BIASING RECEPTOR-MEDIATED SIGNALING IN METASTATIC BREAST CANCER

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Medicinal Chemistry and Molecular Pharmacology

West Lafayette, Indiana

December 2020

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Dedicated to all who have supported me throughout my life

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The epidermal growth factor receptor (EGFR) is a well-recognized proto-oncogene and mediator of cancer cell growth and proliferation. Emerging evidence suggests the paradoxical role of EGFR activation, unique to metastatic breast cancer cells. Previously studies elucidated the role of EGFR mediated activation of Signal Transducer and Activator of Transcription 1, STAT1, is required to induce apoptosis in cells with increased metastatic potential. In this current study, we evaluate the effects of cells with mutations leading to aberrations in phosphatidylinositol 3-kinase, PI3K/ AKT/protein kinase B signaling. Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA) is a key regulator of the PI3K/Akt pathway and is one of the most commonly mutated genes in breast cancer patients. Utilizing human breast cancer cells, MDA-MB-468 and BT-20s, characterized by amplified protein levels of EGFR and mutations in the PI3K signaling, we demonstrate activation of EGFR with EGF leads to increased pSTAT1 expression. We further demonstrate biased EGFR signaling toward STAT1 activation by pharmacological inhibition of downstream kinase activity with MEK1/2 inhibitor, trametinib, and the AKT inhibitor, Uprosertib or in combination with the PI3Kα specific inhibitor Alpelisib, trade name, PIQRAY. Combination MEK and Akt signaling inhibition followed by EGF stimulation show marked increase in apoptotic activity and decreased cell viability. Moreover, we demonstrate changes in EGFR mediated apoptosis in murine breast cancer cell line derived from metastatic lung nodules, delineate from cells derived from the primary tumor. These finding support the notion of differential evolution of cancer cells as they metastasize to secondary organs. Furthermore, EGFR expression was observed to be vital in EGF mediated pSTAT1 in human breast cancer cell lines. To this end we explored alternative stimulators of pSTAT1 using interferon activation. Addition of INFγ led to robust pSTAT1 in cells that did not respond to EGF and levels of pSTAT1 were not attenuated with our

combination treatment. Together, our findings demonstrate the differential role of EGFR expression and signaling in metastatic cells and tolerance for novel combination therapies for patients of late stage breast cancer.

CHAPTER 1. INTRODUCTION

1.1 Breast Cancer

Breast cancer remains the most diagnosed cancer in woman and a leading cause of cancer related deaths in the United States. According to the National Cancer Institute, Surveillance, Epidemiology, and End Results Program (SEER), there was an estimated 271,270 new cases and 42,260 breast cancer related deaths in the year 2019.(Siegel, Miller, & Jemal, 2019) Furthermore, the cost of treating breast cancer in the United States in 2010 was $16.5 million and by 2020 this cost is estimated to rise to $20.5 billion. (Feinstein et al., 2015) These staggering cases in incidence and increase in economic impact highlights the importance in the study of this highly heterogenous disease. Risk factors in the development and progression of breast cancer include age, genetic factors, reproductive factors, hormonal imbalance, and obesity. The identification of these risk factors aids in general health screening and early detection of the disease. Additionally, these risk factors play a role in therapeutic efficacy and treatment outcome.(Lee, Kruper, Dieli-Conwright, & Mortimer, 2019)

Traditional clinical classification of breast cancer includes tumor size, histological grade, patient's age, in addition to estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2 or erb-B2) overexpression status. Histological pathology of noninvasive neoplasms are divided into two major types: lobular carcinoma in situ and ductal carcinoma in situ. While invasive breast cancers are divided into ductal and lobular histologic types. (Alkabban & Ferguson, 2020) These classic biological characterizations, however, overlook many other genetic alterations involved in breast cancer development and progression. Subclassifications of breast cancer further delineate unique mutations in each patient.

These subclassifications can be categorized into four intrinsic subtypes; luminal A, luminal B, HER2+ and basal like. (Yersal & Barutca, 2014)

Together, luminal-A and luminal-B tumors make up approximately 65-80% of breast cancer cases and are characterized by their expression of ER, PR, responsive genes. (Yersal & Barutca, 2014) Estrogen receptors (ERs) are transcription factors that when bound to estrogen steroid hormones, migrate to the nucleus, driving expression of genes involved cell proliferation.(Brunetti & Manfioletti, 2019; Tryfonidis, Zardavas, Katzenellenbogen, & Piccart, 2016) Progesterone receptors (PRs) are a member of the nuclear steroid receptor family. Binding of the PR DNA binding domain and the progesterone response elements results in target gene transcription. (Kariagina, Aupperlee, & Haslam, 2008) Deregulation of these hormone receptors lead to increased cell survival and proliferation resulting in solid tumor formation. Luminal A tumors are characterized by higher levels of ER and lower level proliferation gene, *Ki-67*, making it a good candidate for endocrine treatments. In contrast, luminal B tumors can be characterized by high Ki-67 and low PR when HER2- or luminal B-like with HER2 overexpression/amplified and ER+, with any level of Ki-67 and PR. (Inic et al., 2014; Yanagawa et al., 2012) These characteristic differences in luminal A and B subtypes determine disease outcome and drug response. Luminal A tumors show good prognosis, low relapse rate and good response to endocrine therapy, while luminal B tumors are more aggressive, demonstrating lower survival rates and increased reoccurrence rates compared to the luminal A counterpart. (Kennecke et al., 2010)

The Erb-B2 overexpressing or HER 2 positive (non-luminal) subtype is characterized by HER2 over-expressed/amplified and ER/PR-. The *HER2* gene encodes a transmembrane tyrosine kinase receptor belonging to the epidermal growth factor receptor (EGFR or Erb) family. This

family of receptors function by stimulating growth factor mediated signaling pathways involved in cell proliferation, migration, and survival. HER2 does not have an identified ligand and must rely on heterodimerization with other Erb members or homodimerization with itself leading to transphosphorylation of extracellular domains and activation of signaling cascades. Amplification of HER2 enables constitutive activation of these signaling pathways, leading to oncogenic phenotypes. (Gajria & Chandarlapaty, 2011) This subtype of breast cancer accounts for approximately 15-20% of all breast cancer incidence and is observed to be biologically and clinically more aggressive resulting in poor prognosis. (Staaf et al., 2010; Yersal & Barutca, 2014)

Basal-like subtypes incidences can range from approximately 8-37% depending on the proportion of poorly differentiated basal-like cases included in population studies. (Badowska-Kozakiewicz & Budzik, 2016; Boyle, 2012; Yersal & Barutca, 2014) Basal-like breast cancers are identified by a variety of immunohistochemical markers, including epidermal growth factor receptor (EGFR) and cytokeratin; CK5/6/14/17, among others. Additionally, basal-like breast cancers lack of expression of ERα, PgR, HER2 or "luminal" cytokeratins; CK8/18/19. Furthermore, it is characterized by a higher Ki-67 and lower p53 expression leading to overall aggressiveness and poor prognosis compared to any other breast cancer subtypes. (Badowska-Kozakiewicz & Budzik, 2016) Triple negative breast cancers (TNBCs) are characterized by lack of expression of all three of ER, PR, and HER2 and approximately 70-90% of are considered basal-like carcinomas. The lack of these receptors results in non-responsiveness to clinically available endocrine and HER2 targeted therapies. (Rouzier et al., 2005) Additionally, basal-like tumors are much more invasive and lead to metastatic dissemination of the disease.

Genetic factors play a significant role in breast cancer development and progression. In addition to the genes mentioned above, dysregulation in genes that result in genomic instability

common in breast cancer include; *BRCA1, BRCA2, TP53, PTEN* and *CHEK2*. (Badowska-Kozakiewicz & Budzik, 2016; Toft & Cryns, 2011) Breast cancer type 1/2 susceptibility genes (*BRCA1, BRCA2)* are considered tumor suppressors. Disruption of double-strand DNA repair via homologous recombination occurred when there is a loss of heterozygosity in the second BRCA½ allele. The *TP53 encodes* tumor protein p53 which also functions as a tumor suppressor. Disruption of this gene leads to genomic instability and inhibition of cell cycle arrests and apoptosis even in the presence of DNA damage. The *PTEN* gene encodes phosphatase and tensin homolog (PTEN) implicated in cell cycle progression, cell growth and survival. PTEN is responsible for de-phosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that feeds in the for the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway involved in cellular motility, invasion, proliferation and survival. Thus, PTEN acts as a tumor suppressor and absence of this gene leads to continuative activation of the PI3K/Akt pathway and tumorigenesis.(Carbognin, Miglietta, Paris, & Dieci, 2019) Finally, checkpoint kinase 2, encoded by the *CHEK2* gene is a serine/threonine kinase that regulated DNA damage through cell cycle arrest or apoptosis. Activation begins by phosphorylation and dimerization followed by kinase cascade activity. CHEK2 kinase activity regulates transcription factors and proteins including BRCA½ and p53. Like all other tumor suppressors described above, mutations in the *CHEK2* gene often in the form of missense or deleterious mutations will lead to alternations in cell cycle arrest, DNA repair and apoptosis and subsequently cancer cell survival. (Apostolou & Papasotiriou, 2017)

1.2 Metastasis

Metastasis is the main cause of cancer mortalities and development is currently considered incurable. Metastasis occurs when cells from the primary tumor disseminate into the surrounding stroma, invade the vasculature and travel to secondary sites for colonization and spread of the

malignancy. Specific properties or “hallmarks” of a metastatic cell include (1) mobility and invasion (2) modulation of microenvironment (3) plasticity and (4) colonization. (Welch & Hurst, 2019)

Epithelial to mesenchymal transition (EMT) is an embryonic process in which epithelial cells transition into non-epithelial, mesenchymal cells, that are loosely embedded in an extracellular matrix. Characteristics of this transition include loss of loss of “epithelial phenotype” including loss of E-cadherin, type I cadherin that mediates homophilic interactions by forming adhesive bonds in their extracellular region and connecting to actin microfilaments and subsequently loss of cell-cell adherent junction. This process facilitates the migration of cells into the extracellular environment. (Thiery, 2002)

The role of EMT in cancer progression has been proposed to enhance cancer cell invasiveness, survival of circulating tumor cells, generation of cancer stem cells, and promotion of drug resistance. (Saitoh, 2018) Disseminated cancer cells can extravasate in to secondary organs and either remain solitary or dormant in the form of micro-metastasis or they can form a new carcinoma through mesenchymal to epithelial transition (MET). (Thiery, 2002)

Stage 4 metastatic breast cancer (MBC) is considered the most advanced stage of breast cancer and is the second cause of death in woman in the U.S.. Common secondary organs of MBCs colonization include; the bones, brain, liver, lungs, soft tissue, and adrenal glands. (Al-Mahmood, Sapiezynski, Garbuzenko, & Minko, 2018; Budd et al., 2006; Weigelt, Peterse, & van 't Veer, 2005) Triple negative breast cancer, characterized by its metastatic aggressiveness, makes up approximately 10-20% of breast cancer cases.(Al-Mahmood et al., 2018) In addition to the poor outcome associated with TNBC patient, common breast cancer treatments such as endocrine or HER2 targeted therapeutics do not work in these patients due to lack of hormone and HER2

receptors. Therefore, other biomarkers have been explored for therapeutic targeting as opposed to conventional cytotoxic chemotherapies. One such target, extensively studied, is the overexpression of growth factor receptors in TNBCs.(Lehmann & Pietenpol, 2014)

The heterogeneity within metastasized tumor cells play a substantial role in survival and anti-cancer drug sensitivity. This is partial due to the differing microenvironments that encompass the cancer cells. In 1889, Stephen Paget published, "The distribution of secondary growths in cancer of the breast."(Paget, 1989) Here he proposed the "seed and soil" theory of metastasis. The theory suggests that metastasized cancer cells have predispositions to specific organs for secondary colonization due to the favorable interactions between the cancer cells and the metastatic tumor microenvironment (TME). Indeed, the differences within distinct organs niches have been elucidated to aid in cancer cell survival and targeting these niches have become vital in understanding and treating cancer. The tumor microenvironment consists of the extracellular matrix,blood/lymph vessels, a variety of stromal and immune cells and secreted proteins. Stromal cells include fibroblasts, mesenchymal stromal cells, adipocytes, blood and lymphatic vascular networks.(Roma-Rodrigues, Mendes, Baptista, & Fernandes, 2019) Immune cells include immune cells such as T and B lymphocytes, natural killer cells and macrophages (Figure. 1). These tumor associated stromal cells physically envelop the cancer cells secreting factors into the microenvironement that can be taken up by cancer cells, influencing cancer cell growth, proliferation, survival and drug response at the secondary tumor site distinct from the primary tumor origin. The tumor cell secretome refers to protumorogeneic factors secreted by tumor and stromal cells into the TME. Major components include cytokines, growth factors, enzymes, glycoproteins, and extracellular vesicles. (Madden, Gorman, Logue, & Samali, 2020)

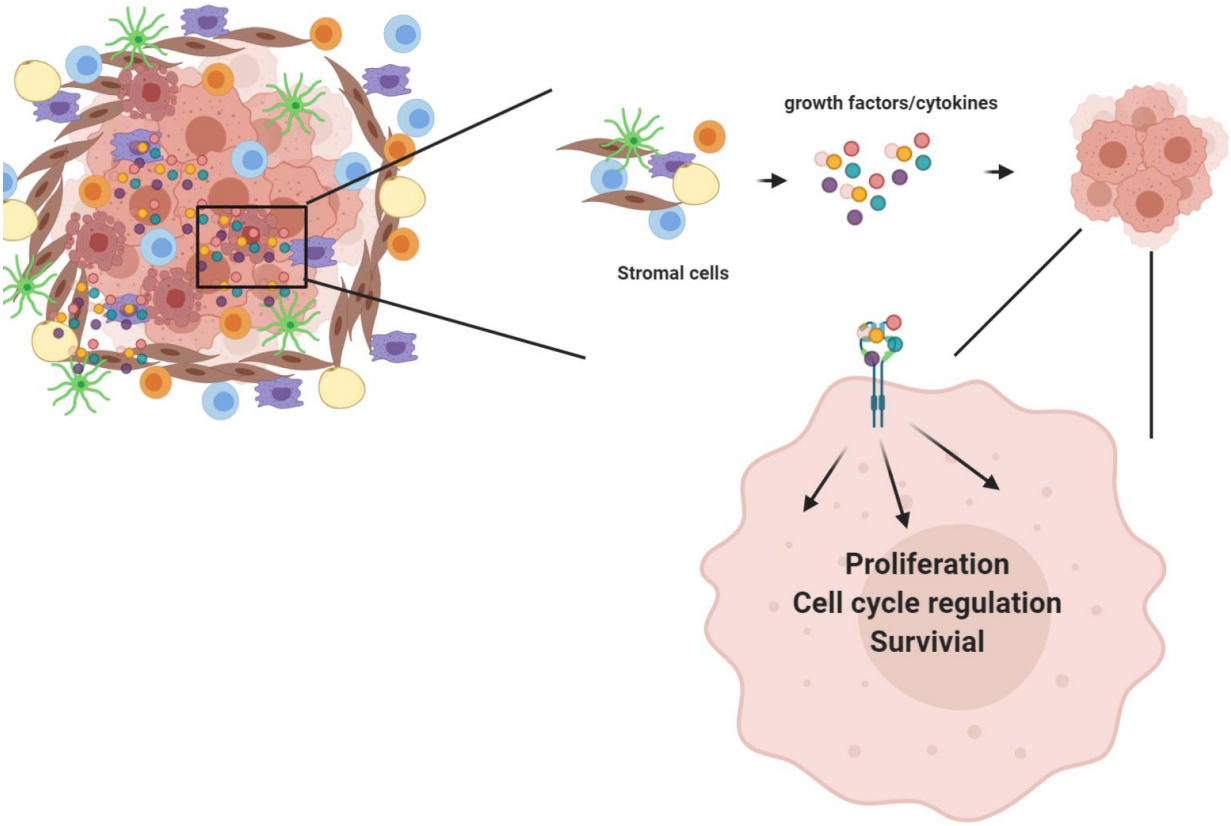


Figure 1 Illustration of tumor microenvironment (TME). Effects of stromal cells and secreted factors in TME that influence cancer cell fate.

1.3 Growth factor receptors

Recptor tyrosine kinases (RTKs) are a single pass transmembrane subtype of tyroine kinases mediated by extracellular growth factors. These receptors posess cytoplasmic enzymatic activity that regulate bioological functions such as cell growth, motility, differentiation, and metabolism.(Du & Lovly, 2018) The general protein structure is comprised of an extracellular ligand binding domain, a single transmembrane helix, and an intracellular region that contains a juxtamembrane regulatory region, a tyrosine kinase domain and a carboxyl terminal tail.(Hubbard, 1999) Upon growth factor ligand binding to the extracellular domain, the receptor dimerizes resulting in conformational changes that enables trans-autophosphorylation of the receptor. This

is followed by recruitment of protiens that contain a src homology-2 (SH2) or phosphotyrosine-binding domains which propogates translocation of docking proteins that activate intracellular signaling pathways.(Pawson, Gish, & Nash, 2001; Schlessinger, 2000) (Figure. 2)

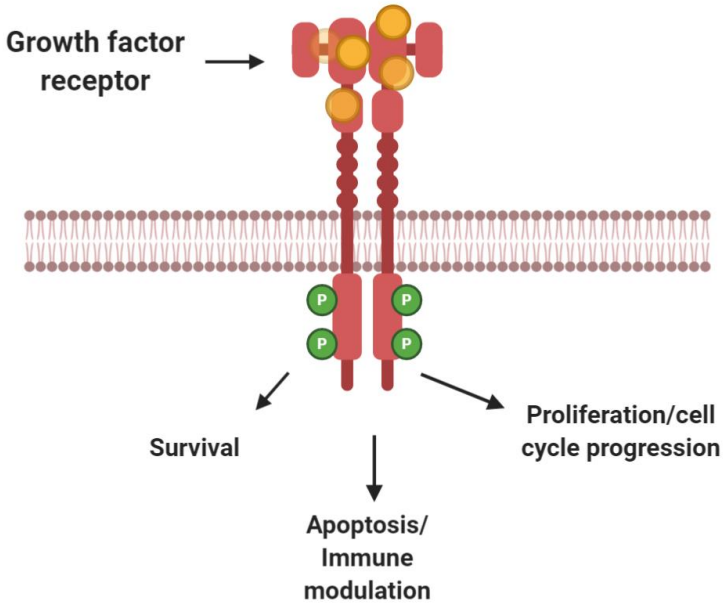


Figure 2. Schematic representation of a prototypical transmembrane growth factor receptor and activation of frequent downstream events involved in cell fate.

Cell surfacce receptors stimulate the exchange of Guanosine-5'-triphosphate (GTP) for Guanosine-5'-diphosphate (GDP) on the small GTPase, Ras. Ras is activated by the guanine nucleotide exchange factor, Sos, via translocation to the plasma membrane and stimulation of the exchange of GTP for GDP with the help to the adaptor protein, Grb2. A complex forms with Grb2 and Sos via its SH3 domains followed by translocation to the activated RTK through binding of the SH2 domain of Grb2 to the specific phosphorylated tyrosine sites of the receptor and close

proximity to the plasma membrane. The GTP-bound state of Ras interacts with several effector proteins including Raf and phospholipid, PI-3 kinase (PI3K) to stimulate intracellular processes.

Activated Raf stimulates mitogen-activated protein kinase, MAP-kinase-kinase (MAPKK, MEK), through phosphoylation of a serine residue in the activation loop. MAPKK then phosphorylates MAPK (ERK) on threonine and tyrosine residues at the activation-loop. Activated MAPK phosphorylates cytoplasmic and membrane linked substrates. Additionally MAPK is translocated into the nucleus where it phosphorylates and activates transcription factors. (Schlessinger, 2000) Tramscriptionaltargets of MAPK include transcription factors such as cAMP response element-binding protein (CREB), transcriptional regulator Myc-like (c-Myc) and nuclear factor kappa B (NF-κB). (Braicu et al., 2019) These trasncription factors regulate expression of genes involved in cell growth and survival, involved in the development and advancment of several malignancies.

Activated PI3K phosphorylates PtdIns(4)P and PtdIns(4,5)P2, resulting in second messengers, PtdIns(3,4)P2 and PtdIns(3,4,5)P3, mediated membrane translocation of signaling proteins. These siganling proteins include the non–receptor protein kinases such as the Ser/Thr kinase PDK1 and PKB, among many others.(Schlessinger, 2000) The Ser/Thr kinases, PKB, also known as Akt, engages with PtdIns(3,4,5)P3, leading to phophorylation by PDK1.(Manning & Toker, 2017) Targets of activatied Akt include forkhead box O 1(FoxO1), Glycogen synthase kinase-3 (GSK-3), and mammalian target of rapamycin (mTOR). These protein play a critival role in cellular metabolism, reconstruction of the cytoskeleton and cell survival.(Xu, Na, Li, & Chen, 2020)

Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathway is involved in the immune system for direct communication from transmembrane signals

to the nucleus. This pathway is generally activated by cytokines and cytokine receptor-associated JAK molecules phosphorylate the intracellular tail of their receptors and create docking sites for STAT transcription factors. STATs phosphorylation by JAKs activates STATs and induces their transfer to the nucleus to regulate gene expression.(Hojjat-Farsangi, 2016) Among the STAT members, STAT3 is vital for several RTK signaling pathways, including EGFR. Converfsly, STAT1, critical for interferon signaling, is a regulator of apoptosis. (Seif et al., 2017)

Abnormal growth factor receptor activity are drivers of many types of cancers. In breast cancer overexpression of various types of RTKS including ErbB family members (EGFR/Her2/Her3), vascular endothelial growth factor receptors (VEGFRs), hepatocyte growth factor receptor(c-Met), platelet-derived growth factor receptors (PDGFRs), insulin-like growth factor receptors (IGFRs), and fibroblast growth factor receptors (FGFRs).(Butti et al., 2018; Du & Lovly, 2018)

Protein tyrosine kinase inhibitors (TKIs), also known as tyrphostins (tyrosine phosphorylation inhibitors) are small molecule inhibitors of abbarrent receptor tyrosine kinase activity. A general mechanism includes binding to the intracellular ATP binding domain and inhibiting downstrea, phosphorylation events.

Receptor tyrosine kinase inhibitors can be classified according to their targets; EGFR TKIs (e.g., gefitinib and erlotinib), VEGFR TKIs (e.g., sunitinib and sorafenib), and FGFR TKIs. Additionally, TKIs can be catagorized into generations; first-generation TKIs (e.g. gefitinib and erlotinib) are reversible/competitive ATP inhibitors and are mostly single-targeted, whereas the second-generation TKIs (e.g., afatinib, lapatinib, and neratinib) and other newer generations of TKIs (e.g., osimertinib) are mostly irreversible/covalent binding and are multitargeted.(Agrawal, Gutteridge, Gee, Nicholson, & Robertson, 2005; Sequist, 2007)

Specific targeting of cytoplasmic, non-receptor tyrosine kinases to interfere with tumor cell functions have become highlighted to enhance personalized medicine in mutiple cancer models. As mentioned in previous sections, dysreulation of signaling pathwasy such as MAPK-ERK/PI3K-AKT/JAK-STAT are main contributors to tumorgensis.(Figure. 3) These cellular signaling pathways have dominated recent studies of breast cancer biology, and inhibitors of these pathways have formed a focus of numerous clinical trial.

Mitogen-activated protein kinase kinase enzymes (MEK) inhibitors are used in targeting MAPK/ERK pathway. Trametinib (GSK1120212) is the first clinically approved MEK inhibitor. It is a selective TKI of MEK1/MEK2 and is an allosteric, non-ATP-competitive inhibitor.(Hojjat-Farsangi, 2016) In primary cell culture, trametinib induces cell apoptosis via caspase activity. In melanoma patients, treatment with trametinib resulted in a statistically significant and clinically meaningful improvement in progression-free survival compared to standard chemotherapy. Additionally trametinib resulted in an improved overall response rate and progression-free survival in melanoma patients compared with cytotoxic treatments. (Cheng & Tian, 2017; Menzies & Long, 2014)

Small molecule phosphatidylinositol-3-kinase (PI3K) inhibitors include; include PI3K/mTOR inhibitors, pan-PI3K inhibitors, and isoform-selective PI3K inhibitors. Alpelisib (BYL719) is a selective PI3Kα isoform inhibitor, exhibiting anti-proliferation of breast cancer cell lines harboring PIK3CA mutations and inhibition of various downstream signaling components of the PI3K/Akt pathway. Additionally, alpelisib displayed dose-dependent antitumor activity in tumor xenograft models. (Fritsch et al., 2014) In clinical trials, alpelisib treatment in combination with fulvestrant, an estrogen receptor antagonist, prolonged progression-free survival in patients with PIK3CA-mutated, HR-positive, HER2-negative advanced breast cancer who had previously

received endocrine therapy and was recently approved by the Food and Drug Administration, under the trade name PIQRAY, for postmenopausal women, and men, with hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative, PIK3CA-mutated, advanced or metastatic breast cancer.(Fabrice André et al., 2019)

Serine/threonine kinase Akt, also known as protein kinase B or PKB, inhibitors include competitive, allosteric and irreversible inhibitors. Potency against individual AKT isoforms and selectivity profiles delineate clinical success of different AKT inhibitors. (Kostaras et al., 2020) Uprosertib (GSK2141795) is a selective, ATP-competitive, Akt inhibitor and displayed increased potency in cell lines with an activated AKT pathway (via PI3K/PTEN mutation or loss).(Dumble et al., 2014) In cell models, Uprosertib, preferentially inhibits proliferation, induces cell cycle and growth arrest as well as enhances chemotherapy induced apoptosis. (Cheraghchi-Bashi et al., 2015) Furthermore, combination with the MEK inhibitor, trametinib, resulted in increased anti-tumor efficacy in a model of pancreatic cancer. (Dumble et al., 2014)

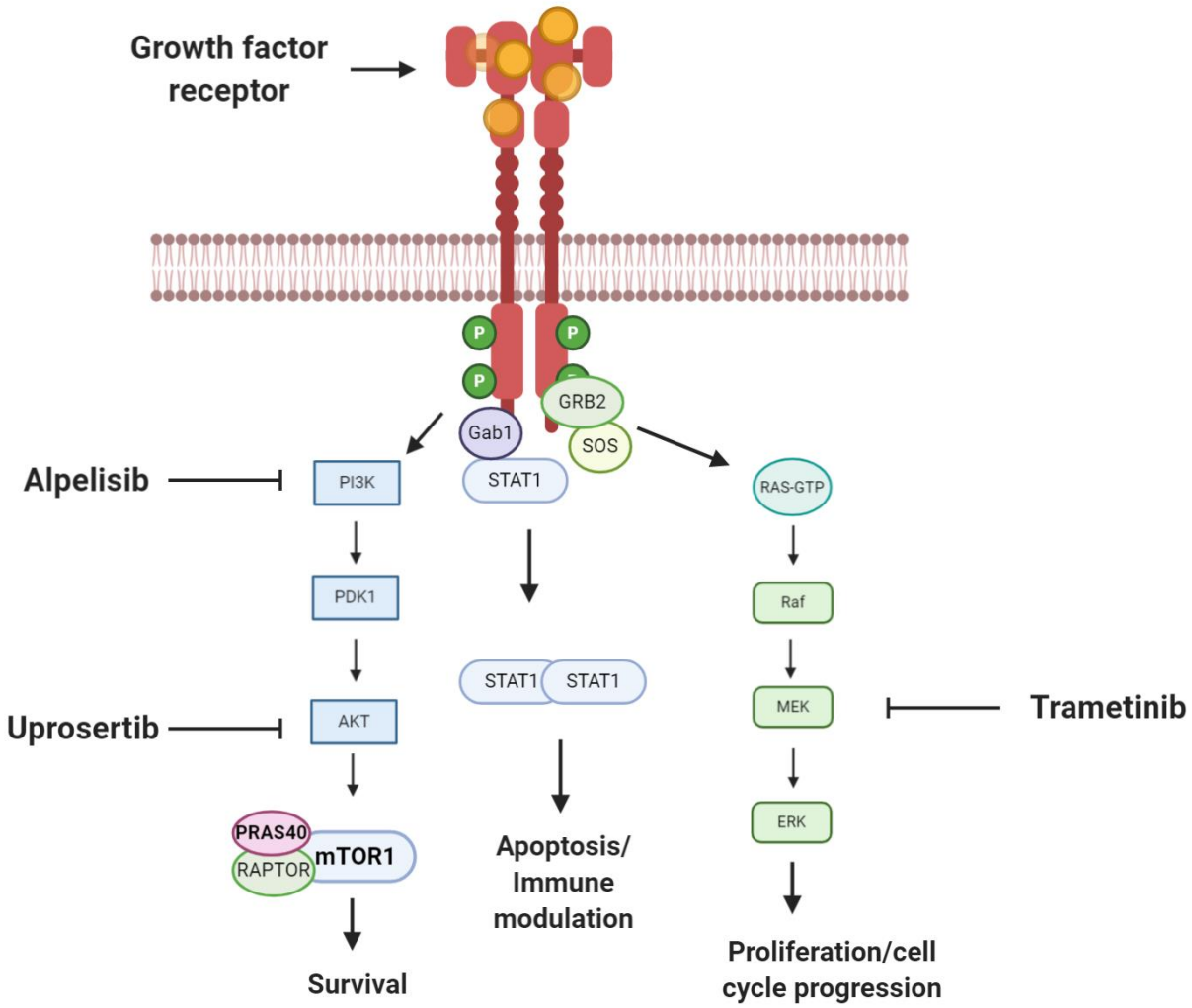


Figure 3. Schematic representation of prototypical growth factor receptor mediated signaling cascades (PI3K/AKT/MTOR, STAT, MAPK) leading to differential cell fate and examples of common clinically used inhibitors of cytoplasmic kinase activity.

1.4 Metastatic Triple Negative Breast cancer treatment

Traditional chemotherpauetic treatments continue to pose adverse affects in patients due to their highly cytoxic nature and non-specificity. Although patients with TNBC respond well to initial chemotherapy treatment, reoccurance of the disease becomes far more frequent than other breast cancers treated with chemotherapy. Furthermore, viable and effective targeted treatment for the metastatic disease remains to be fully elucidated. Thus, targeted therpies have become

extensivly explored and are becoming attractive therapuetic tatic in combating metastatic triple negactive breast cancer.

Despite these recent advances, targted therapy continues to lack efficacy and clinical resistance remains to be fully resolved. Recent approaches to advance efficacy and prevent resistance have been implemented using a combination of tyrosine kinase inhibitors or with chemotherapy agents. Combination of trametinib with several other small molecule kinase inhibitors have been studied in several trials (Table 1). Many of these trials combine inhibitors of phosphatidylinositol-3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) that does not induce apoptosis in vitro but induces an antiproliferative effect without any radiologic response in most treated patients. For example, combination of the trametinib with buparlisib (a PI3K inhibitor) was evaluated in a phase Ib dose-escalation clinical trial in NSCLC patients with RAS- or BRAF-mutants, ovarian and pancreatic cancers.(Bedard et al., 2015) Additionally, a phase I trial, with trametinib in combination with afuresertib (a AKT inhibitor) were evaluated in patients with solid tumors and multiple myeloma. (Tolcher et al., 2015) Trametinib has been combined with Uprosertib (an AKT inhibitor) in patients with solid tumors likely to be sensitive to MEK and/or AKT inhibition in a phase I dose escalating trial.(Tolcher et al., 2020) Additionally, in metastatic triple negative breast cancer patients, trametinib and Uprosertib (Akt Inhibitor) combination have been evaluated Although combination treatment displayed more effectiveness compared to that of monotherapy, unfortunally, all trials mentioned above lacked optimal saftey, tolerability and significant efficacy in their respective cancer models. These observations, prompt further evaluation of the pharmacology of these small molecule inhibitors various cancer models. Ongoing trials include trametinib combination with Alpelisib (a PI3Kα specific inhibitor) in patients with Progressive Refractory Meningiomas (NCT03631953). Exploration in this

combination prompt interest in breast cancer evaluations as PI3Kα mutations make up a large percentage of breast cancer patients. In 2019, the Food and Drug Administartion (FDA) approved Alpelisib (PIQRAY) use in patients with advanced-stage hormone-receptor-positive, HER2-negative breast cancer with PIK3CA mutation.(F. André et al., 2019)

Table 1. Clinical trials of AKT/PI3K inhibitors in combination with MEK inhibitors.

AKT/PI3K Inhibitors	MEK/ERK inhibitors	Diseases/Conditions	Clinical Trials.gov Identifier Number
Afuresertib (GSK2110183)	Trametinib (GSK1120212)	Multiple myeloma or solid tumors	NCT01476137
Uprosertib (GSK2141795)	Trametinib	Recurrent or persistent endometrial cancer	NCT01935973
Uprosertib	Trametinib	Acute Myeloid Leukemia	NCT01907815
Uprosertib	Trametinib	Melanoma	NCT01941927
Uprosertib	Trametinib	Metastatic Triple-Negative Breast Cancer	NCT01964924
MK-2206	Selumetinib (AZD6244)	Colorectal Neoplasms	NCT01333475
Ipatasertib (RG7440)	Cobimetinib	Locally advanced or metastatic cancer	NCT01562275
Ipatasertib	Cobimetinib	Breast cancer and ER+ breast cancer	NCT03395899
MK-2206	Selumetinib	Advanced colorectal carcinoma	NCT01333475
MK-2206	Selumetinib	Lung cancer and thymic malignancies	NCT01306045
Alpelisib	Trametinib	Meningioma	NCT03631953
BKM120	Trametinib	Advanced and Selected Solid Tumors	NCT01155453
Copanlisib	Refametinib (BAY86-9766)	Neoplasms	NCT01392521

CHAPTER 2. METHODS AND MATERIALS

2.1 Cell lines and reagents

Murine NMuMG, human MDA-MB-468, BT-20, and BT-549 cells were purchased from ATCC. NMuMG and isogenic derivatives, MDA-MB-468, MDA-MB-231, and BT-549s were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. BT-20 cells were cultured in Eagle's minimal essential medium (EMEM) containing 10% FBS and 1% penicillin/streptomycin. NMuMG cells and their metastatic isogenic derivatives also received 10 μg/ml of insulin in the culture media. Cells were maintained at 37C and 5% CO2. Inhibitors, growth factors and cytokines were added to cells as indicated in figures.

Table 2. List of compounds, growth factors and cytokines

Name	Target	Concentration	Supplier
Trametinib (GSK1120212, trade name Mekinist)	MEK1/2	As indicated	Selleckchem
Uprosertib (GSK2141795)	Pan-AKT	As indicated	Selleckchem
Alpelisib (BYL719, brand name Piqray)	PI3Kα	As indicated	Selleckchem
EGF	-	As indicated	Goldbio
INFγ	-	As indicated	

2.2 Protein extraction, SDS-PAGE & Western Blot

Cells were lysed with RIPA lysis buffer supplemented with NA_3 VO_4 and a protease inhibitor cocktail. Protein determination was measured using the Pierce BCA protein assay kit (Thermo Scientific) following manufactures protocol. Whole-cell lysates were resolved by SDS-

PAGE and transferred to PVDF membranes using standard methods. Primary antibodies were incubated at 4 °C overnight: mouse anti-rabbit p-STAT1(Tyr701), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Phospho-Akt (Ser473), Phospho-PRAS40 (Thr246) from Cell Signaling ; all at 1:1000 dilution. Blots were developed using anti-rabbit or mouse IgG, horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescent substrate reagent. Protein bands were visualized with the Chemidoc MP Imaging system (Bio-Rad).

Table 3. List of antibodies, dilutions, and suppliers.

Antibody	Dilution	Supplier
p-STAT1 (Y701)	1:1000	Cell Signaling Technologies
p-STAT1 (S727)	1:1000	Cell Signaling Technologies
t-STAT1	1:1000	Cell Signaling Technologies
p-STAT3 (Y701)	1:1000	Cell Signaling Technologies
t-STAT3	1:1000	Cell Signaling Technologies
p-ERK ½ (Thr202/Tyr204)	1:1000	Cell Signaling Technologies
t-ERK	1:1000	Cell Signaling Technologies
p-AKT (Ser473)	1:1000	Cell Signaling Technologies
t-AKT	1:1000	Cell Signaling Technologies
p-PRAS40 (Thr246)	1:1000	Cell Signaling Technologies
Tubulin	1:1000	Developmental Studies Hybridoma Bank

2.3 Cell viability and Apoptosis

Generation of dose response curves involved plating the cells over night for attachment, followed by drug treatment, diluted in DMEM supplemented with 10% FBS, for indicated concentration and time points. In brief, cells treated with TKIs and EGF combination involved,

EGF stimulation and/or kinase inhibition followed by drug/media aspiration and wash with PBS before addition of 50 uL of CellTiter-Glo assay reagent to each well and incubation at 37C for 30 mins. Cell viability was measured using the CellTiter-Glo assay (Promega) according to the manufacturer's instructions. Caspase 3/7 activity was quantified using the Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions. In brief, following EGF stimulation and/or kinase inhibition, 30uL of Caspase-Glo 3/7 reagent was added directly to each well and incubated at room temperature for 30 mins. For both caspase activity and cell viability, luminescence was measured using the Promega GloMax plate reader.

2.4 Statistical Analysis

Data in figures are expressed as mean ± standard deviation and were analyzed using GraphPad Prism version 8.0 (La Jolla, CA). GraphPad Prism was used to fit the sigmoidal concentration-response curves via one-way analysis of variance (ANOVA) followed by Tukey Kramer posthoc. Significance of caspase activity and cell viability was analyzed using two-way analysis of variance (ANOVA) followed by Tukey Kramer posthoc. For all statistical analysis, means were indicated to be statistically different when $p < 0.05$.

CHAPTER 3. BIASING SIGNALING PATHWAYS IN METASTATIC BREAST CANCER

3.1 Introduction

3.1.1 Growth factor and cytokine mediated cell death

It has long been documented that growth factors are multifaceted and depending on the presence of other signaling molecules present can be stimulatory or inhibitory in cell growth and proliferation.(Sporn & Roberts, 1988) Biphasic growth factor induced signal-response profiles have been shown in multiple cancer cell models. For instance, transforming growth factor- β (TGF-β) function switches from being growth suppressive in primary tumors to growth promoting in metastatic ones.(Roberts & Wakefield, 2003; Tian & Schiemann, 2009) Similarly, interleukin 6 (IL-6), platelet-derived growth factor (PDGF), and hormones such as estrogen and androgen have all been shown to inhibit cancer growth under particular condition, both *in vitro* and in animal models.(Hedayati et al., 2016; Kim, Upadhyay, Li, Palmer, & Deuel, 1995; Minami et al., 1996) Furthermore, high level estrogen therapy has even been explored clinically in metastatic breast cancer patients.(Mahtani, Stein, & Vogel, 2009)

3.1.2 Epidermal growth factor receptor (EGFR) and the EGFR paradox

The epidermal growth factor receptor (EGFR) is a well establish protooncogene in cancer development and progression. Overexpression in breast cancer has been associated with decreased prognosis in the basal subtype of breast cancer and is overexpressed in 70-80% of basal-like TNBCs (Lehmann & Pietenpol, 2014; Masuda et al., 2012; Sainsbury, Farndon, Needham, Malcolm, & Harris, 1987). EGFR downstream pathways including PI3K/AKT/mTOR, MAPK/ERK and JAK/STAT pathways, playing substantial roles in proliferation, migration, and

cancer cell survival (Huang et al., 2019; F. Wang et al., 1998). Overexpression of EGFR is more common in highly metastatic triple negative breast cancer compared to other breast cancer subtypes (Corkery, Crown, Clynes, & O'Donovan, 2009; Viale et al., 2009). EGFR targeted therapies include small molecule tyrosine kinase inhibitors and monoclonol antibodies (Liu et al., 2019; Maennling et al., 2019). Despite these advances, EGFR targeted therapies have not significantly improved breast cancer patient outcomes (Baselga et al., 2013; Carey et al., 2012; Trédan et al., 2015). As one potential explaination for this, studies from our lab and others have shown that EGF induces apoptosis and inhibits cell growth in metastatic breast cancer (R. Ali, Brown, Purdy, Davisson, & Wendt, 2018; Remah Ali & Wendt, 2017; Armstrong et al., 1994; Högnason et al., 2001). Indeed, cells derived from pulmonary metastases are inherently resistant to EGFR inhibition and undergo robust growth inhibition in response to EGF (Wendt et al., 2015). Previous studies in the Wendt lab have demonstarted upstream growth factor receptor activation followed by hijacking subsequent signaling cascades, using a model of EGFR biased model and showing EGF-induced apoptosis dependent on signal transducer and activator of transcription 1 (STAT1)(R. Ali et al., 2018). Therefore, as opposed to overt blockade of EGFR signaling, pharmacological rewiring of downstream networks may promote apoptotic outcomes and have a more promising therapuetic result in late stage breast cancer.

3.1.3 Signal transducer and activator of transcription 1 (STAT1)

Signal transducer and activator of transcription 1 (STAT1) is a mediator of several biological responses including tumor cell surveillance, immune response and control of cell growth by stimulation of growth factors and cytokines. Activiation of STAT1 has been demonstarted to enhance antiproliferative activity mediated by caspase induced apoptosis.(Shuai, Liao, & Song, 1996; Sironi & Ouchi, 2004; Stephanou & Latchman, 2003) Furthermore, interferon (INF)

mediated STAT1 activation promotes genes involved in antiviral response, immune modulation, and cytotoxicity. (Khodarev, Roizman, & Weichselbaum, 2012) Therapeutic rewiring of STAT1 activation may serve in biasing growth factor mediated apoptosis. Prevous studies have elucidated the importance of STAT1 in mediation of EGF induced apoptotic signatures.(R. Ali et al., 2018)

3.1.4 Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)

Over 30% of all breast cancer patients harbor mutations in the PIK3CA gene, which encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K), a family of lipid kinases (Koboldt et al., 2012; Madsen, Vanhaesebroeck, & Semple, 2018). Thus, PI3K and its association with mammalian target of rapamycin (mTOR) signaling pathway represents one of the most commonly dysregulated signaling pathway in breast cancer. Activation of this pathway has been linked to proliferation, metastasis and resistance to endocrine and chemotherapy (Bhattacharvva et al., 2011; Mosele et al., 2020; Pang et al., 2014; Paplomata & O'Regan, 2014). Furthermore, aberrations of tumor suppressor and inhibitor of PI3K/AKT/mTOR signaling, phosphatase and tensin homolog (PTEN), results in marked decreased patient prognosis (Depowski, Rosenthal, & Ross, 2001; Saal et al., 2005). These and other data have led to the development of several inhibitors of PI3K and PI3K mediated downstream enzymes. Uprosertib is a potent oral inhibitor of the Akt1, Akt2, and Akt3 active sites, and thus inhibits Akt activity and downstream proliferative effects. Recent approval of a PI3Kα specific inhibitor for patients with PIK3CA-Mutated,(Fabrice André et al., 2019) Hormone Receptor–Positive Advanced Breast Cancer. Unfortunately, current data in advanced breast cancer demonstrate that monotherapy inhibition of PI3K lacks significant efficacy (Verret, Cortes, Bachelot, Andre, & Arnedos, 2019). These findings have driven recent clinical trials aimed at establishing an approach using Uprosertib

(NCT01964924) or Alpelisib (NCT03631953) in combination with inhibition of the MAPK pathway with Trametinib.

3.1.5 Hypothesis and directed aims

We hypothesize receptor mediated apoptotic signatures are increased in breast cancer cells derived from metastatic origins. The focus of this thesis will explore the pharmacology of cytoplasmic tyrosine kinase inhibitor on hijacking upstream receptor activated signaling in metastatic breast cancer cells and evaluation of effects of TKIs on receptor mediated (1) signaling protein activation (2) apoptosis activity and (3) cell viability in response to EGF or INFγ stimulation.

3.2 Results

To address the clinical impact of PIK3CA mutations on breast cancer, we performed a search on the cBioPortal for Cancer Genomics database. Specific query in to the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) study (Pereira et al., 2016) comprised of 2509 primary breast tumors revealed a 38.9% frequency of PIK3CA mutations, the highest of any other gene mutation profiled. Breast cancer samples display high incidence of PIK3CA mutations and PI3K activating events with H1047R/Y on exon 20 near the end of the catalytic domain, which increases interaction of p110α with lipid membranes, being the most frequently mutated site. (Fig. 4A)

PAM50 are intrinsic molecular subtypes defined by mRNA expression of 50 genes and are prognostic in hormone-receptor positive postmenopausal breast cancer.(Pu et al., 2020) Claudin-low tumors further reflect characteristics of intrinsic subtype and are distinguished by low genomic instability, mutational burden and proliferation levels, and high levels of immune and stromal cell

infiltration.(Fougner, Bergholtz, Norum, & Sørlie, 2020) In order to better observe the clinical attributes of PIK3CA mutants in the different breast cancer subtypes, we plotted Pam50 + Claudin-low subtype number samples against PIK3CA mutations vs. wildtype, further divided into breast cancer subtype. The luminal A subtype was revealed to have the highest frequency of PIK3CA missense mutations compared to wildtype. The basal type breast cancer is commonly associated with high EGFR levels and poor prognosis. Within these 203 patient samples of basal subtype, 14.77% were revealed to have PIK3CA missense mutations. These results validate targeting of EGFR mediated Akt signaling is relevant in both PIK3CA mutants and PIK3CA wildtype breast cancer. (Fig. 4C)

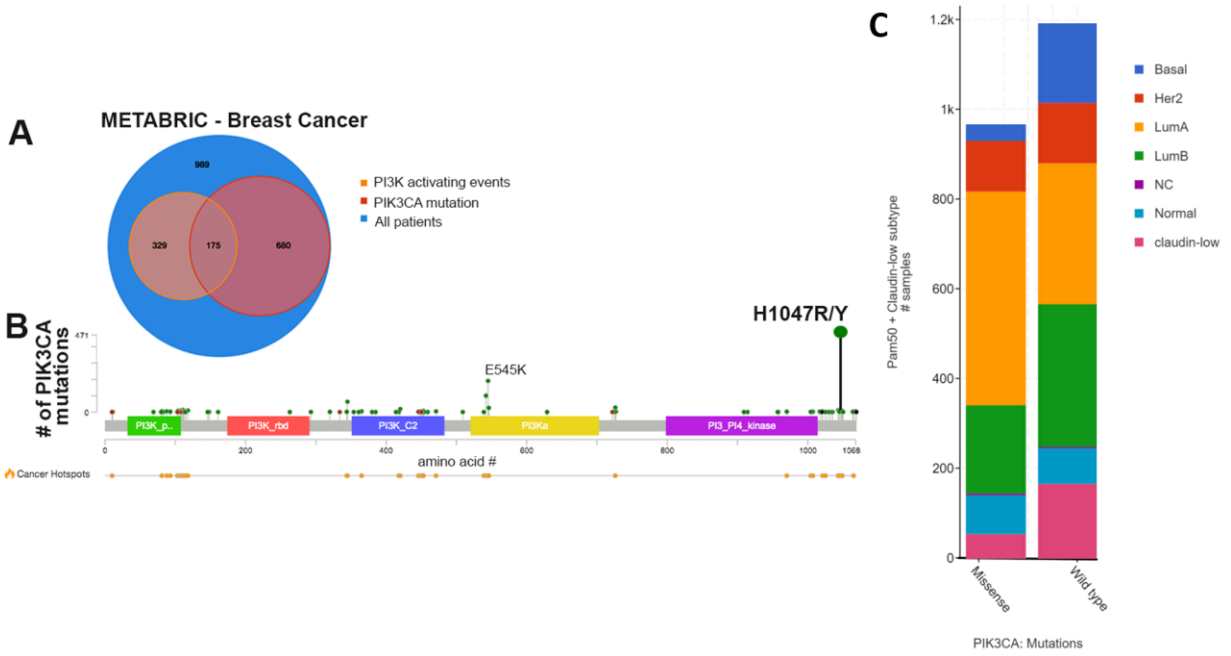


Figure 4. Clinical attributes of PIK3CA missense mutations vs. wildtype in breast cancer patients separated by breast cancer subtype. Data generated using cBioPortal and query of METABRIC data set. (Pereira et al., 2016)

Previous Studies from the Wendt lab have demonstrated EGFR overexpression can transform normal murine mammary cells (Balanis et al., 2013; Balanis, Yoshigi, Wendt,

Schiemann, & Carlin, 2011; Wendt et al., 2010, 2015). Furthermore, cells isogenic cells of the metastatic origin were inherently resistant to EGFR inhibition compared to non-metastatic leading to the investigation of alternative mechanisms to combat the metastatic disease. We demonstrated that treatment with the MEK1/2 inhibitor, Trametinib, leads to biased EGFR signaling as Erk1/2 phosphorylation is blocked but activation of STAT1 is unaffected upon EGF ligand stimulation.(R. Ali et al., 2018) Furthermore, these studies revealed that STAT1 is a primary mediator in biasing EGFR induced apoptosis in a model of EGFR transformed metastatic murine breast cancer cells. In the current study we sought to further bias EGFR signaling toward an apoptotic event by implementing dual kinase inhibition of Akt signaling via addition of Uprosertib, a selective, ATP-competitive, and orally bioavailable Akt inhibitor. Uprosertib inhibits multiple AKT substrate phosphorylation levels, including GSK3β, PRAS40, FOXO and Caspase 9 resulting in inhibited cell growth in human breast cancer cells and prostate adenocarcinoma cells. Additionally, it has been documented that Uprosertib increases cisplatin induced apoptosis in vitro and decreased tumor volume in vivo in a model of ovarian cancer with a dysregulated AKT pathway. (Cheraghchi-Bashi et al., 2015; Dumble et al., 2014) Several clinical studies have implemented dual kinase inhibition in multiple cancer models. (Table. 1) This concept has been highly proposed to overcome clinically observed drug resistance. In our current model we propose that dual kinase inhibition will consolidate EGF: Trametinib mediated biased signaling in breast cancer cell models with abberant AKT signaling.

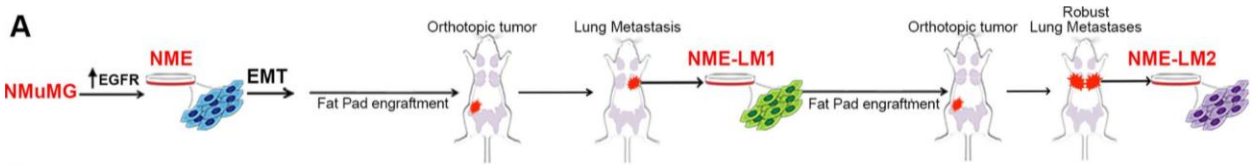


Figure 5. Schematic representation of isogenic murine EGFR-transformed lung-metastatic breast cancer progression series. Ali R. et al 2018

Table 4 List of human breast cancer cell lines with corresponding PIK3CA/PTEN mutation status and EGFR overexpression. Adapted from Meric-Bernstam F., et al. Clinical Cancer Research., 2012

Cell Line	PIK3CA Mutation status	PTEN status	EGFR over expression
BT-20	C1616G:P539R; A3140G:H1047R	wt	+
BT-549	wt	codon 274, GTA AAT to TAA AT, stop; 822delG:L295X, protein blot null; 823delG:V275fs*1	-
MDA-MB-468	wt	codon 70, 44 bp deletion, frameshift; Hom. IVS4+1G>T (A72fsX5), protein blot null	+
MDA-MB-231	wt	wt	-
MCF-7	G1624A:E542K; G1633A:E545K	wt	-

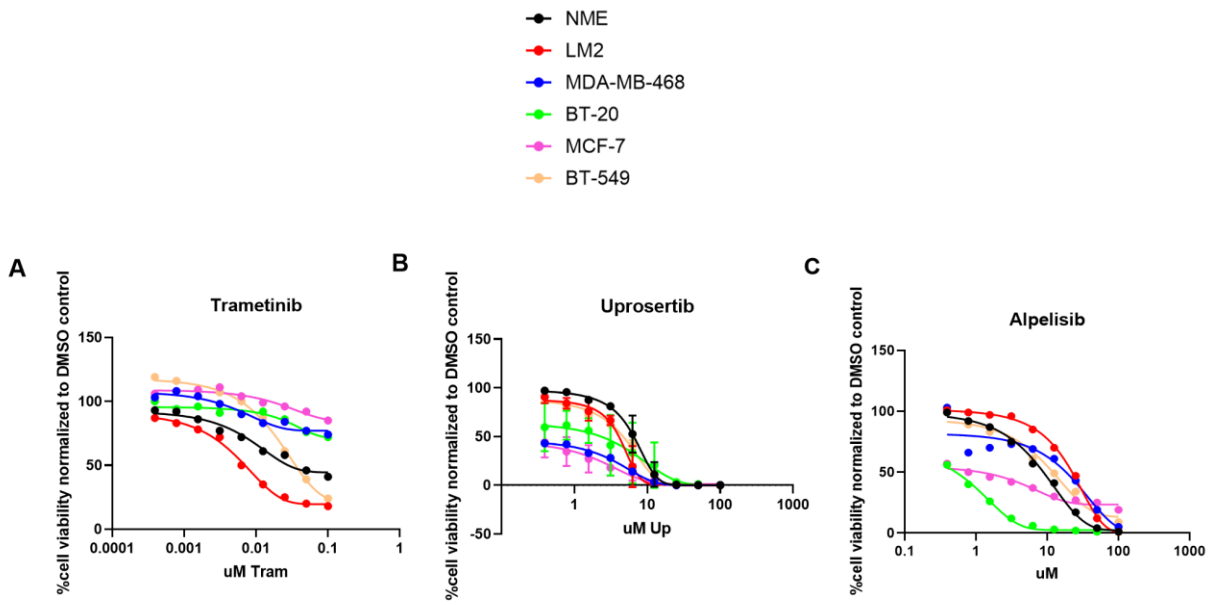


Figure 6. Dose Response in EGFR transformed murine NME and metastatic lung derived (LM2) and human breast cancer cell lines with wither PIK3CA mutations (BT-20 & MCF-7) or PTEN null (MDA-MB-468 & BT-549) to A. the MEK inhibitor Trametinib, B. AKT inhibitor, Uprosertib, or C. the PI3Kα specific inhibitor, Alpelisib. Cells we plated in a 96 well plate at 2,000 cells per well and treated with inhibitors at indicated concentrations in full growth media for 72 hours. Cell viability was determined using the Cell titer Glo assay from Promega suing manufacturer's instructions.

To observe the differential response to the MEK inhibitor, Trametinib, the direct Akt inhibitor, Uprosertib, and PIK3CA specific inhibitor, Alpelisib, in cells of increasing metastatic potential and EGFR overexpression, we utilized our previously developed model of EGFR-driven transformation of mammary epithelial cells.(R. Ali et al., 2018) (Figure. 5) Furthermore, to observe the effects of PI3K/AKT activating mutations, we focused on the triple negative breast cancer cell lines of metastatic origins, MDA-MB-468 which has a PTEN homozygous deletion as well as BT-20 cells which have homozygous PIK3CA mutation, both of which harbor amplified EGFR.(Chavez, Garimella, & Lipkowitz, 2010) (Table4) Dose response to Trametinib showed increased sensitivity in isogenic EGFR-driven transformed mammary epithelial cells (NME and

NME-LM2), whereas MDA-MB-468 and BT-20s showed minimal response to the MEK inhibitor. (Fig.6A) Conversely, when treated with the Akt inhibitor, Uprosertib (Fig. 6B) and Alpelisib (Fig. C), all cell lines showed dose dependent decrease in cell viability, wherein the two PIK3CA mutant cell lines; BT-20 and MCF-7 displayed increased sensitivity compared to the NME isogenic lines and PTEN null lines. These results validate the specificity of the inhibitors to their intended mutant gene targets.

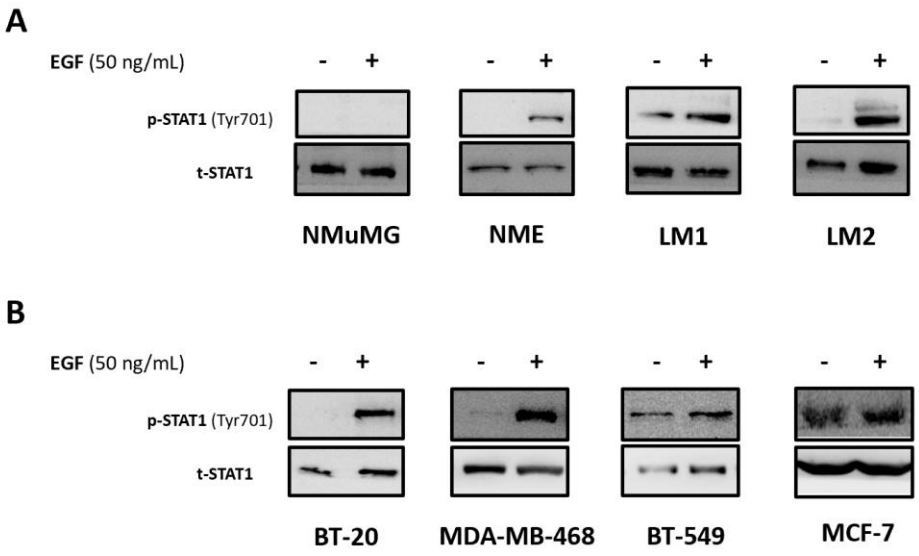


Figure 7. EGFR mediated STAT1 activation in isogenic murine breast cancer cells and human breast cancer cells of differing metastatic capacities. A. Isogenic murine breast cancer cells; NMuMG (non-tumorigenic), NME (EGFR transformed, non-metastatic), LM1(metastatic), LM2 (metastatic) and B. Human breast cancer cell lines BT-20 & MCF-&(PIK3CA mutants) and MDA-MB-468 & BT-549 (PTEN null) treated with 50ng/mL EGF in full growth DMEM for 30 minutes before protein collection and analysis via western blotting for STAT1 at the Tyr701 phosphorylation site and total STAT1 as loading control.

To evaluate the response of EGF in the different breast cancer cell lines, we treated cells with EGF and assessed levels of phosphorylated STAT1. Western blotting revealed metastatic cells responded to EGF with increase pSTAT1 compared to non-metastatic NMEs and non-

tumorigenic, NMuMG relative to non-treated (Figure. 7A) despite having lower EGFR ecpression. These results are consistent with our previous findings that EGFR functions as an apoptotic driver in metastatic cells.(R. Ali et al., 2018; Remah Ali & Wendt, 2017) Furthermore, evaluation of EGF response in human breast cancer cell lines revealed differential phosphorylation of STAT1 by EGF is dependent on EGFR overexpression status. To observe the role of EGFR in cells with aberrant PI3K/AKT/mTOR signaling, we compared human breast cancer cells MCF-7, BT-549, MDA-MB-468 and BT-20. The latter two of which exhibit EGFR overexpression. MCF-7 and BT-20 cells harbor PIK3CA mutations while MDA-MB-468 and BT549 cells harbor PTEN mutations. Here we observed that EGF stimulation has no effect on the levels of pSTAT1 expression in MCF-7s and BT-549s which do not have overexpression of EGFR. (Fig 7B) These findings demonstrate the differences in murine versus human cell line sensitivity to ligands, invoking careful evaluation to response seen in non-human models.

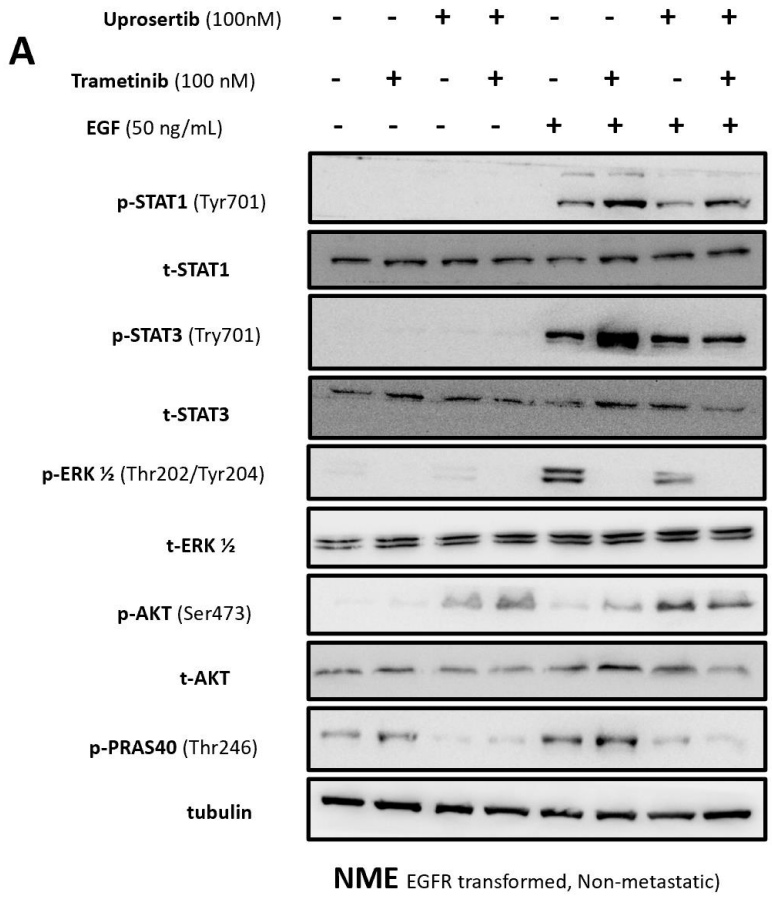
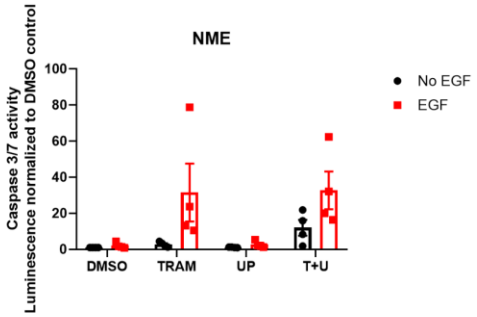


Figure 8. Response of EGFR transformed NME or metastatic LM2 to EGF and cytoplasmic kinase inhibitors. A/D. Signaling profile. Cells were serum starved for 6 hours following a pretreatment of kinase inhibitors for 2 hours before stimulation with EGF for 30 mins. Subsequent evaluation of signaling proteins through phosphorylation of STAT1, STAT3, ERK1/2, AKT, and PRAS40 with respective total protein or tubulin expression for control. B/E. Caspase 3/7 activity was measured using the Caspase Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 10,000 cells per well. The following day, cells were treated with indicated treatments for 24 hours before luminescence read. C/F. Cell viability was measured using the Cell Titer Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 2,000 cells per well. The following day, cells were treated with indicated treatments for 48 hours in full growth media before luminescence read.

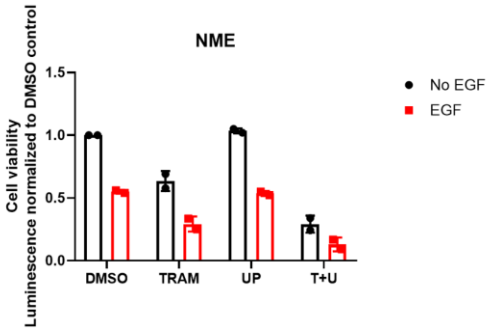
Figure 8 continued

B



Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
No EGF			
DMSO vs. TRAM	No	ns	0.9979
DMSO vs. UP	No	ns	>0.9999
DMSO vs. T+U	No	ns	0.6803
TRAM vs. UP	No	ns	0.9984
TRAM vs. T+U	No	ns	0.7821
UP vs. T+U	No	ns	0.6899
EGF			
DMSO vs. TRAM	Yes	*	0.0294
DMSO vs. UP	No	ns	>0.9999
DMSO vs. T+U	Yes	*	0.0224
TRAM vs. UP	Yes	*	0.0337
TRAM vs. T+U	No	ns	0.9994
UP vs. T+U	Yes	*	0.0258

C



Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
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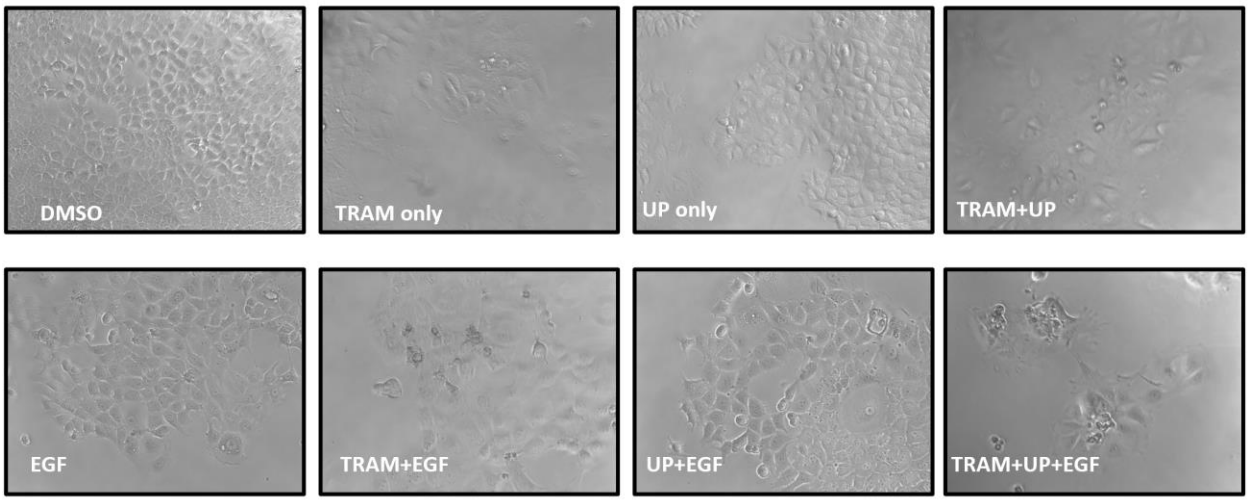
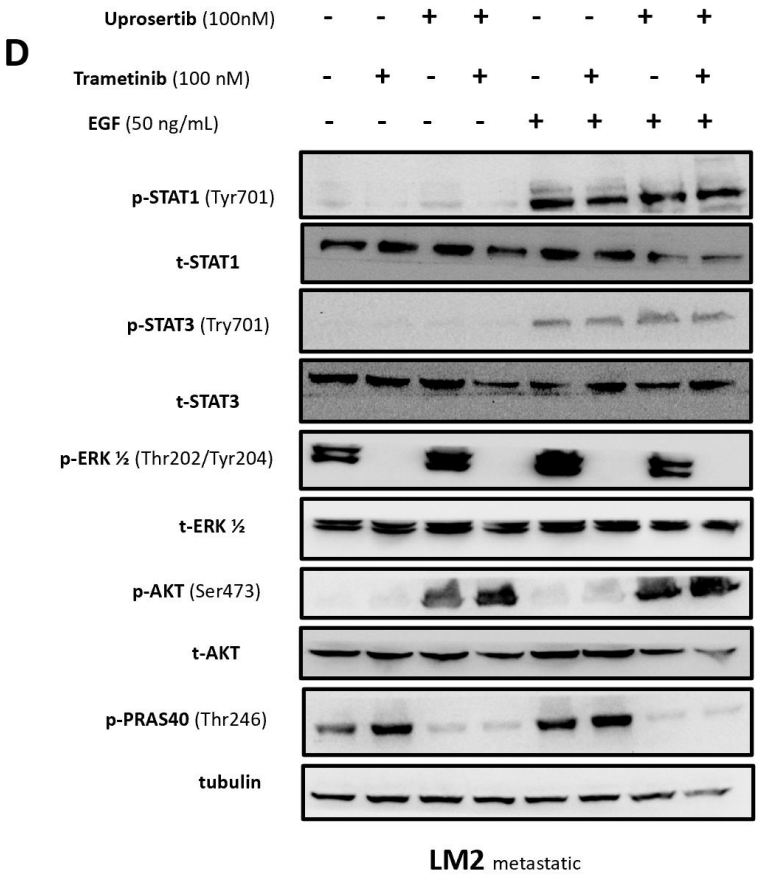
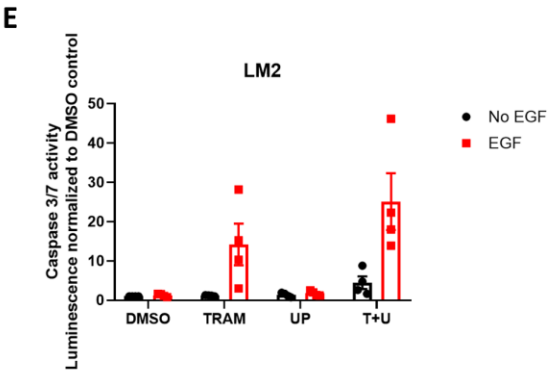
DMSO vs. TRAM	Yes	***	0.0003
DMSO vs. UP	No	ns	0.8837
DMSO vs. T+U	Yes	****	<0.0001
TRAM vs. UP	Yes	***	0.0002
TRAM vs. T+U	Yes	***	0.0005
UP vs. T+U	Yes	****	<0.0001
EGF			
DMSO vs. TRAM	Yes	**	0.0030
DMSO vs. UP	No	ns	0.9908
DMSO vs. T+U	Yes	***	0.0001
TRAM vs. UP	Yes	**	0.0043
TRAM vs. T+U	Yes	*	0.0391
UP vs. T+U	Yes	***	0.0001



NME

Figure 8 continued





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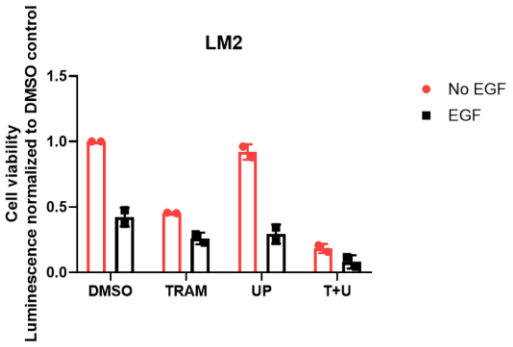
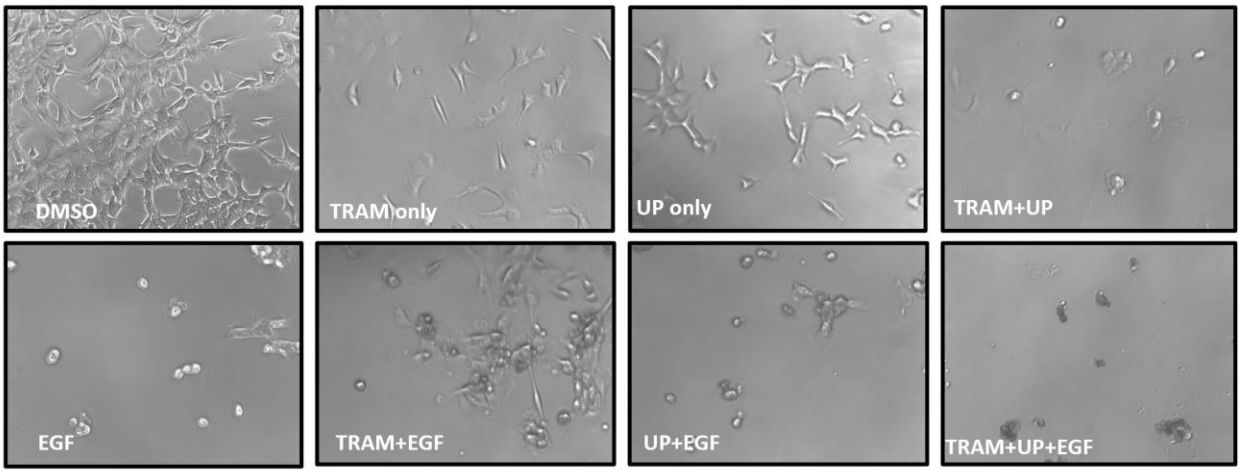
No EGF			
DMSO vs. TRAM	No	ns	>0.9999
DMSO vs. UP	No	ns	0.9998
DMSO vs. T+U	No	ns	0.8715
TRAM vs. UP	No	ns	>0.9999
TRAM vs. T+U	No	ns	0.8904
UP vs. T+U	No	ns	0.9059
EGF			
DMSO vs. TRAM	Yes	*	0.0424
DMSO vs. UP	No	ns	0.9995
DMSO vs. T+U	Yes	***	0.0001
TRAM vs. UP	No	ns	0.0534
TRAM vs. T+U	No	ns	0.1058
UP vs. T+U	Yes	***	0.0002

Figure 8 continued

F



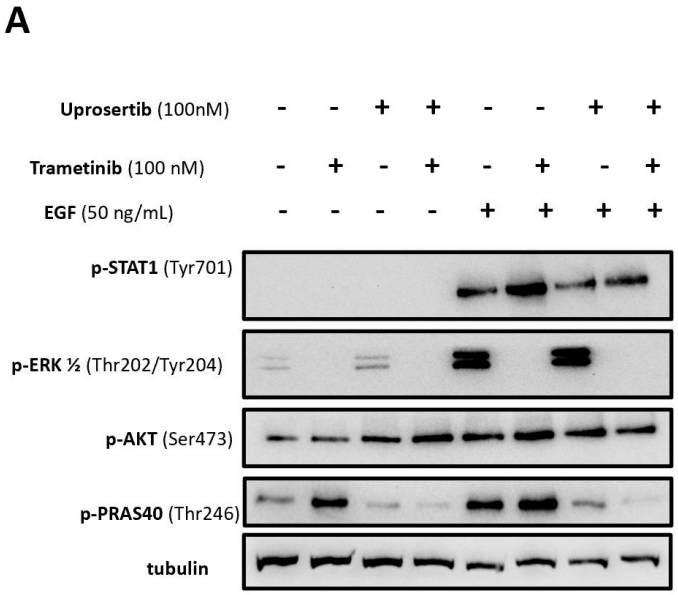
Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
No EGF			
DMSO vs. TRAM	Yes	****	<0.0001
DMSO vs. UP	No	ns	0.4313
DMSO vs. T+U	Yes	****	<0.0001
TRAM vs. UP	Yes	****	<0.0001
TRAM vs. T+U	Yes	**	0.0027
UP vs. T+U	Yes	****	<0.0001
EGF			
DMSO vs. TRAM	Yes	*	0.0439
DMSO vs. UP	No	ns	0.1118
DMSO vs. T+U	Yes	***	0.0006
TRAM vs. UP	No	ns	0.9080
TRAM vs. T+U	Yes	*	0.0285
UP vs. T+U	Yes	*	0.0117

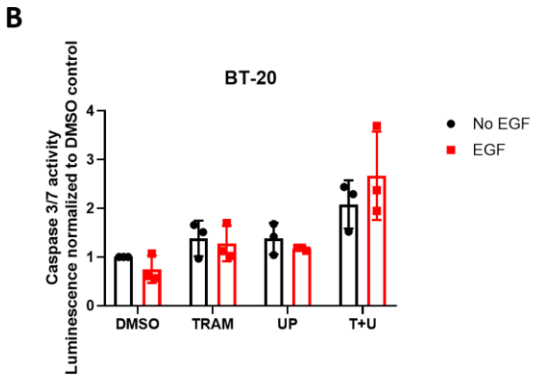


LM2

To evaluate the effects of dual kinase inhibition on EGF mediated STAT1 activation and apoptotic activity in non-metastatic vs. metastatic cells we compared isogenic murine mammary epithelial cells transformed with EGFR, NME, to the metastatic LM2. Previous reports indicate that LM2 cells of metastatic origin loose EGFR expression compared to the EGFR transformed NMEs. However, LM2s response to EGF ligand stimulation is not attenuated.(R. Ali et al., 2018) Consistent with our previous findings, we observed increased phospho-STAT1 expression with EGF stimulation in NME-LM2 cells harboring increased metastatic potential via western blot. Treatment with Trametinib and/or Uprosertib, did not affect EGF induced p-STAT1 expression, while still inhibiting p-ERK and p-PRAS40 (Figure. 8 A/D). PRAS40 is a direct substrate of Akt and acts as an inhibitor subunit of the mTORc1 complex. Phosphorylated PRAS40 by AKT results in its degradation and liberation from PRAS40 allows for mTORc1 substrate recruitment and activation. ("Determination of mTORC1 Complex Structures Reveals Regulatory Mechanisms," 2018; L. Wang, Harris, Roth, & Lawrence, 2007). To evaluate apoptosis, we measured caspase 3/7 activity using the Caspase-Glo 3/7 assay. Here we observed increased caspase 3/7 activity following EGF stimulation which was further propagated with addition of Trametinib and/or Uprosertib. (Figure. 8 B/E) As previously demonstrated, Trametinib induced caspase 3/7 activity require EGFR stimulation with EGF to observed increased caspase 3/7 response. Increased caspase activity is translated to decreased cell viability with the combination of EGF stimulation and Trametinib and/or Uprosertib treatment. (Figure. 8 C/F) In non-tumorigenic cells, dual kinase inhibition also increased caspase activity but the effects were not propagated by EGF stimulation. (Appendix. 1) Together, these findings suggest that dual kinase inhibition not only diminishes expression of signaling proteins involved in growth and survival pathways but with joint EGFR activation, can increase apoptotic signatures and decrease cell survival. Furthermore, as NMEs are

sensitive to EGFR inhibition, special emphasis of significance can be suggested in the metastatic LM2 cells and their response to EGF stimulation and dual kinase inhibition to overcome resistance to EGFR inhibitors.



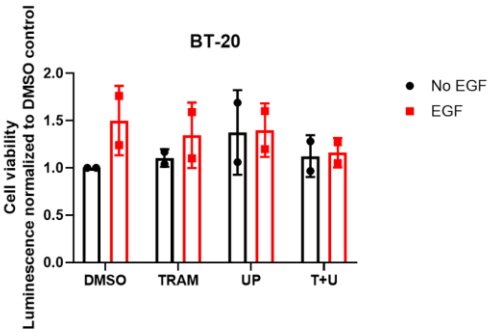


Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
No EGF			
DMSO vs. TRAM	No	ns	0.7144
DMSO vs. UP	No	ns	0.7116
DMSO vs. T+U	Yes	*	0.0351
TRAM vs. UP	No	ns	>0.9999
TRAM vs. T+U	No	ns	0.2370
UP vs. T+U	No	ns	0.2388
EGF			
DMSO vs. TRAM	No	ns	0.4631
DMSO vs. UP	No	ns	0.6465
DMSO vs. T+U	Yes	***	0.0003
TRAM vs. UP	No	ns	0.9889
TRAM vs. T+U	Yes	**	0.0062
UP vs. T+U	Yes	**	0.0033

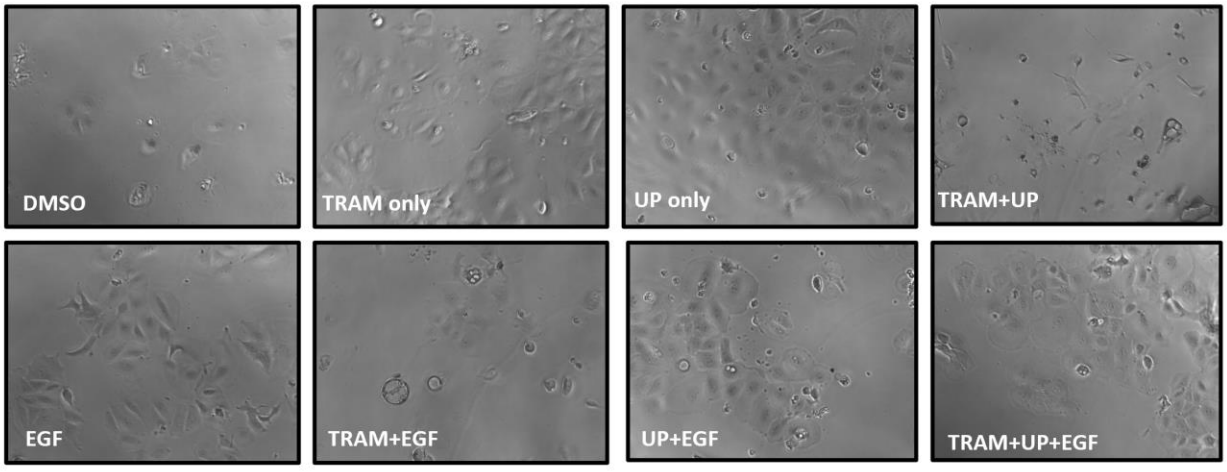
Figure 9. Response of PIK3CA mutant, BT-20 and PTEN null MDA-MB-468 human triple negative breast cancer cells to EGF and cytoplasmic kinase inhibitors. A. Signaling profile. Cells were serum starved for 6 hours following a pretreatment of kinase inhibitors for 2 hours before stimulation with EGF for 30 mins. Subsequent evaluation of signaling proteins through phosphorylation of STAT1, ERK1/2, AKT, and PRAS40 with tubulin expression for control. B. Caspase 3/7 activity was measured using the Caspase Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 10,000 cells per well. The following day, cells were treated with indicated treatments for 24 hours before luminescence read. C. Cell viability was measured using the Cell Titer Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 2,000 cells per well. The following day, cells were treated with indicated treatments for 72 hours in full growth media before luminescence read.

Figure 9 continued

C



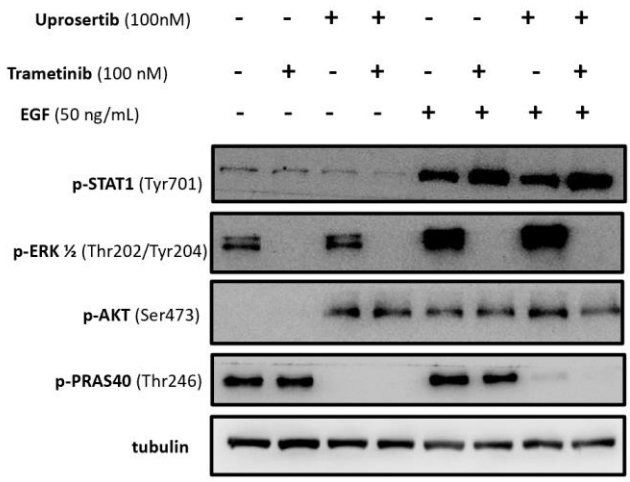
Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
No EGF			
DMSO vs. TRAM	No	ns	0.9802
DMSO vs. UP	No	ns	0.5588
DMSO vs. T+U	No	ns	0.9683
TRAM vs. UP	No	ns	0.7677
TRAM vs. T+U	No	ns	0.9999
UP vs. T+U	No	ns	0.8028
EGF			
DMSO vs. TRAM	No	ns	0.9414
DMSO vs. UP	No	ns	0.9828
DMSO vs. T+U	No	ns	0.6289
TRAM vs. UP	No	ns	0.9970
TRAM vs. T+U	No	ns	0.9065
UP vs. T+U	No	ns	0.8222



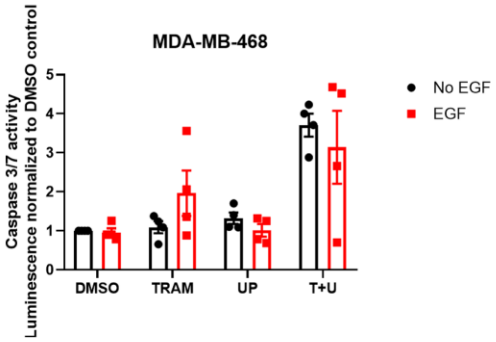
BT-20

Figure 9 continued

D



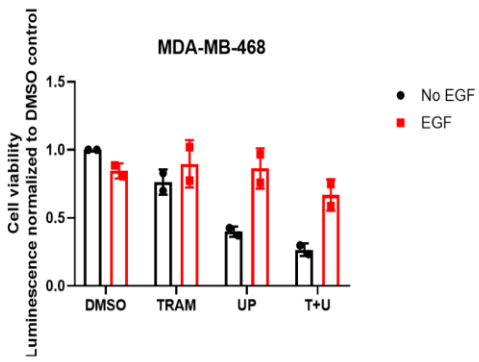
E



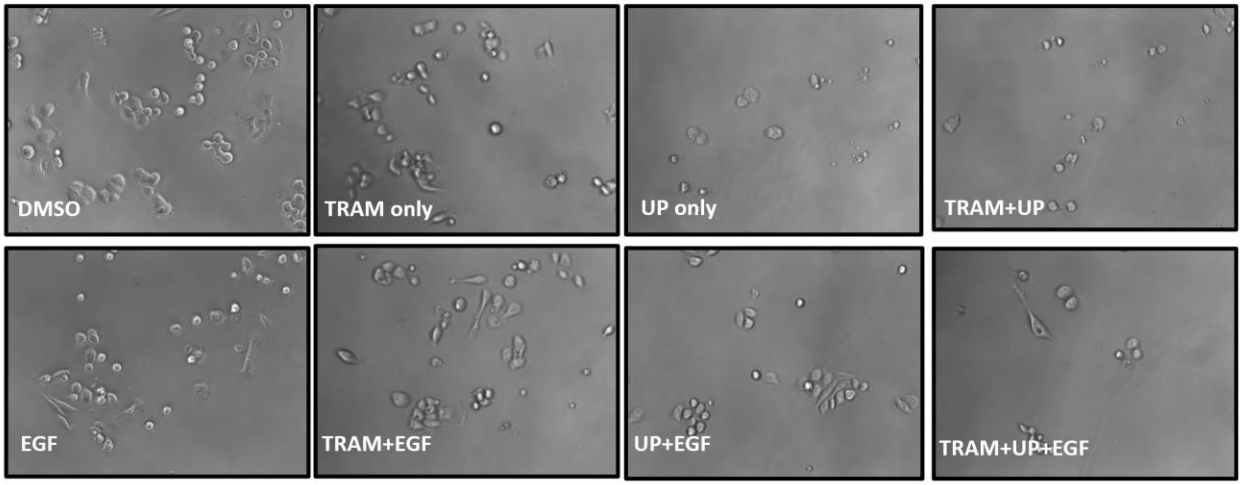
Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
No EGF			
DMSO vs. TRAM	No	ns	0.9986
DMSO vs. UP	No	ns	0.9457
DMSO vs. T+U	Yes	***	0.0006
TRAM vs. UP	No	ns	0.9788
TRAM vs. T+U	Yes	***	0.0009
UP vs. T+U	Yes	**	0.0024
EGF			
DMSO vs. TRAM	No	ns	0.3412
DMSO vs. UP	No	ns	0.9998
DMSO vs. T+U	Yes	**	0.0056
TRAM vs. UP	No	ns	0.3833
TRAM vs. T+U	No	ns	0.2160
UP vs. T+U	Yes	**	0.0069

Figure 9 continued

F



Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
No EGF			
DMSO vs. TRAM	No	ns	0.1648
DMSO vs. UP	Yes	**	0.0015
DMSO vs. T+U	Yes	***	0.0004
TRAM vs. UP	Yes	*	0.0272
TRAM vs. T+U	Yes	**	0.0048
UP vs. T+U	No	ns	0.5734
EGF			
DMSO vs. TRAM	No	ns	0.9560
DMSO vs. UP	No	ns	0.9983
DMSO vs. T+U	No	ns	0.3483
TRAM vs. UP	No	ns	0.9856
TRAM vs. T+U	No	ns	0.1813
UP vs. T+U	No	ns	0.2838



MDA-MB-468

In the BT-20 cells, we were able to observe contestant increased EGF induced p-STAT1 expression via western blot as previously demonstrated. (Figure. 9 A) Caspase 3/7 activity did not show significant difference in any treatment groups but showed increased trend when cells were treated with all three combination of EGF, Trametinib and Uprosertib. (Figure. 9 B/C) In MDA-MB-468 cells, EGF induction of pSTAT1 expression was also observed relative to non-stimulation. (Figure. 9 D) Caspase activity was not effected by mono-treatments of Trametinib or Uprosertib but was increased when both inhibitors were combined. Furthermore, addition of EGF was able to increase Trametinib induced caspase activity, again, suggesting that EGFR activation playing a significant role in apoptosis. (Figure. 9 E/F) Together, these results suggest that EGFR overexpression and activation is critical for ligand induced pSTAT1 expression and subsequently enhanced Trametinib and Uprosertib induced apoptosis in triple negative breast cancer cell lines with genetic abnormalities.

In summary we demonstrate sustained EGF-induced STAT1 phosphorylation and induction of EGF-induced apoptosis in the presence of combined pharmacological inhibition of both MEK1/2 and AKT in human breast cancer cells with high EGFR expression and in our model of isogenic murine breast cancer cells of differing metastatic capacities. Our results here expand the concept of biased growth factor signaling as a potential mechanism for the therapeutic benefits observed with cytoplasmic kinase inhibitors in metastatic breast cancer.

Due to recently observed toxicity associated with Trametinib/Uprosertib combination in patients, (Tolcher et al., 2020) , we opted for the PI3Kα specific inhibitor, Alpelisib. Alpelisib or PIQRAY, is currently a FDA approved drug in use in combination with Fulvestrant in HR+/HER2- advanced breast cancer patients with PIK3CA mutations. Here, we show Alpelisib displayed specific cell death response in cell lines with PIK3CA mutations, BT-20 and MCF-7. (Appendix.

4) Additionally, signaling assays using western blotting, shows no significant effect on pSTAT1 expression in presence of Alpelisib alone or in combination with Trametinib, while still effectively inhibiting pAKT and pPRAS40 in EGF induced pAKT and pPRAS40. These results indicate the specificity of Alpelisib in PIK3CA mutants and tolerance in our model for potential use in biasing growth factor activated caspase induction.

To further expand the concept of hijacking ligand mediated signaling activation, we opted to explore effect in the human breast cancer cell line, MDA-MB-231, which is a RAS mutant and does not have aberrations in AKT signaling. MDA-MB-231 cells showed minimal STAT1 response to EGF stimulation, an observation consistently observed in human cell lines that do not have an overexpression of EGFR such as MCF-7 and BT-549. (Figure. 10 A) These observations led us to evaluate factors other than EGF for STAT1 activation. Interferon γ, INF-γ, is a known activator of immune cell activation. Upon INF-γ stimulation STAT1 is activated and induction of caspase activity is observed. Addition of INF-γ in human breast cancer cell lines MDA-MB-231 and BT-549 displayed robust pSTAT1 expression (Figure. 10 B/D) and caspase activity. (Figure. 10 C/E) Interestingly, in both MDA-MB-231 and BT-549 cells combination of Trametinib and Alpelisib along with INF-γ stimulation displayed reduced caspase activity levels compared to mono-kinase inhibition with Alpelisib. This effect may be due to the lowered cell viability in the MDA-MB-231 cells due to the combination of the multiple compounds (Appendix 6). In the BT-549 cells, however, combination treatment with dual kinase inhibition displayed minimal effect in both caspase activity or cell viability. Further exploration into the role of PTEN null consequences in the BT-549 cells will need to be conducted to exclude effects of biased signaling with the dual kinase inhibition. These findings may lead to expansion in the use of kinase inhibitors for patients without specific receptor overexpression to overcome current mono-kinase resistance and lack of

efficacy in some patients. Although, toxicity profiles will need to be carefully optimized and evaluated.

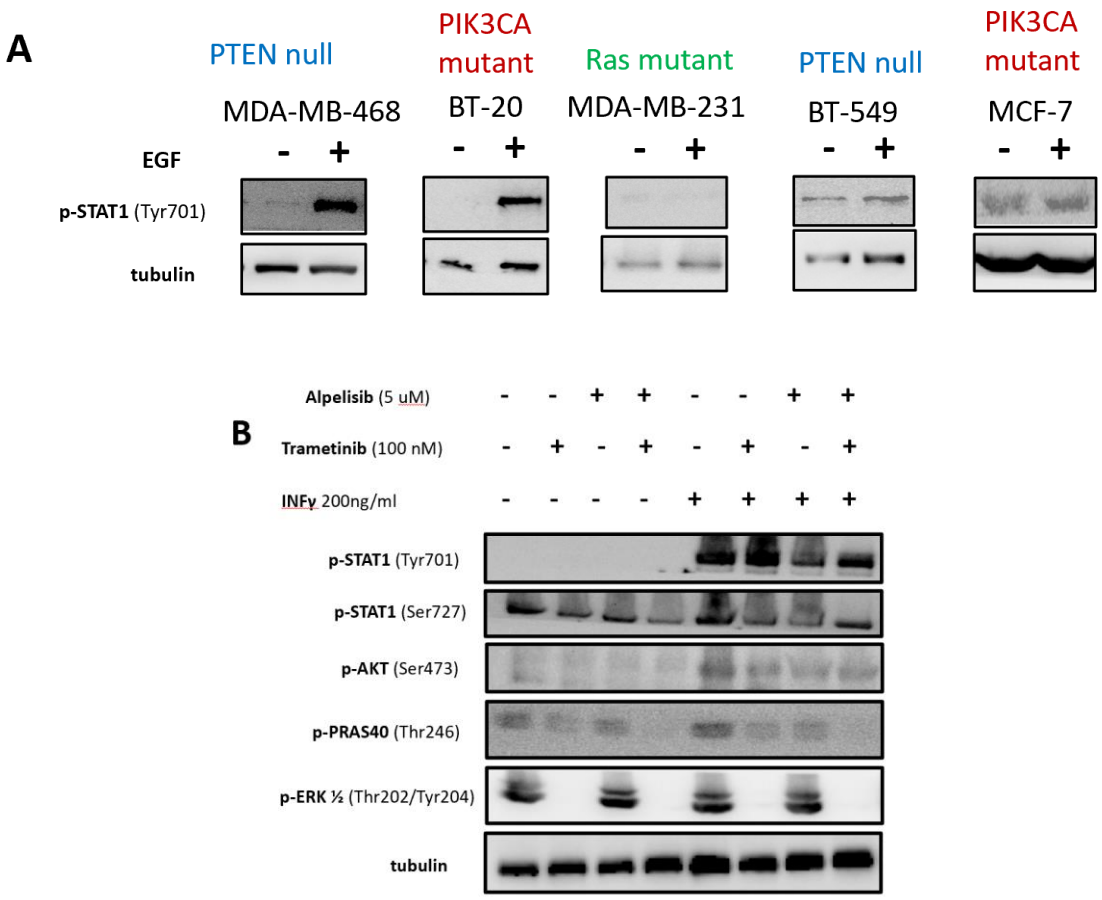
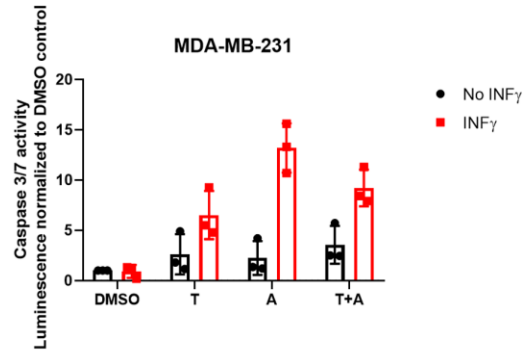


Figure 10. Differing response of human breast cancer cells to EGF vs. INFγ. A. Comparison of breast cancer lines of different mutation status in response to EGF. Cells were treated with EGF for 30 mins in full growth media followed by protein collection and probing for phosphorylated STAT1. B. Signaling profile of MDA-MB-231(B) and BT-549(C) in response to Trametinib, Alpelisib or dual kinase inhibition for 2 hours, followed by INFγ stimulation for 30 mins. C. Caspase 3/7 activity was measured using the Caspase Glo assay (Promega) following manufacturers protocol. MDA-MB-231 (C) or BT-549 (F) were seeded in 96 well plates with 10,000 cells per well. The following day, cells were treated with indicated treatments for 24 hours before luminescence read.

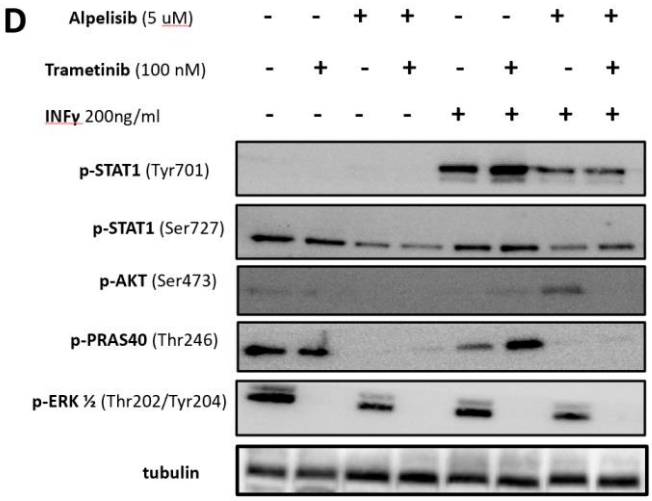
Figure 10 continued

C

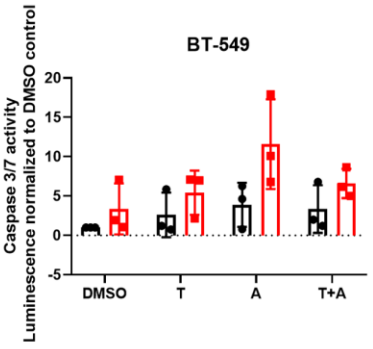


Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
No INFγ			
DMSO vs. T	No	ns	0.6921
DMSO vs. A	No	ns	0.8275
DMSO vs. T+A	No	ns	0.3359
T vs. A	No	ns	0.9943
T vs. T+A	No	ns	0.9184
A vs. T+A	No	ns	0.8118
INFγ			
DMSO vs. T	Yes	**	0.0076
DMSO vs. A	Yes	****	<0.0001
DMSO vs. T+A	Yes	***	0.0002
T vs. A	Yes	**	0.0017
T vs. T+A	No	ns	0.2951
A vs. T+A	No	ns	0.0666

D



E



Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
No INFγ			
DMSO vs. T	No	ns	0.9215
DMSO vs. A	No	ns	0.6789
DMSO vs. T+A	No	ns	0.7988
T vs. A	No	ns	0.9582
T vs. T+A	No	ns	0.9917
A vs. T+A	No	ns	0.9964
INFγ			
DMSO vs. T	No	ns	0.8528
DMSO vs. A	Yes	*	0.0254
DMSO vs. T+A	No	ns	0.5979
T vs. A	No	ns	0.1161
T vs. T+A	No	ns	0.9663
A vs. T+A	No	ns	0.2497

3.3 Discussion

Metastatic lesions harbor a high degree of molecular changes distinct from the primary tumor, demanding an objective assessment of the level of evidence provided by currently recognized biomarkers derived from primary tumor tissues (Bidard, Pierga, Soria, & Thiery, 2013; Cejalvo et al., 2017; Welch & Hurst, 2019). Furthermore, patients of the triple negative subtype lack effective treatment options and reoccurrence and drug resistance remain a clinical hurdle. Elucidating specific signatures involved in metastatic progression is fundamental in developing therapeutic interventions to overcome drug resistance and combat the late-stage disease. The breast cancer progression model used here are intrinsically resistance to EGFR inhibitors. Clinical observations in patients with EGFR-mutant lung cancers reported a lack of efficacy of EGFR inhibitors, erlotinib. Studies utilized the JAK/STAT inhibitor, ruxolitinib as alternative targeting of abberant EGFR signaling as pre-clinical evidence of the JAK1/2:STAT3 pathway involvement in acquired resistance to EGFR inhibitors. Unfortunately, treatment efficacy was not beneficial. (Yu et al., 2017) Ruxolitinib inhibits JAK1/2 signaling, which may affect STAT molecules including STAT1. As previously reported and as shown in the data above, STAT1 plays a critical role in apoptosis in metastatic cells. Thus, clinical inefficacy of to these inhibitors may be contributed to its effects on STAT1. Here we show, as oppose to overt inhibition of EGFR, activation and subsequent cytoplasmic kinase inhibition and biasing activated signaling, may be an alternative therapeutic approach in metastatic cells resistant to EGFR inhibitors.

PIK3CA mutation occur in over 30% of breast cancer patients. (Koboldt et al., 2012; Madsen et al., 2018; Mosele et al., 2020) Recent FDA approval for PI3Kα-specific inhibitor, Alpelisib, for advanced breast cancer, displayed increased overall survival in patients with PIK3CA mutation, hormone receptor positive and HER2 negative.(Fabrice André et al., 2019) These emerging advances pave the foundations for targeting PI3K activating mutations in

advanced metastatic breast cancer. Our preliminary data with the use of the FDA approved PIK3CA inhibitor, Alpelisib, increased sensitivity in cells with aberrant PI3K/AKT/mTOR signaling (MDA-MB-468 and BT-20s) pathways. In cells with wildtype PI3K(MDA-MB-231) we showed that with dual kinase inhibition and ligand stimulation we can increase apoptotic signatures, expanding use of Alpelisib in non-PIK3CA mutants. Furthermore, we use the direct AKT inhibitor, Uprosertib, which showed substantial response in NME-LM2 cell apoptosis and cell viability in combination with EGF and Trametinib treatment. These results suggest that the idea of high heterogeneity and organ tropism mechanisms of metastatic cells require distinct identification and treatment options relative to the primary tumor.

Furthermore, we show that EGFR overexpression is critical in EGF induced p-STAT expression. Interestingly, in EGFR overexpressing lines; MDA-MB-468 and BT-20s, harboring PTEN and PIK3CA mutations respectively, evaded EGF:TKI induced apoptosis and cell growth inhibition, while still expressing high levels of EGF induced pSTAT1. Recent studies have shown that Uprosertib resistance may be induced via increased lactate acidosis in a colon cancer model.(Barnes et al., 2020) Metabolic reprogramming and increase lactic acid in the tumor microenvironment is a hallmark of cancer cells. Studies in triple negative breast cancer models have suggested that EGFR signaling to increase aerobic glycolysis.(Lim et al., 2016) These studies together with the data observed here, may suggest that Uprosertib sensitivity in these cells can be enhanced to support increased EGF mediated apoptosis.

The tumor cell secretome is an emerging factor driving chemoresistance. Pro tumorigenic factors secreted by both cancer cells and stromal cells aid in cell growth and proliferation as well as drug sensitivity. However, the idea of growth factor and cytokine induced cell death is well documented to be highly context dependent.(Sporn & Roberts, 1988) For instance, TGF-β

function switches from being growth suppressive in primary tumors to growth promoting in metastatic ones.(Roberts & Wakefield, 2003; Tian & Schiemann, 2009) Similarly, interleukin 6 (IL-6), platelet-derived growth factor (PDGF), and hormones such as estrogen and androgen have all been shown to inhibit cancer growth under particular condition, both *in vitro* and in animal models.(Hedayati et al., 2016; Kim et al., 1995; Minami et al., 1996) Furthermore, high level estrogen therapy has even been explored clinically in metastatic breast cancer patients.(Mahtani et al., 2009) Here we show the growth factor, EGF, to increase caspase activity and cell death in cells overexpressing EGFR and aberrant PI3K/AKT/mTOR signaling (MDA-MB-468 and BT-20s) as well as in our model of EGFR-transformed mammary epithelial cells isolated from lung metastases (NME-LM2).

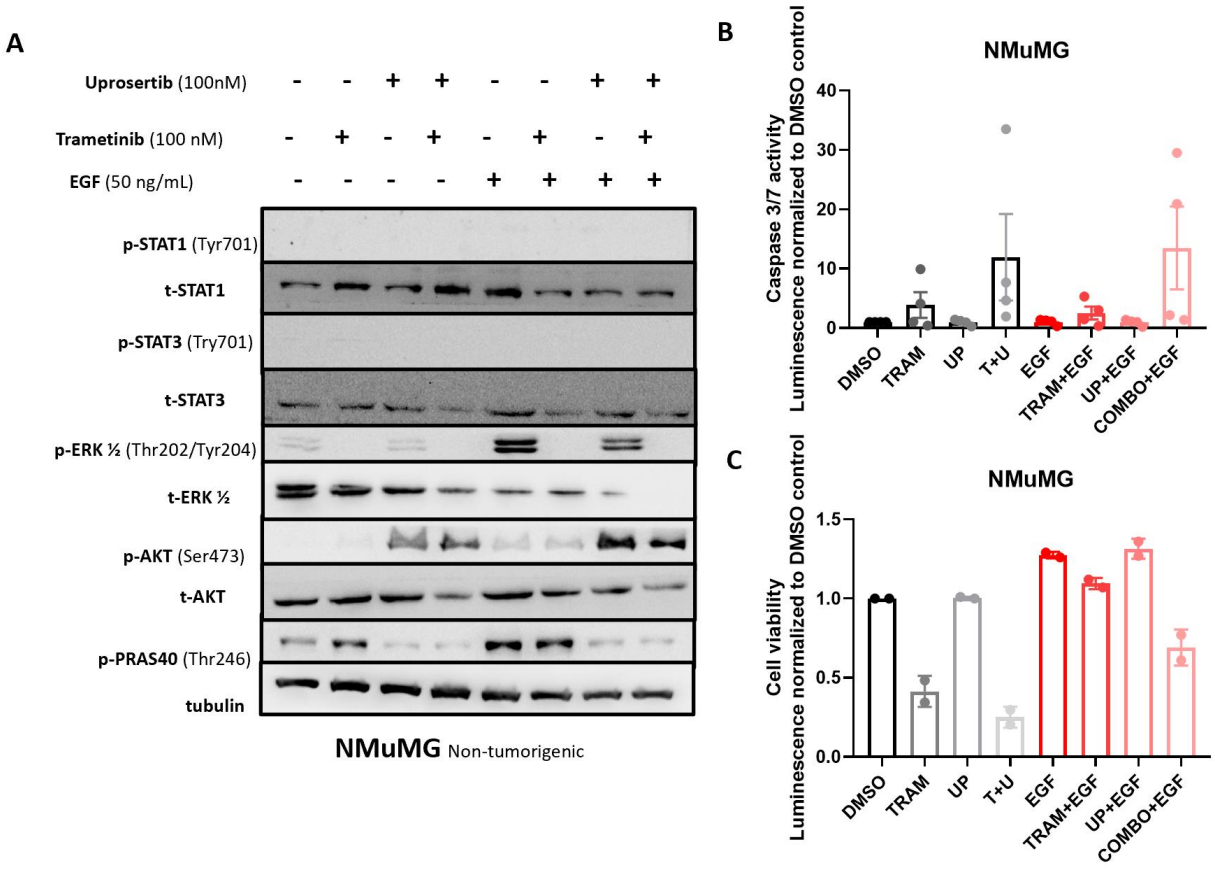
Interferon-gamma (IFN-γ) plays a primary role in immune cell activation. This cytokine is secreted by primarily by activated T cells and natural killer (NK) cells, promoting; macrophage activation, antiviral and antibacterial immunity, antigen presentation, activation of the innate immune system, and immune cell proliferation and apoptosis. (Tau & Rothman, 1999) Additionally, IFN-γ plays a major anti-tumorigenic role by influencing expression of the MHC class I molecules in tumor cells as well as induce tumor cell apoptosis via TNF-alpha related ligands such as TRAIL/Apo2L, Fas/FasL, XIAP associated factor-1 (XAF-1), caspase-4, caspase-8, dsRNA activated protein kinase (PKR), 2'5'A oligoadenylate synthetase (OAS), death activating protein kinases (DAP kinase) (Chawla-Sarkar et al., 2003) Recent studies have demonstrated long-range sensing of T-cell secreted IFN-γ in the TME. (Hoekstra et al., 2020; Thibaut et al., 2020) These emerging studies suggest mechanisms of modulation to distant cancer cells. In our current study we show IFN-γ mediated STAT1 as a driver of caspase activity and dual kinase inhibition can bias IFN-γ mediated signaling. Furthermore, these results suggest that biasing receptor

mediated signaling can be expanding to breast cancer cell lines that do not have overt receptor overexpression. Together with these recent studies, we can exploit TME modulation and bias growth factor and cytokine mediated signaling for novel approaches to inhibit metastatic tumor cell growth. Synergistic drug interactions have long been touted in cancers with multiple genetic mutations or resistance to mono-kinase or chemotherapy alone. These emerging studies will help expand mechanisms of combinatorial drug synergy and redefine guidelines on diagnosis and tailoring of systemic treatments for patients with metastatic breast cancer.

3.4 Future directions

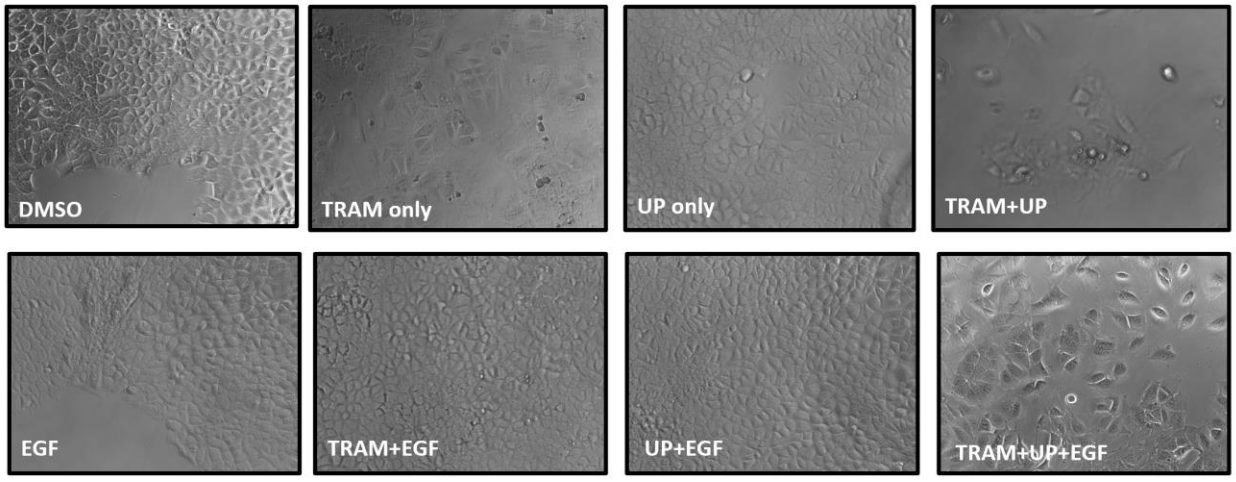
Dual kinase inhibition continues to emerge as a possible cancer treatment strategy, although signs of resistance mechanisms are arising. Previous pharmacological studies as well as our results, elucidate the differences in cancer cell signaling and sensitivity to growth factors, cytokines and kinase inhibitors. Manipulation of growth factor and cytokine mediated signaling to better understand sensitivity to inhibitors in combination studies need to be expanded in a variation of breast cancer cell lines of different genetic background. Furthermore, we can explore alternative routes of receptor mediated apoptosis in combination with kinase inhibition. Several routes of INFα/β/γ induced apoptosis are well characterized and can be explored in our cell signal biasing model. Future studies will also need to translate these finding into *in vivo* models, optimize dosing conditions, and evaluate efficacy of treatments in primary tumor growth and metastatic progression.

APPENDIX

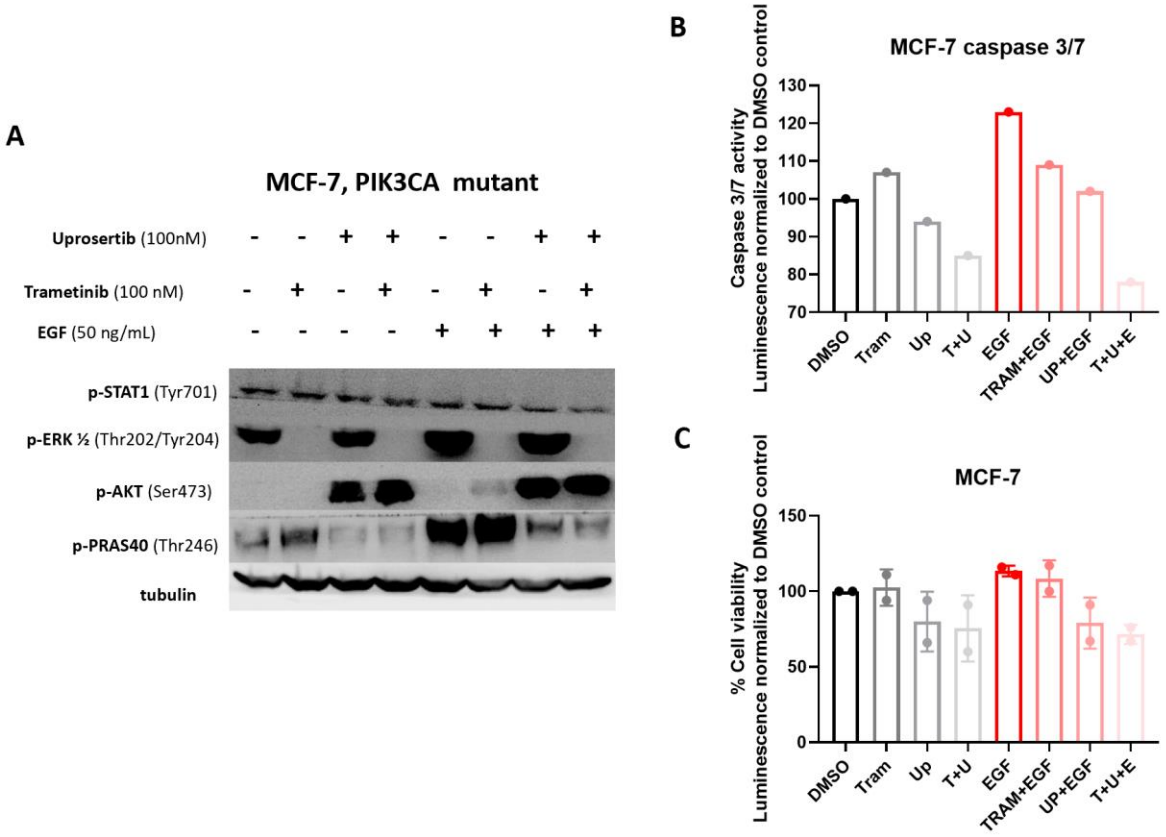


Appendix 1. Response of EGF in non-tumorigenic NMuMG mammary epithelial cells show no increase in pSTAT1 or differences in caspase and cell viability following EGF stimulation. A. Signaling profile. Cells were serum starved for 6 hours following a pretreatment of kinase inhibitors for 2 hours before stimulation with EGF for 30 mins. Subsequent evaluation of signaling proteins through phosphorylation of STAT1, STAT3, ERK1/2, AKT, and PRAS40 with respective total protein or tubulin expression for control. B. Caspase 3/7 activity was measured using the Caspase Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 10,000 cells per well. The following day, cells were treated with indicated treatments for 24 hours before luminescence read. C. Cell viability was measured using the Cell Titer Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 2,000 cells per well. The following day, cells were treated with indicated treatments for 48 hours in full growth media before luminescence read

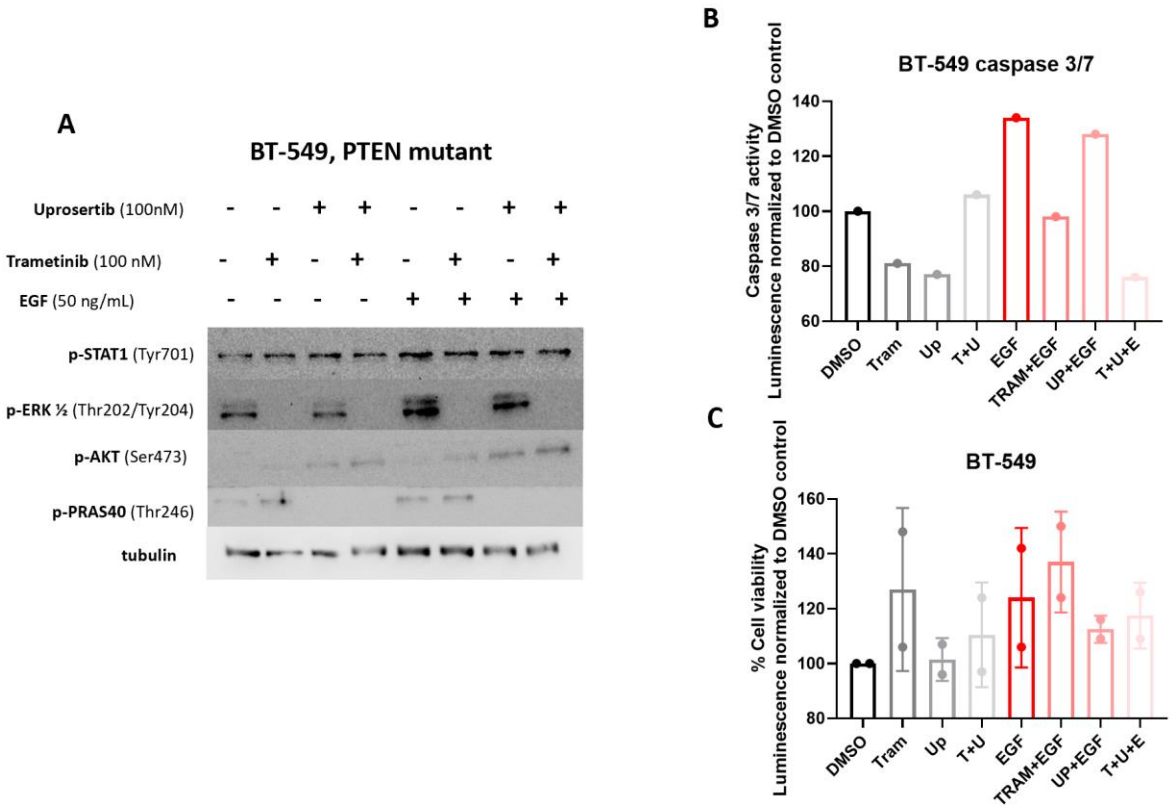
Appendix 1 continued



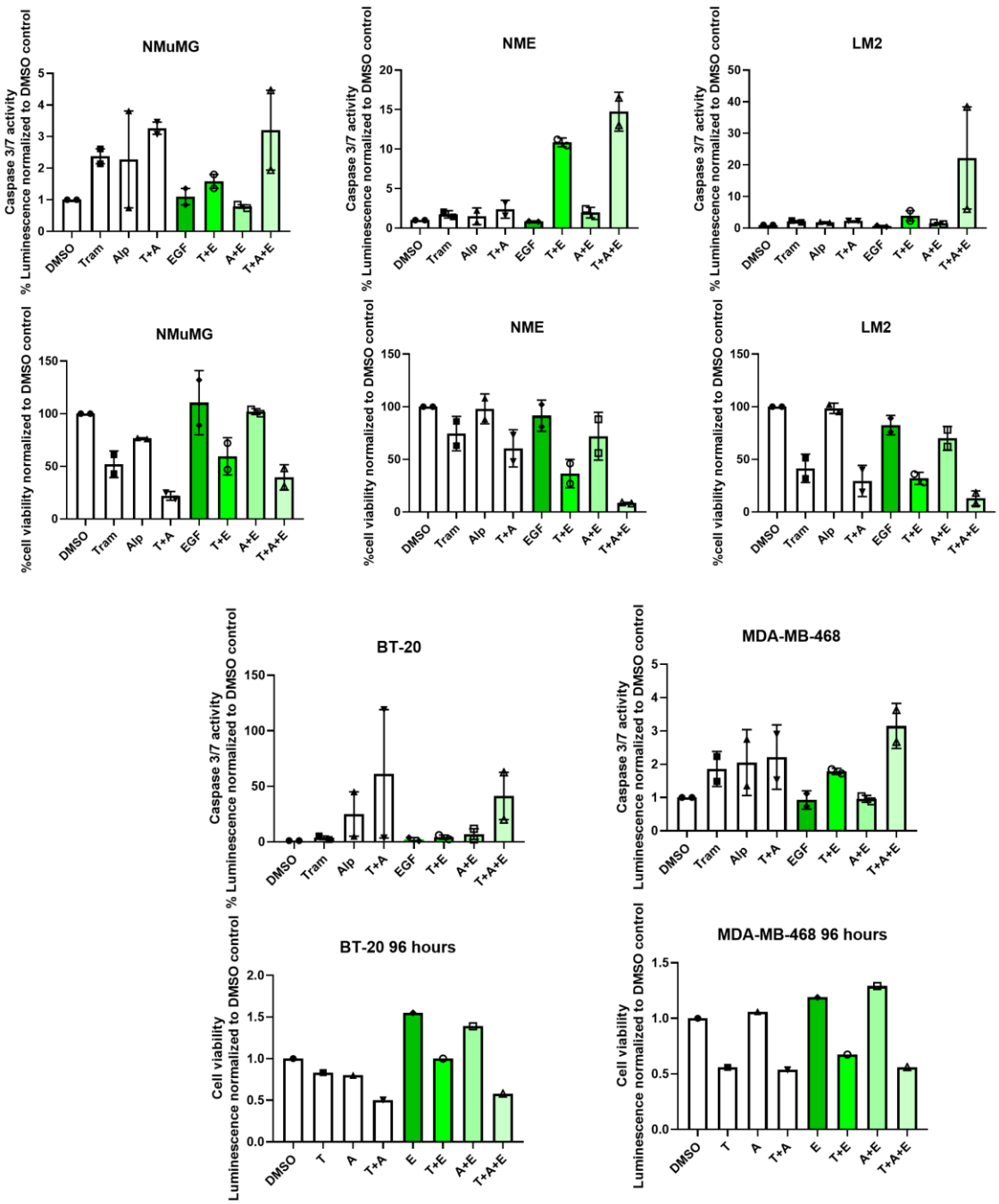
NMuMG



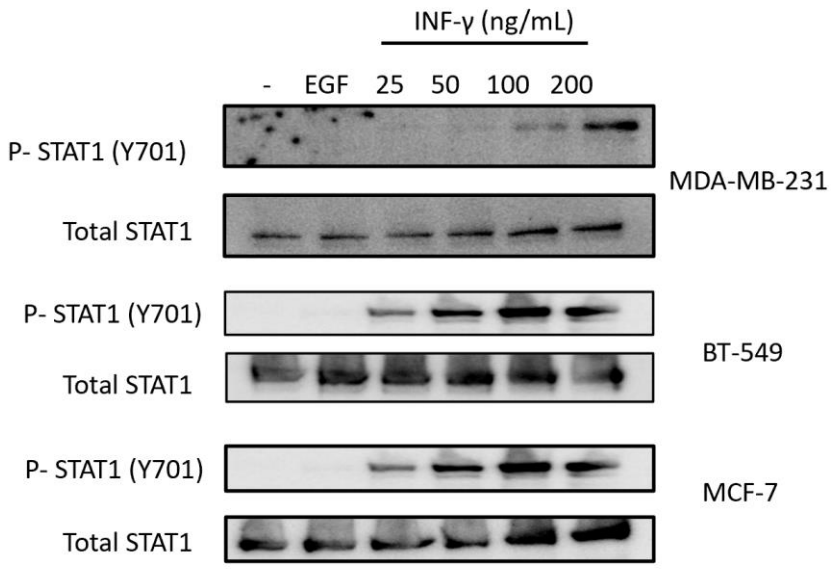
Appendix 2 Response of EGF in HR +/HER-, PIK3CA mutant, MCF-7 cells show no increase in pSTAT1 or differences in caspase and cell viability following EGF stimulation. Caspase activity is inconclusive as MCF-7 cells are caspase-3 deficient A. Signaling profile. Cells were serum starved for 6 hours following a pretreatment of kinase inhibitors for 2 hours before stimulation with EGF for 30 mins. Subsequent evaluation of signaling proteins through phosphorylation of STAT1, STAT3, ERK1/2, AKT, and PRAS40 with tubulin expression for control. B. Caspase 3/7 activity was measured using the Caspase Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 10,000 cells per well. The following day, cells were treated with indicated treatments for 24 hours before luminescence read. C. Cell viability was measured using the Cell Titer Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 2,000 cells per well. The following day, cells were treated with indicated treatments for 48 hours in full growth media before luminescence read.



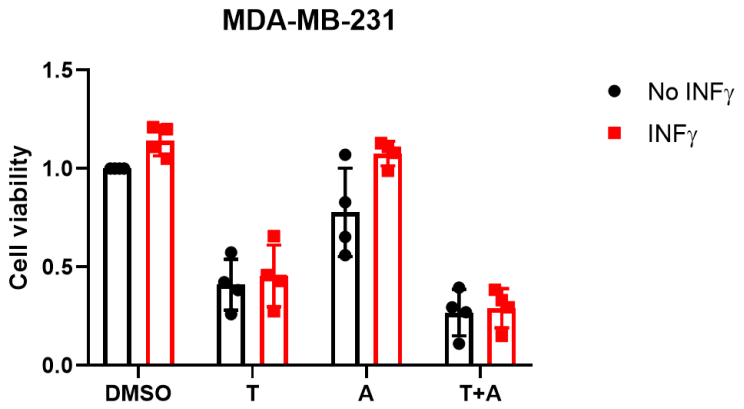
Appendix 3. Response of EGF in triple negative, PTEN null, BT-549 cells show no increase in pSTAT1 or differences in caspase and cell viability following EGF stimulation. Sensitivity to inhibitors so not correlate to EGF addition A. Signaling profile. Cells were serum starved for 6 hours following a pretreatment of kinase inhibitors for 2 hours before stimulation with EGF for 30 mins. Subsequent evaluation of signaling proteins through phosphorylation of STAT1, STAT3, ERK1/2, AKT, and PRAS40 with tubulin expression for control. B. Caspase 3/7 activity was measured using the Caspase Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 10,000 cells per well. The following day, cells were treated with indicated treatments for 24 hours before luminescence read. C. Cell viability was measured using the Cell Titer Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 2,000 cells per well. The following day, cells were treated with indicated treatments for 48 hours in full growth media before luminescence read



Appendix 4. Caspase and cell viability of murine isogenic cells; NMuMG, NME and LM2 as well as BT-20 and MDA-MB-468 to combination of Trametinib and PI3Kα specific Alpelisib followed by EGF stimulation. Caspase 3/7 activity was measured using the Caspase Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 10,000 cells per well. The following day, cells were treated with indicated treatments for 24 hours before luminescence read. Cell viability was measured using the Cell Titer Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 2,000 cells per well. The following day, cells were treated with indicated treatments for 48 hours or 96 hours in full growth media before luminescence read.



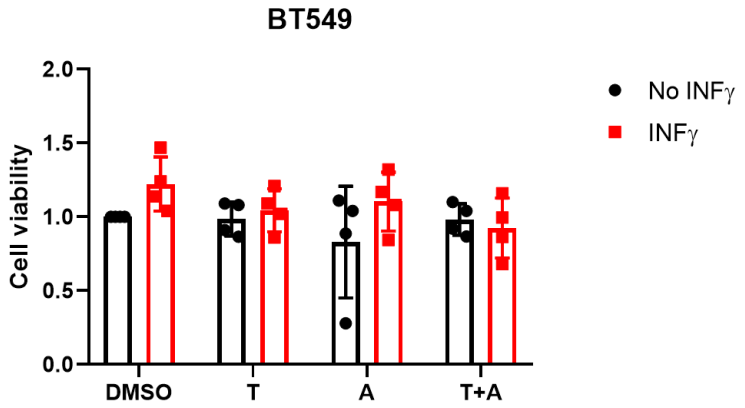
Appendix 5.Western blot showing pSTAT1 signaling response to EGF vs. INFγ stimulation. Cells that did not result in EGF mediated pSTAT1 are sensitive to INFγ induced pSTAT1. These results expand biased signaling from EGFR mediation to INF γ. Further studies will need to elucidate INFγ stimulation with dual kinase inhibition in various cell models. Cells were serum starved for overnight following stimulation with EGF or INFγ for 30 mins before cell lysate collection.



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
No INFγ					
DMSO vs. T	0.5900	0.3458 to 0.8342	Yes	****	<0.0001
DMSO vs. A	0.2220	-0.02216 to 0.4662	No	ns	0.0840
DMSO vs. T+A	0.7315	0.4873 to 0.9757	Yes	****	<0.0001
T vs. A	-0.3680	-0.6122 to -0.1238	Yes	**	0.0019
T vs. T+A	0.1415	-0.1027 to 0.3857	No	ns	0.3982
A vs. T+A	0.5095	0.2653 to 0.7537	Yes	****	<0.0001
INFγ					
DMSO vs. T	0.6873	0.4431 to 0.9314	Yes	****	<0.0001
DMSO vs. A	0.06525	-0.1789 to 0.3094	No	ns	0.8811
DMSO vs. T+A	0.8520	0.6078 to 1.096	Yes	****	<0.0001
T vs. A	-0.6220	-0.8662 to -0.3778	Yes	****	<0.0001
T vs. T+A	0.1648	-0.07941 to 0.4089	No	ns	0.2708
A vs. T+A	0.7868	0.5426 to 1.031	Yes	****	<0.0001

Appendix 6. Cell viability of human breast MDA-MB-231 and BT-549 cells treated with combination of Trametinib and PI3Kα specific Alpelisib followed by INFγ stimulation compared to mono-kinase inhibition. Cell viability was measured using the Cell Titer Glo assay (Promega) following manufacturers protocol. Cells were seeded in 96 well plates with 2,000 cells per well. The following day, cells were treated with indicated treatments for 72 hours in full growth media before luminescence read.

Appendix 6 continued



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
No INFγ					
DMSO vs. T	0.01350	-0.3667 to 0.3937	No	ns	0.9997
DMSO vs. A	0.1710	-0.2092 to 0.5512	No	ns	0.6079
DMSO vs. T+A	0.01800	-0.3622 to 0.3982	No	ns	0.9992
T vs. A	0.1575	-0.2227 to 0.5377	No	ns	0.6673
T vs. T+A	0.004500	-0.3757 to 0.3847	No	ns	>0.9999
A vs. T+A	-0.1530	-0.5332 to 0.2272	No	ns	0.6869
INFγ					
DMSO vs. T	0.1775	-0.2027 to 0.5577	No	ns	0.5791
DMSO vs. A	0.1188	-0.2614 to 0.4989	No	ns	0.8243
DMSO vs. T+A	0.2978	-0.08242 to 0.6779	No	ns	0.1632
T vs. A	-0.05875	-0.4389 to 0.3214	No	ns	0.9734
T vs. T+A	0.1203	-0.2599 to 0.5004	No	ns	0.8189
A vs. T+A	0.1790	-0.2012 to 0.5592	No	ns	0.5725

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