Cellular lysates were analyzed with western blot analysis. Protein levels were obtained for pBad S112 (A and B), pERK1/2 (C), p90RSK (D), and GAPDH was used as a loading control.
Figure 4-9. Synergy summary.
Proposed synergy mechanism in MDA-MB-231 TNBC cells. Ipatasertib and XMD8-92 treatment decrease the phosphorylation of Bad at two separate sites which induces apoptosis. Additionally, XMD8-92 inhibits BRD4, which leads to p21 expression and a decrease in proliferation.

4.3.3 ERK5 KO enhances conventional therapy Paclitaxel in MDA-MB-231 cells

To tie together the relevance of ERK5 KO in MDA-MB-231 cells, we examined the effect of adding conventional chemotherapy Paclitaxel. Significant differences in viability between MDA-MB-231 WT and MDA-MB-231 ERK5 KO were observed beginning at 1 nM and continuing to 10 nM (Figure 4-10). The IC$_{50}$s between the two cell lines were also significantly different by approximately 7 fold; 5.9 nM for MDA-MB-231 WT and 0.89 nM for MDA-MB-231 ERK5 KO (Figure 4-10). These results are similar to previous results that showed ERK5 knockdown, with shRNA, enhanced microtubule inhibitor efficacy in breast cancer cell lines (Al-Ejeh et al. 2014, Ortiz-Ruiz et al. 2014).
**Figure 4-10.** ERK5 KO enhances sensitivity to conventional therapy Paclitaxel in MDA-MB-231 TNBC cells. MDA-MB-231 WT and ERK5 KO cells were treated with Paclitaxel for 72 hours under 5% FBS stimulation. Cell viability was determined using the MTT assay. *P<0.05 vs 231 WT cell line. IC$_{50}$ values were determined with Microsoft excel.

**4.4 Discussion**

Figure 4-2 demonstrates the synergy of Ipatasertib plus XMD8-92. 1:1 and 1:3 ratios achived CIs below one (synergistic). Whereas, 3:1 combinations of Ipatasertib to XMD8-92 were antagonistic (CI>1). These results were similar to previous studies, which examined combinations of Akt and MEK1/2 inhibitors and observed synergy at 1:2, 1:4, and 1:8 ratios and antagonism with 4:1 and 8:1 ratios in lung cancer cell lines (Meng et al. 2010). However, in our study we were able to achieve synergy while sparing ERK1/2 activity, thus indicating a distinct inhibitory pattern. Another distinction is our study is the type of synergy. Synergy obtained from dual PI3K and MEK1/2 is considered to be parallel. While synergy obtained from BRAF and MEK1/2 is considered to be serial (Yin et al. 2014). We propose the synergy between Akt and ERK5 to be serial rather than parallel due to the feedback loop between the two (Chapter 3) and the modifications of anti-apoptotic protein such as Bad (Figure 4-9). However, these aspects primarily describe synergy with respect to viability. Therefore the question becomes: Is
Ipatasertib+XMD8-92 synergistic with respect to invasion, proliferation, and colony formation? The answer is more complicated due to the involvement of BRD4.

According to our earlier work, Wright et al. 2018, inhibition BRD4 in MDA-MB-231 cells does play an important role in the antiproliferative effect of Ipat+XMD by reducing Ki67 expression and inducing p21 expression. This was shown in the IF images in Figure 4-3. Due to the mesenchymal morphology observed in Figure 4-3 we were concerned with invasion in the combination group. In our study, we did not observe increased invasion (Figure 4-4A). Additionally, the mesenchymal shape of the cell may be explained by the inhibition of BRD4 which promotes that shape but is not necessarily more invasive (Andrieu and Denis 2018). Another contribution to the cell shape could be the prolonged expression of p21. In Wright et al. 2018, p21 expression was significantly increased at 24 hours in groups containing XMD8-92 or the BRD4 inhibitor CPI-203. Prolonged p21 expression has been associated with senescence in TP53 mutant tumors and not necessarily an invasive phenotype (Chang et al. 1999). However, new concern arises if the cells are dormant and still have the ability to form tumors. Therefore, we examined the effect of dual inhibition on colony formation. In the 7 day period the combination was much more effective than either drug alone (Figure 4-4B). These results are in line with previous work that has shown ERK5 knock down disrupts colony-stimulating factor signaling (Dong et al. 2001; Rovida et al. 2008). Taken together, BRD4 appear to contribute to the anti-proliferative effects of Ipatasertib+XMD8-92 while ERK5 is involved in colony formation.

To further elucidate the synergy mechanism we utilized the MDA-MD-231 ERK5 KO cell line. Knocking out ERK5 only had a modest effect on cell viability when treated with Ipatasertib and shifted the IC\textsubscript{50} from 10.4 μM (231 WT) to 6.6 μM with (231 ERK5 KO) (Figure 4-5B).
Addition of the BRD4 inhibitor CPI203 in the MDA-MB-231 ERK5 KO reduced cell viability to similar levels seen with the Ipatasertib+XMD combination in 231 WT cells (Figure 4-2B and Figure 4-6B). Next, we sought to examine the effect of ERK5 KO in the pro-apoptotic protein, Bad. Interestingly, the phosphorylation of pBad S112 was decreased to near basal levels and was not responsive to EGF even at multiple time points (Figure 4-7A and Figure 4-8B). Furthermore, the decrease in pBad S112 did not correspond to a decrease in ERK1/2 and p90RSK (Known to phosphorylate pBad S112). BIM is a BH3 protein that promotes cell death in response to cellular stress (Bouillet et al. 1999). The role combined with paclitaxel is more complicated since paclitaxel effects the mitotic phosphorylation of BIM$_{EL}$ via CDK1 phosphorylation, which leads to polyub of BIM$_{EL}$ and degradation via the proteasome. In this case, in HCT116 cells was independent of ERK1/2 and ERK5 (Gilley et al. 2012). Therefore, it is possible that the decrease in pBad S112 is due to CDK1 downregulation due to the ERK5 KO. Further studies are needed to evaluate the role of CDK1 in the ERK5 KO cells.
Chapter 5: Evaluation of Ipatasertib plus XMD8-92 in PTEN altered TNBC

5.1 Introduction

PTEN is a tumor suppressor gene that is frequently altered in TNBC (Chavez et al. 2010). Loss of PTEN enhances cancer cell survival and proliferation via the PI3K/Akt/mTOR signaling axis. Various mechanisms contribute to PTEN alteration: loss of function point mutations, hetero or homozygous deletion on chromosome 10, suppression via miRNA, degradation due to stress, and epigenetic suppression (Laugher et al. 2001, Li N et al. 2017, Shao et al. 2017). PTEN is altered in approximately 35% of TNBCs and less frequently in HER2+ (10%) and luminal A/B (5%) IDCs (The cancer genome atlas 2012). PTEN loss in TNBCs occurs primarily due to homozygous deletion while HER2+ breast cancers lose PTEN in response to Trastuzumab treatment (Li N et al. 2017). Clinically, PTEN and PI3K mutation status correlate with Trastuzumab resistance and decreased response rates in patients with HER2+ breast cancer (Rimawi et al. 2018). Mechanistically, when PTEN is downregulated HIF-1a remains stable in the hypoxic conditions generated by Trastuzumab treatment (Aghazadeh et al. 2017). The stabilization of HIF-1a facilitates the expression of genes such as VEGF, a prompter of angiogenesis (Laugher et al. 2001). In summary, PTEN status is widely considered an “actionable” alteration due to its potential for targeted therapy at both the pre-clinical and clinical levels.

In our initial studies, MDA-MB-231 (TNBC; PTEN WT) cells treated with XMD8-92 exhibited increased levels of pAkt (S473). Groups treated with both Ipatasertib and XMD8-92 appeared to “capture” more pAkt (S473) than either drug alone (Wright et al. 2018). These results were remarkable since Ipatasertib is generally considered non-effective in PTEN WT cancer cell lines (Lin J et al. 2013). Therefore, we propose that XMD8-92 sensitizes the PTEN
WT MDA-MB-231 cells by modulating PTEN expression. We will evaluate the enhancement of Ipatasertib by XMD8-92 in two PTEN mutant TNBCs: BT549 (Basal B) and MDA-MB-468 (Basal A) cells.

5.2 Hypothesis
Ipatasertib plus XMD8-92 will decrease viability in PTEN mutant or PTEN inhibited TNBC cell lines: MDA-MB-468 (mut), BT549 (mut), and MDA-MB-231 (PTENi).

5.3 Cellular models
MDA-MB-468 cells are derived from pleural effusions and are adenocarcinomas in origin. MDA-MB-468 cells are Basal A and grow in “grape-like” clusters in 2D cell culture and have round morphology (Kenny et al. 2007). MDA-MB-468 cells harbor TP53 and PTEN driver mutations (Hollestelle et al. 2009). Additionally, MDA-MB-468 cells have a rare amplification of EGFR (1-6% occurrence, Filmus et al. 1985, Bhargava et al. 2005). MDA-MB-231 cells are also derived from pleural effusions and are adenocarcinomas in origin. MDA-MB-231 cells are Basal B and grow in stellate patterns in 2D cell culture (Neve et al. 2006, Kenny et al. 2007, Kao et al. 2009). MDA-MB-231 cells are PTEN wild type and harbor TP53 and KRAS driver mutations (Hollestelle et al. 2009). BT-549 cells are derived from primary tumors and are invasive ductal carcinomas (IDCs). BT-549 cells are Basal B and grow in stellate patterns in 2D culture and have similar morphology as MDA-MB-231 cells (Neve et al. 2006, Kenny et al. 2007, Kao et al. 2009). BT-549 cells harbor TP53 and PTEN driver mutations (Hollestelle et al. 2009). Lastly, MCF10A cells were used as a normal tissue control. MCF-10A cells are derived from normal breast tissue and are Basal B subtype (Neve et al. 2006, Kao et al. 2009). This cell line does not harbor any driver mutations as seen in the other TNBC cell lines and therefore is
considered “normal”. However, MCF-10A cells can be progressively transformed by KRas and HRas mutations (Santner et al. 2001).

5.4 Results

5.4.1 PTEN degradation in MDA-MB-231 TNBC cells

The Ipat+XMD combination in MDA-MB-231 (Basal B) cells exhibited increased pAkt and decreased PTEN levels (Wight et al. 2019; Figure 5-1A and B). Additionally, HIF-1a expression was significantly increased in the Ipat+XMD combination group. These data are consistent with previous work that showed HIF-1a stabilization with concurrent PTEN downregulation (Laughner E et al. 2001, Safyeh Aghazadeh et al. 2017, Azimi et al. 2017). We observed similar results in the MCF-10A (Basal B) cell line (Figure 5-2). The decrease in PTEN and increase in pAkt was only seen at the 1.3 uM concentration in the MCF-10A cell line (Figure 5-2B). At the 1.3 uM concentration viability was modestly reduced by 22% in the MCF-10A cells (Figure 5-2A). These results were line with Lin J et al. 2013 who showed PTEN KO in MCF-10A cells sensitized Ipatasertib treatment. Next, we attempted to establish if PTEN inhibition was the primary source of Ipatasertib efficacy in a PTEN WT TNBC cell line. We treated MDA-MB-231 with a PTEN inhibitor (PTENi) and added Ipatasertib in combination. The PTEN inhibitor alone increased both pAkt and pS6 1.5 and 1.3 fold over control, respectively (Figure 5-3 B and C). Ipatasertib behaved as expected and increased pAkt 1.8 fold over control while reducing pS6 activity by 60% with respect to control. Lastly, the combination of PTENi and Ipatasertib increased pAkt 2.2 fold and decreased pS6 activity by 75% (Figure 5-3 B and C). Although the principle of the assay was a success, the PTENi+Ipat combination did not affect viability more than Ipatasertib alone (Figure 5-3A). The inhibition of PTEN did not
enhance the effect of Ipatasertib. Therefore, PTEN status in the MDA-MB-231 cell line was not the main factor of efficacy of the Ipat+XMD combination.

Figure 5-1. Ipat+XMD condition induces PTEN degradation in MDA-MB-231 TNBC cells. MDA-MB-231 cells were treated with Ipatasertib and XMD8-92 alone or in combination for 24 hours under 25ng/mL EGF stimulation or 5% FBS for 24 hours. Cellular lysates were analyzed with ELISA (left) and western blot (right). MDA-MB-468 (PTEN null) lysates were used for the PTEN ELISA and GAPDH was used as a loading control for the western blot (A and B). *P<0.05, **P<0.01, and ***P<0.001 vs control. ##P<0.01 vs drugs alone.
Figure 5-2. Ipat+XMD decreases PTEN expression and increases pAkt in MCF10A cells. MCF10A cell lines were treated with Ipat+XMD at various concentrations for 72 hours under 20ng/mL EGF stimulation (A). Cells were visualized with an EVOSfl inverted microscope under a 10x objective. Cell lysates were obtained for Ipat and XMD treated cells for 24 hours under 20ng/mL EGF stimulation (B). PTEN, pAkt, and GAPDH were visualized with western blot.
Figure 5.3. Ipatasertib efficacy is not enhanced by inhibited PTEN in MDA-MB-231 cells. MDA-MB-231 cells were treated with PTENi and Ipatasertib alone or in combination for 72 hours under 5% FBS stimulation (A) In the combination group 100 nM PTENi was used. *P<0.05 vs PTENi alone. Cellular lysates were collected and analyzed with western blot (B and C). Levels of pAkt (S473) and pS6 (S240/244) were determined. *P<0.05, **P<0.01 vs DMSO+FBS control, # vs Ipat and PTENi alone.

5.4.2 Effect of Ipatasertib plus XMD8-92 in Basal B (mesenchymal) PTEN mutant BT-549 cell line

The BT549 cell line was selected because it is a basal B TNBC (similar to MDA-MB-231). However, the BT549 cell line harbors a PTEN mutation, whereas the MDA-MB-231 cell line does not.

Both Ipatasertib and XMD8-92 alone reduced viability in a concentration dependent manner. Ipatasertib was more potent with an IC₅₀ of 8.1 μM while XMD8-92 had an IC₅₀ of 44 μM (Figure 5-4A). In 1:1 combinations, concentrations 3,3 and 10,10 μM decreased viability significantly more than each drug alone (Figure 5-4A). The combinations were also synergistic with CIs of 0.44 and 0.28, respectively. Combinations of 1:5 also achieved synergy while maintaining minimal collateral toxicity with respect to MCF-10A cells (Figure 5-4B). In particular the 1,5 μM combination decreased viability by 70% in BT-549 cells vs 30% in the MCF-10A cells. Additionally, the 1,5 μM combination was synergistic with a CI of 0.23. Lastly, the 1:5 combinations did not enhance the effect of 100 pM Paclitaxel in BT-549 cells (Figure 5-4C). Each drug alone was similar to the control’s proliferative index however; the combination did decrease the Ki67/Hoechst ratio. Ki67/Hoechst Control (60%) Ipat (58%) XMD (52%) and
I+XMD (39%). Both XMD alone or in combination increased expression of the cell cycle inhibitor p21 by 1.5 and 2.1 fold, respectively (Figure 5-5). Taken together, these data suggest the combination of Ipatasertib and XMD8-92 decreases proliferation in BT549 cells.

Both Ipatasertib and XMD8-92 contributed to the decrease in pBad S112: Ipat (77% of control) XMD (67% of control) I+XMD (54% of control) (Figure 5-6A). Additionally, the decrease in pBad S112 corresponded to an increase in cleaved caspase-3: 4.9 fold vs control for Ipatasertib and 7.3 fold vs control for the combination (Figure 5-6B). These results were similar to what was previously observed in the MDA-MB-231 cell line (also a Basal B TNBC cell type). Genetic ablation of ERK5 in BT-549 cell was attempted by Dr. Matthew Burow’s lab but was unsuccessful. Therefore, ERK5 appears to be critical for BT-549 cell survival.

Concerning kinase activity, XMD8-92 worked as expected and decreased the phosphorylated levels of ERK5 alone 38% and 64% in combination (Figure 5-7A). Interestingly, Ipatasertib also decreased pERK5 although the response was not statistically significant (P<0.0502). However, it is possible that Ipatasertib’s effect on ERK5 in BT549 cells is biologically significant. For example, pBad S112 decreased in the presence of Ipatasertib alone, and this could be indirect due to its inhibition of ERK5 (Figure 5-6A). Also, the combination of Ipatasertib and XMD8-92 inhibited pERK5 apparently more than either inhibitor alone (again not significant; P<0.085 but could be biologically relevant). Across all treatment groups, pERK1/2 signaling was not affected (Figure 5-7B). As expected, pAkt levels were increased in the presence of Ipatasertib alone or in combination 2 fold and 2.8 fold over control, respectively (Figure 5-7C). Downstream of Akt, pS6 levels were decreased by 80% in presence of Ipatasertib and 83% in the combination group (Figure 5-7D).
Figure 5-4. 1:5 ratios of Ipat:XMD were effective in BT-549 TNBCs while exhibiting minimal collateral toxicity in MCF-10A cells. BT-549 TNBC cells were treated with Ipatasterib and XMD8-92 at 1:1 and 1:5 ratios for 72 hours under 5% FBS stimulation (A and B). *P<0.05 vs drugs alone. Additionally, MCF-10 cells were treated with 1:5 combinations for 72 hours under manfg instructions (B). *P<0.05 vs MFC10. Lastly, BT-549 cells were treated with 100pM Paclitaxel for 24 hours followed by Ipat+XMD for 48 hours (C). Cell viability was determined using the MTT assay.
Figure 5-5. Ipat+XMD decreases proliferation marker Ki67 and induces p21 expression in BT-549 cells.

BT-549 cells were treated with Ipatasertib and XMD8-92 for 24 or 72 hours under 5% FBS stimulation. Cells were analyzed via immunofluorescence for Ki67 (A). Western blot was performed on cellular lysates after 24 hours of treatment and p21 levels were obtained (B). Cell counts were obtained by counting Hoechst positive cells (C) and proliferative cells were determined with Ki67 (D). *P<0.05, **P<0.01, and ***P<0.001 vs control. #P<0.05 vs drugs alone.
Figure 5-6. Both Ipatasertib and XMD8-92 decreased pBad S112 while Ipat increased Cleaved caspase-3 in BT-549 cells. BT549 cells were treated with Ipatasertib and XMD8-92 for 1 hour prior to 50ng/mL EGF for 4 hours. Cellular lysates were analyzed with an ELISA for pBad S112 (A). *P<0.05 and **P<0.01 vs EGF control. BT549 cells were treated for 48 hours followed by analysis via ELISA for Cleaved Caspase-3 (B). ***P<0.001 and ****P<0.0001 vs control. ##P<0.01 vs both drugs alone.

Figure 5-7. Ipatasertib plus XMD8-92 combination inhibits respective kinases while sparing ERK1/2 activity in BT549 cells. BT549 cells were treated with Ipatasertib and XMD8-92 alone or in combination for 1 hour prior to treatment with 5% FBS for 24 hours. Cellular
lysates were analyzed with western blot and levels of pERK5 (A), pERK1/2 (B), pAkt (C), and pS6 (D) were obtained. GAPDH was used as a loading control. *P<0.05, and **P<0.01 vs control. Representative western blot (E).

5.4.3 Effect of Ipatasertib plus XMD8-92 in Basal A (basal) PTEN mutant MDA-MB-468 cell line

MDA-MB-468 cells are basal A and harbor a homozygous PTEN deletion (Hollestelle et al. 2009). When treated with Ipatasertib alone, cell viability was decreased in a concentration dependent manner with an IC\textsubscript{50} of 5.4 \mu M (Figure 5-8A). XMD8-92 on the other hand, was not able to reduce viability enough to determine an IC\textsubscript{50} value. Therefore combination index (CI) values for synergy were not calculated. We decided to use 1:5 ratios of XMD8-92 due to the success in the BT-549 (Figure 5-4 B) cell line and previous reports of ERK1/2 activity spared at 5 \mu M XMD8-92 (Yang et al. 2010). There were no significant differences in viability until the 1,5 \mu M combination: 40% viable in MDA-MB-468 and 71% viable in MCF10A (Figure 5-8B). With respect to proliferation, only the combination group reduced Ki67/Hoechst ratio compared to control (Figure 5-9A). Morphologically, the combination appeared to disrupt the “grape- like” cluster of MDA-MB-468 cells (Figure 5-9A). Additionally, the 1:5 combination appeared to increase p21 expression 1.8 fold over control however; this was not statistically significant (P<0.07) (Figure 5-9B). Ipatasertib decreased pBad S112 both alone 37% and in combination with XMD8-92 41% vs control (Figure 5-10A). XMD8-92 only modestly decreased pBad S112 by 16% however, this was not significant vs control. Surprisingly, Cleaved caspase-3 levels were not elevated above control in any of the treatment groups (Figure 5-10B); which was counter what was observed in MDA-MB-231 and BT-549 cells. Interestingly, Ipatasertib decreased pERK5 levels alone 64% and in combination 85% vs control (Figure 5-11A). XMD8-92 increased pAkt levels 3.7 fold over control (Figure 5-11C). Lastly, pERK1/2 levels were
significantly increased in the XMD8-92 alone (2 fold over control) and combination groups (1.5 fold over control) (Figure 5-11B).

Figure 5-8. Ipatasertib potentiates XMD8-92 in 1:5 ratio in Basal A TNBC MDA-MB-468 cells. MDA-MB-468 cells were treated with Ipatasertib and XMD8-92 at 1:1 and 1:5 ratios for 72 hours under 5% FBS stimulation (A and B). Additionally, MCF-10 cells were treated with 1:5 combinations for 72 hours under 20ng/mL EGF stimulation (B). *P<0.05 vs MFC10. Lastly, BT549 cells were treated with 100pM Paclitaxel for 24 hours followed by Ipat+XMD for 48 hours (C). Cell viability was determined using the MTT assay.
Figure 5-9. Ipatasertib plus XMD8-92 decreases Ki67 expression in MDA-MB-468 cells. MDA-MB-468 cells were treated with Ipatasertib and XMD8-92 for 24 or 72 hours under 5% FBS stimulation. Cells were analyzed via immunofluorescence for ki67 (A). Western blot was performed on cellular lysates from 24 hours of treatment and p21 levels were obtained (B). Cell counts were obtained by counting Hoechst positive cells (C) and proliferative cells were determined with Ki67 (D). *P<0.05, **P<0.01, and ***P<0.001 vs control.
Figure 5-10. Ipatasertib decreases pBad s112 but fails to induce apoptosis in MDA-MB-468 cells. MDA-MB-468 cells were treated with Ipatasertib and XMD8-92 for 1 hour prior to 50ng/mL EGF for 4 hours. Cellular lysates were analyzed with an ELISA for pBad S112 (A). MDA-MB-468 cells were treated for 48 hours followed by analysis via ELISA for Cleaved Caspase-3 (B).
Figure 5-11. Ipatasertib decreased pERK5 while XMD8-92 increased pERK1/2 in MDA-MB-468. MDA-MB-468 cells were treated with Ipatasertib and XMD8-92 alone or in combination for 1 hour prior to treatment with 5% FBS for 24 hours. Cellular lysates were analyzed with western blot and levels of pERK5 (A), pERK1/2 (B), and pAkt (C) were obtained. GAPDH was used as a loading control. *P<0.05, and **P<0.01 vs control. Representative western blot (D).
5.5 Discussion

PTEN is mutated in approximately 30% of all cancers and is frequently altered in TNBC (Chavez et al. 2010, Bailey et al. 2018). PTEN is extensively studied as an oncogenic driver and therapies targeting PI3K/Akt/mTOR are implemented with the aim of precision oncology treatment. For example, Ipatasertib is primarily indicated for locally advanced TNBC patents with PTEN alterations (Phase III trial; NCT03337724). Targeted agents are also investigated in patents with treatment-induced loss of PTEN. The PI3Kα inhibitor Alpelisib (BYL719) worked synergistically with lapatinib in HER2+ PTEN deficient breast cancer tumors in vivo (Zhang et al. 2016). Alpelisib is currently in phase II clinical trials for Her2+ breast cancer and metastatic breast cancer (MBC) (NCT03706573). Although PTEN status is an important pre-clinical and clinical benchmark, our study demonstrates the variability of treatment response across three different TNBCs.

In our preliminary studies (Chapter 3) we observed elevated levels in pAkt in the presence of XMD8-92. This led us to examine the PTEN status in the PTEN WT MDA-MB-231 cell line. Interestingly, XMD8-92 treatment decreased PTEN levels and increased HIF-1α levels in MDA-MB-231 cells (Figure 5-1). These results are similar to previous studies that observed PTEN degradation under stress conditions (Laugher et al. 2001, Aghazadeh et al. 2017). One concern of the Ipat+XMD treatment is that it could sensitize PTEN WT tissues to Ipatasertib via PTEN degradation. To address this, we examined Ipat+XMD in the MCF-10A cell line (Basal B). PTEN levels were decreased in the MCF-10A cell line (Figure 5-2). However, the viability was only modestly reduced by 22% (Figure 5-2). One factor that may explain the preferential toxicity in TNBC vs MCF-10A is that MDA-MB-231 cells express pBad S112 at higher levels than MCF-10A (Stickles et al. 2015).
Next, we mimicked the conditions induced by XMD8-92 by inhibiting PTEN in MDA-MB-231 cells. Functionally, the assay performed as intended: pAkt was increased (Figure 5-3 B and C). However, the increased pAkt conditions alone were not enough to increase Ipatasertib efficacy in the viability assay (Figure 5-3 A). Therefore, ERK5 appears to play a crucial role in the synergy mechanism.

Across all three TNBC cell lines the Ipatasertib plus XMD8-92 combination decreased viability via synergy (MDA-MB-231 and BT-549) or potentiation (MDA-MB-468). Additionally, the combination decreased the proliferation marker Ki67 expression in each cell type (Figure 5-5 and Figure 5-9). The mechanism of synergy was determined to be pBad inhibition followed by caspase activation in the MDA-MB-231 and BT-549 cell lines (Figure 4-7 and Figure 5-6). The MDA-MB-468 cell line did not appear to activate caspases under the conditions tested (Figure 5-10). The performance of Ipat+XMD is summarized in table 5-1 below.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>PTEN status</th>
<th>Ipat+XMD</th>
<th>Mechanism</th>
</tr>
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<td>MDA-MB-231</td>
<td>Basal B</td>
<td>WT</td>
<td>Synergy</td>
<td>Caspase activation</td>
</tr>
<tr>
<td>BT549</td>
<td>Basal B</td>
<td>Null</td>
<td>Synergy</td>
<td>Caspase activation</td>
</tr>
<tr>
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<td>Basal A</td>
<td>Null</td>
<td>Potentiation</td>
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</tbody>
</table>

Table 5-1. Summary of Ipat+XMD combination in TNBC cell lines.

The kinase activity, pBad signaling, and caspase activation in BT-549 cells were similar to the MDA-MB-231 cells (Chapter4). The MDA-MB-468 cells, on the other hand, behaved very differently. MDA-MB-468 cells treated with Ipat+XMD did not exhibit increased cleaved caspase activity. Perhaps this is due to the unique receptor expression profile of MDA-MB-468 cells. MDA-MB-468 cells overexpress cMET. cMET is the receptor for HGF and is observed in many cancers. In addition to activating growth factor signaling, cMET contains a domain that inhibits caspases (Jung et al. 2012). The Ipat+XMD combination in MDA-MB-468 cells also
activated pERK1/2 signaling (Figure 5-11). This was in contrast with MDA-MB-231 and BT-549 cell both of which pERK1/2 were spared. Another unique feature of MDA-MB-468 cells is that they have an EGFR amplification (Chavez et al. 2010). This may be what was responsible for the increased pERK1/2 activity in the XMD8-92 treated groups. Therefore, inhibiting EGFR may be needed to achieve full efficacy since ERK1/2 appears to be essential to survival in this cell line (She et al. 2005). Sohn et al. 2014, achieved synergy with EGFR and cMET inhibitor combinations in MDA-MB-468 cells. In their study, MDA-MB-468 cells had hyperactive pMET and pEGFR vs MDA-MB-231 cells. This led to increased downstream signaling in the presence of EGF in the MDA-MB-468 cells. Therefore, EGFR and cMET may need to be inhibited for Ipat+XMD to achieve an apoptotic effect.
Chapter 6: Characterization of novel MEK5 and dual MEK5/Akt inhibitors in breast cancer cells

6.1 Introduction

Chapters 3, 4, and 5 all underscored the promise of ERK5 inhibition as a potential target for TNBC. Furthermore, ERK5 inhibition in combination with Akt inhibition was synergistic in TNBC and relatively non-toxic in a normal tissue control. However, the tools used to study MEK5/ERK5 signaling are limited and there is a need to develop novel, selective inhibitors of the MEK5/ERK5 pathway. Additionally, there are currently no known compounds that serve as a dual MEK5/ERK5 and PI3K/Akt inhibitor. Therefore, in this chapter, we will explore the synthesis of selective MEK5 and dual MEK5/Akt inhibitors in hopes of creating a targeted therapy for TNBC.

Aberrations in the MAPK pathway have been extensively studied due to their role in tumor formation, metastasis, chemo-resistance, and angiogenesis. In particular, BRAF mutations, a kinase upstream of MEK, leads to hyperactivation of MEK1/2 and are considered driver mutations in various cancers (Bailey et al. 2018). Both BRAF and MEK1/2 have been successfully targeted in melanoma with FDA approved molecules Vorafinib and Trametinib respectively (Rheault et al. 2013, Salama and Kim 2013, Zhao and Adjei 2014). Additionally, Trametinib has recently been approved to treat V600E BRAF mutant lung cancer patients (Odogwu et al. 2018). As such, Trametinib serves as an example of successful precision oncology at the clinical level.

MEK5/ERK5 is a less studied pathway compared to MEK1/2/ERK1/2. However, it has been identified as a key oncogenic signaling modulator in many cancer types (Hoang et al. 2017). High MEK5 (50%) expression or ERK5 (20%) expression was observed in breast tumors and correlated with decreased disease free survival (Hsieh et al. 2005, Montero et al. 2009).
ERK5 has also been linked to breast tumor kinase (Brk), which is overexpressed in 86% of IDCs (Drew et al. 2012). In MCF-7 cells (ER+) ERK5 mediated chemoresistance, evasion of apoptosis, and enhanced survival (Weldon et al. 2002, Antoon et al. 2013). In BT474 cells (triple positive) ERK5 is constitutively active however introduction of a dominant negative form of ERK5 decreased proliferation (Esparis-Ogando et al. 2002). In MDA-MB-231 (TNBC) cells ERK5 mediated proliferation via CDK1 and p21 expression (Perez-Madrigal et al. 2012).

Current strategies to target the MEK5/ERK5 have relied on a limited number of inhibitors. The ATP competitive inhibitors BIX02188 and BIX02189 were shown to have potent activity against MEK5 (in cell free assays) with IC$_{50}$s of 4.3nM and 1.5nM, respectively. Neither displayed activity against MEK1/2 or EGFR, thus indicating selectivity for MEK5 over similar kinases (Tatake et al. 2008). BIX02189 treatment in colorectal cancer cells decreased proliferation, although only at high concentrations (Pereira DM, et al. 2016). XMD8-92 is an ATP competitive inhibitor of ERK5 with an IC$_{50}$ (Kd) of 80nM (Yang et al. 2010). Inhibition of ERK5 with XMD8-92 decreased proliferation in HeLa cells by inducing p21 expression (Yang et al., 2010). XMD8-92 has also shown promise in prostate and pancreatic cancers. However, the effects of XMD8-92 have come under scrutiny since the molecule has been shown to inhibit BRD4 with moderate affinity (Li et al. 2016). Therefore, AX15836 was developed as a selective ERK5 inhibitor with an IC$_{50}$ of 8 nM vs ERK5 and 3,600 nM vs BRD4 (Li et al. 2016).

Our lab in collaboration with Dr. Patrick Flaherty, a medicinal chemist at Duquesne, began the search for new MEK5 inhibitors using benzimidazole based compounds. However, these compounds were unable to selectively inhibit MEK5/ERK5 activity in HEK293 cells (Flaherty et al. 2010). Therefore, our focus shifted towards type III non-ATP competitive allosteric inhibitors of MEK5. Advantages for type III inhibitors are that they do not have to
compete with ATP (milimolar concentrations in the cell) and are therefore potent (Ataullakanov and Vitvitsky 2002). MEK1/2 inhibitors PD0325901 (0.33 nM IC\textsubscript{50} for MEK) and Trametinib (Table 7-1) were used as lead compounds (Barrett et al. 2008). Our recent work examined the structure activity relationship (SAR) of novel diphenylamine analogs as type III (allosteric) inhibitors of MEK5 (Chakrabarty et al. 2018). In this study, we screened novel diphenylamine and thiophene analogs for MEK5/ERK5, MEK1/2/ERK1/2, and Akt activity in the MDA-MB-231 TNBC cell line. A detailed overview of the screening process is outlined in Figure 6-2. Additionally, results for the novel compound screen are tabulated in table 6-2. Selected (highlighted) compounds were investigated further in the subsequent experiments.
Figure 6-1. Summary of MEK1/2 and MEK5 pathways and inhibitors in cancer. (Thomas Wright, Unpublished work).

<table>
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<th>Compound</th>
<th>Target(s)</th>
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Table 6-1. MEK5 and ERK5 compounds and potencies. Note IC₅₀s are from cell free assays.

6.2 Hypothesis

Novel diphenylamine and thiophene derivatives will provide selective inhibition of MEK5 and MEK5+Akt respectively.

Figure 6-2. Novel compound screening process. Kinase assay: MDA-MB-231 cells were treated with 10 μM novel inhibitors for 1 hour prior to treatment with 50ng/mL EGF for 15 minutes. Cellular lysates were collected and analyzed with western blot. Viability assay: MDA-
MB-231 cells were treated with 10 μM novel inhibitors for 72 hours under 5% FBS stimulation. Cell viability was determined using the MTT assay.

<table>
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<th>Compound</th>
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<th>ERK5 (% activation)</th>
<th>Akt (% activation)</th>
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Table 6-2. Summary of novel kinase inhibitor activity against ERK1/2, ERK5 and Akt.

6.3 Results

6.3.1 Diphenyl amine analogs

Novel diphenylamines described in Chakrabarty et al. 2018 were screened in the MTT viability assay. Only three compounds were able to achieve a significant reduction in viability: SC-1-151 (35%), SC-1-80 (20%), and SC-1-79 (20%) (Figure 6-3). SC-1-151 was further characterized due to its effects in the cellular viability assay and its EMT properties in MDA-MB-231 TNBC cells (Hoang et al. 2016). In our study, SC-1-151 was determined to be a potent inhibitor of ERK1/2 phosphorylation with an IC\textsubscript{50} value of 26 nM in MDA-MB-231 cells (Figure 6-4). SC-1-151 was also shown to inhibit ERK5 phosphorylation by 40% at 10 \mu M (Chakrabarty et al. 2018). Due to the interesting EMT properties of SC-1-151, we sought to determine the
cellular localization of SC-1-151. AJM-1-68 was synthesized: SC-1-151 with a BODIPY linked probe (Figure 6-5A). MDA-MB-231 cells were treated with AJM-1-68 imaged, and cellular lysates were collected. The AJM-1-68 compound appeared to enter the cell (Figure 6-5 B and C). AJM-1-68 remained significantly active in vitro at 1 μM (30% ERK1/2 inhibition) (Figure 6-5 D). The BODIPY dye alone did not affect ERK1/2 activity. Additional Immunofluorescence experiments are needed to precisely determine the localization of AJM-1-68 in vitro. Another interesting feature of SC-1-151 is that it increased pAkt in various experimental settings. This is consistent with previous reports of MEK inhibition induced Akt activation (Aksamitiene et al. 2012). We then proposed SC-1-151 treatment followed by Ipatasertib would “capture” more Akt in the active conformation. Interestingly, increased pAkt was observed in SC-1-151+Ipatasertib in MDA-MB-231 and BT-549 TNBC cell lines (Figure 6-6).

Next, we aimed to generate a novel selective inhibitor of MEK5. SC-1-181 was described in Chakrabarty et al. 2018 as the most selective diphenylamine MEK5 inhibitor to date: 82% ERK5 phosphorylation inhibited at 10 μM. SC-1-181 was further characterized and exhibited an IC_{50} of 115nM vs pERK5 in MDA-MB-231 cells (Figure 6-7C). Additionally, SC-1-181 did not significantly inhibit ERK1/2, thus it was labeled as MEK5 selective (Figure 6-7B). Next, MDA-MB-231 cells were treated with SC-1-181 for 72 hours. Cell viability decreased starting at 30 μM and leveled off at 300 μM (Figure 6-8A). SC-1-181 was then tested in combination with Akt inhibitor Ipatasertib in 1:1 ratios. Significant reductions in viability were observed starting at 1 μM each and leveled off at 10 μM each (Figure 6-8B). Staggered ratios were not examined since no IC_{50} with SC-1-181 was established. Furthermore, the SC-1-181 combination differed from the Ipat+XMD combination (Chapter 4) in that Akt was not activated. Lastly, we were able
to recapitulate the Ipat+XMD combo by using CPI. Ipat+XMD: 63% viability at 0.1,0.1 μM while Ipat+181+CPI: 61% 0.1,0.1,0.1 μM (Figure 4-2 and Figure 6-8C).

**Figure 6-3. Diphenylamine analog viability screen.** MDA-MB-231 cells were treated with 10 μM novel inhibitors for 72 hours under 5% FBS stimulation. Cell viability was determined using the MTT assay. ****P<0.0001, **P<0.01, and *P<0.05 vs control.

**Figure 6-4. SC-1-151 potent novel inhibitor of MEK1/2.** Structure of SC-1-151 (A). MDA-MB-231 cells were treated with various concentrations of SC-1-151 for 1 hour prior to 50ng/mL EGF stimulation for 15 minutes. Western blot analysis of pERK1/2 inhibition by SC-1-151 (B). IC₅₀ of SC-1-151 (C).
Figure 6-5. Cellular localization observed with Bodipy linked SC-1-151 (AJM-1-68) in MDA-MB-231 cells. MDA-MB-231 cells were treated with 10 μM AJM-1-68 (A) for 6 hours prior to visualization with an EVOS-fl inverted microscope (B) 10x and (C) 20x magnification. The excitation wavelength was 509 nm and the emission range was measured from ~500 nm to 600 nm. pERK1/2 activity determined by western blot (D).
Figure 6-6. SC-1-151 and Ipatasertib treatment in MDA-MB-231 (A) and BT549 (B) TNBC cells. (Akshita Bhatt, Unpublished work).

![Figure 6-6](image)

Figure 6-7. SC-1-181 potent novel inhibitor of MEK5. Structure of SC-1-181 (A). MDA-MB-231 cells were treated with various concentrations of SC-1-181 for 1 hour prior to 50ng/mL EGF stimulation for 15 minutes. Western blot analysis of pERK5 inhibition by SC-1-181 (B). IC_{50} of SC-1-181 (C).

![Figure 6-7](image)

Figure 6-8. Novel MEK5 inhibitor SC-1-181 enhances effect of Akt inhibitor Ipatasertib in MDA-MB-231 cells. MDA-MB-231 cells were treated with SC-1-181, Ipatasertib, and CPI203 alone or in combination for 72 hours under 5% FBS stimulation. Viability was determined with the MTT assay. SC-1-181 alone (A). ***P<0.001 and **P<0.01 vs control. SC-1-181 with Ipatasertib (B). ***P<0.001 and ****P<0.0001 vs control. SC-1-181 plus Ipatasertib and CPI203 (C). **P<0.01 vs control; #P<0.05 vs Ipat+CPI and Ipat+SC-1-181.

![Figure 6-8](image)

6.3.2. Thiophenes

Thiophenes are another structural class that has been shown to inhibit MEK at the type III site (Laing et al. 2012). The compound from Figure 6-9A exhibited a 20 nM potency for pERK activity (Laing et al. 2012). 6-9A contains structural features similar to known type III binders of MEK. The docking study revealed a key halogen bond of the 4'Iodo to acyl of Val 127 residue.
This is also consistent with our previous molecules such as SC-1-151 where the 4’Iodo was observed to be a key component of MEK1/2 activity (Chakabarty et al. 2018). Interestingly, the same molecule from 6-9A docked with MEK5 homology model revealed that the 4’Iodo was not needed for MEK5. Therefore, we developed molecules without the 4’Iodo in hopes of MEK5 selectivity (Figure 6-10A). Based on the need for dual inhibition we began to explore other scaffolds that could have dual activity. Another benefit of the thiophene structural class is that they have been shown to inhibit the PI3K/Akt axis (Liu et al. 2011). MG-3-81 (Figure 6-10A) showed promising activity vs pERK5 38% inhibition and pAkt 72% inhibition while sparing pERK1/2 11% inhibition (Figure 6-10C). The efficacy of MG-3-81 was examined in MDA-MB-231 cells. MG-3-81 alone did little to affect cell viability (Figure 6-11A). However, as noted with previous compounds, adding the cytostatic agent CPI had a significant effect that was greater than each drug alone. CPI (77%) MG-3-81 (90%) and Combo (64%) (Figure 6-11B). Even though the response in MDA-MB-231 cells was less than ideal, we began testing in PTEN mutant TNBCs to see if the compound would be more effective. MG-3-81 was more effective in the BT549 cells and reduced viability in a concentration dependent manner 0.1 (23%), 1 (39%), and 10 (65%) (Figure 6-12A). Consistent with previous results MG-3-81 combined with CPI reduced viability more than either drug alone CPI (52%), MG-3-81 (39%) and combo (66%) (Figure 6-12B). Progress continues with the thiophene series as we aim for greater potency. MG-5-5 is a recent compound in the thiophene series that had an IC$_{50}$ of 576 nM for ERK5 and 252 nM for pS6 (downstream target of Akt) (Figure 6-13).
Figure 6-9. Computational analysis of seven-membered thiophene in MEK1 and MEK5 docking studies. Compound from (A) was docked with MEK1 (B; PBD 3SLS) and MEK5 homology model (C). Key interactions with amino acids are denoted. Adapted from Gupta et al. 2018.

Figure 6-10. Novel thiophene MG-3-81 inhibits MEK5 and Akt in MDA-MB-231 cells. MDA-MB-231 cells were treated with MG-3-81 (A) for 1 hour prior to 50ng/mL EGF stimulation for 15 minutes. Docking performed in MOE with MEK5 homology model (B). Enzyme activities listed in (C). Values represent % activation. Adapted from Gupta et al. 2018.
Figure 6-11. Novel dual MEK5/Akt inhibitor MG-3-81 combines with BRD4 inhibitor CPI203 to induce apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were treated with MG-3-81 alone (A) and in combination with CPI203 (B) for 72 hours under 5% FBS stimulation. *P<0.05 and **P<0.01 vs control. #P<0.05 vs drugs alone.

Figure 6-12. Novel Dual inhibitor MG-3-81 decreases viability in PTEN mutant BT549 cells. BT549 cells were treated with MG-3-81 alone (A) or in combination with CPI203 (B) for 72 hours under 5% FBS stimulation (A). **P<0.01 and ***P<0.001 vs control. *P<0.05 vs both drugs alone. BT549 cells cell were treated with MG-3-81 for 1 hour prior to 50ng/mL EGF stimulation for 15 minutes. Cellular lysates were visualized by western blot (C).
Figure 6-13. Novel thiophene MG-5-5 inhibits MEK5 and Akt in BT549 cells. BT549 cells were treated with MG-5-5 (A) for 1 hour prior to 50ng/mL EGF stimulation for 15 minutes. pERK5 and pS6 activity determined by western blot (B). IC\textsubscript{50} values for pERK5 (C) and pS6 (D).

6.3.3. Other Applications of Novel inhibitors in U87 Glioblastoma cells

Glioblastoma is a brain malignancy that currently has no targeted therapy. Frontiers for targeted therapy in GBM are similar to TNBC in that genetic aberrations in the PI3K/Akt have been identified as promising targets. The U87 GBM cell line was selected due to its PTEN and treatment resistance status. The U87 cell line was also used because we observed cross-activation between Akt and MEK5/ERK5 in preliminary studies (Figure 1-14).

U87 glioblastoma (GBM) cells (PTEN mutant) were treated with our most promising leads for MEK5 and MEK5/Akt inhibition: SC-1-181 and MG-3-81, respectively. SC-1-181 was added alone or in combination to TMZ, the standard of care for GBM (Lee 2016). U87 cells are resistant to TMZ and, SC-1-181 was unable to potentiate the conventional therapy (Figure 6-14A). However, SC-1-181 did significantly decrease colony formation at 10 μM (55% of
control) (Figure 6-14B). Additionally, SC-1-181 had an effect on CD133 expression, which is a stem cell marker for GBM (Figure 6-14C). The dual inhibitor, MG-3-81, reduced viability in U87 cell by 75% at 10 μM (Figure 6-15). Overall these findings suggest that the ERK5 plays a role in colony formation and if inhibited along with Akt, reduces viability.

**Figure 6-14.** Novel MEK5 inhibitor SC-1-181 inhibits colony formation in PTEN mutant U87 glioblastoma cells. U87 cells were treated with SC-1-181 alone or in combination with TMZ for 96 hours under 5% FBS stimulation (A). Cell viability determined using the MTT assay. Colony formation was measured after SC-1-181 treatment for 6 days using kit (B). **P<0.01 vs control. Stem cell marker CD133 was observed using western blot (C).**
6.4 Discussion

The inhibitors used to study MEK5/ERK5 are limited and primarily ATP-competitive. In this study, we demonstrated the utility of type III inhibitors as a means to achieve selective MEK5 inhibition. In particular, SC-1-181 was potent and selective for MEK5 over MEK1/2 (Figure 6-7). SC-1-181 also recapitulated the functional results from TNBC cells in chapter 4 (Figure 6-8C). SC-1-181 also exhibited promising activity against stem cells in GBM by reducing CD133 expression and colony formation (Figure 6-14). CD133+ U87 cells are crucial for tumor formation (Lin B et al. 2017). SC-1-181 decreased CD44+ breast cancer stem cells in a TNBC cell line via Jagged1/NOTCH1/MEK5 axis (Ucar et al. 2018). Further studies are needed to determine SC-1-181’s effect on U87 stem cells; preferably a cytometry sorting method that can identify tagged stem cell populations. Despite the success of SC-1-181 improvements are needed to further the current results. Lack of biochemical assays and MEK5 crystal structure are limitations to these studies.
The dual MEK5/Akt inhibitor MG-3-81 showed promising activity against ERK5 and Akt in MDA-MB-231 and BT-549 TNBC cells. In particular, MG-3-81 alone decreased viability in a concentration dependent manner in the PTEN mutant BT-549 cells (Figure 6-12A). Additionally, MG-3-81 enhanced the effects of CPI-203 (BRD4 inhibitor) in BT-549 cells (Figure 6-12B). These results were similar to what was observed in chapter 5. MG-3-81 also decreased viability by 75% in the PTEN mutant U87 glioblastoma cells (Figure 6-15). In Chapter 5 we observed ERK5 inhibition in the presence of the Akt inhibitor Ipatasertib. Similar signaling was observed in neuroblastoma cells where Akt knock out cells had decreased levels of pMEK5 and pERK5. Furthermore, MEKK3 (upstream of MEK5) was shown to be phosphorylated by Akt (Umapathy et al. 2014). Interestingly, we observed similar signaling with Ipatasertib in U87 cells where ERK5 was inhibited. Therefore, we must consider the possibility that our dual inhibitors may be inhibiting ERK5 indirectly. Further studies are required to elucidate the role of each kinase in the dual inhibitory studies.

Additional studies are also needed with the type III inhibitors in mutant models of Akt because mutations to the allosteric site can limit their utility. For example, MK2206, an allosteric inhibitor of Akt, works well on Akt1 wild type enzyme but does not perform as well on E17K mutant Akt forms. The E17K mutation is an activating mutation that occurs in the pleckstrin homology (PH) domain of Akt. In particular, AKT1-E17K has been reported in a variety of cancers (including breast) and is considered an activating mutation (Brugge et al. 2007). In E17K mutant bladder cells MK2206 was unable to inhibit Akt signaling and downstream targets such as pS6. On the other hand Ipatasertib, an ATP site binder, (GDC-0068) remained effective even in the mutant cell line (Cheng et al. 2015). Therefore we will explore ATP competitive scaffolds for selective MEK5 and dual MEK5/Akt inhibition in future studies.
Chapter 7: Evaluation of Ipatasertib plus XMD8-92 in tamoxifen resistant MCF-7 cells

7.1 Introduction

Approximately 70% of invasive ductal carcinomas (IDC) are considered estrogen receptor alpha (ER) and/or progesterone receptor (PR) positive, and their growth is driven by estrogen (Johnston et al. 2003). Initially, ER+ cancers are responsive estrogen receptor antagonists such as tamoxifen or aromatase inhibitors (AI) such as letrozole. However, over the course of treatment (often 5 years or longer) patients frequently develop resistance (Creighton et al. 2009). Tamoxifen and AI treated patients progress over time due to de-novo or acquired resistant mechanisms (Clarke et al. 2001, Bachelot et al. 2012). Acquired Tamoxifen resistance occurs through several mechanisms: Loss of ER expression, mutations in ligand binding domain, alterations to co-activators and co-repressors, aberrant cellular kinase signaling, metabolic reprogramming, and stem cell dynamics (Ring and Dowsett 2004, Chang 2012). ER expression is suppressed through epigenetic modifications such as hypermethylation of histone deacetylation in vitro (Sharma et al. 2005, Zhou et al. 2007). Mutations in the ligand binding domain are another aspect of lost ER function. Mutations to ER are more frequent in vitro and have limited prognostic utility clinically (Anderson et al. 1997). Tamoxifen is a selective estrogen receptor modulator (SERM) meaning it acts as an agonist in some tissues (uterus) and as an antagonist in others (breast). The context of tamoxifen activity is highly dependent on the expression levels of co-activator and co-repressors in a tissue (Webb et al. 1998). Therefore, an imbalance between co-activator/co-repressor may lead to a shift in Tamoxifen’s activity, thus conferring resistance. The PI3K and MAPK pathways have been shown to contribute to resistance by phosphorylating ER at Ser 118 (ERK1/2) and Ser 167 (Akt) (Creighton et al. 2008). This induces ligand independent activation of ER that leads to expression of ER related
genes. Additionally, the PI3K and MAPK pathways contribute to resistance by enhancing survival and promoting proliferation on E2 ligand independent mechanisms.

Several clinical trials are under way to address the challenge of tamoxifen resistance. Preclinical combinations of CDK4/6 inhibitor, Palbociclib, plus tamoxifen were synergistic in vitro (Finn et al. 2009). Palbociclib is currently in a phase II trial combined with tamoxifen with the aim of becoming a first line therapy (NCT02668666). Pan PI3K inhibitor Alplisib and PI3K (β sparing) inhibitor Taselisib are currently under investigation in Phase I trials as an adjuvant to tamoxifen therapy (NCT02058381 and NCT02285179). The MAPK inhibitor LY2228820 was also investigated in combination with tamoxifen; however the trial was terminated due to low enrollment (Campbell et al. 2013, NCT02322853). New evidence suggests that MEK5/ERK5, a member of the MAPK family, is a key component in the proliferation and survival of therapy resistant cancers (Drew et al. 2012, Hoang et al. 2017). MEK5/ERK5 has been shown to promote ER alpha driven transcription in ER+ breast cancers and actin reorganization and metastasis in ER- breast cancers (Erdogan et al. 2014). Furthermore, MEK5/ERK5 promotes hormone independent tumorigenesis in breast cancer (Antoon et al. 2013).

Our initial results indicate that combinations of PI3k/Akt and MEK5/ERK5 blockade are promising because they inhibit both the pro-proliferative and pro-metastatic functions in MCF-7 cells (ER+) (Chapter 3). We propose each pathway contributes to the resistant disease state: PI3k/Akt/mTOR (proliferation) and MEK5/ERK5 (survival). Therefore, we hypothesize acquired tamoxifen resistant MCF-7 cells will remain sensitive to the dual inhibition of the PI3K/Akt and MEK5/ERK5 pathways. The long-term goal of our study is to elucidate the roles of PI3k/Akt/mTOR and MEK5/ERK5 in endocrine resistant breast cancer and determine the efficacy of a dual inhibition strategy.
Hypothesis 7.2
The combination of Akt and ERK5 inhibition will remain effective in an acquired tamoxifen resistant MCF-7 cell line.

7.3 Results
7.3.1 Establishment of MCF-7 tamoxifen resistant cell line.

Cells were treated for 6 months with 100nM 4OH tamoxifen in charcoal stripped FBS and phenol red free medium. Phenol red free medium was used because phenol red has been shown to be estrogenic (Rabenoelina et al. 2002). After 6 months the WT and TamR cell lines were functionally tested for 4-OH Tamoxifen sensitivity (Figure 7-1A). The WT cell line remained sensitive with an IC$_{50}$ of 455nM while the TamR cell line exhibited a 12 fold higher IC$_{50}$ of 5.46 µM (Figure 6-1A). Additionally, MCF-7 cells treated with 100nM of 4OH Tamoxifen had a 2% proliferative fraction (Ki67/Hoechst) whereas, TamR cells under the same conditions 65% (Ki67/Hoechst) of cells were actively proliferating (Figure 7-1C). The latter suggests the cells were still entering into the cell cycle despite 4OH Tamoxifen being present. Lastly, the morphology of the TamR cells also corresponded to the resistant phenotype: TamR cells undergo EMT (Figure 7-1C) (Yuan et al. 2015). Next, we performed saturation binding on MCF-7 WT and TamR cells using [3H]Estradiol to determine if the affinity for the estrogen receptor has changed in the resistant model. Both cell lines exhibited similar affinities: 2.2 nM for MCF-7 and 1.1nM for TamR. Also, the Bmax was apparently larger in TamR vs WT 21.4 vs 4.8, respectively. However, due to the high variability of Bmax in these experiments the values were not significantly different.
Figure 7-1. Generation of 4-OH Tamoxifen resistant MCF-7 cell line. The ER+ MCF-7 cell line was treated with 100nM 4OH Tamoxifen for 6 months under 5% Charcoal stripped FBS. Cell viability was determined by the MTT assay (A). Proliferation was determined by staining cells with ki67 and analysis via Immunofluorescence (B) MCF-7 WT (C) MCF-7 TamR.
Both MCF-7 WT and TamR cell lines had similar affinity for $[^{3}H]$Estradiol (E2). MCF-7 WT (A) and TamR (B) were treated with increasing concentrations of $[^{3}H]$Estradiol and saturation binding curves were obtained. Experiment and Analysis carried out with the assistance of Dr. Witt-Enderby and students from methods class (GPSC 572). n=4 for WT and n=5 for TAMR. Composite saturation binding curves shown.

### 7.3.2 Ipat+XMD decreased viability and ER-ERE complex formation

Once we had established the TamR cell line, we moved to test our hypothesis of combination therapy in TamR cells. Based on our initial success with the Akt inhibitor, Ipatasertib, we decided to continue using it throughout the TamR studies. Ipatasertib alone maintained similar potency in both the WT and TamR cell lines with IC$_{50}$ values of 6.1 and 8.1 uM, respectively (Figure 7-3A). The ERK5 inhibitor, XMD8-92, was less effective in the TamR cell line with an IC$_{50}$ of 10.1 uM vs 4.0 uM in the WT cells (Figure 6-3B). Next, we investigated 1,3 uM of Ipat:XMD in both MCF-7 WT and TamR cells. XMD8-92 alone and in combination significantly decreased Hoechst and Ki67 positive cells vs control (Figure 7-4). However, only the combination decreased the Hoechst/Ki67 ratio with respect to control. In TamR cells, Ipatasertib, XMD8-92 and the combination all significantly decreased Hoechst positive cells (Figure 7-4B). Interestingly, the morphology of the WT cells appeared to be mesenchymal in cells treated with XMD8-92 (Figure 7-4B). This is consistent with previous work that showed BRD4 inhibition induced EMT morphology (Yuan et al. 2015).
Next, we examined the binding of ERα to ERE in the presence of Estradiol (E2) with an ELISA. In the MCF-7 WT cell line, 4-OH tamoxifen worked as expected by decreasing the ER-ERE complexes in the presence of 10 nM E2 (Figure 7-6A). 4-OH Tamoxifen did not significantly reduce the ER-ERE complexes in the TamR cell line in the presence of 10 nM E2 (Figure 7-6C). Also, the basal level of ER-ERE complexes was elevated in the TamR cells (Figure 7-6C). In the experimental groups, XMD8-92 alone or in combination with Ipatasertib significantly decreased ER-ERE complexes under 10 nM E2 stimulation in the MCF-7 WT cells (Figure 7-6B). BRD4 has been shown to influence ER related gene expression in MCF-7 cells (Figure 7-5, Nagarajan et al. 2014). Therefore we used to BRD4 inhibitor CPI-203 to determine its effects on ER-ERE complex formation in our model. CPI-203 significantly reduced ER-ERE complex formation under 10 nM E2 stimulation in the MCF-7 WT cells (Figure 6-6B). Interestingly, XMD8-92 alone or in combination with Ipatasertib decreased ER-ERE complex formation only in the presence of 4-OH and E2 (Figure 7-6D).

Figure 7-3. MCF-7 and TamR sensitivities to Ipatasertib and XMD8-92. MCF-7 Wt and TamR cells were treated with increasing concentrations of Ipatasertib (A) and XMD8-92 (B).
Cell viability determined using the MTT assay. IC\textsubscript{50} values determined using Microsoft excel. *P<0.05 unpaired t-test.

**Figure 7-4. Ipat+XMD combination decreased proliferation in both MCF-7 Wt and TamR cell lines.** Cells were treated with 1 \( \mu \text{M} \) Ipat and 3 \( \mu \text{M} \) XMD alone or in combination for 72 hours. Proliferation was determined by staining cells with Ki67 and analysis via Immunofluorescence (A,C,D) MCF-7 WT (B,E,F) MCF-7 TamR. Added Ki67 and Hoechst.
Figure 7-5. BRD4 role in ER gene expression. Adapted from: Nagarajan et al. 2014. Reproduced with permission; License number: 4570520291836.

Figure 7-6. XMD and CPI decreases ERE-ER binding complexes in MCF-7 WT cells. MCF-7 WT (A and B) and TamR (C and D) cells were treated with compounds prior to lysis and nuclear extraction. Nuclear extracts were analyzed for active Era complexes with an ERE-ELISA ELISA assay. ***P<0.001 vs E2 control MCF-7 WT. ^^^P<0.001 vs E2 matched control.
7.3.3 XMD8-92 combined with Taselisib decreased viability in MCF-7 and TamR cells

Although an IC$_{50}$ with Ipatasertib was obtained (Figure 7-3A), a full concentration response with IC$_{75}$ is needed to determine synergy. Therefore, we investigated XMD8-92 in combination with Taselisib, a clinically relevant PI3Ka inhibitor. Taselisib decreased viability in MCF-7 WT and TamR cells with IC$_{50}$s of 734 nM and 1.05 uM, respectively (Figure 7-7A and C). 1:1 combinations with XMD8-92 decreased viability in both MCF-7 and TamR cell lines (Figure 7-7 B and D). In particular, 100 nM each in the MCF-7 and 300 nM each in the TamR cells were synergistic combinations with CIs of 0.16 and 0.32, respectively.

Figure 7-7. XMD8-92 synergistically combines with PI3Ka inhibitor Taselisib in MCF-7 and TamR cells. MCF-7 (top) and TamR (bottom) cells were treated with Taselisib alone (A and C) or in combination with XMD8-92 (B and D). Cell viability determined using the MTT assay. *P<0.05, **P<0.01, ***P<0.001 vs control.
7.4 Discussion

We successfully established an acquired tamoxifen resistant model based on viability, proliferation, ligand binding, and ER-ERE complex formation. First, the viability of TamR cells was 12 fold different than the WT cell line (Figure 7-1A). Additionally, TamR cells remained proliferative even in the presence of 4-OH Tamoxifen (Figure 6-1C). Binding of estradiol (E2) was similar in both WT and TamR cell lines with Kds of 2.2 nM and 1.1 nM, respectively (Figure 7-2). These results were similar to literature values of E2 binding to ER in MCF-7 cells: 1.8 nM Kd (Brooks et al. 1973). 4-OH Tamoxifen was able to partially abolish the effects of E2 by reducing ER-ERE complexes in MCF-7 Cells (Figure 7-6A). Again, These results were similar to Kling et al. 1998 who showed that ER-Tam complexes had a lower affinity for EREs than ER-E2 complexes. In the TamR cells, 4OH-Tamoxifen was unable to abolish the effects of E2 stimulation (Figure 7-6C).

Both Ipatasertib and XMD8-92 were able to achieve IC50s in MCF-7 and TamR cells (Figure 7-3). However, Ipatasertib was unable to reduce viability past 50% and therefore limited synergy interpretations for these cell lines. We utilized 1,3 uM combinations of Ipat:XMD in MCF-7 and WT cell lines. The combination significantly decreased Ki67/Hoechst ratio (Figure 7-4A and B). The combination also induced a morphological changes in the MCF-7 WT cell line. One concern was that this phenotype was more invasive however our results from chapter 3 demonstrated that LY+XMD MCF-7 cells migrated less than the control. However, additional studies are needed with a Boyden chamber invasion assay to confirm that the cells are inert. Another feature of the combination was inhibiting ER-ERE complex formation (Figure 7-6 B and D). In the WT cell line XMD8-92 and CPI-203 (both have BRD4 inhibition properties) significantly decreased ER-ERE complexes under E2 stimulation (Figure 7-6B). In the TamR
cell line XMD8-92 decreased ER-ERE complexes but only when 4-OH Tamoxifen was present (Figure 6-6D). Perhaps this was due to the altered conformation of helix 12 of ER with 4OH tamoxifen binding long term (Shiau et al. 1998). Additional studies are needed to determine if the effects of XMD8-92 on ER-ERE complex formation affect the expression of ER related genes. Additionally, the acquired resistant model has a few caveats such that passaging the cells over a long period of time creates a large amount of genetic drift and sequencing would, therefore, be needed to determine the extent of the drift and if the kinase pathways we are studying are altered. However, it must be said that the PI3K pathway would likely not be altered since mutations in the pathway are generally mutually exclusive.

Lastly, the results from his study have implications for other forms of acquired resistance. Our results in chapter 3 indicate that the luminal B (HER2+), triple positive, BT474 cell line was sensitive to dual PI3K/ERK5 inhibition. More specifically, the combination significantly reduced both viability and migration. Perhaps the combination would also be effective in a Trastuzumab (a HER2 antagonist) resistant BT474 model. Additional studies are needed to elucidate the role of PI3K/AKt and MEK5/ERK5 dual inhibition in acquired HER2+ resistance.
Chapter 8 Conclusions and Future Directions

Targeting PI3K/Akt and MEK5/ERK5 was effective across a diverse panel of cell lines. The mechanism of synergy or potentiation was not the same for each condition. However, the decrease in viability was universal. For example, the Basal B TNBCs MDA-MB-231 and BT-549 appeared to undergo apoptosis via cleaved caspase-3 activation (Chapters 4 and 5). Conversely, Basal A TNBC, MDA-MB-468, did not undergo apoptosis perhaps due to its expression of cMET and EGFR. In the tamoxifen resistant cell line the Ipatasertib plus XMD8-92 worked unexpectedly by inhibiting ER-ERE complex formation. Lastly, novel MEK5 and MEK5/Akt inhibitors were able to recapitulate the functional results from chapters 4 and 5. Results from these studies are a promising start for targeted TNBC and tamoxifen resistant therapy. However, several questions remain and will shape the direction of future experiments.

Animal models are needed to examine the potential therapeutic benefit and collateral toxicity of these cellular based studies. Using clinical compounds with known PK/PD will expedite future animal studies. Additionally, using patient derived xenografts (PDXs) will also accelerate the translational potential of these studies. Our collaborators at Tulane and LSU have access to TNBC patient samples pretreated with chemotherapy. These samples are converted to PDX models, which are a realistic representation of TNBC. However, a limitation of these studies is that the PDXs require an immune deficient mouse model. Therefore there is a need to investigate in immune competent mouse models as well. Additionally, Ipatasertib has recently been shown to enhance the effects of Immunotherapy in TNBC patients. Roche announced the results of the phase 1b trial of Ipatasertib and atezolizumab (PD-L1 inhibitor) plus paclitaxel for locally advanced TNBC. The trial showed promise with a 73% response rate with patients receiving Ipatasertib plus atezolizumab (NCT03800836). Also, the fact that ERK5 inhibition and
our novel MEK5 inhibitor did not affect immune response underscore the potential of dual PI3K/Akt and MEK5/ERK5 inhibition.

Although many of the cell lines in this study share similar “driver” mutations, there were marked divergences in cellular signaling. To address this future studies characterizing the upstream kinases of MEK5: MEKK2 and MEKK3 should be conducted. Also, the role of ERK1/2 in EMT needs to be further evaluated. Lastly, in these studies, drugs were simultaneously applied for treatment. Future studies should examine the temporal component of the combination strategy.

In summary, the results of this dissertation indicate, for the first time, the therapeutic utility of combined PI3K/Akt and MEK5/ERK5 inhibition in breast cancer.
Chapter 9 References


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Filmus, J., Pollak, M.N., Cailleau, R., et al. (1985). MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. *Biochemistry Biophysics Research Communications*, 128, 898-905.


Fremin, C., & Meloche, S. (2010). From basic research to clinical development of MEK1/2 inhibitors for cancer therapy. *Journal of Hematology and Oncology, 3*(8).


Wang, J., Lou, P., Lesniewski, R., & Henkin, J. (2003). Paclitaxel at ultra low concentrations...


## Appendix A Literature Compounds

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<tr>
<th>Structure</th>
<th>Compound ID</th>
<th>Target</th>
<th>Potency (IC&lt;sub&gt;50&lt;/sub&gt;)</th>
</tr>
</thead>
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<td><img src="image1.png" alt="Structure" /></td>
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<td>ERK5, BRD4</td>
<td>130nM, 190nM</td>
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<tr>
<td><img src="image2.png" alt="Structure" /></td>
<td>AX15836 (AX)</td>
<td>ERK5</td>
<td>8 nM</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure" /></td>
<td>U0126 (UO)</td>
<td>MEK1/2</td>
<td>60nM, 70nM</td>
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<tr>
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<td>MEK1/2</td>
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</tr>
<tr>
<td><img src="image5.png" alt="Structure" /></td>
<td>CPI-203 (CPI)</td>
<td>BRD4</td>
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</tr>
<tr>
<td>Structure</td>
<td>Compound ID</td>
<td>Target</td>
<td>Potency (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
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<tr>
<td>-----------</td>
<td>-------------</td>
<td>--------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td><img src="image" alt="LY294002" /></td>
<td>LY294002 (LY)</td>
<td>Pan PI3K</td>
<td>500-570 nM</td>
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<tr>
<td><img src="image" alt="Pictilisib" /></td>
<td>Pictilisib (Pict)</td>
<td>Pan PI3K</td>
<td>3 nM, 33 nM, 75 nM</td>
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<td><img src="image" alt="Tasellisib" /></td>
<td>Tasellisib (Tas)</td>
<td>PI3K alpha</td>
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</tr>
<tr>
<td><img src="image" alt="Ipatasertib" /></td>
<td>Ipatasertib (Ipat)</td>
<td>Akt 1/2/3</td>
<td>5 nM, 18 nM, 8 nM</td>
</tr>
<tr>
<td><img src="image" alt="MK-2206" /></td>
<td>MK-2206 (MK)</td>
<td>Akt 1/2/3</td>
<td>8nM, 12nM, 65nM</td>
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<tr>
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<td>ER alpha</td>
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<tr>
<td><img src="image" alt="Paclitaxel" /></td>
<td>Paclitaxel (Tax)</td>
<td>Microtubule</td>
<td>0.1 pM</td>
</tr>
<tr>
<td>Structure</td>
<td>Compound ID</td>
<td>Target</td>
<td>Potency (IC₅₀)</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>--------</td>
<td>---------------</td>
</tr>
<tr>
<td><img src="image1" alt="Temozolomide Structure" /></td>
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<td>Alkylating agent</td>
<td>Variable</td>
</tr>
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<td>VO-Ohpic trihydrate (PTENi)</td>
<td>PTEN</td>
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</table>
Cavanaugh Laboratory Procedures

By Thomas Wright
Mellon 414

Duquesne University
**Procedure: Splitting Cells**

Originated: 08/2014 by TDW

Updated: 08/2018 by TDW

**Splitting cells**

- Remove medium via vacuum
- Add 2 mL TryPLE (pre-heated to 37 C) and place in the incubator for 2 minutes*
- Remove plate from incubator and wash plate with 5 mL medium (pre-heated to 37 C) collect the washes with 15 mL centrifuge tube
- Centrifuge for 7 min at 3,000 rpm
- Remove supernatant via vacuum and re-suspend cell pellet with the proper mL based on table below:

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type, Tissue</th>
<th>Medium Type</th>
<th>Split Ratio</th>
<th>mL for split</th>
<th>Incubator conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>Triple negative, Breast</td>
<td>DMEM.F-12</td>
<td>1:4</td>
<td>4</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>MDA-MB-23 ERK5 KO</td>
<td>Triple negative, Breast</td>
<td>DMEM.F-12</td>
<td>1:4</td>
<td>4</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>BT549</td>
<td>Triple negative, Breast</td>
<td>1640</td>
<td>1:4</td>
<td>4</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Triple negative, Breast</td>
<td>L-15</td>
<td>1:4</td>
<td>4</td>
<td>No CO₂</td>
</tr>
<tr>
<td>MCF-7</td>
<td>ER+, Breast</td>
<td>DMEM.F-12</td>
<td>1:3</td>
<td>3</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>MCF-7 TAMR</td>
<td>ER+, Breast</td>
<td>DMEM - ph red</td>
<td>1:3</td>
<td>3</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>BT474</td>
<td>Triple positive, Breast</td>
<td>Hybricare</td>
<td>1:4</td>
<td>4</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>MCF-10*</td>
<td>Normal fibroblast, Breast</td>
<td>MEBM bullet kit</td>
<td>1:3 or 1:6</td>
<td>3 or 6</td>
<td>5% CO₂</td>
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<tr>
<td>U-87 MG</td>
<td>Glioblastoma, Brain</td>
<td>EMEM</td>
<td>1:2 to 1:5</td>
<td>2 or 5</td>
<td>5% CO₂</td>
</tr>
</tbody>
</table>

* Add 1 mL for the cell suspension to a 10 cm plate and add 9 mL fresh medium to make a final volume of 10 mL
* Place cells in incubator

**Alternate “quick” procedure:**

- Remove medium via vacuum
- Add 2 mL TryPLE (pre-heated to 37 C) and place in the incubator for 2 minutes
- Remove 1.5 mL TryPLE and suspend cells in volume indicated in table above
- Add 1 mL for the cell suspension to a 10 cm plate and add 9 mL fresh medium to make a final volume of 10 mL

Place cells in incubator
Procedure: Thawing and Freezing cells

Originated: 08/2014 by TDW
Updated: 08/2018 by TDW

Thawing cells

- Remove cells from N$_2$(I) canister. Make sure to select cells from the Brown section (cancer cells). Also, denote the vial(s) removed in the binder.
- Gently loosen the cap to allow N$_2$ to escape. Partially submerge the vial in the 37°C water bath until the cells are loosened.
- Pour cells into 10 mL of chilled medium (on ice) (Use a 15 mL vial). Wash cryovial to ensure all cells have transferred. Centrifuge the mixture for 7 minutes @ 3000 rpm and 4°C.
- A cell pellet should be seen at the bottom of the conical vial. Remove the supernatant layer via vacuum. Avoid disturbing the cell pellet.
- Suspend the cells by adding about 5 mL of medium previously heated to 37°C. Gently work the pellet up and down in the pipet to ensure the full pellet is broken up.
- Place the 5 mL from the previous step onto a 10cm plate then wash the vial with an additional 5 mL of medium. Add the washings to the plate.
- Place the cells in the incubator 5% CO$_2$ and 37°C.

Freezing cells

- Label cryovials with the following
  - Initials and date
  - Passage and Cell type
  - 5% DMSO

- Prepare the following solutions
  - Reconstitution medium (1.6 mL Base medium + 0.4 mL FBS)
  - Cryo medium (1.8 mL Base medium + 0.2 mL DMSO)

- Place solutions on ice and add 0.5 Cryo medium to cryo vial and place on ice
  - Proceed as in splitting cells except for the following changes: Centrifuge cells at 4°C
  - Resuspend cells in 0.5 mL Reconstitution medium
  - Add resuspended cells to the cryo vial

- Place cryo vial in freezer container and freeze overnight at -80°C
- Move cryo vial to liquid nitrogen in the Brown (cancer) section
Procedure: Treatment with kinase inhibitors

Originated: 08/2014 by TDW
Updated: 08/2018 by TDW

Cell Culture: Splitting for treatment

- Split cells as directed in “Splitting cells”
- Re-suspend the cell pellet
- Count the cells with a Hemocytometer. Example: 2 μL of cell suspension and 18 μL Trypan Blue solution. The Cytometer will uptake the sample (about 10 μL) via capillary action. Expect 2 to 7.5 million cells per 10cm plate.
- Dilute cells according to cell count to achieve the proper seeding density shown in the table below

<table>
<thead>
<tr>
<th>Plate type</th>
<th>Seeding density</th>
<th>Volume per well (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 cm (60 mm)</td>
<td>1,000,000</td>
<td>4</td>
</tr>
<tr>
<td>6 well plate (35 mm)</td>
<td>500,000</td>
<td>2</td>
</tr>
<tr>
<td>12 well plate</td>
<td>250,000</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: Seeding densities may vary by cell line. As a rule of thumb treat cells once they are 70-90% confluent; which may occur 24 hours or later after seeding.

Cell treatment with MEK inhibitors (initial screen)

- Prior to treatment, allow cells to attach and grow for 24 hours (70-90% confluent)
- Remove the medium via vacuum and add base media containing 0.1% BSA and no FBS to each plate. Serum starve the cells for 16-24 hours
- Treat the cells with MEK inhibitors by adding 2 μL of a 10 mM stock (final concentration of 10 μM if done in 6 well plate with 2 mL of volume). Incubate for 30 minutes at 5% CO₂ and 37°C
- Stimulate cells with 2 μL EGF 100ng/μL stock (final concentration 100ng/mL if done in 6 well plate with 2 mL of volume). Incubate for 15 minutes at 5% CO₂ and 37°C
- Remove medium via vacuum and place plates on ice
- Wash cells with 2 mL (6 well format) chilled 1X PBS and remove the washings via vacuum
- Prepare lysis buffer:
  - 1x RIPA buffer
  - 10 μL Protease inhibitor cocktail per 1 mL 1x RIPA buffer
  - Keep contents on ice
- Add lysis buffer for 5 minutes

<table>
<thead>
<tr>
<th>Plate type</th>
<th>Lysis buffer volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 cm (60 mm)</td>
<td>200</td>
</tr>
<tr>
<td>6 well plate (35 mm)</td>
<td>150</td>
</tr>
<tr>
<td>12 well plate</td>
<td>75</td>
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</tbody>
</table>

- Scrape each plate (forcefully) and collect lysates in chilled 1.5 mL centrifuge tubes.
- Centrifuge the lysates for 10 minutes @ 10,000 rpm and 4°C.
- Decant the supernatant into three vials:
  - 3.5 μL protein assay
  - 48 μL western
Note: This procedure is primarily intended to serve as an initial screen for novel compounds at a 10 μM concentration. Refer to decision tree below for additional testing.

**Kinase Activity**

- Significant response? P<0.05 vs control
  - No
    - Troubleshoot To rule out false negatives
  - Yes
    - Further Characterization With concentration response and additional time points
      - Try additional cell lines
      - Examine alternate pathways
      - Isolated enzyme assay

**Example study:**
1. Determine concentration response: 10 nM, 30 nM, 100 nM, 1μM, 3 μM, 10 μM, 30 μM, and 100 μM
2. Examine selected concentration(s) (preferably an IC_{50}) at various time points such as 30 min, 1 hr, 4 hr, 8 hr, and 24 hr.
Procedure: Bradford protein assay (10x)

Originated: 08/2014 by TDW

Updated: 08/2018 by TDW

Protein assay

- Sample Prep: 3.5 μL + 31.5 μL water
- Standard prep:

<table>
<thead>
<tr>
<th>Protein conc (mg/mL)</th>
<th>Bovine γ-globulin (μL)</th>
<th>Lysis Buffer (μL)</th>
<th>H₂O (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.5</td>
<td>31.5</td>
</tr>
<tr>
<td>0.1</td>
<td>3.5</td>
<td>3.5</td>
<td>28</td>
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<tr>
<td>0.2</td>
<td>7</td>
<td>3.5</td>
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<tr>
<td>0.5</td>
<td>17.5</td>
<td>3.5</td>
<td>14</td>
</tr>
</tbody>
</table>

- Bradford reagent: 3 mL Bradford reagent + 12 mL water. Filter.
- Add 10 μL of each sample and standard to a 96 well plate. Plate both standards and samples in triplicate.
- Add 200 μL Bradford reagent to each well.
- Scan using microplate reader in room Mellon 454.
- Microplate reader:
  - Start up computer and turn on instrument (Computer user name/password: allen/cavanaugh)
  - Open "Workout 2.0"
  - Select "Run existing protocol"
  - Select "Cavanaugh Bradford 10X" from the menu
  - Edit sample arrangement if necessary (if your plate layout is different)
  - Click "run"
  - Export to excel
  - Check the R² (should be greater than or equal to 0.95)
  - Save in "Cavanaugh Lab folder"
  - Email copy
  - Shut down computer

- Use spreadsheet program to determine amount of protein to load onto western blot gels.

Note: Nuclear extracts require either a x40 or x60 dilution in order to be measured in the linear range
Procedure: Western blot (Immunoblot)

Originated: 10/2014 by TDW

Updated: 08/2018 by TDW

Western blot

- Place 1.5 mm plates and spacer plates into gel casting stand.
- Running gel (10% gel):
  - 8 mL water
  - 6.6 mL 30% Acrylamide mix (AKA “protogel” stored in 4°C)
  - 5.0 mL 1.5M Tris
  - 200 µL 10% SDS
  - 200 µL 10% APS (stored in -20°C)
  - 8 µL TEMED
- Pour 7.5 mL for each gel and add 1 mL 85% Isopropyl alcohol.
- Allow to stand for 20 minutes.
- Prepare samples by adding 12 µL of loading dye (9:1 ratio of Loading buffer to 2-mercaptoethanol) to the 48 µL western blot lysate. Boil samples at 90 °C for 10 minutes.
- Pour off Isopropyl alcohol and wash with water.
- Stacking gel:
  - 4.1 mL water
  - 1 mL 30% Acrylamide mix
  - 0.75 mL 1M Tris
  - 60 µL 10 % SDS
  - 60 µL 10% APS
  - 6 µL TEMED
- Add 2.5 mL for each gel and quickly insert combs (either 15 or 10 well but make sure they are 1.5 mm diameter).
- Allow to stand for 15 minutes.
- Remove combs and wash wells gently with water.
- Place the gels into the running buffer apparatus.
- Running buffer:
  - 810 mL water
  - 90 mL 10X Tris
- Load the corresponding volume from the Bradford protein assay to each well. (Load 0.5 µL marker)
- Run gel for 2 hours at 100 volts and 400 mAmps
  - For 4-15% precast gels 15 min at 90 volts and 1 hour at 120 volts
- Transfer the gel to a nitrocellulose membrane buffered by sponges and filter paper and place in transfer apparatus. (Don’t forget to add an ice pack)
- Transfer buffer:
  - 560 mL water
  - 160 mL Methanol
  - 80 mL 10X Tris
- Run for 1 hour at 100 volts and 400 mAmps (up to 750 mAmps for 2 transfers)
- For 4-15% precast gels 45 minutes at 100 volts and 750 mAmperes
  - Cut excess portions of the membrane with a razor (if needed)
  - Incubate in a LiCOR box with 5 mL 1X PBS for 5 minutes. Discard wash.
  - Block membrane with 5 mL Blocking buffer for 1 hour.
  - Save the 5 mL blocking buffer to prepare ERK ½ antibody.
  - Apply primary antibodies for t-ERK 5, p/t-ERK ½, and GAPDH (or others as needed).
  - Incubate overnight in the cold room by Dr. Cavanaugh’s office.

Membrane washing and antibody application

Immediately after transferring the gel to a membrane, place the membrane in a black box with ~5 mL 1x PBS.

Remove the PBS and add ~5 mL blocking buffer to the box. Block the membranes for 1 hour (incubator speed 3-5) (you can reuse this 5 mL of blocking buffer to make the primary antibody).

Primary antibody application:
  (1:1000)
  5 μL antibody
  5 mL blocking buffer
  10 μL tween

Add the primary antibody and incubate overnight in the cold room (locked door by Dr. Cavanaugh’s office).

List of Primary antibodies: incubate overnight in the cold room.
- t-ERK 5 (Goat-anti rabbit; Red channel)
- t-ERK ½ (Goat-anti mouse; Green channel)
- p-ERK ½ (Goat-anti rabbit; Red channel)
- t-Akt (Goat-anti rabbit; Red channel)
- p-Akt (Goat-anti mouse; Green channel)
- GAPDH (Green channel)

Before washes, pour the primary antibodies into a tube and store them in the +4. Wash membranes three times with 1x PBS + 0.2% tween at 10 minute intervals. (incubation speed 7).

Secondary antibody application:
  (1:10,000)
  0.5 μL secondary (either anti-rabbit 680nm or anti-mouse 800nm)
  5 mL blocking buffer
  10 μL tween

Add the secondary antibody and incubate at speed 3 for 1 hour. Remove the secondary antibody and store it in the +4.

Wash membranes three times with 1x PBS + 0.2% tween at 10 minute intervals. (incubation speed 7).

One last wash with 1x PBS.
Removing antibodies

Stripping Buffer
4 mL stripping buffer
16 mL H₂O

Apply stripping buffer for 7 minutes (incubation speed 7)

Wash three times with 1x PBS at 10 minutes intervals.

Repeat steps from "antibody application" starting with blocking for 1 hour.

Reusing antibodies and stability

1. t-ERK 5- always use fresh blocking buffer to prepare and use approximately 4 mL (since the ERK 5 portion of the membranes is smaller). This antibody is stable for up to one month in the 4°C and can be reused 2 to 3 times.

2. p/t-ERK ½ and tubulin- these antibodies are more robust than ERK 5 and appear to have greater longevity. As mentioned above, you can reuse the 5 mL from the blocking step to prepare this antibody. Also, it only takes about 0.5 µL of tubulin to elicit a robust response so keep this in mind when trying to conserve resources. This antibody is stable for up to one month in the 4°C and can be reused 4 to 5 times.

3. p/t-Akt- This antibody is robust like ERK ½ however it cannot be co-administered with tubulin because of the similarity in weight. This antibody is stable for up to one month in the 4°C and can be reused 4 to 5 times.

Odyssey Imaging system

- Before scanning membranes wash membranes in 1X PBS (no tween).
- Take membranes to the Leak lab (sign up before hand via google calendar: Odyssey@Duquesne password:licorimaging).
- Open "image studio" program
  Insert flash drive
  Click the Image Studio icon
  Select work area or create new if needed
  Save work area in flash drive. Do not save on desktop.
  Select "Odyssey" instrument when prompted. Enter "Duquesne" as the password
  Place membranes on the imager surface and Roll out bubbles
  Adjust the dimensions of the image area
  Adjust the imager intensity to the appropriate levels (start with 5.0 for each channel)
  While the imager is running, adjust the gamma to 1.0 in the "curves" tab
  Save run after imaging is completed
  Save run
  Remove membranes from image surface and clean with isopropanol
  Eject flash drive
Procedure: Scratch assay and Invasion assay

Originated: 10/2014 by TDW

Updated: 08/2018 by TDW

Scratch assay

- Proceed as in "cell splitting", count cells, and seed at a density of 200,000 cells/well in a 12 well plate
- Allow cells to attach overnight
- Prior to treatment: remove medium via vacuum, make scratch with a 200 µL pipet tip, mark underside of plate with a dot next to the scratch and wash with 250 µL 1x PBS
  - Example:

![Diagram of a circle with markings](image)

- Discard wash and add treatments to each well (1 mL/well)
- Take initial images with the EVOS microscope under transmitted light and 10x magnification
  - Example save file: DMSO 0hr (231) exp 1
- Take additional images depending on the time point
  - Example save file: DMSO 24hr (231) exp 1
- Wound closure: (border at 24 hr – border at 0 hr)/(border at 0 hr) x 100
  - Calculate with either power point or Image J software
Invasion assay

- Kit obtained from Cell Biolabs (Product# CBA-111)
- 24 well plate and 12 basement membrane inserts were brought to room temperature for 10 minutes and were rehydrated with 300 μL Base medium with no FBS for 1 hr
- The media was removed prior to adding 270 μL of the cell suspension in base medium with no FBS (500,000 cells/well) and 30 μL of the 10x inhibitor solution (1:10 dilution will make 1x final concentration) to the basement membrane insert
- 500 μL of the base medium plus 10% FBS was added to the bottom layer
- Plate was incubated for 48 hours
- The medium was aspirated from the inner layer and the insert was transferred to a well with 225 μL of cell detachment solution for 30 minutes at 37
- 75 μL of the Cytoquant/4x Lysis buffer solution was added to each well and incubated for 20 minutes at room temperature
- 200 μL of the resulting mixture was transferred to a blacked out Fluorescence plate and measured at 485 nm using a micro plate reader
Procedure: MET

Originated: 07/2015 by TDW

Updated: 08/2018 by TDW

**MET morphology**

- Proceed as in "cell splitting", count cells, and seed at a density of 5,000 cells/well in a 96 well plate (2,500 cells/well for 7 day)
- Allow cells to attach and grow for 24 hours before treatment with compounds
- Treat for 3, 5, or 7 days
- After treatment, remove medium by inverting plate and flicking (like ELISA washes) and wash with 100 µL 1x PBS
- Discard wash and add 50 µL 4% PFA in 1x PBS to each well
- Allow cells to fix for 15 minutes on the desktop shaker
- Remove fixative and wash with 100 µL 1x PBS
- Discard wash and add 25 µL of 0.1% crystal violet in 90% MeOH
- Stain cells for 15 minutes on desktop shaker
- Remove stain and wash 3 times with 100 µL 1x PBS
- After washes add 100 µL 1x PBS
- Image plate under transmitted light using the EVOS imager with 10x magnification
- Store plate +4 °C
Procedure: MTT

Originated: 04/2015 by TDW

Updated: 08/2018 by TDW

MTT

- Proceed as in “cell splitting” and count cells to determine the proper dilution for plating

- Plate cells in a 96 well plate at a density of 5,000 cells per well
  - For single treatment 90 μL per well
  - For dual treatment 80 μL per well
  - Use a multichannel pipet to plate cells from a sterile through

- Allow cells to attach overnight

- Once cells have attached, begin preparing the samples:
  - Example: Add 10 μL of a 10 mM solution to 1 mL of medium. Add 10 μL of the solution to the appropriate well on the plate (final volume should be 100 μL and final concentration of 10 uM). Solutions may be stored at +4 C for one week

- Place in the incubator for the duration of the treatment (24 hrs, etc.)

- After the time point has passed, add 10 μL of a 5.5 mg/mL MTT solution (final concentration 0.5 mg/mL) and return plate to the incubator for 3 hours

- After 3 hours, remove the medium-MTT via vacuum and add 100 μL DMSO per well

- Place the plate in foil and shake for 10 min (incubation speed 6)

- Analyze the plate with the plate reader in room 454 using “MTT abs570” Workout 2.0 program or “S70Abs” 1420 Workstation program

- Export results to excel file, save file, and begin data analysis
  - In each file name include: treatment, concentration, time point, cell line, and experiment number
  - Example file name: MTT LY 10 nM to 100 uM 24hr (231) exp1
Trouble shooting MTT assay:

1. Surfactant effect:
   It is important to examine cell detachment due to a surfactant effect. If there are floating cells in
   the well, it is possible that they are not dead (cancer cells can grow in an anchorage independent
   manner). If this is the case, try centrifuging the 96 well plate before analysis (the O’Donnell lab
   has a centrifuge that can accomplish this; 5 min at 500 rpm). Alternatively, the medium from each
   well can be aspirated and the floating cells can be analyzed for viability using the Trypan blue
   assay.

2. Low absorbance values:
   Some cell lines do not metabolize the MTT reagent efficiently and hence produce lower signals
   (0.1 AU). In these cases, perform a calibration curve using several seeding densities: 1,250, 2,500,
   5,000, and 10,000 cells/well. Make sure the cell density used in your experiments is at least 3x
   greater than the background. It may also be necessary to adjust the time point of the assay to
   ensure a large enough population of cells is present to produce adequate signal.

3. Low signal in drug treatment groups but high cell counts:
   It is possible drug treatments can decrease the functional capacity of the metabolic enzymes
   responsible for MTT metabolism. In this case, it is necessary to run a plate in parallel for crystal
   violet staining and cell counting. This additional experiment adds structural data to help interpret
   the MTT results.
Procedure: IF/IHC

Originated: 05/2017 by TDW

Updated: 08/2018 by TDW

IF/IHC

- Proceed as in "cell splitting", count cells, and seed at a density of 5,000cells/well in a 96 well plate
- Allow cells to attach before treatment with compounds
- After treatment, remove medium and wash with 1x PBS
- Discard wash and add 50 μL 4% PFA in 1x PBS to each well
- Allow cells to fix for 15 minutes on the desktop shaker
- Remove fixative and wash with 100 μL 1x PBS
- Discard wash and add 50 μL Blocking buffer to each well for 1 hour at room temperature
  - 705 μL 1x PBS
  - 750 μL LiCOR block
  - 50 μL Triton-X
  - Total volume: 1.5 mL enough for 30 wells. Adjust proportions for larger volumes
- Remove block and add 50 uL primary antibody to each well; incubate overnight at +4 °C
  - 705 μL 1x PBS
  - 750 μL LiCOR block
  - 50 μL Triton-X
  - 2 μL primary antibody
- Remove primary and wash 3 times with 100 μL 1x PBS for 5 minutes each wash
- Discard wash and add 50 μL secondary antibody to each well for 1.5 hours at room temperature
  - 705 μL 1x PBS
  - 750 μL LiCOR block
  - 50 μL Triton-X
  - 2 μL Secondary antibody (AlexaFlour 555 nm anti rabbit; RFP or AlexaFlour 488 nm anti mouse; GFP)
  - 20 μL Hoechst stain
- Remove secondary and wash 3 times with 100 μL 1x PBS for 5 minute each wash
- After final wash add 100 μL 1x PBS to each well
- Image plate using the EVOS imager with 10x to 40x magnification

Store plate at +4 °C
Procedure: Trypan blue assay

Originated: Adapted from Witt-Enderby lab 07/2015

Updated: 08/2018 by TDW

Trypan blue

The following procedure will enable you to accurately determine the cell viability. Cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemacytometer. If cells take up trypan blue, they are considered non-viable.

1. Determine the cell density of your cell line suspension using a hemacytometer.
2. Prepare a 0.4% solution of trypan blue in buffered isotonic salt solution, pH 7.2 to 7.3 (i.e., phosphate-buffered saline).
3. Add 0.1 mL of trypan blue stock solution to 1 mL of cells.
4. Load a hemacytometer and examine immediately under a microscope at low magnification.
5. Count the number of blue staining cells and the number of total cells. Cell viability should be at least 95% for healthy log-phase cultures.

\[
\% \text{ viable cells} = \left(1.00 - \frac{\text{(Number of blue cells)}}{\text{(Number of total cells)}}\right) \times 100
\]

To calculate the number of viable cells per mL of culture, use the formula below. Remember to correct for the dilution factor.

\[
\text{Number of viable cells} \times 10^4 \times 1.1 = \text{cells/mL culture}
\]
Pull down assay procedure

Originated: 04/2015 by TDW
Updated: 08/2018 by TDW

Cell culture:
- MDA-MB-231 cells were plated in 60mm plates at a density of 250,000 cells/mL (4mL total).
- Cells were allowed to attach overnight and serum starved for 24 hours prior to treatment. Cells were treated with 4 uL EGF (100ng/ul stock) for 20 minutes at 37C and 5% CO2.
- Medium was removed via vacuum and plates were placed on ice.
- Cells were washed with iced 4mL PBS (1x).

- Lysis buffer was prepared (10 mL):
  - 9.56 mL Water
  - 100 uL 5M PIPES (50 mM)
  - 100 uL 5M NaCl (50 mM)
  - 100 uL 0.5M MgCl2 (5 mM)
  - 100 uL 0.5M EGTA (5 mM)
  - 10 uL Tergitol NP40 (0.1%)
  - 10 uL Triton x-100 (0.1%)
  - 10 uL Tween 20 (0.1%)
  - 1 EDTA free protease inhibitor (1 tablet per 10 mL)

- 750 uL lysis buffer added to each plate.
- Plates scraped and collected in a 1.5 mL centrifuge tube.
- Lysates were spun for 10 min at 10,000 rpm and 4C.

- Protein concentration was determined by using a Bradford protein assay.

Pre incubation:
- 0.5 mL streptavidin beads (4mg/mL)
- 0.5 mL 0.01 mM probe A in PBS (H9 10 mM stock 2 uL in 2 mL PBS)
- Allowed to incubate for 30 min at room temp.
- A magnet was applied and the probe A solution was poured off.
- The beads were washed with 1x PBS and the wash was discarded.

- Beads were incubated with 500 uL cell lysate for 1 hr at room temp.
- Washes x3 @ 10 min intervals:
  - Plate A:
    - Lysis buffer 1.5 mL
    - 375 uL 0.5 mM MgCl2 (100 mM)
  - Plate B:
    - Lysis buffer 1.5 mL
    - 375 uL 0.5 mM MgCl2 (100 mM)
    - 46.5 uL 10 mM F7 (10 uM)
- Washes were discarded by applying a magnet and pouring off the supernatant.
- The final wash pellet was resuspended in 50 μL Lamml buffer (with Beta mercapto ethanol).
- The proteins were eluted off the beads by heating for 5 min at 95°C (or desthiobiotin).
- The beads were spun on a desk top centrifuge and a magnet was applied while the Lamml buffer was poured off.

- A 12% SDS gel was poured and the sample was loaded onto a 10 well gel (66 μL max).
- The gel was run for 3.5 hours at 110 volts.

- Coomassie stain:
  - Gel was fixed with 20 mL solution of 45:45:10 water-methanol-acetic acid and allowed to incubate overnight.
  - Gel was treated with 20 mL of a 0.25 mg/mL coomassie solution for 1 hour.
  - Gel was treated with a destain solution of 45:45:10 water-methanol-acetic acid and allowed to incubate overnight (at least 16 hours).

- Various portions of the gel were excised for Mass spec analysis.
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Method validation

Figure 1 MTT Calibration 72 hours.

Figure 2 Correlation between MTT and cell count (Crystal violet).

Figure 3 Sample Immunofluorescence. MDA-MB-231 cells treated with DMSO. 10x Magnification. Green= Tubulin. Red= Ki67, and Blue= Hoechst.
## Appendix C Novel Compounds

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<th>pERK5 (% activation)</th>
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