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Sylvia Shabaya Gayle Date

Novel Therapeutic Strategies for HER2-overexpresssing breast cancer

By

Sylvia Shabaya Gayle
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Molecular & Systems Pharmacology

_____
Rita Nahta, PhD Advisor

_____
Haian Fu, PhD Committee Member

_____
Harold Saavedra, PhD Committee Member

_____
Roy Sutliff, PhD Committee Member

Accepted:

_____
Lisa A. Tedesco, PhD Dean of the Graduate School

_____
Date

Novel Therapeutic Strategies for HER2-overexpresssing breast cancer

By

Sylvia Shabaya Gayle
B.S. University of Akron, 2005
M.S., University of Akron, 2008

Advisor: Rita Nahta, PhD

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Abstract

This dissertation seeks to uncover mechanisms by which resistance to lapatinib develops and proposes novel therapeutic strategies for patients with HER2-overexpressing breast cancer. The standard targeted therapy for HER2-overexpressing breast cancer is the HER2 monoclonal antibody, trastuzumab. Although effective, many patients eventually develop trastuzumab resistance. The dual EGFR/HER2 small molecule tyrosine kinase inhibitor lapatinib is approved for use in trastuzumab-refractory metastatic HER2-positive breast cancer. However, lapatinib resistance is also a problem as most patients with trastuzumab-refractory disease do not benefit from lapatinib for long. Understanding the mechanisms underlying lapatinib resistance may ultimately facilitate development of new therapeutic strategies for HER2-overexpressing breast cancer. Our results indicate reduced sensitivity to lapatinib is associated with an inability of lapatinib to inhibit the two main signaling pathways downstream of HER2, MEK/ERK and PI3K/Akt/mTOR. We genetically and pharmacologically blocked MEK/ERK signaling and evaluated lapatinib response by trypan blue exclusion, anchorage-independent growth assays, flow cytometric cell cycle and apoptosis analysis, and in tumor xenografts. Our results suggest MEK inhibition increases response to lapatinib. In addition, Western blots, immunofluorescence, and immunohistochemistry demonstrated the combination of MEK inhibitor plus lapatinib reduced nuclear expression of the MEK/ERK downstream proto-oncogene FOXM1. Other trypan blue, transfection, anchorage-independent growth assays, and xenograft studies evaluating the role of PI3K/Akt/mTOR in lapatinib response demonstrated transfection of constitutively active Akt reduced lapatinib sensitivity, while kinase-dead Akt increased sensitivity. Knockdown of 4EBP1 also increased lapatinib sensitivity, in contrast to p70S6K knockdown, which did not affect response to lapatinib. Pharmacologic inhibition of mTOR increased lapatinib sensitivity and reduced phosphorylated Akt levels in cells that showed poor response to single-agent lapatinib, including those transfected with hyperactive Akt. The collective findings presented herein provide insight into the mechanisms by which resistance to lapatinib is achieved, and suggest potential therapeutic strategies that could be of benefit to patients with HER2-overexpressing breast cancer.

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List of Abbreviations

IHC- immunohistochemical
FISH- fluorescent in situ hybridization
EGFR-epidermal growth factor receptor
HER2-human epidermal growth factor receptor 2
HER3- human epidermal growth factor receptor 3
HER4- human epidermal growth factor receptor 4
TGFa-transforming growth factor alpha
EGF-epidermal growth factor
SH2-src homology 2
JAK-Janus kinas
STAT-signal transducer and activator of transcription
PLC- phosphoinositide-specific phospholipase C
PTEN-phosphatase and tensin homolog
PI3K-phosphatidylinositol 3-kinases
SGLT- sodium glucose co-transporters
DAG -diacyl glycerol
IP3-inositol 1,4,5-triphophate
PIP2-phosphatidylinositol 4,5-biphosphate
PIP3-phosphatidylinositol 3,4,5-triphosphate
PLC-phosphoinositide-specific phospholipase C
JNK1-c-Jun-N-terminal kinases
IGF-IR- insulin like growth factor receptor
CDK2-cyclin dependent kinase 2
HRG-heregulin
IGF-insulin-like growth factor
MAPK-mitogen activated protein kinase pathway
IGFBP-IGF binding proteins
FAK-focal adhesion kinase
DMSO-dimethyl sulfoxide
DPBS- Dulbecco's phosphate buffered saline
PI-propidium iodide
PCR-polymerase Chain Reaction
SDS-sodium dodecyl sulfate
siRNA-small interfering RNA

Chapter 1

Introduction and Background

Portions of this chapter were previously published.
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1. EGFR receptor tyrosine kinase family and HER2-overexpressing breast cancers

i. HER2 and breast cancer

Breast cancer is the most common occurring cancer in women, with 1.5 million cases reported in a 2008 study (Ferlay et al, 2010). Of these, almost one third resulted in a fatality, making breast cancer the most common cause of cancer death in women. Although there have been advances in breast cancer detection and therapy, approximately 40,000 women in the US die annually (Ferlay et al, 2010). These findings demonstrate the importance of understanding the mechanisms by which breast cancer progresses so effective treatments can be developed.

Breast cancer is a heterogenous disease that can be characterized by molecular biomarkers. Two of the main molecular profiles observed in breast cancer are the expression of the estrogen receptor, and amplification of the human epidermal growth factor receptor 2 (HER2) (Slamon et al, 1987). HER2 is overexpressed in approximately 25% of human metastatic breast cancers and is an important regulator of cell growth and differentiation during embryogenesis, as well as during puberty for mammary development (Slamon et al, 1987). HER2 is located at chromosomal location 17q21, with amplification yielding up to 20 fold increases in gene copy expression in breast cancer patients (Slamon et al, 1987; Schechter et al, 1985). In many cases, HER2-overexpression is a result of HER2 gene amplification that leads to increased HER2 mRNA production and increased synthesis and expression on the cell surface (Hynes et al, 1993).

HER2 was first discovered in the early 1980s by Shih et al, who found that NIH

3T3 cells became transformed after a transfection with DNA from a novel transmissible gene obtained from rat neuroblastomas (Shih et al, 1981). Subsequently, the group identified a phosphoprotein with a molecular weight of 185 kilodaltons from the sera of the injected mice (Padhy et al, 1982). The nucleic acid of this newly discovered neu oncogene was found to be related to the epidermal growth factor receptor, and homologous to the erb-B oncogene, as well as the neu-associated tumor antigen p185 (Shechter et al, 1984). Two other groups independently identified the erb-B related genes HER2 and c-erbB2, with further analysis revealing that neu, HER2, and c-erbB2 are identical genes mapping on the same chromosome location (Coussens et al, 1985; Semba et al, 1985; Schechter et al, 1985).

Since the discovery of HER2, researchers have focused on learning more about its onocogenic properties in order to develop effective therapies for patients. The transforming version of neu (neuT) is an experimental tool developed to study HER2. NeuT possesses a single point mutation that changed a valine in the transmembrane domain to a glutamic acid (V664E) (Bargmann et al, 1986; Coussens et al, 1985; Brandt et al, 2001). The transfection of neuT into NIH3T3 as well as mammary epithelial cells resulted in transformation, while the transfection of wild-type neu did not result in transformation. The mechanism behind the transforming ability of neuT was determined to be a result of increased receptor dimerization leading to increased kinase activity (Weiner et al, 1989). This neuT data suggested therapeutic treatments that disturb receptor dimerization and/or inhibit kinase activity would yield the most favorable responses in patients with HER2-overexpressing breast cancer.

Further investigation of HER2/neu revealed that while a mutation is required for

oncogenic properties in rats, HER2/neu is oncogenic in humans in the absence of any mutations (Hudziak et al, 1987). Overexpression of the HER2 gene enables activation of survival pathways, and therefore serves as an oncogenic driver in breast cancer. In addition, the amplification of the *HER2* gene is a significant predictor of reduced overall survival and shorter time to relapse in patients with early-stage breast cancer (Slamon et al, 1987). Hence, HER2 has become an important therapeutic target for this subtype of breast cancers. When a breast cancer diagnosis is made, HER2 status is routinely assessed by either immunohistochemical (IHC) analysis of HER2 protein expression, or fluorescent in situ hybridization (FISH) analysis of *HER2* gene copy number in a breast tumor biopsy (Sauter et al, 2009).

FISH allows for the quantitation and visualization of target DNA sequences through a microscope (Trask et al, 2002). A cloned piece of the genome conjugated to a reporter molecule is used as a probe. Following denaturation, the probe hybridizes to its complementary sequence in the chromosomal DNA thereby tagging it with a fluorescent reagent. The use of fluorescent tags is an advantage over the isotopic labels previously used because of their ease of detection, stability, higher resolution, and capability to simultaneously locate several DNA sequences. CEP17 is used as an internal control and also to account for chromosome 17 aneusomy for HER2 amplification analysis (Wolf et al, 2007). A HER2/CEP17 ratio <1.8 is categorized as the absence of HER2 amplification, while a HER2/CEP17 ratio >2.2 is positive for HER2 gene amplification. A ratio between 1.8 and 2.2 is classified as equivocal, which can result in further HER2 amplification analysis by IHC. IHC detects cell surface HER2 protein using antibodies conjugated to either an enzyme like peroxidase that can catalyze a color producing

reaction, or a flourophore. Results are scored as 0, 1+,2+, or 3+ and reflect the percentage and quality of membranes staining of tumor tissue (Meijer et al, 2011). Tumors with staining scores of 0 and 1+ are categorized as HER2 negative, while a 2+ score is equivocal, and a score of 3+ is categorized as HER2-positive. Evidence of increased expression or amplification of *HER2* warrants further consideration when determining clinical treatment options.

ii. The role of the EGFR Family in HER2-overexpressing breast cancer

HER2 (erbB2/neu) is a member of the type I transmembrane tyrosine kinase receptor family which includes epidermal growth factor receptor (EGFR), HER3, and HER4. Each of these cell surface receptors has an extracellular ligand-binding domain and a transmembrane-spanning domain (Margolis et al, 1989; Fig 1-1). HER2 has three major regions: the extracellular amino-terminal region made up of domains I to IV, the hydrophobic transmembrane domain that contains the juxtamembrane domain, tyrosine kinase, and C-terminal tail, and the carboxy-terminal kinase domain (Margolis et al, 1989). Studies by Margolis et al, 1989 in which they substituted the C-terminus of HER2 for the C-terminus of EGFR revealed that HER2 authophosphorylation tyrosine sites are located within the C-terminus, and are identical to EGFR phosphorylation sites. The HER2 phosphorylation sites identified were: Y1248, Y1023, Y1112, Y1127, Y1139, Y1196, Y1221, and Y1222 (Hazan et al, 1990). Further research revealed mutant HER2 lacking the intracellular domains is still capable of interacting with EGFR and wildtype HER2, thereby providing evidence that the extracellular domains are adequate for dimerization (Qian et al, 1994, 1996; Nahta et al, 2012).

All EGFR family receptors, except HER2, bind specific ligands that induce conformational changes and receptor homo-or hetero-dimerization. Even though no ligand has been identified for HER2, ligand binding to other EGFR family members results in HER2 heterodimerization (Graus-Porta et al, 1997). Several HER family ligands have been identified, including transforming growth factor alpha (TGFa), epidermal growth factor (EGF), and the heregulins (Nielsen et al, 2008). In addition, all except HER3 contain an intracellular tyrosine kinase domain. Interestingly, HER2 kinase function is not necessary for homodimerization (Penuel et al, 2002). Studies in which HER2 kinase function is abrogated as a result of a Lys732 mutation in the HER2 ATP-binding pocket revealed that HER2 homodimerization was not significantly inhibited (Penuel et al, 2002). In contrast, a mutation in the C-terminal residues 996 to 998 resulted in an impairment of autophoshorylation and inhibited HER2 homodimerization.

Dimerization of EGFR family members occurs upon ligand binding and results in kinase activation and receptor auto- or trans- phosphorylation. The phosphorylated tyrosine residues serve as docking sites for src homology 2 (SH2) and phosphotyrosine binding domain (PTB) containing proteins. These docking sites link the receptors to multiple cell survival and proliferation pathways including the phosphatidylinositol-3 kinase (PI3K) and MEK/ERK pathways (Spector et al, 2009; Graus-Porta et al, 1997). HER2 heterodimers have increased ligand binding affinity and increased catalytic activity relative to other heterodimer complexes making HER2 the preferred dimerization partner for the other EGFR family members (Spector et al, 2009; Graus-Porta et al, 1997). In

particular, the HER2-HER3 heterodimer has the strongest kinase activity and transforming ability, as HER3 possesses multiple PI3K docking sites in its cytoplasmic tail.

Patients who are diagnosed with HER2-overexpressing breast cancer have a poor prognosis and overall survival when compared to patients with other subtypes of breast cancer (Eccles et al, 2001). To fully understand the mechanisms behind the oncogenic properties of HER2 in breast cancer, we have to know and take into account the other members of the EGFR family as well as the downstream signaling pathways.

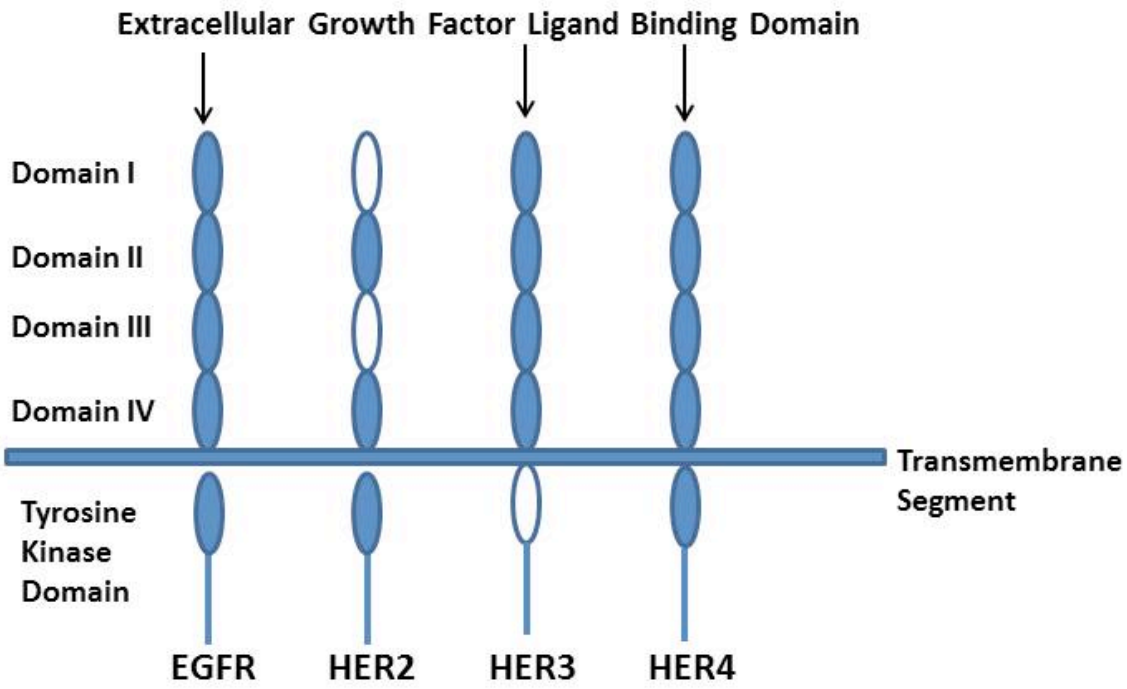

Extracellular Growth Factor Ligand Binding Domain
Domain I
Domain II
Domain III
Domain IV
Transmembrane Segment
Tyrosine Kinase Domain
EGFR
HER2
HER3
HER4

Fig. 1-1. HER/erbB family of growth factor receptors. The four members of the EGFR family are illustrated. The inactive ligand-binding domains of HER2 and the inactive kinase domain of HER3 are denoted with an open oval. Trastuzumab binds to domain IV of the extracellular region of HER2.

i. Signaling pathways downstream to EGFR Family

Although the role of EGFR activation in tumor growth has been established for years, targeted therapy designed to inhibit EGFR signaling has yielded only modest clinical success in breast cancer patients. The limited clinical benefit observed is partially due to the highly complicated nature of EGFR signaling. These signaling pathways downstream to the EGFR family are some of the most dysregulated molecular pathways in human cancers and are best known for their signaling functions as cell surface receptors. However, receptor cleavage and intracellular signaling function have been observed in normal as well as cancerous cells. EGFR family member activation leads to the induction of signal transduction pathways including: PLC, JAK2-STAT3, SGLT1, Ras/Raf/MEK and PI3K/Akt/mTOR, with the two most common being Ras/Raf/MEK and PI3K/Akt/mTOR.

i. JAK/STAT

The Janus kinas (JAK)/ signal transducer and activator of transcription (STAT) pathway regulates gene expression, cellular activation, and proliferation (Bromberg et al, 2000). Four JAK (JAK1-4) and seven STAT (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) proteins are encoded by the human genome (Watowich et al, 1996). Following ligand binding to the EGFR family members, receptor-associated JAKs are activated, resulting in SH2 and SH3 domains. STAT binds to the SH2 and SH3 sites and is phosphorylated by the JAKs (Imada et al, 2000). Upon activation, STAT proteins dimerize and translocate to the nucleus and bind to conserved enhancer elements

within the promoters of responsive genes. The JAK/STAT pathway is important to mention because it is downstream of EGFR family members, but its contribution to the neoplastic effects observed in HER2-overexpressing breast cancer upon EGFR activation have not been researched extensively.

ii. PLC

Eukaryotic phosphoinositide-specific phospholipase C (PLC) isozymes consist of a related set of soluble proteins ranging in mass from 85-150 kilodaltons, and function to cleave the polar head group from inositol phospholipids (Rebecchi et al, 2000). The core structure is conserved in PLCs and includes: a pleckstrin homology domain, a C2 domain, four tandem EF hand domains, and a split triose phosphate isomerase (TIM) barrel which is split in two halves referred to as the X and Y domains. PLC isoforms include four beta, two gamma, and four delta forms, as well as multiple mammalian splice variants. The mammalian delta isoform closely resembles the PLC found in yeasts, slime molds, and filamentous fungi (Koyanagi et al, 1998). EGFR family activation of PLC results in the selective hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) on the glycerol side of the phosphodiester bond (Kadamur et al, 2012). PIP2 regulates multiple cellular processes including PLC activity, and acts as a substrate for phosphatidylinositol 3,4,5-triphosphate (PIP3) which plays roles in Akt activation (Cantley et al, 2002).

These effects make controlling PIP2 cellular concentrations by PLC depletion an important cellular process that is well regulated (Hilgemann et al, 2007; Falkenburger et

al, 2010). PLC activation by EGFR family members yields diacyl glycerol (DAG), and inositol 1,4,5-triphophate (IP3) (Kadamur et al, 2012). DAG serves as the substrate for the regulatory molecule: phosphatidic acid synthesis, and remains membrane bound to mediate the stimulation of various enzymes and structural proteins by binding to a conserved C1 domain. In contrast to DAG, IP3 diffuses through the cytosol to bind calcium channels (Kadamur et al, 2012). In addition, IP3 gates an endoplasmic reticulum Ca2+ channel that plays roles in regulating cytoplasmic calcium concentrations. IP3 is also the rate-limiting substrate for inositiol polyphosphate synthesis which plays roles in protein kinase activation, transcription, and mRNA processing. The contribution that this EGFR downstream pathway plays has not been a focus in HER2-overexpressing breast cancer, and so it is unknown.

iii. SGLT

The sodium glucose co-transporters (SGLT) are part of the expanded solute carriers SLC5A family, and are mostly found in the brush-border membranes of small intestine and proximal convoluted tubule of the kidney (Zhao et al, 2005). Members of this gene family of transporters are responsible for active transport of vitamins, amino acids, and sugars (Wright, et al 1994). SGLT has multiple isoforms, but SGLT1 and SGLT2 have been studied the most extensively. SGLT1 is the first member of the SGLT family, and plays an integral role in transporting glucose across cellular membranes (Hanabata et al, 2012;Diez-Sampedro et al, 2009).

Upon ligand binding to the EGFR family members, a physical interaction between the receptor and SGLT results in SGLT1 stabilization (Hanabata et al, 2012). Stabilized

SGLT is then able to mediate a kinase-independent process that allows glucose uptake into cancer cells. Also, studies in oral squamous cell carcinoma revealed a statistically significant SGLT and EGFR co-expression (Hanabata et al, 2012). Recently, Hanabata et al further investigated the EGFR family member/SGLT interaction by overexpressing EGFR and monitoring glucose uptake in cancer cells deprived of an energy supply. Their results demonstrated that the physical interaction between EGFR and SGLT promotes glucose uptake and prevents the cell death of cancer cells that have been exposed to an energy shortage. A role for SGLT has yet to be determined in HER2-overexpressing breast cancer cells, but it is important to discuss this pathway as it is activated upon EGFR family member stimulation.

iv. The PI3K/Akt/mTOR pathway

Phosphatidylinositol 3-kinases (PI3Ks) are intracellular signal transducer enzymes that function to phosphorylate the 3' hydroxyl group of the inositol ring of phosphatidylinositol and phosphoinositides (Engelman et al, 2006;Martiniez-Marti et al, 2012). PI3K activity leads to the initiation of multiple intracellular signaling pathways that regulate cellular processes such as proliferation, growth, apoptosis, and motility. Studying this pathway is important as PI3K mutation or amplification has been impacted in multiple cancers including HER2-overespressing breast cancer (Berns et al, 2007).

The PI3K family consists of eight catalytic isoforms divided into three classes according to their substrate preference and sequence homology (Engelman et al, 2006). Class Ia PI3Ks are most clearly linked to human cancers (Yuan et al, 2008). Class Ia PI3Ks are heterodimers composed of a p85 regulatory subunit and a p110 catalytic

subunit (Huang et al, 2007). The p85 regulatory subunit is important in PI3K activation by receptor tyrosine kinases and has a common core structure that contains a p110-binding domain flanked by two SH2 domains. The p85 regulatory subunit is encoded by three genes: PIK3R1, PIK3R2, and PIK3R3. PIK3R1 can lead to the formation of two shorter isoforms, p55alpha and p50 alpha, which are both similar in core structure to the other isoforms. The p110 catalytic subunit has three isoforms, p110 alpha, p110 beta and p110Rho, which are encoded by PIK3CA, PIK3CB, and PIK3CD respectively (Cantley et al, 2002). P110 alpha has multiple domains consisting of an N-terminal domain that binds p85 alpha, a C2 domain that binds cellular membranes, a helical domain, and a kinase catalytic domain (Martineze-Marti et al, 2012; Vanhaesebroeck et al, 1999; Cantley et al, 2002).

There are multiple ways that PI3K can be activated, including the activation of EGFR family members and G-protein coupled receptors. G protein coupled receptors stimulate PI3K by activating the p110 beta catalytic subunit directly (Katso et al 2001). The PI3K mechanism of action of interest involves ligand binding to the extracellular region of EGFR family members leading to receptor dimerization and tyrosine residue phosphorylation. Subsequently, the intermolecular inhibition of the p110 catalytic subunit is relieved by the direct binding of Ras to the p110 beta catalytic subunit, thereby stimulating PI3K, or by binding of the p85 regulatory subunit binding to phosophotyrosine residues. This recruits PI3K to the cell membrane where the PI3K substrate, PIP2 resides (Zhao et al, 2008). Activation of the p110 catalytic subunit allows it to phosphorylate PIP2 on the 3'OH to produce PIP3, initiating further downstream signaling cascades. One of the main functions of PIP3 is the activation of the protein

serine/threonine kinase Akt.

Akt is a key regulator of cell survival processes and functions to phosphorylate and activate factors such as the mammalian target of rapamycin (mTOR), forkhead transcription factors, and mdm2 to name a few (Vivanco et al, 2002; Franke et al, 1997). It is important to note that this pathway is negatively regulated by the tumor suppressor phosphatase and tensin homolog on chromosome 10 (PTEN) (Courtney et al, 2010). PTEN limits PI3K activity by dephoshorylating PIP3 to PIP2, thereby abrogating PI3K signaling.

PI3K activation is important not only because it is one of the main signaling pathways downstream to the EGFR family, but also because it plays roles in the development of resistance to HER2 targeted therapies (Gayle et al, 2012; Eichorn et al, 2009).

An effector of PI3K/Akt is the 289KDa serine/threonine kinase mammalian target of rapamycin (mTOR) signaling protein that is highly conserved and plays roles in cell proliferation, survival and motility (Lang et al, 2010). Signaling from this molecule is initiated upon EGFR family member activation that leads to PI3K/Akt phosphorylation and activation. PI3K/Akt functions to inhibit the TSC1/TSC2 complexes, thereby preventing Rheb from inhibiting mTOR. MTORC1 (mTOR, Raptor, mLST8/GBL and PRAS40) and mTORC2 (mTOR, RICTOR, mLST8/GBL, SIN1, and PROTOR/PRR5) are the two distinct complexes through which mTOR exerts cellular effects. The complexes have different functional roles, with mTORC1 having been implicated in cell cycle progression, motility, and protein biosynthesis through its phosphorylation of

substrates 4EBP1 and S6K, while mTORC2 regulates cytoskeleton organization (Wullschleger et al, 2005). Allosteric mTORC1 inhibitors like rapamycin target the PI3K/AKT/mTOR pathway, but have some effects on mTORC2 upon prolonged exposure (24 hours) although the mechanism is unknown (Akcaknat et al, 2007).

Preclinical in vivo studies in which mice were treated with single agent trastuzumab, the mTOR inhibitor rapamycin, or a combination of trastuzumab plus rapamycin showed that the combination was more effective at inducing tumor regression than either of the single agent treatments (Miller et al, 2009). In cell culture experiments using the rapamycin analogue RAD001, a greater amount of growth inhibition was observed with combination mTOR inhibition plus HER2-targeting than with either drug alone. It is thought that the inability of trastuzumab to completely inhibit mTOR signaling can permit synergy of trastuzumab with mammalian target of rapamycin (mTOR) inhibitors to prevent the growth of HER2 cancer cells. In fact, phase I trials have indicated that in patients resistant to trastuzumab, combinatorial everolimus (mTOR inhibitor) and trastuzumab could be a promising treatment (Jerusalem et al, 2011).

Trastuzumab partially decreased PI3K activity, but not mTOR activity (Miller et al, 2009). Increased PI3K signaling is a validated mechanism of trastuzumab resistance, but its association with lapatinib resistance is yet to be determined due to conflicting data (Eichhorn et al, 2008; O'Brien, 2010). Patients with HER2-overexpressing breast cancer who have developed resistance to trastuzumab may be given the dual EGFR/HER2 tyrosine kinase inhibitor lapatinib. Response to single agent lapatinib is less than 25%, indicating cross-resistance between trastuzumab and lapatinib (Blackwell et al, 2010; Eichhorn et al, 2008).

As with trastuzumab treatment, the small subset of patients who initially responded to lapatinib eventually developed resistance, at which point there is no standard therapeutic approach available. Combination mTOR inhibitor plus lapatinib has been found to have clinical benefit (Jerusalem et al, 2011). It is thought that the inability of trastuzumab to completely inhibit PI3K/Akt/mTOR signaling may permit escape from growth inhibition; mTOR inhibitors would thus synergize with trastuzumab to prevent the continued growth of HER2-dependent cancer cells.

v. The MEK/ERK pathway

Similarly to the PI3K/Akt pathway, the MEK1 and MEK2 (MEK) pathway is one of the main downstream signaling pathways to the EGFR family. MEK as well as ERK1 and ERK2 (ERK) are part of the mitogen activated protein kinase (MAPK) family. Upon activation by extracellular molecules such as cytokines, hormones, and growth factors, these proteins can initiate intracellular signaling cascades that lead to increased cell proliferation, differentiation and survival (Raman et al, 2007). Other MAPK family members found in humans include: c-Jun-N-terminal kinases (JNK1, JNK2, JNK3), p38s (p38a, b,c,d), and ERK5.

Upon activation of the EGFR family, the Grb2-SOS signaling axis is activated therefore leading to the GTPase Ras being activated and serving as an adaptor that recruits the Raf kinase to the membrane (Malumbres et al, 2003; McKay et al, 2007). Raf kinases (A-Raf, B-Raf, and C-Raf) are a family of serine/threonine kinases that participate in a signaling cascade of phosphorylation events resulting in MEK 1 and MEK2 activation (MEK1/2).

In humans, MEK proteins consist of a trifunctional N-terminal sequence, a protein kinase domain, and a C-terminal (Fischmann et al, 2009). The N-terminal is comprised of approximately 70 amino acids that make up an inhibitory segment, a nuclear export sequence, and a segment that plays roles in binding ERK substrates. The N-terminal lobe is significantly smaller than the C-terminal lobe and is made up of a five stranded antiparallel beta-sheet and an alpha-C helix (Taylor et al, 2011). The large C-terminal lobe is mostly alpha helical, but it also contains a catalytic residue that plays roles with the phosphoryl transfer from ATP to the ERK1/2 substrates (Roskoski et al, 2012).

MEK phosphorylates ERK1/2 on their TEY sequence, allowing for ERK1/2 nuclear translocation (Robbins et al, 1993). ERK1 and ERK2 sequences are over 80% identical, share substrates, and have similar functions (Lloyd et al, 2006). ERK1/2 phosphorylates substrates bound via two main docking sites, the common docking and the ERK1/2 docking sites (Gibbs et al, 1991). Substrates containing D-domain docking sites bind to ERK1/2 via the Common Docking site and substrates containing the DEF docking site bind to ERK1/2 via the ERK Docking site. Although the MEK/ERK pathway has been implicated in HER2-overexpressing breast cancer, it is not currently an approved therapeutic target. Further evidence supporting the clinical benefits of targeting this pathway is necessary.

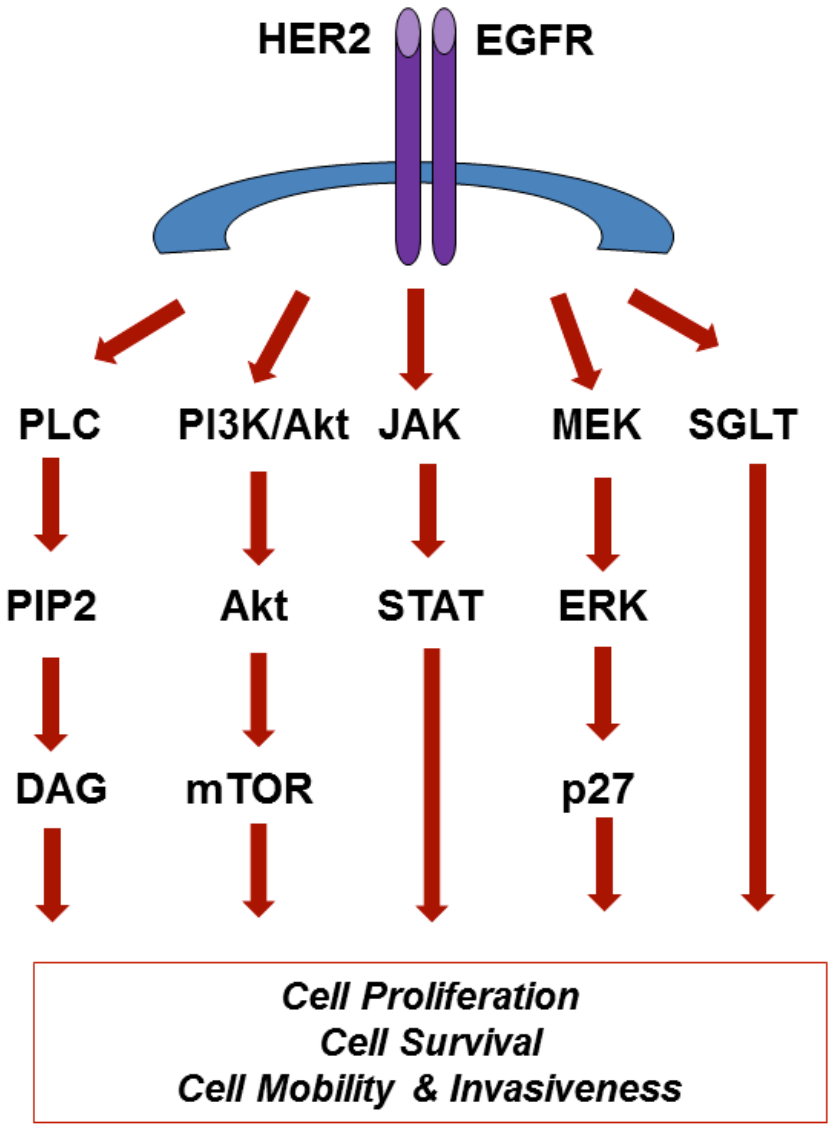

HER2
EGFR
PLC
PI3K/Akt
JAK
MEK
SGLT
PIP2
Akt
STAT
ERK
DAG
mTOR
p27
Cell Proliferation
Cell Survival
Cell Mobility & Invasiveness

Fig. 1-2. HER/EGFR signaling pathways. Upon activation, HER2 and EGFR activate the PLC, PI3K/Akt, JAK/STAT, MEK, and SGLT pathways resulting in cell proliferation, survival, mobility, and invasiveness.

2. HER2 Targeted Therapies

The two main therapeutic approaches developed in an effort to inhibit HER2 signaling are: the inhibition of the receptor with antibodies that bind the extracellular domain, and the inactivation of the tyrosine kinase with small tyrosine kinase inhibitors that diffuse through the cellular membrane to gain access to the intracellular domain (Ocana et al, 2008). Small molecule tyrosine kinase inhibitors can bind to the ATP binding pocket either in a competitive reversible fashion, like lapatinib, or in a noncompetitive irreversible fashion, like neratinib. Antibodies that have shown the most promise clinically are trastuzumab and pertuzimab. Both strategies have demonstrated clinical benefit in a subset of patients, but the development of resistance is still an issue (Ocana et al, 2006).

i. Irreversible pan-HER kinase inhibitors

Irreversible pan-HER inhibitors were developed in an effort to increase the effectiveness of current HER2 targeted agents by preventing the activation of alternative signaling pathways. Irreversible pan-HER inhibitors abrogate receptor signaling by covalently binding to the receptor kinase active site, usually reacting with a nucleophilic cysteine residue (Cohen et al, 2005). Irreversible pan-HER inhibitors were first used in hematological cancers, but have been found to also benefit patients with HER2-dependent breast cancers (Ocana et al, 2009). Currently, the irreversible pan-HER kinase inhibitors that have shown the most promise and thus will be discussed here are neratinib, canertinib (CI-1033), and BIBW-2992 (Tovok).

A. Neratinib (HKI-272)

Preclinical data showed Neratinib (HKI-272) inhibited growth both in cells that

overexpressed HER2, as well as those that had EGFR sensitizing and resistance associated mutations (Kwak et al; Rabindran et al, 2004 2005). A phase I study of advanced solid tumors focused on 25 participants to evaluate Neratinib efficacy. Results showed 32% of participants had partial responses, 64% had poor response, and 4% had stable disease progression. Adverse effects observed were diarrhea, nausea, vomiting, and anorexia. In addition, a multinational multicenter phase II trial in women with HER2-positive metastatic breast cancer evaluated the efficacy of neratinib in two groups: a group previously treated with trastuzumab, and a trastuzumab naïve group (Burstein et al, 2009). Results revealed progression-free survival at 16 weeks for trastuzumab-pretreated and naïve patients was 60% and 77%, respectively, and median time to progression was 23 weeks and 40 weeks, respectively (Burstein et al, 2009). The major adverse effect observed in 85% of the participants, diarrhea, was made manageable by the use of anti-diarrheal agents and neratinib dose modification. Currently, neratinib is being evaluated in combination with the ant-mitotic vinorelbine in phase II studies, and in combination with lapatinib and the prodrug capecitabine that is converted to 5-flourouracil in phase III studies (ClinicalTrials.gov: NCT00706030).

B. Canertinib

Canertinib is an irreversible inhibitor of all HER proteins developed by Pfizer. Results from a phase I dose escalation trial consisting of 32 patients with advance tumors demonstrated that canertinib treatment did not provide an objective response (Rixe et al, 2009). Response to canertinib was higher in patients with HER2-positive breast cancer, although toxicity at the most effective dose was limiting and unacceptable, and so it is

currently not an agent that researchers are focused on.

C. BIBW-2992 (Tovok)

In contrast to canertinib, BIBW-2992, has been demonstrated to provide an objective response. Developed by Boehringer Ingelheim Pharmaceuticals, BIBW-2992 is an oral irreversible small molecule tyrosine kinase inhibitor against EGFR and HER2. A phase II trial in which 41 patients with HER2-positive breast cancer who were not responsive to trastuzumab revealed that BIBW-2992 could be a promising treatment (Hickish et al, 2009). Results demonstrated four of the 41 patients had a partial response, while 14 patients had stable disease progression. Adverse effects observed with treatment were rash and diarrhea.

ii. Reversible pan-HER kinase inhibitors

A. Lapatinib

Lapatinib (Tykerb), the first dual inhibitor of the EGFR and HER2 tyrosine kinases, binds to the receptor ATP binding pocket, thereby preventing receptor phosphorylation and subsequent activation of downstream pathways. A trial of single-agent lapatinib in trastuzumab-refractory advanced breast cancer showed a 12.8% response rate with a median time to progression in the single-agent trial of approximately 4 months (Blackwell et al, 2009). As with trastuzumab treatment, patients who initially responded to lapatinib eventually developed resistance, making it necessary to elucidate mechanisms by which resistance develops.

A Phase III trial comparing lapatinib plus capecitabine with capecitabine alone

indicated the combination treatment was associated with improved overall response rate versus single agent capecitabine (22% vs 14%) and significantly increased median TTP (8.4 vs 4.4 months) in patients with pre-treated HER2-over-expressing advanced disease (Geyer et al, 2006). Lapatinib was subsequently approved by the FDA in 2007 for use in combination with capecitabine for treatment in patients with advanced or metastatic breast cancer whose tumors overexpress HER2 and who have previously been treated with an anthracycline, taxane, and trastuzumab (Medina et al, 2008). Thus, lapatinib is an important and effective therapy for a subset of HER2-over-expressing metastatic breast cancers that have progressed on trastuzumab. However, similar to trastuzumab, the median duration of response to lapatinib was less than one year, and a majority of trastuzumab pre-treated patients (almost 80%) failed to respond to lapatinib.

Combination trastuzumab plus lapatinib treatment has been shown to induce apoptosis in part via down-regulation of survivin in cell culture and animal models (Xia et al, 2005). Initial phase I data suggested the combination is well-tolerated and elicits partial or complete responses in a subset of patients who have progressed on prior trastuzumab therapy (Storniolo et al, 2008). The combination has been tested clinically in advanced phase trials in patients who have progressed on trastuzumab-based regimens. Progression-free survival and quality of life were improved in patients treated with the combination versus lapatinib alone (Wu et al, 2011). EGF104900 showed the combination was superior to lapatinib alone in the trastuzumab-resistant setting, with a clinical benefit rate of 24.7% versus 12.4% (Blackwell et al, 2010). A potentially important mechanism of action for this drug combination is the lapatinib induced accumulation of inactive HER2 dimers via reduced receptor ubiquitination, providing

increased pharmacologic target for trastuzumab- mediated antibody-dependent cellular cytotoxicity (Scaltriti et al, 2009). Combining trastuzumab with lapatinib offers a chemotherapy-free option for treating HER2-positive trastuzumab- resistant disease.

iii. HER2 targeted Antibodies

A. Pertuzumab

Pertuzumab, an anti-HER2 monoclonal antibody, targets an extracellular epitope distinct from what is targeted by trastuzumab. Pertuzumab binds HER2 near the center of domain II, sterically blocking a binding pocket necessary for receptor dimerization and signaling (Franklin et al, 2004). In contrast, trastuzumab does not significantly inhibit HER2 interaction with other erbB receptors. Nahta et al, were the first to show combining pertuzumab with trastuzumab results in synergistic inhibition of proliferation of HER2-overexpressing breast cancer cells (Nahta et al, 2004a). Trastuzumab increased pertuzumab-mediated disruption of HER2 dimerization with EGFR and HER3, and further reduced pertuzumab-mediated inhibition of PI3K signaling (Nahta, 2004a). Phase II data shows combining trastuzumab with pertuzumab in patients who have progressed on prior trastuzumab regimens achieves a clinical benefit rate of 50%, objective response rates of 24%, and median progression-free survival of 5. 5 months (Baselga et al, 2010a).

A potential mechanism of synergy between pertuzumab and trastuzumab is non-overlapping mechanisms by single agents, trastuzumab-mediated inhibition of p95HER2 cleavage and pertuzumab-mediated disruption of dimerization (Scheuer et al, 2009). Clinical evaluation of pertuzumab and trastuzumab (CLEOPATRA) is currently being conducted in an international, randomized, double-blind, placebo-controlled phase III

trial. HER2-positive breast cancer patients with locally recurrent or metastatic disease will be randomized to receive either docetaxel, trastuzumab, and pertuzumab, or docetaxel, trastuzumab, and placebo. Progresion-free survival will be assessed to determine the efficacy of combination pertuzumab plus trastuzumab in the trastuzumab-refractory setting (Baselga et al, 2010b).

B. Trastuzumab

Trastuzumab (Herceptin) is a recombinant humanized monoclonal anti-HER2 antibody developed by Genentech. It is approved by the US Food and Drug Administration for treatment in patients with metastatic HER2-overexpressing breast cancer (Goldenberg et al, 1999). Trastuzumab is a purified recombinant DNA-derived humanized monoclonal antibody produced using hamster ovaries in medium containing the antibiotic gentamicin. The antibody contains human regions with complementary regions of murine 4D5 antibody (Stancovski et al, 1991). Trastuzumab selectively binds to domain IV of the extracellular region of HER2, and has been administered either as a single agent or in combination with paclitaxel or carboplatin, resulting in a significant inhibition of tumors that overexpress HER2. The mean half-life of trastuzumab is 5.8 days, with steady state of serum achieved when there is a mean trough of 79pg/mL and peak concentration of 123pg/mL (Baselga et al, 1996).

Early preclinical and clinical studies investigating the effectiveness of trastuzumab provide evidence it is clinically beneficial. In a single arm clinical study consisting of 222 patients with HER2-overexpressing breast cancer, single agent trastuzumab treatment

administered intravenously with a loading dose of 4mg/kg followed by weekly 2mg/kg doses resulted in a response rate of 14% (Cobleigh et al, 1998). In another study, 469 metastatic HER2-overexpressing breast cancer patients were treated with either paclitaxel and cyclophosphamide, or anthracycline and cyclophosphamide, both in the absence or presence of trastuzumab (Slamon et al, 1998). Study results showed patients with trastuzumab as a part of their treatment regimen had a significantly better overall response when compared to patients treated with chemotherapy alone, with the greatest benefit observed in the paclitaxel plus trastuzumab group. Common adverse effects observed with trastuzumab treatment include fever, chills, pain, asthenia, vomiting, diarrhea, headache, dyspnea, rhinitis, and insomnia.

Ten years later, trastuzumab is still the first line treatment for HER2 positive breast cancer (Aebi et al, 2010, Cardoso et al, 2010). Recent studies show trastuzumab treatment provides a significant benefit for patients with various stages of breast cancer, with the addition of trastuzumab to neoadjuvant chemotherapy resulting in a 33% increase in overall survival and reduced recurrence rate when administered in early stage breast cancer (Petrelli et al, 2010). In addition, disease free and overall survival are both increased to about 36% by trastuzumab adjuvant treatment, with the risk of recurrence reduced to about 40% (Dahabreh et al, 2008). For patients with metastatic breast cancer, the addition of trastuzumab to chemotherapy results in approximately a 50% increase in the time to progression and approximately a 40% increase in the time to failure, resulting in a 20% improvement in overall survival when compared to chemotherapy alone (Slamon et al, 2001).

A novel preparation of trastuzumab is the drug conjugate trastuzumab-DM1, which

is trastuzumab conjugated to a microtubule-depolymerizing drug called maytansinoid (Lewis Phillips et al, 2008). Trastuzumab-DM1 blocks growth of both trastuzumab-naive and trastuzumab-refractory HER2-overexpressing breast tumors in vivo (Lewis Phillips et al, 2008), and retains the mechanistic activity of unconjugated trastuzumab (Junttila et al, 2010). Antibody- dependent cellular cytotoxicity is induced by trastuzumab-DM1, and tumor growth of trastuzumab-resistant cells is blocked by trastuzumab-DM1 due to induction of apoptosis and mitotic catastrophe (Barok et al, 2011). A phase I dose-escalation study in patients who progressed on trastuzumab showed a clinical benefit in 15 of 24 patients, including objective responses in 5 patients (Krop et al, 2010). A phase II study of trastuzumab-DM1 in patients with trastuzumab-refractory HER2-positive breast cancer showed objective response of 25.9% and a median progression-free survival of 4.6 months (Burris et al, 2011). Thus, trastuzumab-DM1 HER2 antibody-chemotherapy conjugate is a promising treatment for HER2-positive breast cancer that progressed on prior HER2-directed therapies.

4. Mechanisms of resistance to HER2 Targeted Therapies

i. Mechanisms of resistance to trastuzumab

Trastuzumab is an effective treatment in HER2-overexpressing breast cancer, but has limited clinical benefit due to the frequent development of refractory disease within a year. Considerable research has been dedicated to understanding the mechanisms by which trastuzumab resistance develops. In addition to HER2 mediated mechanisms of resistance, the overexpression of the EGFR family members, EGFR, HER3, and HER4, has been linked to trastuzumab resistance. Extensive research has been performed to

elucidate the role EGFR plays in the development of trastuzumab resistance, and will be discussed in detail. Also to be discussed in detail is the role of the insulin like growth factor receptor (IGF-IR) in trastuzumab resistance. IGF-IR is not part of the EGFR family, but has been demonstrated to physically interact with HER2 and result in a decreased response to trastuzumab (Lu et al, 2001). Other mechanisms of trastuzumab resistance of importance discussed here will include p27 downregulation, mTOR pathway signaling, and PTEN deficiency/increased Akt activity.

Although the mechanisms by which trastuzumab treatment produces anti-cancer effects have not been elucidated, research has revealed that they include:

1. Trastuzumab binding to the extracellular region of HER2, thereby blocking intracellular signaling. This has been shown to lead to up-regulation of the cyclin dependent kinase inhibitor $p27^{kip\ 1}$, which inhibits activation of the cyclin E/ cyclin dependent kinase 2 (CDK2) complex, resulting in G1 cell cycle arrest (Lane et al, 2001; Le et al, 2003).
2. Trastuzumab induced membrane localization and activity of phosphatase and tensin homolog (PTEN), thereby inhibiting the PI3K pathway and cellular proliferation.
3. Trastuzumab induced rapid dissociation of the non-receptor tyrosine kinase Src from HER2, thereby reducing Src activity and resulting in PTEN de-phosphorylation and translocation to the membrane.
4. Trastuzumab binding to the HER2 juxtamembrane domain of HER2 resulting in antibody downregulated expression of HER2 (Cuello et al, 2006).
5. Trastuzumab selectively blocking ligand-independent HER2/HER3

heterodimerization (Junttila et al, 2009).

6. Trastuzumab binding to HER2 and leading to a proteolytic cleavage of the extracellular domain that results in decreased levels of the more active p95 HER2 (Molina et al, 2009).
7. Trastuzumab triggering of immune-mediated responses against HER2-overexpressing cells by engaging Fc receptors on immune effector cells leading to antibody-dependent cellular toxicity (Clynes et al, 2000; Arnould et al, 2006).

Of the several mechanisms discussed above, the first three listed trastuzumab mechanisms involving p27/CDK2, PTEN, and Src will be discussed in further detail.

A. p27/CDK2

Trastuzumab induces G1 arrest by several mechanisms including increased expression of cyclin-dependent kinase inhibitor p27kip1, which inhibits cyclin E/cdk2 and cyclin A/cdk2 complexes and blocks cell cycle progression through S phase (Lane, 2001; Le, 2003). Trastuzumab induces p27kip1expression by suppressing expression of proteins that sequester p27kip1, which also results in increased interaction between p27kip1 and cdk2, leading to cdk2 inactivation (Lane, 2001).

It was previously reported (Nahta et al, 2004b) that cells with acquired trastuzumab resistance showed increased proliferation, reduced p27kip1 expression, reduced p27kip1-cdk2 interaction, and increased cdk2 activity relative to parental trastuzumab-sensitive cells. Transfection of wild-type p27kip1 increased trastuzumab sensitivity in cells with acquired resistance (Nahta et al, 2004b). Yakes et al, (Yakes, 2002), showed knockdown

of p27kip1 reduced trastuzumab sensitivity in HER2-overexpressing breast cancer cell lines, further supporting a requirement of p27kip1 expression for optimal response to trastuzumab.

Post-translational modification of p27kip1 occurs primarily by phosphorylation, with subsequent protein ubiquitination and degradation. Preliminary data supporting ubiquitin-proteasome degradation of p27kip1 as a mechanism of p27kip1 down-regulation in trastuzumab resistance includes our finding that proteasome inhibitor MG132 induced p27 expression and reduced viability of resistant cells (Nahta et al, 2004b). Further, Cardoso et al. (Cardoso et al, 2006) showed that the proteasome inhibitor bortezomib induced p27kip1 and increased the efficacy of trastuzumab in HER2-overexpressing breast cancer cells.

PI3K inhibition has been shown to induce p27kip1 expression, and is believed to contribute to p27kip1 down-regulation and acquired trastuzumab resistance. In addition to observing reduced p27kip1 levels in models of acquired resistance, our data indicates p27kip1 expression is down- regulated post-transcriptionally in cells with primary trastuzumab resistance (Fig. 1-2). Cyclin E expression is regulated by HER2 expression status, in that HER2 knockdown resulted in reduced cyclin E level and reduced cyclin E-associated kinase activity (Mittendorf et al, 2010). In addition, HER2-overexpressing breast cancers that also show increased cyclin E expression have lower 5 year disease-free survival versus those that have lower cyclin E levels (Mittendorf et al, 2010).

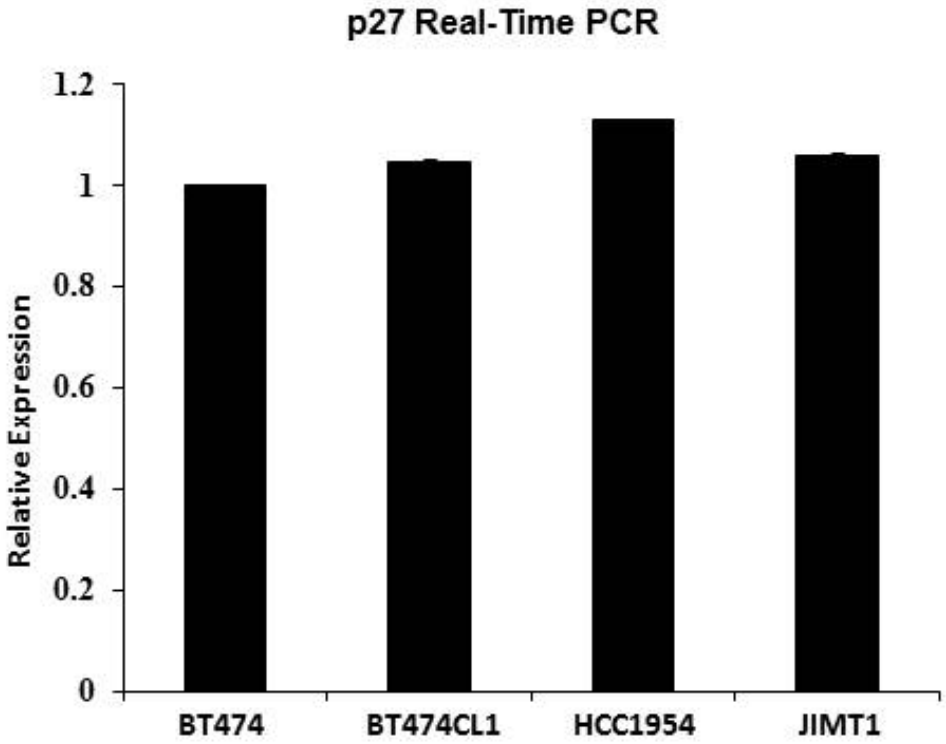

p27 Real-Time PCR
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
BT474
BT474CL1
HCC1954
JIMT1

Fig. 1-3. p27 expression in models of intrinsic (primary resistance). BT474 and acquired resistant clone BT-HRc1 and primary resistant HCC1954 and JIMT-1 cells were examined by real-time PCR for p27 transcript which was normalized to ribosomal protein, large, P0 (RPLPO) housekeeping gene.

B. PTEN deficiency/increased Akt activity

HER2 signaling is initiated upon receptor dimerization, which induces phosphorylation of tyrosine residues within the receptor cytoplasmic domain. The phosphorylated residues serve as docking sites for adaptor proteins and link the receptor to downstream survival pathways including the PI3K/Akt/mTOR axis (Spector et al, 2009). The PI3K pathway is frequently hyperactivated in many cancers. An association between oncogenic PI3K mutations and trastuzumab resistance was found in a study examining HER2- overexpressing tumors from patients with trastuzumab-refractory disease (Berns et al, 2007). About 25% of tumors analyzed had PIK3CA mutations, and reduced PTEN expression was present in 22% of the tumors.

Two important studies suggest increased PI3K signaling correlates with reduced response of HER2-over-expressing metastatic breast cancers to trastuzumab Nagata et al, 2004; Berns et al, 2007). Constitutive PI3K signaling has been noted to occur in solid tumors, due to either loss of the phosphatase and tensin homolog (PTEN) phosphatase, a negative regulator of PI3K activity (Eng et al, 2003) or hyper-activating mutations in *PIK3CA*, which encodes the p110 alpha catalytic subunit of PI3K (Samuels et al, 2004).

Among 47 primary tumors obtained from patients treated with trastuzumab plus taxane, IHC indicated that protein expression of PTEN was reduced in approximately 36% of tumors (Nagata et al, 2004). Patients whose tumors showed PTEN staining intensity of less than half of what is observed in mammary tissues of healthy individuals were defined as PTEN- deficient; response rates to trastuzumab plus taxane were 35.7% in PTEN-deficient patients versus 66.7% for patients defined as PTEN-positive (normal level). A statistically significant trend was noted such that the probability of responding

to trastuzumab was reduced as PTEN staining decreased. In contrast, although approximately 43% of patients treated with taxane showed PTEN loss, there was no correlation between response to taxanes alone and PTEN staining. Thus, the study concluded that PTEN deficiency is associated with reduced response to trastuzumab. The study did not address the exact mechanism by which PTEN protein expression was reduced (i.e., gene level or post- transcriptional). However, previous studies have demonstrated that although loss of heterozygosity at PTEN chromosomal region 10q23 occurs in approximately 41% of sporadic breast carcinomas (Singh et al, 1998), somatic mutation in the *PTEN* gene is extremely rare, estimated to occur in <5% of sporadic breast tumors (Eng et al, 2003). Thus, reduced expression of the PTEN protein in breast cancer may be due to post- transcriptional events (e.g., increased protein degradation).

A large-scale RNA interference genetic screen identified *PTEN* as the top short hairpin RNA (shRNA) out of 24,000 shRNA to confer trastuzumab resistance *in vitro* (Berns et al, 2007). The investigators then examined 55 tumor samples from patients treated with trastuzumab alone (6 patients) or trastuzumab plus chemotherapy (49 patients) for PTEN expression by IHC and also for *PIK3CA* mutation status by direct sequencing or SNP-based analysis. Reduced PTEN expression was noted in 22% of tumor samples, and while these patients showed a trend for worse PFS, this was not statistically significant. *PIK3CA* mutations were found in exons 20 and 9 in 25% of a total of 14 tumors which were analyzed. PTEN loss and *PIK3CA* mutation rarely occurred together. Shorter PFS was noted in patients with *PIK3CA* mutation, with borderline statistical significance (p=0.052). When the authors grouped patients according to whether tumors showed “activated PI3K,” de- fined as presence of either

PIK3CA mutation or PTEN loss, versus "non-activated PI3K," PFS was significantly shorter for patients with activated PI3K signaling, with multivariate analysis showing hazard ratio = 1.9.

Both of these studies indicate that increased PI3K signaling promotes resistance to trastuzumab. Mechanisms by which PI3K is activated may differ, and include PTEN loss (reduced negative regulation of PI3K signaling) and oncogenic activating PI3K mutation. The first study (Nagata et al, 2004) showed statistically significant difference in response to trastuzumab with reduced expression of PTEN, whereas the second study (Berns et al, 2007) did not. The discrepancy in results could possibly be due to differences in the chemotherapy included with trastuzumab in each study, as the second study (Berns et al, 2007) included a heterogeneous population treated with various chemotherapy agents versus the first study (Nagata et al, 2004) including only combination with a taxane. Mutations in *PIK3CA* are found in 25%-30% of all human breast cancers (Karakas et al, 2006). This is consistent with the number of *PIK3CA* mutations observed by Berns *et al*, 2007. Overall, the conclusion of both studies is that PI3K activation predicts for resistance to trastuzumab. Thus, combination analysis of PTEN loss at the protein level and *PIK3CA* mutational status at the gene level may be a useful predictive assay for determining which patients will respond to trastuzumab-based therapy.

C. Src rapid dissociation

Trastuzumab treatment of HER2-overexpressing breast cancer cells results in inhibition of Src non-receptor tyrosine kinase (Nagata et al, 2004). Src inhibition appears to be important to trastuzumab-mediated anti-cancer activity, as increased Src signaling is

associated with trastuzumab resistance (Mitra et al, 2009; Liang et al, 2010; Zhang et al, 2011). One mechanism leading to increased Src activity appears to be a variant of HER2, HER2Delta16 (Mitra et al, 2009), which shows increased oncogenic activity. Local disease progression involved HER2Delta16 in 89% of breast cancer patients with HER2-positive tumors (Mitra et al, 2009). Transfection of MCF7 or NIH3T3 cells with HER2 delta 16 promoted receptor dimerization, invasion, and trastuzumab resistance (Mitra et al, 2009).

The oncogenic properties of HER2Delta16 were mediated through direct interaction of HER2Delta16 with Src kinase. Activated Src kinase was found in 44% of HER2Delta16-positive breast carcinomas (Mitra et al, 2009). Dual targeting of HER2Delta16 plus Src with dasatinib resulted in Src inactivation, destabilization of HER2Delta16, and decreased tumorigenicity (Mitra et al, 2009). In addition, Src activation via Jak2 has been shown to reduce trastuzumab activity (Liang et al, 2010). Recombinant human erythropoietin activated Jak2-Src signaling and inactivated PTEN in HER2-positive cells (Liang et al, 2010). Combined treatment with recombinant human erythropoietin plus trastuzumab reduced response to trastuzumab in cell culture and *in vivo* models. Further, shorter progression-free and overall survival was found in patients with HER2-positive breast cancer treated concurrently with erythropoietin and trastuzumab (Liang et al, 2010). Src was also shown to be activated in primary and acquired trastuzumab resistance as a consequence of PTEN loss (Zhang et al, 2011). Finally, src-targeted therapy blocked growth of trastuzumab-resistant tumors *in vivo* (Zhang et al, 2011). Therefore, Src activation may occur via multiple mechanisms, ultimately abrogating sensitivity to trastuzumab. Combining Src-targeted therapy with

trastuzumab may offer benefit to patients with HER2-overexpressing breast cancer.

D. EGFR-overexpression

Studies have revealed that EGFR amplification plays roles limiting trastuzumab's efficiency at modulating EGFR family member activation and interaction on the cell surface (Diemier et al, 2005). The inhibitory effects of trastuzumab on cell proliferation both in the presence and absence of Epidermal growth factor (EGF) and Heregulin (HRG) were evaluated using HER2 overexpressing BT474 cells that have low levels of EGFR, and HER2 overexpressing SKBR3 cells that have amplified EGFR (Brockhoff et al, 2001). In both BT474 and SKBR3 cells, EGF resulted in a strong phosphorylation of EGFR at Y1173 when compared to untreated cells. Trastuzumab slightly diminished the EGF induced phosphorylation in BT474 cells, but not in SKBR3 cells. In addition, trastuzumab treatment induced a slight HER2 Y887 phosphorylation in BT474, but a strong phosphorylation in SKBR3 cells both in the presence and absence of EGF and HRG; this effect was not observed with single agent EGF and HRG treatment. All combinations of ligand and trastuzumab treatment were not able to induce phosphorylation at Y1112 in BT474 cells, in contrast to SKBR3 cells which had a strong induction of phosphorylation at Y1112 with EGF both in the presence and absence of trastuzumab. The addition of trastuzumab resulted in a strong induction of phosphorylation of Y1248 in BT474 and SKBR3, both in the presence and absence of EGF and HRG. However, the trastuzumab induced effect in SKBR3 is 14 fold more in the absence of growth factors. Investigation of changes in EGFR and HER2 dimerization upon EGF and trastuzumab treatment revealed an increase in EGFR/HER2 dimerization

in BT474 cells.

BT474 cells have a considerable amount of EGFR/HER2 hetero-dimerization in the absence of EGF, with addition of EGF addition resulting in an increase both in the absence and presence of trastuzumab. On the other hand, in SKBR3, EGF treatment resulted in an attenuation of EGFR/HER2 physical interaction, with HRG and trastuzumab treatment not significantly disrupting the interaction. In both cell lines, trastuzumab treatment induced HER2 homo-dimerization independently of EGF and HRG. These experiments evaluating response in BT474 and SKBR3 cells provided evidence that trastuzumab more effectively inhibits cell proliferation when EGFR expression is low. Increased EGFR expression leads to a decreased response to trastuzumab suggesting a role for the EGFR/HER2 heterodimers in trastuzumab resistance.

E. IGF-IR

The association of increased IGF-IR activity with the development of trastuzumab resistance in HER2-overexpressing breast cancer makes IGF-IR an important target. A subset of HER2/IGF-IR-overexpressing cells were found to be less sensitive to the growth inhibitory effects of trastuzumab when compared to HER2-overexpressing cells that do not overexpress IGF-IR (Lu et al, 2001). Taking this data into account, researchers have been working toward the goal of developing agents that target IGF-IR for the past several years with each generation of agents aimed at producing a greater benefit for the patient while decreasing adverse effects.

IGF-IR is a heterotrimeric transmembrane tyrosine kinase receptor that regulates cell metabolism and growth (Chaves et al, 2010), and has been associated with increased risk and maintenance of multiple cancers including HER2-overexpressing breast cancer (Esparis-Ogando et al, 2008; Hankinson et al, 1998; Surmacz et al, 2000). Circulating ligands of the insulin-like growth factor (IGF) system include IGF-I and IGF-II, with IGF-I having the highest affinity for IGF-IR. Upon binding to IGF-IR, a receptor conformational change is induced that leads to tyrosine phosphorylation and activation of several downstream survival signaling pathways such as the Ras/Raf/mitogen activated protein kinase pathway (MAPK), and the PI3K/Akt/mTOR pathway. Activation of these pathways results in cell cycle progression and resistance to apoptosis (Chaves et al, 2011; Adams et al, 2000).

In addition, flow cytometry revealed that after trastuzumab treatment, HER2 overexpressing cells were less likely to progress through the cell cycle and stopped at the G1 phase, while a greater number of HER2/IGF-IR overexpressing cells passed the restriction point and completed the cell cycle. These results demonstrate that IGF-IR interferes with the growth inhibitory actions of trastuzumab, supporting therapeutic strategies that co-target HER2 and IGF-IR. Further, we discovered that signaling interactions exist between IGF-IR and HER2 in trastuzumab-resistant cancers (Nahta et al, 2005; Jin et al, 2008).

Immunoprecipitation and immunoblotting experiments revealed that IGF-I stimulation results in an increase in IGF-IR phosphorylation more rapidly in trastuzumab-resistant cells than in trastuzumab-sensitive cells. Furthermore, IGF-IR heterodimerization with HER2 results in HER2 activation in trastuzumab-resistant cells,

but not in trastuzumab-sensitive cells, indicating crosstalk between the two receptors. Kinase inhibition or antibody blockade of IGF-IR restores trastuzumab sensitivity. Treatment of trastuzumab-resistant breast cancer cells with the highly specific IGF-IR antibody alpha IR3 disrupted the IGF-IR/HER2 heterodimer and increased trastuzumab sensitivity. These results suggest that IGF-IR targeted treatments may be useful in combination with trastuzumab.

An IGF-IR targeted treatment that has previously been considered is the addition of IGF binding proteins (IGFBPs). IGFBPs modulate IGF-IR activity by binding to the IGF ligands thereby sequestering them and preventing ligand-induced receptor activation (Adams et al, 2000). Higher levels of circulating IGF-I have been linked to trastuzumab resistance in HER2-overexpressing breast cancer, with the addition of IGFBP3 decreasing IGF-IR activity, and subsequently resulting in an increased response to trastuzumab (Lu et al, 2001; Jerome et al, 2006). Our gene microarray analysis data indicated that IGFBP3 and IGFBP5 are down-regulated in resistant versus sensitive cells (Table 1-1). However, ELISA of secreted IGFBP3 (Fig. 1-3A) or real-time PCR analysis of endogenous IGFBP3 or IGFBP5 transcript level (Fig. 1-3B,C) failed to show any differences in IGFBP3 or IGFBP5 level in resistant versus parental cells. Thus, our data did not support down-regulation of IGFBP3 or IGFBP5 as a mechanism of increased IGF-IR signaling in trastuzumab resistance.

Table 1-1. Genes that are down-regulated in SKBR3- and BT474-derived acquired trastuzumab-resistant cells versus parental SKBR3 and BT474 cells

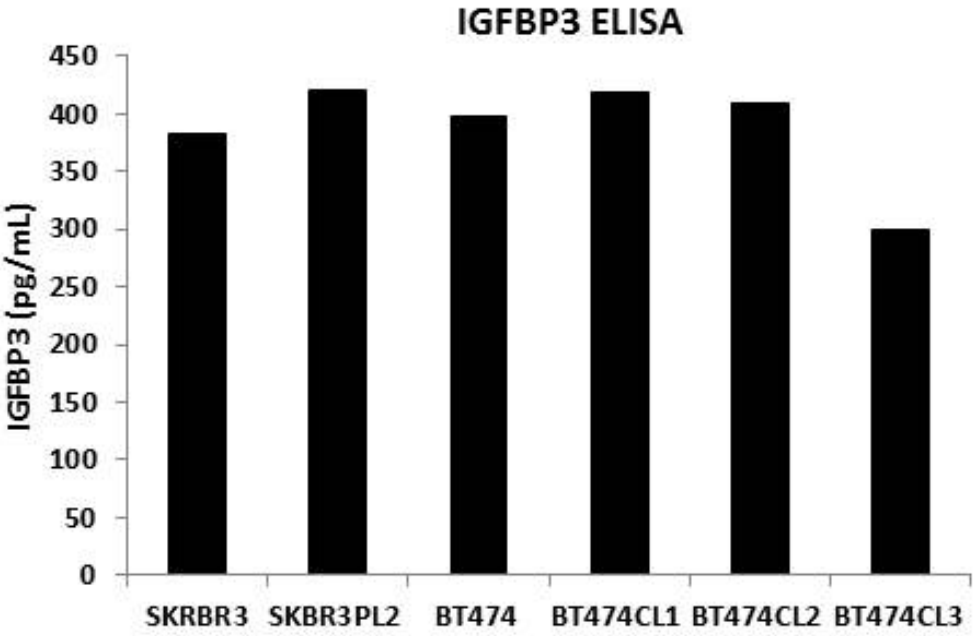
Gene Name	Fold Change	ILMN_GENE	DEFINITION
IGFBP5	-20. 55848937	IGFBP5	Homo sapiens insulin-like growth factor binding protein 5 (IGFBP5), mRNA.
IGFBP5	-20. 0185274	IGFBP5	Homo sapiens insulin-like growth factor binding protein 5 (IGFBP5), mRNA.
IGFBP3	-7. 77282369	IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2, mRNA.
PKIA	-6. 484521044	PKIA	Homo sapiens protein kinase (cAMP-dependent, catalytic) inhibitor alpha (PKIA), transcript variant 7, mRNA.
IGFBP3	-6. 193624741	IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 1, mRNA.
PKIA	-5. 371909749	PKIA	Homo sapiens protein kinase (cAMP-dependent, catalytic) inhibitor alpha (PKIA), transcript variant 6, mRNA.
BASP1	-4. 444496135	BASP1	Homo sapiens brain abundant, membrane attached signal protein 1 (BASP1), mRNA.
HERC6	-4. 048474978	HERC6	Homo sapiens hect domain and RLD 6 (HERC6), mRNA.
FRAS1	-3. 988854857	FRAS1	Homo sapiens Fraser syndrome 1 (FRAS1), mRNA.
THBS1	-3. 966312615	THBS1	Homo sapiens thrombospondin 1 (THBS1), mRNA.

Table 1-1. Genes down-regulated in SKBR3- and BT474-derived acquired trastuzumab-resistant cells versus parental SKBR3 and BT474 cells by 4-fold or more. Gene microarray analysis of genes downregulated in HER2-overexpressing breast cancer resistant versus sensitive cells.

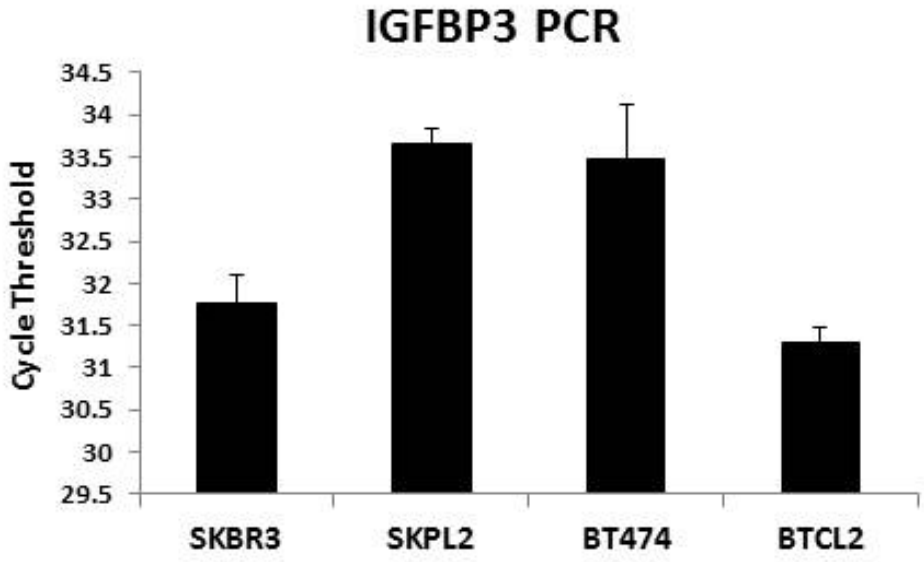
IGF-IR overexpression and crosstalk with HER2 suggests that IGF-IR plays a crucial role in conferring trastuzumab resistance. The molecular signaling pathways by which IGF-IR confers resistance to trastuzumab is not clear, although downstream focal adhesion kinase (FAK) and PI3K/Akt pathway signaling likely play a role (Yang et al, 2010). This data linking IGF-IR to the development of trastuzumab resistance, along with the increased sensitivity to trastuzumab upon IGF-IR inhibition provides a rational for the development of combinatorial HER2 and IGF-IR targeting.

Although a limited amount of research has been done on the effects of IGF-IR signaling on lapatinib resistance, the small amount of information we have suggests that the IGF-IR/HER2 targeting is unnecessary with lapatinib treatment. Contrary to what we would expect, high levels of IGF-IR have been correlated with high sensitivity to lapatinib (Spector et al, 2005). Also, lapatinib was demonstrated to induce cell cycle arrest and apoptosis in trastuzumab sensitive as well as resistant HER2-overexpressing breast cancer cells in the presence of IGF (Spector et al, 2005).

A


IGFBP3 ELISA
IGFBP3 (pg/mL)
450
400
350
300
250
200
150
100
50
0
SKRBR3
SKBR3PL2
BT474
BT474CL1
BT474CL2
BT474CL3

B


IGFBP3 PCR
Cycle Threshold
34.5
34
33.5
33
32.5
32
31.5
31
30.5
30
29.5
SKBR3
SKPL2
BT474
BTCL2

C

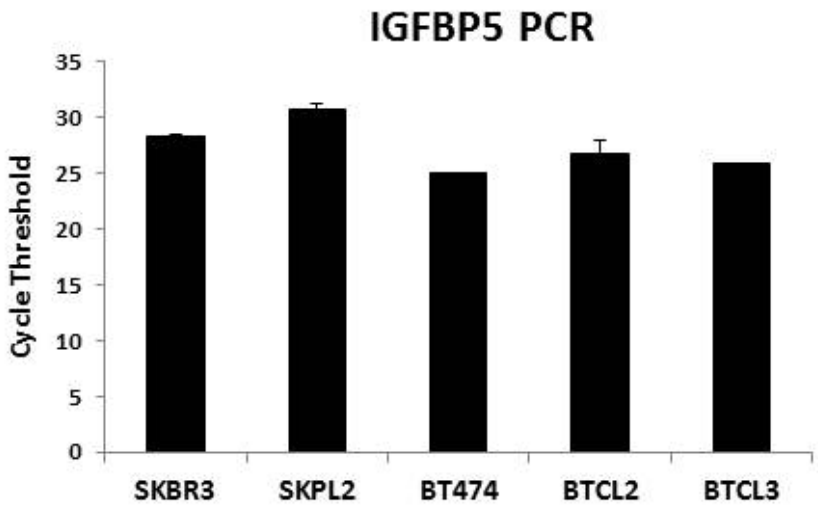

IGFBP5 PCR
Cycle Threshold
35
30
25
20
15
10
5
0
SKBR3
SKPL2
BT474
BTCL2
BTCL3

Fig. 1-4. IGFBP3 and IGFBP5 in resistant and sensitive cells. (A) Secreted IGFBP3 was assessed by ELISA in SKBR3 parental, resistant pool 2, BT474 parental, resistant clone 2 and clone 3 cells. IGFBP3 is shown in pg/mL and was measured in triplicate with reproducible results per line. (B) Real-time PCR analysis of IGFBP3 and (C) IGFBP5 was examined in triplicate per line, with error bars representing standard deviation between replicates. Housekeeping gene RPLPO was measured as an internal control; IGFBP3 and IGFBP5 values are normalized to RPLPO.

ii.Mechanisms of resistance to lapatinib

Further research investigating the mechanisms of resistance to lapatinib is required, but studies have been performed suggesting roles for MEK, Src, AXL, and PTEN deficiency/increased Akt activity,

A. MEK/ERK/BIM

Work by Tanizaki et al (Tanizaki, 2011), linked the MEK/ERK pathway to lapatinib resistance in HER2-overexpressing breast cancers. Their results demonstrated that one of the mechanisms by which lapatinib results in apoptosis involves the induction of the pro-apoptotic protein Bcl-2 interacting mediator of cell death (BIM). Evidence exists that the mechanism by which MEK signaling decreases response to lapatinib involves the downregulation of BIM expression. MEK inhibition by pharmacologic and genetic agents resulted in increased BIM expression, with BIM RNA interference mediated depletion demonstrated to prevent lapatinib induced apoptosis. This data suggests that BIM upregulation contributes to the apoptotic effects observed with lapatinib treatment (Tanizaki et al, 2011).

B. Src

The tyrosine kinase src which was previously discussed as it relates to trastuzumab resistance could also be involved in lapatinib resistance. Preclinical evidence links src activity to decreased lapatinib response (Rexer et al, 2011). Lapatinib resistant cells were demonstrated to have elevated src levels with combination src inhibition plus lapatinib resulting in increased lapatinib sensitivity.

C. AXL

The overexpression of the membrane bound receptor tyrosine kinase AXL is associated with poor prognosis and increased invasiveness in various cancers including breast cancer (Zhang et al, 2008). AXL has been linked to the resistance of multiple small molecule tyrosine kinase inhibitors including lapatinib (Mahadevan et al, 2007; Hong et al, 2008). Liu et al, (Liu, 2009), found an increase of AXL expression in lapatinib resistant HER2-overexpressing BT474 cells, with lapatinib sensitivity restored by AXL inhibition either by siRNA targeted against AXL, or the AXL inhibitor GSK1363089. These findings suggest that AXL inhibition could improve the response to lapatinib in patients with HER2-overexpressing breast cancer.

D. PTEN deficiency/increased Akt activity

The role of Akt in lapatinib resistance is controversial. Researchers like O'Brien et al, 2010, did not find an association between hyperactive PI3K and lapatinib resistance. Wang et al, 2011 found that patients with the loss of PTEN or hyperactive PI3K had lower clinical benefit than patients without hyperactive PI3K. With respect to lapatinib and PI3K signaling, knockdown of *PTEN* did not alter response to lapatinib *in vitro* (Xia et al, 2007). In addition, a phase II trial of lapatinib monotherapy in inflammatory breast cancer demonstrated that PTEN loss was not associated with reduced response to lapatinib, as approximately 70% of responders showed PTEN deficiency (Xia et al, 2007; Johnston et al, 2008). Thus, these studies suggest that PTEN loss does not predict for resistance to lapatinib. In contrast, a genome wide loss-of-function shRNA screen showed that PTEN loss promoted lapatinib resistance *in vitro* (Eichorn et al, 2008). In

addition, *PIK3CA* mutations were associated with lapatinib resistance *in vitro*. Thus, clinical data in inflammatory breast cancer does not support an association between lapatinib response and PTEN status; however, additional *in vitro* data supports further detailed analysis of activated PI3K signaling as a predictor of lapatinib resistance in metastatic breast cancer.

5. Scope of this dissertation

This dissertation aims to use the knowledge by which resistance to lapatinib is acquired to develop novel therapeutic treatments for patients with HER2-overexpressing breast cancer. The PI3K/Akt/mTOR and MEK/ERK pathways have been linked to the development of resistance to HER2 targeted therapies. I propose that combination lapatinib treatment and inhibition of the either the PI3K/Akt/mTOR pathway or combination lapatinib treatment and inhibition of the MEK/ERK pathway will achieve an optimal lapatinib response. The data reveals that reduced lapatinib sensitivity is associated with an inability of lapatinib to inhibit both Akt and MEK/ERK phosphorylation. An investigation of the role of Akt in lapatinib resistance demonstrated that the transfection of constitutively active Akt reduces lapatinib sensitivity, while kinase-dead Akt increases sensitivity. Knockdown of 4EBP1 also increases lapatinib sensitivity, in contrast to p70S6K knockdown, which does not affect response to lapatinib. Further, the pharmacologic inhibition of mTOR using rapamycin or ridaforolimus increases lapatinib sensitivity and reduces Akt phosphorylation levels in cells that show poor response to single agent lapatinib, including those transfected with hyperactive Akt. An evaluation of the role that MEK/ERK plays in lapatinib resistance

demonstrates that genetically and pharmacologically blocking MEK/ERK signaling improves response to lapatinib. Also, combination MEK inhibitor plus lapatinib results in a reduced nuclear expression of the MEK/ERK downstream proto-oncogene FOXM1. In addition, xenograft studies demonstrate that pharmacologic inhibition of MEK increases lapatinib sensitivity in HER2-overexpressing breast cancers that are resistant to trastuzumab and lapatinib. This dissertation provides mechanisms by which resistance to lapatinib is achieved, and strongly supports future clinical trials of combination lapatinib plus mTOR inhibition, as well as combination lapatinib plus MEK inhibition.

Chapter 2. Materials and Methods

1. General Methods

i. Reagents

Lapatinib was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lapatinib for *in vitro* studies was purchased from Santa Cruz, Biotech (Santa Cruz, CA), and dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM. Lapatinib for *in vivo* studies was purchased from the Winship Cancer Institute pharmacy and dissolved in 1% Tween 80 at a stock concentration of 7.5 mg/mL. Rapamycin mTOR inhibitor (Sigma-Aldrich; St. Louis, MO) was supplied as a 2.74 mM solution in DMSO. MK-8669 (ridaforolimus, supplied by Merck through an MTA) was dissolved in DMSO at stock concentration of 10 mM for *in vitro* studies. For *in vivo* studies, MK-8669 was dissolved fresh daily in 10% Tween 80 and 40% PEG-400 in sterile water at stock 1 mg/mL as recommended by Merck. Trastuzumab was purchased from the Winship Cancer Institute pharmacy and dissolved in sterile water at a stock concentration of 20 mg/mL. Selumetinib was purchased from LC Laboratories (Woburn, MA), PD0325901 was purchased from Cayman Chemical (Ann Arbor, MI). Thiostrepton was purchased from Sigma-Aldrich (St. Louis, MO). Lapatinib was dissolved in DMSO at a stock concentration of 10 mM, and thiostrepton was dissolved at a stock concentration of 50 mM. Lapatinib for *in vivo* studies was purchased from the Winship Cancer Institute pharmacy and dissolved in a 1% Tween 80 and 5% hydroxypropyl methylcellulose buffer just prior to use.

ii. Bacterial Transformations

Expression vectors for each gene were used for protein expression in mammalian cells. Transformations were done using MAX Efficiency DH5α cells (Invitrogen; Grand Island, NY) according to the manufacturer's protocol. Briefly, DNA was added to competent cells, incubated on ice for 30 minutes, heat shocked for 45 seconds, followed by incubation on ice for 2 minutes. SOC Medium (Invitrogen) was added, then shaken at 225 rpm 37°C for 1 hour. Reaction containing plasmid DNA was diluted into SOC medium and incubated overnight at 37°C under selective pressure with ampicillin. The following morning, cells were harvested by centrifugation, and purified using a Midi-Prep (Qiagen; Valencia, CA) according to the manufacterer's instructions. Briefly, bacterial pellet was resuspended in buffer P1(50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 g/ml RNase A), lysed in buffer P2 (200 mM NaOH, 1% SDS), and neutralized in buffer P3 (3M potassium acetate pH 5.5). The lysate was incubated in a cartridge that allowed the seperation and precipitation of genomic DNA, proteins, and detergent. Contaminants were removed by washing with buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol), and DNA eluted using buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol). DNA was precipitated by adding 70%, concentrated using a QIA-Precipitator, and washed using 70% ethanol. DNA was eluted with sterile water.

iii. Cell Culture

JIMT-1 cells were purchased from DSMZ (Braunschweig, Germany); all other cell lines were purchased from American Type Culture Collection (Manassas, VA). HCC1419 and HCC1954 cells were maintained in RPMI with 10% fetal bovine serum (FBS); MDA-MB-361 was maintained in RPMI with 20% FBS; JIMT-1, BT474, and MDA-MB-453

were maintained in DMEM with 10% FBS; all cells were maintained in 1% penicillin/ streptomycin and cultured in humidified incubators at 37°C with 5% CO_2.

iv. Cell Cycle Analysis and Apoptosis Detection

Cells were harvested, washed twice with Dulbecco's phosphate buffered saline (DPBS)+10% FBS, fixed in ice-cold 80% ethanol, and stored at -20°C for at least 24 hours. Fixed cells were incubated in 50µL of propidium iodide (PI) buffer (20µg/mL PI (Sigma), 0.1% Triton-X 100, 200µg/mL RNaseA (Promega) in DPBS) for 30 minutes in the dark. The cells were then resuspended in 400µL of DPBS for flow analysis. For apoptosis detection, cells were harvested and stained for Annixin V-FITC and Propidium Iodide using the TACS Annexin V kit (Trevigen; Gaithersburg, MD) according to the manufacter's protocol. All samples were analyzed using a BD FACS Canto II cytometer (BD Biosciences; San Jose, CA) with BD FACS Diva software. All experiments were performed in triplicate.

v. DNA/siRNA Transfection

Cells were plated in antibiotic-free media. The next day, cells were transfected using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) with either 100 nM FOXM1 siRNA (Cell Signaling; Danvers, MA), 100 nM MEK1 siRNA plus 100 nM MEK2 siRNA, 1µg of one of the following plasmids: dominant negative kinase dead Akt1 mutant (pcDNA3-Akt1-K179A), constitutively active Akt1 mutant (pcDNA3-Akt1-T308D/S473D), or pcDNA3 empty vector control (plasmids generously provided by Dr. Keqiang Ye, Emory), 100 nM p70/85 S6 Kinase siRNA II (Cell Signaling), 4EBP1 siRNA (Cell Signaling), or control

siRNA (Santa Cruz Biotechnology) according to the manufacturer's protocol. After 24 hours, cells were treated with vehicle control or lapatinib for an additional 24 hours, at which point cells were counted by trypan blue exclusion. Knockdown was confirmed after 48 hours of transfection by Western blotting. Experiments were repeated twice for reproducibility.

vi. Immnunoflourescence

Cells were plated on glass coverslips in 24-well cell culture plates. After 24 hours, cells were treated with 1000 nM lapatinib, 1000 nM selumetinib, combination 1000 nM lapatinib plus selumetinib, or vehicle control for 24 hours. Cells were fixed with 4% formaldehyde and permeabilized with 0.3% Triton X-100. Coverslips were blocked for one hour in blocking buffer (10% goat serum in TBS-T) and incubated with primary antibody in blocking buffer overnight at 4°C. Antibodies against FOXM1 (Santa Cruz) and p27 (Dako; Carpinteria, CA) were each used at a 1:25 dilution. The next day coverslips were incubated with secondary antibodies for one hour at room temperature, and signal amplification was performed using tyramide (Invitrogen). Cells were mounted using Prolong Gold (Invitrogen) and examined using a Zeiss LSM 510 Meta confocal system.

vii. Immunohistochemistry

IHC was performed on tumor tissues from two animals per treatment group using a standard immunoperoxidase procedure as previously described (Griner et al, 2012). The treatment groups were vehicle diluent, lapatinib (75 mg/kg), selumetinib (50 mg/kg), or

combination lapatinib and selumetinb. The tissue sections on glass slides were deparaffinized by heating at 60^{o}C for 10 minutes, followed by cooling for 30 minutes while in the same buffer. Quenching of the endogenous peroxidase was achieved by incubating slides with 0.3$^{\%}$ H_2O_2 in methanol for 15 minutes. The tissues were then washed with water and PBS/TBS and incubated in 10% swine serum (Dako) for 1 hour to eliminate non-specific background staining. Tissue sections were stained for FOXM1 (Santa Cruz, SC500, dilution 1:100), pERK (Cell Signaling, CS9101, 1:500) Ki67 (Thermo Scientific, SP6, 1:400). Biotinylated anti-rabbit antibody (Dako) was used as secondary antibody, and positive staining was detected by incubation with 3,3 diaminobenzidine solution (DAB + chromogen; Dako) with hematoxylin as a counterstain. The slides were then washed in water and dehydrated by passing through alcohol grades and xylene. The slides were mounted with permanent mounting medium Permount (Fisher Scientific; Pittsburgh, PA). After drying completely, the slides were viewed under a light microscope, and pictures were taken at 10X.

viii. Polymerase Chain Reaction

Total RNA was extracted using the RNeasy purification kit (Qiagen; Valencia, CA) and treated with DNase (Invitrogen). Total RNA was used to prepare cDNA using random primers and the Superscript III first strand synthesis Kit (Invitrogen). Real-time quantitative PCR master mix (4304437, Applied Biosystems; Carlsbad, CA) was used to synthesize cDNA. Primers for RPLPO (Hs99999902_M1), FOXM1 (Hs01073586_M1), p27 (Hs00153277_B1), and survivin (Hs04194392_M1) were obtained from Applied Biosystems (Taq-Man Gene Expression Assays). The RPLPO internal control was used

to normalize samples and compared as arbitrary units, represented as mean +/- SD. Samples were run in triplicate, and experiments were repeated twice to ensure reproducibility.

ix. Western Blotting

Cells were lysed in RIPA buffer (consists of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EDTA , $1^{\%}$ NP-40, $1^{\%}$ sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4 , 1 µg/ml leupeptin; Cell Signaling) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Total protein extracts (30 µg) diluted with 5X sample buffer and boiled for 5 minutes. Proteins were resolved using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with either 10% or 12% gels followed by blotting onto a nitrocellulose membrane. The nitrocellulose blot was incubated for 1 hr in 5% bovine serum albumin followed by an overnight incubation at 4°C with the primary antibody, diluted in 5% bovine serum albumin. Blots were probed overnight using the following antibodies from Cell Signaling: phospho-Thr389 p70S6K clone 1A5 at 1:750; total p70S6K (1:1000), p-S473 Akt-XP used at 1:1000, and polyclonal antibodies against total Akt (1:1000), p-Thr202/Tyr204 p42/p44 ERK1/2 (1:1000), total p42/p44 ERK1/2 (1:1000), and FOXM1 (D12D55) XP (1:1000). β-actin monoclonal AC-15 (Sigma-Aldrich) at 1:10,000 was used as a loading control. Protein bands were detected using the Odyssey Imaging System (Li-Cor Biosciences; Lincoln, NE). Experiments were repeated three times to ensure reproducibility.

x. ELISA

Human insulin growth factor binding protein (IGFBP) 3 and human insulin growth factor binding protein 5 (R&D Systems; Minneapolis, MN) were used according to the manufacturer's directions. Briefly, sample media was incubated in either IGFBP3 or IGFBP5 antibody-coated microplates for 2 hours, then washed and incubated with appropriate antibody conjugated to horseradish peroxidase for 1 hour. After washing, plates were incubated with color reagent (hydrogen peroxide–chromogen mix) for 30 minutes. Optical density of each well was determined using a microplate reader set to 450 nm. The concentrations were calculated according to the standards supplied with the kit by creating a four parameter logistic curve-fit. Samples were run in triplicate, and experiments were repeated 3 times with reproducible results.

2. Biological Assays

i. Cell Proliferation Assay

Cells were plated at 3000 per well in 96-well format, and treated with lapatinib and/or rapamycin or MK-8669 versus DMSO control corresponding to the volume found in the highest dose combination. For another experiment, cells were treated with 20 μg/mL trastuzumab versus untreated control. Six replicates were run per group. After 6 days of treatment, proliferation was measured by MTS assay as directed by the manufacturer (Promega; Madison, WI). Combination index (C.I.) values were determined using the commercial software package Calcusyn (Biosoft, Cambridge, United Kingdom) by the method of Chou and Talalay (Chou and Talalay, 1984). Experiments were repeated on at least two independent occasions to ensure reproducibility.

ii. Trypan Blue

For anchorage-dependent growth, cells were plated at 3 x 10^4 in a 12-well plate format. After 24 hours, media plus drug was added for either 48 hours for the lapatinib plus PD0325901 treatments, and 72 hours for the lapatinib plus selumetinib treatments. Viable cells were then counted by trypan blue exclusion.

iii. Anchorage-Independent Growth

Cells were plated at 1 x 10^4 in 12-well plate format in 250 mL matrigel (BD Biosciences; Franklin Lakes, NJ) diluted 1:1 (media:matrigel). The matrigel-cell suspension was allowed to solidify for 2 hours at 37ºC, followed by the addition of 1 mL of media containing either lapatinib, PD0325901, selumetinib, or DMSO control. In a separate experiment, lapatinib, mTOR inhibitor (rapamycin or MK-8669), combination lapatinib plus mTOR inhibitor, or DMSO control at the same volume found in the drug combination group, was added to the matrigel-cell culture.

Media plus drug was changed twice a week for approximately 2 weeks. Photographs were taken with an Olympus IX50 inverted microscope at 4X magnification. Matrigel was then digested using dispase (BD Biosciences), and viable cells were counted by trypan blue exclusion. Experiments were repeated on at least two independent occasions to ensure reproducibility. For anchorage-dependent growth, cells were plated at 3 x 10^4 in a 12-well plate format. After 24 hours, media plus drug was added for either 48 hours for the lapatinib plus PD0325901 treatments, and 72 hours for the lapatinib plus selumetinib

treatments. Viable cells were then counted by trypan blue exclusion. Experiments were repeated three times for reproducibility.

3. Xenograft Studies

i. Xenograft mouse model

Female athymic *nu/nu* mice (Harlan; Indianapolis, IN) were used to establish flank xenografts by s.c. injection with $1x10^6$ JIMT-1 cells and $50^{\%}$ Matrigel (BD Biosciences). Tumor volumes were calculated as the product of the length, width and height of the tumor measured twice a week with a caliper. In one experiment, animals were administered lapatinib by oral gavage at a dose of 75 mg/kg, and MK-8669 by i.p. injection at a dose of 1 mg/kg. Control mice received vehicle diluent (10% Tween 80, 40% PEG-400 in sterile water). In another experiment, animals were administered vehicle diluent, lapatinib (75 mg/kg), selumetinib (50 mg/kg), or combination lapatinib and selumetinb by oral gavage. All treatments were done daily for 5 days, then off for 2 days, for a total of 17 days. Animals were euthanized by CO_2 inhalation in accordance with institutional IACUC regulations.

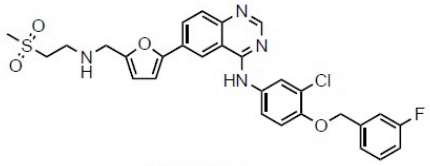
Chapter 3. Pharmacologic Inhibition of mTOR Improves Lapatinib Sensitivity in HER2-overexpressing Breast Cancer Cells with Primary Trastuzumab Resistance

Portions of this chapter were previously published:
Gayle SS., Arnold SL., O'Regan RM., Nahta R. Pharmacologic Inhibition of mTOR Improves Lapatinib Sensitivity in HER2-Overexpressing Breast Cancer Cell with Primary Trastuzumab Resistance. *Anticancer Agents in Medicinal Chemistry.* 2012. 12(2):151-62

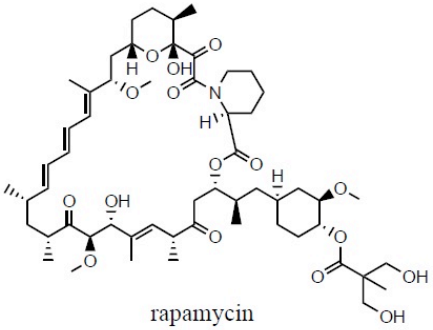
1. Introduction

The *HER2* gene is amplified and overexpressed in approximately 25%-30% of metastatic breast cancers, and is associated with an aggressive clinical course resulting in reduced disease-free and overall survival compared with other breast cancer subtypes (Slamon et al, 1987; Sorlie et al, 2001). Trastuzumab (Herceptin) is a recombinant humanized monoclonal antibody directed against the HER2 extracellular domain. Initial clinical trials of single-agent trastuzumab demonstrated overall response rates ranging from 11% to 21% in patients with HER2-overexpressing metastatic breast cancer (Cobleigh et al, 1999;Baselga et al, 1996). Thus, almost two-thirds of patients demonstrated primary resistance to trastuzumab, although response rates were improved when combined with chemotherapy (Esteva et al, 2002;Slamon et al, 2001).

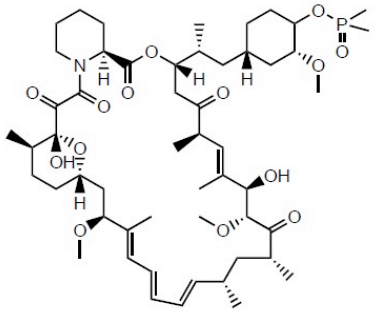
The dual EGFR/HER2 tyrosine kinase inhibitor lapatinib (Tykerb) (Figure 3-1) is approved in combination with capecitabine for use against HER2-overexpressing breast cancers with prior disease progression on trastuzumab and as first-line therapy in combination with letrozole for hormone receptor-positive, HER2-positive metastatic breast cancer. Combination lapatinib plus chemotherapy achieved an overall response rate of 22% and clinical benefit rate of 27%, with median time to progression of 8.4 months (Geyer et al, 2006). As a single agent, lapatinib showed clinical benefit rates ranging from 12.4% to 25% in trastuzumab-pretreated populations (Blackwell et al, 2010; Toi et al, 2009). Thus, lapatinib shows benefit in a subset of trastuzumab-refractory breast cancers, although the majority of trastuzumab-resistant disease shows poor response to lapatinib.



lapatinib



rapamycin



ridaforolimus

Figure 3-1. Chemical structures of kinase inhibitors. Structures for lapatinib, rapamycin, and the rapamycin analogue ridaforolimus (MK-8669) were downloaded from the ChemACX database (CambridgeSoft, Cambridge, MA) and drawn in ChemDraw (CambridgeSoft).

Resistance to trastuzumab has been closely associated with increased PI3K signaling due to either loss of the *PTEN* phosphatase gene (Nagata et al, 2004) or hyper-activating mutations in the *PIK3CA* catalytic subunit of PI3K (Berns et al, 2007). Esteva et al (Esteva et al, 2010) recently showed that phosphorylation of Akt or the mTOR substrate p70S6K were not independently associated with trastuzumab resistance, but when considered together, p-Akt, p-p70S6K, and loss of *PTEN* were strongly associated with poor response to trastuzumab.

A genome-wide loss-of-function short hairpin RNA screen performed to identify mediators of lapatinib resistance showed that loss of *PTEN* or *PIK3CA* mutations also contributed to lapatinib resistance (Eichorn et al, 2008). Further, treatment with a dual inhibitor of PI3K/mTOR inhibited colony formation and proliferation of lapatinib-resistant cells harboring genetic defects in PI3K signaling (Eichorn et al, 2008). In contrast, O'Brien et al. (O'Brien et al, 2010) suggested that lapatinib resistance was not associated with loss of *PTEN* or *PIK3CA* mutations, and that lapatinib could block the hyperactive PI3K signaling associated with trastuzumab resistance. Wang et al. (Wang et al, 2011) examined 57 primary tumor samples from lapatinib-treated patients with HER2-overexpressing breast cancer heavily pretreated with chemotherapy and trastuzumab. Patients with loss of *PTEN* or hyper-activating mutations in *PIK3CA* had a significantly lower clinical benefit rate (36.4% versus 68.6%) and significantly lower overall response rate (9.1% versus 31.4%) in contrast to those patients whose tumors did not show PI3K pathway activation.

Blocking the PI3K pathway with mTOR inhibition has been demonstrated to be beneficial in trastuzumab-resistant cancers. Response rates of more than 40% and disease

control rates of more than 70% were achieved in metastatic HER2-positive breast cancers resistant to trastuzumab and taxanes, when treated with trastuzumab, paclitaxel and everolimus (Andre et al, 2010). The combination of trastuzumab, chemotherapy and everolimus has been demonstrated to be beneficial in patients with HER2-positive metastatic breast cancer, resistant to both trastuzumab and lapatinib. The use of mTOR inhibition, with everolimus, is currently being evaluated in a phase 3 trial of patients with metastatic HER2-positive breast cancers resistant to trastuzumab. Patients entering this trial can have received lapatinib. Therefore, there is a clear need to understand the role of PI3K signaling in HER2-positive breast cancers that are resistant to currently approved HER2-directed agents, including lapatinib.

Thus, although some studies suggest that PI3K pathway activation correlates with reduced response to lapatinib, controversy exists in the literature regarding the role of PI3K/mTOR in lapatinib sensitivity. We examined the ability of lapatinib to inhibit proliferation in multiple HER2-overexpressing breast cancer cell lines that have primary trastuzumab resistance. Lower response to lapatinib was associated with an inability of lapatinib to reduce p-Akt or p-p70S6K levels. Transfection of constitutively active Akt into lapatinib-sensitive cells abrogated response to lapatinib, while kinase-dead Akt improved lapatinib sensitivity, further suggesting that inhibition of Akt phosphorylation is critical to achieving response to lapatinib. In contrast, knockdown of p70S6K alone did not improve response to lapatinib. However, knockdown of 4EBP1 or pharmacologic mTOR inhibition increased lapatinib sensitivity. Inhibition of mTOR reduced p-Akt levels and increased response to lapatinib in cells that showed poor response to single-agent lapatinib, even those transfected with hyperactive Akt. Single agent mTOR

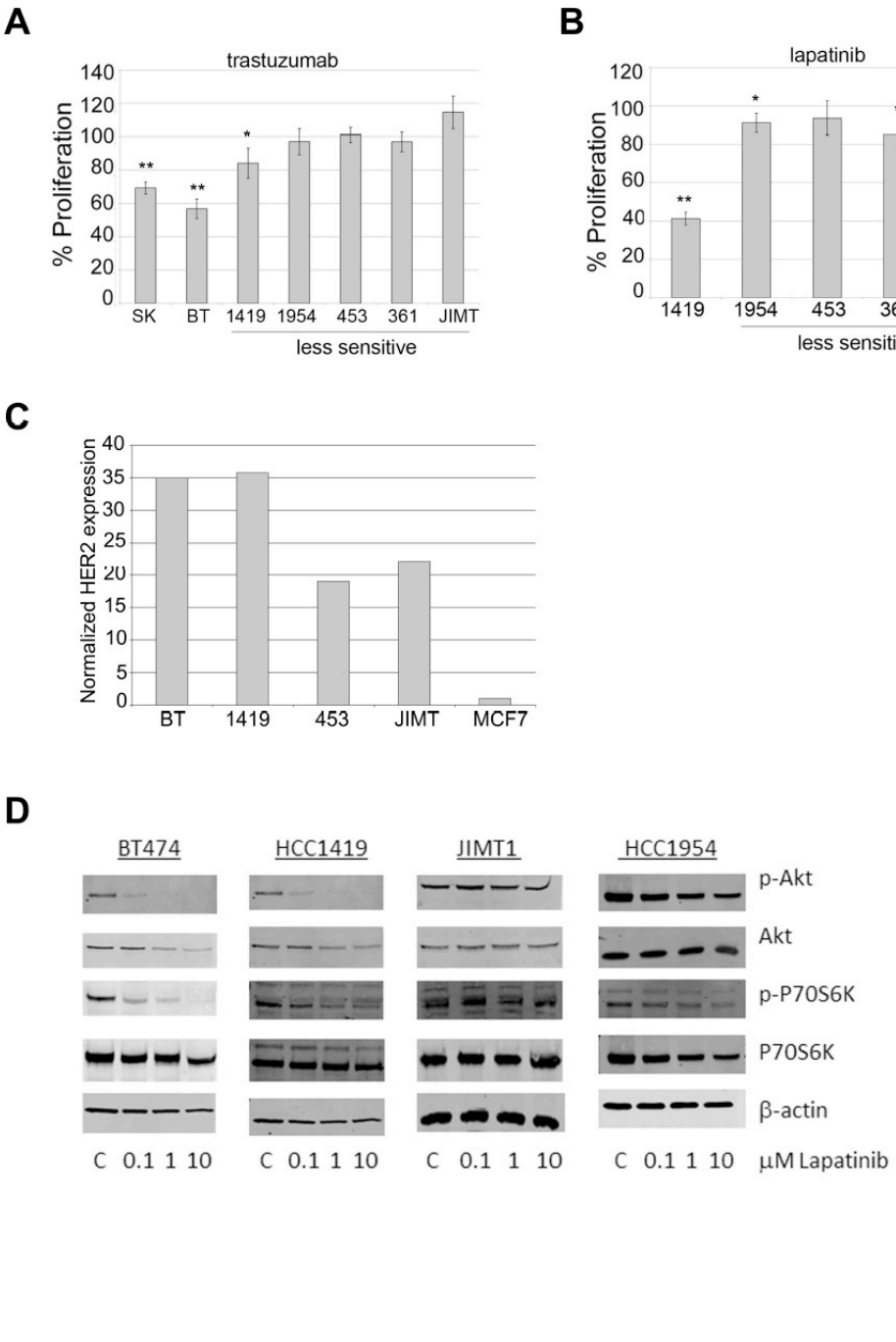
inhibition was associated with feedback signaling activating Akt and Erk1/2, which was overcome by co-treatment with lapatinib. Combination mTOR inhibition plus lapatinib resulted in synergistic growth inhibition of HER2-overexpressing trastuzumab-resistant breast cancer cells and xenografts. Our data indicate that p-Akt is a critical downstream target of lapatinib, whose inhibition must be intact in order to achieve optimal response to lapatinib. In cases where lapatinib alone does not effectively block Akt or p70S6K phosphorylation, our data support strategies that combine lapatinib with mTOR inhibition in the context of primary trastuzumab-resistant HER2-overexpressing breast cancer.

2. Results

i. Analysis of Lapatinib Response in HER2-overexpressing Breast Cancer Cell Lines with Primary Resistance to Trastuzumab

The HER2-overexpressing breast cancer cell lines SKBR3, BT474, HCC1419, HCC1954, MDA453, MDA361, and JIMT-1 were examined for sensitivity to trastuzumab by MTS proliferation assay (Figure 3-2A). A clinically relevant concentration of trastuzumab (20 μg/mL) inhibited proliferation of SKBR3 and BT474 cells. HCC1419 cells showed slightly lower although statistically significant inhibition of proliferation in response to trastuzumab. HCC1954, MDA453, MDA361, and JIMT-1 cells showed primary resistance to trastuzumab. Lapatinib (0.1 μM) inhibited proliferation of HCC1419 cells by 50-60%, but inhibited the remaining four primary trastuzumab-resistant cell lines by only 10-20% (Figure 3-2B). Thus, 4 out of 5 lines (approximately 80%) of cells with trastuzumab resistance also showed poor response to

lapatinib, which mimics clinical response rates to lapatinib in trastuzumab-pretreated patients (Geyer et al, 2006;Blackwell et al, 2010;Toi et al, 2009). To confirm HER2 over-expression in lines used in this study, Western blotting for total HER2 was performed relative to the MCF-7 cell line (Figure 3-2C). In addition to measuring differential response to lapatinib by proliferation assay, Western blotting for p-Akt and p-p70S6K was performed. Lapatinib blocked phosphorylation of Akt and p70S6K in lapatinib-sensitive BT474 and HCC1419 cells, but not in JIMT-1 or HCC1954 cells (Figure 3-2D). These results suggest that inhibition of PI3K and mTOR may be important for achieving inhibition of proliferation in response to lapatinib.


A
trastuzumab
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less sensitive
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lapatinib
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1419
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361
JIMT
less sensitive
C
Normalized HER2 expression
40
35
30
25
20
15
10
5
0
BT
1419
453
JIMT
MCF7
D
BT474
HCC1419
JIMT1
HCC1954
p-Akt
Akt
p-P70S6K
P70S6K
β-actin
C 0.1 1 10
C 0.1 1 10
C 0.1 1 10
C 0.1 1 10
μM Lapatinib

Figure 3-2. Analysis of anti-proliferative activity of lapatinib in primary trastuzumab-resistant HER2-overexpressing breast cancer cell lines. Proliferation was examined by MTS assay in cell lines treated with **(A)** 20 μg/mL trastuzumab, or **(B)** 0.1 μM lapatinib for 6 days. Values represent the average of 6 replicates per group as a percentage of untreated control cells (for trastuzumab) or DMSO-treated cells (for lapatinib). Error bars represent standard deviation between replicates. P-values were determined by t-test; *$p<0.05$, **$p<0.005$. Experiments were repeated three times with reproducible results. A representative immunoblot of total HER2 is shown for all cell lines. **(C)** Total protein lysates of cell lines were examined by Western blotting for total HER2. Bands were quanitated and values were normalized to actin levels. Total HER2 level is shown relative to MCF-7 cell line. **(D)** BT474, HCC1419, JIMT-1, and HCC1954 cells were treated with 0.1, 1 or 10 μM lapatinib, or with DMSO at the volume found in the highest dose of lapatinib (*C*, control) for 48 h. Whole cell protein lysates were immunoblotted for p-S473 Akt, total Akt, p-T389 p70S6K, total p70S6K, or actin loading control. Blots were repeated on at least two separate occasions with reproducible results.

ii. Constitutively Active Akt Reduces Lapatinib Sensitivity, while Kinase Dead Akt Improves Lapatinib Sensitivity

Based on this initial data (Figure 3-2) showing that lapatinib sensitivity correlates with reduced p-Akt in BT474 and HCC1419 cells in contrast to JIMT-1 and HCC1954 cells, we hypothesized that Akt inhibition is important for achieving response to lapatinib. To test this hypothesis, HCC1419 cells, which are relatively sensitive to lapatinib, were transfected with constitutively active Akt double mutant T308D/S473D. Alternatively, JIMT-1 cells, which showed relatively low response to single-agent lapatinib, were transfected with dominant-negative kinase-dead Akt mutant K179A. Transfection was confirmed by blotting for p-Akt and total Akt (Figure 3-3A). In comparison to control transfectants, HCC1419 cells transfected with constitutively active Akt showed significantly reduced sensitivity to lapatinib ($p=0.006$) (Figure 3-3B). Sensitivity to the mTOR inhibitor rapamycin was also reduced in the presence of constitutively active Akt, although this effect did not reach statistical significance ($p=0.08$).

In the presence of hyperactive Akt, combination mTOR inhibition plus lapatinib resulted in a significant reduction in cell viability versus either drug alone ($p=0.01$). These results suggest that combination lapatinib plus rapamycin may be an effective therapeutic strategy in tumors that show elevated PI3K signaling and low response to single-agent lapatinib.

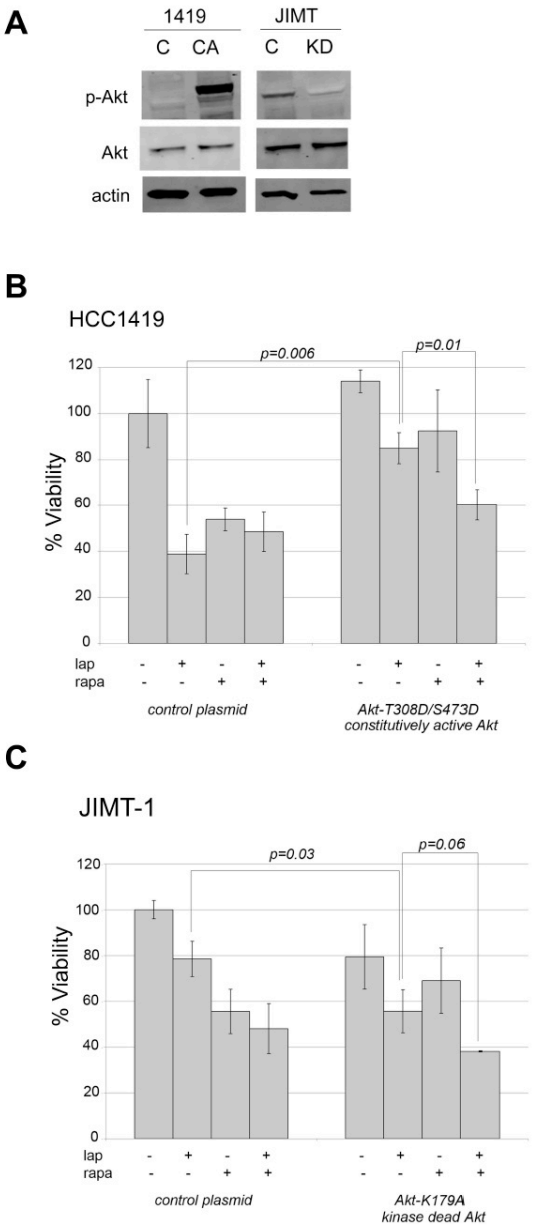
In a background of kinase dead Akt, JIMT-1 cells showed a statistically significant increase in lapatinib sensitivity ($p=0.03$) (Figure 3-3C). In contrast, sensitivity to rapamycin was not significantly affected. The combination of lapatinib plus rapamycin

showed a trend towards reduced cell viability versus single-agent lapatinib, although this did not reach statistical significance (p=0.06). However, JIMT-1 cells did retain sensitivity to this drug combination in the presence of kinase dead Akt. Collectively, these results suggest that Akt activation status affects lapatinib sensitivity. Hyperactive Akt signaling significantly reduced response to lapatinib in HCC1419 cells, whereas kinase dead Akt significantly improved response to lapatinib in JIMT-1 cells. In addition, pharmacologic inhibition of mTOR significantly increased response to lapatinib in HCC1419 cells transfected with constitutively active Akt. Thus, this combination may be effective in HER2-overexpressing breast cancers that show poor response to lapatinib and high baseline Akt activity.

iii. Knockdown of 4EBP1 but not p70S6K Improves Lapatinib Sensitivity

Inhibition of p70S6K phosphorylation correlated with the anti-proliferative activity of lapatinib (Figure 3-2). In addition, mTOR inhibition by rapamycin increased lapatinib activity in JIMT-1 cells (Figure 3-3). Thus, we hypothesized that p70S6K inhibition is critical for achieving response to lapatinib. We transfected lapatinib-resistant JIMT-1 cells with p70S6K siRNA, and confirmed knockdown by Western blot (Figure 3-4A). Surprisingly, knockdown of p70S6K alone was not sufficient to increase the growth inhibitory activity of lapatinib (Figure 3-4B), suggesting that additional signaling molecules regulated by mTOR must be inhibited in order to achieve optimal response to lapatinib. Knockdown of 4EBP1 confirmed by Western blotting (Figure 3-4C) significantly increased the sensitivity of JIMT1 cells to lapatinib (Figure 3-4D). Thus,

mTOR inhibition, particularly 4EBP1 inhibition, increases lapatinib-mediated cytotoxicity.


A
1419
JIMT
C
CA
C
KD
p-Akt
Akt
actin
B
HCC1419
p=0.006
p=0.01
% Viability
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100
80
60
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lap
rapa
control plasmid
Akt-T308D/S473D
constitutively active Akt
C
JIMT-1
p=0.03
p=0.06
% Viability
120
100
80
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40
20
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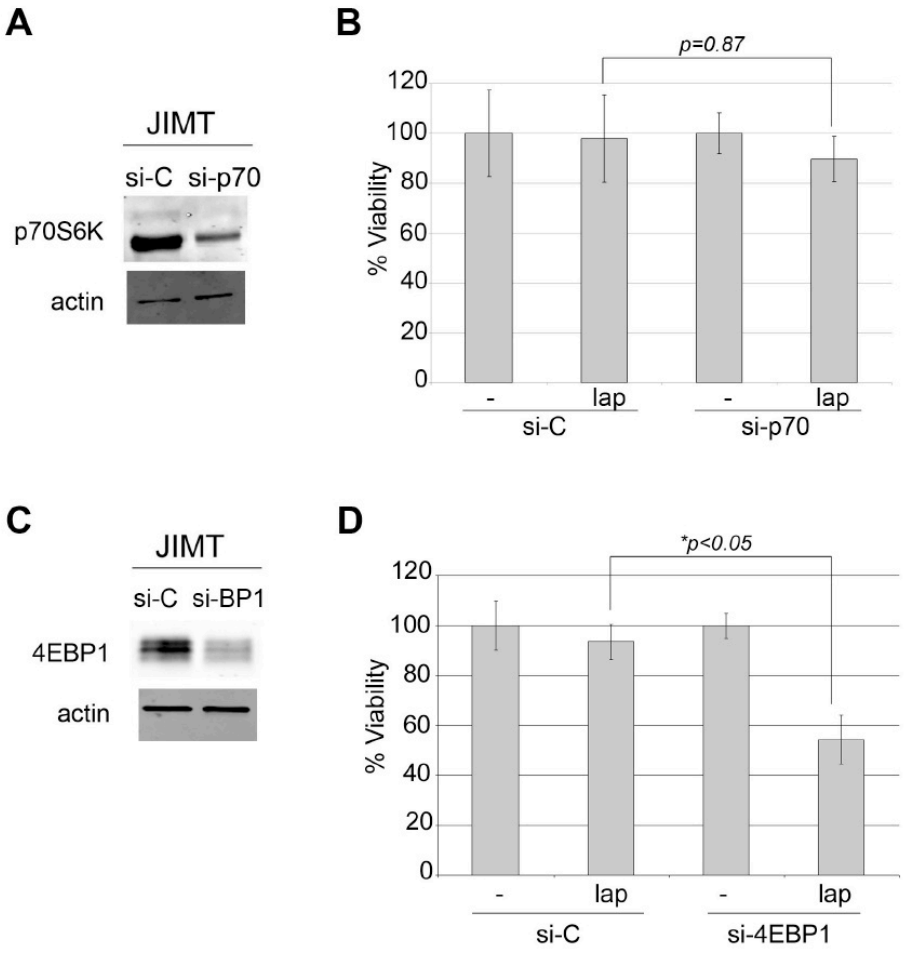
lap
rapa
control plasmid
Akt-K179A
kinase dead Akt

Figure 3-3. Akt activation status affects lapatinib sensitivity. (A) HCC1419 cells were transiently transfected with 1 μg pcDNA3 vector control (C) or pcDNA3-Akt-T308D/S473D plasmid (CA), which expresses constitutively active Akt. JIMT-1 cells were transiently transfected with 1 μg pcDNA3 vector control (C) or pcDNA3-Akt-K179A plasmid (KD), which expresses kinase dead Akt. Total protein lysates were collected after 48 h and immunoblotted for phosphorylated S473 Akt and total Akt to confirm p-Akt level after transfection. **(B)** HCC1419 cells were transiently transfected with 1 μg pcDNA3 vector control (C) or pcDNA3-Akt-T308D/S473D constitutively active Akt plasmid. **(C)** JIMT-1 cells were transiently transfected with 1 μg pcDNA3 vector control (C) or pcDNA3-Akt-K179A kinase dead Akt plasmid. After 24 h transfection, cells were treated for an additional 72 h with DMSO, 10 μM lapatinib, 100 nM rapamycin, or a combination of 100 nM rapamycin plus 10 μM lapatinib. Viable cells were then counted by trypan blue exclusion. Viability is presented as a percentage of DMSO-treated control vector group, and reflects the average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. P-values were determined by t-test for each treatment group in Akt-transfected cells versus corresponding treatment group in control-transfected cells; $p=0.006$ for lapatinib-treated constitutively active Akt-transfected HCC1419 cells versus lapatinib-treated vector control; $p=0.01$ for constitutively active Akt-transfected HCC1419 cells treated with combination lapatinib plus rapamycin versus treated with lapatinib alone; $p=0.03$ for lapatinib-treated, kinase-dead Akt-transfected JIMT-1 versus JIMT-1 lapatinib-treated vector control; no other statistically significant differences were found between treatment groups. Experiments were repeated at least twice with reproducible results


A
JIMT
si-C si-p70
p70S6K
actin
B
p=0.87
120
100
80
60
40
20
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% Viability
-
lap
-
lap
si-C
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JIMT
si-C si-BP1
4EBP1
actin
D
*p<0.05
120
100
80
60
40
20
0
% Viability
-
lap
-
lap
si-C
si-4EBP1

Figure 3-4. Knockdown of 4EBP1 but not p70S6K improves lapatinib sensitivity. **(A)** JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or p70S6K siRNA (si-p70) for 48 h. Total protein lysates were immunoblotted for total p70S6K and actin loading control to confirm knockdown. **(B)** JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or p70S6K siRNA (si-p70) for 24 h, and then treated with DMSO control or 1 μM lapatinib (lap). After 72 h, viable cells were counted by trypan blue exclusion. Viability is presented as a percentage of DMSO-treated si-C group, and reflects the average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. P-values were determined by t-test for p70 knockdown plus lapatinib versus si-C plus lapatinib. Experiments were repeated three times with reproducible results. **(C)** JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or 4EBP1 siRNA (si-BP1) for 48 h. Total protein lysates were immunoblotted for total 4EBP1 and actin loading control to confirm knockdown. **(D)** JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or 4EBP1 siRNA (si-4EBP1) for 24 h, and then treated with DMSO control or 1 μM lapatinib (lap). After 72 h, viable cells were counted by trypan blue exclusion. Viability is presented as a percentage of DMSO-treated si-C group, and reflects the average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. P-values were determined by t-test for 4EBP1 knockdown plus lapatinib versus si-C plus lapatinib. Experiments were repeated three times with reproducible results.

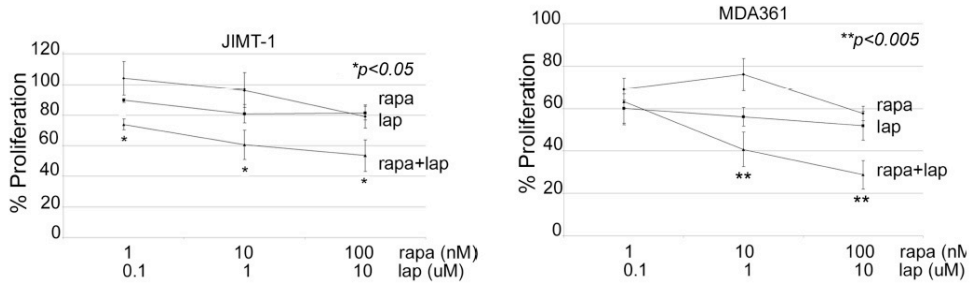
iv. Rapamycin Increases Lapatinib Sensitivity in HER2-overexpressing Breast Cancer Cells that have Primary Trastuzumab Resistance

Pharmacologic mTOR inhibitors appear to improve response to trastuzumab Andre et al, 2010;Chou et al, 1984;Miller et al, 2009). Our data indicates that pharmacologic inhibition of mTOR may also improve response to lapatinib in cells with primary trastuzumab resistance. We performed drug combination analysis to determine whether true synergy is achieved by addition of rapamycin to lapatinib. Treatment of JIMT-1 and MDA361 cells with rapamycin plus lapatinib resulted in significantly reduced proliferation versus either drug alone (Figure 5A). Using the method of Chou and Talalay (Chou & Talalay, 1984) (CalcuSyn; Biosoft), we analyzed data to determine if these drugs act synergistically (Table 3-1). Drug combination index (C.I.) values less than 1.0 were achieved in JIMT-1 cells, indicating strong pharmacologic synergy. Higher C.I. values were measured in MDA361 cells, suggesting lower drug synergy versus what was observed in JIMT-1 cells, although increased benefit was still observed with the combination versus single agent treatments.

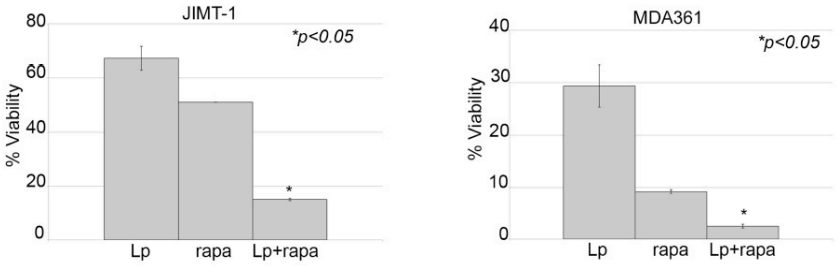
Next, anchorage-independent (AI) colony growth of JIMT-1 and MDA361 cells was examined. Drug combination inhibited AI growth more significantly than either drug alone (Figure 3-5B). In addition, a stronger response to single-agent lapatinib was observed under anchorage-independent conditions, which may be due to differences in 3-dimensional cultures versus adherent cultures or may be due to longer-term treatment (3-4 weeks) versus short-term treatment (6 days) in MTS assays. Western blot analysis showed that combination drug treatment inhibited Akt S473 phosphorylation in JIMT-1,

whereas neither drug was able to do so when given as a single agent (Figure 3-5C). In MDA361 cells, the combination inhibited Akt phosphorylation to a similar degree as individual lapatinib. Treatment with single-agent rapamycin resulted in feedback signaling, as shown by increased phosphorylation of Akt and Erk1/2 in MDA361 cells. Activation of PI3K and MAPK signaling in response to rapamycin has been previously reported (Sun et al, 2005;Wang et al, 2008;Liu et al, 2011), and is thought to be a mechanism driving resistance to pharmacologic mTOR inhibition. Importantly, co-treatment with lapatinib was able to overcome rapamycin-induced feedback signaling, as we previously observed (Liu et al, 2011). Phosphorylation of the mTOR substrate p70S6K remained largely unaffected by the combination of rapamycin and lapatinib in MDA361 cells. However the combination achieved increased inhibition of p-p70S6K in JIMT-1 cells versus single agents. Thus, our data indicate that mTOR inhibition increases lapatinib sensitivity in association with reduced Akt and possibly reduced p70S6K phosphorylation.

A


JIMT-1
*p<0.05
% Proliferation
rapa
lap
rapa+lap
rapa (nM)
lap (uM)
MDA361
**p<0.005
% Proliferation
rapa
lap
rapa+lap
rapa (nM)
lap (uM)

B


JIMT-1
*p<0.05
% Viability
Lp
rapa
Lp+rapa
MDA361
*p<0.05
% Viability
Lp
rapa
Lp+rapa

C

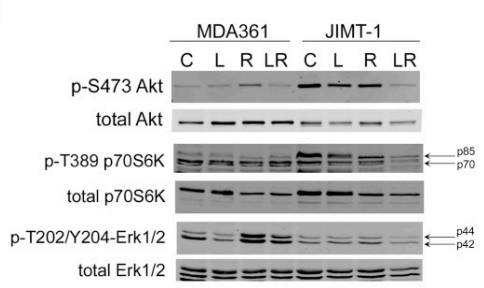

MDA361
JIMT-1
C L R LR C L R LR
p-S473 Akt
total Akt
p-T389 p70S6K
p85
p70
total p70S6K
p-T202/Y204-Erk1/2
p44
p42
total Erk1/2

Figure 3-5. Rapamycin increases lapatinib sensitivity of HER2-overexpressing breast cancer cells with primary trastuzumab resistance. (A) JIMT-1 and MDA361 cells were treated with rapamycin alone, lapatinib alone, or combination rapamycin plus lapatinib at indicated doses for 6 days. Proliferation was then measured by MTS assay. Values represent the average of 6 replicates per group as a percentage of DMSO-treated cells per treatment group. Error bars represent standard deviation between replicates. P-values were determined by t-test for each combination versus corresponding dose of lapatinib; *$p<0.05$, **$p<0.005$. Experiments were repeated twice with reproducible results. **(B)** JIMT-1 and MDA361 cells were plated in matrigel and treated with DMSO, 10 nM rapamycin (rapa), 1 μM lapatinib (Lp), or a combination of 10 nM rapamycin plus 1 μM lapatinib. Media plus drugs were changed every 3 days for approximately 3-4 weeks. Matrigel was dissolved with dispase, and viable cells were counted by trypan blue. Viability is shown as a percentage of the DMSO control group, and reflects an average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. P-value was determined by t-test for combination treatment versus lapatinib alone; *$p<0.05$, **$p<0.005$. **(C)** MDA361 and JIMT-1 cells were treated with DMSO (*C*), 10 μM lapatinib (*L*), 100 nM rapamycin (*R*), or a combination of 100 nM rapamycin plus 10 μM lapatinib (*LR*) for 48 h, lysed for total protein, and immunoblotted for p-S473 Akt, total Akt, p-T389 p70S6K, total p70S6K, p-T202/Y204 Erk1/2, or total Erk1/2.

Table 3-1. Combination Index (C.I.) values for lapatinib + rapamycin (1:10)

Cell line	ED50	ED75	ED90	Dm	m	r
MDA361	0.03638	1849.48693	9.6203e+007	0.63550	-0.36817	0.97157
JIMT	0.20320	0.00555	0.00569	14.51422	-0.20104	0.98597

Table 3-1. Combination Index (C.I.) values for lapatinib + rapamycin (1:10)

Cells were treated with lapatinib (0.1, 1, or 10 μM), rapamycin (1, 10, or 100 nM), or combination lapatinib plus rapamycin. After 6 days, proliferation was measured by MTS assay. The fraction of cells proliferating relative to DMSO control-treated cells was determined, and C.I. values were determined for the combination using CalcuSyn software. C.I. values are listed for effective doses at which 50%, 75%, or 90% (ED50, ED75, and ED90, respectively) of cells were killed. Statistically, drug synergy is defined by C.I. values less than 1.0, and very strong synergy is defined by C.I. values less than 0.1. D_m, the median-effect (ED50) drug concentration; $m < 1$ indicates a negative sigmoidal shape to the dose-effect curve; r states the linear correlation coefficient.

v. Pharmacologic mTOR inhibitor MK-8669 Increases Lapatinib Sensitivity of HER2-overexpressing Breast Cancer Cells

The novel rapamycin analogue, *MK-8669* (AP23573, ridaforolimus*; provided by Merck*), has shown promising tumor inhibitory effects in early phase clinical trials (Perotti et al, 2010;Yardley et al, 2009). We combined MK-8669 with lapatinib in MDA361 and JIMT-1 HER2-overexpressing breast cancer cells. The combination achieved statistically significant improvements in inhibition of proliferation versus either drug alone (Figure 3-6A). Drug combination index values (Table 3-2) showed synergy between lapatinib and MK-8669 in both MDA361 and JIMT-1 cells. Furthermore, combined lapatinib plus MK-8669 dramatically reduced anchorage-independent growth of MDA361 and JIMT-1 cells (Figure 3-6B). Similar to what was observed with combination lapatinib plus rapamycin, the combination of MK-8669 and lapatinib inhibited phosphorylation of Akt in JIMT-1 cells without effect on p70S6K phosphorylation (Figure 3-6C). In contrast, although no change in p-Akt level was detected in MDA361 cells, treatment with the drug combination reduced phosphorylation of the p70 isoform p85 S6K with slight inhibition of p70 phosphorylation. Thus, synergistic growth inhibitory effects of combination MK-8669 plus lapatinib were associated with inhibition of PI3K-Akt-mTOR signaling. Finally, we determined if the combination of MK-8669 and lapatinib was synergistic *in vivo* by treating xenografts of primary trastuzumab-resistant JIMT-1 cells with each drug alone or with the drug combination (Figure 3-6D). Treatment with single-agent lapatinib or MK-8669 resulted in reduced tumor growth versus control. When administered in combination, however, lapatinib plus MK-8669 achieved statistically significant inhibition of tumor growth

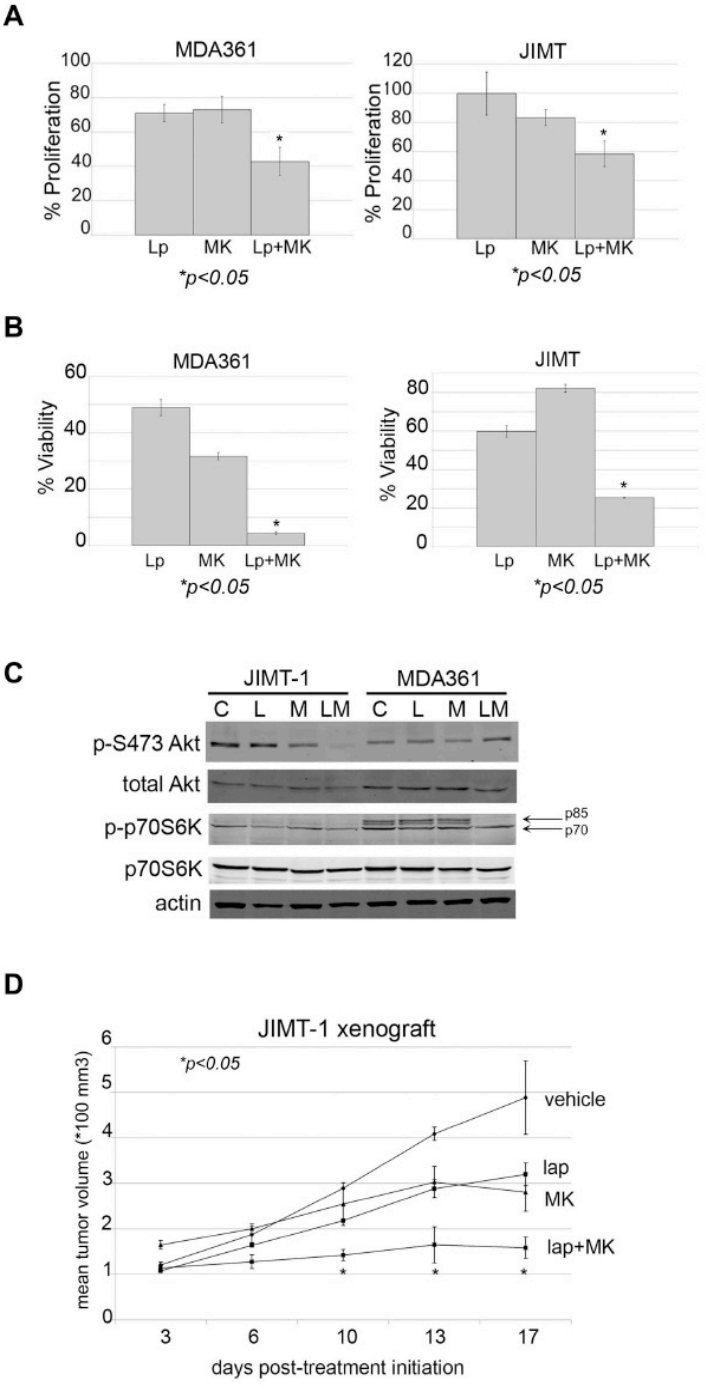
versus either drug alone. Thus, our data support the clinical evaluation of lapatinib in combination with pharmacologic mTOR inhibitors as a potential strategy for inhibiting growth of HER2-overexpressing breast cancers that show resistance to trastuzumab and poor response to single agent lapatinib.

Table 3-2. Combination Index (C.I.) values for lapatinib + MK-8669 (1:10)

Cell line	ED50	ED75	ED90	Dm	m	r
MDA361	1581.46933	3.8312e-012	0.04294	0.01883	-0.043822	0.99312
JIMT	0.01445	0.00286	0.00128	7.70049	-0.29234	0.95796

Table 3-2. Combination Index (C.I.) values for lapatinib + MK-8669 (1:10)

Cells were treated with lapatinib (0.1, 1, or 10 μM), MK-8669 (1, 10, or 100 nM), or combination lapatinib plus MK-8669. After 6 days, proliferation was measured by MTS assay. The fraction of cells proliferating relative to solvent control-treated cells was determined, and C.I. values were determined for the combination using CalcuSyn software. C.I. values are listed for effective doses at which 50%, 75%, or 90% (ED50, ED75, and ED90, respectively) of cells were killed. Statistically, drug synergy is defined by C.I. values less than 1.0, and very strong synergy is defined by C.I. values less than 0.1. D_m, the median-effect (ED50) drug concentration; $m < 1$ indicates a negative sigmoidal shape to the dose-effect curve; r states the linear correlation coefficient.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MDA361
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Lp
MK
Lp+MK
*p<0.05
JIMT
120
100
80
60
40
20
0
Lp
MK
Lp+MK
*p<0.05
B
MDA361
% Viability
60
40
20
0
Lp
MK
Lp+MK
*p<0.05
JIMT
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C
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C L M LM C L M LM
p-S473 Akt
total Akt
p-p70S6K
p85
p70
p70S6K
actin
D
JIMT-1 xenograft
*p<0.05
mean tumor volume (*100 mm3)
6
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2
1
0
3
6
10
13
17
days post-treatment initiation
vehicle
lap
MK
lap+MK

Figure 3-6. Efficacy of combination MK-8669 plus lapatinib in primary trastuzumab-resistant HER2-overexpressing breast cancer cells. **(A)** MDA361 and JIMT-1 cells were treated with 1 μM lapatinib (*Lp*), 10 nM MK-8669 (*MK*), or a combination of 1 μM lapatinib plus 10 nM MK-8669 for 6 days. Proliferation was then measured by MTS assay. Values represent the average of 6 replicates per group as a percentage of DMSO-treated cells. Error bars represent standard deviation between replicates. P-values were determined by t-test for combination treatments versus lapatinib alone for each cell line; *$p<0.05$. Experiments were repeated twice with reproducible results. **(B)** MDA361 and JIMT-1 cells were plated in matrigel and treated with 10 nM MK-8669, 1 μM lapatinib, or a combination of 10 nM MK-8669 plus 1 μM lapatinib. Media plus drugs were changed every 3 days for approximately 3-4 weeks. Matrigel was dissolved with dispase, and viable cells were counted by trypan blue. Viability is presented as a percentage of DMSO control group, and reflects an average of 3 replicates per treatment group. Error bars represent the standard deviation between replicates. P-value was determined by t-test for combination treatment versus lapatinib alone; *$p<0.05$. **(C)** JIMT-1 and MDA361 cells were treated with DMSO (*C*), 10 μM lapatinib (*L*), 100 nM MK-8669 (*M*), or a combination of 10 μM lapatinib plus 100 nM MK-8669 (*LM*) for 48 h, lysed for total protein, and immunoblotted for p-S473 Akt, total Akt, p-T389 p70S6K, total p70S6K, or actin loading control. **(D)** JIMT-1 cells were injected s.c. in the flank of athymic mice. After palpable tumors formed, tumors were treated daily (5 days on, 2 days off) with vehicle control (n=2), 75 mg/kg oral lapatinib (n=2), 1 mg/kg i.p. MK-8669 (n=2), or combination lapatinib plus MK-8669 (n=3). Mean tumor volume (x 100 mm^3) is shown per treatment group, with error bars representing the standard

deviation between replicates. P-value was determined by t-test for combination treatment versus lapatinib alone for each day that measurements were taken; *$p<0.05$.

3. Discussion

Our data indicated that inhibition of Akt is essential in order to achieve optimal response to lapatinib, with hyperactivation of Akt abrogating lapatinib sensitivity. Co-treatment with an mTOR inhibitor significantly improved response to lapatinib, as measured by reduced proliferation, anchorage-independent growth, and *in vivo* tumor growth. Although many HER2-overexpressing breast cancers are initially sensitive to trastuzumab, many recur and all metastatic cancers eventually develop resistance. Lapatinib has been approved for use in trastuzumab-refractory breast cancers, although response rates to single agent lapatinib are low. Our findings suggest that pharmacologic inhibition of mTOR should be tested in combination with lapatinib in HER2-overexpressing breast cancers exhibiting resistance to trastuzumab. Further, our results suggest that p-Akt levels may be measured as a marker of response in patients whose cancers are treated with lapatinib.

Resistance to trastuzumab has been strongly associated with increased PI3K signaling (Nagata et al, 2004;Berns et al, 2007;Esteva et al, 2010); however, conflicting data exists regarding the relationship between resistance to lapatinib and Akt activity. Eichhorn et al. (Eichorn et al, 2008) showed that *PTEN* loss or dominant activating *PIK3CA* mutations (E545K and H1047R) reduced lapatinib sensitivity *in vitro* and *in vivo*. In contrast, data presented by O'Brien et al. (O'Brien et al, 2010) suggested that there is not any association between increased PI3K signaling and response to lapatinib. Our data indicated that HCC1954, JIMT-1, MDA361, and MDA453 cells, all of which possess activating *PIK3CA* mutations and primary resistance to trastuzumab, also exhibit reduced sensitivity to lapatinib. In contrast, HCC1419 cells, which express wild-type

PIK3CA, showed higher response to lapatinib. Thus, hyperactive PI3K signaling due to activating mutations in *PIK3CA* appeared to reduce lapatinib sensitivity (although cells were not fully resistant). These data are consistent with that of O'Brien et al. (O'Brien, 2010), in that cells with activating *PIK3CA* mutations are not completely resistant to lapatinib, although our results indicate that these cells do show reduced sensitivity to lapatinib and resistance to trastuzumab. Reduced sensitivity to lapatinib was associated with an inability to block phosphorylation of Akt and p70S6K. In addition, overexpression of constitutively active Akt reduced response to lapatinib, while kinase-dead Akt improved sensitivity to lapatinib. Thus, our data support a direct association between Akt activity, the ability to inhibit p-Akt levels, and sensitivity to lapatinib. In contrast, although inhibition of p70S6K phosphorylation correlated with lapatinib sensitivity, knockdown of p70S6K alone was not sufficient to improve response to lapatinib. P70S6K is one downstream target of mTOR. Since inhibition of mTOR was sufficient to improve lapatinib sensitivity, our data suggest that other downstream effectors of mTOR must be inhibited in order to achieve optimal response to lapatinib. Indeed, knockdown of the mTOR substrate 4EBP1 resulted in a statistically significant improvement in the response to lapatinib. Thus, mTOR inhibition, and in particular, reduced expression and function of 4EBP1, is likely to achieve optimal response to lapatinib.

PI3K/Akt signaling controls expression of multiple cell cycle and apoptotic regulators. Sensitivity to lapatinib has been associated with modified expression of many of these proteins, consistent with the concept that PI3K inhibition is required for optimal response to lapatinib. Reduced expression of FOX03a transcription factor downstream of

PI3K inhibition has been reported in lapatinib-sensitive cells, leading to increased p27 and estrogen receptor transcription (Hegde et al, 2007) Inhibition of PI3K-survivin and MEK-Erk-Bim signaling has also been associated with lapatinib-mediated apoptosis (Xia et al, 2006;Tanizaki et al, 2011).

In addition to inhibiting kinase activities of EGFR and HER2, lapatinib has been shown to block nuclear translocation of these receptors (Kim et al, 2009). Nuclear HER2 acts as a transcription factor, inducing expression of cell cycle regulators such as thymidylate synthase, which is required for DNA synthesis. Lapatinib suppresses DNA replication in part by blocking TS transcription due to inhibition of HER2 translocation to the nucleus (Kim et al, 2009). Thus, inhibition of multiple PI3K-dependent cell cycle and apoptotic pathways appears to be required for lapatinib sensitivity. In addition, PI3K-independent mechanisms such as inhibition of HER2 nuclear localization may contribute to achieving complete response to lapatinib.

Survival outcomes for patients with HER2-overexpressing breast cancer have dramatically improved with the introduction of trastuzumab. Despite its efficacy, however, a subset of tumors show primary resistance and many may develop acquired resistance. Additional HER2-targeted agents, such as the dual EGFR/HER2 kinase inhibitor lapatinib, have shown promise clinically. However, many trastuzumab-pretreated cancers fail to respond to lapatinib therapy, and all eventually develop resistance. While these cancers are initially addicted to HER2 signaling, it remains possible that cancers that fail trastuzumab and/or lapatinib therapy have become addicted to additional signaling pathways, such as the PI3K/Akt-mTOR pathway, consistent with the concept of “oncogenic switch” (Stommel et al, 2007; Valbrega et al, 2011).

Our current data and recent clinical trials (Andre et al, 2010;,Morrow et al, 2011) support inhibition of mTOR as a potentially effective strategy for treating breast cancers that are resistant to trastuzumab, suggesting some level of dependence of these cancers on this molecular pathway. Single-agent rapamycin has been shown to induce feedback signaling via increased PI3K and/or MAPK signaling (Sun et al, 2005;Wang et al, 2008;Liu et al, 2011). However, this compensatory feedback signaling activated by rapamycin appears to be overcome by co-treatment with lapatinib. Collectively, the experiments support further study of combination lapatinib plus mTOR inhibition as a treatment approach in HER2-overexpressing breast cancers that show poor response to trastuzumab or lapatinib.

Akt phosphorylation can be activated by multiple upstream molecular alterations, including HER2 overexpression; thus, PI3K-Akt inhibition has been explored as a strategy for improving outcome of *HER2*-amplified breast cancers. Preclinical work has shown that PI3K inhibitors improve response to trastuzumab in trastuzumab-resistant breast cancer cells (Lu et al, 2007;Ozbay et al, 2010). Clinically, PI3K inhibitors were slower to evolve as targeted therapies in breast cancer due to selectivity issues; however, inhibitors of downstream mTOR have shown great promise in the context of refractory HER2-overexpressing breast cancer. Phase II trial of the mTOR inhibitor ridaforolimus plus trastuzumab in patients with HER2-positive trastuzumab-refractory metastatic breast cancer showed early evidence of anti-cancer activity with 2 partial responses reported out of 22 patients enrolled (Yardley et al, 2009). Our results support additional trial of ridaforolimus plus lapatinib in trastuzumab-refractory disease. Phase 1 and 2 trials combining the mTOR inhibitor, everolimus, with trastuzumab with or without

chemotherapy in patients with trastuzumab-resistant HER2-positive metastatic breast cancer showed encouraging results (Andre et al, 2010;Morrow et al, 2011;Jerusalem, 2011). A retrospective analysis of two phase 1 trials was performed to determine the safety and efficacy of everolimus in combination with trastuzumab-based chemotherapy in patients who had received prior treatment with both trastuzumab and lapatinib (Jerusalem et al, 2011;Baselga et al, 2011). The overall response rate (ORR) was higher in patients who had not received lapatinib (31%), compared to those who had received lapatinib (18%). Time to progression (TTP) was shorter in patients who had received lapatinib (29 weeks) compared to those who did not receive lapatinib (41 weeks).

These findings may be a reflection of heavier pretreatment in the lapatinib group. Despite the reduced ORR and TTP, the clinical benefit was approximately equal in both groups: 89% in the lapatinib pre-treated group and 84% in the lapatinib-free group. These results are important, as they suggest that patients who have progressed on both trastuzumab and lapatinib in the metastatic setting may derive clinical benefit from combination trastuzumab plus mTOR inhibitor. These results are being confirmed in ongoing phase 3 trials in first-line and trastuzumab-resistant settings. In summary, our results strongly support future clinical trials of combination lapatinib plus mTOR inhibitor in the context of metastatic breast cancers that show resistance to trastuzumab and poor sensitivity to single-agent lapatinib. Given the results presented here and the encouraging results of recent trials (Baselga et al, 2010), future studies should also examine combination treatments of trastuzumab, lapatinib, and mTOR inhibitor as first-line therapy in HER2-overexpressing breast cancers that are resistant to available HER2-targeted agents.

Chapter 4. Sustained MEK Signaling Confers Lapatinib Resistance Through FOXM1

Portions of this chapter were previously published:
Gayle SS., Castellino RC. Buss MC., Nahta R. Sustained MEK Inhibition increases lapatinib sensitivity via modulation of FOXM1. *Current Medicinal Chemistry.* 2013. (Epub ahead of publication)

1. Introduction

The human epidermal growth factor receptor (HER) tyrosine kinase family is comprised of four members: HER1 (epidermal growth factor receptor, EGFR), HER2, HER3, and HER4. Upon ligand binding, receptor dimerization activates multiple signaling pathways including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways (Bubil et al, 2007;Citri et al, 2006;Yarden et al, 2001). HER2 is overexpressed in about 20% of metastatic breast cancers and is associated with a poor prognosis (Eccles et al, 2001;Slamon et al, 1987). Trastuzumab (Herceptin), a monoclonal antibody targeted against the extracellular domain of HER2, is a highly effective treatment for patients with HER2-overexpressing breast cancers. However, as is the case with many targeted therapies, a majority of patients will eventually develop resistance to trastuzumab, resulting in disease progression.

The dual EGFR/HER2 tyrosine kinase inhibitor lapatinib (Tykerb) is approved for use in trastuzumab-refractory tumors. Lapatinib binds to the ATP-binding pocket of HER2, thereby preventing receptor phosphorylation and subsequent activation of downstream pathways. However, response to single agent lapatinib in trastuzumab-resistant cancers is less than 25% (Blackwell et al, 2010;Eichorn et al, 2008). Understanding the molecular mechanisms that lead to lapatinib resistance may ultimately improve the clinical benefit that patients received from lapatinib-based treatment. Previous studies have focused primarily on the role of PI3K/mTOR signaling in lapatinib resistance (Brunner et al, 2011;Gayle et al, 2012). However, inhibition of MEK appears to also play a critical role in mediating lapatinib cytotoxicity. Tanizaki et al. (Tanizaki et al, 2011) showed that lapatinib-mediated inhibition of MEK is critical for induction of

apoptosis. Lapatinib treatment of HER2-overexpressing cells inhibited MEK/ERK signaling, leading to up-regulation of the pro-apoptotic protein Bcl-2-interacting mediator of cell death (BIM). Further, transfection of oncogenic Ras into HER2-overexpressing, lapatinib-sensitive SKBR3 and BT474 cells has been shown to activate MEK/ERK signaling and abrogate response to lapatinib (Zoppoli et al, 2010). The Forkhead box transcription fraction M1 (FOXM1) is downstream of the MEK signaling pathway and its up-regulation is associated with HER2 overexpression (Bektas et al, 2008; Lam et al, 2006; Huang et al, 2007). Upon MEK/ERK phosphorylation, FOXM1 is activated and translocates to the nucleus to stimulate proliferation and cell survival by modulating expression of cell cycle and apoptosis regulators including the cyclin-dependent kinase inhibitor $p27^{kip1}$ and the anti-apoptotic protein surviving (Wang et al, 2010).

In the current study, we examine the role of MEK/ERK signaling in HER2-positive trastuzumab-resistant cells that exhibit reduced response to lapatinib. We demonstrate that an inability to block ERK phosphorylation is associated with reduced response to lapatinib. Pharmacologic inhibition or knockdown of MEK in combination with lapatinib induced cell cycle arrest and/or apoptosis in association with reduced expression of nuclear FOXM1 in resistant cells. Further, knockdown or pharmacologic inhibition of FOXM1 increased response of resistant cells to lapatinib, whereas transfection of FOXM1 into sensitive cells resulted in resistance. Finally, co-treatment of xenografts of HER2-positive, trastuzumab-resistant breast cancer cells with lapatinib and the MEK inhibitor selumetinib suppressed tumor growth, reduced Ki-67 staining, suppressed ERK phosphorylation, and reduced expression of FOXM1 in comparison to xenografts treated with single agents or vehicle control. Thus, MEK inhibition should be

studied further as a strategy for increasing response to lapatinib in HER2-positive breast cancers that have progressed on trastuzumab.

2. Results

i. Sustained MEK/ERK signaling is associated with reduced sensitivity to lapatinib

The ability of lapatinib to suppress ERK phosphorylation was examined in the HER2-overexpressing breast cancer lines BT474, HCC1419, JIMT-1, and MDA361 (Figure 1A). Lapatinib achieved dose-dependent inhibition of ERK phosphorylation in BT474 and HCC1419 cells, which we previously showed are sensitive to lapatinib (Gayle et al, 2012). In contrast, concentrations of lapatinib as high as 1000 nM failed to reduce ERK phosphorylation in JIMT-1 and MDA361 cells, which we previously showed have a lower sensitivity to lapatinib (Gayle et al, 2012).

Next, Western blots for phosphorylated and total ERK protein were performed in BT474, HCC1419, JIMT-1, and HCC1954 cell lysates to determine if lapatinib-resistant cells show increased baseline signaling of this pathway. However, levels of phosphorylated ERK were similar in lapatinib-sensitive and lapatinib-resistant lines (Figure 4-1B). Thus, sustained phosphorylation of ERK is associated with reduced response to lapatinib but does not appear to be due to endogenous hyper-activation of MEK signaling

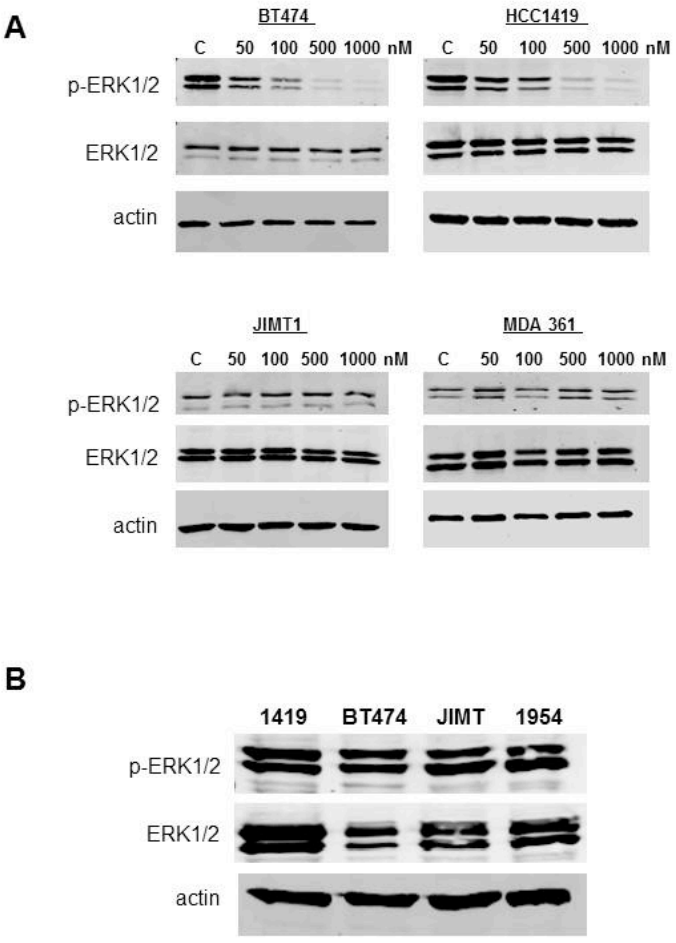
A
BT474
HCC1419
C 50 100 500 1000 nM
C 50 100 500 1000 nM
p-ERK1/2
ERK1/2
actin
JIMT1
MDA 361
C 50 100 500 1000 nM
C 50 100 500 1000 nM
p-ERK1/2
ERK1/2
actin
B
1419 BT474 JIMT 1954
p-ERK1/2
ERK1/2
actin

Figure 4-1. Sustained MEK Signaling in lapatinib-resistant cells. (A) BT474, HCC1419, JIMT-1, and MDA361 cells were treated with the control vehicle (C), 50, 100, 500, or 1000 nM lapatinib for 24 hours. Whole cell protein lysates were immunoblotted for p-ERK, total ERK or actin loading control. Blots were repeated at least three times with reproducible results. Lapatinib did not reduce ERK phosphorylation in JIMT-1 and MDA361 cells. **(B)** Untreated BT474, HCC1419, JIMT-1, and HCC1954 cells were lysed and immunoblotted for basal p-ERK, total ERK and actin loading control. Blots were repeated at least three times with reproducible results. No differences in baseline phosphorylated ERK were observed between lines.

ii. Pharmacologic MEK inhibition increases sensitivity to lapatinib

To investigate whether MEK inhibition improves response to lapatinib, we treated lapatinib-resistant JIMT-1 cells with 1000 nM lapatinib plus either 10 nM or 1000 nM of the MEK inhibitor PD0325901 (PD) (Liu et al, 2008). The combination of PD and lapatinib inhibited ERK phosphorylation to a greater degree than single agents (Figure 4-2A). Furthermore, although PD at 1000 nM induced Akt phosphorylation, lapatinib co-treatment overcame this compensatory signaling.

Next, the combination of lapatinib plus MEK inhibition was tested in clonogenic growth assays (Figure 4-2B). Growth of JIMT-1 cells in matrigel was partially reduced by lapatinib alone (Figure 4-2C). This is in contrast to adherent growth assays, in which lapatinib did not suppress growth of JIMT-1 cells, and may be due to prolonged exposure to the drug (3 weeks versus 3 days in adherent assays). However, PD alone did not significantly reduce growth of JIMT-1 cells. The most striking result was that co-treatment with PD plus lapatinib almost completely blocked survival of JIMT-1 cells in matrigel. Colonies that were still visible showed reduced colony size and number versus single agent treatment groups.

Next, we combined lapatinib with the MEK inhibitor, selumetinib (Bekaii-Saab et al, 2011;O'Neil et al, 2011), which is being developed for clinical use. Co-treatment of HCC1419, JIMT-1, and HCC1954 cells with selumetinib plus lapatinib resulted in a statistically significant inhibition of ERK phosphorylation in comparison to single agent treatments (Figure 4-3A). Lapatinib alone significantly reduced survival of BT474 cells (Figure 4-3B), whereas the viability of adherent JIMT-1 cells was not affected by lapatinib or selumetinib as single agents. In contrast, combined treatment with lapatinib

plus selumetinib significantly reduced survival of JIMT-1 cells in a dose-dependent manner. Similar to the combination of PD and lapatinib, co-treatment with selumetinib plus lapatinib significantly decreased JIMT-1 colony size and number growth in matrigel (Figure 4-3C). Finally, cell cycle profiling of JIMT-1 cells showed that the combination of selumetinib and lapatinib induced G1 arrest (Figure 4-3D) and reduced the percentage of cells in S phase by half (Table 4-1). Thus, co-treatment with a MEK inhibitor improves lapatinib sensitivity in HER2-positive breast cancer cells.

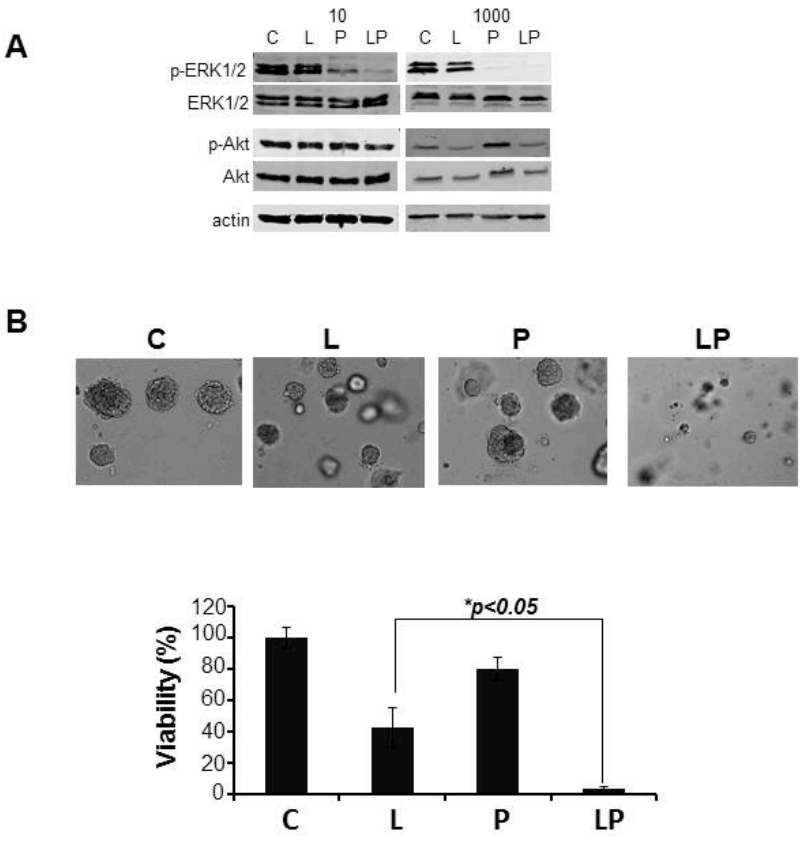
A
10
1000
C L P LP C L P LP
p-ERK1/2
ERK1/2
p-Akt
Akt
actin
B
C
L
P
LP
*p<0.05
Viability (%)
120
100
80
60
40
20
0
C
L
P
LP

Figure 4-2. MEK inhibition by PD0325901 (PD) increases lapatinib (L) response. **(A)** In the blots to the left, JIMT-1 cells were treated with 1000 nM L, 10 nM PD, combination 1000 nM L and 10 nM PD, or vehicle control (C) for 24 hours. In the blots to the right, JIMT-1 cells were treated with 1000 nM L, 1000 nM PD, combination 1000 nM L and 1000 nM PD, or vehicle control (C) for 24 hours. Whole cell protein lysates were immunoblotted for p-ERK, total ERK, p-Akt, total Akt, or actin loading control. Blots were repeated at least twice with reproducible results. Combined lapatinib plus PD suppressed phosphorylation of ERK to a greater degree than either drug alone and suppressed compensatory up-regulation of Akt phosphorylation. **(B)** JIMT-1 cells were plated in matrigel and treated with vehicle control, 1000 nM L, 10 nM PD, or a combination of 1000 nM L and 10 nM PD. Media plus drugs were changed every 3 days for approximately 2 weeks. Representative photos of colony growth in each treatment group are shown at 4× magnification. Graph: Matrigel from the growth assays was dissolved with dispase, and the number of cells was counted by trypan blue. Viability is presented as a percentage of DMSO control, and reflects an average of three replicates per treatment group. Error bars represent the standard deviation between replicates. Experiments were repeated at least three times with reproducible results. Lapatinib plus PD reduced growth in matrigel versus lapatinib alone; *$p<0.05$.

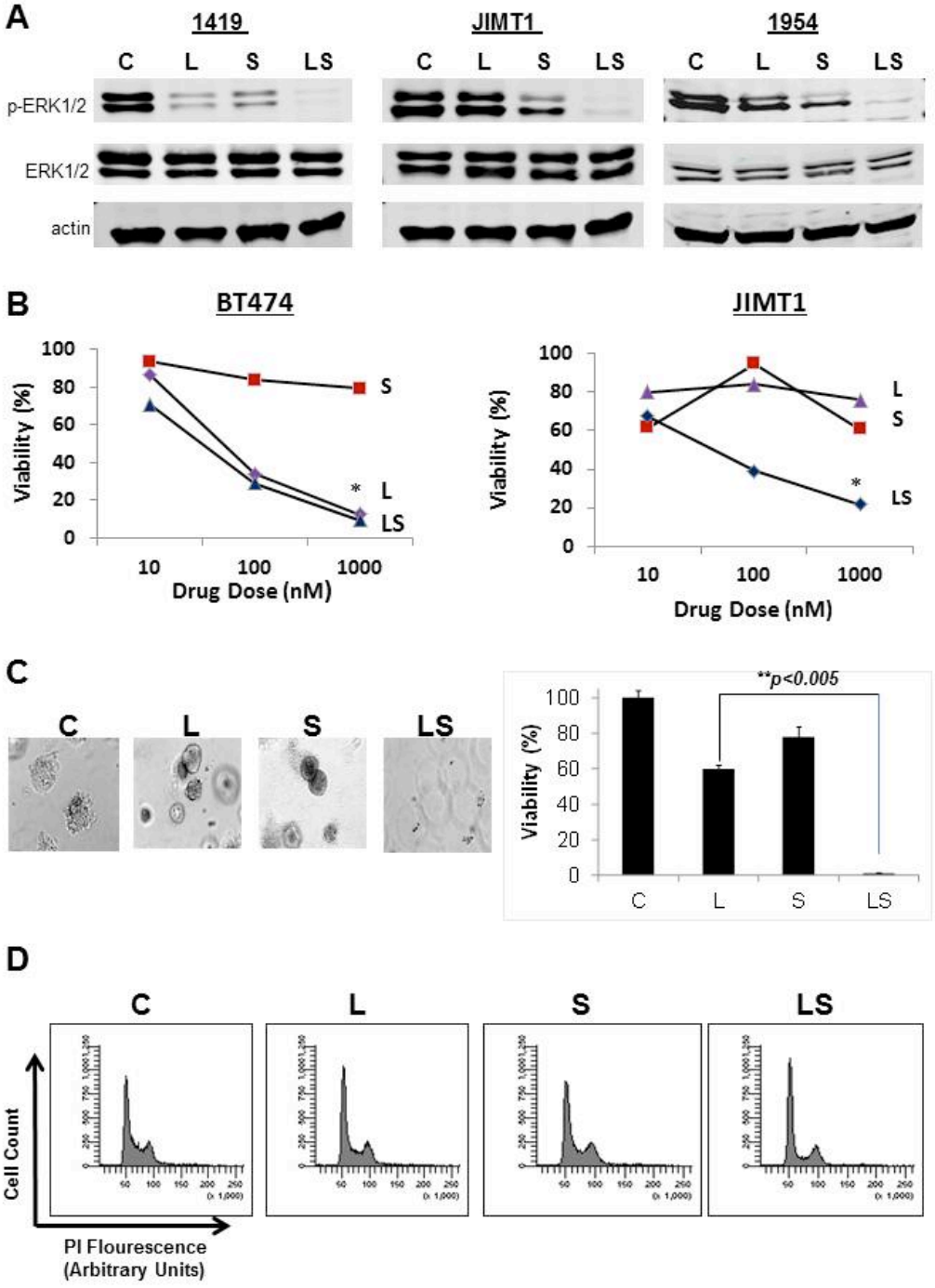
A
1419
JIMT1
1954
C
L
S
LS
p-ERK1/2
ERK1/2
actin
B
BT474
JIMT1
Viability (%)
Drug Dose (nM)
10
100
1000
S
L
LS
*
C
**p<0.005
D
Cell Count
PI Flourescence
(Arbitrary Units)

Figure 4-3. MEK inhibition by selumetinib (S) increases lapatinib (L) response. (A) HCC1419, JIMT-1, and HCC1954 cells were treated with the control vehicle (DMSO), 1000 nM L, 1000 nM S, or a combination of 1000 nM L and 1000 nM S (LS) for 24 hours. Whole cell protein lysates were immunoblotted for p-ERK, total ERK, or actin loading control. Blots were repeated on at least three separate occasions with reproducible results. Combined lapatinib plus selumetinib suppressed phosphorylation of ERK to a greater degree than either drug alone. **(B)** BT474 and JIMT-1 cells were plated at 3 x 10^4 in a 12-well plate format. After 24 hours, cells were treated with either L (10, 100, or 1000 nM), S (10, 100, or 1000 nM), or combination L plus S. Viable cells were counted by trypan blue exclusion after 72 hours and are reported as a percentage of control cells. Experiments were repeated twice with reproducible results. Lapatinib plus selumetinib reduced survival of JIMT-1 cells versus lapatinib or selumetinib alone; *$p < 0.05$. **(C)** JIMT-1 cells were plated in matrigel and treated with vehicle control DMSO, 1000 nM L, 10 nM S, or a combination of 1000 nM L and 10 nM S (LS). Media plus drugs were changed every 3 days for approximately two weeks. Representative photos of colony growth in each treatment group are shown at 4× magnification. Matrigel was dissolved with dispase, and viable cells were counted by trypan blue. Viability is presented as a percentage of DMSO control group, and reflects an average of three replicates per treatment group. Error bars represent the standard deviation between replicates. Experiments were repeated twice with reproducible results. Lapatinib plus selumetinib reduced growth of JIMT-1 cells in matrigel versus lapatinib alone; **$p<0.005$. **(D)** JIMT-1 cells were treated with DMSO control (C), 1000 nM L, 1000 nM S, or LS for 48 hours, fixed, stained with propidium iodide, and analyzed by flow

cytometry. Cell cycle profiles are displayed. Triplicate cultures were run per treatment group, and the experiment was repeated three times.

Table 4-1. Combination MEK inhibition and lapatinib treatment results in G0/G1 cell cycle arrest and apoptosis.

	%G0/G1	%S	%G2/M
C	55±0.48	18±0.08	28±0.54
L	54±0.78	16±0.18	30±0.63
S	57±1.66	15±0.78	29±0.89
LS	63±0.19	9±0.62	28±0.78

Table 4-1. Combination MEK inhibition and lapatinib treatment results in G0/G1 cell cycle arrest and apoptosis. Quantification of mean percentages + standard error among triplicates of JIMT1 cells treated with vehicle control DMSO, 1000nM lapatinib (L), 10nM selumetinib (S), or a combination of 1000nM L and 10nM S (LS) and analyzed by flow cytometry after fixing and staining with propidium iodide.

iii. Knockdown of MEK in combination with lapatinib induces apoptosis of JIMT-1 cells

Since the combination of pharmacological MEK inhibition and lapatinib suppressed ERK phosphorylation and cellular proliferation, we examined the effects of MEK knockdown on lapatinib resistance. Knockdown of MEK was achieved by simultaneously transfecting siRNA oligonucleotides against MEK1 and MEK2 and was confirmed by Western blotting (Figure 4-4A). The combination of lapatinib plus MEK knockdown reduced phosphorylation of ERK in JIMT-1 cells (Figure 4-4A) and significantly reduced cell survival (Figure 4-4B). Flow cytometric analysis of annexin-stained cells showed increased apoptosis of cells that were transfected with MEK1/2 siRNA and treated with lapatinib (Figure 4-4C). Apoptosis was induced in almost 63% of cells treated with lapatinib plus MEK knockdown, which was approximately 2-fold higher than when cells were treated with lapatinib and control siRNA (Table 4-2).

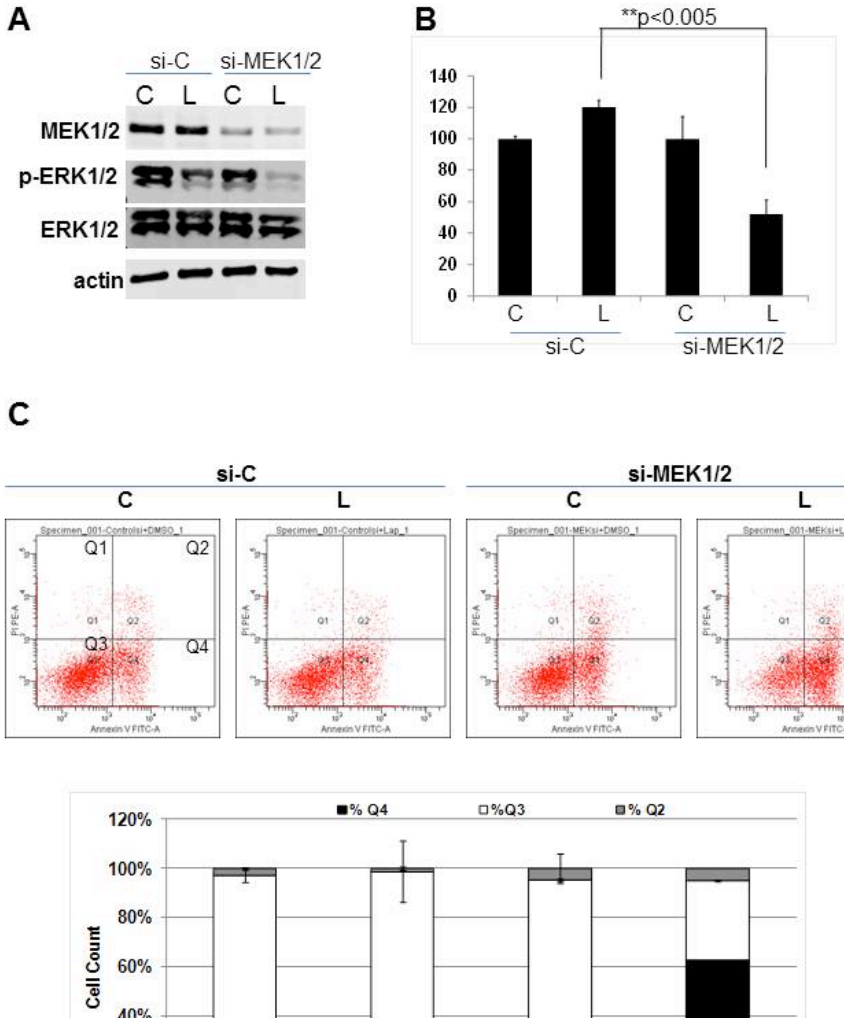
A
si-C
si-MEK1/2
C
L
C
L
MEK1/2
p-ERK1/2
ERK1/2
actin
B
**p<0.005
140
120
100
80
60
40
20
0
C
L
C
L
si-C
si-MEK1/2
C
si-C
si-MEK1/2
C
L
C
L
Q1
Q2
Q3
Q4
% Q4
%Q3
% Q2
120%
100%
80%
60%
40%
20%
0%
Cell Count
si-C
Control
si-C
Lapatinib
MEK-si
Control
MEK-si
Lapatinib

Figure 4-4. MEK knockdown increases lapatinib (L) response. (A) JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or MEK1/2 siRNA (si-MEK) for 24 hours, and then treated with DMSO control (C), or 1000 nM L for 48 hours. Total protein lysates were immunoblotted for MEK1/2, p-ERK, total ERK, and actin loading control to confirm knockdown. Knockdown of MEK was confirmed. The combination of MEK knockdown plus lapatinib suppressed ERK phosphorylation to a greater degree than MEK knockdown or lapatinib alone. **(B)** JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or MEK1/2 siRNA for 24 hours and then treated with DMSO control (C) or 1000 nM L. After 48 hours, surviving cells were counted by trypan blue exclusion. Viability is presented as a percentage of DMSO-treated, si-C-transfected cells and reflects the average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. P-values were determined by t-test for MEK1/2 knockdown plus lapatinib versus control siRNA plus lapatinib. MEK knockdown plus lapatinib showed significantly reduced cell survival versus lapatinib and control siRNA; $^{**}p<0.005$. Experiments were repeated at least three times with reproducible results. **(C)** JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or MEK1/2 siRNA (si-MEK) for 24 hours and then treated with vehicle control (C) or 1000 nM L for 24 hours. Cells were stained with annexin V-FITC and propidium iodide and analyzed by flow cytometry. The top panels show dot plots of the cells. Quadrant 2 (Q2) shows late apoptotic cells (positive for both Annexin V-FITC and propidium iodide staining). Quadrant 3 (Q3) shows normal viable cells. Quadrant 4 (Q4) shows early apoptotic cells (Annexin V-FITC positive). The bottom graph shows quantification of the mean

percentages of cells in each quadrant with Q4 showing increased apoptosis in cells treated with lapatinib plus selumetinib.

iv. Co-treatment with selumetinib and lapatinib alters FOXM1 expression

FOXM1 functions as a transcription factor in the nucleus where it represses expression of cell cycle inhibitors including $p27^{kip1}$. Immunofluorescence was performed to determine the cellular localization of FOXM1 in different treatment groups. FOXM1 was localized to the nucleus and cytoplasm in the majority of control HCC1419 cells (Figure 4-5A). Treatment with lapatinib, selumetinib, or the combination of drugs did not alter FOXM1 localization, i.e., all cells still showed FOXM1 staining in the nucleus and cytoplasm. In contrast, FOXM1 was exclusively nuclear in JIMT-1 cells treated with vehicle control alone. Single agent lapatinib or selumetinib did not alter this localization. However, upon treatment with the combination of lapatinib plus selumetinib, FOXM1 was detected in both the nucleus and cytoplasm of the majority of JIMT-1 cells, indicating that co-treatment induced translocation of FOXM1 into the cytoplasm (Figure 4-5B). Nuclear and cytoplasmic fractioning confirmed that JIMT-1 cells expressed FOXM1 primarily in the nucleus at baseline, and that lapatinib alone or selumetinib alone did not alter this localization (Figure 4-5C). Combination lapatinib plus selumetinib reduced expression of FOXM1. Consistent with these findings, the transcript level of the FOXM1 target $p27^{kip1}$, which is normally repressed by FOXM1, was induced by lapatinib by was dramatically more increased upon co-treatment with lapatinib plus selumetinib (Figure 4-5D). These results suggest that combining lapatinib with MEK inhibition results in reduced FOXM1 nuclear staining, reduced FOXM1 expression, and reduced transcript level of target gene p27kip1, consistent with the induction of growth arrest.

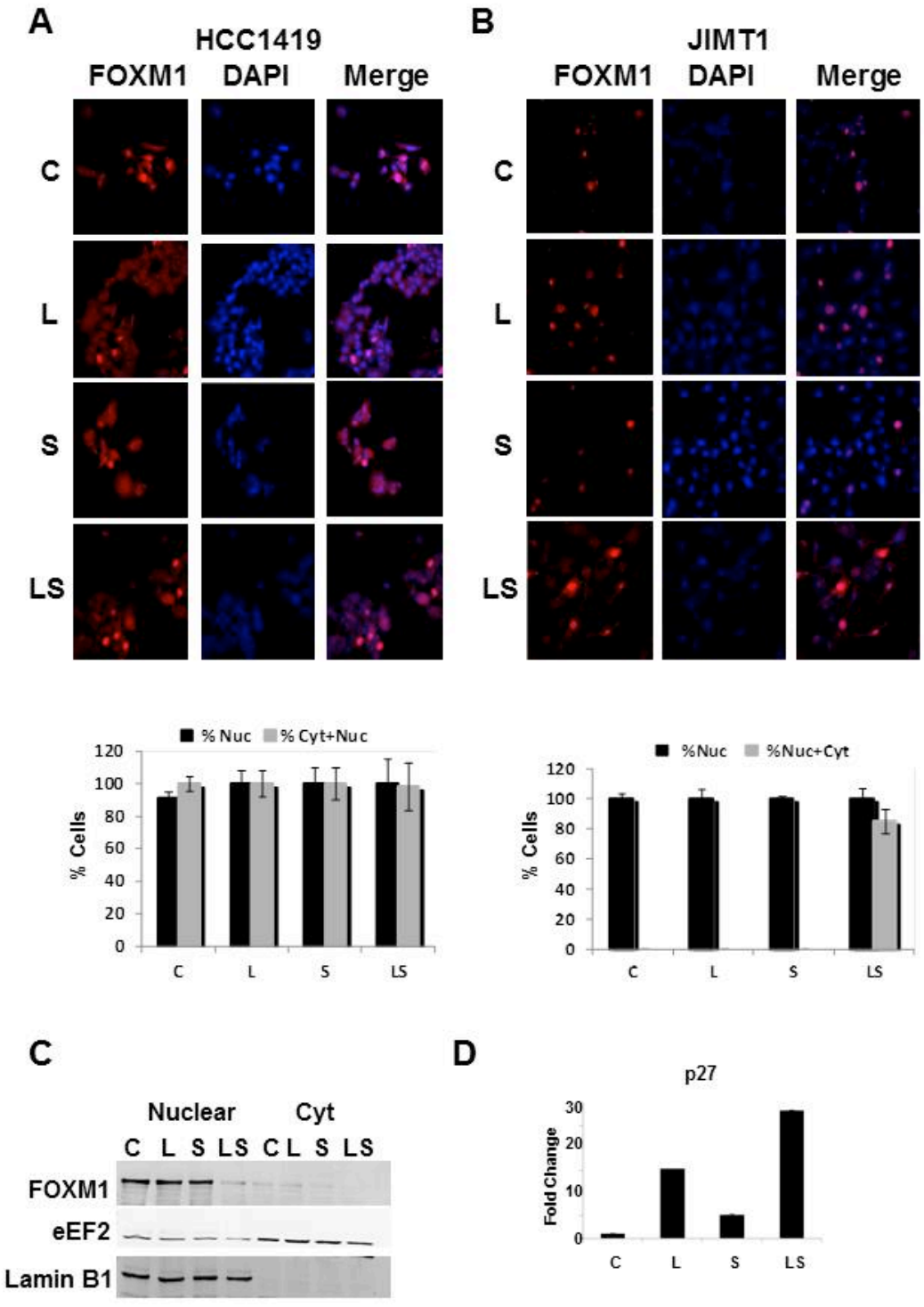
A
HCC1419
FOXM1
DAPI
Merge
C
L
S
LS
B
JIMT1
FOXM1
DAPI
Merge
C
L
S
LS
% Nuc
% Cyt+Nuc
% Cells
120
100
80
60
40
20
0
C
L
S
LS
%Nuc
%Nuc+Cyt
% Cells
120
100
80
60
40
20
0
C
L
S
LS
C
Nuclear
Cyt
C L S LS C L S LS
FOXM1
eEF2
Lamin B1
D
p27
Fold Change
30
20
10
0
C
L
S
LS

Figure 4-5. FOXM1 localization and expression in response to lapatinib plus selumetinib treatment. (A) JIMT-1 cells were plated on glass coverslips for 24 hours before being treated with vehicle control, 1000 nM L, 1000 nM S, or a combination of 1000 nM L plus 1000 nM S (LS) for 24 hours. Immunofluorescence was performed to determine the cellular location of total FOXM1. Cells were observed at a magnification of 40×. Graphs are a quantification of the IF data from 10 samples per treatment demonstrating the percentage of cells expressing FOXM1 in the nucleus or nucleus plus cytoplasm versus the percentage of cells expressing FOXM1 in the cytoplasm or cytoplasm plus nucleus. The data indicated that lapatinib and selumetinib did not alter localization of FOXM1 in lapatinib-sensitive HCC1419 cells. In contrast, JIMT-1 cells, which expressed FOXM1 primarily in the nucleus at baseline, showed an increased percentage of cells staining positive for FOXM1 in the cytoplasm when treated with lapatinib plus selumetinib. **(C)** Western blot analysis detecting FOXM1 from the nuclear and cytoplasmic fractions of JIMT-1 cells treated with DMSO control, 1000 nM lapatinib (L), 1000 nM selumetinib (S), or combination LS for 24 hours. Lamin B (nuclear) and eEF2 (cytoplasmic) were used as fractionation controls. The combination reduced expression of nuclear FOXM1. **(D)** Real-time PCR was performed for FOXM1 downstream target p27 in JIMT-1 cells that had been treated with DMSO control, 1000 nM L, 1000 nM selumetinib S, or combination LS for 24 hours. Values reflect the fold change in transcript normalized to RPLPO housekeeping gene. Lapatinib induced p27 transcript levels. The combination caused an even greater induction in p27 levels.

Table 4-2. Combination MEK inhibition and lapatinib treatment results in increased apoptosis in lapatinib-resistant cells.

	% Late Apoptotic	% Normal	% Early Apoptotic
si-C +C	2.87±0.33	69±2.58	27.84±1.5
si-C+L	1.14±0.97	55.3±10.53	27.8±11.5
si-MEK+C	4.69±6	68.5±0.63	36±0.32
si-MEK +L	4.9±0	32.2±0.32	62.9±1.88

Table 4-2. Combination MEK inhibition and lapatinib treatment results in increased apoptosis in lapatinib-resistant cells. Quantification of mean percentages + standard error in triplicates of JIMT-1 cells transfected with 100 nM control siRNA (si-C) or MEK1/2 siRNA (si-MEK) for 24 hours and then treated with DMSO control (C) or 1000nM Lap for 48 hours. Cells were analyzed by flow cytometry for Annexin V-FITC and propidium iodide staining.

v. FOXM1 knockdown increases lapatinib sensitivity

To gain additional evidence that modulation of FOXM1 improves response to lapatinib, we knocked down FOXM1 and assessed cell survival. FOXM1 knockdown resulted in significantly decreased cell viability on its own. A significantly greater reduction in viability was observed when FOXM1 knockdown was combined with lapatinib compared to lapatinib treatment in FOXM1 expressing cells (Figure 4-6A).

The thiazole antibiotic thiostrepton selectively reduces FOXM1 mRNA and protein expression in a dose- and time-dependent manner (Bhat et al, 2009; Kwok et al, 2008). FOXM1 down-regulation by thiostrepton was found to induce apoptosis and repress migration and metastasis in breast cancer cells. Treatment of JIMT-1 cells with thiostrepton resulted in a significant decrease in viability (Figure 4-6B). The combination of thiostrepton and lapatinib treatment resulted in significantly reduced colony survival of HCC1954 and JIMT-1 cells grown in matrigel (Figure 4-6C). Conversely, transfection of a FOXM1 expression plasmid into lapatinib-sensitive HCC1419 cells abrogated lapatinib-induced cytotoxicity (Figure 4-6C). These data suggest that FOXM1 expression levels regulate sensitivity to lapatinib, and that down-regulation of FOXM1 improves lapatinib sensitivity.

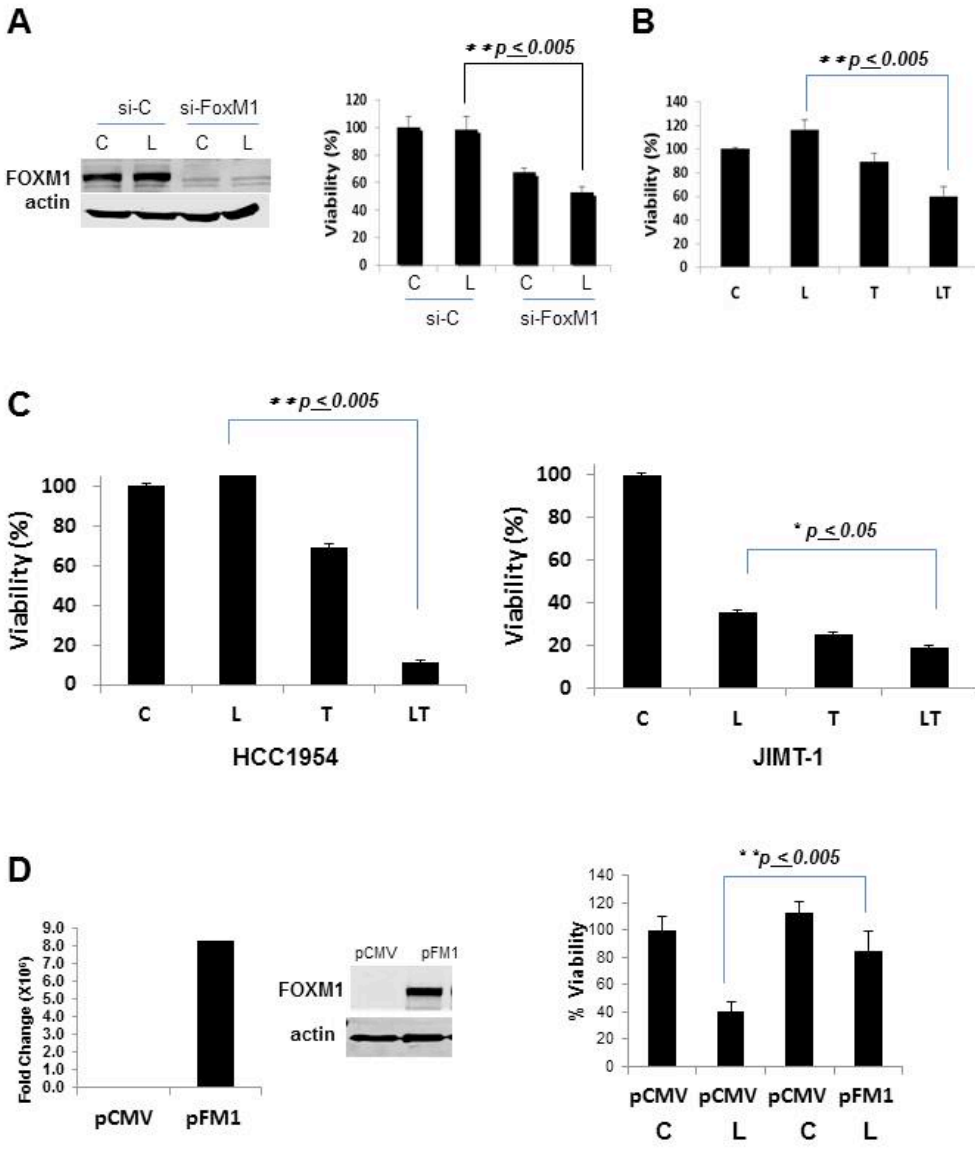
A
si-C
si-FoxM1
C
L
C
L
FOXM1
actin
**p ≤ 0.005
Viability (%)
si-C
si-FoxM1
B
**p ≤ 0.005
Viability (%)
C
L
T
LT
C
**p ≤ 0.005
Viability (%)
HCC1954
*p ≤ 0.05
JIMT-1
D
Fold Change (X10⁶)
pCMV
pFM1
pCMV
pFM1
FOXM1
actin
**p ≤ 0.005
% Viability
pCMV
pCMV
pCMV
pFM1
C
L
C
L

Figure 4-6. Modulation of FOXM1 expression affects lapatinib sensitivity. (A) JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or FOXM1 siRNA (si-FM1) for 24 hours, and then treated with DMSO control vehicle (C) or 1000 nM Lapatinib (L) for 24h. Total protein lysates were immunoblotted for FOXM1 to confirm knockdown and actin. Alternatively, after 48 hours of drug treatment, surviving cells were counted by trypan blue exclusion. Viability is presented as a percentage of DMSO-treated control siRNA-transfected cells and reflects the average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. P-values were determined by t-test for FOXM1 knockdown plus L versus si-C plus L; **$p<0.005$. Experiments were repeated three times with reproducible results. FOXM1 knockdown reduced viability of JIMT-1 cells, with addition of lapatinib further reducing cell survival. **(B)** JIMT-1 cells were plated at 3×10^4 in a 12-well plate format. After 24 hours, cells were treated with DMSO control, 1000 nM L, 2000 μM thiostrepton (T), or combination L plus T. Viable cells were counted after 48 hours by trypan blue exclusion and are reported relative to control cells. Error bars represent standard deviation between replicates. Experiments were repeated three times with reproducible results. P-values were determined by t-test for L treatment versus FOXM1 inhibition plus L. Viability was significantly reduced by lapatinib plus thiostrepton versus lapatinib alone; **$p<0.005$. **(C)** HCC1954 and JIMT-1 cells were plated in matrigel and treated with control, 1000 nM L, 2000 nM T, or combination 1000 nM L and 2000 nM T. Media plus drugs were changed every 3 days for approximately 2 weeks. Matrigel was dissolved with dispase, and viable cells were counted by trypan blue. Viability is presented as a percentage of DMSO control, and reflects an average of 3 replicates per treatment group. Error bars represent the standard

deviation between replicates. Experiments were repeated three with reproducible results. P-value was determined by t-test for combination treatment versus lapatinib alone. Clonogenic growth was significantly reduced by lapatinib plus thiostrepton versus either agent alone in HCC1954 (**p<0.005) and JIMT-1 (*p<0.05) cells. **(D)** In the graph on the left, real-time PCR was performed for FOXM1 in HCC1419 cells that had been transfected with either 5μg pCMV-FOXM1 plasmid (pFM1) or control CMV plasmid (pCMV). Values reflect the fold change in transcript normalized to RPLPO housekeeping gene. In blot to the right, HCC1419 cells were transfected with either pCMV (C) or 2μg pFM1 for 24 hours. Total protein lysates were immunoblotted for FOXM1 to confirm overexpression and actin loading control. In the graph on the right, HCC1419 cells were transfected with 2 μg pCMV or pFM1 for 24 hours and then treated with DMSO control (C) or 1000 nM lapatinib (L) for another 48 hours. Viability measured by trypan blue exclusion is presented as a percentage of DMSO-treated pCMV and reflects the average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. Experiments were repeated three times with reproducible results. P-values were determined by t-test for FOXM1 plus L versus vector control plus L; **p<0.005

vi. The combination of selumetinib plus lapatinib suppresses tumor growth of JIMT-1 xenografts

Next, JIMT-1 cells were injected to form flank xenografts in athymic mice. Mice were treated with vehicle control, 75mg/kg oral lapatinib, 50mg/kg oral selumetinib, or a combination of lapatinib and selumetinib. Co-treatment with lapatinib plus selumetinib achieved a statistically significant greater inhibition of tumor growth in comparison to

either drug alone (Figure 4-7A). Tumors from the combination treatment group showed lower levels of the proliferation marker Ki67, decreased phosphorylation of ERK, and reduced FOXM1 expression (Figure 4-7B). These results support the concept that MEK inhibition plus lapatinib suppresses phosphorylation of ERK and FOXM1 expression, consistent with in vitro results. Further, the results suggest that the combination of MEK inhibitor selumetinib plus lapatinib suppresses growth of trastuzumab-resistant HER2-overexpressing breast cancer.

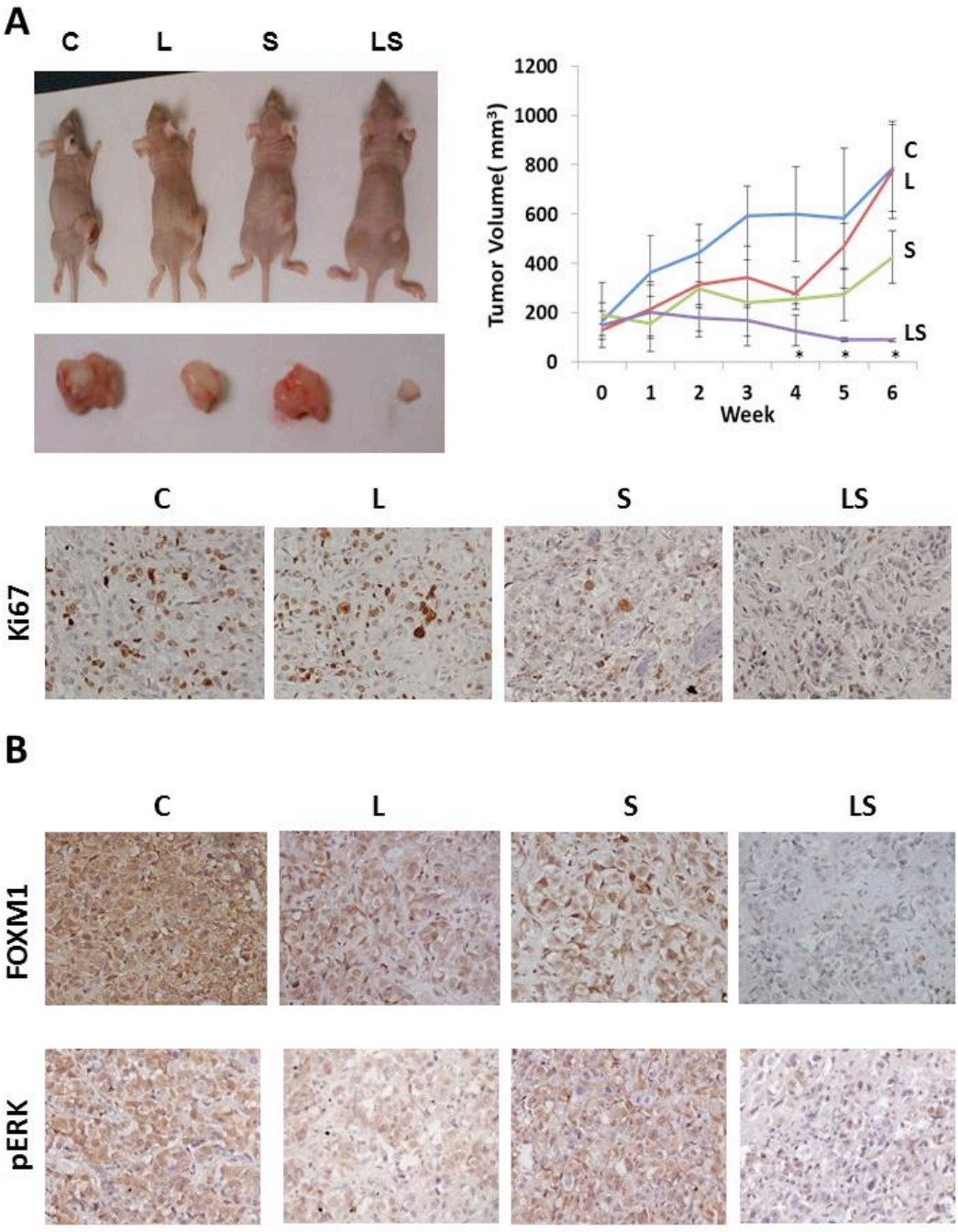
A
C
L
S
LS
Tumor Volume(mm³)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6
Week
C
L
S
LS
Ki67
B
FOXM1
pERK

Figure 4-7. The combination of selumetinib plus lapatinib suppresses tumor growth of JIMT-1 trastuzumab-resistant HER2-overexpressing breast cancer xenografts. JIMT-1 cells were injected s.c. in the flank of athymic mice. After palpable tumors formed, tumors were treated daily (5 days on, 2 days off) with vehicle control (n=3), 75 mg/kg oral lapatinib (n=3), 50 mg/kg oral selumetinib (n=3), or combination lapatinib plus selumetinib (n=3). **(A)**. The top panel is a representative picture of an animal from each treatment group and the bottom panel is a representative picture of the excised tumor from one animal in each treatment group. In the graph to the right, mean tumor volume is shown per treatment group, with error bars representing the standard deviation between replicates. P-value was determined by t-test for combination treatment versus lapatinib alone for each week that measurements were taken; *$p<0.05$. Bottom IHC shows a representative picture of an animal from each treatment group stained for Ki67. **(B)** Staining for FOXM1 and pERK in a representative picture of an animal from each treatment group. All IHC photos were taken at 10× magnification.

3. Discussion

HER2 overexpression is frequently observed in metastatic breast cancer and is associated with aggressive disease and poor prognosis (Slamon et al, 1987,1989). The dual EGFR/HER2 small molecule tyrosine kinase inhibitor, lapatinib, has been approved for use in HER2-overexpressing breast cancers that have progressed on trastuzumab. Response rates to single agent lapatinib range between 12 % and 25%, making it necessary to elucidate the precise mechanisms that confer resistance (Blackwell et al, 2009. 2010;Toi et al, 2009). Our data demonstrate that sustained MEK/ERK signaling results in poor lapatinib response despite similar baseline levels of phosphorylated ERK. Combination MEK inhibition and lapatinib treatment suppressed MEK/ERK signaling, increasing overall cytotoxicity, apoptosis, and inhibition of tumor growth relative to the single agent treatments. Combination MEK inhibition and lapatinib treatment also led to a greater decrease in ERK phosphorylation and reduced expression of the proto-oncogene FOXM1 when compared to either single agent treatment. These findings provide potential rationale for combining pharmacologic inhibition of MEK with lapatinib in HER2-overexpressing breast cancers that have progressed on trastuzumab.

In an effort to better understand the mechanisms behind growth inhibition of adherent and matrigel-suspended cells observed with combination MEK inhibition and lapatinib treatment, we performed cell cycle analysis experiments. Our results showed that pharmacological MEK inhibition combined with lapatinib treatment promoted G0/G1 cell cycle arrest and decreased the percentage of cells in S phase. Induction of $p27^{kip1}$ in the combination treatment group versus either single agent groups was consistent with induction of G1 arrest and reduced proliferation. Similarly, addition of

lapatinib to cells that had been transfected with small interfering RNA (siRNA) against MEK1 and MEK2 (MEK1/2) also reduced ERK phosphorylation and viability. In contrast to pharmacological inhibition, however, MEK1/2 knockdown plus lapatinib induced a significant level of apoptosis. Thus, it is possible that off-target effects of MEK inhibition elicit different cellular effects than specific knockdown of MEK1 and MEK2. In vivo pharmacological inhibition of MEK effectively increased lapatinib response, suppressing proliferation (as measured by Ki-67 staining) and leading to inhibition of tumor growth, supporting in vitro findings.

The forkhead transcription factors FOX01, FOX03a, and FOXM1 are downstream of MEK/ERK and play pivotal roles in tumorigenesis. FOX01 and FOX03a are upstream negative regulators of FOXM1 and behave as tumor suppressors. In contrast, FOXM1 functions as an oncogene. FOXM1 expression decreased upon treatment with the combination of MEK inhibition and lapatinib when compared to either single agent treatment. These data suggest that FOXM1 is a critical downstream effector of MEK involved in conferring lapatinib resistance.

FOXM1 has been implicated in multiple human cancers including breast, lung, esophageal, and pancreatic cancer (Wang et al, 2010; Fu et al, 2008; Hui, et al, 2012). In breast cancer, FOXM1 expression is associated with poor prognosis and has been tightly correlated with HER2 status (Bektas et al, 2008;Francis et al, 2009; Zhao et al, 2012). The role of MEK-FOXM1 has not previously been investigated in HER2-overexpressing breast cancer, but this pathway has been targeted in ovarian cancer with promising results. Chan et al. showed that ERK/FOXM1 inhibition by U0126 or thiostrepton resulted in decreased cancer cell growth *in vitro* as well as *in vivo* in ovarian cancer

models (Chan et al, 2012). Our current data support inhibition of MEK-FOXM1 in combination with lapatinib as a potentially effective strategy for HER2-overexpressing breast cancers that are resistant to trastuzumab, suggesting a role for this pathway in lapatinib resistance. Our data indicate that pharmacologic and genetic inhibition of FOXM1 increases lapatinib sensitivity in resistant HER2-overexpressing breast cancer cells, providing evidence for the role of FOXM1 in lapatinib resistance. Indeed, overexpression of FOXM1 in lapatinib-sensitive cells abrogated sensitivity to lapatinib. The present study is the first to show that decreased expression of FOXM1 due to MEK inhibition is required for optimal lapatinib response in trastuzumab-resistant HER2-overexpressing breast cancers.

We also showed that combinatorial MEK/ERK inhibition and lapatinib treatment results in decreased FOXM1 nuclear expression. This finding is consistent with published data demonstrating that FOXM1 is ubiquitously expressed in proliferating cells and is dependent on MEK/ERK signaling for activation and nuclear translocation (Wang et al, 2010; Leung et al, 2001). Our data showed a positive correlation between decreased FOXM1 expression and induction of the FOXM1 target $p27^{kip1}$ after treatment with combination lapatinib and MEK inhibition. Thus, induction of p27 may serve as a biomarker of lapatinib response. Additional FOXM1 downstream signaling molecules could be involved in the growth arrest and apoptosis induced by this combination. Future studies will investigate the role of FOXM1 downstream effectors in lapatinib resistance.

Our *in vivo* data with xenografts of lapatinib-resistant HER2-overexpressing breast cancer cells showed that co-treatment with a MEK inhibitor increases response to lapatinib, as demonstrated by suppressed tumor growth when compared to either single

agent treatment. Tumor suppression was associated with decreased phosphorylation of ERK, reduced FOXM1, and decreased staining for the proliferation marker Ki-67. These data indicate the importance of simultaneously inhibiting MEK-FOXM1 signaling with lapatinib treatment in HER2-overexpressing breast cancers that have progressed on trastuzumab.

Chapter 5

Conclusions

i. Summary & conclusions

My research provides evidence that the MEK/ERK and PI3K/Akt pathways mediate the development of resistance to lapatinib. I propose models whereby optimal response to lapatinib is achieved with the combinatorial inhibition of either the MEK/ERK pathway, or PI3K/mTOR pathway (Figure 5-1, 2). Significantly, these models reveal some of the signaling proteins that are involved in conferring lapatinib resistance and provide other targets that should be inhibited along with EGFR/HER2 for an optimal response in HER2-overexpressing breast cancer patients who have progressed on trastuzumab.

The preceding chapters provide evidence for the roles of PI3K/mTOR and MEK/ERK in lapatinib resistance. In chapter 3, our data demonstrates that reduced sensitivity to lapatinib is associated with an inability of lapatinib to inhibit Akt and p70S6K phosphorylation. Our interest in the role PI3K/mTOR was sparked by conflicting published data about the involvement of this pathway in the development of lapatinib resistance. Our goal was to elucidate the role PI3K/mTOR plays in lapatinib response and determine whether it should be considered as a co-target along with EGFR/HER2 inhibition by lapatinib.

Our data demonstrated that transfection of constitutively active Akt reduced lapatinib sensitivity, while kinase-dead Akt increased sensitivity. Knockdown of 4EBP1 also increased lapatinib sensitivity, in contrast to p70S6K knockdown, which did not affect response to lapatinib. Pharmacologic inhibition of mTOR using rapamycin or ridoforolimus increased lapatinib sensitivity and reduced phospho-Akt levels in cells that showed poor response to single-agent lapatinib, including those transfected with

hyperactive Akt. Finally, combination mTOR inhibition plus lapatinib resulted in synergistic inhibition of proliferation, reduced anchorage independent growth, and reduced in vivo tumor growth of HER2-overexpressing breast cancer cells that have primary trastuzumab resistance.

Our data suggest that PI3K/mTOR inhibition is critical for achieving optimal response to lapatinib. Collectively, these experiments support evaluation of lapatinib in combination with pharamacologic mTOR inhibition as a potential strategy for inhibiting growth of HER2-overexpressing breast cancers that show resistance to trastuzumab and poor response to lapatinib.

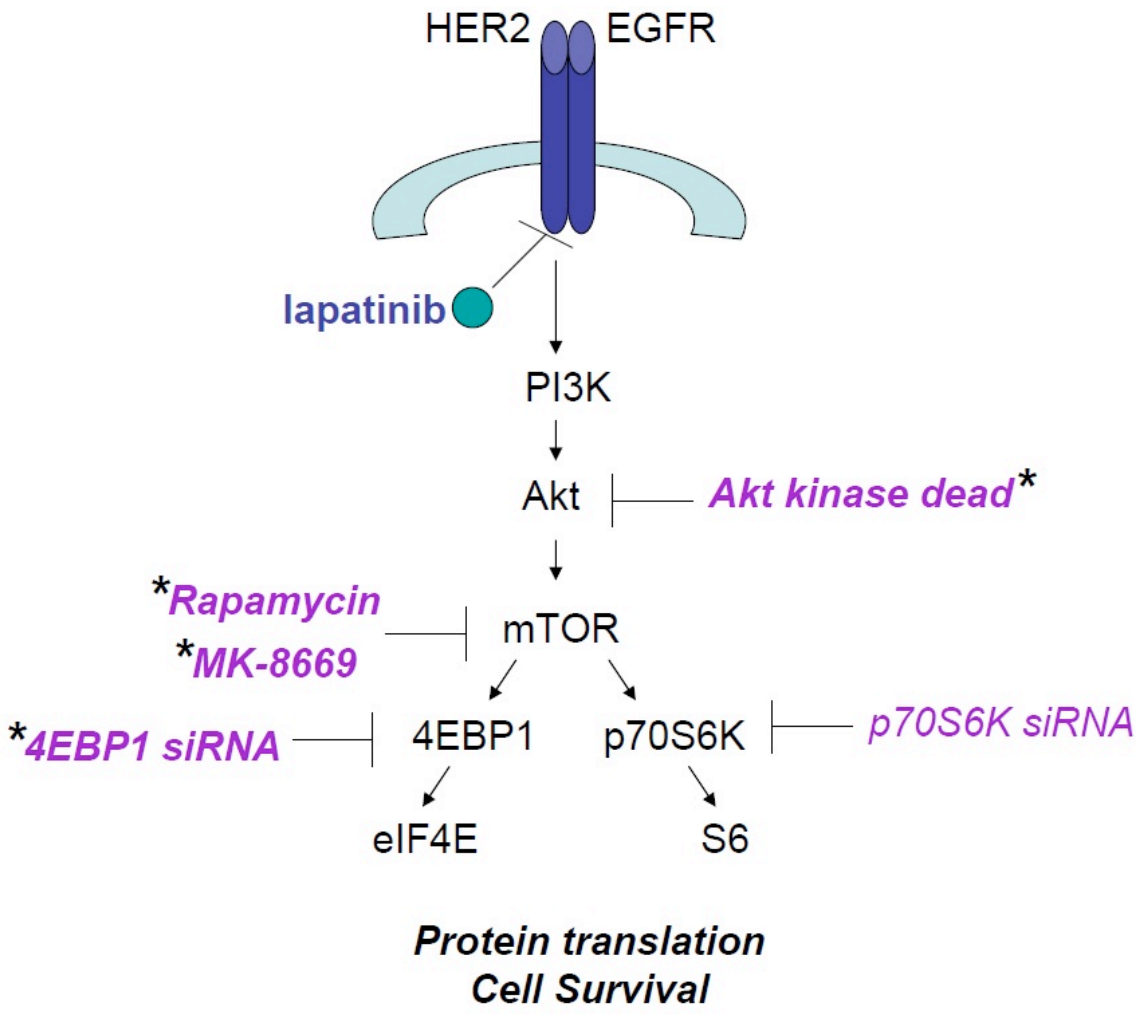
HER2
EGFR
lapatinib
PI3K
Akt
Akt kinase dead
*Rapamycin
*MK-8669
mTOR
*4EBP1 siRNA
4EBP1
p70S6K
p70S6K siRNA
eIF4E
S6
Protein translation
Cell Survival

Figure 5-1. Proposed model and summary. Lapatinib, a dual EGFR/HER2 kinase inhibitor, inhibits cell proliferation and survival in part by blocking PI3K/Akt/mTOR signaling. Based on our data, we propose that lapatinib is unable to block proliferation when PI3K/mTOR is not inhibited. Genetic or pharmacologic strategies that improved sensitivity to lapatinib (marked with an asterisk) included expression of dominant negative kinase-dead Akt, knockdown of 4EBP1, and mTOR inhibition by rapamycin or MK-8669. In contrast, knockdown of p70S6K alone did not increase the anti-proliferative activity of lapatinib.

In chapter 4, we investigate the other main downstream pathway to EGFR and HER2, MEK/ERK. Our data indicates that MEK/ERK inhibition increases lapatinib-mediated cytotoxicity in resistant HER2-overexpressing breast cancer cells. We genetically and pharmacologically block MEK/ERK signaling and evaluate lapatinib response. Combinatorial MEK inhibition and lapatinib treatment reduces phosphorylated ERK more than single agent treatment. In addition, the combination of MEK inhibitor plus lapatinib reduces nuclear expression of the MEK/ERK downstream proto-oncogene FOXM1. We deemed this observation important due to the involvement of FOXM1 in various cancers including HER2-overexpressing breast cancer.

We went on to show that genetic knockdown of MEK increases lapatinib-mediated cell cycle arrest or apoptosis in JIMT-1 and MDA361 cells. Finally, *in vivo* data demonstrates that combined pharmacological inhibition of MEK plus lapatinib suppresses tumor growth and reduces expression of FOXM1 in HER2-overexpressing breast cancers that are resistant to trastuzumab and lapatinib. Our data suggest that FOXM1 contributes to lapatinib resistance downstream of MEK signaling, and supports further study of pharmacological MEK inhibition to improve response to lapatinib in HER2-overexpressing trastuzumab-resistant breast cancer.

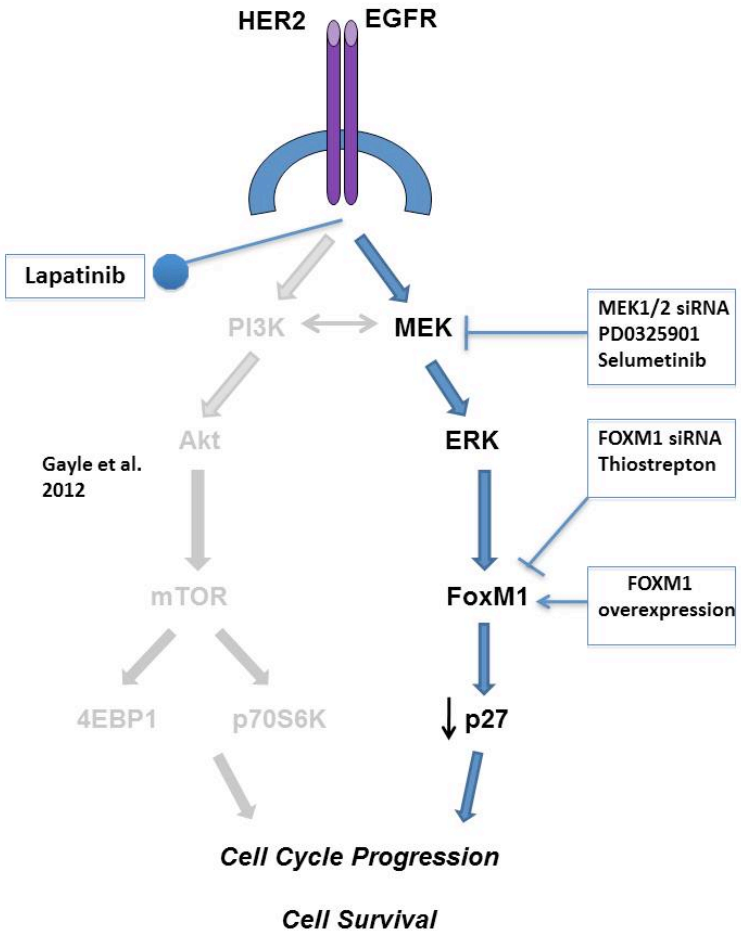
HER2
EGFR
Lapatinib
PI3K
MEK
MEK1/2 siRNA
PD0325901
Selumetinib
Akt
ERK
Gayle et al.
2012
FOXM1 siRNA
Thiostrepton
mTOR
FoxM1
FOXM1
overexpression
4EBP1
p70S6K
p27
Cell Cycle Progression
Cell Survival

Figure 5-2. Proposed model and summary. Lapatinib, a dual EGFR/HER2 kinase inhibitor, inhibits cell proliferation and survival by blocking the PI3K/Akt/mTOR (previously demonstrated by Gayle et al,2012) and MEK/ERK pathways. Based on our data, sustained activation of the MEK/ERK pathway in the presence of lapatinib is associated with reduced response to lapatinib. In this study, we inhibit MEK1/2 genetically using siRNA and pharmacologically using PD0325901 and selumetinib. FOXM1 was inhibited genetically using siRNA, pharmacologically using thiostrepton, and overexpressed using a FOXM1-CMV plasmid.

ii. Implications to the field and clinic

Significant advances have been made in HER2 targeting resulting in a greater number of patients benefiting clinically. In addition to trastuzumab, the FDA has approved HER2 targeted agents such as lapatinib for the treatment of HER2-overexpressing breast cancers. This study was necessary because it investigated the contribution of PI3K/mTOR and MEK to the development of lapatinib resistance and proposes novel drug combinations that can potentially be beneficial for HER2-overexpressing breast cancer patients. The results provide additional drug targets that should be inhibited in combination with lapatinib treatment. While combination treatments have the potential for a greater clinical benefit, increased toxicity is always a concern. Adverse effects observed clinically with lapatinib, MEK inhibition, or PI3K inhibition include diarrhea, rash, nausea and fatigue (Burris et al, 2005;Flaherty et al, 2012;Wang et al, 2007). Clinically, doses of lapatinib plus either MEK inhibiter or PI3K inhibiter will have to be adjusted to yield the greatest clinical benefit with the lowest adverse effects.

In addition to providing evidence for combination treatments, our research is important because it provides information that could potentially be used to develop biological predictors of response or resistance to determine which patients are most likely to benefit from lapatinib treatment. Genomic and proteomic approaches could be used for molecular profiling in an effort to determine a personalized medicine treatment regimen. For example, the identification of trastuzumab refractory HER2-overexpressing patients with PTEN loss or hyperactive Akt could be used to predict a benefit from combination lapatinib and mTOR inhibition treatment. Alternatively, trastuzumab refractory HER2-

overexpressing patients with FOXM1 overexpression could benefit from combination MEK inhibition and lapatinib treatment. Eventually, the creation of a molecular signature of lapatinib response or resistance may allow the best HER2-targeted combination of therapies to be utilized.

iii. Future Directions

Based on our results, future studies investigating the role of HER3 and HER4 in lapatinib resistance could yield new drug targets in HER2-overexpressing breast cancer. Our results demonstrated that knocking down 4EBP1 which has an inhibitory interaction with eukaryotic translation initiation factor 4E (eIF4E), results in an increased response to lapatinib. The phosphorylation of 4EBP1 by mTOR results in the dissociation of 4EBP1 from eIF4E, freeing eIF4E to play roles in cap-dependent translation (Wullschleger et al, 2005). The increased response to lapatinib observed upon 4EBP1 inhibition suggests an increased dependence of the cell on signaling from receptor tyrosine kinases such as HER3 and HER4. Elucidating the role of HER3 & HER4 could provide additional targets that can be inhibited in an effort to prevent activation of signaling pathways that lead to lapatinib resistance.

Other future studies include evaluating the role of FOXM1 downstream effectors such as survivin in the development of lapatinib resistance in acquired versus primary resistance. Also, FOXM1 inhibition should be investigated *in vivo* in combination with lapatinib treatment in a trastuzumab-refractory setting.

Finally, studies examining combinatorial PI3K/mTOR and MEK inhibition plus lapatinib treatment should be investigated. Lapatinib is the first line of therapy for HER2-

overexpressing breast cancer patients who have progressed on trastuzumab. Patients who respond poorly to lapatinib are left without a standard therapy, making it important for us to elucidating the roles of PI3K/mTOR and MEK in lapatinib resistance. Our data indicates that an optimal lapatinib response will be achieved when signaling of the two main downstream pathways to EGFR/HER2 is abrogated. This novel drug combination has the potential to yield a greater clinical benefit in the sub-set of HER2-overexpressing breast cancer patients who have progressed on trastuzumab and have a poor response to lapatinib.

References

Adams TE, Epa VC, Garrett TP, & Ward CW. Structure and function of the type 1 insulin-like growth factor receptor. *Cell Mol Life Sci*. 2000. 57 (7):1050-93

Andre, F.; Campone, M.; O'Regan, R.; Manlius, C.; Massacesi, C.; Sahmoud, T.; Mukhopadhyay, P.; Soria, J. C.; Naughton, M.; Hurvitz, S. A. Phase I study of everolimus plus weekly paclitaxel and trastuzumab in patients with metastatic breast cancer pretreated with trastuzumab. *J Clin Oncol.* 2010**.** *28* (34):5110-5115.

Akcakanat A, Singh G, Hung MC & Meric-Bernstam F. Rapamycin regulates the phosphorylation of Rictor. *Biochemical and Biophysical Research Communications* 2007. 362: 330–333.

Barok M, Tanner M, Koninki K, & Isola J. (2011). Trastuzumab-DM1 causes tumor growth inhibition by mitotic catastrophe in trastuzumab-resistant breast cancer cells in vivo. Breast *Cancer Res.* 2011. 13:(2):R46

Bargmann, C I, M C CHung, and R A AWeinberg. "Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185". *Cell* 1986. 45 (5):649-657.

Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, & Norton L. (1996). Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol*. 1996. 14 (3): 737-44

Baselga, J.; Tripathy, D.; Mendelsohn, J.; Baughman, S.; Benz, C. C.; Dantis, L.; Sklarin, N. T.; Seidman, A. D.; Hudis, C. A.; Moore, J.; Rosen, P. P.; Twaddell, T.; Henderson, I. C.; Norton, L. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol.* 1996**.** *14* (3): 737-744.

Baselga, J.; Bradbury, I.; Eidtmann, H.; Di Cosimo, S.; Aura, C.; De Azambuja, E.; Gomez, H.; Dinh, P.; Fauria, K.; Van Dooren, V.; Paoletti, P.; Goldhirsch, A.; Chang, T-W.; Lang, I.; Untch, M.; Gelber, R. D.; Piccart-Gebhart, M.; on Behalf of the NeoALTTO Study Team. First Results of the NeoALTTO Trial (BIG 01-06/EGF 106903): A Phase III, Randomized, Open Label, Neoadjuvant Study of Lapatinib, Trastuzumab, and Their Combination Plus Paclitaxel in Women with HER2-Positive Primary Breast Cancer. In: *Cancer Res*, Proceedings of the Thirty-Third Annual CTRC-AACR San Antonio Breast Cancer Symposium, San Antonio, TX. 2010. 70 (24): S3-3.

Bekaii-Saab T, Phelps MA, Li X, Saji M, Goff L, Kauh JS, et al. Multi-institutional phase II study of selumetinib in patients with metastatic biliary cancers. *J Clin Oncol*. 2011. 29:2357-63.

Bektas N, Haaf A, Veeck J, Wild PJ, Luscher-Firzlaff J, Hartmann A, et al. Tight correlation between expression of the Forkhead transcription factor FOXM1 and HER2 in human breast cancer. *BMC Cancer*. 2008. 8:42.

Berns K, Horlings HM, Hennessy BT, *et al*. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell.* 2007. 12 (4): 395-402.

Bhat UG, Halasi M, Gartel AL. Thiazole antibiotics target FoxM1 and induce apoptosis in human cancer cells. *PLoS One*. 2009. 4:e5592.

Bublil EM, Yarden Y. The EGF receptor family: spearheading a merger of signaling and therapeutics. *C urrent opinion in cell biology*. 2007. 19:124-34.

Blackwell KL, Pegram MD, Tan-Chiu E, Schwartzberg LS, Arbushites MC, Maltzman JD, et al. Single-agent lapatinib for HER2-overexpressing advanced or metastatic breast cancer that progressed on first- or second-line trastuzumab-containing regimens. *Ann Oncol*. 2009. 20:1026-31.

Blackwell KL, Burstein HJ, Storniolo AM, Rugo H, Sledge G, Koehler M, et al. Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. *J Clin Oncol*. 2010. 28:1124-30.

Brandt R, Wong AM, Hynes NE. Mammary glands reconstituted with Neu/ErbB2 transformed HC11 cells provide a novel orthotopic tumor model for testing anti-cancer agents. *Oncogene*. 2001. 20:5459–5465.

Brunner-Kubath C, Shabbir W, Saferding V, Wagner R, Singer CF, Valent P, et al. The PI3 kinase/mTOR blocker NVP-BEZ235 overrides resistance against irreversible ErbB inhibitors in breast cancer cells. *Breast Cancer Res Treat.* 2011. (129):387-400.

Burris HA., Hurwitz HI., Dee EC., Dowlati A., Blackwell KL., O'Neil B., Marcom PK., Ellis MJ., Overmoyer B., Jones SF., Harris JL., Deborah AS., Koch KM., Stead A., Mangum S., Spector NL. Phase I Safety, Pharmacokinetics, and Clinical Activity Study of Lapatinib (GW72016), a Reversibl Dual Inhibitor of Epidermal Growth Factor Receptor Tyrosine Kinases, in Heavily Pretreated Patients With Metastatic Carcinomas. *JCO.* 2005. 23:5305-5313

Burris HA 3rd, Rugo HS, Vukelja SJ, Vogel CL, Borson RA, Limentani S, Tan-Chiu E, Krop IE, Michaelson RA, Girish S, Amler L, Zheng M, Chu YW, Klencke B, & O'Shaughnessy JA. (2011). Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer after prior HER2-directed therapy. *J Clin Oncol.* 2011. 29 (4):398-405

Cantley, Lewis C. The phosphoinositide 3-kinase pathway. *Science.* 2002. 296 (5573):1655-1657.

Cardoso F, Durbecq V, Laes JF, Badran B, Lagneaux L, Bex F, Desmedt C, Willard-Gallo K, Ross JS, Burny A, Piccart M, & Sotiriou C. (2006). Bortezomib (PS-341, Velcade) increases the efficacy of trastuzumab (Herceptin) in HER-2-positive breast cancer cells in a synergistic manner. *Mol Cancer Ther. 2006.* 5(12):3042-51

Chan D, Ngan H. Abstract 2159: Targeting GRB7/ERK/FOXM1 signaling cascade is a promising therapeutic approach in ovarian cancer. Cancer research. 2012;72.

Chaves J, Saif MW. IGF system in cancer: from bench to clinic. *Anticancer Drugs*,. 2011. 22 (3):206-12

Chou, T. C.; Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul.* 1984**.** *22*:27-55.

Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. *Nature reviews Molecular cell biology*. 2006. 7:505-16.

Cobleigh, M. A.; Vogel, C. L.; Tripathy, D.; Robert, N. J.; Scholl, S.; Fehrenbacher, L.; Wolter, J. M.; Paton, V.; Shak, S.; Lieberman, G.; Slamon, D. J. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol.* 1999**.** *17* (9) 2639-2648.

Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, PatonV, Shak S, Lieberman G, & Slamon DJ. (1999). Multinational study of the efficacyand safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol*. 17(9):2639-48

Coussens, L, et al. "Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene." *Science.* 1985. 230 (4730):1132-1139.

Courtney K D, Corcoran R B, Engelman J A. The PI3K pathway as drug target in human cancer. *Journal of clinical oncology* 2010. 28(6):1075-1083.

Daez-Sampedro A.. "Involvement of amino acid 36 in TM1 in voltage sensitivity in mouse Na+/glucose cotransporter SGLT1." *The journal of membrane biology.* 2009. (227.2):57-66.

D'Alessio, A.; De Luca, A.; Maiello, M. R.; Lamura, L.; Rachiglio, A. M.; Napolitano, M.; Gallo, M.; Normanno, N. Effects of the combined blockade of EGFR and ErbB-2 on signal transduction and regulation of cell cycle regulatory proteins in breast cancer cells.*Breast Cancer Res Treat,* 2010**,** *123* (2), 387-396.

Eccles SA. The role of c-erbB-2/HER2/neu in breast cancer progression and metastasis. *J Mammary Gland Biol Neoplasia.* 2001. 6:393-406.

Eichhorn PJ, Gili M, Scaltriti M, Serra V, Guzman M, Nijkamp W, et al. Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235. *Cancer Res*. 2008. 68:9221-30.

Eng C. PTEN: one gene, many syndromes. *Hum Mutat* 2003. 22(3): 183-98.

Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet*. 2006. 7:606–619.

Engelman J A. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nature Reviews Cancer.* 2009. 9(8):550-562.

Esparís-Ogando A, Ocaña A, Rodríguez-Barrueco R, Ferreira L, Borges J & Pandiella A. (2008). Synergic antitumoral effect of an IGF-IR inhibitor and trastuzumab on HER2-overexpressing breast cancer cells. *Ann Oncol*. 2008. 19(11):1860-9

Esteva, F. J.; Valero, V.; Booser, D.; Guerra, L. T.; Murray, J. L.; Pusztai, L.; Cristofanilli, M.; Arun, B.; Esmaeli, B.; Fritsche, H. A.; Sneige, N.; Smith, T. L.; Hortobagyi, G. N. Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. *J Clin Oncol.* 2002. *20* (7):1800-1808.

Esteva, F. J.; Guo, H.; Zhang, S.; Santa-Maria, C.; Stone, S.; Lanchbury, J. S.; Sahin, A. A.; Hortobagyi, G. N.; Yu, D. PTEN, PIK3CA, p-AKT, and p-p70S6K status: association with trastuzumab response and survival in patients with HER2-positive metastatic breast cancer. *Am J Pathol.* 2010**.** *177* (4):1647-1656.

Falkenburger H, Jensen B, and BertilHille. Kinetics of PIP2 metabolism and KCNQ2/3 channel regulation studied with a voltage-sensitive phosphatase in living cells. *The Journal of general physiology.* 2010. (135)2:99-114

Ferlay, Jacques, et al. "Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008." *International journal of cancer.* 2010. 127(12):2893-2917.

Flaherty KT, Robert C, Hersey P, Nathan P, Garbe C, Milhem M, Demidov LV, Hassel JC, Rutkowski P, Mohr P, Dummer R, Trefzer U, Larkin JM, Utikal J, Dreno B, Nyakas M, Middleton MR, Becker JC, Casey M, Sherman LJ, Wu FS, Ouellet D, Martin AM, Patel K, Schadendorf D, METRIC Study Group. Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med.* 2012. 367:107–114

Francis RE, Myatt SS, Krol J, Hartman J, Peck B, McGovern UB, et al. FoxM1 is a downstream target and marker of HER2 overexpression in breast cancer. *Int J Oncol.* 2009. 35:57-68.

Franke T F, Yang S I, Chan T O, Datta K, Kazlauskas A, Morrison D K, Kaplan D R, Tsichlis P N. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell.* 1995. 81(5):727-736.

Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, & Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell. 2004.* 5(4): 317-28

Fu Z, Malureanu L, Huang J, Wang W, Li H, van Deursen JM, et al. Plk1-dependent phosphorylation of FoxM1 regulates a transcriptional programme required for mitotic progression. *Nat Cell Biol.* 2008. 10:1076-82.

Fischmann T O, Smith C K, Mayhood T W, Myers J E, Reichert P, Mannarino A, Carr D, Zhu H, Wong J, Yang R, Le H V, Madison V S. Crystal structures of MEK1 binary and ternary complexes with nucleotides and inhibitors. *Biochemistry* 2009. 48(12):2661-2674.

Gayle SS, Arnold SL, O'Regan RM, Nahta R. Pharmacologic Inhibition of mTOR Improves Lapatinib Sensitivity in HER2-Overexpressing Breast Cancer Cells with Primary Trastuzumab Resistance. *Anti-cancer agents in medicinal chemistry*. 2012. 12:151-62.

Geyer, C. E.; Forster, J.; Lindquist, D.; Chan, S.; Romieu, C. G.; Pienkowski, T.; Jagiello-Gruszfeld, A.; Crown, J.; Chan, A.; Kaufman, B.; Skarlos, D.; Campone, M.; Davidson, N.; Berger, M.; Oliva, C.; Rubin, S. D.; Stein, S.; Cameron, D. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med.* 2006**.** *355* (26): 2733-2743.

Gibbs JB, Schaber MD, Marshall MS, Scolnick EM, Sigal IS. Identification of guanine nucleotides bound to *ras*-encoded proteins in growing yeast cells. *J. Biol. Chem.* 1987 262:10426–10429.

Griner SE, Joshi JP, Nahta R. Growth Differentiation Factor 15 Stimulates Rapamycin-Sensitive Ovarian Cancer Cell Growth and Invasion. *Biochem Pharmacol*. 2012. 85 (1):46-58

Hazan R, Margolis B, Dombalagian M, Ullrich A, Zilberstein A, Schlessinger J. Identification of autophosphorylation sites of HER2/neu. *Cell Growth Differ.* 1990. 1(1):3–7.

Hegde, P. S.; Rusnak, D.; Bertiaux, M.; Alligood, K.; Strum, J.; Gagnon, R.; Gilmer, T. M. Delineation of molecular mechanisms of sensitivity to lapatinib in breast cancer cell lines using global gene expression profiles. *Mol Cancer Ther*. 2007. *6* (5):1629-1640.

Hilgemann, Donald W, et al. "Molecular control of cardiac sodium homeostasis in health and disease." *Journal of cardiovascular electrophysiology*. 2006. suppl :17 S47-S56.

Graus Porta D, Beerli R R, Daly J M, Hynes N E. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO journal* 1997. 16(7):1647-1655.

Hanabata, Yasuko, et al. "Coexpression of SGLT1 and EGFR is associated with tumor differentiation in oral squamous cell carcinoma." *Odontology.* 2012. 100.2:156-163.

Hudziak RM, Schlessinger J, Ullrich A. Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3T3 cells. *Proc Natl Acad Sci.* 1987. 84(20):7159–716

Huang H, Tindall DJ. Dynamic FoxO transcription factors. *J Cell Sci.* 2007. 120:2479-87.

Huang C, Mandelker D, Schmidt Kittler O, Samuels Y, Velculescu V E, Kinzler K W, Vogelstein B, Gabelli S B, Amzel L M. The structure of a human p110alpha/p85alpha complex elucidates the effects of oncogenic PI3Kalpha mutations. *Science.* 2007. 318 (5857):1744-1748.

Hui MK, Chan KW, Luk JM, Lee NP, Chung Y, Cheung LC, et al. Cytoplasmic Forkhead box M1 (FoxM1) in esophageal squamous cell carcinoma significantly correlates with pathological disease stage. *World journal of surgery.* 2012. 36:90-7.

Imada, K, and W J JLeonard. "The Jak-STAT pathway." *Molecular immunology.* 2000. 37 (1-2):1-11.

Jerome L, Alami N, Belanger S, Page V, Yu Q, Paterson J, Shiry L, Pegram M, & Leyland- Jones B. (2006). Recombinant human insulin-like growth factor binding protein 3 inhibits growth of human epidermal growth factor receptor-2-overexpressing breast tumors and potentiates herceptin activity in vivo. *Cancer Res.* 2006. 66(14):7245-52

Jerusalem G., Fasolo A., Dieras V., Cardoso F., Bergh J., Vittori L., Zhang Y., Massacesi C., Sahmond T., Gianni L. Phase I trial of oral mTOR inhibitor everolimus in combination with trastuzumab and vinerelbine in pre-treated patients with HER2-overexpressing metastatic breast cancer. *Breast Cancer Res Treat* 2001. 125:447-455

Jerusalem, G.;, Fasolo, A.; Dieras, V.; Cardoso, F.; Bergh, J.; Vittori, L.; Zhang, Y.; Massacesi, C.; Sahmoud, T.; Gianni, L. Phase I trial of oral mTOR inhibitor everolimus in combination with trastuzumab and vinorelbine in pre-treated patients with HER2-

overexpressing metastatic breast cancer. *Breast Cancer Res Treat*. 2011**.** *125* (2), 447-455.

Jin Q & Esteva FJ. (2008). Cross-talk between the ErbB/HER family and the type I insulinlike growth factor receptor signaling pathway in breast cancer. *J Mammary Gland Biol Neoplasia*. 2008. 13 (4):485-98

Johnston S, Trudeau M, Kaufman B, *et al*. Phase II study of predic- tive biomarker profiles for response targeting human epidermal growth factor receptor 2 (HER-2) in advanced inflammatory breast cancer with lapatinib monotherapy. *J Clin Oncol*. 2008. 26(7): 1066-72.

Junttila TT, Li G, Parsons K, Phillips GL, & Sliwkowski MX. (2010). Trastuzumab-DM1 (TDM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer. *Breast Cancer Res Treat.* 2010. [Epub ahead of print])

Kadamur, Ganesh, and Elliott MRoss. "Mammalian phospholipase C." *Annual Review of Physiology* 2013. 75(2013):127-154.

Kalin TV, Wang IC, Ackerson TJ, Major ML, Detrisac CJ, Kalinichenko VV, et al. Increased levels of the FoxM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice. *Cancer Res*. 2006. 66:1712-20

Karakas B, Bachman KE, Park BH. Mutation of the PIK3CA on- cogene in human cancers. *Br J Cancer.* 2006. 94(40): 455-9.

Kim IM, Ackerson T, Ramakrishna S, Tretiakova M, Wang IC, Kalin TV, et al. The Forkhead Box m1 transcription factor stimulates the proliferation of tumor cells during development of lung cancer. *Cancer Res*. 2006. 66:2153-61.

Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J. and Waterfield, *M. D.* Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis and cancer. *Annu. Rev. Cell Dev. Biol.* 2001. *17*:*615* -675.

Kim, H. P.; Yoon, Y. K.; Kim, J. W.; Han, S. W.; Hur, H. S.; Park, J.; Lee, J. H.; Oh, D. Y.; Im, S. A.; Bang, Y. J.; Kim, T. Y. Lapatinib, a dual EGFR and HER2 tyrosine kinase inhibitor, downregulates thymidylate synthase by inhibiting the nuclear translocation of EGFR and HER2. *PLoS One,* 2009**.** *4* (6): e5933.

Krop IE, Beeram M, Modi S, Jones SF, Holden SN, Yu W, Girish S, Tibbitts J, Yi JH, Sliwkowski MX, Jacobson F, Lutzker SG, & Burris HA. (2010). Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. *J Clin Oncol.* 2010. 28 (16):2698-704

Koyanagi, M, et al. "Phospholipase C cDNAs from sponge and hydra: antiquity of genes involved in the inositol phospholipid signaling pathway." *FEBS letters.* 1998. 439(1-2):66-70.

Kwok JM, Myatt SS, Marson CM, Coombes RC, Constantinidou D, Lam EW. Thiostrepton selectively targets breast cancer cells through inhibition of forkhead box M1 expression. *Mol Cancer Ther*. 2008. 7:2022-32.

Lam EW, Francis RE, Petkovic M. FOXO transcription factors: key regulators of cell fate. *Biochemical Society transactions*. 2006. 34:722-6.

Lang S.A, Hackl C., Moser C., Fichtner-Feigl S., Koehl G.E., Schlitt H.J., Geissler E.K., Stoeltzing O. Implication of Rictor in the mTOR inhibitor-mediated induction of insulin-like growth factor-I receptor (IGF-IR) and human epidermal growth factor receptor-2 (HER2) expression in gastrointestinal cancer cells. *Biochimica et Biophysica Acta. 2010.* 803:435–442.

Leung TW, Lin SS, Tsang AC, Tong CS, Ching JC, Leung WY, et al. Over-expression of FoxM1 stimulates cyclin B1 expression. *FEBS Lett*. 2001. 507:59-66.

Lewis Phillips GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, Blättler WA, Lambert JM, Chari RV, Lutz RJ, Wong WL, Jacobson FS, Koeppen H, Schwall RH, Kenkare- Mitra SR, Spencer SD, & Sliwkowski MX. (2008). Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res*.2008. 68 (22):9280-90

Liang K, Esteva FJ, Albarracin C, Stemke-Hale K, Lu Y, Bianchini G, Yang CY, Li Y, Li X, Chen CT, Mills GB, Hortobagyi GN, Mendelsohn J, Hung MC, & Fan Z. (2010). Recombinant human erythropoietin antagonizes trastuzumab treatment of breast cancer cells via Jak2-mediated Src activation and PTEN inactivation. *Cancer Cell.* 2010. 18(5):423-35

Liu D, Xing M. Potent inhibition of thyroid cancer cells by the MEK inhibitor PD0325901 and its potentiation by suppression of the PI3K and NF-kappaB pathways. *Thyroid.* 2008. 18:853-64.

Lu, C. H.; Wyszomierski, S. L.; Tseng, L. M.; Sun, M. H.; Lan, K. H.; Neal, C. L.; Mills, G. B.; Hortobagyi, G. N.; Esteva, F. J.; Yu, D. Preclinical testing of clinically applicable strategies for overcoming trastuzumab resistance caused by PTEN deficiency. *Clin Cancer Res.* 2007**.** *13* (19), 5883-5888.

Liu,T.; Yacoub, R.; Taliaferro-Smith, L. D.; Sun, S. Y.; Graham, T. R.; Dolan, R.; Lobo, C.; Tighiouart, M.; Yang, L.; Adams, A.; O'Regan, R. M. Combinatorial effects of lapatinib and rapamycin in triple-negative breast cancer cells. *Mol Cancer Ther.* 2011. *10* (8), 1460-1469.

Lloyd A C. Distinct functions for ERKs? *Journal of biology.* 2006. 5(5):13-13.

Malumbres M, Barbacid M. RAS oncogenes: the first 30 years. *Nat. Rev. Cancer*. 2003. 3:459–465.

Margolis B, Rhee SG, Felder S, Mervic M, Lyall R, Levitzki A, Ullrich, A, Zilberstein A, Schlessinger J. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell.* 1989. 57:1101–1107

Martinez-MartÃƒ A, Felip E. PI3K Pathway in NSCLC. *Frontiers In Oncology* 2011. 1:55-55.

McKay M M, Morrison D K. Integrating signals from RTKs to ERK/MAPK. Oncogene 2007. 26(22):3113-3121.

Medina PJ, Goodin S. Lapatinib: a dual inhibitor of human epidermal growth factor receptor tyrosine kinases. Clin Ther. 2008.30(8):1426-47.

Meijer SL.,Wesseling J.,Smit VT., Nederlof PM., Hooijer GKJ., Ruijter H., Arends JW., Kliffen M., Van Gorp JM., Sterk L., Van de Vijver MJ. HER2 Gene Amplification in Patients with Breast Cancer with Equivocal IHC Results. J Clin Pathol. 2011. 64(12):1069-1072

Miller, T. W.; Forbes, J. T.; Shah, C.; Wyatt, S. K.; Manning, H. C.; Olivares, M. G.; Sanchez, V.; Dugger, T. C.; de Matos Granja, N.; Narasanna, A.; Cook, R. S.; Kennedy, J. P.; Lindsley, C. W.; Arteaga, C. L. Inhibition of mammalian target of rapamycin is required for optimal antitumor effect of HER2 inhibitors against HER2-overexpressing cancer cells. *Clin Cancer Res.* 2009**.** *15* (23):7266-7276.

Mitra D, Brumlik MJ, Okamgba SU, Zhu Y, Duplessis TT, Parvani JG, Lesko SM, Brogi E, & Jones FE. (2009). An oncogenic isoform of HER2 associated with locally disseminated breast cancer and trastuzumab resistance. *Mol Cancer Ther.* 2009. 8(8): 2152-62

Mittendorf EA, Liu Y, Tucker SL, McKenzie T, Qiao N, Akli S, Biernacka A, Liu Y, Meijer L, Keyomarsi K, & Hunt KK. (2010). A novel interaction between HER2/neu and cyclin E in breast cancer. *Oncogene 2010.* 29(27):3896-907

Morrow, P. K.; Wulf, G. M.; Ensor, J.; Booser, D. J.; Moore, J. A.; Flores, P. R.; Xiong, Y.; Zhang, S.; Krop, I. E.; Winer, E. P.; Kindelberger, D. W.; Coviello, J.; Sahin, A. A.; Nunez, R.; Hortobagyi, G. N.; Yu, D.; Esteva, F. J. Phase I/II Study of Trastuzumab in Combination With Everolimus (RAD001) in Patients With HER2-Overexpressing Metastatic Breast Cancer Who Progressed on Trastuzumab-Based Therapy. *J Clin Oncol,* 2011**.** 29(23):3126-3132.

Nahta R, Hung MC, & Esteva FJ. (2004a). The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. *Cancer Res* 2004. 64(7):2343-6

Nahta, R.; O'Regan, R. M. Evolving strategies for overcoming resistance to HER2-directed therapy: targeting the PI3K/Akt/mTOR pathway. *Clin Breast Cancer.* 2010**.***10 Suppl 3*, S72-S78.

Nahta,R. Molecular Mechanisms of Trastuzumab-Based Treatment in HER2-Overexpressing Breast Cancer. *Oncology.* 2012. 428062-428062.

Nahta R, Takahashi T, Ueno NT, Hung MC, & Esteva FJ. (2004b). P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. *Cancer Res.* 2004. 64(11):3981-6

Nagata Y, Lan KH, Zhou X, *et al*. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastu- zumab resistance in patients. *Cancer Cell* 2004. 6(2):117-27.

Nielsen DL, Anderson M, & Kamby C. HER2-targeted therapy in breast cancer. Monoclonal antibodies and tyrosine kinase inhibitors. *Cancer Res*. 2009. 35 (2):121-36.

O'Brien NA, Browne BC, Chow L, Wang Y, Ginther C, Arboleda J, et al. Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. *Mol Cancer Ther*. 2010.9:1489-502.

Ozbay, T.; Durden, D. L.; Liu, T. O'Regan, R. M.; Nahta, R. In vitro evaluation of pan-PI3-kinase inhibitor SF1126 in trastuzumab-sensitive and trastuzumab-resistant HER2-over-expressing breast cancer cells. *Cancer Chemother Pharmacol.* 2010**.** *65* (4):697-706.

O'Neil BH, Goff LW, Kauh JS, Strosberg JR, Bekaii-Saab TS, Lee RM, et al. Phase II study of the mitogen-activated protein kinase 1/2 inhibitor selumetinib in patients with advanced hepatocellular carcinoma. *J Clin Oncol*. 2011.29:2350-6.

Padhy, L C, Shih C, Cowing D, Finkelstein R, Weinberg RA. "Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas." *Cell*.1982. 28(4):865-871.

Penuel, Elicia, Robert WAkita, and Mark XSliwkowski. Identification of a region within the ErbB2/HER2 intracellular domain that is necessary for ligand-independent association. *Journal of biological chemistry* 2002. 277(32):28468-28473.

Perotti, A.; Locatelli, A.; Sessa, C.; Hess, D.; Vigano, L.; Capri, G.; Maur, M.; Cerny, T.; Cresta, S.; Rojo, F.; Albanell, J.; Marsoni, S.; Corradino, I.; Berk, L.; Rivera, V. M.; Haluska, F.; Gianni, L. Phase IB study of the mTOR inhibitor ridaforolimus with capecitabine. *J Clin Oncol.* 2010**.** *28* (30):4554-4561.

Qian X, LeVea CM, Freeman JK, Dougall WC, Greene MI. Heterodimerization of epidermal growth factor receptor and wild-type or kinase-deficient Neu: a mechanism of interreceptor kinase activation and transphosphorylation. *Proc Natl Acad Sci.* 1994.91(4):1500–1504

Raman M, Chen W, Cobb M H. Differential regulation and properties of MAPKs. *Oncogene* 2007.26(22):3100-3112.

Rebecchi, M J, and S N NPentyala. "Structure, function, and control of phosphoinositide-specific phospholipase C." Physiological reviews 2000. 80(4):1291-1335

Robbins D J, Zhen E, Cheng M, Xu S, Vanderbilt C A, Ebert D, Garcia C, Dang A, Cobb M H. Regulation and properties of extracellular signal-regulated protein kinases 1, 2, and 3. Journal of the American Society of Nephrology 1993.4(5):1104-1110.

Roskoski R. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacological research 2012.66(2):105-143.

Samuels Y, Wang Z, Bardelli A, *et al*. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004. 304(5670): 554.

Sauter G, Moch H, Moore D,*et al.* Heterogeneity of erbB-2 gene amplification in bladder cancer. *Cancer Res*. 1993. 53:2199–2203

Sauter G, Lee J, Bartlett JM, *et al*. Guidelines for human epidermal growth factor receptor 2 testing: Biologic and methodologic considerations. *J Clin Oncol* 2009. 27(8): 1323-33.

Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, Smith DJ, Landolfi S, Ramon y Cajal S, Arribas J, & Baselga J. (2009). Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. *Oncogene* 2009.28(6):803-14

Schechter, A L, Stern DF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, Weinberg RA. The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. *Nature*. 1984. 312(5994):513-516.

Scheuer W, Friess T, Burtscher H, Bossenmaier B, Endl J, & Hasmann M. (2009). Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models. *Cancer Res.* 2005. 69(24) 9330-6

Semba, K, et al. A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. *Proceedings of the National Academy of Sciences of the United States of America* 1985 82(19):6497-6501.

Shih, C, Padhy LC, Murray M, Weinberg RA. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature.1981.* 290(5803):261-264.

Singh B, Ittmann MM, Krolewski JJ. Sporadic breast cancers exhibit loss of heterozygosity on chromosome segment 10q23 close to the Cowden disease locus. *Genes Chromosomes Cancer.* 1998. 21(2): 166-71.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu *Oncogene Science*. 1987. 235:177-82.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*. 1989.244:707-12.

Slamon, D. J.; Leyland-Jones, B.; Shak, S.; Fuchs, H.; Paton, V.; Bajamonde, A.; Fleming, T.; Eiermann, W.; Wolter, J.; Pegram, M.; Baselga, J.; Norton, L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Me.,* 2001**.** *344* (11):783-792.

Sorlie, T.; Perou, C. M.; Tibshirani, R.; Aas, T.; Geisler, S.; Johnsen, H.; Hastie, T.; Eisen, M. B.; van de Rijn, M.; Jeffrey, S. S.; Thorsen, T.; Quist, H.; Matese, J. C.; Brown, P. O.; Botstein, D.; Eystein Lonning, P.; Borresen-Dale, A. L. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci.* 2001**.** *98* (19):10869-10874.

Spector, Neil L, and Kimberly LBlackwell. "Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer." *Journal of clinical oncology* 2009 27(34):5838-5847.

Stommel, J. M.; Kimmelman, A. C.; Ying, H.; Nabioullin, R.; Ponugoti, A. H.; Wiedemeyer, R.; Stegh, A. H.; Bradner, J. E.; Ligon, K. L.; Brennan, C.; Chin, L.; DePinho, R. A. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. *Science.* 2007**.** *318* (5848):287-290.

Storniolo AM, Pegram MD, Overmoyer B, Silverman P, Peacock NW, Jones SF, Loftiss J, Arya N, Koch KM, Paul E, Pandite L, Fleming RA, Lebowitz PF, Ho PT, & Burris HA 3rd. (2008). Phase I dose escalation and pharmacokinetic study of lapatinib in combination with trastuzumab in patients with advanced ErbB2-positive breast cancer. *J Clin Oncol 2008.* 26(20):3317-23

Sun, S. Y.; Rosenberg, L. M.; Wang, X.; Zhou, Z.; Yue, P.; Fu, H.; Khuri, F. R. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res.* 2005**.** *65* (16):7052-7058.

Tanizaki J, Okamoto I, Fumita S, Okamoto W, Nishio K, Nakagawa K. Roles of BIM induction and survivin downregulation in lapatinib-induced apoptosis in breast cancer cells with HER2 amplification. *Oncogene*. 2011. 30:4097-106.

Tanizaki, J.; Okamoto, I.; Fumita, S.; Okamoto, W.; Nishio, K.; Nakagawa, K. Roles of BIM induction and survivin downregulation in lapatinib-induced apoptosis in breast cancer cells with HER2 amplification. *Oncogene.* 2011**.** *30* (39):4097-4106.

Toi M, Iwata H, Fujiwara Y, Ito Y, Nakamura S, Tokuda Y, et al. Lapatinib monotherapy in patients with relapsed, advanced, or metastatic breast cancer: efficacy, safety, and biomarker results from Japanese patients phase II studies. *Br J Cancer*. 2009.101:1676-82.

Trask B. Human cytogenetics:46 chromosomes, 46 years and counting. *Nature Reviews Genetics*, 2002. 3 (10)769-78.

Valabrega, G.; Capellero, S.; Cavalloni, G.; Zaccarello, G.; Petrelli, A.; Migliardi, G.; Milani, A.; Peraldo-Neia, C.; Gammaitoni, L.; Sapino, A.; Pecchioni, C.; Moggio, A.; Giordano, S.; Aglietta, M.; Montemurro, F. HER2-positive breast cancer cells resistant to trastuzumab and lapatinib lose reliance upon HER2 and are sensitive to the multitargeted kinase inhibitor sorafenib. *Breast Cancer Res Treat.* 2011**.** *130* (1):29-40.

Vanhaesebroeck, B. and M.D. Waterfield, *Signaling by Distinct Classes of Phosphoinositide 3-Kinases.* Experimental Cell Research. 1999. 253(1):239-254.

Vivanco, Igor, and Charles LSawyers. "The phosphatidylinositol 3-Kinase AKT pathway in human cancer." *Nature Reviews. Cancer* 2 (7):489-501.

Wang D, Boerner SA, Winkler JD, LoRusso PM Clinical experience of MEK inhibitors in cancer therapy. *Biochim Biophys Acta.* 2007. 1773:1248–1255

Wang, X.; Hawk, N.; Yue, P.; Kauh, J.; Ramalingam, S. S.; Fu, H.; Khuri, F. R.; Sun, S. Y. Overcoming mTOR inhibition-induced paradoxical activation of survival signaling pathways enhances mTOR inhibitors' anticancer efficacy. *Cancer Biol Ther.* 2008**.** *7* (12):1952-1958.

Wang Z, Ahmad A, Li Y, Banerjee S, Kong D, Sarkar FH. Forkhead box M1 transcription factor: a novel target for cancer therapy. *Cancer treatment reviews*. 2010. 36:151-6.

Wang, L.; Zhang, Q.; Zhang, J.; Sun, S.; Guo, H.; Jia, Z.; Wang, B.; Shao, Z.; Wang, Z.; Hu, X, PI3K pathway activation results in low efficacy of both trastuzumab and lapatinib. *BMC Cancer.* 2011**.** *11*: 248-258.

Watowich, S S, et al. "Cytokine receptor signal transduction and the control of hematopoietic cell development." Annual review of cell and developmental biology 1996. (12):91-128.

Weiner LM, Holmes M, Richeson A, Godwin A, Adams GP, Hsieh-Ma ST, Ring DB, Alpaugh RK. Binding and cytotoxicity characteristics of the bispecific murine monoclonal antibody 2B1. J Immunol. 1993. 1,151(5):2877–2886

Wolf AC, Hammond EH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane, LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM, and Hayes DF. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. J Clin Oncol 2007. 25(1):1-28.

Wright E M, Hirayama BA, Loo DDF, Turk E, Hager K Intestinal sugar transport. In *Physiology of the Gastrointestinal Tract*. 1994. 3:1751-1772

Wu, Y.; Amonkar, M. M.; Sherrill, B. H.; O'Shaughnessy, J.; Ellis, C.; Baselga, J.; Blackwell, K. L.; Burstein, H. J., Impact of lapatinib plus trastuzumab versus single-agent lapatinib on quality of life of patients with trastuzumab-refractory HER2+ metastatic breast cancer. *Ann Oncol.* 2011. [Epub ahead of print].

Wullschleger S., Loewith R., Oppliger W., Hall M.N. Molecular organization of target of rapamycin complex 2 *J. Biol. Chem*. 2005. 280:30697-30704.

Xia, W.; Bacus, S.; Hegde, P.; Husain, I.; Strum, J.; Liu, L.; Paulazzo, G.; Lyass, L.; Trusk, P.; Hill, J.; Harris, J.; Spector, N. L. A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. *Proc Natl Acad Sci.* 2006. *103* (20):795-7800.

Xia, W.; Bisi, J.; Strum, J.; Liu, L.; Carrick, K.; Graham, K. M.; Treece, A. L.; Hardwicke, M. A.; Dush, M.; Liao, Q.; Westlund, R. E.; Zhao, S.; Bacus, S.; Spector, N. L. Regulation of survivin by ErbB2 signaling: therapeutic implications for ErbB2-overexpressing breast cancers. *Cancer Res*. 2006. *66* (3):1640-1647.

Xia W, Husain I, Liu L, *et al*. Lapatinib antitumor activity is not dependent upon phosphatase and tensin homologue deleted on chromosome 10 in ErbB2 overexpressing

breast cancers. *Cancer Res.* 2007. 67(3):1170-5.

Xia W, Gerard CM, Liu L, Baudson NM, Ory TL, & Spector NL. (2005). Combining lapatinib (GW572016), a small molecule inhibitor of ErbB1 and ErbB2 tyrosine kinases, with therapeutic anti-ErbB2 antibodies enhances apoptosis of ErbB2-overexpressing breast cancer cells. *Oncogene.* 2005. 24 (41):6213-21

Yang XH, Flores LM, Li Q, Zhou P, Xu F, Krop IE, & Hemler ME. (2010). Disruption of laminin-integrin-CD151-focal adhesion kinase axis sensitizes breast cancer cells to ErbB2 antagonists. *Cancer Res.* 2010. 70 (6):2256-63

Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, & Arteaga CL. (2002). Herceptininduced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. *Cancer Res. 2002.* 62(4):4132-41

Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nature reviews Molecular cell biology. 2001;2:127-37.
Zhao F, Lam EW. Role of the forkhead transcription factor FOXO-FOXM1 axis in cancer and drug resistance. 2012. *Frontiers of medicine*. 6:376-80.

Yardley, D.; Seiler, M.; Ray-Coquard, I.; Melichar, B.; Hart, L.; Dieras, V.; Barve, M.; Melnyk, A.; Dorer, D.; Turner, C.; Dodion, P. Ridaforolimus (AP23573; MK-8669) in Combination with Trastuzumab for Patients with HER2-Positive Trastuzumab-Refractory Metastatic Breast Cancer: A Multicenter Phase 2 Clinical Trial. In: *Cancer Res,* Proceedings of the 32nd Annual CTRC-AACR San Antonio Breast Cancer Symposium, San Antonio, TX. 2009. 69 (24 Supplement): 3091.

Yuan T L, Cantley L C. PI3K pathway alterations in cancer: variations on a theme. *Oncogene*. 2008. 27(41):5497-5510.

Zhang S, Huang WC, Li P, Guo H, Poh SB, Brady SW, Xiong Y, Tseng LM, Li SH, Ding Z, Sahin AA, Esteva FJ, Hortobagyi GN, & Yu D. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. *Nat Med* 2011 17:(4):461-9

Zhao FQ, McFadden TB, Wall EH, Dong B, Zhen YC. Cloning and expression of bovine sodium/glucose cotransporters . *Journ Dairy Sci*. 2005. 88 (8):2738-2748

Zhao, L, and P K KVogt. "Class I PI3K in oncogenic cellular transformation." *Oncogene* 2008. 27:(41):15486-5496.

Zoppoli G, Moran E, Soncini D, Cea M, Garuti A, Rocco I, et al. Ras-induced resistance to lapatinib is overcome by MEK inhibition. *Current cancer drug targets*. 2010. 10:168-75