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The Covalent JNK Inhibitor, JNK-IN-8, Synergizes with Lapatinib to Cause Cell Death in Basal-like Breast Cancer Cell Lines

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by

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The Covalent JNK Inhibitor, JNK-IN-8, Synergizes with Lapatinib to Cause Cell Death in Basal-like Breast Cancer Cell Lines

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The University of Texas at Austin, 2015

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Basal-like and Claudin-low breast cancers have the worst prognosis and represent 15-20% of breast cancers diagnosed each year. Endocrine and molecularly targeted therapies are ineffective for these tumors due to lack of Estrogen Receptor (ER) or Human Epidermal Growth Factor Receptor 2 (HER2) overexpression, leaving chemotherapy as the only option for treatment. New molecularly targeted therapies for this subtype are urgently needed.

High expression of c-Jun N-terminal Kinase 2 (JNK2) in human basal-like breast cancers significantly correlates with decreased disease-free survival. In mouse models, JNK2 promotes basal-like tumor progression, increases Epidermal Growth Factor Receptor (EGFR)-mediated migration, upregulates Epithelial and Mesenchymal (EMT) gene expression, and promotes metastasis.

Hypothesis: Due to the central role of JNK in Basal-like tumor progression, JNK inhibition may be therapeutic in Basal-like breast cancer, especially when combined with the EGFR/HER2 inhibitor lapatinib (Tykerb™).

Treatment with the covalent JNK inhibitor, JNK-IN-8, synergizes with lapatinib to cause cell death, while these drugs as single agents have little effect on cell viability. Our studies suggest that JNK1 and HER2 are the key targets in this response.

Intracellular response to lapatinib and JNK-IN-8 combination treatment significantly reduces DNA binding activity of Nuclear Factor kappa B (NFκB), and induces a 10-fold increase in Reactive Oxygen Species (ROS) accumulation that is cytotoxic. We hypothesize that inhibition of NFκB signaling by JNK-IN-8 and lapatinib causes ROS accumulation that triggers apoptosis. However, the combination of JNK-IN-8 and lapatinib leads to a higher degree of cell killing than pharmacologic inhibition of canonical NFκB signaling alone. Mechanisms involving JNK-IN-8 and lapatinib regulation of non-canonical NFκB members will be explored in the future. An additional mechanism of synergy could involve inhibition of AP-1 transcriptional activity which may exacerbate NFκB transcriptional inhibition through cooperation of these transcription factors at various promoters.

JNK-IN-8 formulation for *in vivo* studies will enable us to determine whether JNK-IN-8 and lapatinib synergize in mouse models of Basal-like breast cancer. Success here will legitimize human studies with this combination to establish whether there will be a therapeutic benefit for patients with Basal-like breast cancers whose lack of molecularly targeted therapies represents an unmet medical need.

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Chapter 1: Introduction

1.1 A Brief History of Breast Cancer

Although tumors of the breast have been described since ancient medicine, it was not until the mid-eighteenth century that surgical removal of a breast tumor was suggested for the treatment of breast cancer. Surgeons also discovered the benefits of removing the surrounding lymphatic tissue to prevent spread of the disease. This was the standard of treatment for women with breast cancer all the way through to the twentieth century. Beginning in the 1930s, total mastectomy was performed often followed by radiation therapy. Later, other radical procedures such as the removal of the ovaries and adrenal glands to block regrowth of breast cancer by estrogen stimulation were developed. In the 1950's, consideration for other forms of treatment became paramount due the fact that even radical mastectomy followed by radiation was only able to cure breast cancer in less than two thirds of patients [1]. Around this time, advances in cell biology were quickly dispelling older schools of thought about causes of cancer and how it spreads. In the 1970's, two classes of genes important to cancer were described, the oncogenes and tumor suppressor genes. As more and more of these genes were described for different cancers, it became clear that therapies targeting these specific gene products would be the next wave of cancer treatments. One of the first targeted therapies for breast cancer, trastuzumab (Herceptin™), is an inhibitory monoclonal antibody that binds the oncogene HER2 to block downstream signaling [2]. Since the advent of trastuzumab and other targeted therapies, such as tamoxifen which inhibits the activity of the ER in mammary epithelial cells, five-year survival rates rose steadily between 1992 and 2008, including rates for patients with stage IV disease [3].

1.2 BREAST CANCER SUBTYPES

1.2.1 CLINICAL OUTCOMES

Recently, researchers discovered that human breast tumors could be subtyped through genetic profiling, and that each subtype carried its own clinical significance. This discovery enabled others to determine what treatments best suit the different subtypes based on their gene expression patterns. The luminal subtype is distinguished by expression of ER and other genes such as GATA-3 and cytokeratins 8 and 18 that are expressed by luminal cells in the normal mammary gland. This subtype is further classified into Luminal A and Luminal B tumors. Luminal B tumors express lower levels of ER and a unique gene set whose function is ill defined. These groups are further differentiated by their drastic differences in disease-free survival and time to metastatic events with Luminal B tumors having a much worse outcome than Luminal A tumors.

A third subtype is the HER2-enriched group (ERBB2+ in **Figure 1.1**) which represents tumors over-expressing ERBB2/HER2. Typically, tumors in this group have an amplification of the HER2 gene which causes substantial increase in protein expression. Overexpression of HER2 had before been known as an indicator of poor prognosis, and this patient group, without intervention, typically shows the shortest disease-free survival and time to metastasis.

Basal-like subtypes show expression of proteins reminiscent of basal cells in the normal mammary gland such as cytokeratins 5 and 6, and are negative for expression of ER, Progesterone Receptor (PR), and lack HER2 overexpression. This subtype frequently shows mutation in p53 and/or BRCA1 tumor suppressor genes. Typically they overexpress high levels of EGFR, a family member and binding partner of HER2 (See **Chapter 1.3.2.2.1**). Patients with Basal-like tumors have shorter disease-free survival and time to metastasis similar to the HER2-enriched subtype. Finally, a very small

number of tumors express genes akin to those present in normal mammary gland tissue, including expression patterns of adipose tissues and immune cells. These tumors are dubbed the Normal Breast-like group and their clinical prognosis falls somewhat in between Luminal A and Basal-like groups sometimes mirroring that of the Luminal B subtype (**Figure 1.1**)([4-7]).

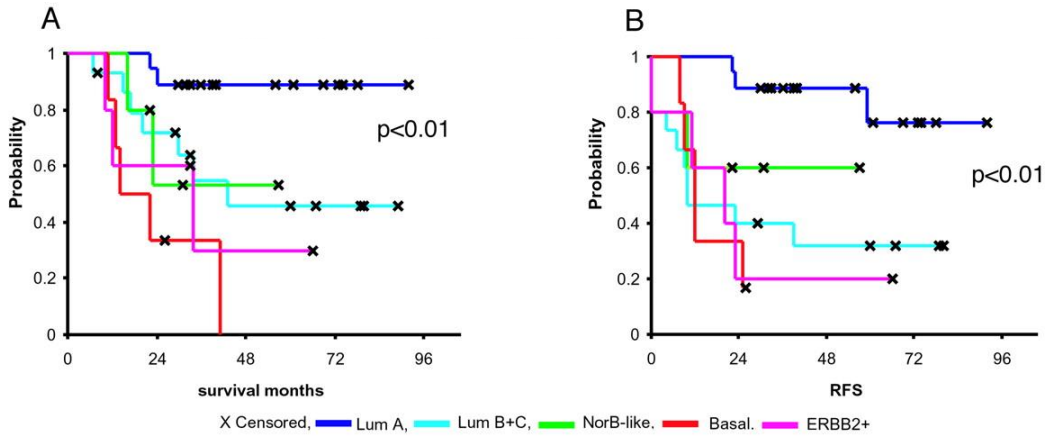


Figure 1.1: Clinical Outcomes of Five Breast Cancer Subtypes
Overall (A) and Relapse-free Survival (B) was analyzed for five breast cancer subtypes. This illustrates the stratification of these tumor subtypes based on clinical outcome (Adapted from [5]).

The Claudin-low subtype (not present in **Figure 1.1**) was later differentiated from Basal-like tumors in that they lack expression of E-cadherin, a cell-to-cell adhesion protein, and express genes related to epithelial to mesenchymal transition (EMT). These tumors are also rich in cancer stem cell and Tumor Initiating Cell (TIC) gene signatures making them the most poorly differentiated subtype. Although they show many similarities to the Basal-like tumor subtype, they show relatively low proliferation profiles in comparison, and so it is not surprising that their overall survival and relapse free survival falls in between Luminal A and Basal-like subtypes, mirroring that of the

Luminal B tumors [8, 9]. Although in another study, the disease-free survival for the Claudin-low subtype was not significantly different from the Basal-like subtype [10].

1.2.2 Cell Lines

In addition to subtyping primary breast tumors, human breast cancer cell lines fall into the stratifications of Luminal, Basal-like, and HER2-enriched groups. Within the Basal-like subtype, cell lines may be differentiated into Basal A and Basal B groups, with the Basal B group being characterized by increased expression of EMT genes, such as Transforming Growth Factor Beta (TGFβ), loss of cytokeratin 5 expression, and increased expression of TIC and stem cell-associated markers, similar to the Claudin-low subtype of tumors. This subtype of breast cancer cell lines includes the highly aggressive MDA-MB-231 and MDA-MB-436 cells [11]. Because of the conserved genetic profiling apparent in breast cancer cell lines compared to primary breast tumors, the use of these cell lines as appropriate models for studying subtype characteristics and suitable treatment options is validated.

1.3 Current Treatment Options for Breast Cancer

1.3.1 Chemotherapy

Chemotherapy to treat breast cancer may be used prior to tumor removal to decrease the size of the primary tumor (neoadjuvant therapy), or after removal of the tumor (adjuvant therapy) to ensure elimination of any remaining cancer cells that could cause relapse. Examples of chemotherapy drugs include doxorubicin, an intercalator which disrupts DNA replication to the point of cellular toxicity, and paclitaxel which causes toxicity by disrupting cell division through excessive tubulin assembly [12].

The effectiveness of chemotherapy differs between the breast cancer subtypes. One study showed that for neoadjuvant chemotherapy containing doxorubicin and paclitaxel, the HER2-enriched and Basal-like subtypes showed very high response rates with 45% of tumors showing pathologic complete response (pCR). Luminal tumors and Normal Breast-like tumors showed very low response rates of 6% and 0%, respectively. Interestingly, gene changes after treatment did not overlap between the HER2-enriched and Basal-like groups, indicating separate mechanisms of response to chemotherapy [13]. These differences hold true for neoadjuvant treatment with other chemotherapy drugs [14-16], as well as with adjuvant treatment, where five-year disease-free survival of patients with ER-negative tumors was 22.8% higher for those receiving chemotherapy than those who were not, versus 7% for patients with ER-positive tumors [14].

While Basal-like and HER2-enriched (ER-negative) tumors showed increased benefit from the use of adjuvant chemotherapy long-term (compared to no adjuvant treatment), they actually had significantly worse disease free survival and overall survival rates compared to ER-positive Luminal tumors at five years post-surgery. This phenomenon was restricted to ER-negative patients with residual disease after neoadjuvant treatment, and so it was surmised that the residual disease of Basal-like and HER2-enriched tumors is more aggressive than residual disease of patients with ER-positive tumors despite the use of chemotherapy [15, 16].

In light of the shortcomings of chemotherapy for treating breast cancer, including high rates of relapse and lower quality of life due to side-effects, the impetus for finding more effective and less toxic treatments continues to motivate researchers in the area of drug discovery.

1.3.2 Molecularly Targeted Therapies

1.3.2.1 Hormone Receptor Targeted Drugs

Since the discovery of common gene signatures and protein expression patterns for tumor subtypes, it became apparent that targeting one or more of these shared proteins would be therapeutic for that subtype.

One of the earliest of these drugs was tamoxifen, which competes with the hormone estrogen for binding to its receptor, ER. This prevents downstream transcription of ER target genes in breast epithelial cells. The use of tamoxifen is effective as a single agent to reduce mortality and recurrence in women with ER-positive breast cancers (reviewed in [17, 18]). Studies of the clinical effectiveness of tamoxifen in conjunction with chemotherapy for long-term adjuvant treatment saw only a modest increase in benefit, taking into consideration the lower quality of life brought about by the use of chemotherapy. However, the use of chemotherapy after tamoxifen is recommended for patients with metastatic disease and those whose tumors acquire resistance to tamoxifen despite initial ER-positive status (reviewed in [19]).

Since the advent of tamoxifen, other drugs such as Fulvestrant, which degrades ER, and aromatase-inhibitors, such as letrozole which inhibits estrogen synthesis, have been developed and are in use for ER-positive breast cancer therapy.

1.3.2.2 EGFR Family Targeted drugs

1.3.2.2.1 ERBB Signaling

About 20% of all breast cancers diagnosed fall under the HER2-enriched category. The HER2 receptor is a tyrosine kinase receptor that is in the same family with EGFR, HER3, and HER4. Each receptor has different affinities for various ligands. EGF, Amphiregulin, and TGF-α specifically bind EGFR. Betacellulin, epiregulin, and heparin-

binding growth factor bind both EGFR and HER4. Neuregulins (NRG) bind HER3 and HER4, with NRG-1 and NRG-2 binding both HER3 and HER4, and NRG-3 and NRG-4 specifically binding HER4 (reviewed in [20, 21]).

HER2 is unique since there are no known ligands that bind its extracellular domain. However, HER2 is the most potent ERBB receptor in that it is the preferred binding partner for both EGFR and HER3, with the HER2/HER3 heterodimer being the most potent activator of proliferation and transformation [22-25]. This is due to the unique structure of HER2 where the conformation of the extracellular domain resembles ligand-activated conformations of other HER extracellular domains (**Figure 1.2**) [21]. It is, therefore, available for dimerization without the need for ligand binding, which explains its lack of known ligands. HER2's ability to homodimerize to activate signaling gives some insight as to why HER2-enriched tumors are so aggressive.

Once dimerized, the kinase domains of these receptors trans-phosphorylate tyrosine residues that serve as binding sites for downstream effectors. HER3 is unique in this realm as it has no intrinsic kinase activity, but heterodimerization with other ERBB members still results in phosphorylation of both receptors. Once phosphorylated, proteins such as SHP1/2, Shc, Grb2, Grb7, and the intracellular kinases Src and phosphatidyl inositol 3-kinase (PI3K) are recruited and activated to induce the Akt, STAT, RAS/RAF/ /MAPK, and JNK pathways. Gene expression is affected through transcription factors such as c-fos, c-jun, c-myc, STAT, NF-κB, and Ets family members.

These receptors are highly consequential in that they affect a wide range of processes including growth, survival, differentiation/EMT, and transformation. The level at which these processes occur depends largely on which receptors and which signaling pathways are activated (reviewed in [20, 21]).

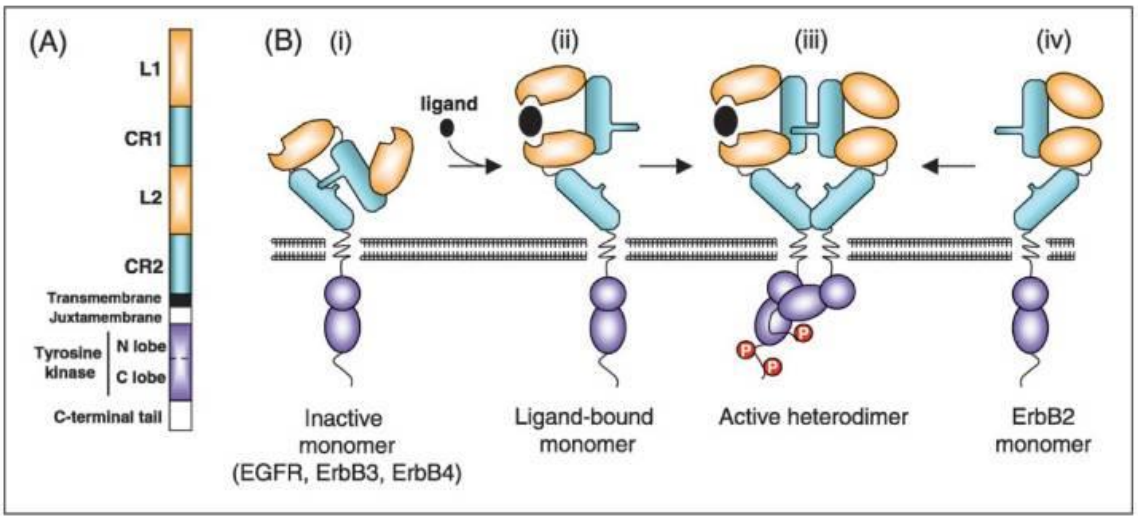


Figure 1.2: ERBB Receptor Domains and Structure
(A) Linear representation of conserved ERBB receptor domains. L1 and L2 are leucine-rich domains that mediate ligand binding. CR1 and CR2 are cysteine-rich domains that mediate receptor dimerization. (B) Typical folding structure of the ERBB receptors in various states with (i-iii) representing an ERBB receptor that recognizes extracellular ligands and has an intact tyrosine kinase domain, such as EGFR. The unique structure of HER2 is represented in (iv) [21].

1.3.2.2.2 INHIBITORY MONOCLONAL ANTIBODIES

The first class of drugs targeting HER2 were monoclonal antibodies that bind specific residues on the extracellular domain of the receptor and inhibit its downstream signaling. Trastuzumab was the first to show substantial benefit in patients whose tumors were HER2-enriched, and was approved for wide use in the treatment of these cancers. This drug showed effectiveness as a single agent and in combination with the chemotherapy drug cisplatin. It amazingly showed the greatest effectiveness in patients who had relapsed after one or two rounds of chemotherapy [26].

1.3.2.2.3 TYROSINE KINASE INHIBITORS

Tyrosine kinase inhibitors (TKIs) are small molecules that either compete with ATP for binding in the catalytic pocket of an enzyme (Type I), or bind adjacent site when the enzyme is inactive (Type II) [27, 28]. Some TKIs bind transiently to the target protein (reversible), and some TKIs bind covalently (irreversible). Because many proteins involved in transformation and tumor growth are kinases, these drugs are very attractive as therapeutic molecules.

Since trastuzumab showed that HER2 inhibition is a valid method for treating HER2-enriched breast cancers, TKIs were also developed to target HER2 by different mechanisms than therapeutic antibodies to synergize with trastuzumab, or overcome cases of trastuzumab resistance. In addition, TKIs have some advantages over the use of therapeutic monoclonal antibodies. For one, the small size allows cell permeability and improved penetration of the blood-brain barrier [29]. TKIs are useful in overcoming trastuzumab resistance because binding the intracellular kinase domain can inhibit the action of a mutated form of HER2, the truncated p85 unit, that has no extracellular domain for trastuzumab to bind, but is still capable of potent signaling [30]. Finally, TKIs have higher oral bioavailability which is more desirable for patients [29].

Lapatinib was the first FDA-approved TKI and is currently indicated for HER2-enriched breast cancers. Lapatinib is a reversible inhibitor of both EGFR and HER2, making it a very effective drug at blocking signaling downstream of these receptors. Lapatinib has clinical efficacy both as a single agent as well as in combating resistance in tumors treated with trastuzumab [31-33]. The benefit of combination therapy with lapatinib and capecitabine in tumors that had progressed despite treatment with trastuzumab or other chemotherapy drugs was significant, without much added toxicity from administration of capecitabine [34]. Lapatinib was also shown to combat tamoxifen

resistance, as signaling through the ERBB receptors is one mechanism of resistance to anti-estrogen drugs [35]. Human cell lines established from HER2-enriched breast tumors also show sensitivity to lapatinib [36, 37].

1.3.2.2.4 TKI FAILURES IN BASAL-LIKE BREAST CANCER

Basal-like and Claudin-low subtypes represent 15-20% of all breast cancers diagnosed. These tumors do not overexpress HER2, ER, or PR. For this reason, none of the aforementioned molecularly targeted drugs show efficacy for this tumor subtype. However, most of these tumors highly express EGFR [6] which can be targeted by TKIs.

Clinical trials with EGFR-directed TKIs began with the reversible inhibitors erlotinib and gefitinib. These trials involved women with advanced stage cancers whose tumors were resistant to one or more chemotherapy regimens. Neither drug showed efficacy as a single agent. Of the few patients that did show complete response, not all were of the Basal-like subtype indicating that the lack of efficacy was not due to inclusion of tumors from other subtypes. Surprisingly, immunohistochemical (IHC) analysis of tumors after treatment showed that EGFR phosphorylation was inhibited, however, pAkt and Ki67 remained high, indicating the presence of compensatory pathways or a lack of reliance on EGFR [38, 39]. Although Basal-like breast cancers generally have no amplification or overexpression of HER2, low levels, as seen by IHC, may still heterodimerize with HER3 or HER4 leading to continued downstream signaling to ERBB targets such as Akt in the presence of EGFR TKIs.

Sadly, lapatinib has also shown a lack of efficacy in treating Basal-like breast cancers, despite inhibiting EGFR and HER2, and no correlation was observed between the effectiveness of lapatinib and level of EGFR expression [40]. Lapatinib also does

little to slow the growth or cause apoptosis of Basal A or Basal B classified breast cancer cell lines [36].

In light of these shortcomings, new molecularly targeted therapies for Basal-like breast cancers are urgently needed.

1.3.2.2.5 MECHANISMS OF LAPATINIB RESISTANCE

Using breast cancer cell lines such as the HER2-amplified, SK-BR-3 cell line, researchers have been able to delineate some mechanisms of lapatinib sensitivity. For most sensitive cell lines (IC_{50} 0.01-1.00μM), lapatinib efficiently decreases tyrosine phosphorylation of EGFR and HER2 while increasing apoptosis. This correlates with loss of Akt and ERK1/2 phosphorylation at Ser473 and Tyr202/Tyr204, respectively. Resistant cell lines show inhibition of EGFR and HER2 phosphorylation after lapatinib treatment, but no changes are seen in ERK or Akt phosphorylation [36]. Other researchers found that Akt-mediated inhibitory phosphorylation of FOXO3A (a transcription factor that induces expression of pro-apoptotic genes) is downregulated in sensitive cell lines after lapatinib treatment, and that FOXO3A mRNA is upregulated significantly. Other gene expression changes observed in SK-BR-3 lapatinib-sensitive cells include downregulation of Akt1 and MAPK9 (JNK2) transcripts by 25-fold. These changes are not seen in resistant, Basal-like cell lines such as MDA-MB-231 and MDA-MB-468 cells [41]. Using quantitative phosphoproteomics, one group found that only a very small portion of proteins change phosphorylation status with lapatinib in the SK-BR-3 cancer cell line. Of those, major temporal and concentration-dependent dephosphorylation is seen with ERK2(T185/Y187), c-Jun(S73), and JunD(S100) indicating that reduced phosphorylation of these proteins correlates with lapatinib induced cell death [42]. Importantly, diminished phosphorylation of Akt by lapatinib

downregulates survivin expression, a potent anti-apoptotic protein [43, 44]. This is vital to the operation of lapatinib and correlates most strongly with its antitumor efficacy [45, 46]. High expression of survivin in breast cancers is usually associated with ER/PR negative tumors and HER2-overexpression. Survivin is independently and very strongly linked to poor prognosis [47-49].

Because lapatinib-resistant cells show inhibition of the targeted proteins (EGFR and HER2) but maintain downstream signaling, it stands to reason that there is compensatory signaling or mutation that restores phosphorylation of downstream kinases like ERK and Akt or restore activation of transcription factors like c-Jun and JunD.

Intrinsic resistance can be caused by mutations in any pathway essential for lapatinib sensitivity. For example, aberrations in the PI3K/Akt/mTOR signaling pathway can result in an inability to deactivate Akt. Loss of Phosphatase and Tensin Homolog (PTEN), by promoter methylation or gain of function mutations in PI3K, is frequent in breast cancers (about 30% and 18% to 40%, respectively) and can result in constitutively active Akt [50, 51].

Acquired resistance to TKIs usually occurs through upregulation of a compensatory pathway. Increased ER signaling after treatment with lapatinib in HER2-enriched, ER+ cell lines causes overexpression of AXL receptor tyrosine kinase which induces lapatinib resistance [52]. Lapatinib-induced ER signaling also restores expression of survivin [53]. However, in the SK-BR-3 HER2-amplified cell line, acquired resistance to lapatinib is associated with survivin expression despite pAkt and pERK downregulation, suggesting that another compensatory pathway, other than ER, can restore survivin expression [53]. Overexpression of the MET receptor is also implicated in EGFR directed TKI resistance, and treatment of Basal-like breast cancer cell lines with EGFR directed TKIs and a MET inhibitor is synergistic [54-56].

In SK-BR-3 cells, reduced phosphorylation of Akt due to treatment with HER2 TKIs results in increased expression and phosphorylation of HER3 [57, 58]. Increased signaling through HER3 rescues downstream effectors, thus, sidestepping the effects of the TKIs [59].

1.4 C-JUN N-TERMINAL KINASES

1.4.1 JNK ISOFORMS

C-Jun N-Terminal Kinases (JNKs), members of the mitogen-activated protein kinases (MAPKs), are highly activated by cellular stresses and were initially labeled "stress-activated protein kinases" [60]. Of the three JNK genes (JNK1-3), JNK1 and JNK2 are ubiquitously expressed whereas JNK3 expression pattern is believed to be more limited. Each gene encodes several splice-variants that result in 10 isoforms with molecular weights around 46 and 54 kDa [61]. These kinases are phosphorylated by the MAPK Kinases (MKK) 4 and MKK7 on tyrosine and threonine residues [62]. Once phosphorylated, JNKs affect substrates that exist in the cytosol, or they may translocate into the nucleus to phosphorylate nuclear targets [63, 64] .

Although many believe that JNK1 and JNK2 act in a compensatory fashion for some processes, there is much literature describing differing and even opposing functions of the two JNK isoforms. For one, they differentially regulate their favored target, c-Jun [65]. C-Jun is preferentially phosphorylated by JNK1 after JNK activation, whereas JNK2 decreases c-Jun stability in un-stimulated cells [66]. Although, activated JNK2 may act redundantly with JNK1 with regards to being a positive regulator of c-Jun expression [67]. Using genetic deletion of JNK1 or JNK2, loss of JNK2 in primary mouse embryonic fibroblasts increases cell proliferation, whereas loss of JNK1 reduces proliferation [66]. Our lab has observed similar effects in three-dimensional cultures of

primary mammary epithelial cells. In this case, jnk2-/- primary mammary epithelial acini grow faster than wildtype and show precocious hollowing/differentiation [68]. Conversely, jnk1-/- acini grow much more slowly than wildtype (unpublished data). Furthermore, in relation to migration and invasion, one study showed that Doublecortin is preferentially phosphorylated by JNK2, rather than JNK1, leading to neurite outgrowth [69]. Finally, in a model of obesity, only systemic deletion of JNK1 resulted in increased insulin sensitivity, via enhanced signaling through Insulin Receptor, and weight loss [70].

1.4.2 JNK IN VARIOUS CELLULAR PROCESSES

JNKs are serine/threonine kinases with a variety of substrates. The actions of JNK proteins have been linked to signaling downstream from extracellular stimuli such as UV stress and heat-shock, binding of pro-inflammatory cytokines and activation by Toll-receptor ligands after pathogen invasion (making them regulators of various immune responses). JNKs also respond to intracellular stress such as protein misfolding [71]. Still other studies show JNK activation downstream of non-stress related signaling such as growth factor binding, alongside pathways such as Ras/Raf/ERK, PI3K/Akt/mTOR, and Jak/STAT [72]. This places JNK in the center of signaling for survival, differentiation and cell migration as well as apoptosis.

1.4.2.1 AP-1 TRANSCRIPTION FACTOR

Of the JUN substrates, JNKs activate c-Jun very strongly and JunD much less so. JunB is not a substrate of JNK, although it is a strong binding partner for JNK2 [61, 73]. This is because JunB possesses the moieties for binding to JNK but not for substrate recognition by JNK. JunB recruits JunD, which lacks the JNK docking domain but

contains substrate recognition residues, adding an extra level of regulation to these substrates [73].

C-Jun is phosphorylated by JNK on serine63 and serine73. Once phosphorylated, c-Jun translocates into the nucleus and its transcriptional activity increases when it dimerizes with itself or other binding partners to form the AP-1 transcription factor. Jun and ATF transcription factors, also substrates of JNK [74], homo- and hetero-dimerize with members of the Fos and MAF family members to form AP-1. Homodimers of Jun and heterodimers of Jun and Fos bind the canonical TPA-Responsive Element (TRE) to enhance transcription of target genes. Other heterodimers preferentially bind non-canonical AP-1 response elements such as the cAMP-Response Element (CRE) (**Figure 1.2**).

Interestingly, many studies have reported that inactivated JNK, especially JNK2, binds its associated transcription factors and causes their degradation, while phosphorylation enhances not only their action but also protein stability [75-77]. JNK also phosphorylates the E3-ligase, Itch, on multiple sites to enhance protein degradation of its targets including c-Jun [78] and JunB [79].

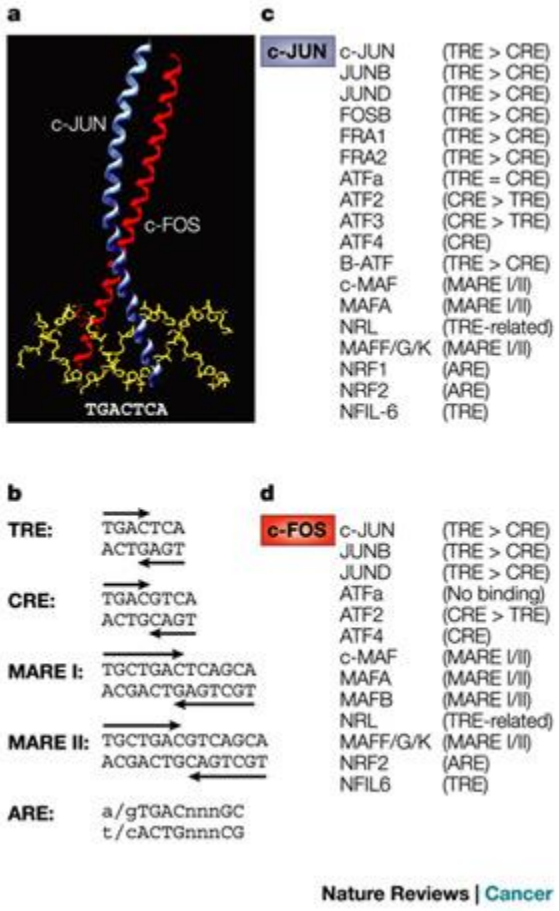


Figure 1.3: AP-1 Binding Partners and Response Elements
(A) c-Jun and c-Fos forming an AP-1 heterodimer at the TRE DNA response element. (B) Response element sequences. (C) c-Jun binding partners and their preferred response elements. (D) c-Fos binding partners and their preferred response elements [80].

AP-1 containing c-Jun is a positive regulator of cellular proliferation through induction of target genes including cyclinD1 and c-MYC and inhibition of p53 expression [81-83]. C-Jun and c-Fos were both discovered as viral oncoproteins whose expression transforms various cell types. They also play roles in the progression of many different cancers (reviewed in [80]).

C-Jun also regulates apoptosis by increasing expression of pro-apoptotic proteins such as Bak and TNF-α [84]. Interestingly, JunB antagonizes c-Jun in many of these instances [81, 83, 85]. JunD can be phosphorylated by ERK proteins downstream of EGFR activation facilitating crosstalk between JNK and ERK pathways [86]. JunD is involved in survival signaling after JNK phosphorylation by upregulating Cellular Inhibitor of Apoptosis Protein 2 (c-IAP2) in the presence of activated NF-κB [87].

In breast cancer, a study using tissues from 72 primary tumors of various subtypes and 37 adjacent, non-tumor breast tissues analyzed mRNA expression of the various AP-1 family members. While Fra-1, Fra-2, JunB, and JunD were significantly higher in breast tumors compared to normal adjacent tissues, c-Jun and c-Fos were found to be significantly lower. These findings correlated with protein expression analysis except c-Jun, likely due to its regulation at the level of protein stability. Interestingly, high levels of Fra-1 significantly associated with Basal-like breast tumors. No other Jun or Fos proteins were found to have significantly different expression between subtypes [88]. However, a later study by the same group showed significant correlation of c-Jun protein expression with Basal-like breast cancer cell lines compared to HER2-enriched or luminal cell lines[89]. In Basal-like breast cancers high Fra-1 was significantly associated with lower distant metastasis-free survival [89].

Knockdown of Fra-1 or c-Jun by siRNA in Basal-like cell lines resulted in decreased proliferation and cell invasion both in vitro and in a zebrafish model of metastasis [89, 90]. C-Jun, as a part of AP-1, is responsible for EpCAM mediated migration of MDA-MB-231 basal-like breast cancer cells [91].

As a part of AP-1, c-Jun is implicated in transformation and proliferation of various cancers [80], and in breast cancer associates most strongly with the more aggressive, Basal-like subtype. Pharmacologic inhibition of JNK activity may therefore

be beneficial in treating Basal-like breast cancers through decreased phosphorylation of c-Jun and AP-1 transcriptional activity.

1.4.2.2 JNK IN GROWTH FACTOR SIGNALING

Like AP-1, high expression of JNK is also correlated with poor prognosis. In patients with infiltrating ductal carcinoma, high expression of phosphorylated JNK1 or 2 was a significant, independent indicator of poor prognosis [92]. In another study using 469 tumors of all grades and subtypes, high expression of JNK2 trended toward poorer disease-free survival without reaching significance, but in an analysis of only Basal-like breast cancers, high expression of JNK2 significantly decreased disease-free survival [93]. This may be due not only to JNKs activation of AP-1 but also JNK's conveyance of signaling responses downstream of growth factor receptors that promote transformation, cell growth, and survival.

In a mouse model of breast cancer where Basal-like 4T1.2 cells were injected orthotopically into the mammary fatpad, JNK2 positively regulates expression of Grb2 Associated Binding Protein (GAB2), an adaptor protein that binds to and conveys signals downstream of many Receptor Tyrosine Kinases (RTKs). Downregulation of GAB2 by JNK2 knockdown resulted in decreased phosphorylation of Akt after treatment with HGF, EGF, and insulin, displaying the importance of JNK to the successful induction of these pathways [94]. In a Luminal breast cancer cell line, MCF7, JNK was activated after stimulation of Insulin-like Growth Factor-1 Receptor (IGF-IR) [95, 96], but JNK phosphorylates Insulin Receptor Substrate 1 (IRS-1) in an inhibitory fashion after activation by TNF-α or other pro-inflammatory cytokines. This event decreases insulin sensitivity with the *in vivo* consequences of inducing diabetes associated insulin resistance [70, 97, 98].

JNK affects intracellular signaling by regulating other protein kinases. JNK phosphorylates Akt1 at Thr450. Like Ser473, phosphorylation of Thr450 may prime Akt to be phosphorylated by PDK1 at its major catalytic site, Thr308 [99].

JNK may well prove to be an important mediator of breast cancer cell signaling due to its actions downstream of many growth factor receptors. Because mechanisms of growth factor receptor TKI resistance often involve upregulation of compensatory signaling pathways (See **1.3.2.2.5)**, JNK inhibition may be beneficial in overcoming resistance to these drugs or increasing their effectiveness.

1.4.2.3 JNK IN DIFFERENTIATION

Breast cancer subtypes, discussed in **Chapter 1.2**, differ in their expression of genes important for normal mammary differentiation, and higher grade tumors with worse prognosis are enriched in Epithelial to Mesenchymal Transition (EMT) and stem-cell related gene expression. In the previously mentioned study where phosphorylated JNK1 and JNK2 were indicators of poor prognosis, they were also associated with younger age at diagnosis and higher grade tumors displaying de-differentiation [92].

TGFβ can cause EMT in multiple cell types through activation of JNK [100-102]. In a mouse model of breast cancer where p53-/;jnk2-/- mouse cells expressing either GFP-JNK2 or GFP were injected into the mammary fatpad of wildtype mice, GFP-JNK2 caused the growth of poorly differentiated tumors expressing higher TGFβ1 and lower ER and E-cadherin compared to GFP controls. GFP-JNK tumors grew significantly more than the well-differentiated GFP tumors that appeared growth arrested. Analysis of the cell lines revealed high expression of EMT related genes and low expression of E-cadherin in the GFP-JNK2 expressing cells, along with a high percentage of TICs. The GFP cells were more luminal, expressing markers such as GATA-3 and BRCA1.

Knockdown of JNK2 in the human Basal-like cell line MDA-MB-231 resulted in a significant increase in $EpCAM^{HI}$ (more epithelial-like) cells that expressed greater amounts of BRCA1 protein, which is often mutated or downregulated in human Basal-like breast cancers and cell lines [68]. In a separate study, Basal-like MDA-MB-468 cells transfected with a constitutively active JNK (SAPKβ-MKK7) adopted a more spindle-like cell shape, reminiscent of mesenchymal cells, and upregulated the mesenchymal markers Vimentin and Fibronectin [103].

Through JNK inhibition, increased differentiation in breast cancers might increase the effectiveness of certain drugs, as de-differentiated tumors expressing many stem-cell and EMT-related genes are drug resistant [104, 105]. Increasing differentiation may also lead to increased survival, as dedifferentiated tumors show a higher propensity to metastasize [106].

1.4.2.4 JNK IN MIGRATION/INVASION AND METASTASIS

A hallmark of EMT is increased ability of cells to migrate toward and invade into the extracellular matrix surrounding glands. This is the first step in metastasis and one of the reasons why patients with Basal-like breast cancers experience shorter survival.

In the 4T1.2 mouse mammary cancer cell line, which is considered Basal-like, knockdown of JNK2 increased expression of an adaptor protein called EPS8 and decreased migration of those cells three-fold. High expression of EPS8 caused association with RN-Tre to inhibit internalization of activated EGFR (commonly overexpressed in Basal-like breast cancers) which inhibited EGFR-mediated migration [93]. Knockdown of JNK2 in 4T1.2 cells also decreased invasion in vitro and significantly increased survival in mice after injection into the mammary fatpad. After sacrifice, mice injected with knocked-down JNK2 cancer cells showed significantly less

lung metastasis. Intra-cardiac injection of shJNK2 expressing cells resulted in significantly less metastatic rib lesions compared to control cells [94].

In human Basal-like MDA-MB-231 cells, Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL)-induced migration resulted in JNK phosphorylation and was inhibited by SP600125 [107]. With the same cell line, chemokine-mediated migration and metastasis to the lungs from the mammary gland was shown to be regulated by JNK's phosphorylation of Paxillin [108] which is a part of focal adhesions. JNK localizes to membrane ruffles at the front of migrating cells stimulated by growth factors, and here it phosphorylates Paxillin at Ser178 to facilitate migration [109].

These data indicate that JNK inhibition may protect against metastasis of human breast cancers, specifically of the Basal-like subtype, in addition to beneficial effects in the primary tumor.

1.4.2.5 APOPTOTIC PROTEINS AS JNK SUBSTRATES

Despite facilitating signaling downstream of growth factor binding and being a positive regulator of AP-1, ironically JNK can also promote apoptosis through regulation of apoptotic proteins.

Through phosphorylation of 14-3-3 proteins, JNK indirectly regulates protein translocation. 14-3-3 family members bind phospho-motifs on proteins in the cytosol and sequester them from the nucleus by masking nuclear or other organelle localization signals. This occurs frequently with proteins involved in apoptosis. JNK phosphorylation of 14-3-3 causes its dissociation from Bax. Unbound Bax enters the mitochondria to induce apoptosis [110]. Phosphorylation by JNK of 14-3-3-bound Bad facilitates its pro-apoptotic function [111]. JNK also directly phosphorylates apoptosis-related proteins. JNK phosphorylated Bcl-2 on Ser70 has been described as both enhancing and

inactivating [112, 113]. JNK phosphorylation of Thr47 and Thr115 of Bcl-xL diminishes its pro-survival function [114], and JNK phosphorylation of Bax [115] and several Bim family members [116, 117] enhances their pro-apoptotic functions. JNK also phosphorylates p53 on Ser6 and Thr81 to stabilize it and facilitate apoptosis [118, 119]. Lastly, through phosphorylation of the E3-ligase Itch, JNK enhances degradation of c-FLIP, a caspase-9 inhibitor [120]. Collectively, these functions place JNK as a key mediator of cell death.

1.4.3 JNK INHIBITORS

Significant effort has been focused on developing JNK inhibitors for the treatment of immune related human diseases including fibrosis. While no agents have shown clinical efficacy yet, some of these agents are popular in laboratory research.

Although JNK activates apoptosis in some models, breast cancer data implicate JNK as an independent indicator of poor prognosis [88, 89, 92, 93]. The roles of JNK in growth factor signaling, de-differentiation, and metastasis likely outweigh its pro-apoptotic functions in breast cancer, making JNK an attractive target for therapeutic inhibition. Thus, our lab and others have explored the potential therapeutic effectiveness of JNK inhibitors in breast cancer models.

The first commercially available JNK inhibitor, SP600125, was released in 2001 by Celgene/Signal Pharmaceuticals. SP600125 is an ATP-competitive reversible inhibitor with an anthrapyrazolone structure. It showed high selectivity for JNK1 and JNK2 with IC_{50} values of 40nM each which were many fold lower than for other kinases tested, including other MAPKs ERK1 and p38 kinase. One of the earliest studies in cells involved use of SP600125 in Jurkat T cells where the observed concentration necessary for inhibition of c-Jun phosphorylation at Ser63 was 5-10uM (Reviewed in [121]).

Since then, countless research articles using SP600125 to study the involvement of JNK in cellular mechanisms have been published, including many using Basal-like breast cancer cells in mice and humans. A study in 2005 showed that SP600125 decreases proliferation of the MDA-MB-231 and MDA-MB-468 Basal-like breast cancer cell lines [122]. More recently, a 2012 study showed that Growth Hormone (GH) treatment of MDA-MB-231 cells protects against apoptosis induced by doxorubicin. They used SP600125 to prove that JNK downregulation by GH treatment is the mechanism by which this protection occurs [123].

However, in 2003, a study showed that SP600125 had equal or higher affinity to 13 other kinases at the concentration of 10uM [124]. Inhibition of some of these off target kinases such as Aurora B and Aurora C could easily explain decreased proliferation of cells treated with SP600125. Therefore, much misleading information may be published in the literature when authors attribute effects seen with SP600125 to JNK inhibition alone.

Peptide inhibitors of JNK have been developed using knowledge of the structure of JNK-Interacting Protein (JIP)-family scaffold proteins. JIPs bring together members of the protein kinase signaling cascade that phosphorylates JNK. Downstream of Rac activation, the Mixed Lineage Kinases (MLK) -2 and -3 phosphorylate MKK7 and/or MKK4 which then phosphorylate JNK [125]. MLK, MKK and JNK bind to JIP and close proximity facilitates the phosphorylation cascade. Interestingly, overexpression of full length JIP or just the JNK interacting domain of JIP were shown to bind JNK in the cytosol and prevent JNKs phosphorylation of downstream targets such as c-Jun. These peptides are cell permeable and are quite selective for JNK and its upstream kinases MLK and MKK. Use of various JIP-derived inhibitory peptides in many disease models including ischemia and cell line models of Alzheimer's showed encouraging results.

Discovery of other JNK scaffolds such as Daxx and JAMP might help develop inhibitory peptides specific to certain JNK isoforms (Reviewed in [126]). Currently, the clinical use of peptides is rather limited due to *in vivo* instability.

A new pharmacophor of ATP site-directed JNK inhibitors has recently been developed that covalently bind JNK. Of these, JNK-IN-8 was found to be the most selective with the highest affinity for all three JNK isoforms. JNK-IN-8 covalently binds Cys154 in JNK3's catalytic site and Cys116 in the catalytic sites of both JNK1 and JNK2. In the HEK-293-T cell line, this molecule potently inhibits phosphorylation of c-Jun at Ser63 after three hours of treatment at a concentration of 3uM, with no effect on similar kinases like Msk1 and p38 kinase.

Significantly, a highly selective covalent inhibitor of JNK may be more useful in a therapeutic sense in that covalent binding of the drug ensures that smaller doses would need to be administered to ensure complete inhibition of the target. In a molecular sense, the availability of a single residue on JNK that can be mutated to render the drug incapable of optimal binding will help researchers determine JNK-IN-8 selectivity in their own models, to conclude with confidence that the induced phenotypes are specifically due to JNK inhibition [127].

1.5 Dissertation Outline and Objectives

JNK has central roles in differentiation, migration, survival, and growth factor signaling in Basal-like breast cancer. Our hypothesis is that JNK inhibition will be therapeutic in treating Basal-like breast cancer, especially in combination with the EGFR/HER2 inhibitor lapatinib. With this work, we hope to establish the legitimacy of pharmacologically co-targeting EGFR/HER2 and JNK as a therapy for patients with Basal-like breast cancers. **Chapter 1**, an introduction to EGFR/HER2 and JNK-related

literature, aims to provide a framework from which one can logically deduce how the targeting of EGFR/HER2 and JNK would be beneficial to the treatment of Basal-like breast cancer.

The remaining chapters provide data using lapatinib and JNK-IN-8, alone or in combination, to induce cell death in human Basal-like breast cancer cell lines. **Chapter 2** provides evidence that while JNK-IN-8 and lapatinib do not have a significant effect on cell viability alone, their combination causes a synergistic decrease in cell viability in several established Basal-like breast cancer cell lines, as well as in one HER2-amplified cell line. Through various methods, decreased cell viability is shown to involve apoptosis.

To deduce the mechanism by which synergistic apoptosis occurs with these drugs, it was first necessary to determine whether the intended drug targets were involved or whether off-target effects were producing the phenotype. **Chapter 3**, therefore, focuses on genetic manipulation of EGFR, HER2, JNK1, and JNK2 to see how reducing expression of individual drug targets compares to pharmacologic inhibition. Through these experiments it is revealed that HER2 and JNK1 are the main targets of lapatinib and JNK-IN-8, respectively.

Once the primary drug targets were defined, a link between EGFR/HER2 and JNK needed to be established. **Chapter 4** reveals that treatment with lapatinib causes an increase in cell viability that is independent of changes in previously implicated receptors and intracellular kinases. However, JNK activity is induced by long-term treatment with lapatinib resulting in an increase in nuclear-associated c-Jun. Overexpression of JNK further increases cell viability in the presence of lapatinib. Conflicting results involving inhibition of the AP-1 transcription factor call into question the involvement of c-Jun,

implicating other JNK targets or non-JNK regulated AP-1 members in the cell death phenotype.

Chapter 5 delineates a mechanism for apoptosis involving the unchecked accumulation of intracellular ROS. ROS scavenging completely rescues cell death caused by combination treatment with JNK-IN-8 and lapatinib. A possible explanation for ROS accumulation is explored concerning JNK-IN-8 and lapatinib regulation of the NFκB transcription factor.

Chapter 2: The Combination of JNK-IN-8 and Lapatinib Causes Synergistic Cell Death in Basal-like Breast Cancer Cell Lines

2.1 Introduction

Effective treatments for patients with Basal-like or Claudin-low breast cancers are urgently needed. JNK and c-Jun have emerged as indicators of poor prognosis in Basal-like breast cancers [89, 92, 93] and studies from our own lab have confirmed the importance of JNK2 in mouse mammary tumor cell differentiation, growth, and metastasis [94]. EGFR is overexpressed in most Basal-like and Claudin-low breast cancers [6], but EGFR-targeted inhibitors have been mystifyingly inept [38-40]. Because JNK signals downstream of many growth factor receptors including EGFR [72, 93] we hypothesized that JNK-IN-8 might increase the effectiveness of lapatinib either by further blocking EGFR/HER2 downstream signaling, or by inhibiting signal transduction from a compensatory pathway that renders lapatinib ineffective. In either case, we set out to determine whether JNK-IN-8 might increase the effectiveness of lapatinib in sensitive cells, such as the HER2-overexpressing cell line SK-BR-3, or in cells that are intrinsically resistant to lapatinib, such as the Basal-like cell lines MDA-MB-231, MDA-MB-436, and HCC1569.

2.2 Methods

2.2.1 Cell Lines and Reagents

MDA-MB-436 and HCC1569 cell lines were a kind gift from the lab of Dr. Calvin Kuo at Stanford University. They were maintained in RPMI media (# SH30027.01 HyClone, Logan, UT) supplemented with 10% Fetal Bovine Serum (FBS) (#100-106,

Gemini Bio Products, Sacramento, CA) at 37°C in a 5% CO_2 atmosphere. The MDA-MB-231 cell line was purchased from ATCC (Manassas, VA) and maintained in Improved MEM (#A10488-01, Gibco, Grand Island, NY) supplemented with 10% FBS and 10µg/mL Humulin insulin (#HI-310, Eli Lilly & Co., Indianapolis, IN). JNK-IN-8 (#420150 Millipore, Darmstadt, Germany) and lapatinib (#L-4804 LC Labs, Woburn, MA) were dissolved in dimethyl sulfoxide (DMSO) and kept at -20° in small aliquots.

2.2.2 WESTERN BLOT

Cells were lysed in Radio Immunoprecipitation Buffer (RIPA) (50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA) and 80µg of protein was separated using SDS-PAGE. Proteins were then transferred to 0.22µm nitrocellulose and blocked in 5% non-fat powdered milk in TBS-T (Tris base, NaCl, pH 7.6 plus 0.05% Tween 20) for one hour, shaking at room temperature. Nitrocellulose blots were then incubated overnight at 4°C on a rocking platform in the presence of primary antibody. Phospho-c-Jun(Ser63)(#9261) and phospho-JNK(Thr183/Tyr185)(#9251) antibodies were purchased from Cell Signaling (Danvers, MA) and used at a dilution of 1:500. After removal of primary antibody by washing in TBS-T, the blots were incubated in HRP-conjugated, anti-rabbit IgG secondary antibody (#7074 Cell Signaling Danvers, MA) for one to two hours at room temperature at a 1:1000 dilution. Proteins were visualized using ECL 2 (#80196 Thermo Scientific Grand Island, NY) to produce fluorescent signal that was detected by scanning with the Storm 860 Imager (GE Amersham Pittsburgh, PA). Band densitometries were calculated using Image J software (National Institutes of Health Bethesda, MD).

2.2.3 CELLULAR VIABILITY ASSAY USING MTT

Cells were plated at a density of 3000 cells/well in 96-well, flat bottom plates. After being allowed to attach overnight, cells were treated for various timepoints. After treatment, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (#475989 Calbiochem, San Diego, CA) was added to a final concentration of 0.5mg/ml from 5mg/ml stock in PBS. Cellular metabolism reduces this tetrazole to insoluble formazan crystals. After four hours of incubation, the media + MTT was gently removed. Formazan crystals were dissolved using DMSO and absorbance at 590nm was read using the Synergy II Plate Reader (BioTek Winooski, VT).

2.2.4 SYNERGY CALCULATION

Raw absorbance values were normalized to the vehicle control wells and represented as a % decrease in absorbance from the vehicle control (control = 0 effect, 2% decrease in absorbance = .02 effect). These values were entered into the CompuSyn software (ComboSyn, Paramus, NJ) as "effect" along with the concentrations of both JNK-IN-8 and lapatinib used to obtain that value. Effect values for increasing concentrations of each drug alone were used to calculate the median-effect, which was then used to determine whether the effect caused by a combination concentration was synergistic [128]. Combination Index plots and values were generated by the CompuSyn software.

2.2.5 ANNEXIN V APOPTOSIS ASSAY

Apoptosis was measured by Annexin V and propidium iodide (PI) positivity using the "Rapid-binding protocol" from the Calbiochem Annexin V-FITC kit (#PF032 San

Diego, CA). Fluorescence from the Annexin V antibody or PI was read using the Guava EasyCyte™ flow cytometer (Millipore, Darmstadt, Germany). Analysis and generation of two-color plots were performed using the FlowJo 10 software (Ashland, OR).

2.2.6 STATISTICS

All statistical analyses were performed using Prism5 software (GraphPad LaJolla, CA). One-way or Two-way ANOVA was performed with Bonferroni's Multiple Comparison post test. Student's t-test was used to obtain statistical significance between groups. For all graphs: (*≤.05, **≤.01, ***≤.001, ****≤.0001).

2.2.7 LIGHT MICROSCOPY

An Olympus CKX41 (Center Valley, PA) upright light microscope with a phase-contrast filter was used to visualize cells in culture. Photographs of cells were taken at 1000X using QCapturePro imaging software (QImaging, Surrey, BC, Canada).

2.3 RESULTS

2.3.1 JNK-IN-8 EFFECTIVELY INHIBITS C-JUN PHOSPHORYLATION

The investigators that created the JNK-IN-8 compound achieved almost total inhibition of Ser63 phosphorylation of c-Jun at 3μM treatment for three hours in the HEK-293-T cell line overexpressing IL-1R. These cells were treated with anisomycin to induce JNK-mediated c-Jun phosphorylation. They also show a change in mobility of JNK after addition of JNK-IN-8 due to covalent binding of the drug [127]. We obtained similar results using a mouse mammary cancer cell line that is p53-/-. Using UV

irradiation to stimulate phosphorylation of JNK and c-Jun, we obtained about 50% inhibition of c-Jun phosphorylation after three hours incubation with 3μM JNK-IN-8, and 92% inhibition with 5μM JNK-IN-8 (**Figure 2.1A**). Even with low concentrations of JNK-IN-8, such as 0.1μM, we obtained decreased mobility of phosphorylated JNK (Thr183/Tyr185), indicating covalent binding of the drug (**Figure 2.1B**).

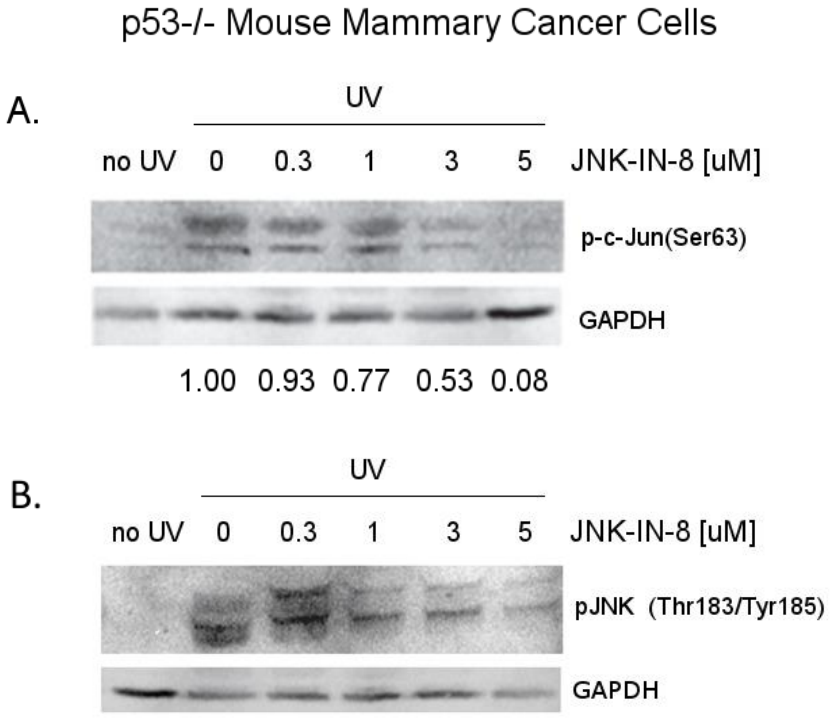


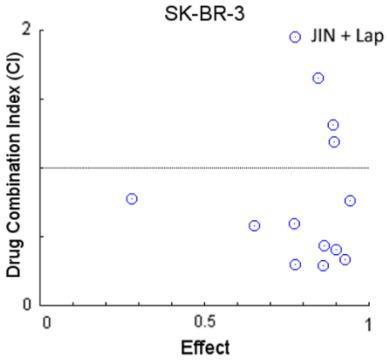
Figure 2.1: JNK-IN-8 Inhibits c-Jun Phosphorylation and Causes JNK Mobility Shift

(A) Phosphorylation of c-Jun (Ser63) by western blot after UV irradiation and/or JNK-IN8 treatment. Relative densitometry values are below GAPDH. (B) Phosphorylation of JNK (Thr183/Tyr185) after UV and/or JNK-IN-8 treatment showing mobility shift in all isoforms.

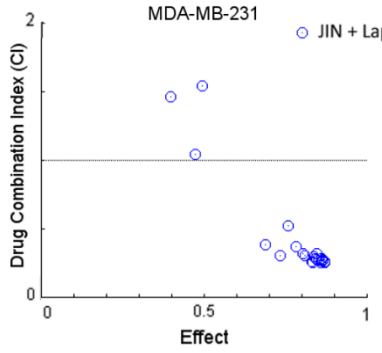
2.3.2 DECREASED CELL VIABILITY CAUSED BY JNK-IN-8 AND LAPATINIB IS SYNERGISTIC

To determine if JNK-IN-8 sensitizes breast cancer cell lines to lapatinib, cells were seeded to about 50% confluency in microplates and allowed to grow in the presence of either vehicle alone (DMSO), JNK-IN-8 alone, lapatinib alone, or JNK-IN-8 with lapatinib. MTT was used to measure cellular metabolism and cell viability. Various combinations of concentrations of JNK-IN-8 and lapatinib were tested. Ranges of values tested for JNK-IN-8 were between 0.1uM and 20uM based on observed inhibition of c-Jun phosphorylation at 5uM (**Figure 2.1A**). Ranges of lapatinib concentrations tested were based on published IC_{50} values, except for the MDA-MB-436 cell line for which no published values could be found but were presumed to be similar to the MDA-MB-231s. Published IC_{50} values cell lines are as follows: 0.05-0.2 uM for SK-BR-3, 5-10uM for MDA-MB-231 [36, 129], and about 3uM for HCC1569 [130]. For each graph in **Figure 2.2**, the blue circles represent a different concentration combination of JNK-IN-8 and lapatinib. The x-axis shows the combination effect (which is the % decreased metabolism as measured by formazan absorbance at 590nm), and the y-axis shows the combined drug index (CI). CIs less than one (the y-axis center) are synergistic concentrations, CIs equaling one are additive, and CIs greater than one are antagonistic.

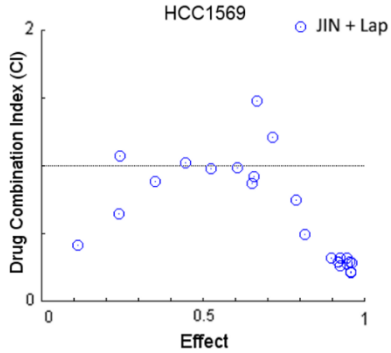
For all four cell lines, the combination of JNK-IN-8 and lapatinib synergistically decreased cell metabolism, especially at concentrations causing greater than 50% effect (**Figure 2.2**). This was true despite the demonstrated sensitivity of the SK-BR-3 cells to lapatinib and the insensitivity of the other cell lines.



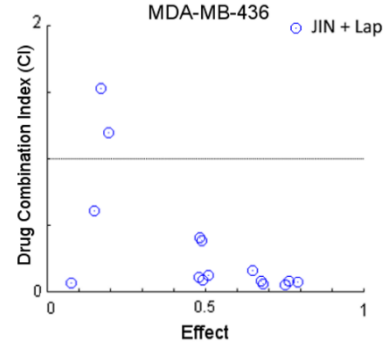
Lap [uM]	JIN [uM]	Effect	CI
0.1	1	0.77855	0.30271
0.1	5	0.86309	0.29227
0.1	10	0.86435	0.43653
0.1	20	0.92997	0.33993
1	1	0.84669	1.65447
1	5	0.89464	1.19266
1	10	0.89338	1.3177
1	20	0.94511	0.76371



Lap [uM]	JIN [uM]	Effect	CI
3	5	0.27122	2.77453
3	6	0.3991	1.46891
3	7	0.47558	1.04540
3	8	0.69087	0.38652
3	10	0.73586	0.30489
5	3	0.49679	1.54709
5	5	0.78406	0.37099
5	6	0.80431	0.32428
5	7	0.83226	0.26402
5	8	0.83515	0.25845
5	10	0.81105	0.31127



Lap [uM]	JIN [uM]	Effect	CI
1	0.1	0.24418	1.07535
1	1	0.35321	0.88972
1	5	0.52470	0.98091
1	10	0.65758	0.92165
1	20	0.89835	0.31942
3	0.1	0.22033	3.59279
3	1	0.32936	2.30854
3	5	0.65190	0.87166
3	10	0.81658	0.49667
3	20	0.92561	0.26293



Lap [uM]	JIN [uM]	Effect	CI
3	20	0.65133	0.16575
10	0.1	0.15133	0.61469
10	5	0.51211	0.12484
10	10	0.67676	0.08253
10	20	0.76513	0.08391
20	5	0.68281	0.05884
20	10	0.75182	0.05752
20	20	0.79177	0.07351
20	0.1	0.49274	0.08897
20	1	0.48063	0.11419

Figure 2.2: JNK-IN-8 and Lapatinib Synergistically Decrease Cell Viability

Combination Index (CI) plots and effect and CI value tables for various combination concentrations of JNK-IN-8 and lapatinib. Points represent a specific combination of JNK-IN-8 and lapatinib concentrations plotted by their CI and effect based on MTT viability results. CI<1 is synergistic. Analysis was done using CompuSyn software [131].

2.3.3 Decreased Cell Metabolism Caused by JNK-IN-8 and Lapatinib is due to Apoptosis

In order to determine whether the cause of decreased cell viability from JNK-IN-8 and lapatinib combination was due to increased apoptosis, cells were assayed for expression of Annexin V on the cell surface and the ability of propidium iodide to cross the cell membrane. Early apoptotic cells express Annexin V on their cell surface while later apoptotic cells will express Annexin V and lose integrity of the cell membrane, allowing propidium iodide to enter and stain DNA.

A single combination of synergistic concentrations was chosen for the MDA-MB-231 and HCC1569 cell lines from values that obtained about 70% effect. In **Figure 2.3A**, graphs represent percentages of cells that were Annexin V and/or propidium iodide positive. For both the MDA-MB-231 and HCC1569 cell lines, apoptosis was significantly higher than the control or either drug alone, indicating that the synergistic effect seen in JNK-IN-8 and lapatinib combination is due to apoptosis.

MDA-MD-231 cells were also assayed for expression of the proliferation marker Ki67 using immunofluorescence. After treatment with vehicle (DMSO), JNK-IN-8 (JIN), lapatinib (Lap), or the combination (J+L) for 72 hours, cells were fixed and probed with Ki67 primary antibody. In brightfield, mitotic cells are bright and round compared to the flat, fully attached cells. In the combination treatment, although many cells are round, membrane blebbing is seen indicating apoptosis (**Figure 2.3B arrows**). As evidenced by fluorescent microscopy, remnants from apoptotic cells (red patches without DAPI staining) are seen in the combination treatment, however, cells that are still alive display nuclear Ki67 positivity to a similar degree as vehicle and other single treatments (**Figure 2.3B**)

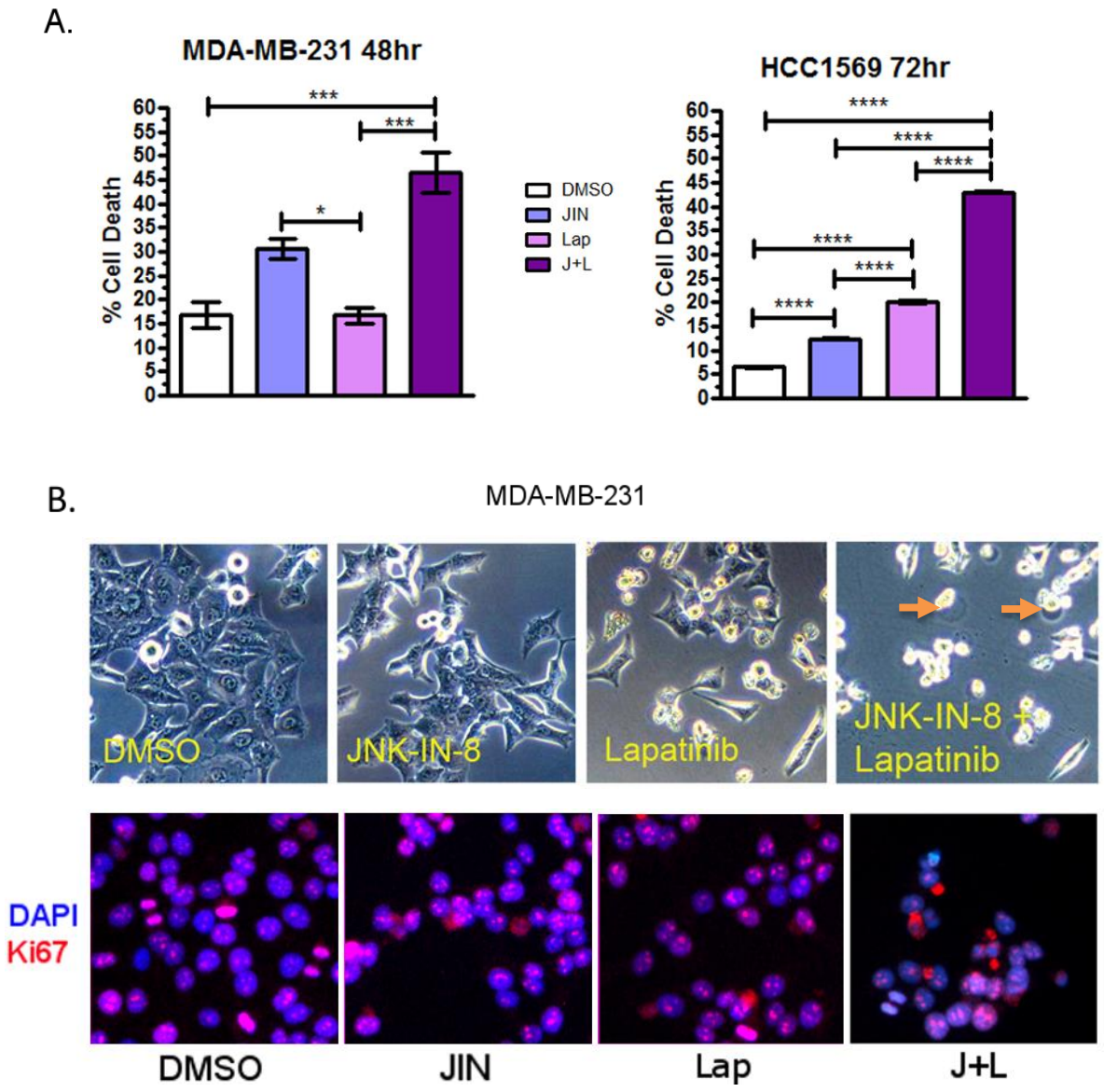


Figure 2.3: Treatment with JNK-IN-8 and Lapatinib Causes Apoptosis

(A) MDA-MB-231 or HCC1569 cells were treated with 3uM lapatinib (Lap) and/or 5uM JNK-IN-8 (JIN) and 1uM Lap and/or 3uM JIN, respectively. Annexin V and propidium iodide mean positivity were assayed at 48 or 72 hours after treatment ± SEM (*≤.05, **≤.01, ***≤.001, ****≤.0001). (B) Bright field images (1000x) and fluorescence (6000x) of MDA-MB-231 cells treated with DMSO, 5uM JIN, 3uM Lap, or combination for 72 hours. Blue channel = DAPI, Red channel = Ki67.

2.4 DISCUSSION

Here we have identified a novel effect where addition of the JNK inhibitor, JNK-IN-8, increases the effectiveness of lapatinib in killing Basal-like breast cancer cells. These drugs act synergistically and significantly increase in apoptosis when compared to vehicle or either drug alone. However, proliferation remains high in cells not undergoing apoptosis. Presumably, JNK-IN-8 could synergize with lapatinib by inhibiting the same pathway that signals survival or each could inhibit a separate survival pathway (**Figure 2.4)**

However, in order to address these issues, it was first important to confirm whether the mechanism actually involves both JNK and EGFR/HER2, or whether these phenotypes are due to the inhibition of off-target kinases by one or both drugs.

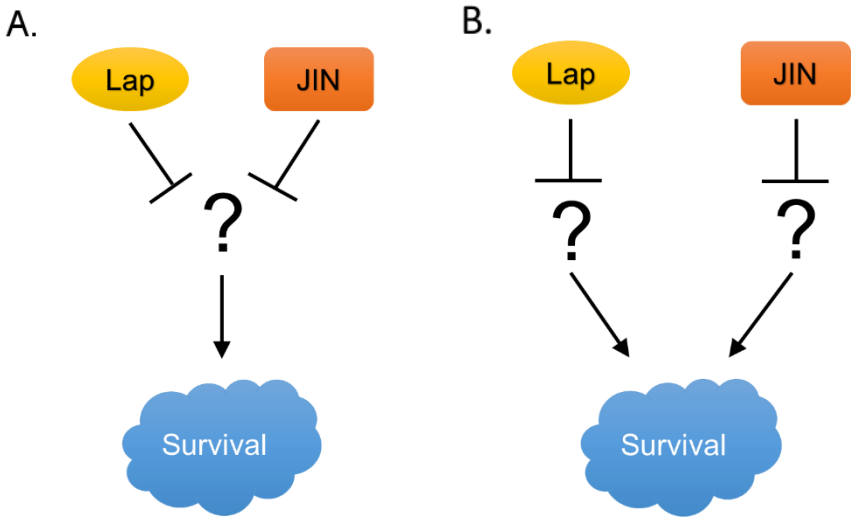


Figure 2.4: Possible Mechanisms of Cell Death by JNK-IN-8 and Lapatinib
(A) Lapatinib and JNK-IN-8 may synergize through inhibition of the same pathway needed for cell survival. (B) Lapatinib and JNK-IN-8 may synergize through inhibition of two different pathways needed for cell survival.

Chapter 3: The Mechanism of Synergy Between JNK-IN-8 and Lapatinib Relies on HER2 and JNK1

3.1 JNK-IN-8 and Lapatinib Synergy Relies More Heavily on Lapatinib Specific Inhibition of HER2 Rather than EGFR

3.1.1 Introduction

EGFR is generally highly expressed in Basal-like breast cancers and cell lines [6, 7, 11, 132], yet these tumors remain mostly resistant to treatment with EGFR inhibitors alone [38, 39], calling into question the reliance of these tumors, or cell lines, on EGFR for growth or survival. Because HER2 is generally absent or expressed at low levels in Basal-like breast cancer cell lines, it would not be impractical to conclude that lapatinib effects in Basal-like models are due to off-target inhibitions, of which some have been described [133, 134]. It was imperative to assess whether inhibition of EGFR and/or HER2 is important for synergy with JNK-IN-8 in order to give a "jumping-off point" from which to narrow down possible mechanisms of synergy.

3.1.2 Methods

3.1.2.1 Cell Lines and Reagents

HEK-293-T cells were obtained from ATCC (Manassas, VA) and were maintained in DMEM with high glucose (#SH30243.01 HyClone, Logan, UT) supplemented with 10% FBS at 37°C in 5% CO_2. Gefitinib (#H018 AK Scientific, Union City, CA) and Erlotinib (#10483 Cayman Chemical, Ann Arbor, MI) were dissolved in DMSO for a stock concentration of 20mM and kept at -20°C in small aliquots. Neratinib (#52150 SelleckChem, Houston, TX) was dissolved in DMSO for a stock concentration

of 3mM. Epidermal Growth Factor (EGF) (#100-15 PeproTech, Rocky Hill, NJ) was dissolved in PBS for a stock concentration of 100µg/mL.

3.1.2.2 MTT ASSAY AND SYNERGY CALCULATION

The MTT assays and synergy calculations were carried out as in **2.2.3** and **2.2.4.** Statistics were calculated using the same methods as in **2.2.6**.

3.1.2.3 TARGET GENE KNOCKDOWN WITH RETROVIRAL SHRNA

HEK-293-T cells were plated at $1x10^6$ cells per 60mm dish on poly-lysine coated plates and allowed to attach overnight. The next day, six total µg of DNA was mixed with 6uL of Enhancer™ Reagent and complexed with 6uL of Lipofectamine® 3000 (#L3000001 Life Technologies, Grand Island, NY) per the manufacturer's protocol. For the DNA mixture, 3µg of shRNA plasmid and 3µg of pCL-Eco (Open Biosystems Lafayette, CO) was used. This mixture was allowed to sit for 10 minutes and added drop-wise to HEK-293-T cells. Target MDA-MB-231 cells were plated the day after transfection at 250,000 cells per 60mm dish. At 48 hours and 72 hours after transfection, media containing retrovirus was taken off the HEK-293-T cells and filtered through a 0.45µM syringe filter to remove HEK-293-T packaging cells. Polybrene (Hexadimethrine bromide) (#107689 Sigma, St. Louis, MO) at 8ug/mL was added to increase viral transduction efficiency and this mixture was added drop-wise to the MDA-MB-231 target cells. After 72 hours of incubation in viral media, fresh media containing G418 (#29065A Santa Cruz Biotechnology Dallas, TX) at 800µg/mL was added to select for cells expressing the shRNA constructs. Target gene knockdown was assessed by western blot. ShRNA retroviral plasmids: shEGFR (targeting sequence: CTGTGCAGAATCCTGTCTATC), shHER2 (targeting sequence:

GGGAGAGAGTTCTGAGGATTG) and shNC (control) (targeting sequence: CAACAAGATGAAGAGCACCAA) sequences in the pSUPERIOR.retro.neo.gipz vector were obtained from Dr. Dennis Hughes at the MD Anderson Cancer Center (Houston, TX).

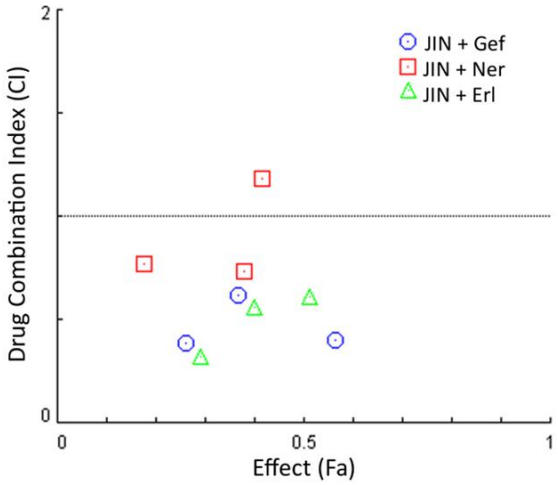
3.1.2.4 WESTERN BLOT

Western blot methods follow **2.2.2** except for the following changes: Cells were lysed in TDL Buffer (1% TritonX-100, 10mM Tris-HCL (pH=7.4) 150mM NaCl) and 100µg of protein was loaded for HER2 blots and 40µg of protein was loaded for EGFR blots. EGFR (#4267), pEGFR (#4407), and pHER2 (#2241) antibodies were purchased from Cell Signaling (Danvers, MA) and used at a dilution of 1:1000. HER2 (#284) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX) and used at a dilution of 1:600.

3.1.3 RESULTS

3.1.3.1 JNK-IN-8 SYNERGIZES WITH OTHER ERBB TKIS TO DECREASE CELL VIABILITY

To interrogate the importance of EGFR and HER2 as lapatinib targets in Basal-like breast cancer cells, we first tested for synergy between JNK-IN-8 and other EGFR or EGFR/HER2 inhibitors. MDA-MB-231 cells were treated with three concentrations of the reversible EGFR inhibitors gefitinib and erlotinib based around published IC_{50} values for breast cancer cell lines [135-138] in combination with 5uM JNK-IN-8. The EGFR/HER2 covalent inhibitor neratinib at values near the IC_{50} for MDA-MB-231s [139] was also used. For almost all of the concentrations tested, JNK-IN-8 was synergistic with these drugs in decreasing cell viability by MTT assay (**Figure 3.1**)



Gef(uM)	JIN(uM)	Effect	CI
1.0	5.0	0.26155	0.38638
5.0	5.0	0.36957	0.61823
10.0	5.0	0.56386	0.40275
Ner(uM)	**JIN(uM)**	**Effect**	**CI**
0.1	5.0	0.17731	0.77213
0.5	5.0	0.38044	0.73586
1.0	5.0	0.41780	1.1822
Erl(uM)	**JIN(uM)**	**Effect**	**CI**
1.0	5.0	0.29348	0.31939
5.0	5.0	0.40285	0.56377
10.0	5.0	0.51223	0.6134

Figure 3.1: JNK-IN-8 is Synergistic with Other ERBB Inhibitors

MDA-MB-231 cells were treated with 5uM JNK-IN-8 (JIN) and either 1, 5, or 10µM gefitinib (Gef), 0.1, 0.5, or 1µM neratinib (Ner), and 1, 5, or 10µM erlotinib (Erl). After 72 hours, MTT was added to assess cell viability. MTT assay values were entered into CompuSyn to test for synergistic effect. The table shows concentrations tested, their effect as described by % decreased cell viability, and their combination index.

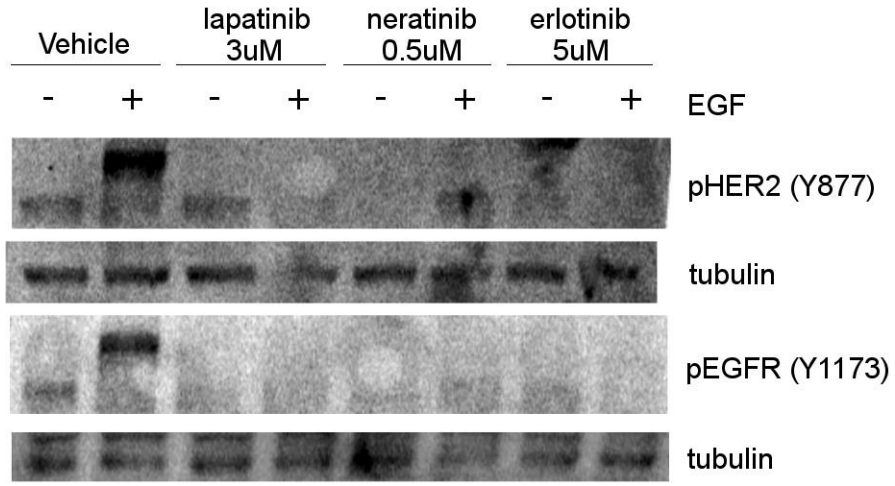


Figure 3.2: Other ERBB Inhibitors Efficiently Inhibit EGFR and HER2

MDA-MB-231 cells were serum starved overnight and treated with vehicle alone, 3µM lapatinib, 0.5µM neratinib or 5µM erlotinib for 6 hours. EGF ligand at a concentration of 100ng/mL was added to the cells five minutes before lysis. Western blot analysis of lysates shows inhibited phosphorylation of EGFR (Y1173) and HER2 (Y877) for all ERBB inhibitors.

At the intermediate concentrations tested for erlotinib and neratinib, these drugs showed similar potency as lapatinib in inhibiting EGFR and HER2 phosphorylation (**Figure 3.2**). Although erlotinib targets only EGFR, HER2 phosphorylation was still inhibited indicating that lack of EGFR phosphorylation inhibits transactivation of HER2 and that EGFR is the preferred binding partner for HER2 in these cells. These data support the requirement to inhibit EGFR/HER2 to synergistically inhibit cell viability in combination with JNK-IN-8.

3.1.3.2 KNOCKDOWN OF HER2 BUT NOT EGFR PARTIALLY RESCUES SYNERGY BETWEEN JNK-IN-8 AND LAPATINIB

Although EGFR is overexpressed in most Basal-like breast cancers and cell lines [6, 7, 11], one study showed that the effectiveness of lapatinib has no correlation with EGFR expression, but rather expression of HER2 was a better indicator of sensitivity to lapatinib [36]. Our lab (**Figure 3.3A**) and others show low level expression of HER2 in Basal-like breast cancer cell lines [11, 36, 140] despite the "triple-negative" moniker which refers to a lack of HER2 overexpression, not necessarily a complete lack of expression [141]. In order to determine whether EGFR or HER2 is more important for synergy between JNK-IN-8 and lapatinib, we used stably transfected shRNA to knockdown EGFR or HER2 expression in MDA-MB-231 cells. Almost 90% knockdown was achieved for EGFR, and similar knockdown was achieved for HER2 (**Figure 3.3A**). For both the shEGFR and shHER2 cells, knockdown caused a significant decrease in cell viability by MTT in full media compared to control cells after 72 hours (**Figure 3.3B)**. Visually, this effect appeared to be due to a slower growth rate rather than decreased viability. These cells were treated with 4μM lapatinib and/or 6μM JNK-IN-8 for 72 hours in full media. MTT was used to assay viability. Neither shEGFR nor shHER2 cells

showed increased sensitivity to JNK-IN-8 treatment after 72 hours but both cell lines did show significantly increased cell viability in the presence of lapatinib. ShHER2 cells were also somewhat resistant to treatment with JNK-IN-8 and lapatinib, significantly rescuing decreased cell viability. Cells with knocked down EGFR showed no resistance to the combination treatment (**Figure 3.3C**).

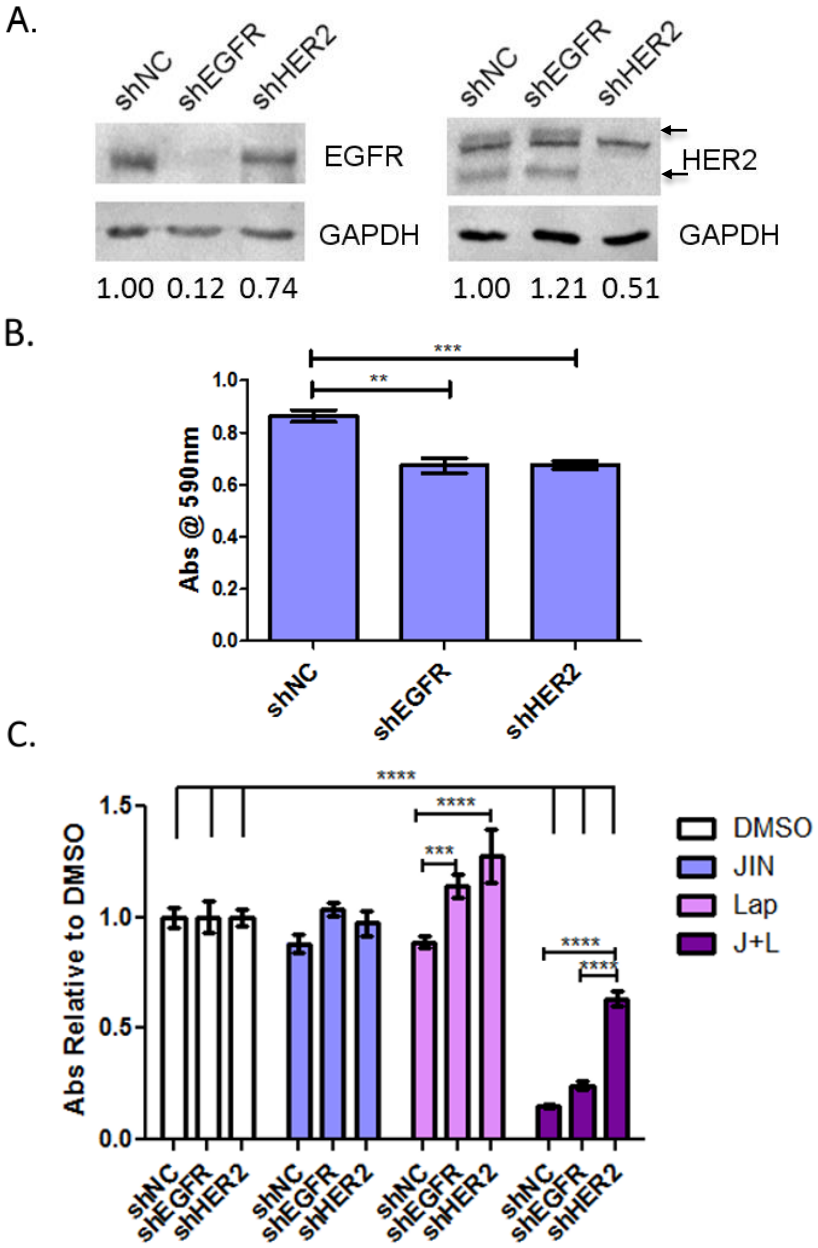


Figure 3.3: HER2 is Required for Maximum Synergy between JNK-IN-8 and Lapatinib

(A) MDA-MD-231 cells stably expressing either shNC (non-silencing control), shEGFR, or shHER2 and their relative densitometries to shNC for EGFR and HER2 expression by western blot. The non-specific band in the HER2 blot was included in densitometry calculation due to its close proximity to HER2. (B) Cell lines with indicated shRNA plasmids were untreated or (C) treated with vehicle (DMSO), 4μM lapatinib (Lap) and/or 6μM JNK-IN-8 (JIN) for 72 hours. Cell viability was assayed using MTT and bars represent (B) absorbances at 590nm or (C) normalized to the corresponding vehicle control ± SEM (*≤.05, **≤.01, ***≤.001, ****≤.0001).

3.1.4 DISCUSSION

Treatment of MDA-MB-231 cells with JNK-IN-8 and either erlotinib, gefitinib, or neratinib revealed synergy between these drugs in their ability to decrease cell viability, similar to the ability of JNK-IN-8 to synergistically act with lapatinib in these cells. Although erlotinib and gefitinib are selective for EGFR only, treatment with erlotinib showed equivalent inhibition of HER2 phosphorylation to lapatinib and neratinib which are selective for both EGFR and HER2. This is likely due to a lack of transactivation of HER2 by EGFR when EGFR is inhibited by these drugs. The seemingly total inhibition of HER2 transactivation indicates that very little dimerization is occurring with HER3 or HER4.

Because erlotinib, gefitinib, and neratinib target EGFR and/or HER2 at different residues and have affinities for different off-target kinases, it is likely that synergy between JNK-IN-8 and these drugs is due to the inhibition of their specific targets, EGFR and HER2, and not through off-target effects.

Knockdown of either EGFR or HER2 did not, however, increase these cells' sensitivity to JNK-IN-8. This could be caused either by a small amount of residual receptor or compensatory action of the other receptor, highlighting the importance of inhibiting both EGFR and HER2 to create synergy with JNK-IN-8. Interestingly, knockdown of either EGFR or HER2 caused an increase in growth in the presence of lapatinib. This is reminiscent of the fact that parental MDA-MB-231 cells have greater cell viability in the presence of lapatinib compared to vehicle treated cells. These data could indicate that both loss of signaling through EGFR and/or HER2 by RNA interference (RNAi)-mediated knockdown or pharmacologic inhibition may activate a stress-related cell survival pathway causing increased survival.

In the presence of both JNK-IN-8 and lapatinib, knockdown of HER2 but not EGFR partially rescued the synergistic decrease in cell viability, designating HER2 as the more important target of lapatinib for synergy with JNK-IN-8 (**Figure 3.3**). The likely reason for this is that when EGFR levels in the system are decreased long-term, as with stable shRNA transfection, signaling through HER2 remains high because of its dimerization with HER3 and HER4, making cells sensitive to lapatinib plus JNK-IN-8 combination. However, when HER2 levels are decreased in the system, dependence on EGFR is also decreased, and either HER3 and HER4 or other compensatory pathways become responsible for the bulk of growth and survival signaling, rendering lapatinib ineffective.

3.2 JNK-IN-8 AND LAPATINIB SYNERGY RELIES MORE HEAVILY ON JNK-IN-8'S SPECIFIC INHIBITION OF JNK1 RATHER THAN JNK2

3.2.1 INTRODUCTION

Several studies have shown disparate actions of JNK1 and JNK2. Within different contexts these gene products can be compensatory or even antagonistic [61, 66, 70, 100]. Using mouse models of breast cancer, our lab has shown independent functions of JNK2 that promote tumor cell growth, de-differentiation, and metastasis [64, 68, 93, 94].

Like lapatinib and other kinase inhibitors, JNK-IN-8 may have off target effects that could cause synergy with lapatinib. We set out to determine whether JNK-IN-8's synergy with lapatinib was due to specific inhibition of JNK1 and/or JNK2 using shRNA directed gene knockdown and introduction of mutant isoforms that cannot covalently bind JNK-IN-8.

3.2.2 METHODS

3.2.2.1 CELL LINES AND REAGENTS

HEK-293-T cells were obtained from ATCC (Manassas, VA) and were maintained in DMEM with high glucose (#SH30243.01 HyClone, Logan, UT) supplemented with 10% FBS at 37°C in 5% CO_2. WT JNK1 and JNK2 and Cys116Ser MUT JNK1 and JNK2 plasmids were gifts from Dr. Kevin Dalby (The University of Texas at Austin, Austin, TX).

3.2.2.2 CELLULAR METABOLISM ASSAY USING MTT

MTT assays were carried out as in **2.2.3.** Statistics were calculated using the same methods as in **2.2.6**.

3.2.2.3 TARGET GENE KNOCKDOWN WITH LENTIVIRAL SHRNA

Production of lentivirus containing shRNA plasmids was similar to **3.1.2.3** except for plasmids containing lentiviral *gag, pol*, and *env* genes (Open Biosystems Lafayette, CO) were added at 1µg each instead of 3µg of pCL-Eco. Transduction of MDA-MB-231 cells with lentivirus was also similar except for puromycin (#BP2956-100 Fisher Scientific Waltham, MA) at 2.5µg/mL was used to select for cells stably expressing shRNA constructs. shRNA lentiviral plasmids: shJNK1 (NM_002750), shJNK2 (NM_139068) , and GipZ (control) (# RHS4346) sequences in the pGIPZ.puro backbone were obtained from Open Biosystems (Lafayette, CO).

3.2.2.4 WESTERN BLOT

Western blot methods follow **2.2.2** except for the following changes: 100µg of protein was loaded for each gel. JNK1 (#3708) and total JNK (#9258) antibodies were obtained from Cell Signaling (Danvers, MA) and used at a dilution of 1:1000. JNK2 (#7345) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX) and used at a dilution of 1:750. FLAG (#F1804) antibody was purchased from Sigma-Aldrich (St. Louis, MO) and used at a dilution of 1:1000. GAPDH (#6C5) antibody was purchased from Advanced Immunochemical (Long Beach, CA) and used at a dilution of 1:2500 for 20 minutes at room temperature. JNK2, FLAG, and GAPDH blots were incubated in HRP-conjugated anti-mouse IgG secondary antibody (#2005 Santa Cruz Biotechnology Dallas, TX) at a 1:1000 dilution. JNK1 and total JNK blots were incubated in anti-rabbit IgG secondary antibody (#7074 Cell Signaling Danvers, MA) at a 1:1000 dilution.

3.2.2.5 TRANSIENT PLASMID EXPRESSION

MDA-MB-231 cells were seeded to a density of 400,000 cells per 60mm dish and 4,000 cells per well in a 96-well plate. In serum-free media, 8µg or 0.08µg of WT JNK1 and JNK2 or Cys116Ser MUT JNK1 and JNK2 sequences in pcDNA.3.FLAG plasmids were mixed with 8µl or 0.08µl of Enhancer™ Reagent and then complexed with 8µl or 0.08µl of Lipofectamine 3000® for 60mm dishes or 96-well plates, respectively. Because cell numbers were scaled linearly between 60mm dishes and 96-well plates, DNA and transfection reagent mixes were made as a master mix. 250µl or 2.5µl of the DNA/Lipofectamine complexes were added to 60mm dishes and 96-well plates respectively. Cells were treated 24 hours after transfection. WT and MUT JNK1 and JNK2 plasmids were gifts from Dr. Kevin Dalby (The University of Texas, Austin).

3.2.3 Results

3.2.3.1 The Presence of JNK1 is Necessary for Maximum Synergy Between JNK-IN-8 and Lapatinib

Using shRNA by lentiviral transduction to stably knockdown JNK1 and/or JNK2 we were able to obtain 95% and 90% gene expression inhibition of JNK1 and JNK2, respectively. Simultaneous knockdown of both JNKs was difficult to achieve. Most clones or populations tested had very good knockdown in one isoform or the other, but no cells had strong knockdown in both. The highest achievable double knockdown was about 60% knockdown of JNK1 with about 75% knockdown of JNK2 (**Figure 3.4A**). Neither knockdown of JNK1, JNK2, or both led to any significant changes in cell growth after 72 hours in full media by MTT assay (**Figure 3.4B**). Further, neither single nor double knockdown cells showed increased sensitivity to lapatinib alone. However, JNK1 knockdown, and to a lesser extent combined JNK1 and JNK2 knockdown, significantly rescued cell viability when JNK-IN-8 and lapatinib are used in combination. Knockdown of JNK2 alone had no effect on synergy between JNK-IN-8 and lapatinib (**Figure 3.4C).**

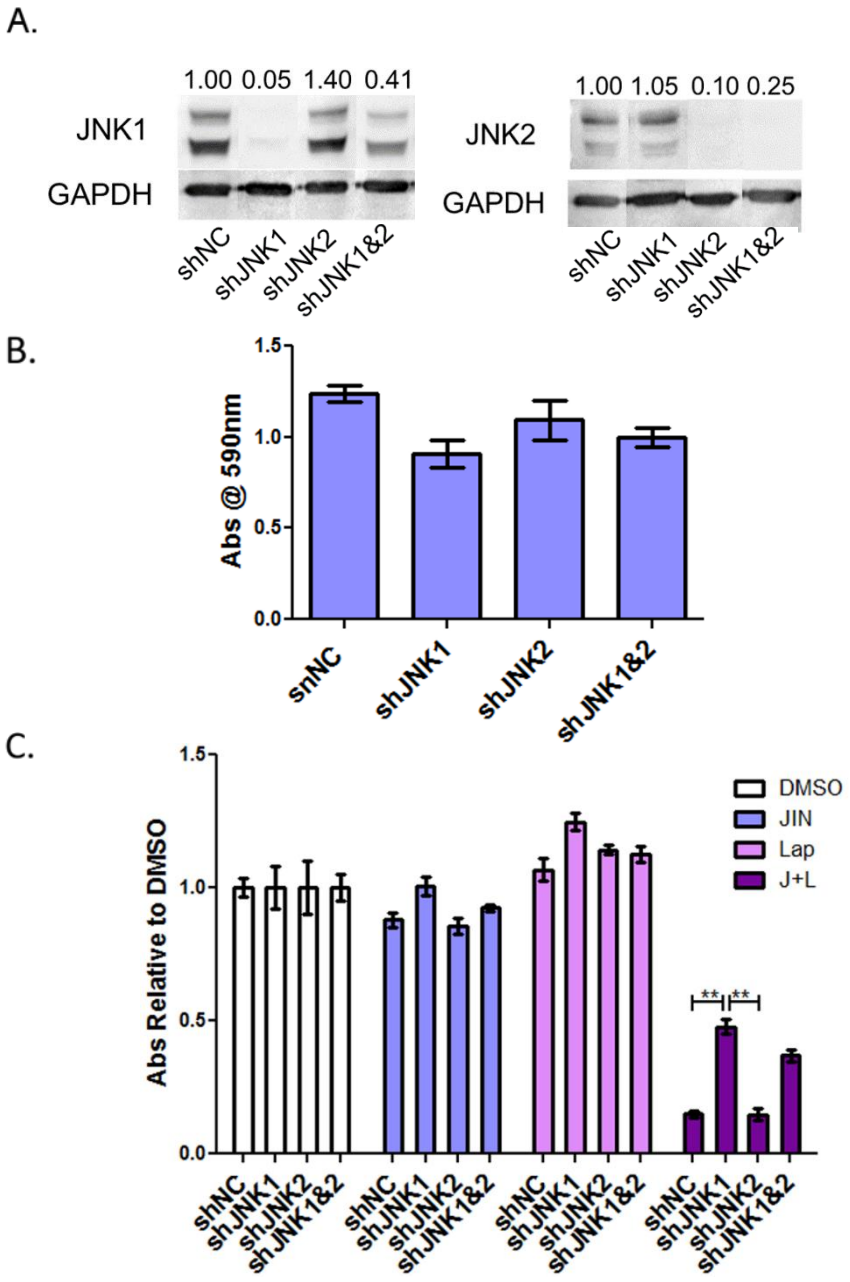


Figure 3.4: JNK1 is Required for Maximum Synergy between JNK-IN-8 and Lapatinib

(A) MDA-MD-231 cells stably expressing either shNC (non-silencing control), shJNK1, or shJNK2 and their relative densitometries to shNC for JNK1 and JNK2 expression by western blot. (B) Cell lines with indicated shRNA plasmids were untreated or (C) treated with vehicle (DMSO), 4μM lapatinib (Lap) and/or 6μM JNK-IN-8 (JIN) for 72 hours. Cell viability was assayed using MTT and bars represent (B) absorbances at 590nm or (C) normalized to the corresponding vehicle control ± SEM (*≤.05, **≤.01, ***≤.001, ****≤.0001).

3.2.3.2 Overexpression of Mutant JNK Isoforms Diminishes the Effectiveness of JNK-IN-8 and Rescues Synergy Between JNK-IN-8 and Lapatinib

JNK-IN-8 exerts its inhibitory action on JNK1 and JNK2 isoforms by bonding covalently to Cysteine 116 in the ATP-binding pocket. As shown in the Zhang 2012 publication, this mutation results in 100-fold increase in IC_{50} for JNK2 inhibition without interfering with substrate binding affinity [127]. Using the HEK-293-T cells, we overexpressed wildtype or mutant JNK1 and JNK2 to determine whether the presence of the mutant forms would rescue cell death with JNK-IN-8 and lapatinib treatment. First, we evaluated the sensitivity of the HEK-293-T cell line to JNK-IN-8, lapatinib, and the combination. The HEK-293-T cell line was used for its high transfection efficiency. Although they are somewhat more sensitive to lapatinib alone compared to the Basal-like breast cancer cell lines, they show an increased effect from JNK-IN-8 and lapatinib together (**Figure 3.5**).

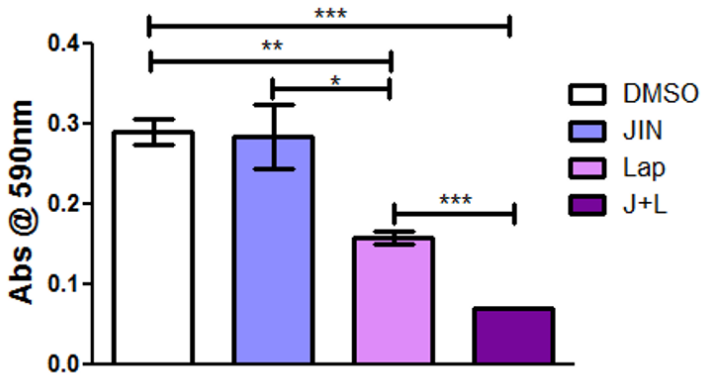


Figure 3.5: HEK-293-T Cells Respond to JNK-IN-8 and Lapatinib Combination

HEK-293-T cells were treated with vehicle (DMSO), 5μM lapatinib (Lap) and/or 5μM JNK-IN-8 (JIN) for 72 hours. Cell viability was assayed using MTT and bars represent mean absorbances ± SEM at 590nm (*≤.05, **≤.01, ***≤.001, ****≤.0001).

Transfection with wildtype (WT) and Cys116Ser mutant (MUT) JNK1 and JNK2 plasmids resulted in an increase in resistance to lapatinib compared to the parental cell lines, which partially diminished the additive effect of JNK-IN-8 and lapatinib in this cell line. However, expression of the MUT JNK plasmids significantly rescues the combination effect of JNK-IN-8 and lapatinib compared to cells expressing the WT plasmids, and abolishes significant differences between lapatinib alone and JNK-IN-8 plus lapatinib (**Figure 3.6A**). Both WT and MUT JNK isoforms were tagged with the FLAG epitope, and could be detected separately from endogenous JNK using a FLAG directed antibody. Western blots show that in cells where WT plasmids were overexpressed, presence of JNK-IN-8 results in a mobility shift in both JNK isoforms due to covalent binding with the drug. This mobility shift is not seen in cells transfected with MUT JNK plasmids, showing that JNK-IN-8 cannot covalently bind with JNK1 or JNK2 harboring the Cys116Ser mutation. The "Parental" lane contains lysate from untransfected HEK-293-T cells and serves as a negative control for the FLAG antibody. However, western blot with a total JNK antibody shows that expression of WT and MUT JNK1 and JNK2 is extremely high compared to the amount of endogenous JNK shown in the "Parental" lane. This may be the cause of increased resistance to lapatinib in these cells compared to parental. Still, phosphorylation of c-Jun at Ser63 is inhibited by JNK-IN-8 in the WT transfected cells despite the high level of JNK. Phosphorylation of c-Jun is not inhibited in MUT transfected cells, consistent with JNK-IN-8's inability to form covalent bonds with Cys116Ser mutant JNK isoforms (**Figure 3.6B**).

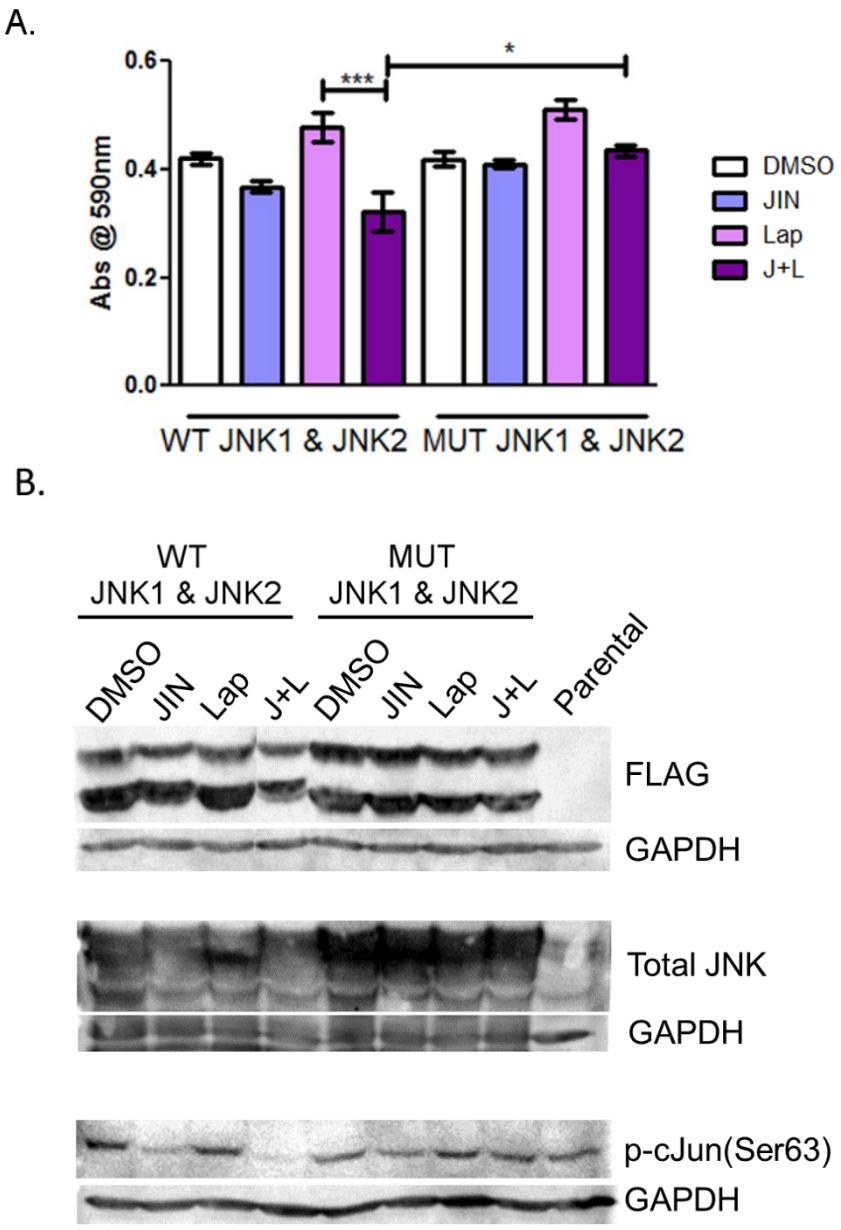


Figure 3.6: Cys116Ser Mutation in JNK1 and JNK2 Rescues the Effect of JNK-IN-8 and Lapatinib Combination

HEK-293-T cells were transiently co-transfected with plasmids containing wildtype (WT) JNK1 and JNK2 or plasmids containing JNK1 and JNK2 with a Cys116Ser mutation (MUT). Twenty four hours after transfection, cells were treated with vehicle (DMSO), 8μM JNK-IN-8 (JIN), 5μM lapatinib (Lap), or a combination of the two (J+L). Seventy two hours after treatment, (A) cell viability was assayed using MTT. Bars represent mean absorbances ± SEM at 590nm (*≤.05, **≤.01, ***≤.001, ****≤.0001). (B) A portion of transfected and treated cells was lysed for western blot and probed with FLAG, total JNK, phospho c-Jun(Ser63), and GAPDH (loading control).

3.2.4 DISCUSSION

Using large panels of kinases to assay selectivity of common kinase inhibitors, several authors have shown the immensity of off-target effects with these of drugs [124, 142]. In order to discover the mechanism of synergy between JNK-IN-8 and lapatinib, it was first important to determine whether inhibition of these drugs' intended targets was necessary, or whether the cell death phenotype was caused by off-target inhibition. Once the relevant targets of inhibition are known, then potential mechanisms can be pin-pointed. Because JNK-IN-8 targets multiple kinases of the same family, we also found it important to determine the relative necessity of the individual kinases, JNK1 or JNK2.

To this end, expression of JNK1 and JNK2 was knocked down individually and in combination using stably transfected shRNAs. This experiment revealed that only knockdown of JNK1 rescued synergy between JNK-IN-8 and lapatinib. This indicates the necessity for JNK1 presence in the system, and the inability of JNK2 to compensate. To further illustrate this point, a cell population displaying modest knockdown of both genes, 40% for JNK1 and 75% for JNK2, did not rescue cell death as well as JNK1 knockdown alone. JNK1 knockdown in this cell line is only 40%, much less than the 90% achieved in the JNK1 alone knockdown, and since JNK2 knockdown alone had no rescue effect, the decreased rescue in the double knockdown is probably due to a greater expression of JNK1, showing a dose-response of JNK1 presence.

In a separate experiment, mutagenesis was used to demonstrate the specificity of JNK-IN-8 and show the importance of JNK inhibition in synergy with lapatinib. Plasmids containing wildtype JNK1 and JNK2 sequences or mutant (Cys116Ser) JNK1 and JNK2 were co-transfected into HEK-293-T cells. Mutant JNK isoforms cannot form a covalent bond with JNK-IN-8 at Cys116, diminishing its capacity to inhibit JNK. Therefore, introduction of these proteins into cells should rescue the synergistic effects of JNK-IN-8

and lapatinib combination if JNK1 and JNK2 are the relevant targets. This experiment showed that expression of mutated JNK1 and JNK2 significantly rescued cell viability caused by JNK-IN-8 and lapatinib treatment. If the synergistic effect was due largely to off-target inhibition by JNK-IN-8, then no significant rescue would have been seen. Expression of mutant JNK isoforms also rescued c-Jun phosphorylation as expected.

Within the experimental model, another interesting facet was introduced. The HEK-293-T cell line displayed low levels of endogenous JNK and was more sensitive to lapatinib treatment than the breast cancer cell lines, although combination with JNK-IN-8 still increased lapatinib effectiveness. When the WT or MUT JNKs were introduced, however, resistance to lapatinib increased dramatically compared to the untransfected cell line. This hints at a mechanism whereby lapatinib-induced viability is mediated through JNK, and indicates the necessity for specific JNK inhibition in synergy between JNK-IN-8 and lapatinib (**Figure 3.7**).

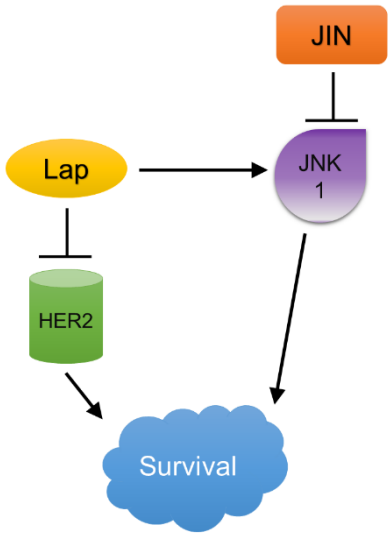


Figure 3.7: Specific Targets of Lapatinib and JNK-IN-8 Synergy
Lapatinib and JNK-IN-8 specifically target HER2 and JNK1, respectively, to cause synergistic cell death. Overexpressed JNK causes increased viability in the presence of lapatinib.

Chapter 4: Treatment with Lapatinib Affects JNK Activation and Signaling

4.1 Introduction

Typical mechanisms of acquired lapatinib resistance involve upregulation of tyrosine kinase receptors that compensate for lost signaling [53, 57, 58, 143]. In breast cancer, JNK signals downstream of various RTKs, such as EGFR and IGF-IR [93-95, 100, 144], as well as cytokine receptors [100, 145]. If Basal-like breast cancer cells are resistant to lapatinib because of residual ERBB or other compensatory receptor signaling, it stands to reason that JNK may be transducing these signals to AP-1 and other targets. It could also be the case that JNK-IN-8, through affecting transcription, might downregulate compensatory receptors or transducers of receptor signaling. After establishing the specific involvement of HER2 and JNK1 in the mechanism of synergy between JNK-IN-8 and lapatinib, we wanted to determine if JNK-IN-8 has distal effects on ERBB or other signaling pathways, or whether lapatinib affects JNK activation and downstream signaling.

4.2 Methods

4.2.1 Reagents

Epidermal Growth Factor (EGF) was purchased from PeproTech (#100-15 Rocky Hill, NJ) and used at a final concentration of 100ng/mL. Neuregulin1 (NRG1) was purchased from BioVision (#4711-50 Milpitas, California) and used at a final concentration of 30ng/mL.

4.2.2 CELLULAR VIABILITY ASSAY USING MTT

MTT assays were carried out as in **2.2.3.** Statistics were calculated using the same methods as in **2.2.6**.

4.2.3 IMMUNOFLUORESCENCE

Cells were plated at 25% confluency in 8-well chamber slides and treated for 48 and/or 72 hours. Media was removed, cells were washed twice with cold PBS and fixed in ice-cold methanol:acetone 1:1 for 10 minutes at -20°C. Fixative was removed and cells were washed twice more with cold PBS before incubation for 12 minutes on ice in permeabilization buffer (0.05% TritonX-100 in PBS). Cells were blocked in 5% normal goat serum in PBS for one hour at room temperature. Phospho-JNK(Thr183/Tyr185) (#9251) and c-Jun (#9165) primary antibodies were purchased from Cell Signaling (Danvers, MA) and used at dilutions of 1:300 and 1:500, respectively. After overnight incubation in primary antibody at 4°C, cells were washed and incubated with AlexaFluor568 (#A11011) at a dilution of 1:2000 for one hour at room temperature. Cells were washed three times in PBS and slides were mounted using VectaShield containing DAPI (#H1200 Vector Laboratories, Burlingame, CA). Fluorescent images were taken at 4000x or 6000x using a Leica DM4000B (Buffalo Grove, IL) upright fluorescent microscope. Leica Application Suite v3.7 (Leica, Buffalo Grove, IL) software was used to capture images. Quantification of c-Jun positive nuclei was performed by hand, counting five separate fields of total nuclei and c-Jun positive nuclei to obtain the percent positive nuclei.

4.2.4 WESTERN BLOT

Western blot methods follow **2.2.2** except for the following changes: 100µg of protein was loaded for gels probed with Phospho-c-Jun(Ser63) (#9261), total c-Jun (#9165), pHER2(Tyr877) (#2241), HER3(#4754), pMet(Tyr1234/Tyr1235) (#3129), Met(#3127), pAkt(Ser473) (#4060), pERK(Thr202/Tyr204) (#4377), PDK1(#5662), pSrc(Tyr416) (#6943), pSTAT3(Ser727) (#9136), and p-p38 kinase (Thr180/Tyr182) (#9211) which were obtained from Cell Signaling (Danvers, MA). Phospho antibodies were used at a dilution of 1:500 and total antibodies were used at a dilution of 1:1000. Forty micrograms of protein was loaded for EGFR (#4267) and pEGFR(Tyr1173) (#4407) blots, both from Cell Signaling. HER2 antibody (#284) (Santa Cruz Biotechnology, Dallas, TX) was used at a 1:600 dilution. GAPDH (#6C5) antibody was purchased from Advanced Immunochemical (Long Beach, CA) and used at a dilution of 1:2500 for 20 minutes at room temperature. Primary antibodies made in mouse were incubated with HRP-conjugated anti-mouse IgG secondary antibody (#2005 Santa Cruz Biotechnology, Dallas, TX) at a 1:1000 dilution. Primary antibodies made in rabbit were incubated in anti-rabbit IgG secondary antibody (#7074 Cell Signaling, Danvers, MA) at a 1:1000 dilution.

4.2.5 LUCIFERASE REPORTER ASSAY

4.2.5.1 TRANSIENT PLASMID EXPRESSION

MDA-MB-231 cells and MDA-MB-436 cells were seeded to a density of 300,000 cells per 60mm dish and treated with JNK-IN-8 and/or lapatinib. At 24 hours after treatment, cells were transfected. In serum-free media, 6µg of pGL3-basic.3xAP-1 (Addgene) and 3µg CMV-β-galactosidase were mixed with 9µl of Enhancer™ Reagent

and then complexed with 9µl of Lipofectamine 3000®. Lipofectamine/DNA complexes were added dropwise to 60mm dishes containing the target cells and swirled to mix.

4.2.5.2 LUCIFERASE ASSAY

Cells were washed twice with cold PBS and lysed using Reporter Lysis Buffer (#E4030 Promega Madison, WI). Lysate was transferred to black 96 well plates (#3915 Corning, Corning, NY) in triplicate. A BioTek (Winooski, VT) Synergy 2 plate reader with injectors was used to inject 100µL of luciferin in Luciferase Assay Buffer (#E4030 Promega, Madison, WI). Signal was incorporated for 10 seconds immediately after injection and reported as Relative Luciferase Units (RLU).

4.2.5.3 B-GALACTOSIDASE ASSAY

Lysates in Reporter Lysis Buffer were added to clear, round bottom 96 well plates in triplicate. ONPG (o-nitrophenyl-β-D-galactopyranoside) (#369-07-3 Research Products International Corp. Mt. Prospect, IL) substrate was dissolved at 4mg/mL in ONPG buffer (0.120M Na_2HPO_4, 0.08M NaH_2PO_4, 2mM $MgCl_2$, 100mM β-mercaptoethanol) and added to lysates at equal volume. The plate was incubated at 37°C until sufficient yellow color was developed (30 minutes to 1 hour). Absorbance at 420nm was read immediately after injection of 1M sodium carbonate at twice the volume of lysate+ONPG Buffer using the BioTek (Winooski, VT) Synergy 2 plate reader.

4.3 RESULTS

4.3.1 TREATMENT WITH LAPATINIB RESULTS IN INCREASED CELL VIABILITY AFTER ERBB-SPECIFIC ACTIVATION

MDA-MB-231 cells were treated with DMSO, JNK-IN-8, lapatinib, or both for 72 hours in serum-free media and EGF or Neuregulin 1 (NRG1). Both growth factors increased cell viability, as measured by MTT assay, compared to serum starved cells. Lapatinib further increased cell viability in the presence of both growth factors, while the JNK-IN-8 and lapatinib combination resulted in synergistically decreased viability as seen in full media (**Figure 4.1**).

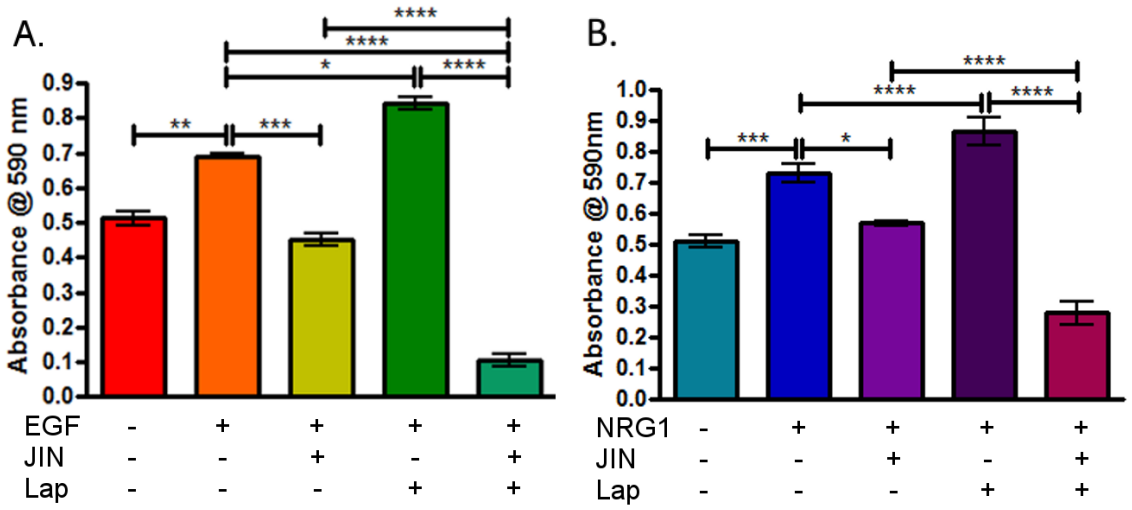


Figure 4.1: JNK-IN-8 and Lapatinib Synergize in the Presence of ERBB Ligands Alone

MDA-MB-231 cells were serum starved and then treated with either (A) EGF (100ng/mL) or (B) NRG1 (30ng/mL) in conjunction with 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap). Seventy two hours after treatment, cell viability was assayed using MTT. Bars represent mean absorbances ± SEM at 590nm (*≤.05, **≤.01, ***≤.001, ****≤.0001).

This further verifies the specificity of lapatinib in synergy with JNK-IN-8 since the phenotype is still present when other receptor pathways are stimulated only at a basal level. This also indicates that specific inhibition of signaling downstream of EGFR/HER2 or other ERBB heterodimers by lapatinib induces a growth, proliferation or survival signal that increases cellular metabolism. This effect could involve changes in other receptors or intracellular kinases including JNK.

4.3.2 JNK-IN-8 Plus Lapatinib Does not Downregulate Expression of Growth Factor Receptors or Intracellular Kinases

Common mechanisms of lapatinib, or other EGFR and/or HER2 inhibitor, resistance involves upregulation of compensatory signaling pathways, often at the receptor level (See **1.3.2.2.5**). Increased signaling through a compensatory pathway would explain increased viability after lapatinib treatment.

We looked at expression and phosphorylation levels of the ERBB receptors and receptors that are involved in ERBB crosstalk in order to determine whether JNK-IN-8 might downregulate expression of compensatory receptors responsible for lapatinib resistance. Phosphorylation and/or total levels of EGFR, HER2, HER3, and MET were relatively unchanged by any treatment alone or in combination (**Figure 4.2**).

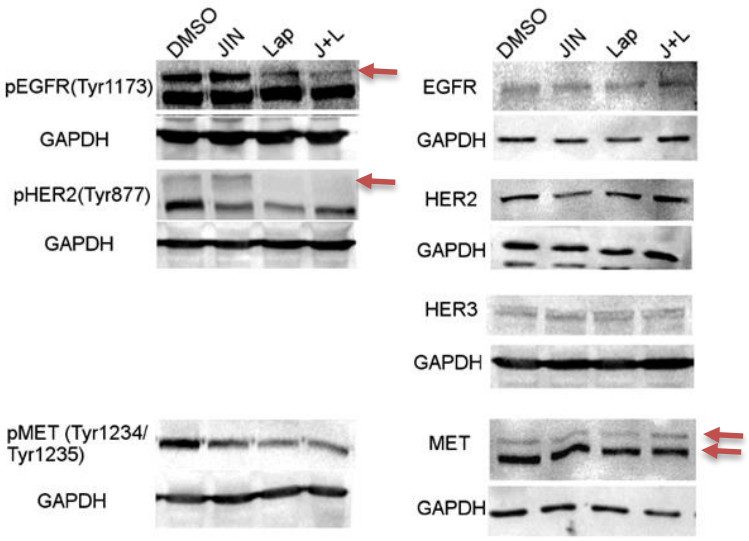


Figure 4.2: JNK-IN-8 and Lapatinib do not Change Phosphorylation or Expression Levels of Growth Factor Receptors

MDA-MB-231 cells were treated with either vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap). Forty-eight hours after treatment in full media, cells were lysed in TDL buffer and separated using SDS-PAGE. Antibodies used for western blot are labeled.

Acquired resistance to TKIs such as lapatinib can involve changes in intracellular kinase signaling by expression or phosphorylation. In addition, changes in receptors that were not assayed should be observable by their effect on downstream kinases. Downstream from growth factor RTK activation, major transducers of signal include Ras/MAPK(ERK), PI3K/Akt, Src, and JAK/STAT pathways [21, 146, 147]. Some affects from JNK-IN-8 and/or lapatinib treatment included downregulation of pAkt in the MDA-MB-231 and HCC1569 cell lines, and downregulation of the kinase upstream of Akt, PDK1, in the HCC1569 cells. However, these changes were not consistent amongst cell lines (**Figure 4.3**).

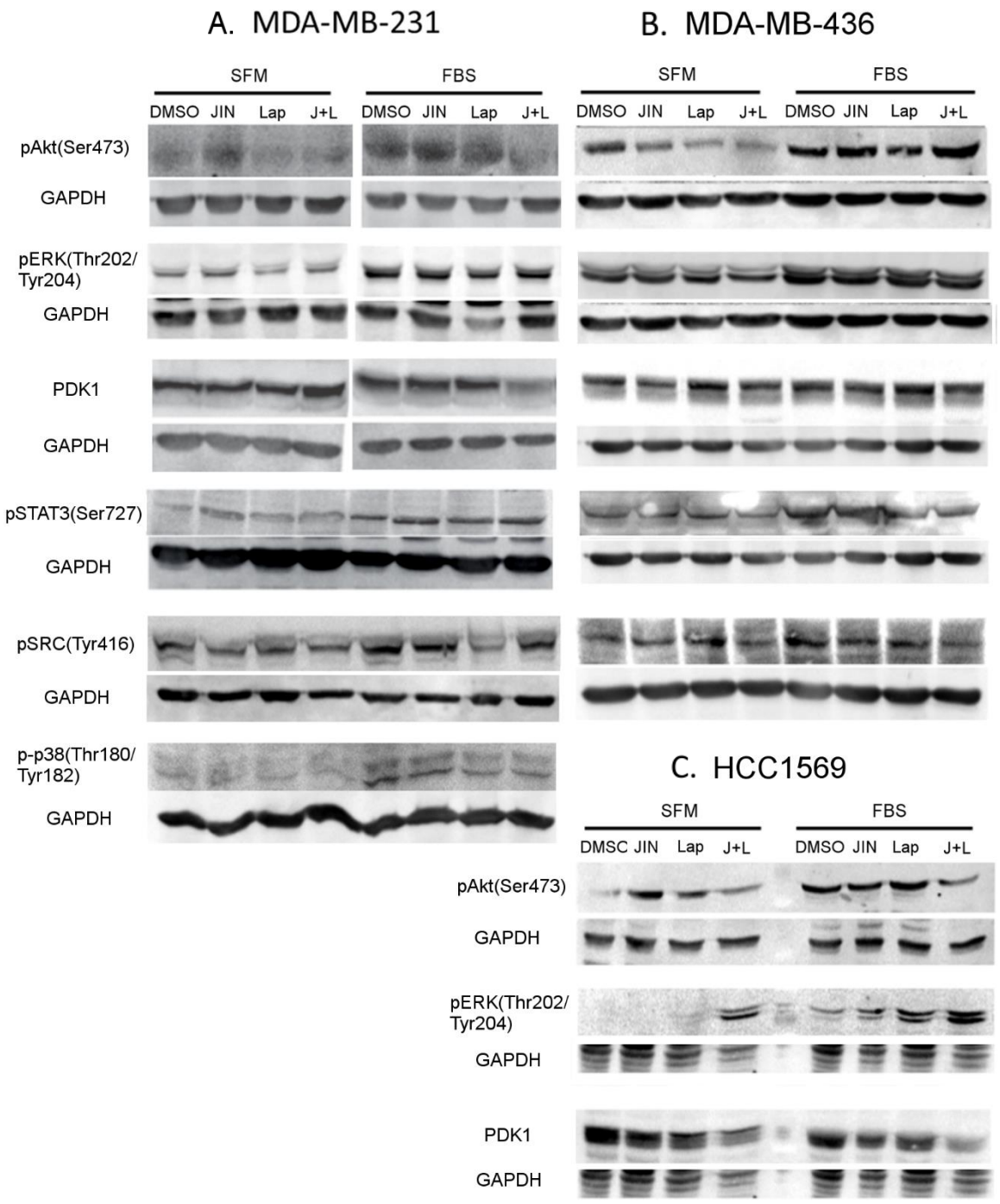


Figure 4.3: JNK-IN-8 and Lapatinib do not Consistently Change Phosphorylation or Expression Levels of Intracellular Signaling Kinases

(A) MDA-MB-231, (B) MDA-MB-436, or (C) HCC1569 cells were treated with (A) either vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap), (B) DMSO, 5μM JIN and/or 8μM Lap, or (C) DMSO, 1μM JIN and/or 1μM Lap. Forty-eight hours after treatment in full media, cells were lysed in RIPA buffer and separated using SDS-PAGE. Antibodies used for western blot are labeled.

4.3.3 Treatment with Lapatinib Increases Nuclear Accumulation of JNK and c-Jun

Despite the classical view that JNK is primarily required for apoptosis after growth factor, cytokine, or stress signaling [63, 96, 148-150], some studies have shown that JNK participates in survival signaling after these stimuli [94, 103, 151]. Identification of high JNK and c-JUN expression as indicators of bad prognosis in breast cancer [89, 92, 93] suggests the importance of JNK in a survival or proliferation role.

After treatment with lapatinib for 72 hours, phosphorylated JNK accumulates in the nucleus of MDA-MB-231 cells. Phosphorylated JNK remains high in the cells treated with JNK-IN-8 and lapatinib because the JNK inhibitor does not prevent JNK phosphorylation, it only prevents JNK from phosphorylating its downstream targets (**Figure 4.4A**). Consistent with this result, nuclear c-Jun levels increase in the lapatinib treated cells, and are significantly lower in cells treated with JNK-IN-8 (**Figure 4.4B**). While JNK-IN-8 only prevents phosphorylation of c-Jun by JNK, the c-Jun immunofluorescence appears to show an overall decrease in c-Jun abundance. Western blot confirmed decreased levels of phosphorylated and total c-Jun in JNK-IN-8 treated cells, while lapatinib increased total and phospho-c-Jun in the MDA-MB-231 cells, confirming the result seen in **Figure 4.4**. However, the effect of lapatinib on c-Jun was not consistent between all cell lines (**Figure 4.5**).

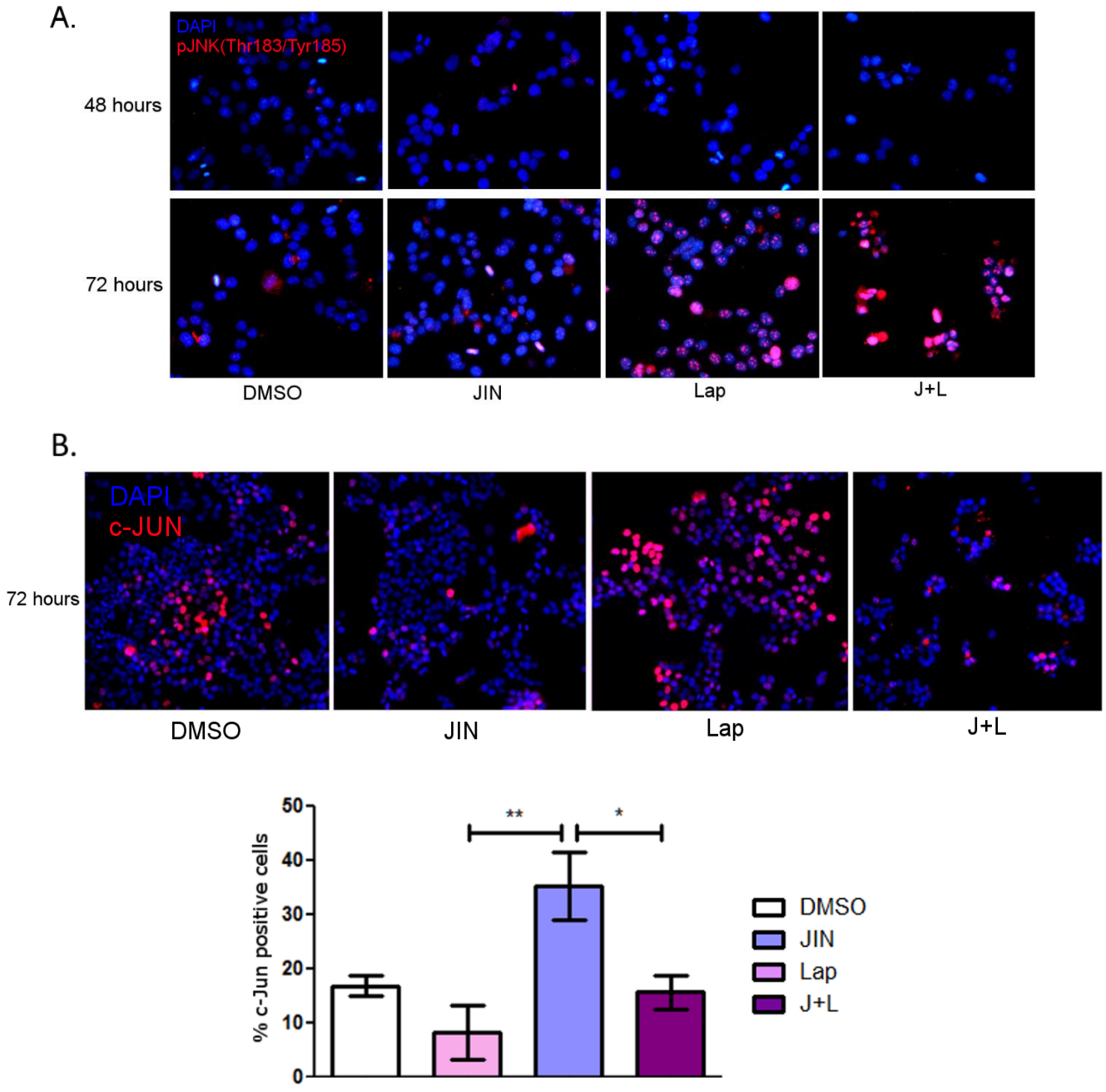


Figure 4.4: Lapatinib Increases Nuclear Accumulation pJNK and c-Jun

MDA-MB-231 cells were treated with vehicle (DMSO), 6μM JNK-IN-8 (JIN), and/or 4μM lapatinib (Lap) for (A) 48 and 72 hours or (B) 72 hours. Cells were then fixed and probed with (A) phospho-JNK(Thr183/Tyr185) or (B) c-Jun for immunofluorescence. Nuclei were counterstained with DAPI. The bar graph in (B) represents the quantification of percent c-Jun positive nuclei. (*≤.05, **≤.01, ***≤.001, ****≤.0001).

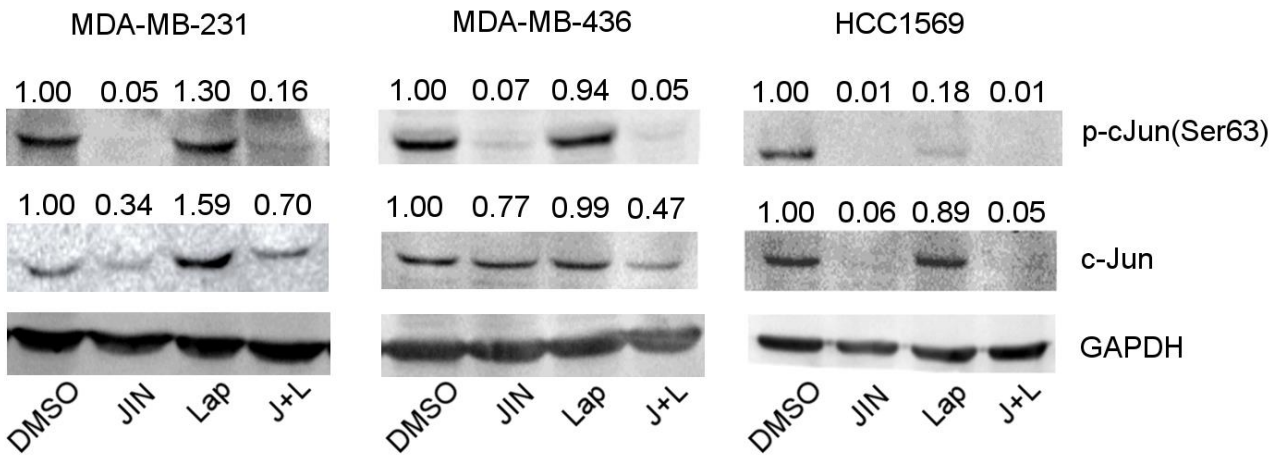


Figure 4.5: JNK-IN-8 Decreases Phospho- and Total c-Jun Levels

(A) MDA-MB-231, (B) MDA-MB-436, or (C) HCC1569 cells were treated with (A) either vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap), (B) DMSO, 5μM JIN and/or 8μM Lap, or (C) DMSO, 1μM JIN and/or 1μM Lap for 72 hours in full media and then lysed. Western blots were performed using antibodies against phospho-c-Jun (Ser63) and total c-Jun. Band densitometries are shown relative to DMSO for each blot.

4.3.4 AP-1 ACTIVITY DOES NOT FOLLOW C-JUN EXPRESSION PATTERNS

The JNK target, c-Jun, binds with members of the Fos and ATF family of proteins to form the AP-1 transcription factor (reviewed in **1.4.3.1).** Considering effects on phosphorylation and expression levels of c-Jun in the presence of JNK-IN-8 and lapatinib, we hypothesized that transcriptional activity of AP-1 would be diminished as well. MDA-MB-231 cells were transfected with a luciferase reporter gene under the control of a minimal promoter containing synthetic, canonical AP-1 sites. They were also transfected with a constitutively expressed β-galactosidase gene as a transfection control. Transfected cells were then treated in full media with JNK-IN-8 and/or lapatinib and then assayed for luciferase activity. Unexpectedly, AP-1 driven luciferase activity did not follow c-Jun expression patterns, and also differed between cell lines. In the MDA-MB-231 cells, AP-1 activity increased significantly after addition of JNK-IN-8, and decreased significantly after treatment with lapatinib. In contrast, both lapatinib and JNK-IN-8

decreased AP-1 activity in the MDA-MB-436 cells. However, both cell lines showed a decrease in AP-1 activity in the JNK-IN-8 plus lapatinib treatment that was significantly more than in vehicle or either drug alone (**Figure 4.4**).

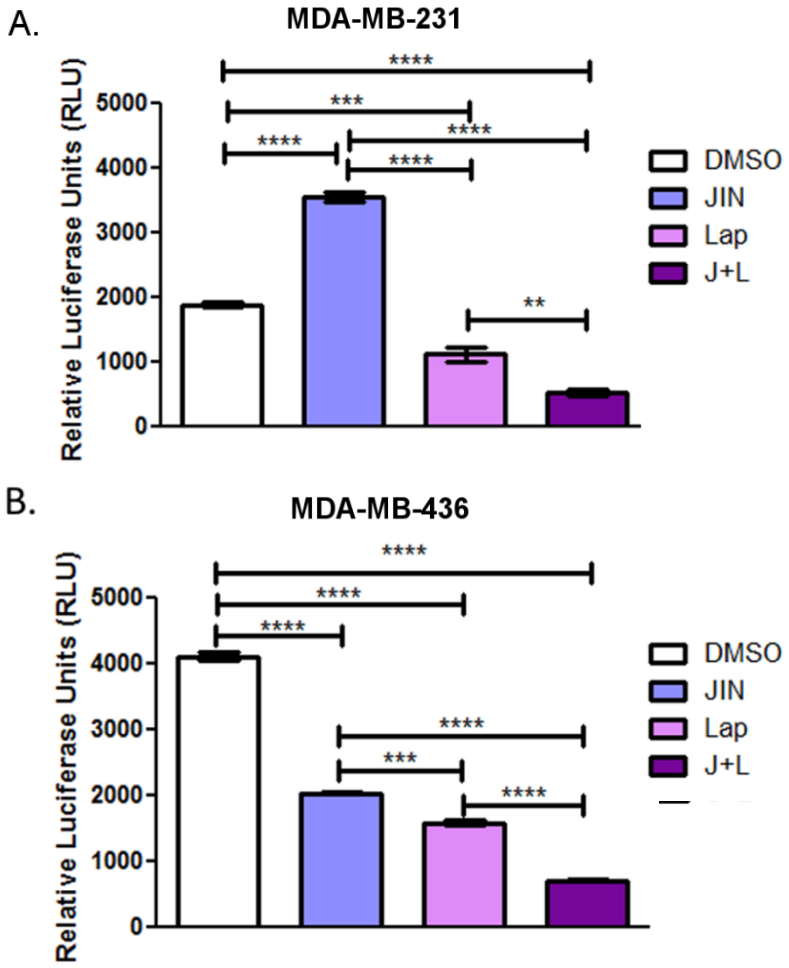


Figure 4.6: AP-1 Luciferase Reporter Activity After JNK-IN-8 and Lapatinib Treatment

(A) MDA-MB-231 and (B) MDA-MB-436 cells were treated with (A) either vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap), or (B) DMSO, 5μM JIN and/or 8μM Lap for 72 hours in full media. After 24 hours of treatment, they were also transfected with a luciferase reporter plasmid under the control of three tandem, canonical AP-1 sites, and a constitutively expressed β-galactosidase plasmid. Cells were lysed at 72 hours after treatment and assayed for luciferase and β-galactosidase activity. Bar graphs show luciferase values normalized to β-galactosidase activity (absorbance at 420nm). (*≤.05, **≤.01, ***≤.001, ****≤.0001).

4.4 DISCUSSION

In Basal-like breast cancer cell lines, treatment with lapatinib causes an increase in cell viability by MTT despite being synergistic in causing apoptosis with JNK-IN-8. This could indicate that inhibition of signaling through EGFR/HER2 may cause upregulation of other ERBB dimers or compensatory pathways that stimulate survival. However, **Figure 4.2** demonstrated that no consistent changes in ERBB or Met receptor expression or phosphorylation were seen after treatment. In addition, **Figure 4.3** explored phosphorylation and expression levels of intracellular kinases involved in major signaling pathways. No consistent changes between cell lines were demonstrated for any treatment.

Figure 4.4 points to JNK as a likely transducer of the survival signal because JNK was highly phosphorylated and localized in the nucleus after lapatinib treatment. This led to an increase in nuclear c-Jun. As a member prominent member of the AP-1 transcription factor, c-Jun has been implicated in survival signaling and proliferation [144, 152]. This data also harkens back to **Figure 3.2.3** where expression of JNK1 and JNK2 increased viability in the presence of lapatinib in the HEK-293-T cell line.

These data explain why the use of JNK-IN-8 increases sensitivity to lapatinib. **Figure 4.1** revealed that lapatinib increases cell viability after exposure to single growth factors, EGF and NRG1, so the stimulating effect of lapatinib is present when EGFR and the other ERBB receptors are stimulated at high levels. In addition, JNK-IN-8 still synergizes with lapatinib in the presence of single growth factors, indicating that non-ERBB receptor stimulation is unnecessary for this effect. Taken together, these data indicate that inhibition of EGFR and HER2 lead to survival signaling through activation of JNK signaling.

A readout of AP-1 transcriptional activity showed inconsistencies between cell lines in relation to effects on c-Jun. Luciferase assay of AP-1 transcriptional activity

showed increased activity in cells treated with JNK-IN-8, despite the fact that total and phosphorylated c-Jun was decreased. Lapatinib treatment, which caused c-Jun increase in the MDA-MB-231 cells, caused decreased AP-1 activity. In the MDA-MB-436 cells, phosphorylation of c-Jun was almost completely abolished by treatment with JNK-IN-8, while treatment with lapatinib had no effect on c-Jun, however, they showed similar effects on AP-1 (**Figure 4.6**). The only consistent effect comes from combination treatment with JNK-IN-8 and lapatinib, where levels of total and phosphorylated c-Jun are highly decreased in all cells lines as well as AP-1 activity

Figure 4.7 is a diagram of possible connections involved in synergy between JNK-IN-8 and lapatinib. JNK activation after lapatinib treatment is supported by **Figure 3.2.3** and **Figure 4.4** where JNK overexpression increases lapatinib-induced viability, and phospho-JNK accumulates in the nucleus in lapatinib treated cells. Lapatinib activates AP-1 through a non-JNK target, and JNK activates AP-1, probably through c-Jun. However, lapatinib alone decreases AP-1 despite activating JNK, indicating AP-1 activation by HER2 is more potent. However, AP-1 contribution to survival is unknown. Regardless, a definite link between lapatinib resistance and JNK activity/expression has been established, validating the use of JNK-IN-8 to increase the effectiveness of lapatinib.

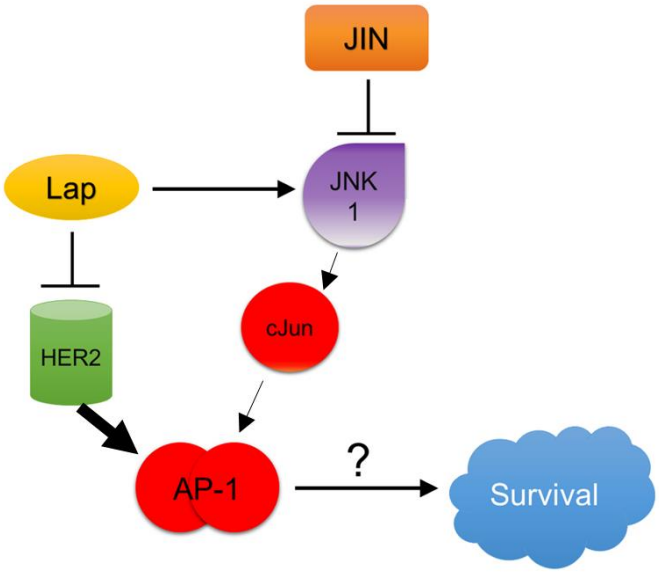


Figure 4.7: JNK-IN-8 and Lapatinib Synergy May Involve AP-1 Inhibition

A possible mechanism for JNK-IN-8 and lapatinib synergy where inhibition of separate AP-1-activating mechanisms downstream of HER2 and JNK1 may lead to decreased survival.

Chapter 5: Treatment with JNK-IN-8 and Lapatinib Results in Cytotoxic Levels of Oxidative Stress that Causes Cell Death

5.1 INTRODUCTION

As a stress induced kinase, JNK can activate apoptosis after UV-irradiation [148, 151] and exposure to pro-inflammatory cytokines such as TNF-α [150]. Mechanisms of the pro-apoptotic functions of JNK include translocation to the mitochondria to induce cytochrome c release[148] such as after UV irradiation. After TNF-α exposure, JNK activation leads to degradation of the caspase inhibitor c-FLIP [120].

Like JNK, NFκB is activated after cellular stress and cooperates with JNK to mediate survival or apoptosis after TNF-α binding [150, 153-155]. Generally, NFκB activation is associated with survival following cellular stress due to its activation of transcription of multiple anti-apoptotic proteins, such as Bcl-2 family members, XIAP, and c-FLIP, and genes responsible for redox regulation after stimulation by oxidative stress [156, 157]. NFκB and JNK are tightly linked particularly during exposure to TNF-α and oxidative stress which occur concomitantly. After TNF-α binding, TNF-associated Factor (TRAF) 2 and TRAF5 are recruited to TNF receptors resulting in rapid phosphorylation and nuclear translocation of NFκB [158, 159]. TNF-α induces pro-apoptotic JNK through accumulation of ROS that inhibit JNK's phosphatases and activate Apoptosis Signal-regulating Kinase 1 (ASK1), an upstream activator of JNK [155, 160-162]. Low levels of ROS act as signal transducing molecules while high levels are cytotoxic, leading to apoptosis and necrosis [163]. NFκB exerts survival action both by activating transcription of anti-apoptotic Bcl-2 family member proteins and by downregulating pro-apoptotic JNK activation and ROS accumulation. NFκB

downregulates JNK through its transcriptional targets. Expression of Superoxide Dismutase (SOD) and others scavenge potentially cytotoxic ROS and inhibit ROS-induced JNK activation after TNF-α [153, 155]. Expression of GADD45β directly binds MKK7 which decreases its ability to phosphorylate JNK [164]. ROS accumulation after TNF-α may also activate NFκB as a part of a negative feedback loop, but the mechanisms are complex due to initiatory and inhibitory actions of ROS on upstream NFκB pathway members [165].

Interestingly, the status of NFκB can modulate whether JNK activation is pro- or anti-apoptotic. By knocking out p65 or overexpressing an Inhibitor of kappa B (IκB) super repressor, some cells were resistant to apoptosis after TNF-α exposure. These cells exhibited a prolonged activation of JNK (due to loss of inhibition by NFκB) that was anti-apoptotic. The use of SP600125 rescued cell death after TNF-α exposure by suppressing sustained JNK activation [166]. A study comparing Leukemic Stem Cells (LSC) to normal Hematopoietic Stem and Progenitor Cells (HSPC) revealed a context-dependent function of this pathway, where inhibition of both JNK and NFκB was synergistic in killing LSCs, but inhibiting JNK made HSPCs resistant to NFκB inhibitors [167]. This discovery indicates that co-inhibition of JNK and NFκB may present a therapeutic advantage over other treatments due to its selective killing of cancer cells in this model.

In breast cancer cells, links exist between EGFR/HER2 signaling and NFκB activation. High levels of NFκB activation are found in ER-negative breast tumors expressing HER2 [168], and lapatinib treatment of HER2-overexpressing cells decreased NFκB consensus sequence binding [169]. Treatment of HER2-overexpressing cells with heregulin increased NFκB consensus sequence binding, and HER2-targeted therapy resistance was NFκB mediated. Combined treatment with lapatinib and an NFκB

inhibitory peptide caused synergistic cell death in lapatinib resistant clones [170]. Although not as upregulated as in HER2-overexpressing breast cancer cells, Basal-like breast cancers and cell lines also show greatly enhanced NFκB activity compared to ER-positive tumors [168, 170, 171].

In Basal-like breast cancer cell lines, survival after lapatinib treatment may depend on NFκB and/or JNK signaling. We sought to determine whether JNK-IN-8 synergizes with lapatinib through modulation of NFκB or its survival mechanisms including prevention of oxidative stress.

5.2 METHODS

5.2.1 REAGENTS

NFκB inhibitor BAY11-7082 (#B5556, Sigma, St. Louis, MO) was reconstituted in DMSO to a concentration of 10mM. ROS detection reagent DCDFA (2',7' – dichlorofluorescin diacetate) was purchased from Abcam (#ab113851, Cambridge, MA). N-acetyl cysteine (NAC)(#A9165), Ascorbic Acid(#A5960), and Catalase(#C-40) were purchased from Sigma-Aldrich (St. Louis, MO). The p5xNFκB plasmid was a gift from Dr. Linda DeGraffenried (The University of Texas at Austin).

5.2.2 CELLULAR VIABILITY ASSAY USING MTT

MTT assays were carried out as in **2.2.3.** Statistics were calculated using the same methods as in **2.2.6**.

5.2.3 WESTERN BLOT

Western blot methods follow **2.2.2** except for the following changes: 100µg of RIPA-lysed protein was loaded for gels probed with IKKα(#7606 Santa Cruz Biotechnology, Dallas, TX), IKKβ(#2684 Cell Signaling, Danvers, MA) and p65(Ser536)(#3033, Cell Signaling, Danvers, MA). Phospho- antibodies were used at a dilution of 1:500 and total antibodies were used at a dilution of 1:250. GAPDH (#6C5) antibody was purchased from Advanced Immunochemical (Long Beach, CA) and used at a dilution of 1:2500 for 20 minutes at room temperature. Primary antibodies made in mouse were incubated with HRP-conjugated anti-mouse IgG secondary antibody (#2005 Santa Cruz Biotechnology, Dallas, TX) at a 1:1000 dilution. Primary antibodies made in rabbit were incubated in anti-rabbit IgG secondary antibody (#7074 Cell Signaling, Danvers, MA) at a 1:1000 dilution.

5.2.4 LUCIFERASE REPORTER ASSAY

5.2.4.1 TRANSIENT PLASMID EXPRESSION

MDA-MB-231 cells and MDA-MB-436 cells were seeded at 100,000 cells/well in 12-well plates and treated with JNK-IN-8 and/or lapatinib. Cells were transfected in serum-free media with 1.25µg of p5xNFκB-Luc and 0.5µg CMV-β-galactosidase. Using a 1:1:1 ratio of DNA : Enhancer™ : Lipofectamine 3000®. Lipofectamine/DNA complexes were added dropwise to wells containing the target cells and swirled to mix.

5.2.4.2 LUCIFERASE ASSAY

Cells were washed twice with cold PBS and lysed using Reporter Lysis Buffer (#E4030 Promega Madison, WI). Lysate was transferred to black 96-well plates (#3915 Corning, Corning, NY) in triplicate. A BioTek (Winooski, VT) Synergy2 plate reader

with injectors was used to inject 100µL of luciferin in Luciferase Assay Buffer (#E4030 Promega, Madison, WI). Signal was incorporated for 12 seconds immediately after injection.

5.2.4.3 B-GALACTOSIDASE ASSAY

Lysates in Reporter Lysis Buffer were added to clear, round bottom 96-well plates in triplicate. ONPG (o-nitrophenyl-β-D-galactopyranoside) (#369-07-3 Research Products International Corp. Mt. Prospect, IL) substrate was dissolved at 4mg/mL in ONPG buffer (0.120M Na_2HPO_4, 0.08M NaH_2PO_4, 2mM $MgCl_2$, 100mM β-mercaptoethanol) and added to lysates at equal volume. The plate was incubated at 37°C until sufficient yellow color was developed (30 minutes to one hour). Absorbance at 420nm was read immediately after injection of 1M sodium carbonate at twice the volume of lysate+ONPG Buffer using the BioTek (Winooski, VT) Synergy2 plate reader.

5.2.5 GENERAL OXIDATIVE STRESS (ROS) DETECTION ASSAY

Cells were seeded at 2,750 cells/well in black 96-well plates with clear bottoms. After treatment with JNK-IN-8 and/or lapatinib (or H_20_2 for positive control) for the indicated times, DCDFA ROS detection reagent was added to each well to a final concentration of 10µM in phenol red-free media. After a 45 minute incubation in DCDFA, plates were immediately read for fluorescence signal at 495nm/529nm excitation/emission. Fluorescence levels were normalized to cell number by MTT assay. MTT reagent was incubated after fluorescence reading.

5.3 RESULTS

5.3.1 JNK-IN-8 AND/OR LAPATINIB DECREASE NFκB TRANSCRIPTIONAL ACTIVITY

Cells were treated for various time points with either JNK-IN-8, lapatinib, or the combination of both and tested for NFκB transcriptional activation of a luciferase reporter gene. The graph shows that in as little as six hours after treatment, JNK-IN-8 and JNK-IN-8 plus lapatinib significantly inhibit NFκB transcriptional activity. By 12 hours, JNK-IN-8, lapatinib, and the combination all significantly inhibit NFκB transcriptional activity with the combination treatment having more effect than either inhibitor alone. This trend continues into 48 hours after treatment where NFκB transcriptional activity continues to drop for all three treatments. In the MDA-MB-231 cells, values are less than half of the vehicle treated cells for JNK-IN-8 and lapatinib alone, and negligible for the combination treatment. In MDA-MB-436 cells, JNK-IN-8 and lapatinib have less effect alone, but the effect on NFκB transcriptional activity by the combination treatment is very pronounced as with the MDA-MB-231 cells (**Figure 5.1**). Together, these data indicate that JNK-IN-8 and lapatinib inhibit NFκB transcriptional activity in multiple basal-like breast cancer cell lines and combination treatment further reduces it beyond either treatment alone.

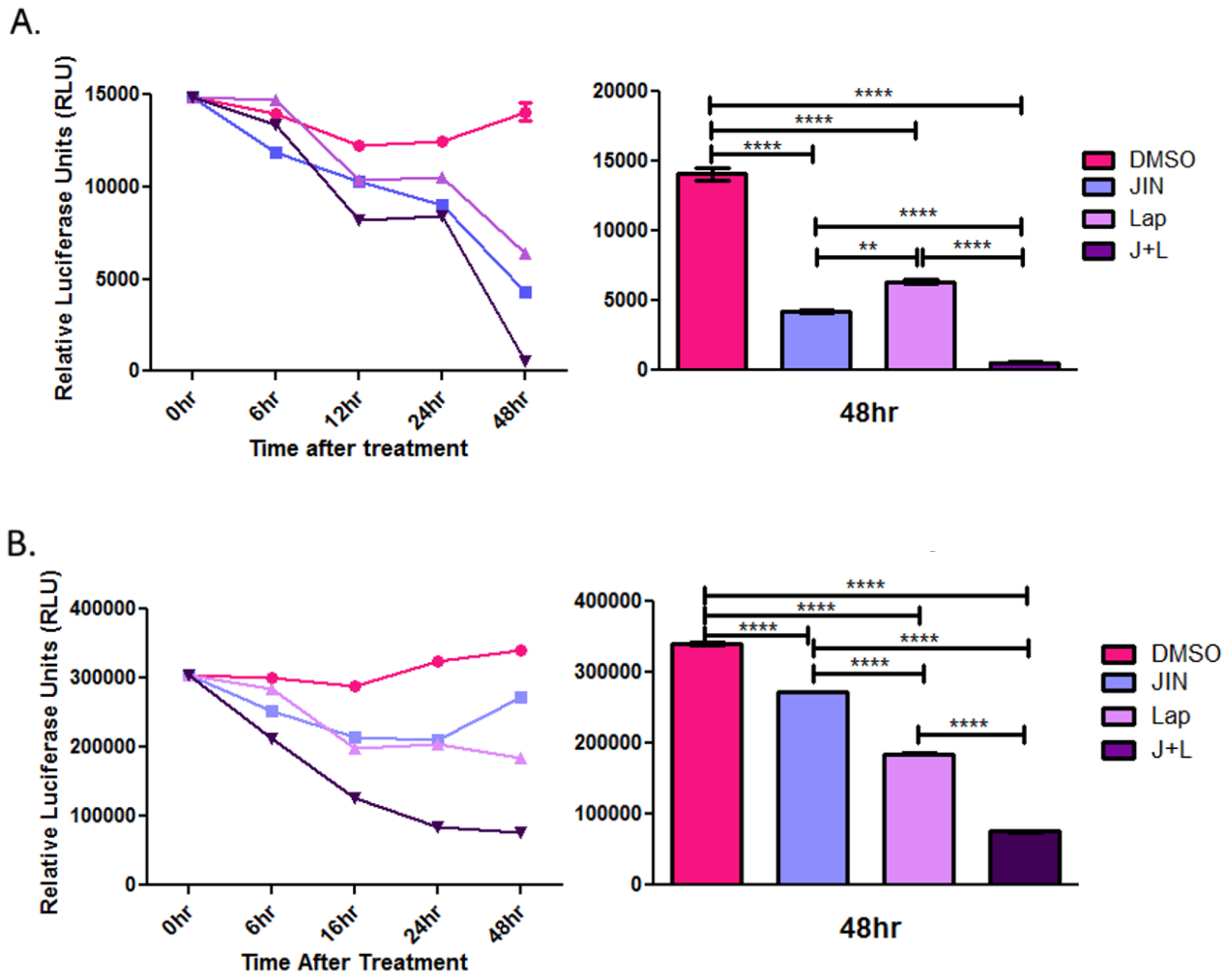


Figure 5.1: NFκB Luciferase Reporter Activity During JNK-IN-8 and Lapatinib Treatment

(A) MDA-MB-231 and (B) MDA-MB-436 cells were treated with (A) either vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap), or (B) DMSO, 5μM JIN and/or 8μM Lap for various time points in full media. They were also transfected with a luciferase reporter plasmid under the control of five tandem, NFκB consensus sites, and a constitutively expressed β-galactosidase plasmid. Cells were lysed and assayed for luciferase and β-galactosidase activity. Line graph points and bar graphs represent luciferase values normalized to β-galactosidase activity (absorbance at 420nm). (*≤.05, **≤.01, ***≤.001, ****≤.0001).

5.3.2 Basal-like Breast Cancer Cells are Sensitive to NFκB Inhibition but a Population of Cells is Resistant

Due to the inhibition of NFκB transcriptional activity by JNK-IN-8 and lapatinib, we wondered whether Basal-like breast cancer cells would be sensitive to NFκB inhibition using a pharmacologic inhibitor. If the NFκB-specific inhibitor successfully restricted NFκB activation and caused cell death, we may consider that the mechanism of cell killing by JNK-IN-8 and lapatinib is due to their combined inhibition of NFκB transactivation. We also wanted to combine an NFκB-specific inhibitor with JNK-IN-8 and/or lapatinib to determine whether maximal cell death could be achieved with the NFκB-specific inhibitor alone, or whether it must be combined with one of the other treatments.

Using data from previously published studies [172], 2.5 or 5μM BAY11-7082 treatment of MDA-MB-231 cells is sufficient to eliminate p65/p50 consensus sequence binding (using electrophoretic mobility shift assay) at 48 and 72 hours. BAY 11-7082 (BAY) is an irreversible inhibitor of IκB phosphorylation resulting in the inability of p65 to translocate to the nucleus [173]. Using 2.5 or 5μM BAY, it is evident that the MDA-MB-231 cells are sensitive to pharmacologic inhibition of NFκB. Using 2.5μM BAY, cells continue to grow albeit at about 50% of the viability of vehicle treated cells. The combination of BAY with JNK-IN-8 is not significantly different than the effect seen with BAY alone by 72 hours, however, lapatinib seems to increase viability in the presence of BAY just as it does compared to vehicle treated cells. The combination of 2.5μM BAY with JNK-IN-8 and lapatinib results in a curve that is extremely similar to JNK-IN-8 plus lapatinib (**Figure 5.2A**). Using 5μM BAY, there is a large drop in cell viability after 24 hours of treatment, but a population of cells recovers and continues to

grow into the 72 hour time point. Cell death caused by JNK-IN-8 and lapatinib combination shows the opposite effect where cell death increases over time, resulting in lower cell viability at 72 hours than with BAY alone. Combination of BAY with JNK-IN-8 and lapatinib prevents the growth of cells resistant to BAY and results in equivalent decreased cell viability when compared to JNK-IN-8 and lapatinib combination alone. Combination of JNK-IN-8 with BAY partially, but significantly, restricts growth of cells resistant to BAY (**Figure 5.2B**).

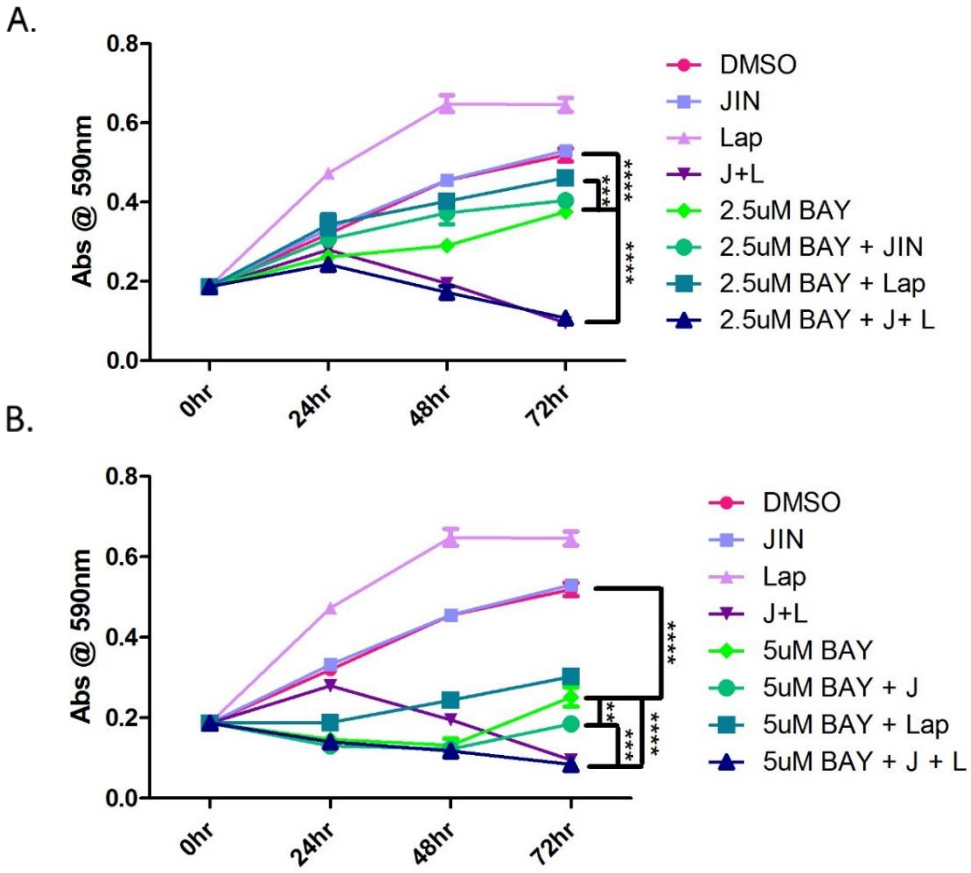


Figure 5.2: Cells are More Sensitive to JNK-IN-8 plus Lapatinib than to BAY alone

MDA-MB-231 cells were treated with either vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap), and (A) 2.5μM BAY or (B) 5μM BAY for various time points in full media. Line graphs represent MTT values over time. (*≤.05, **≤.01, ***≤.001, ****≤.0001).

5.3.3 JNK-IN-8 AND LAPATINIB MAY NOT DECREASE NFκB TRANSCRIPTIONAL ACTIVITY SOLELY THROUGH THE CANONICAL PATHWAY

There are various members of the NFκB transcription factor family. In the canonical pathway, activation of Toll-like or TNF receptors results in phosphorylation and activation of IκB Kinases (IKK) α, β, and γ. This complex phosphorylates Inhibitor of kappa B (IκB). In inactive cells, IκB is bound to heterodimers of p65 (RelA) and p50 that make up the NFκB transcription factor. This keeps NFκB sequestered in the cytoplasm, unable to activate transcription. When IKK phosphorylates IκB, IκB is poly-ubiquitinated and degraded, facilitating nuclear translocation of NFκB where it activates transcription.

In the non-canonical pathway, RelB is sequestered in the nucleus by p100. Activation of Receptor Activator of NFκB (RANK) or Lymphotoxin β Receptor (LTβR) results in the activation of NF-κB-inducing kinase (NIK) which then activates IKKα homodimers. These phosphorylate p100 leading ubiquitin-mediated degradation. Approximately half of the protein is processed resulting in the p52 protein which is now the binding partner of RelB which will translocate to the nucleus [174].

One study showed that both canonical and non-canonical NFκB heterodimers bind the same consensus sequence with equal affinity [175], however, temporal control of subunit expression, phosphorylation state, and interaction with co-activators in vivo results in differential activation of certain gene sets by the two heterodimers [176].

To check that our working concentrations of BAY were indeed preventing NFκB transcriptional activation, as with JNK-IN-8 and lapatinib combination, we treated cells with 2.5 and 5.0μM BAY for 24, 48, and 72 hours and quantified luciferase activity of our NFκB driven luciferase reporter gene. As shown in other publications, BAY

decreases NFκB consensus sequence binding over time [172], but at maximum, only somewhat less than 50% transcriptional inhibition could be reached (**Figure 5.3**). This was puzzling as a large amount of cell death was achieved with these concentrations.

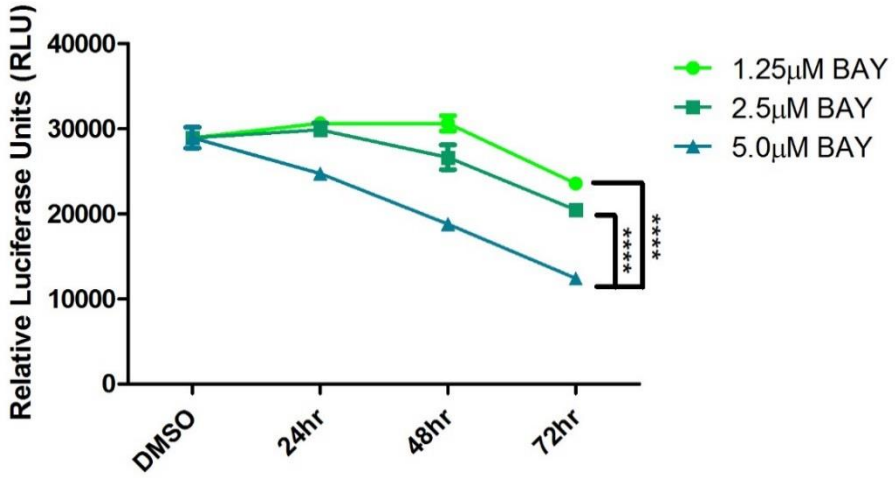


Figure 5.3: NFκB Luciferase Reporter Activity is not Fully Abrogated by BAY Treatment

MDA-MB-231 cells were treated with vehicle (DMSO), 1.25, 2.5, or 5.0μM BAY for various time points in full media. They were also transfected with a luciferase reporter plasmid under the control of five tandem, NFκB consensus sites, and a constitutively expressed β-galactosidase plasmid. Cells were lysed and assayed for luciferase and β-galactosidase activity. Line graph points represent luciferase values normalized to β-galactosidase activity (absorbance at 420nm). (*≤.05, **≤.01, ***≤.001, ****≤.0001).

A lack of transcriptional activity inhibition by BAY, despite a high level of toxicity, could indicate that a large amount of non-canonical NFκB signaling is driving transcriptional activity at NFκB consensus sites. If cell death in this line were due to NFκB inhibition alone, than BAY should not exert such effectiveness at cell killing at these concentrations. This added toxicity is likely due to the off-target effects of BAY 11-7082 [177-180].

Regardless of toxicity, cells recover after treatment with BAY as seen with the MDA-MB-231 cells and after other forms of NFκB inhibition [166]. If JNK-IN-8 and

lapatinib combination decreases non-canonical NFκB signaling, this would explain why cells treated with BAY, JNK-IN-8, and lapatinib do not recover growth, and how JNK-IN-8 and lapatinib combination causes a much more drastic decrease in NFκB transcriptional activation compared to BAY alone.

By western blot, expression of the strictly canonical IKKβ is decreased after treatment with JNK-IN-8 alone or in combination with lapatinib. Conversely, phosphorylation of p65 at ser536, a transcriptional activating site phosphorylated by IKKβ [181], was increased by about 50% in the JNK-IN-8 and lapatinib combination treatment (**Figure 5.4A**). Expression of IKKα, involved in both canonical- and non-canonical signaling, was decreased by JNK-IN-8 or lapatinib and somewhat more in the combination treatment (**Figure 5.4B**).

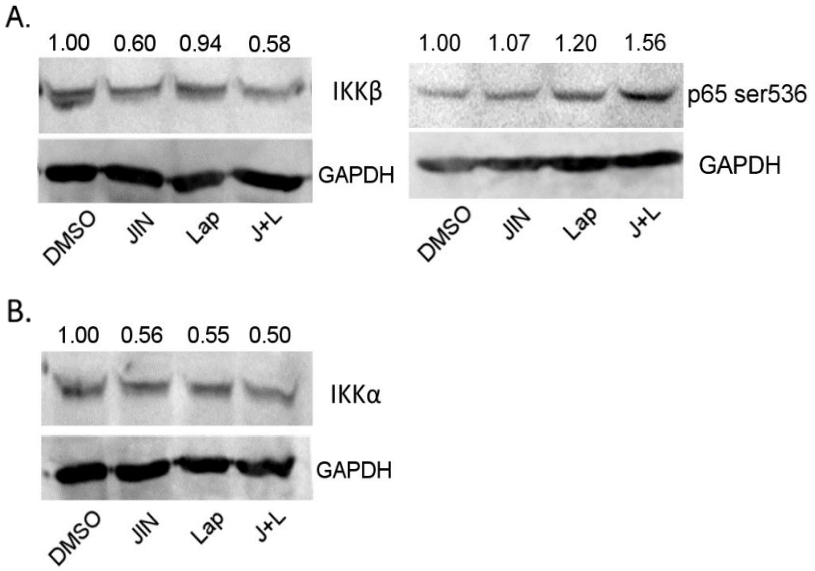


Figure 5.4: JNK-IN-8 and Lapatinib Affect Expression of Canonical- and Non-canonical NFκB Pathway Members

MDA-MB-231 cells were treated with either vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap) for forty-eight hours after treatment in full media. Cells were lysed in RIPA buffer and probed with antibodies against (A) members of the canonical NFκB pathway members IKKβ and phosphor-p65, and (B) a member of the non-canonical NFκB pathway, IKKα.

5.3.4 JNK-IN-8 and Lapatinib Combination Results in Cytotoxic ROS Accumulation

Consistent with NFκB's negative regulation of ROS through transcriptional targets such as Ferritin Heavy Chain (FHC) and Mn^{+} SuperOxide Dismutase (Mn-SOD) [153, 155], the combination of JNK-IN-8 and lapatinib, which potently inhibits NFκB, causes ROS accumulation over time to levels that are several fold higher than vehicle control (**Figure 5.5**). JNK-IN-8 alone significantly increases ROS above control but only at the 72 hour timepoint (**Figure 5.8**).

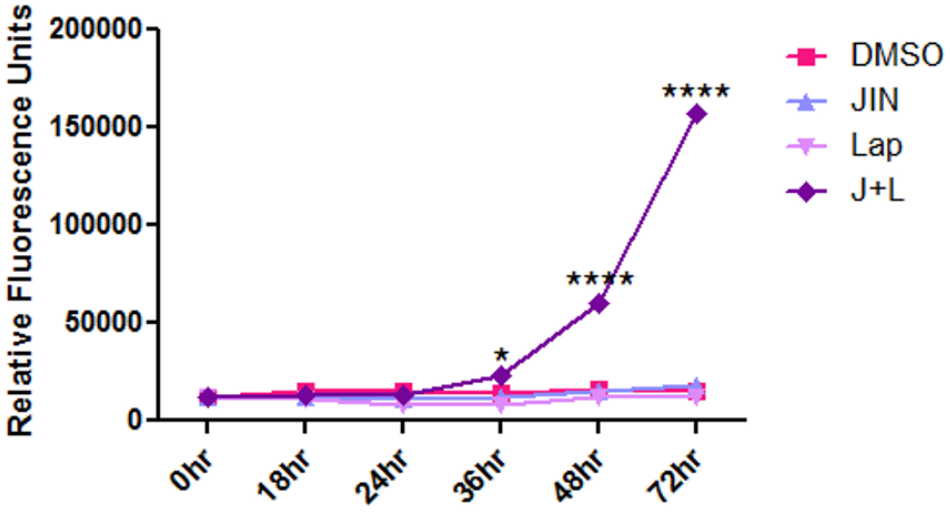


Figure 5.5: JNK-IN-8 and Lapatinib Combination Causes ROS Accumulation
MDA-MB-231 cells were treated with either vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap) for various time points in full media. At the indicated time point, DCDFA was added and incubated for 45 minutes prior to fluorescence quantification. The bar graph represents fluorescence values normalized to MTT assay absorbance.

Treatment of cells with 5μM BAY for 48 hours causes a significant increase in ROS compared to control. However, consistent with MTT assay results showing that JNK-IN-8 and lapatinib combination showed greater effect on cell viability than BAY alone (**Figure 5.2B**), the increase in ROS was significantly less than with JNK-IN-8 and lapatinib combination (**Figure 5.6**).

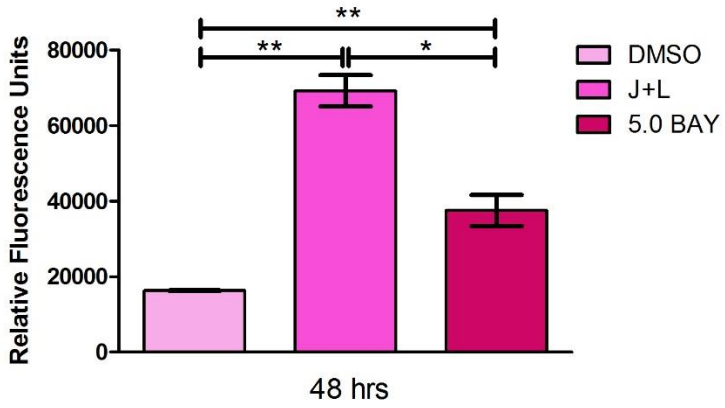


Figure 5.6: JNK-IN-8 and Lapatinib Increases ROS Greater than with BAY Alone

MDA-MB-231 cells were treated with either vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap), or 5μM BAY for 48 hours in full media. At the indicated time point, DCDFA was added and incubated for 45 minutes prior to fluorescence quantification. The bar graph represents fluorescence values normalized to MTT assay absorbance.

In order to determine whether ROS accumulation is responsible for decreased cell viability or is merely as consequence of apoptosis, cells were treated with JNK-IN-8 and lapatinib in addition to various ROS scavenging molecules including N-Acetyl Cysteine (NAC), ascorbic acid, and catalase. NAC is a precursor for glutathione and is a source of sulfhydryl groups that can be oxidized by ROS. Ascorbic acid possesses hydroxyl groups that may be oxidized and contributes to the regeneration of reduced vitamin E. Finally, catalase breaks down hydrogen peroxide into water and oxygen [182].

Treatment of cells with NAC along with JNK-IN-8 and lapatinib for 72 hours resulted in complete rescue of cell viability by MTT. Ascorbic acid also significantly rescued cell viability, although not nearly to the degree that NAC did, whereas catalase was ineffective (**Figure 5.7A**). Interestingly, the timing of NAC addition was important to the degree of rescue. NAC treatment at the same time as JNK-IN-8 and lapatinib resulted in complete rescue whereas addition of NAC after addition of JNK-IN-8 and lapatinib resulted in diminishing responsiveness. Noticeably, there was no significant change when cells were treated 24 hours after JNK-IN-8 and lapatinib, but about a 50% drop-off in rescue when cells are treated 48 hours after JNK-IN-8 and lapatinib. However, rescue from JNK-IN-8 and lapatinib combination was still significant at this time point (**Figure 5.7B**). This corresponds with the ROS time course showing that ROS accumulation begins between 24 and 48 hours after JNK-IN-8 and lapatinib treatment. **Figure 5.8** shows that NAC significantly reduces ROS associated with H_2O_2 treatment. Although 5mM NAC is too low to counter such a large increase as with H2O2 treatment, it is able to fully rescue ROS accumulation in cells treated with JNK-IN-8 and lapatinib. This is consistent with NAC's rescue of decreased cell viability. These data reveal that ROS accumulation is the cause of decreased cell viability after JNK-IN-8 and lapatinib combination.

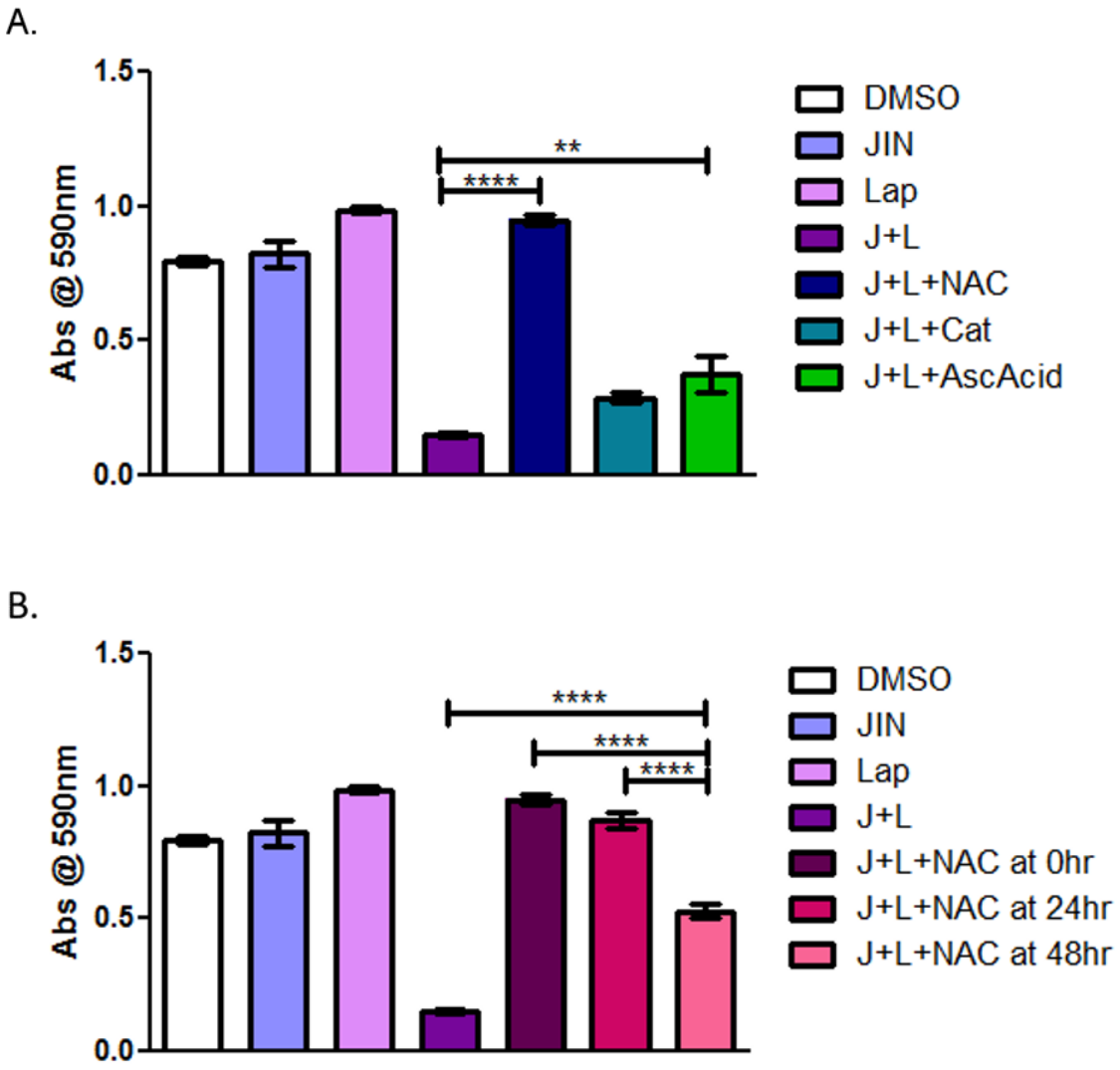


Figure 5.7: ROS Scavengers Rescue Decreased Viability in Cells Treated with JNK-IN-8 and Lapatinib

(A) MDA-MB-231 cells were treated with vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap), or JNK-IN-8 and lapatinib (J+L) with 5mM NAC, 500 U/mL catalase, or 100μg/mL ascorbic acid (AscAcid) for 72 hours in full media. (B) MDA-MB-231 cells were treated with vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap) or JNK-IN-8 and lapatinib (J+L) with 5mM NAC. NAC was either added along with JNK-IN-8 and lapatinib (NAC at 0hr), 24 hours after JNK-IN-8 and lapatinib (NAC at 24hr), or 48 hours and after JNK-IN-8 and lapatinib (NAC at 48hr). Cell viability at 72 hours after addition of JNK-IN-8 and/or lapatinib was assayed using MTT. Bars represent mean absorbances ± SEM at 590nm (*≤.05, **≤.01, ***≤.001, ****≤.0001).

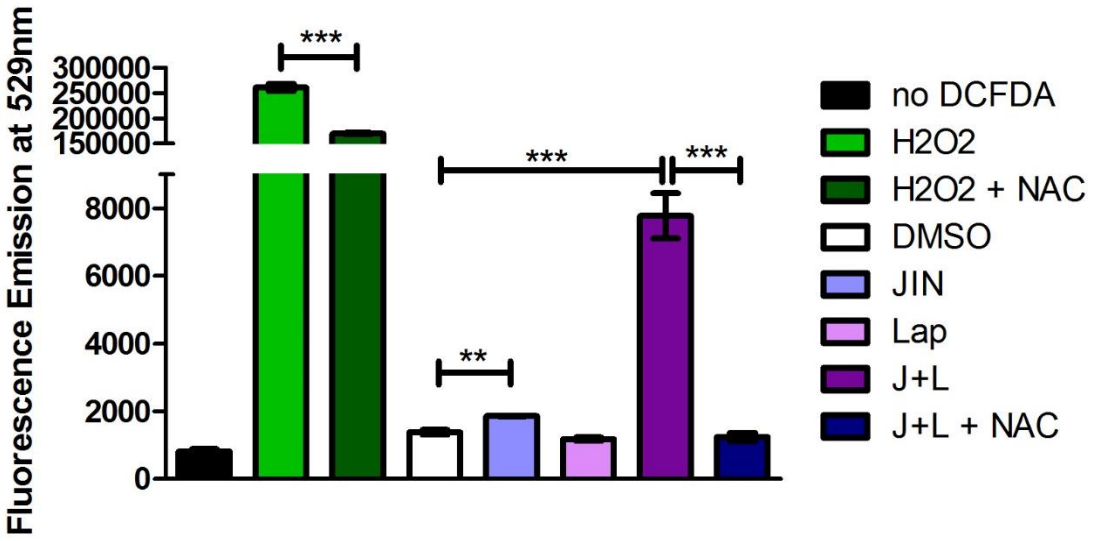


Figure 5.8: NAC Rescues Cytotoxic ROS Levels Caused by JNK-IN-8 and Lapatinib

MDA-MB-231 cells were treated with vehicle (DMSO), 6μM JNK-IN-8 (JIN) and/or 4μM lapatinib (Lap), or JNK-IN-8 and lapatinib (J+L) with 5mM NAC for 72 hours in full media. Negative control cells were not treated with DCDFA. For positive control, cells were pre-treated with 5mM NAC for 24 hours (or vehicle) and then treated with 15mM H2O2 for 45 minutes before assay. Fluorescence of oxidized DCDFA (DCF 2', 7' –dichlorofluorescein) was detected at 485nm/529nm excitation/emission. Bars represent mean fluorescence ± SEM at 590nm (*≤.05, **≤.01, ***≤.001, ****≤.0001).

5.4 DISCUSSION

JNK-IN-8 and lapatinib combination potently inhibits transcriptional activation by the pro-survival transcription factor, NFκB. This effect begins to occur very shortly after addition of the drugs and continues up to 48 hours, when significant apoptosis begins to occur. Although NFκB usually inhibits JNK activation through the mechanisms described in **Chapter 5.1,** JNK inhibition may negatively affect NFκB through a feedback loop. Lapatinib also contributes to the inhibition of NFκB transcriptional activity through unknown mechanisms, but this may explain upregulation of nuclear phosphorylated JNK in lapatinib treated cells (**Figure 4.4**) considering NFκB's inhibition of JNK.

Using the NFκB inhibitor BAY 11-7082, we discovered that although inhibition of NFκB may be part of the mechanism by which JNK-IN-8 and lapatinib combination kills Basal-like breast cancer cells, BAY alone is not sufficient to reach the level of cell killing that JNK-IN-8 and lapatinib combination does. Mysteriously, though, BAY treatment does not inhibit NFκB transcriptional activity as effectively as JNK-IN-8 and lapatinib combination. The inconsistencies here may lie in the mechanism of NFκB inhibition by BAY 11-7082.

Studies using BAY 11-7082 have shown that it is a potent inhibitor of IκB phosphorylation, but the actual mechanism is not well understood. Some papers describe BAY as an inhibitor of IKKs due to the fact that they are responsible for IκB phosphorylation, but no direct evidence is provided, and some studies have shown that IKK knockdowns do not affect cell viability to the degree that BAY treatment does [177]. Another study argued that IκB phosphorylation inhibition was an indirect effect of BAY's effects on E2 ubiquitin conjugating enzymes [180], and inhibition of IKK phosphorylation was also an indirect effect of this phenomenon.

Although it is unclear whether BAY actually effects IKK activation or action, inhibition of IκB phosphorylation should not affect signaling through non-canonical NFκB transcription factors since they do not interact with IκB. Residual transcriptional activation at NFκB consensus sites by non-canonical members could explain incomplete abrogation of NFκB luciferase activity by BAY treatment, and could explain why cells are not completely sensitive to BAY and can recover growth. If JNK-IN-8 and lapatinib combination achieves almost complete abrogation of NFκB transcriptional activity by inhibition of both canonical and non-canonical signaling, this may also explain why BAY treated cells cannot recover in the presence of JNK-IN-8 and lapatinib. Consistent with NFκB's ability to inhibit ROS, data showing that ROS accumulation is greater with JNK-IN-8 and lapatinib combination than with BAY alone further supports a role for this combination in decreasing NFκB signaling through mechanisms insensitive to BAY, which may only inhibit canonical transcription. JNK-IN-8 and lapatinib effects on IKKα and IKKβ expression could easily explain the mechanism by which both canonical and non-canonical NFκB signaling is reduced by these drugs (**Figure 5.9**).

ROS accumulation rises to about 10-fold higher levels than control after 72 hours of treatment with JNK-IN-8 and lapatinib. This level of oxidative stress leads to decreased viability in the MDA-MB-231 cells (**Figure 5.9**) as evidenced by the ability of NAC, and to a lesser extent other ROS scavengers, to rescue cell viability in response to JNK-IN-8 and lapatinib. This rescue was dependent on the time at which NAC was added to the cells. If NAC was added 48 hours after treatment with JNK-IN-8 and lapatinib, cell viability rescue was only about half of the rescue observed when NAC was added at the same time as JNK-IN-8 and lapatinib. This is consistent with ROS accumulating at 36 hours and reaching four-fold higher levels by 48 hours after treatment with JNK-IN-8 and

lapatinib. NAC completely rescued ROS accumulation in cells treated with JNK-IN-8 and lapatinib consistent with the rescue of cell viability.

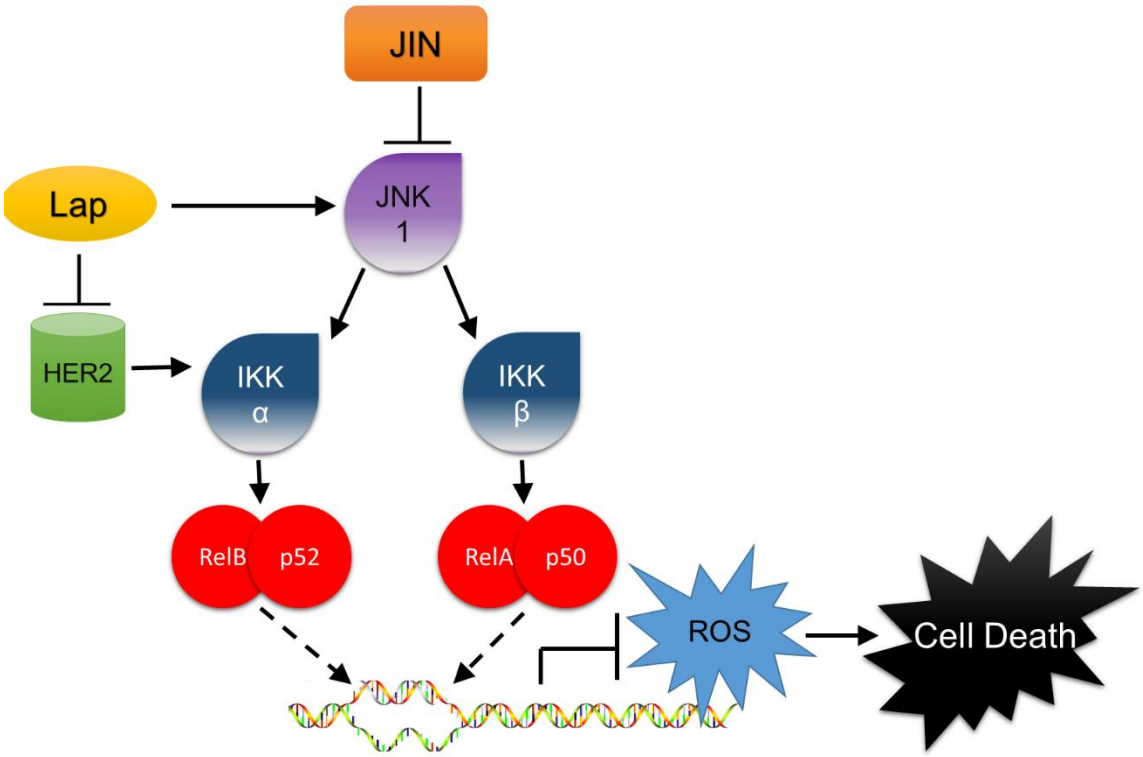


Figure 5.9: JNK-IN-8 and Lapatinib Synergize to Cause Cell Death through ROS Accumulation

ROS accumulation due to combination treatment with JNK-IN-8 and lapatinib leads to cell death. The mechanism of ROS accumulation may involve decreased transcription of NFκB targets due to inhibition of both canonical and non-canonical NFκB transcription factors. JNK-IN-8 inhibits IKKα and β while lapatinib inhibits IKKα.

Chapter 6: Summary and Future Directions

Treatment of Basal-like breast cancers with EGFR or dual EGFR/HER2-directed inhibitors has been unsuccessful despite effective action against the drugs' intended targets [38-40]. Due to JNKs roles downstream of growth factor receptors and other stimuli, we hypothesize that JNK inhibition in Basal-like breast cancer might increase the effectiveness of the dual EGFR/HER inhibitor lapatinib by diminishing compensatory signaling.

Presented in this work we have established a possible therapeutic benefit for the combination of lapatinib and JNK-IN-8 in the treatment of Basal-like breast cancers using established, Basal-like breast cancer cell lines as models. JNK-IN-8 and lapatinib, working mainly through their inhibition of JNK1 and HER2, respectively, synergize in the induction of apoptosis in these cell lines.

JNK-IN-8 synergizes with lapatinib by induction of cytotoxic oxidative stress through the accumulation of ROS. Although normally present at low levels in cells for signaling purposes [183-187], large amounts of ROS can be cytotoxic [188]. Only when JNK-IN-8 and lapatinib are combined do we observe a 10-fold increase in ROS accumulation that begins at about 36 hours after treatment. NAC-mediated depletion of ROS rescued cell death in a time-dependent manner. Interestingly, neither JNK-IN-8 nor lapatinib alone have much effect on ROS levels, although JNK-IN-8 increases ROS significantly at 72 hours of treatment.

Mechanisms of ROS accumulation in this model are yet to be explored. For future experiments, we would like first to determine whether excess ROS is being generated or whether ROS is increasing due to lack of scavengers. Sources of intracellular ROS include the electron transport chain in mitochondria where superoxides may be produced,

and various enzymes such as NADPH oxidases, nitric oxide synthases, and cyclooxygenases, etc. that produce various forms of ROS [189]. Quantitative PCR to assay expression levels of these enzymes will reveal whether they may be responsible for ROS accumulation after JNK-IN-8 and lapatinib treatment. If one or more of these enzymes is upregulated in the combination treatment, we will knockdown the enzyme using RNAi in the presence of JNK-IN-8 and lapatinib to determine whether decreased expression rescues ROS accumulation and cell death.

ROS accumulation may occur in cells undergoing treatment with JNK-IN-8 and lapatinib because of inhibition of NFκB transcriptional activity. After exposure to TNF-α, ROS are produced and NFκB is activated. During this time, JNK is also activated. Transcription of NFκB target genes deplete the ROS pool and inhibit JNK activation, so a likely scenario is that inhibition of NFκB leads to a decrease in expression of ROS scavengers. NFκB increases expression of SOD1 and SOD2 [190-192], which change superoxides into O_2 or H_2O_2, and Glutamate Cysteine-ligase [193] which is important for glutathione (a potent scavenger) biosynthesis. Quantitative PCR measurement of these scavengers will help to determine whether NFκB inhibition increases ROS accumulation through decreased expression of NFκB target genes. If one or more of these genes is downregulated after JNK-IN-8 and lapatinib treatment, we will overexpress the gene and determine whether overexpression rescues ROS accumulation and cell viability. Due to NFκB's inhibition of ROS and the involvement of JNK in this signaling pathway, it is easy to imagine how JNK inhibition may restrict NFκB activation through a negative feedback mechanism that would result in ROS accumulation.

MDA-MB-231 cells were particularly sensitive to pharmacologic inhibition of NFκB by BAY 11-7082, but the observed cell death was not as complete as with JNK-IN-8 and lapatinib combination. This was probably due to the fact that BAY 11-7082

only showed about 50% inhibition of NFκB transcriptional activity by luciferase reporter assay (**Figure 5.3**). Because BAY 11-7082 may only affect canonical NFκB signaling through its inhibition of IκB phosphorylation, it's possible that non-canonical NFκB signaling may drive transcription from NFκB consensus sites in the presence of BAY 11-7082. Inhibition of canonical- and non-canonical signaling by JNK-IN-8 and lapatinib would explain the greater effectiveness of these drugs at abrogating NFκB transcriptional activity and cell death when compared to BAY 11-7082 treatment alone.

Future studies into this mechanism will involve determining whether JNK-IN-8 and lapatinib affect the canonical and/or non-canonical NFκB signaling pathways, and whether decreased NFκB transcriptional activity is a direct cause of ROS accumulation and/or cell death. First, BAY will be combined with an inhibitor of NFκB Inducing Kinase (NIK), which specifically inhibits non-canonical signaling. If addition of the NIK inhibitor alone, or combination with BAY, accomplishes abrogation of NFκB transcriptional activity and equivalent cell death, then we may conclude that JNK-IN-8 and lapatinib have some effect on non-canonical NFκB signaling. This may be in addition to effects on the canonical pathway.

As pharmacologic inhibitors frequently have off-target effects, mRNA knockdown of specific NFκB transcription factors, canonical and non-canonical, will also be used. If decreased NFκB signaling is solely responsible for ROS accumulation and cell death, then knockdown of p65 (canonical) and/or RelB (non-canonical) should phenocopy treatment with JNK-IN-8 and lapatinib. Due to data using BAY and JNK-IN-8 and lapatinib inhibition of both IKKα and IKKβ, we anticipate that only knockdown of RelB and p65 together will completely rescue ROS accumulation and/or cell death.

Rescue of ROS and cell death due to JNK-IN-8 and lapatinib will be attempted by overexpressing p65, RelB, IKKα, and/or IKKβ. If a decrease in canonical NFκB

signaling is solely responsible for ROS accumulation or cell death after treatment with JNK-IN-8 and lapatinib, then overexpression of p65 should rescue these effects [194-196]. Overexpression of RelB would be used for the same purpose except to reveal whether a decrease in non-canonical NFκB signaling is responsible for ROS accumulation and/or cell death. If it is necessary to overexpress both p65 and RelB, then we may conclude that JNK-IN-8 and lapatinib cause ROS accumulation and cell death by inhibiting both pathways. Because JNK-IN-8 and lapatinib decreased the expression of IKKα and IKKβ (**Figure 5.4**), re-expression of either or both of these proteins will reveal whether their expression regulation is the mechanism by which JNK-IN-8 and lapatinib decrease signaling to NFκB transcription factors. If their overexpression cannot rescue cell death in the presence of JNK-IN-8 and lapatinib, then decreased NFκB transcriptional activation may be coincidental. It could be the case, however, that decreased NFκB signaling exacerbates or is exacerbated by the loss of another transcription factor or binding partner.

Another potential mechanism to be explored is the importance of AP-1 cooperation with NFκB (**Figure 6.1**). Although AP-1 transcriptional activation data were somewhat unexpected in that they did not follow c-Jun status, AP-1 activity was abrogated in the combination treatment in both the MDA-MB-231 and MDA-MB-436 cells. Many promoters, including those for the cytokines IL-6, IL-8, and CXCL8 [197, 198], as well as promoters for microRNAs [199], contain consensus binding sequences for both NFκB and AP-1. Frequently, authors cite the ability of AP-1 to enhance the transcriptional activity of p65. This may occur through physical interaction (directly or within a complex) that affects chromatin structure to enhance DNA availability for transcription [200-203].

Although this question is harder to answer considering the sheer number of proteins that may be involved in the AP-1 transcription factor, there are a few clues that may help to narrow down the relevant players. In our AP-1 luciferase assay, c-Jun status did not correspond with AP-1 transcriptional activity, so it's unlikely that c-Jun is involved in this mechanism. However, lapatinib greatly decreased AP-1 activity despite increasing JNK activation. This indicates that HER2 activates AP-1 through a non-JNK AP-1 target. These facts heavily implicate the fos transcription factors including c-fos, fosb, fra-1, and fra-2, which have been linked to signaling downstream of EGFR/HER2 through ERK [204-206]. To define the role of AP-1 in JNK-IN-8 and lapatinib induced cell death, ROS accumulation, or NFκB inhibition, expression levels of the fos transcription factors will be assayed. Once a candidate fos has been identified, meaning one whose expression is decreased by lapatinib treatment alone and in combination with JNK-IN-8, that protein will be overexpressed to determine whether it rescues any of the above phenotypes. It may be the case that NFκB transcription factors must be co-transfected with fos transcription factors to obtain full rescue, if the mechanism of cell death is dependent on eliminating cooperation between the two.

Finally, a true therapeutic benefit for combination treatment with JNK-IN-8 and lapatinib cannot be acknowledged without *in vivo* testing using mouse models of breast cancer. Currently, JNK-IN-8 is not formulated for *in vivo* studies, but future work with this compound aims to complete formulation in order to test the effectiveness of the JNK-IN-8 and lapatinib combination. It may also be the case that addition of an NFκB inhibitor would prove beneficial even on its own, however, impairment of specific survival functions of NFκB may be preferable to total NFκB blockade, such as through inhibition with JNK-IN-8 and lapatinib, because of severe immunosuppressive defects that occur after long-term treatment [153, 207]. Regardless, if JNK-IN-8 and lapatinib are

able to synergize to treat Basal-like mammary tumors in mice, then the impetus for human testing is not far off.

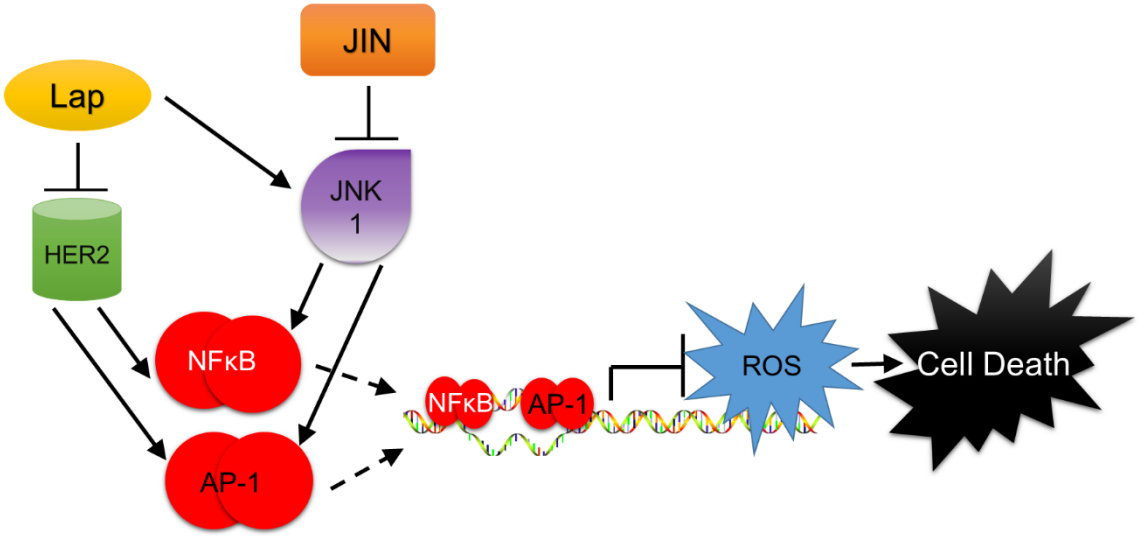


Figure 6.1: JNK-IN-8 and Lapatinib May Synergize to Cause Cell Death Inhibition of NFκB and AP-1 Cooperation

JNK-IN-8 and lapatinib abrogate NFκB and AP-1 transcriptional activity. Since these transcription factors often cooperate at promoters of the same target genes, inhibition of both may be required for JNK-IN-8 and lapatinib synergistic cell death.

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