Role of X-Linked Inhibitor of Apoptosis Protein in Therapeutic Resistance of Inflammatory Breast Cancer Cells

by
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Duke University

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Mike Datto

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Neil Spector

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology in the Graduate School of Duke University

2010
ABSTRACT
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Abstract

Apoptotic dysregulation is a hallmark of cancer cells. The inability of cancer cells to undergo apoptosis may lead to therapeutic resistance. Inflammatory breast cancer (IBC) is a highly aggressive subtype of breast cancer that is often characterized by ErbB2 overexpression and ErbB2 activation. ErbB-targeting is clinically relevant using trastuzumab (anti-ErbB2 antibody) and lapatinib (small molecule ErbB1/2 inhibitor). However, acquired resistance is a common outcome even in IBC patients who show an initial clinical response, which limits the efficacy of these agents. Little is known about the molecular mechanisms of therapeutic resistance in IBC cells. We hypothesized that apoptotic dysregulation leads to therapeutic resistance of IBC cells to therapeutic agents, including ErbB-targeting agents. To determine whether apoptotic dysregulation and changes in anti-apoptotic proteins leads to resistance of IBC cells to therapeutic agents, we performed a variety of in vitro-based studies using agents that are used in the clinic to treat IBC patients. The sensitivity of both ErbB2 overexpressing and ErbB1 activated IBC cells to various therapeutic agents was evaluated using various cell death and apoptosis assays, and anti-apoptotic protein expression post-treatment was determined using western blot analysis. The overarching theme observed was that x-linked inhibitor of apoptosis protein (XIAP) expression inversely correlated with sensitivity of cells to therapeutic agents with various mechanisms of action, including TNF-related apoptosis
inducing ligand (TRAIL), doxorubicin, cisplatin, paclitaxel, and two ErbB-targeting agents: trastuzumab and a lapatinib-analog (GW583340). Moreover, there was a specific and marked overexpression of XIAP in cells with de novo resistance to trastuzumab and with acquired resistance to GW583340. The observed overexpression was identified to be caused by IRES-mediated XIAP translation. Stable XIAP overexpression using a lentiviral system reversed sensitivity to therapeutic agents (TRAIL and GW583340) in parental IBC cells. Moreover, XIAP downregulation in cells resistant to therapeutic agents (TRAIL, trastuzumab, and GW583340) resulted in decreased viability and increased apoptosis, demonstrating that XIAP is required for survival of cells with resistance to these agents. A novel mechanism of GW583340 oxidative stress-induced mediated apoptosis was identified, and resistant cells had increased antioxidant expression and capability. Interesting, inhibition of XIAP function overcame this increase in antioxidant potential, demonstrating a new function for XIAP in oxidative stress-induced apoptosis. These studies establish the feasibility of development of an XIAP inhibitor that potentiates apoptosis for use in IBC patients with resistance to therapeutic agents.
Dedication

To my parents, who always believed in me, always stood by me, and let me grow in my own way.
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<td>2-ME,</td>
<td>2-methoxyestradiol</td>
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<td>5-FU,</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ADCC,</td>
<td>antibody dependent cellular toxicity</td>
</tr>
<tr>
<td>ALS2CR2,</td>
<td>Amyotrophic lateral sclerosis 2 chromosome region candidate 2</td>
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<tr>
<td>AMPK,</td>
<td>AMP-activated protein kinase</td>
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<td>AP-1,</td>
<td>activator protein 1</td>
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<td>APAF-1,</td>
<td>apoptotic protease activating factor 1</td>
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<td>BIR,</td>
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<td>CCS,</td>
<td>copper chaperone for superoxide dismutase</td>
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<td>IAP</td>
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NAIP, neuronal apoptosis inhibitory protein
NFκB, nuclear factor kappa B
Nrf2, NF-E2-related factor 2
NQO1, NAD(P)H:quinone oxidoreductase
PKC, protein kinase c
PR, progesterone receptor
PI3K, phosphoinositide 3-kinase
PMA, phorbol myristyl acetate
PP2A, protein phosphatase 2
PTEN, phosphatase and tensin homolog
RING, really interesting new gene
ROS, reactive oxygen species
RR, response rate
SH2, Src homology 2
SOD1, superoxide dismutase 1
SOD2, superoxide dismutase 2
STAT5, signal transducer and activator of transcription 5
TAD, trans-activating domain
TAK1, transforming growth factor β–activated kinase 1
TMRE, tetramethylrhodamine, ethyl ester
TNF, tumor necrosis factor
TRAIL, TNF-related apoptosis inducing ligand
Trx2, thioredoxin-2
TGF-β, transforming growth factor beta
UTR, untranslated region
VEGF, vascular endothelial growth factor
XAF-1, XIAP-associated factor 1
XIAP, x-linked inhibitor of apoptosis protein
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1. Introduction

Introduction to the Problem

At the inception of the research detailed in this dissertation, very little was known about the resistance mechanisms of inflammatory breast cancer (IBC) cells to therapeutic agents that are commonly used to treat patients. Because a number of microarray studies looking at IBC vs. non-IBC samples hinted that the dysregulation of the apoptotic pathway was potentially a key factor in the pathogenesis of IBC (Bertucci et al., 2004; Boersma et al., 2008; Nguyen et al., 2006; Bertucci, 2004 #268; Ryan et al., 2006; Turpin et al., 2002; Van Laere et al., 2006; Wang et al., 2010) (and evasion of apoptosis is a hallmark of cancer cells (Hanahan and Weinberg, 2000)), we decided to evaluate the apoptotic signaling pathways in response to therapeutic agents in the only two commercially available IBC cell lines (SUM190 and SUM149). We were particularly interested in understanding the apoptotic pathway dysregulations, which protein(s) was most important for the resistance phenotype, and how best to target these proteins/pathways to lead to apoptosis of IBC cells. We hypothesized that either XIAP or survivin may be important because: 1) they are members of the inhibitor of apoptosis (IAP) family, which can inhibit both intrinsic and extrinsic pathways of apoptosis (Liston et al., 2003); 2) both can be regulated by nuclear factor kappa B (NFκB) (Kawakami et al., 2005; Stehlik et al., 1998), which is highly upregulated in IBC tumor samples (Van Laere et al., 2006); and 3) both can lead to more aggressive disease (Mehrotra et al., 2010) and therapeutic resistance in breast cancer (Schimmer and Dalili, 2005; Schimmer
et al., 2006; Xia et al., 2006a; Xia et al., 2006b). We therefore had three goals, which will be addressed in this dissertation:

**Objective 1:** Evaluation of sensitivity of IBC cells to commonly-used therapeutic agents.

**Objective 2:** Determination of the key apoptotic protein(s) involved in therapeutic resistance to ErbB-targeting agents in an IBC cellular model.

**Objective 3:** Determination of the mechanism of apoptotic dysregulation in IBC cells resistant to ErbB2 targeting.
**Introduction to Apoptosis**

To understand why apoptosis may be important in resistance of IBC cells to therapeutic agents, we must first be aware of the apoptotic signaling pathways and how they are regulated under normal conditions. The summary of the literature in this section will address the apoptotic pathways, what is known about apoptotic dysregulation in cancer, and the protein we decided to evaluate for this research detailed in this dissertation.

1.1.1 Apoptotic Pathways

Apoptosis (or programmed cell death) is an energy-dependent mechanism that is distinct from other types of cell death, including necrosis, lysosomal cell death, mitotic catastrophe, and autophagy (Jin and El-Deiry, 2005). Cellular apoptosis can be executed by four main pathways: 1) Intrinsic/mitochondrial pathway, which is activated by cellular stresses such as DNA damage, oxidative stress, starvation, and many chemotherapies used for cancer treatment. This pathway is mediated by the increase in mitochondrial membrane permeability, which releases proapoptotic proteins into the cytoplasm that foster the formation of the apoptosome and subsequent activation of caspase 9 (Wang, 2001); 2) Extrinsic/death receptor pathway, which is mediated by the binding of members of the TNF receptor family (called death receptors) to their cognate ligand. This causes formation of the death inducing signaling complex (DISC) and subsequent activation of caspase 8 (Sartorius et al., 2001); 3) Endoplasmic reticulum (ER) stress, which releases calcium ions and activates caspase 12 (Faitova et al., 2006; Szegedi et al., 2003; Szegedi et al., 2006); and 4) Granzyme B activation of caspases.
(Lord et al., 2003). What is noteworthy is that all four pathways converge at the level of the caspases, the executioners of apoptosis (Denault and Salvesen, 2002). Caspases are cysteine proteases that are in the cell as inactive zymogens (called procaspases) and are activated upon apoptotic stimuli by cleavage of the large and small subunits. Active initiator caspases (caspases 2, 8, 9, and 10) go on to cleave the executioner caspases (caspases 3, 6, and 7), resulting in an amplification of the death signal. Executioner caspases have many substrates, including cellular structural components and many key survival and anti-apoptotic signaling proteins, and cleavage of these substrates results in the hallmark morphologic and biochemical changes associated with apoptosis. These include cell shrinking and condensation of the chromatin, membrane blebbing, and appearance of apoptotic bodies (Elmore, 2007), which occur due to the cleavage of DNA into oligonucleotides and proteins that change the integrity and shape of the cytoplasm and organelles. In contrast to necrosis, which is not energy-dependent and also causes an inflammatory response and potential damage to surrounding healthy tissue (Jin and El-Deiry, 2005), apoptosis is an energy-dependent process that does not induce an inflammatory response; therefore, the surrounding healthy tissue is not damaged. Thus, apoptosis induction is preferable to necrosis in causing cancer cell death.

### 1.1.2 Apoptotic Dysregulation in Cancer

Apoptosis is a highly-regulated programmed cellular death pathway that is necessary for many normal cellular processes such as limb generation in development (Zuzarte-Luis and Hurle, 2002) and deletion of self-reactive immune cells (Rathmell and Thompson, 2002). However, it is now clear that dysregulation of apoptosis is
pathogenic, and the inability of a cell to undergo apoptosis is a hallmark of cancer (Evan and Vousden, 2001; Hanahan and Weinberg, 2000). This inability of cancer cells to undergo apoptosis is in part due to the inactivation of caspases by their negative regulators.

1.1.3 Negative Regulators of Apoptosis

As with all cellular processes, apoptosis is highly regulated to ensure cells only undergo death in response to apoptotic stimuli. There are two classes of anti-apoptotic proteins that negatively regulate apoptosis: 1) Bcl-2 anti-apoptotic proteins and 2) inhibitor of apoptosis proteins (IAPs). Bcl-2 anti-apoptotic proteins’ central functions are to guard mitochondrial integrity and bind to the pro-apoptotic members of the Bcl-2 family (such as Bax and Bid) to inhibit the initiation of apoptosis through the intrinsic/mitochondrial pathway (Deveraux et al., 2001). On the other hand, IAPs have been shown to directly interact with and inhibit the activation of procaspases, and unlike the Bcl-2 proteins, IAPs can inhibit both the intrinsic and extrinsic pathways of apoptosis (Cory and Adams, 2002; Coultas and Strasser, 2003), making them potentially more potent inhibitors of therapeutic agent-induced apoptosis. Therefore, we decided to narrow down our research to evaluate the IAPs.

1.1.4 Inhibitor of Apoptosis Proteins

There are 8 characterized IAP family members to date. These include X-linked inhibitor of apoptosis protein (XIAP), Livin, Survivin, NAIP, cIAP1, cIAP2, Apollon, and hILP2 (Fraser et al., 2003). The IAP proteins are highly conserved and consist of 1-3 baculovirus IAP repeats (BIRs) (Reed et al., 2004), which are critical for their function.
In addition, XIAP (Galban and Duckett), cIAP1&2 (Bertrand et al., 2008; Cheung et al., 2008; Choi et al., 2009; Sekine et al., 2008), and livin (Ma et al., 2006) have a RING domain that consists of an E3 ubiquitin ligase that is used for both ubiquitination of other proteins and autoubiquitination (Ni et al., 2005; Vaux and Silke, 2005). These proteins have been shown to have multiple functions including direct inhibition of caspases, cell cycle regulation, pro-survival signaling and have been shown to be both prognostic and diagnostic biomarkers for cancer (Capalbo et al., 2007; Chen et al., 2009; Gazzaniga et al., 2003; Grzybowska-Izydorczyk et al.; Haferkamp et al., 2008; Kempkensteffen et al., 2007; Kim et al., 2005a; Kren et al., 2004; Li et al., 2007; Liao et al., 2009; Liston et al., 2003; Liu et al., 2009a; Margulis et al., 2008; Mizutani et al., 2007; Nakayama and Kamihira, 2002; Psyrri et al., 2006; Qi et al., 2008; Ramp et al., 2004; Seligson et al., 2007; Shen et al., 2009; Sun, 2006; Tamm et al., 2004a; Tamm et al., 2004b; Wang et al., 2007; Xiang et al., 2006).

1.1.5 X-linked inhibitor of apoptosis protein

In particular, XIAP is the most potent caspase inhibitor known to date. XIAP can inhibit caspases -3, -7, and -9, which comprise members of both the intrinsic and extrinsic pathways of apoptosis (Eckelman et al., 2006), with a Kᵢ of 7x10⁻¹⁰, 2x10⁻¹⁰, and 1x10⁻⁹ M for caspases 3, 7, and 9, respectively (Salvesen and Duckett, 2002). In addition to its caspase binding properties, XIAP is a potent pro-survival signaling molecule, which will be discussed in Section 1.1.6.
1.1.5.1 Expression and Structure

Unlike many of the other IAPs, which have a more specific expression pattern, XIAP is ubiquitously expressed in most cells of the adult human body (Liston et al., 1996). It is generally localized in the cytoplasm (Aird et al., 2010; Ferreira et al., 2001b; Liston et al., 2001; Vischioni et al., 2006); however, a few studies have shown nuclear localization of XIAP (Holcik and Korneluk, 2001; Liston et al., 2001). It is still unclear what the function of nuclear XIAP is, although this suggests XIAP may have cell cycle regulatory functions. Although it is expressed in most normal cell types, XIAP is often upregulated in cancer cells (Fong et al., 2000; Tamm et al., 2000), including breast cancer (Jaffer et al., 2007), through mechanisms that will be discussed in the following sections, which can lead to resistance of cells to a variety of therapeutic agents (Gimenez-Bonafo et al., 2009). Therefore, targeting XIAP for cancer therapy remains a promising strategy (Devi, 2004; Schimmer et al., 2006).

The structure of XIAP (Fig. 1.1) is crucial for its function. It has two 5’ UTRs, one short and one long (Gu et al., 2009; Riley et al.) (Fig. 1.1), that lead to cap- and IRES-dependent translation of the XIAP mRNA, respectively (Riley et al.). The XIAP protein is 57 kD and contains three BIR domains and a RING domain (Duckett et al., 1996) (Fig. 1.1). BIR domains are characteristic cysteine- and histidine-rich domains that chelate zinc (Hinds et al., 1999). They are 70-80 amino acids in length and have a core domain sequence of $C(X)_2C(X)_8W(X)_3D(X)_5H(X)_6C$, where X is any amino acid (Hunter et al., 2007). The presence of these domains is what classifies the IAP family, and their sequence homology is highly conserved (Uren et al., 1998). The linker region between
BIR1 and 2 is where XIAP binds to caspases 3 and 7 (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001), and BIR3 is where XIAP binds to caspase 9 (Shiozaki et al., 2003). The RING domain has been demonstrated to be an active E3 ubiquitin ligase [recently reviewed in (Galban and Duckett)], which can ubiquitinate other substrates (MacFarlane et al., 2002; Morizane et al., 2005; Suzuki et al., 2001) or autoubiquitinate itself (Yang et al., 2000). The ability of XIAP to bind to and inhibit caspases and ubiquitinate other proteins are some of the main mechanisms of how XIAP is both a potent anti-apoptotic and pro-survival protein.
Figure 1.1: DNA, RNA, and protein structure of XIAP.

XIAP DNA, RNA, and protein structures are shown (not to scale). The XIAP DNA sequence has 7 exons, multiple transfection factor (TF) binding sites, and is 54,256 bp. The XIAP mRNA is 6.6 kB and has a short and long 5' UTR [the long 5' UTR is where the internal ribosomal entry site (IRES) is found]. The XIAP protein is 57 kD and has 3 baculoviral IAP repeats (BIR) and a really interesting new gene (RING) domain, which is the E3 ubiquitin ligase.
1.1.5.2 Regulation of XIAP

Because upregulation of XIAP can lead to therapeutic resistance, it is important to determine how XIAP is regulated (transcriptionally, translationally, and post-translationally). Elucidation of these mechanisms may lead to better and more potent XIAP inhibitory strategies. Below is a discussion of the main ways that the XIAP gene, mRNA, and protein are regulated.

1.1.5.3 Transcriptional Regulation of XIAP

Increased transcription of XIAP can lead to therapeutic resistance of cancer cells. XIAP can be controlled at the transcriptional level by a number of transcription factors: positively by NFκB (Stehlik et al., 1998), STAT5 (Mohapatra et al., 2003), and Sp1 (Lee et al., 2006) and negatively by p53 (Sasaki et al., 2000). Very little is known about the specifics of how STAT5, Sp1, and p53 regulate XIAP in different cellular scenarios. However, studies have indicated that targeting the ability of these transcription factors to bind to the XIAP promoter may help to sensitize cancer cells to therapeutic agents (Bruno et al., 2008; Dai et al., 2005; Lee et al., 2006; Mohapatra et al., 2003; Ougolkov et al., 2007).

The most widely studied transcription factor of XIAP is nuclear factor kappa B (NFκB), which consists of dimeric complexes that can translocate to the nucleus during different cellular contexts and bind to cognate sequences in the promoters of a variety of genes (Dixit and Mak, 2002). The first study to demonstrate the positive regulation of NFκB on XIAP was done by Stehlik et al. in 1998 (Stehlik et al., 1998). Although NFκB
is a transcription factor for both pro- and anti-apoptosis gene products (Burstein and Duckett, 2003), numerous studies have demonstrated that activation of NFκB and its subsequent increase in XIAP mRNA levels leads to resistance of a variety of cancer cells to multiple different therapeutic agents, including paclitaxel (Lin et al., 2004), adriamycin (Lin et al., 2004), TRAIL (Braeuer et al., 2006; Samanta et al., 2004), doxorubicin (Bruno et al., 2008), cisplatin (Bruno et al., 2008; Gordon et al., 2007), and HDACi (Dai et al., 2005). Interestingly, some reports have indicated that increased NFκB activation leads to increased pro-apoptotic protein expression and subsequent cancer cell apoptosis (Aggarwal et al., 2005; Chovolou et al., 2007; Kang et al., 2009). Why increased NFκB activation leads to cell death or cell survival in different models is not currently understood. Furthermore, a recent study by Braeuer et al. (Braeuer et al., 2006) demonstrated that constitutively active NFκB, and not induced NFκB, leads to resistance of cancer cells to TRAIL through the increase in XIAP transcription. Other studies have identified signaling further upstream of NFκB that also mediates XIAP transcription via its NFκB binding sites. These include overexpression of Cyr61 (Lin et al., 2004), a matrix associated protein (Chen and Du, 2007; Leask and Abraham, 2006; Perbal, 2004); Che1 (Bruno et al., 2008), a DNA damage response protein (Halazonetis and Bartek, 2006; Passananti et al., 2007); MEKK3 (Samanta et al., 2004), an activator of the MAPK signaling pathway (Dhillon et al., 2007); and GSK3β (Ougolkov et al., 2007), a protein with multiple functions in development and metabolism (Kim and Kimmel, 2000; Rayasam et al., 2009). Importantly, in all the above studies, knockdown of NFκB activity or XIAP itself sensitized the cancer cells to therapy-induced apoptosis.
Data from our lab (Appendix, Fig. 9.9) have shown that both the IBC (inflammatory breast cancer) cell lines SUM149 and SUM190 have high phospho-NFκB expression, and these cells have decreased sensitivity to commonly used therapeutic agents compared to non-IBC breast cancer cell lines (Table 5&6). In IBC, NFκB activation has been shown to be highly upregulated (Lerebours et al., 2008; Van Laere et al., 2006), which may be one of the reasons for the increased therapeutic resistance of these tumors. Interestingly, a clonal population of GW583340 (a lapatinib analog)-resistant cells (Aird et al., 2010), displayed lower phospho-NFκB expression compared to the parental counterparts (unpublished). Additionally, we showed that the increase in XIAP was not transcriptional (to be discussed in Chapter 6), suggesting that in this setting, NFκB does not play a role in XIAP-mediated therapeutic resistance.

### 1.1.5.4 Translational Regulation of XIAP

Unlike most cellular mRNAs, XIAP can be regulated at the translational level as well as the transcriptional level (Holcik, 2003). In 1999, Robert Korneluk’s group identified that XIAP has an unusually long 5’ UTR (1.7 kb), and this 5’ UTR contains an internal ribosomal entry site (IRES) (Holcik et al., 1999) (Fig. 1.1). IRES elements are generally thought of as viral elements (Balvay et al., 2009); however, a handful of cellular RNAs important for the survival of cells have also been identified to have these elements in their 5’ UTRs (Stoneley and Willis, 2004). Importantly, a handful of studies, including those done in our lab (Chapter 6), have now shown that XIAP can be translated during periods of cellular stress (serum starvation, irradiation, therapeutic treatment) to overcome apoptosis normally induced by these agents (Aird et al., 2010; Holcik et al.,
Moreover, it has been shown that transient transfection of XIAP with the IRES element, and not a construct without the 5’ UTR, is better able to protect cells from apoptosis induction (Holcik et al., 1999). This indicates the regulation of the XIAP IRES is highly significant in the context of therapeutic resistance.

Although it has been over 10 years since the XIAP IRES was first identified, there is an ongoing debate about whether the XIAP IRES is active because several studies have shown that there is a cryptic promoter in the bicistronic plasmids that have traditionally been used to study this phenomenon in cell cultures (Baranick et al., 2008; Bert et al., 2006; Saffran and Smiley, 2009). However, recent evidence has suggested that XIAP has two 5’ UTRs, one short (323 nt) and one long (1.7 kb) (Gu et al., 2009; Riley et al.), and more stringent experiments (Van Eden et al., 2004) have demonstrated that the XIAP IRES is in fact actively translated during periods of cellular stress (Riley et al.). A recent study from our lab (Chapter 6) has shown that in GW583340-resistant IBC cells, the XIAP IRES is more active than in their parental counterparts (Aird et al., 2010), and studies are currently ongoing to elucidate the mechanism of XIAP IRES upregulation.

Very little is known about the regulation of the XIAP IRES in the context of dying cells and therapeutic resistance. No mutations have been identified in this sequence (Holcik et al., 2000b), and only a few RNA binding proteins have been shown to physically interact with the XIAP IRES to positively or negatively regulate its activity (Gu et al., 2009; Holcik et al., 2003; Holcik and Korneluk, 2000; Lewis et al., 2007). La autoantigen, the first RNA binding protein demonstrated to bind to the XIAP IRES, is
cleaved when cells are undergoing apoptosis (Ayukawa et al., 2000; Rutjes et al., 1999), which does not allow its nuclear translocation. Therefore, it is abundant in the cytoplasm where it can bind to the XIAP IRES and facilitate XIAP translation to inhibit cell death. Although there is no evidence to associate increased La autoantigen-XIAP IRES binding with therapeutic resistance, inhibition of this interaction decreases XIAP IRES activity, which may be a novel method to induce apoptosis in drug resistant cells with high XIAP IRES mediated translation.

Another known RNA binding protein, hnRNPC1/2, has also been shown to accumulate in the cytoplasm in pre-apoptotic cells (Lee et al., 2004) where it can bind to the XIAP IRES (Holcik et al., 2003) to potentially overcome the death signals. No research has yet been conducted to correlate hnRNPC1/2 expression and drug resistance in cancer, but one recent study in a neuronal model has indicated that there is increased hnRNPC1/2 expression in ischemic brains, which led to increased XIAP expression (Spahn et al., 2008). This report indicates that hnRNPC1/2 can be induced during cellular stress, which may lead to increased XIAP IRES-mediated translation and therapeutic resistance of cancer cells.

Finally, MDM2 has been shown to positively regulate the IRES-mediated translation of XIAP (Gu et al., 2009). MDM2, which is often overexpressed in cancers (Leach et al., 1993; Lianes et al., 1994; Muthusamy et al., 2006; Reifenberger et al., 1993) and is known to destabilize p53 (Fuchs et al., 1998), can lead to therapeutic resistance of cancer cells (Cocker et al., 2001; Gu et al., 2002; Gu et al., 2009; Hayashi et al., 2006; Johnston et al., 1997; Kondo et al., 1995; Suzuki et al., 1998). Although most
of the work in therapeutic resistance has been to look at the interaction between MDM2 and p53, MDM2 is also dephosphorylated by the cyclin G1-PP2A complex during times of cellular stress (Meek and Knippschild, 2003), and this dephosphorylated form remains in the cytoplasm where it can interact with the XIAP IRES to cause resistance to therapeutic agents. Blockade of this interaction in vitro can lead to cell death. Currently, MDM2 antagonists, such as nutlin-3a (Shangary and Wang, 2009; Vassilev, 2007), are being used in preclinical models and clinical trials. In vitro, it has been demonstrated that nutlin-3a is able to overcome resistance to therapeutic agents (Kojima et al., 2006; Michaelis et al., 2009; Peirce and Findley, 2009; Van Maerken et al., 2009), and it is interesting to speculate whether this is in part due to the disruption of the MDM2-XIAP interaction. More work needs to be done to identify whether this is a potential mechanism of the MDM2 small molecule inhibitors.

In addition to the three known positive regulators of the XIAP IRES, one RNA binding protein, hnRNP A1, has been shown to negatively regulate XIAP IRES-mediated translation (Lewis et al., 2007). Like La autoantigen and MDM2, hnRNP A1 is localized in the cytoplasm during periods of cellular stress (Allemand et al., 2005; van der Houven van Oordt et al., 2000) and also binds to the core RNP binding site [-34 to – 62 (Holcik and Korneluk, 2000)]; however, it does not compete with La autoantigen for binding, suggesting that its regulation of the XIAP IRES occurs through another mechanism. Interesting, hnRNP A1 positively regulates the FGF2 IRES (Bonnal et al., 2005) and in addition to the XIAP IRES, negatively regulates the IRES elements of VEGF, c-myc, and apaf-1 (Bonnal et al., 2005; Cammas et al., 2007). The mechanism of how and why this
RNA binding protein differentially regulates these IRES elements is still unknown. Further research to understand how these cellular RNA binding proteins can regulate the XIAP IRES may lead to novel therapeutic strategies to avoid increased XIAP expression during cellular stress.

1.1.5.5 Post-Translational Regulation and XIAP Stability

In addition to both the transcriptional and translational regulation of XIAP, it can also be regulated post-translationally, and most of the mechanisms of post-translational mechanism lead to a decrease in autoubiquitination and subsequent degradation of the XIAP protein via the proteasome. The stabilization of XIAP increases its half-life and can lead to resistance to apoptosis-inducing agents.

One of the mechanisms to overcome XIAP autoubiquitination is phosphorylation. Two studies have demonstrated that XIAP can be phosphorylated; one demonstrated that XIAP can be phosphorylated at serine 87 by AKT1 and AKT2 (Dan et al., 2004) and the other by Raf-1 (Tian et al., 2006). This phosphorylation is able to decrease the autoubiquitination of XIAP in the presence of different apoptotic triggers, leading to decreased drug-induced apoptosis. Because AKT is highly active in many cancers, XIAP stabilization via AKT could be a potent anti-apoptotic mechanism. Additionally, many signaling pathways that are dysregulated in cancer cells feed into the AKT pathway, which could modulate the stability of XIAP. For instance, it was recently shown that overexpression of cFLIPs is able to increase XIAP stability via the AKT pathway (Kim et al., 2008), leading to resistance of cells to TRAIL-induced apoptosis. This interaction
between AKT and XIAP could potentially greatly influence the ability of cancer cells to resist the apoptotic-inducing effects of therapeutic agents.

Other proteins have also been shown to increase XIAP stability through decreasing its ubiquitination, such as PKC and Notch. Although it is still unclear how PKC directly regulates XIAP stability, Shi et al. (Shi et al., 2005) demonstrated that PMA, a potent activator of PKC, inhibits ubiquitination induced by TRAIL treatment. Interestingly, this effect was independent of the AKT pathway. Further studies need to be done to determine whether PKC can bind to XIAP and whether it phosphorylates XIAP to inhibit its ubiquitination. However, it is clear that PKC-mediated stabilization of XIAP leads to drug resistance (Bourguignon et al., 2009; Clark et al., 2003; Shi et al., 2005; Trauzold et al., 2001), and inhibition of this phenomenon may lead to sensitization of resistant cancer cells.

Notch-1 has also been recently shown to increase stability of the XIAP protein. Liu et al. (Liu et al., 2007) showed that the half-life of the XIAP protein increased by 4-fold by Notch-1 overexpression. The authors went on to show a direct interaction between the TAD domain of Notch-1 and the RING domain of XIAP, which decreased binding accessibility to E2 ligases, thereby stabilizing the XIAP protein. Although little is currently known about the implications of this interaction, Notch expression has been shown to increase drug resistance (Gu et al.; Lee et al., 2008a; Mehta and Osipo, 2009; Nefedova et al., 2004), and targeting Notch has become a promising strategy, especially to kill cancer stem cells (Rizzo et al., 2008). Interestingly, Notch expression has been shown to modulate the expression of survivin, a member of the IAP family, in a very...
aggressive subset of breast cancer cells (Lee et al., 2008a). Although the authors did not see a change in XIAP protein expression upon Notch transfection, survivin and XIAP potentially form complexes [discussed below (Dohi et al., 2004; Dohi et al., 2007)], and this complex may lead to chemoresistance of these cells. Further studies need to be performed to understand whether targeting Notch in cancer cells would disrupt this complex to lead to apoptosis.

As mentioned above, studies have shown that XIAP and survivin may form a complex, which increases when cells are stressed by therapeutic agents and stabilizes XIAP to prevent apoptosis (Dohi et al., 2004; Dohi et al., 2007). However, another study was unable to detect this complex in the absence of XAF-1 (Arora et al., 2007), a cellular XIAP inhibitor. It is still unclear why there is discordance between these two studies. Both studies demonstrated that survivin degradation can lead to XIAP destabilization, which promotes apoptosis. In contrast, studies from our lab have indicated that inhibition of survivin using siRNA cannot sensitize resistant cells to trastuzumab, a monoclonal antibody to ErbB2, or a lapatinib analog (GW58334), a dual ErbB1/2 tyrosine kinase inhibitor, whereas inhibition of XIAP using siRNA can sensitize cells (Aird et al., 2008) (Chapter 4&5). These results indicate that the XIAP-survivin complex is not the only regulator of XIAP stability, and further research needs to be done to understand how and when inhibition of this complex could lead to apoptosis of drug resistant cancer cells.

It is important to note that increased XIAP stability is not always observed when cells are resistant to therapeutic agents. Data from our lab (Chapter 5) indicates that in GW583340-resistat cells, which have XIAP overexpression, the stability of XIAP is the
same as in the parental counterparts. Thus, only targeting the stability of XIAP may not fully sensitize cells to therapeutic agents.

1.1.6 Multiple Pro-Survival Functions of XIAP

It is well known that XIAP can bind to and inhibit the activity of caspases 3, 7, and 9 (Liston et al., 2003), which can lead to resistance to apoptosis-inducing therapeutic agents (Dean et al., 2007; Devi, 2004; Holcik et al., 2001; Schimmer et al., 2006). Recently, more attention has been paid to the pro-survival and signaling effects of XIAP, which will be discussed below and are illustrated in Figure 1.2.

1.1.6.1 XIAP Activates AKT

Two reports in 2001 demonstrated that in two different cellular models, granulosa cells and ovarian cancer cells, XIAP can affect the activation of AKT (Asselin et al., 2001a; Asselin et al., 2001b). Using XIAP sense and antisense adenoviral vectors, Asselin et al. showed that overexpression of XIAP can lead to increased phosphorylated AKT. Further, decreased XIAP expression decreased p-AKT and caused AKT cleavage in ovarian cancer cells (Asselin et al., 2001a). Similar results were seen in a study from our lab (Chapter 4), where XIAP siRNA significantly decreased p-AKT expression in SUM190 IBC cells (Aird et al., 2008). However, until recently, the mechanism of XIAP regulation of AKT activation was not known. Last year, Van Themsche et al. showed that XIAP can ubiquitinate PTEN (Van Themsche et al., 2009), a phosphatase that negatively regulates AKT by dephosphorylation (Simpson and Parsons, 2001). Ubiquitination of PTEN caused nuclear localization, which disallowed regulation of AKT. Moreover, when XIAP was knocked down, the majority of PTEN was expressed
in the cytoplasm. Both increases in AKT and decreases in PTEN have been shown to cause resistance to therapeutic agents (Mellinghoff et al., 2007; Uzoh et al., 2009; Yuan and Cantley, 2008), including trastuzumab (Clark et al., 2002; Fujita et al., 2006; Koninki et al.; Nagata et al., 2004), an ErbB2 monoclonal antibody (to be discussed more later). Although most studies have not looked at the effect of XIAP on AKT activity or PTEN decrease, it is interesting to speculate that XIAP may play a major role in these areas.

1.1.6.2 XIAP Activates NFκB

It is well known that XIAP is transcriptionally regulated by NFκB (discussed in Section 1.1.5.3). Interestingly, XIAP can also regulate NFκB. The first report to demonstrate the regulation of NFκB by XIAP was done in 2000 by Hofer-Warbinek et al. (Hofer-Warbinek et al., 2000). The authors showed that XIAP overexpression leads to increased NFκB reporter activity and increased nuclear translocation of p65 (Rel A) (Hofer-Warbinek et al., 2000), part of the NFκB DNA binding complex (Moynagh, 2005). This was shown to occur through a decrease in IκBα, a negative regulator of NFκB (Moynagh, 2005), although the mechanism of how this occurs is not known. Further studies confirmed these results (Levkau et al., 2001), and showed that the RING domain of XIAP (the E3 ubiquitin ligase) is necessary for NFκB activation (Lewis et al., 2004). The precise details of the mechanism of this activation are not currently clear. Some studies have indicated that TAK1, a MAPKKK (Yamaguchi et al., 1995), is ubiquitinated by XIAP (Kaur et al., 2005), thereby linking activation of the TGFβ signaling pathway to activation of NFκB. Other studies have shown that XIAP binds to and ubiquitinates MEKK2, which binds to IκBβ to cause biphasic NFκB activation, or a
“second wave” (Winsauer et al., 2008). Additionally, XIAP can ubiquitinate COMMD1 (Burstein et al., 2004), a negative regulator of NFκB (Maine and Burstein, 2007). Finally, a recent study has shown that XIAP-survivin complexes (discussed in Section 1.1.5.5) can increase NFκB activation (Mehrotra et al., 2010). However XIAP activates NFκB, it is clear that upregulation of activated NFκB and translocation of NFκB into the nucleus increases drug resistance (Arlt and Schafer, 2002; Mayo and Baldwin, 2000; Plantivaux et al., 2009; Xia et al., 2010), including resistance to lapatinib, which will be discussed further in Section 1.2.6. In fact, inflammatory breast cancer (IBC; the model used in this dissertation) has been shown to have increased NFκB activation compared to non-IBC tumors {Van Laere, 2006 #254; Van Laere, 2007 #893} (discussed in Section 1.2.3). It is therefore interesting to speculate whether this is due to increased XIAP expression.

1.1.6.3 XIAP Modulates Reactive Oxygen Species

Recent evidence has demonstrated that XIAP can inhibit oxidative stress-mediated apoptosis (Wang et al., 2004) via induction of antioxidants (Kairisalo et al., 2007; Resch et al., 2008; Zhu et al., 2007). In particular, these studies have reported that overexpression of XIAP increases superoxide dismutase 2 (SOD2), thioredoxin 2 (Trx2) both in vitro (neuronal cells) (Kairisalo et al., 2007) and in vivo (Zhu et al., 2007). Further, Resch et al. showed that SOD2, Trx2, NAD(P)H dehydrogenase [quinone] 1 (NQO1), and heme oxygenase 1 (HO-1) are decreased in XIAP knockout cells. Only one study showed the mechanism of XIAP-induced antioxidant expression, via NFκB (Kairisalo et al., 2007), but others speculated that it is either through NFκB or activation
of AP-1, both of which are known to be activated by XIAP expression (Kaur et al., 2005; Levkau et al., 2001).

XIAP can also modulate reactive oxygen species through its ability to regulate copper homeostasis (Brady et al.). A recently study has shown that XIAP can bind to COMMD1 (also known as MURR1) and ubiquitinate it, which causes and increase in intracellular copper levels (Burstein et al., 2004; Mufti et al., 2007). Additionally, XIAP can bind to and ubiquitinate CCS (copper chaperone for superoxide dismutase), leading to specific activation of superoxide dismutase 1 (SOD1) (Brady et al.), a major cellular antioxidant (to be discussed in detail in Chapter 7), although the mechanism by which ubiquitinated CCS leads to increased SOD1 activation is not yet clear.

Although these studies have all been done in normal cells, if this signaling pathway is active in cancer cells, this could have huge implications in drug resistance. Increased antioxidant expression has been shown to lead to drug resistance (Balendiran et al., 2004; Brown et al., 2009; Cho et al., 2008; Hour et al., 2004; Kalinina et al., 2001; Kalinina et al., 2006) through a decrease in chemotherapy-induced oxidative stress (Agostinelli and Seiler, 2006).
Figure 1.2: Multiple pro-survival and anti-apoptotic functions of XIAP.

Besides inhibiting caspase, XIAP has been shown to be a potent pro-survival signaling molecule. Through its RING domain (the E3 ubiquitin ligase, XIAP can ubiquitinate many different proteins (shown above), which leads to changes in downstream survival and apoptosis pathways. Additionally, XIAP can regulate a variety of other proteins, although the mechanism of this is not yet known.
Inflammatory Breast Cancer

1.1.7 Clinicopathological Features of Inflammatory Breast Cancer

Inflammatory breast cancer (IBC) is an aggressive, highly invasive tumor, with the worst clinical outcome among breast cancers that was first described in 1924 (Lee and Tannenbaum, 1924). IBC is defined by the invasion and growth of tumor emboli in dermal lymphatics. Contrary to the earlier belief that IBC is simply a neglected locally advanced breast cancer (LABC), it is now regarded clinicopathologically as distinct from neglected LABC based on aggressive features such as rapid clinical progression (Anderson et al., 2003) and resistance to chemotherapy and radiotherapy (Chu et al., 1980; Rouesse et al., 1986). Prognosis for women diagnosed with IBC is poor with only a 26.7% 10 year overall survival rate compared to 44.8% for non-IBC LABC tumors (Low et al., 2002). Furthermore, women are often diagnosed at a younger age (58 yo for IBC vs. 63 yo for non-IBC), have higher grade tumors at diagnosis (RR of 3.1 for IBC vs. 1.6 in non-IBC), and more lymph node involvement (RR of 4.7 vs. 2.5) (Hance et al., 2005). These characteristics are attributed to estrogen receptor (ER) negativity, which constitutes 80% of IBC tumors (Van den Eynden et al., 2004), and ErbB2 positivity (42.3% IBC vs. 17.5% non-IBC) (Van den Eynden et al., 2004). Loss of ER and expression of ErbB2 have both been previously shown to correlate with lower disease-free survival in breast cancer (Kaufmann, 1996; Slamon et al., 1987). Even with neoadjuvant and multimodality treatment, disease-free survival is still poor (Hortobagyi
et al., 1988; Merajver et al., 1997), and there is an unmet clinical need for new therapeutic strategies.

1.1.8 IBC Treatment Strategies

Although IBC was first described in the early 1900’s (Lee and Tannenbaum, 1924), to date there is still currently no specific IBC therapy (Woodward and Cristofanilli, 2009). However, over the past 3 decades, response and survival have improved due to advances in multimodality treatments (Dawood et al., 2008; Giordano and Hortobagyi, 2003; Woodward and Cristofanilli, 2009). In the 1970’s, doxorubicin-based therapies were used for patients with IBC, and 5-year overall survival rates were exceedingly poor (27%) (Thoms et al., 1989). In the late 1970’s, anthracyclines were introduced into primary systemic regimens, and survival significantly improved (44%) (Baldini et al., 2004). Currently, chemotherapeutic regimens for patients with IBC include: cyclophosphamide, doxorubicin, 5-fluoruracil, and paclitaxel (Dawood et al., 2008; Woodward and Cristofanilli, 2009). In addition to chemotherapeutics, targeted agents are being used more frequently for patients with IBC. Due to the specific biology of IBC tumors and the lower toxicity of these agents, they present a novel strategy for treatment of IBC. These include: ErbB-targeting agents (trastuzumab, lapatinib) and angiogenesis inhibitors (bevacizumab, semaxinib). Although many studies using these agents have not separated overall survival results of IBC and non-IBC patients, combination of these agents with chemotherapeutics has greatly increased response and survival of LABC patients in general (Dawood et al., 2008; Overmoyer, 2010).
In addition to combinatorial chemotherapeutic regimens, locoregional therapy including surgery and radiation is highly important for patients with IBC to improve outcome and overall survival (Singletary, 2008; Woodward and Buchholz, 2008; Woodward and Cristofanilli, 2009). Mastectomy has been shown to be especially important for patients who are responders to neoadjuvant chemotherapy (Fields et al., 1989; Fleming et al., 1997), and the cleaner the margins, the better the outcome for patients (Curcio et al., 1999). Finally, radiation therapy is aggressively used for IBC patients. In fact, many institutes now give twice-daily doses up to a total of 60-66 Gy (Liao et al., 2000; Liauw et al., 2004; Pisansky et al., 1992). Along with chemotherapy, surgery and radiation can significantly increase overall survival and disease-free survival of patients with IBC (Singletary, 2008).

1.1.9 Apoptotic Dysregulation in IBC

Recent studies comparing the molecular characteristics of IBC vs. non-IBC tumors (summarized in Table 1) have shown that apoptotic dysregulation may be one key differences between IBC and other LABCs. Nguyen, et al. have shown that an apoptotic factor is increased in IBC tumors vs. non-IBC tumors (Nguyen et al., 2006). In addition, another study demonstrated increased expression of ALS2CR2 (Bertucci et al., 2004), which interacts with XIAP to inhibit JNK-mediated apoptosis (Sanna et al., 2002). Moreover, numerous microarray studies comparing IBC to non-IBC tumors have shown that activation of the NFκB pathway is common in IBC tumors (Van den Eynden et al., 2004; Van Laere et al., 2007a; Van Laere et al., 2005; Van Laere et al., 2006; Van Laere et al., 2007b). Importantly, many of the IAP family members are transcriptional targets
of the NFκB transcription factor (Deveraux and Reed, 1999) (discussed in Section 1.1.5.3), and XIAP can even act upstream of NFκB (Hofer-Warbinek et al., 2000; Jin et al., 2009; Levkau et al., 2001) (discussed in Section 1.1.6.2). These studies indicate that apoptotic dysregulation may play a central role in the pathogenesis, aggressiveness, and resistance of IBC cells to therapeutic agents. Therefore, understanding the mechanisms of apoptotic dysregulation would be important both to understand the etiology of the disease and to identify novel therapeutic strategies for IBC patients.
Table 1: Recent studies evaluating non-IBC vs. IBC tumor samples that demonstrate apoptotic dysregulation.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Gene/Pathway</th>
<th>Patient Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nguyen DM, et al., Clin Cancer Res: 2006; 12(17).</td>
<td>Increased Bel-2 family member</td>
<td>89 IBC patients (84% naïve samples)</td>
</tr>
<tr>
<td>Bertucci F, et al., Cancer Res: 64, 8558-8565.</td>
<td>Increased ALS2CR2 expression</td>
<td>37 IBC patients all pretreatment biopsies</td>
</tr>
<tr>
<td>Boersma BJ, et al., Int J Cancer:122,1324-1332.</td>
<td>Cell growth, G2M transition</td>
<td>52 IBC patients (most on neoadjuvant therapy)</td>
</tr>
<tr>
<td>Turpin E, et al., Oncogene:49, 7593-97.</td>
<td>Up to 60% mutated p53</td>
<td>161 IBC patients</td>
</tr>
<tr>
<td>Van Laere S, et al., Breast Cancer Res and Treatment:93, 237-246.</td>
<td>Activated NFkB</td>
<td>16 IBC patients all pretreatment biopsies</td>
</tr>
<tr>
<td>Wang J, et al., Human Pathology: 2010.</td>
<td>80% XIAP positivity</td>
<td>42 patients with invasive triple-negative disease</td>
</tr>
</tbody>
</table>
1.1.10 ErbB2 Signaling in Inflammatory Breast Cancer

ErbB receptor signaling (Feldman et al., 2007) is largely regulated by a family of peptide ligands (epidermal growth factor family members) that bind to their cognate ErbB receptors (ErbB1, ErbB3, ErbB4), with ErbB2 lacking an exogenous ligand (Hynes and Lane, 2005). Binding of ligand to its cognate ErbB receptor(s) triggers the formation of receptor homo- or heterodimers (with ErbB2 the preferred heterodimeric partner) and induces autophosphorylation of tyrosine residues within the cytoplasmic tail of the receptor. Lacking an exogenous ligand, ErbB2 is transactivated by its heterodimeric partner and subsequently phosphorylated. ErbB2 contains six tyrosine autophosphorylation sites that serve as docking sites for phosphotyrosine-binding domain and/or Src homology 2 (SH2) containing proteins, which in turn activate downstream MAPK-Erk1/2 and PI3K-Akt proliferation and survival signaling pathways (Hynes and Lane, 2005). Both pathways in turn activate the anti-apoptotic signaling pathway including XIAP and survivin (Asanuma et al., 2005; Jin and El-Deiry, 2005). Therefore, downregulation of this signaling pathway may be able to overcome the apoptotic dysregulation seen in IBC cells.

1.1.11 ErbB2 Targeting Agents

Increased overexpression of ErbB2 in IBC patients has made it an attractive therapeutic target in this patient population. Due to the high overexpression of ErbB2 in many breast cancers, the ErbB2 receptor has been an important target for cancer therapeutics. There are only two ErbB2 targeting agents used in the clinic to date:
Trastuzumab (Herceptin) is an FDA-approved humanized monoclonal antibody, which binds to the extracellular domain of the ErbB2 receptor. Although this is an immune strategy and trastuzumab causes an immune-mediated anti-tumor effect (antibody dependent cellular cytotoxicity, ADCC) (Carter et al., 1992; Clynes et al., 2000), there is still much debate about the exact anti-cancer activity of trastuzumab. In addition to ADCC, trastuzumab has been shown to down-regulate the receptor (Baselga et al., 2001; Sliwkowski et al., 1999), cause G1 arrest (Lane et al., 2001), inhibit downstream signaling through PI3K (Nagata et al., 2004), inhibit ErbB2 ectodomain cleavage (Molina et al., 2001), and suppress angiogenesis (Hardee et al., 2009; Izumi et al., 2002; Klos et al., 2003; Petit et al., 1997). To date, the anti-signaling effects of trastuzumab on cancer cells remain somewhat unclear. Trastuzumab has been used as a monotherapy for patients with ErbB2-overexpressing breast cancers (Cobleigh et al., 1999) and is used in various combinations, with objective response rates between 15 and 26% (Cobleigh et al., 1999; Vogel et al., 2002). However, subsets of women with ErbB2 overexpressing tumors do not respond and resistance is common (Nahta and Esteva, 2006; Nahta et al., 2006).

Lapatinib (Tykerb) is an FDA-approved small molecule inhibitor that reversibly binds to the ATP pocket of ErbB1 and ErbB2, thereby inhibiting phosphorylation and downstream signaling through PI3K/AKT and MAPK (Xia et al., 2002). Recent data suggest that lapatinib’s mechanism of action is more multifactorial than was first appreciated. Studies have demonstrated that lapatinib affects survivin stability (Xia et al., 2006b), multidrug resistance proteins (Dai et al., 2008), efflux and uptake transporters
(Polli et al., 2008), and cell metabolism (Spector et al., 2007). In a recent clinical trial, Lapatinib showed a 50% clinical response rate in ErbB2-overexpressing IBC patients (Spector et al., 2006); however, acquired resistance is a common outcome even in those patients who show an initial clinical response (Burris et al., 2005; Chen et al., 2008). Therefore, understanding the mechanisms of acquired lapatinib resistance in IBC cells is highly significant.

1.1.12 Resistance to ErbB2 Targeting Agents

Although both trastuzumab and lapatinib have been major breakthroughs for breast cancer therapy, as mentioned above, naïve therapeutic resistance is common and even those patients initially responsive to trastuzumab or lapatinib rapidly develop acquired resistance. Therapeutic resistance to trastuzumab has been well characterized by many labs and just as trastuzumab has been shown to have many anti-tumor effects, resistance to trastuzumab is also varied. Loss of PTEN (Nagata et al., 2004), co-expression of insulin like growth factor receptor 1 (IGF-1R) (Lu et al., 2001), presence of truncated ErbB2 (p95) (Scaltriti et al., 2007), and expression of the cell surface associated protein Muc-1 (Nagy et al., 2005) have all been shown to contribute to therapeutic resistance to trastuzumab. Importantly, no mechanisms of resistance have been previously reported in IBC cell lines.

Therapeutic resistance to lapatinib has not been as extensively studied. Unlike trastuzumab, the presence of PTEN (Xia et al., 2007), co-expression of IGF-1R (Nahta et al., 2007), and presence of truncated ErbB2 (p95) (Xia et al., 2004) do not seem to contribute to lapatinib resistance. There are a number of known mechanisms of
resistance to lapatinib, most of which have been elucidated in ER+ breast cancer models. One is in an ER-dependent model where p-AKT is completely downregulated which lifts its repression of FOXO3a (Xia et al., 2006a), a member of the forkhead box transcription factors (Birkenkamp and Coffer, 2003). This in turn upregulates ER activity, which includes maintaining high levels of survivin, a member of the IAP family. This same model showed increased Rel A activation (Xia et al., 2010), which is a transcription factor of the IAPs [discussed in Section 1.1.5.3 (Lee and Collins, 2001)]. Another ER-positive lapatinib-resistance model showed increased activation of AXL (Liu et al., 2009b), which is another receptor tyrosine kinase (Linger et al., 2008) that has been shown to correlate with ER expression (Berclaz et al., 2001). However, because most IBC patients are ER negative (Van den Eynden et al., 2004), these models may not mimic the clinical situation for IBC patients. Finally, a study in colorectal cells (HCT116) showed that the anti-apoptotic protein Mcl-1 is increased in cells with acquired lapatinib resistance (Martin et al., 2008). The majority of these studies support the idea that dysregulation of the apoptotic signaling pathway plays a key role in the resistance of cancer cells to lapatinib.

**Research Objectives Outlined in this Dissertation**

The goals of this dissertation are to examine the sensitivity of two IBC cell lines to commonly used therapeutic agents and to understand the mechanisms apoptotic dysregulation of these cells and how this leads to therapeutic resistance.
1.1.13 **Objective 1: Evaluation of sensitivity of IBC cells to commonly-used therapeutic agents. (Discussed in Chapter 3)**

Objective 1 aims to characterize the IBC cellular model in regards to sensitivity to agents that are commonly used in the clinic to treat IBC patients. There are currently very few studies to report the sensitivity of these cell lines to various therapeutic agents; thus, these results will provide insight into the pathway dysregulation of the IBC cells. These results will be compared to breast cancer cellular models that have been well documented in the literature.

1.1.14 **Objective 2: Determination of the key apoptotic protein(s) involved in therapeutic resistance to ErbB-targeting agents in an IBC cellular model. (Discussed in Chapters 3-5)**

Objective 2 aims to elucidate the mechanism(s) of therapeutic resistance in the IBC cellular models to two ErbB-targeting agents: trastuzumab and a lapatinib analog. These studies will be the first to describe mechanisms of resistance to trastuzumab and lapatinib in IBC cells. These results will be compared and contrasted with reports on mechanisms of resistance to these agents in non-IBC cell models.

1.1.15 **Objective 3: Determination of the mechanism of apoptotic dysregulation in IBC cells resistant to ErbB2 targeting. (Discussed in Chapters 6-7)**

Objective 3 aims to elucidate the mechanism of apoptotic dysregulation that experiments in Chapters 3-5 characterize. The results will allow for a better understanding of apoptotic dysregulation in the IBC cellular model and will provide insights into novel therapeutic combinations that may be useful for IBC patients with acquired resistance to ErbB-targeting agents.
2 Materials and Methods

Cell Culture

The molecular characteristics of all cell lines used in this dissertation are summarized in Table 2. SUM149, SUM159, SUM44, and SUM190 breast cancer cells were obtained from Asterand, Inc. (Detroit, MI). SKBR3, MCF-7, MDA-MB-231, and BT474 cells were obtained from American Type Culture Collection (Manassas, VA). Ham’s F-12 nutrient mixture, McCoy’s 5a medium, Eagle’s Minimal Essential Media, and Leibovitz's L-15 media were purchased from Gibco (Carlsbad, CA). DMEM was purchased from Mediatech (Herdon, VA). Insulin, hydrocortisone, HEPES, penicillin, streptomycin, ethanolamine, transferring, sodium selenite, and EGF were all purchase from Sigma Chemical Co. (St. Louis, MO). SUM149 and SUM159 cells were routinely cultured in Ham’s F-12 nutrient mixture supplemented with 5 \( \mu \)g/ml Insulin, 1 \( \mu \)g/ml Hydrocortisone, 10 mM HEPES, 10 units/ml Penicillin, 10 \( \mu \)g/ml Streptomycin, and 5% fetal bovine serum. SUM44 and SUM190 cells were cultured in Ham’s F-12 nutrient mixture supplemented 5 \( \mu \)g/ml Insulin, 1 \( \mu \)g/ml hydrocortisone, 5 mM Ethanolamine, 10 mM HEPES, 5 \( \mu \)g/ml Transferrin, 10 nM Triiodo Thyronine, 50 nM Sodium Selenite, 10 units/ml Penicillin, 10 \( \mu \)g/ml Streptomycin and 2% fetal bovine serum. Twenty-four hours after subculturing, media was changed to serum free conditions. SKBR3 cells were routinely cultured in modified McCoy’s 5a medium with 1.5 mM L-glutamine adjusted to contain 2.2 g/L sodium bicarbonate, 10 units/ml Penicillin, 10\( \mu \)g/ml Streptomycin, and 10% fetal bovine serum. MCF-7 cells were routinely cultured in Minimum essential
medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1mM sodium pyruvate and supplemented with 0.01 mg/ml bovine insulin, 10 units/ml Penicillin, 10 µg/ml Streptomycin, and 10% fetal bovine serum. BT474 cells were routinely cultured in DMEM, 10 units/ml Penicillin, 10 µg/ml Streptomycin, and 10% fetal bovine serum. MDA-MB-231 cells were routinely cultured in Leibovitz's L-15 medium with 2 mM L-glutamine, 10 units/ml Penicillin, 10 µg/ml Streptomycin and 10% fetal bovine serum. Cells were kept in a 5% CO₂ and 95% air humidified incubator at 37°C.
Table 2: Phenotype of breast cancer cells used in this dissertation.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source</th>
<th>ErbB1</th>
<th>ErbB2</th>
<th>ER/PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUM190</td>
<td>Primary inflammatory breast</td>
<td>Low</td>
<td>Overexpressed</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUM149</td>
<td>Primary inflammatory breast</td>
<td>Activated</td>
<td>Low</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUM44</td>
<td>Malignant pleural effusion</td>
<td>-</td>
<td>Low</td>
<td>+/+</td>
</tr>
<tr>
<td>SUM159</td>
<td>Anaplastic carcinoma</td>
<td>Low</td>
<td>Low</td>
<td>-/-</td>
</tr>
<tr>
<td>SKBR3</td>
<td>Non-IBC Adenocarcinoma Pleural Effusion</td>
<td>Low</td>
<td>Overexpressed</td>
<td>-/-</td>
</tr>
<tr>
<td>BT474</td>
<td>Invasive Ductal Carcinoma</td>
<td>Low</td>
<td>Overexpressed</td>
<td>+/+</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Non-IBC Adenocarcinoma Pleural Effusion</td>
<td>Low</td>
<td>-</td>
<td>+/+</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Non-IBC Adenocarcinoma Pleural Effusion</td>
<td>Low</td>
<td>Low</td>
<td>-/-</td>
</tr>
</tbody>
</table>
2.1.1 Generation of GW583340-Resistant IBC Cell Lines

Laboratory grade lapatinib analog (GW583340; Sigma) was dissolved in DMSO. Acquired resistance to GW583340 was selected in SUM190 and SUM149 cells (referred to as rSUM190 and rSUM149) by culturing cells in normal growth media supplemented with increasing concentrations of GW583340 (0.25-2.5 µM and 0.25-7.5 µM, respectively). Initially, marked cell death and decrease in cell growth was observed in the cells. However, after two weeks of each increase in drug concentration, small colonies of viable cells were observed, which were cultured until confluence before the next increase in drug concentration. This was continued for a minimum of 3 months. From then on, both rSUM190 and rSUM149 cells were routinely cultured in 2.5 µM and 7.5 µM GW583340, respectively.

2.1.2 Generation of Stable XIAP Overexpressing and Knockdown IBC Cell Lines

SUM149 cells stably expressing wtXIAP and FG9 GFP vector control and SUM190 cells stably expression shXIAP and FG12 GFP vector control were generated using a lentiviral expression system (kindly provided by Dr. Colin Duckett, University of Michigan). Briefly, HEK294T cells were transfected using polyethylenimine with 5 µg of pHCMV, pRRE, and pRSVrev (Qin et al., 2003), which drive the expression of lentiviral structural proteins, and 5 µg of pFG9 EF1 XIAP WT silent mutation hyg, pFG9 EF1a hygro GFP (Galban et al., 2009), FG12 H1 shXIAP, and FG12 H1 sheGFP (Brady et al.). Twenty-four hours post-transfection, media was changed. Forty hours post-transfection, the virus-containing media on the HEK293T cells was collected and filtered
through a 0.45 mm Millex HV PVDF filter unit (Millipore) onto cells [with 25 mM polybrene (Sigma)] After four hours, fresh media was added and cells were incubated for an additional forty-eight hours at 37°C, 5% CO₂. Stable cells were selected by the addition of hygromycin B (Invitrogen; 200 mg/mL).

**Determination of Cell Proliferation/Viability/Apoptosis**

2.1.3 **MTT Assay**

Cells were seeded in a 96-well plate (Corning Incorporated, Corning, NY) and allowed to reach 70% confluence. Culture medium was aspirated and changed the day of treatment. After treatment with agents, culture media was aspirated and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) was added at a concentration of 5 mg/ml. Cells were incubated at 37°C until the MTT reaction caused the formation of granulated purple coloration. Excess reagent was aspirated and replaced with 100 µl DMSO (Sigma) in each well. Absorbance was read at 550 nm in a BioRad plate reader (Hercules, CA).

2.1.4 **Trypan Blue Exclusion Assay**

Cell viability was determined by the trypan blue exclusion assay. Cells were trypsinized and resuspended in medium to achieve a homozygous mixture. An aliquot of cell suspension was mixed with an equal volume of 0.4% trypan blue solution (Sigma). Cell numbers were recorded in 10 µl of the resultant mixture using a hemocytometer.
2.1.5 7-AAD Staining

For some experiments cell death was ascertained by staining with 7-AAD (Invitrogen, Carlsbad, CA) for 30 min. At least twenty-five thousand events were collected on a FACScalibur flow cytometer (Beckton Dickinson) and analyzed using Cellquest software (Beckton Dickinson).

2.1.6 TMRE Staining

After incubation to induce apoptosis, cells were harvested and incubated for 30 min with 500 nM TMRE (Molecular Probes)] at 37°C. Cells were then washed twice with 1% BSA/PBS and analyzed for fluorescence by flow cytometry. At least 25,000 events were collected on a FACScalibur flow cytometer (Beckton Dickinson) and analyzed using Cellquest software (Beckton Dickinson).

2.1.7 Annexin-V/PI Doubling Staining

Cells were treated and then stained for Annexin V and PI using the Annexin V Biotin Kit (Beckman Coulter, Fullerton, CA) as per the manufacturer’s instructions. Total cell death is presented as the sum of Annexin V+/PI-, Annexin V+/PI+, and Annexin V-/PI+ cells. Alternatively, for some experiments cell death was ascertained by staining with 7-AAD (Invitrogen, Carlsbad, CA) for 30 min. At least 25,000 events were collected on a FACScalibur flow cytometer (Beckton Dickinson) and analyzed using Cellquest software (Beckton Dickinson).
2.1.8 Cell Cycle Analysis

Cells were treated to induce apoptosis and harvested. Cells were then centrifuged for 3 min at 1200 rpm and washed twice with PBS. Cells were fixed overnight at 4 °C in 70% EtOH. The next day, cells were washed twice with 1% BSA/PBS and then incubated with 1 ml propidium iodide (PI) staining solution (0.5 µg/ml PI, 10 µM EDTA, 0.5 µg/ml RNAse A, and 0.06% Triton-X 100) for 1 h at room temperature. Cells were again washed twice with 1% BSA/PBS and analyzed by flow cytometry.

2.1.9 Nucleosome Enrichment ELISA

Cells were seeded in 96-well plates (Corning Incorporated). Embelin (50 µM) and staurosporine (5 µM) were made in regular growth media. DMSO (at the same concentration as drug treatments) was used as a vehicle control. After 20 h incubation, nucleosome enrichment was determined by the Cell Death Detection ELISAPLUS (Roche Applied Science; Mannheim, Germany) as per the manufacturer’s instructions. Nucleosome enrichment was calculated by: (mU sample – Blank)/ (mU untreated – Blank) *100.

2.1.10 Caspase Activity Assays

Cells were seeded in 6-well plates (Corning Incorporated), and the next day cells were treated with embelin (50 µM) for 4 h in regular growth media. DMSO (at the same concentration as embelin) was used as a vehicle control. After incubation, caspase 9 activity was determined in 3 µg total cell lysates using the Caspase-Glo® Assay
(Promega) as per the manufacturer’s instructions. Peak light intensity of treatment wells was normalized to DMSO.

**Western Immunoblot Analysis**

Cells were harvested and immediately lysed in NP40 cell lysis buffer (BioSource, Camarillo, CA) with fresh protease inhibitor cocktail (Sigma) and 1 mM PMSF. Protein concentration was determined by the Pierce BCA Protein Assay Kit (Rockford, IL). Equal amounts of cell lysates were then subjected to SDS-PAGE under reducing conditions. Before loading onto the gel all lysates were boiled for 5 min and immediately cooled on ice. The protein was transferred onto Immobilon-P membrane (Millipore, Billerica, MA) previously soaked in methanol and transfer buffer by the TRANS-BLOT SD semi-dry transfer cell (BioRad). After the transfer process was complete, the membranes were allowed to dry, resoaked in methanol, and incubated with blocking buffer (5% dry nonfat milk in 1 X TBS-0.1% Tween 20) for 1 h at room temperature. Membranes were incubated with primary antibodies (listed in Table 3) overnight at 4°C. Membranes were washed three times with wash buffer (1 X TBS-0.1% Tween 20) for 10 min each and subsequently incubated with appropriate secondary antibody conjugated with horseradish peroxidase (1:1000 dilution) for 1 h at room temperature. Membranes were again washed three times in wash buffer for 15 min each and bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Signals were developed after exposure to Kodak BioMax XAR X-ray film (X-Omat films, Eastman Kodak Co., Rochester, NY). Actin or GAPDH immunodetection was conducted to serve as a loading control. This was done by stripping the same membrane in stripping buffer
[100 mmol/L 2-mercaptoethanol -2% SDS -62.5 mM Tris-HCl (pH 6.7)] at 50°C for 30 min followed by washing and blocking procedure as described above. Densitometric analysis was performed using the NIH ImageJ software.
Table 3: Antibodies used in this dissertation.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Cat. #</th>
<th>Amount Protein</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Santa Cruz</td>
<td>sc-1615</td>
<td>n/a</td>
<td>1:2000</td>
</tr>
<tr>
<td>AKT</td>
<td>Cell Signaling</td>
<td>9272</td>
<td>20 µg</td>
<td>1:1000</td>
</tr>
<tr>
<td>AMPK</td>
<td>Cell Signaling</td>
<td>2532</td>
<td>20 µg</td>
<td>1:1000</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Santa Cruz</td>
<td>sc-7382</td>
<td>20 µg</td>
<td>1:1000</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Cell Signaling</td>
<td>9662</td>
<td>20 µg</td>
<td>1:1000</td>
</tr>
<tr>
<td>ErbB1</td>
<td>Cell Signaling</td>
<td>2232</td>
<td>20 µg</td>
<td>1:1000</td>
</tr>
<tr>
<td>ErbB2</td>
<td>Cell Signaling</td>
<td>2242</td>
<td>20 µg</td>
<td>1:1000</td>
</tr>
<tr>
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<tr>
<td>GAPDH</td>
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<td>1:1000</td>
</tr>
<tr>
<td>p-AKT (Ser473)</td>
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<td>4051</td>
<td>20 µg</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-AMPK (Thr172)</td>
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<td>20 µg</td>
<td>1:1000</td>
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<tr>
<td>pan-p-Tyr</td>
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<td>05-321X</td>
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<tr>
<td>p-ErbB2 (Tyr877)</td>
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<td>2241</td>
<td>20 µg</td>
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<tr>
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<td>Cell Signaling</td>
<td>9101</td>
<td>20 µg</td>
<td>1:1000</td>
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<tr>
<td>p-NFkB (Ser536)</td>
<td>Cell Signaling</td>
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<tr>
<td>Procaspase 9</td>
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<td>MS-1145-P0</td>
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</tr>
<tr>
<td>SOD2</td>
<td>BD Biosciences</td>
<td>611580</td>
<td>5 µg</td>
<td>1:1000</td>
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<tr>
<td>Survivin</td>
<td>R&amp;D Systems</td>
<td>AF886</td>
<td>5 µg</td>
<td>1:2000</td>
</tr>
<tr>
<td>XIAP</td>
<td>BD Biosciences</td>
<td>610762</td>
<td>5 µg</td>
<td>1:2000</td>
</tr>
</tbody>
</table>
**Real Time RT-PCR**

RNA was prepared from different cells using the RNeasy minikit (Qiagen, Valencia, CA) with a DNase step. Two micrograms of RNA in twenty microliters was converted to cDNA using iScript cDNA Synthesis (BioRad). Quantitative real-time PCR was performed in a 20 µl reaction mixture containing 10 µl 2 X Power SYBR Green PCR Master Mix (ABI Applied Biosystem; Foster City, CA), 200 nM each primer and 2 µl cDNA product. Amplification was carried out in ABI 7300 real time PCR instrument. After initial denaturation (50°C, 2 min, 10 min at 95°C), amplification was performed with 40 cycles of 15 s at 95°C and 60 s at 60°C. β-actin was used as an internal control. ΔΔCT shows the difference between actin control and survivin or XIAP. Folds (2^ΔΔCT) represent changes normalized to a separate reference cell line (SKBR3). The primers were designed to target XIAP: 5’F-GCAGGGTTTCTTTATCTGG-3’, 5’R-TGTCCCTTTCTGTTCTAAACAG-3’, survivin: 5’F-GCATGGGTGCCCGACGTTG-3’, 5’R-GCTCCGGCCAGAGGCTCAA-3’, and β-actin: 5’F-TCACCACACTGTGCCATCTACGA-3’, 5’R-CAGCGG-AACCGCTCATTGCCAATGG-3’.

**Trastuzumab Binding Assay**

Cells were collected and washed in 100 µl 1% BSA-PBS (per 3 x 10^5 cells) and subsequently incubated in 10 µg/ml trastuzumab on ice for 30 min. Cells were washed twice with 1% BSA-PBS in 3.5ml and suspended 100 µl 1% BSA-PBS. Cells were then incubated with 3 µl of anti-human IgG-PE on ice for 30 min. Cells were washed twice as
before and resuspended in 250 µl of 1% BSA-PBS for analysis by flow cytometry. 25,000 events were collected on a FACScalibur flow cytometer (Becton Dickenson, San Jose, CA) and analyzed using Cellquest software (Becton Dickenson).

**Antibody-Dependent Cellular Cytotoxicity Assay**

Healthy human PBMC were obtained under a Duke IRB-approved protocol and used as effector cells. The effector cells were incubated with IL-2 (1000 U/ml) overnight at 37°C. Target cells (1 x 10⁶) were labeled with 100 µCi chromium for 1 h at 37°C and washed three times with PBS and incubated with 10µg/ml trastuzumab for 1 h at 37°C. Target cells were then incubated with effector cells at a E:T ratio of 80:1 for 4 h at 37°C. After incubation, cells were spun down and supernatant radioactivity was quantified using a liquid scintillation and luminescence counter (model: 1450 MicroBeta Trilux; Wallac, Waltham, MA).

**Transfection of Cells with siRNA**

2.1.11 Transfection of Survivin siRNA

Survivin-specific siRNA and its appropriate nonsilencing control were designed as previously reported (24) and synthesized by Qiagen. Survivin siRNA or control siRNA (Cont. siRNA 3) was transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA; Vehicle 2) as per manufacturer’s instructions. Briefly, cells were seeded into 12 well plates (Corning Incorporated) and allowed to reach 50% confluence. At that time, media was removed and replaced with 500 µl fresh normal growth media. In separate tubes, 100 µl serum-free Opti-MEM (Gibco) media was mixed with 2 µl
transfection reagent and 100 µl serum-free Opti-MEM media was mixed with 100nM siRNA and incubated at room temperature for 5 min. After incubation, tubes were mixed and further incubated for 20 min at room temperature. Mixtures were added directly to wells and plates were gently swirled to mix. Cell viability was determined by trypan blue exclusion assay at 48 h after transfection.

2.1.12 Transfection of XIAP siRNA

XIAP siRNA (Cell Signaling) was transfected into cells as per manufacturer’s instructions. Briefly, cells were seeded into 12 well plates (Corning Incorporated) and allowed to reach 50% confluence. At that time, media was removed and replaced with 500 µl fresh normal growth media. In separate tubes, 100 µl serum-free media was mixed with 2 µl transfection reagent (Vehicle 1) and incubated at room temperature for 5 min. 100 nM XIAP siRNA, negative control siRNA (Cell Signaling; Cont. siRNA 1), or GAPDH siRNA (Ambion, Austin, TX; Cont. siRNA 2) was added to tubes, mixed, and incubated at room temperature for 5 min. Mixtures were added directly to wells and plates were gently swirled to mix. Cell viability was determined by trypan blue exclusion assay at 48 h after transfection.

Immunofluorescent Microscopy

Cells were seeded onto cover slips (VWR, West Chester, PA) in dishes and allowed to reach 70% confluence. Cover slips were washed once with PBS and fixed with 100% methanol at -20°C for 20 min. After fixation, cells were blocked with 1% BSA/PBS at 37°C for 30 min and incubated with XIAP antibody (BD Biosciences) for 1
h at room temperature. Cells were then washed three times with PBS for 5 min and incubated with R-PE-labeled secondary antibody (Southern Biotech, Birmingham, AL) for 1 h at room temperature. For a nuclear counterstain, cells were incubated for 1 min with 0.1 µg/ml Hoechst 33258 (Sigma). Finally, cells were washed three times with PBS, cover slips were inverted onto slides, sealed, and imaged on a Zeiss Axio Observer inverted widefield fluorescence microscope using a 63x/1.40 DIC Plan Apochromat objective. Images were captured on a Hamamatsu ORCA ER CCD camera (Hamamatsu Corporation, Bridgewater, NJ). The system was controlled by MetaMorph Software (Molecular Devices, Downingtown, PA). XIAP staining intensity was measured by using NIH ImageJ software.

**Cloning of XIAP.IRES Plasmid**

The cloning strategy of the XIAP.IRES plasmid is illustrated in Figure 2.1. The luciferase construct was constructed by inserting the 5’UTR of XIAP (kindly provided by Dr. Martin Holcik, University of Ottawa) upstream of luciferase in the pGL3 Basic vector (Promega, Madison, WI) and a CMV promoter upstream of the 5’UTR (pGL3-XIAP.IRES). The negative control constructs [pGL3-XIAP.IRES (cont 1) and pGL3-XIAP.IRES (cont 2)] were constructed by deleting the sequence between the PstI (-800-731) and PstI/HindIII (-800-0) restriction sites, respectively. For transfection of DNA, cells were seeded in their respective media in 24-well plates (Corning Incorporated) and allowed to reach 80-90% confluency. At that time, cells were transfected with 1.5 µg pGL3-hUTR.luc and 0.5 µg pRL-TK (Promega) DNA using Lipofectamine 2000
(Invitrogen) as per the manufacturer’s instructions. Cells were incubated for 24 h and lysed for luciferase activity assay.
pBgal/hUTR/CAT (kind gift from Dr. Martin Holcik) was recloned into a luciferase-based vector. First, the XIAP IRES was cut out (using the Nhe1/Xho1 restriction sites) and inserted into a pGL3 vector (Promega), which had a CMV promoter upstream of luciferase. This served as the XIAP.IRES plasmid. For the negative controls, the sections between the PstI sites (cont 1) or the section between the PstI and the HindIII sites (cont 2) were removed.

Figure 2.1: Cloning strategy of XIAP IRES constructs and negative controls.

pβgal/hUTR/CAT (kind gift from Dr. Martin Holcik, University of Ottawa) was recloned into a luciferase-based vector. First, the XIAP IRES was cut out (using the Nhe1/Xho1 restriction sites) and inserted into a pGL3 vector (Promega), which had a CMV promoter upstream of luciferase. This served as the XIAP.IRES plasmid. For the negative controls, the sections between the PstI sites (cont 1) or the section between the PstI and the HindIII sites (cont 2) were removed.
2.1.13 Luciferase Activity Assay

Cells were lysed for 15 min in 500 µl luciferase lysis buffer (35 mg/ml Tris base, 5 mg/ml CDTA, 10% glycerol, 0.5% Triton-X 100, pH 7.8) and 25 µl of the lysate was added to a 96-well plate (Greiner Bio-One, Monroe, NC). Luciferase activity was determined using a luminometer (Turner Biosystems, Sunnyvale, CA). Firefly or renilla luciferase substrate [1 mM luciferin or coelenterazine (Gold Biotechnology, St. Louis, MO) in 15 mM MgSO$_4$, 15 mM K$_2$HPO$_4$, 4 mM EGTA, 1 mM DTT, 0.1 mM ATP] was added (100 µl) to wells and luciferase activity was read after 10 s.

**Determination of Intracellular Reactive Oxygen Species**

2.1.14 Determination of Cytoplasmic Hydrogen Peroxide-Derived Radicals

Cells were cultured in 6-well plates (Corning Incorporated) in regular growth media until they reached 70-80% confluence. Then, cells were treated with hydrogen peroxide (H$_2$O$_2$) and GW583340 1 and 24 h respectively. After incubation, cells were harvested, washed twice with 1%BSA/PBS, and incubated for 30 min with 10 µM carboxy-H$_2$-DCFDA (Molecular Probes) at 37°C. Cells were then washed twice with 1% BSA/PBS and analyzed for fluorescence by flow cytometry. At least 25,000 events were collected on a FACScalibur flow cytometer (Beckton Dickinson) and analyzed using Cellquest software (Beckton Dickinson).
2.1.15 Determination of Cytoplasmic Superoxide

Cells were cultured in 6-well plates (Corning Incorporated) in regular growth media until they reached 70-80% confluence. Then, cells were treated with paraquat and GW583340 for 24 h. After incubation, cells were harvested, washed twice with 1%BSA/PBS, and incubated for 30 min with 10 μM DHE (Molecular Probes) at 37°C. Cells were then washed twice with 1% BSA/PBS and analyzed for fluorescence by flow cytometry. At least 25,000 events were collected on a FACScalibur flow cytometer (Beckton Dickinson) and analyzed using Cellquest software (Beckton Dickinson).

2.1.16 Determination of Mitochondrial Superoxide

Cells were cultured in 6-well plates (Corning Incorporated) in regular growth media until they reached 70-80% confluence. Then, cells were treated with paraquat and GW583340 for 24 h. After incubation, cells were harvested, washed twice with 1%BSA/PBS, and incubated for 30 min with 10 μM MitoSOX Red (Molecular Probes) at 37°C. Cells were then washed twice with 1% BSA/PBS and analyzed for fluorescence by flow cytometry. At least 25,000 events were collected on a FACScalibur flow cytometer (Beckton Dickinson) and analyzed using Cellquest software (Beckton Dickinson).

Glutathione Assay

Glutathione was assessed using the GSH-Glo™ Glutathione Assay (Promega, Madison, WI) as per the manufacture’s instructions. Briefly, 3 μg total cell lysates were incubated at room temperature for 30 min with 50 μl of prepared GSH-Glo™ Reagent
2X. Then, 100 µl of reconstituted Luciferin Detection Reagent was added, plates were mixed and incubated at room temperature for 15 min, and luminescence was read using a 96-well plate luminometer with a 1 s integration time.
3 Evaluation of Apoptotic Dysregulation in IBC Cells Treated with a Panel of Therapeutic Agents

Introduction

Inflammatory breast cancer (IBC) is the most aggressive form of locally advanced breast cancers (LABC) (Anderson et al., 2003), and overall survival for women with IBC is extremely low (Gonzalez-Angulo et al., 2007) (discussed in Section 1.2). Although IBC was first described in 1924 (Lee and Tannenbaum, 1924), to date little is known about the distinct molecular characteristics of IBC that make these tumors so aggressive and resistant to therapeutic agents (Ferrara, 2008).

Recently, a handful of studies have identified apoptotic dysregulation to be a key factor in the molecular biology of IBC tumors (discussed in Section 1.2.3). As described by Hanahan and Weinberg, (Evan and Vousden, 2001; Hanahan and Weinberg, 2000), apoptotic dysregulation is one of the hallmarks of cancer, and understanding the factors that are involved in this process will allow us to better target cancer cells for death. Microarray studies comparing IBC and non-IBC tumor samples have demonstrated that proteins including p53, activated nuclear factor kappa B (NFκB), BAX (a member of the Bcl-2 family), and ALS2CR2 (which can interact with anti-apoptotic proteins) are upregulated in IBC tumors, demonstrating that apoptosis and cell turnover pathways are important molecular features of IBC (Bertucci et al., 2004; Boersma et al., 2008; Nguyen et al., 2006; Van Laere et al., 2005) (discussed in Section 1.1.9, Table 1).

These studies indicate that overcoming apoptotic dysregulation may be an important factor for therapeutically targeting IBC tumor cells. Many therapeutic agents
have been shown to act in part through modulation of the apoptotic pathway (Lowe and Lin, 2000; Nicholson, 2000; Schmitt and Lowe, 1999). Therefore, if these agents are able to overcome the apoptotic dysregulation of IBC cells, they may be efficacious. Because IBC is such an aggressive disease, many different therapeutic strategies are currently used to treat these patients (discussed in Section 1.2.2). Chemotherapeutic agents that are currently used for treatment of patients with IBC include: doxorubicin, cisplatin, paclitaxel, 5-fluorouracil (5-FU), and cyclophosphamide (Table 4) (Dawood et al., 2008; Woodward and Cristofanilli, 2009). Importantly, all of these agents affect apoptotic pathways (Bhalla, 2003; Minotti et al., 2004; Sampath et al., 2003; Sedletska et al., 2005; Siddik, 2003; Vaux et al., 2003; Zhao et al., 2005a). Other novel therapeutics that are currently in clinical trials affect the death receptor pathway. One example is TNF-related apoptosis-inducing ligand (TRAIL), a classical apoptotic-inducing ligand that binds to death receptors (DR) 4 and 5 and causes subsequent apoptosis through the extrinsic pathway (Kim et al., 2000). Clinical trials using TRAIL have been encouraging, and toxicity to normal cells has been relatively low (Ashkenazi et al., 2008; Papenfuss et al., 2008). One of the most promising characteristics of TRAIL is its ability to directly induce tumor cell death via death receptors so that the status of intracellular sensors such as p53 is less relevant (Papenfuss et al., 2008).

In this results reported in this chapter, we characterized the effects of commonly used chemotherapeutic agents on two different IBC cell lines SUM149 and SUM190 [taken from the primary tumors of IBC patients (Forozan et al., 1999)]. We identified that these cells have differential sensitivity to TRAIL and cisplatin, which inversely
correlates with XIAP expression. Moreover, XIAP overexpression and knockdown studies in sensitive SUM149 cells and resistant SUM190 cells, respectively, demonstrated that XIAP is sufficient and necessary for resistance of IBC cells to TRAIL. Finally, inhibition of XIAP in combination with TRAIL synergistically increased cell death. These data validate XIAP as a potential therapeutic target for potentiating TRAIL-induced apoptosis.

**Sensitivity of IBC Cells to a Panel of Chemotherapeutic Agents**

The first goal was to determine the sensitivity of the two IBC cell lines to commonly used therapeutic agents with different mechanisms of action (Table 4).
Table 4: Therapeutic agents used in this dissertation.

<table>
<thead>
<tr>
<th>Therapeutic Agent</th>
<th>Mechanism of Action</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>DNA cross-linking agent</td>
<td>(Siddik, 2003)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>DNA intercalating agent</td>
<td>(Neidle, 1979)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Microtubule stabilizer</td>
<td>(Horwitz, 1992)</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Death receptor ligand</td>
<td>(Srivastava, 2001)</td>
</tr>
<tr>
<td>5-FU</td>
<td>Pyrimidine analog</td>
<td>(Longley et al., 2003)</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>ErbB2 monoclonal antibody</td>
<td>(Baselga et al., 2001)</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>ErbB1/2 kinase inhibitor</td>
<td>(Xia et al., 2002)</td>
</tr>
</tbody>
</table>

The above therapeutic agents (with shown mechanisms of action) were chosen for investigation in this research because they are currently used in the clinic (or clinical trials in the case of TRAIL) to treat IBC patients.
For this, we used the MTT assay and observed affects on proliferation at both 24 and 72 h time points. Results from these experiments are shown in Table 5. Compared to non-IBC cell lines with similar molecular characteristics (Table 2), there was no obvious trend in resistance or sensitivity of the IBC cells to certain agents. Interesting, the SUM149 cells were relatively insensitive to cisplatin and highly sensitive to TRAIL, whereas the opposite was observed in the SUM190 cells.
Table 5: Sensitivity (IC$_{50}$) of a panel of LABC cell lines to therapeutic agents with different mechanisms of action.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Type</th>
<th>Cell Line</th>
<th>Cisplatin</th>
<th>TRAIL</th>
<th>5-FU</th>
<th>Dox</th>
<th>Taxel</th>
</tr>
</thead>
<tbody>
<tr>
<td>ErbB2 Overexpressing</td>
<td>IBC</td>
<td>SUM190</td>
<td>6.28 +/- 1.57 µM (24 h)</td>
<td>Resistant (72 h)</td>
<td>5.5-6 µg/ml (72 h)</td>
<td>0.38 +/- 0.23 µM (24 h)</td>
<td>Resistant (72 h)</td>
</tr>
<tr>
<td>Non-IBC</td>
<td>SKBR3</td>
<td>43 +/- 7 µM (120 h) (Serova et al., 2006)</td>
<td>Resistant (72 h)</td>
<td>26 µg/ml (72 h) (Chen et al., 2000)</td>
<td>0.8-1 µM (72 h)</td>
<td>16-18 µM (24 h)</td>
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</tr>
<tr>
<td></td>
<td>BT-474</td>
<td>75 +/- 12 µM (120 h) (Serova et al., 2006)</td>
<td>20 ng/ml (40 h) (Dubska et al., 2005)</td>
<td>28.6 µg/ml (72 h) (Chen et al., 2000)</td>
<td>0.76 +/- 0.23 µM (72 h) (Campiglio et al., 2003)</td>
<td>4 µM (48 h) (Kim et al., 2005b)</td>
<td></td>
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<tr>
<td>Triple Negative</td>
<td>IBC</td>
<td>SUM149</td>
<td>8-10 µM (72 h)</td>
<td>85-90 ng/ml (72 h)</td>
<td>0.75-1 µg/ml (72 h)</td>
<td>0.02-0.05 µM (72 h)</td>
<td>0.53-1.06 µM (24 h)</td>
</tr>
<tr>
<td>Non-IBC</td>
<td>MDA-MB-231</td>
<td>200-300 µM (24 h) (Chu et al., 2005)</td>
<td>10% viability (25 µg/ml, 16 h) (Chinnaiyan et al., 2000)</td>
<td>19.3 µg/ml (24 h) (Vazquez-Martin et al., 2007)</td>
<td>50 nM (24h) (Aoudjit and Vuori, 2001)</td>
<td>25 µM (24h) (Menendez et al., 2001)</td>
<td></td>
</tr>
</tbody>
</table>

Using MTT assay, the sensitivity of the above agents was determined in ErbB2 overexpressing IBC cells (SUM190) and ErbB1 activated IBC cells (SUM149) and compared to LABC cells with similar molecular characteristics. Numbers represent IC$_{50}$ (time).
XIAP Expression Inversely Correlates with Sensitivity of IBC Cells to Therapeutic Agents

To determine which IAP correlates with sensitivity of the cells to these agents, we next evaluated XIAP and survivin expression in the SUM190 IBC cells. As shown in Table 5, SUM190 cells are highly sensitive to cisplatin- and doxorubicin-mediated inhibition of cell proliferation. Decrease in proliferation in response to cisplatin and doxorubicin corresponded with a significant downregulation of XIAP (Figure 3.1). XIAP is a potent inhibitor of procaspase 9 activation, and a decrease in XIAP in the drug-sensitive cells corresponded with decrease in procaspase 9 compared to untreated and vehicle treated cells (Figure 3.1). In contrast, XIAP expression remained unchanged in cells treated with TRAIL, to which SUM190 cells were not sensitive (Table 5).
Figure 3.1: Expression of XIAP in SUM190 cells inversely correlates with sensitivity to various therapeutic agents.

Western immunoblot analysis of SUM190 total cell lysates 24 h after treatment with indicated therapeutic agents. Actin was used as a loading control. Phospho blots were stripped and reprobed with corresponding total protein. The results show that XIAP is the only protein whose expression exactly correlates with sensitivity of SUM190 cells to the therapeutic agents (Table 5).
Figure 3.2: XIAP expression inversely correlates with sensitivity to cisplatin.

A) Evaluation of proliferation using the MTT assay of SUM149 and SUM190 cells after 24 h treatment with indicated concentrations of cisplatin, showing that SUM190 cells are highly sensitive to cisplatin and SUM149 cells are less sensitive to cisplatin treatment at 24 h. B) Evaluation of cell cycle using PI staining after 24 h treatment with 50 μM cisplatin, which shows that sensitive SUM190 cells have an increased <2N population, indicating increased apoptosis. C) Western blot analysis of XIAP and caspase 3 24 h post-treatment with indicated concentrations of cisplatin. GAPDH was used as a loading control. Numbers represent densitometric analysis of total protein normalized to actin or GAPDH. XIAP decreased in SUM190 cells in response to cisplatin, which also changes procaspase 9 (inactive) expression. In contrast, there is not change in the less sensitive SUM149 cells.
Similar results were observed in the IBC cells treated with a dose range of cisplatin (Fig. 3.2). SUM149 cells were significantly less sensitive to cisplatin than SUM190 cells, which corresponded with XIAP expression.

**XIAP Expression Correlates with Resistance of IBC Cells to TRAIL**

The efficacy of TRAIL was tested in the SUM190 and SUM149 cells, taken from the primary tumor of IBC patients (Forozan et al., 1999). Data in Figure 3.3 show that the SUM149 cells are sensitive to TRAIL treatment, reaching 50% decrease in proliferation at approximately 100 ng/ml; however, the SUM190 cells are insensitive to TRAIL even up to 72 h (Table 5).

Previous reports have shown that XIAP expression inversely correlates with TRAIL sensitivity in various cancer models, and both XIAP expression and apoptotic dysregulation are important molecular features of IBC cells (Section 1.1.9). Therefore, IBC cells treated with TRAIL were analyzed for XIAP expression. Data in Figure 3.3 demonstrate that SUM149 cells, which are sensitive TRAIL, display a marked decrease in XIAP expression post-TRAIL treatment. This corresponds to cleavage of pro-caspase 3 and its subsequent activation. In contrast, TRAIL-insensitive SUM190 cells showed no decrease in XIAP expression after TRAIL treatment, and therefore pro-caspase 3 was not cleaved. These data demonstrate that XIAP expression inversely correlates with TRAIL sensitivity in IBC cells.
Figure 3.3: XIAP expression inversely correlates with sensitivity to TRAIL.

A) Evaluation of proliferation using the MTT assay of SUM149 and SUM190 cells after 24 h treatment with indicated concentrations of TRAIL, indicating that SUM190 cells are de novo resistant to TRAIL whereas SUM149 cells are sensitive. B) Western blot analysis of XIAP and caspase 3 24 h post-treatment with indicated concentrations of TRAIL. GAPDH was used as a loading control. Numbers represent densitometric analysis of total protein normalized to GAPDH. XIAP decreased in SUM149 cells in response to cisplatin, which also cleaved procaspase 3. In contrast, there is not change in the resistant SUM190 cells.
Exogenous Overexpression of XIAP Reverses Sensitivity of IBC Cells to TRAIL

To determine if XIAP is sufficient for resistance of IBC cells to TRAIL, we generated stably overexpressing wildtype XIAP (wtXIAP) SUM149 cells lines (Figure 3.4) and examined the effects of TRAIL on these cells. Data in Figure 3.4 (panel B, C) shows that SUM149 wtXIAP cells were significantly less sensitive to the cell death-inducing effect of TRAIL than the vector control cells (p<0.05), which corresponded to XIAP expression (Fig. 3.4, panel A). Moreover, caspase 3/7 activity was significantly increased in the SUM149 vector controls cells compared to the SUM149 wtXIAP cells (Fig. 3.4, panel C, p<0.005), demonstrating that overexpression of XIAP leads to a decrease in the sensitivity of IBC cells to TRAIL.
Figure 3.4: Exogenous overexpression of XIAP reverses sensitivity of parental IBC cells to TRAIL.

A) Western blot analysis of XIAP expression in SUM149 cells stably expressing XIAP using a lentiviral system, showing that the cells do indeed have increased XIAP expression. B) Viability analysis of SUM149 stably overexpressing XIAP and vector control cells after 24 h treatment with indicated concentrations of TRAIL. C) Caspase 3/7 analysis of SUM149 stably overexpressing XIAP and vector control cells after 3 h treatment with indicated concentrations of TRAIL. These data indicate that exogenous
overexpression of XIAP can reverse the cell death/apoptosis induced by TRAIL, indicating that XIAP is sufficient to cause TRAIL resistance.

**Knockdown of XIAP Reverses Resistance of IBC Cells to TRAIL**

To determine if XIAP expression is necessary for the resistance of SUM190 cells to TRAIL treatment, we generated stable XIAP knockdowns (shXIAP) using SUM190 cells. Data in Figure 3.5 show that knockdown of XIAP in SUM190 cells made them more sensitive to the death-inducing effects of TRAIL compared to vector control cells (p<0.05).
Figure 3.5: Knockdown of XIAP expression reverses resistance of SUM190 IBC cells to TRAIL.

XIAP western blot analysis (right) and viability (left) of SUM190 with stable knockdown of XIAP and vector controls cells. Using XIAP knockdown in cells with de novo TRAIL resistance, these data show that XIAP is necessary for the survival of IBC treated with TRAIL.
Discussion

In this chapter, we characterized the sensitivity of two IBC cell lines with different molecular characteristics (SUM190: ErbB2 overexpressing, ER/PR-ve; SUM149: ErbB1 activated, ER/PR-ve) to a panel of therapeutic agents with different mechanisms of action (Table 4) that are currently used in the clinic or in trials to treat IBC patients. The results were compared to non-IBC cell lines with similar molecular features, and no distinct trends were observed. Interestingly, SUM149 cells were fairly insensitive to cisplatin and highly sensitive to TRAIL whereas the opposite was observed in the SUM190 cells. This was observed to inversely correlate with XIAP expression. Moreover, XIAP overexpression and knockdown studies demonstrate that XIAP is a critical factor in TRAIL responsiveness.

As discussed in Section 1.2.3, IBC cells may have increased apoptotic dysregulation compared to non-IBC cells (Nguyen et al., 2006; Van den Eynden et al., 2004), which could be why these tumors are so aggressive and less responsive to treatment (El-Tamer et al., 2002; Smoot et al., 2006). Current treatment strategies include multimodality treatment using anthracycline (doxorubicin)-based regimens along with paclitaxel (Dawood et al., 2008; Woodward and Cristofanilli, 2009) (discussed in Section 1.1.8). The results in this chapter indicate that the ErbB2 overexpressing SUM190 cells are highly sensitive to doxorubicin and cisplatin, but much less sensitive to 5-FU, TRAIL, and paclitaxel, which all cause apoptosis (Kaufmann and Earnshaw, 2000). This profile inversely correlated with XIAP and not any of the other pro-survival
or anti-apoptotic proteins, including survivin. This is most likely because survivin is not a true caspase inhibitor (Banks et al., 2000; Eckelman et al., 2006), although there is still controversy surrounding this (Altieri, 2003; Mita et al., 2008). In contrast, SUM149 cells were sensitive to paclitaxel and TRAIL but fairly insensitive to doxorubicin, 5-FU, and cisplatin. Interestingly, this profile also inversely correlated with XIAP expression.

It is intriguing that the two IBC cell lines were affected so differently by cisplatin and TRAIL. Cisplatin is a DNA damaging agent that can also induce apoptosis through upregulation of Fas (Siddik, 2003). In contrast, TRAIL is a natural death receptor ligand, which induces apoptosis through the extrinsic pathway (Ashkenazi et al., 2008; Holoch and Griffith, 2009). Numerous resistance mechanisms have been previously demonstrated for both cisplatin and TRAIL. Resistance mechanisms of cisplatin include p53 dysfunction (Cabelguenne et al., 2000; Houldsworth et al., 1998; Reles et al., 2001; Sarkis et al., 1995), reduced intracellular drug accumulation (Kelland, 1993; Siddik, 2003), increased DNA damage repair (Chao et al., 1991; Kelland et al., 1992; Lai et al., 1988; Sheibani et al., 1989; Siddik et al., 1998), increased thiol-containing molecules (Kelland, 1993), and expression of anti-apoptotic proteins, including XIAP (Asselin et al., 2001a; Asselin et al., 2001b; Ding et al., 2009; Ikeguchi et al., 2002). Resistance mechanisms of TRAIL include changes or mutations in receptors or increased decoy receptors (Pai et al., 1998; Shin et al., 2001; Zhang and Fang, 2005), cFLIP expression (Kataoka et al., 1998; Krueger et al., 2001), epigenetic silencing of caspase 8 (Hopkins-Donaldson et al., 2000a, b), and expression of anti-apoptotic proteins like XIAP (Amantana et al., 2004; Braeuer et al., 2006; Cummins et al., 2004; Fakler et al., 2009;
Makhov et al., 2008; Mori et al., 2007; Roa et al., 2003; Shrader et al., 2007; Vogler et al., 2009). It is noteworthy that as discussed above, expression of XIAP has been shown numerous times to be a key factor in resistance of tumors cells to both cisplatin and TRAIL. The data in this chapter demonstrate the dominance of XIAP expression as a main factor in the sensitivity and resistance of the SUM190 and SUM149 cells to these two agents. To our knowledge, this is the first report to characterize this in an IBC cellular model.

Although stable overexpression of XIAP was able to moderately reverse sensitivity to TRAIL, 40% of cells still underwent cell death. It is unlikely that these cells have simply lost XIAP expression. Instead, this may be due to differences in XIAP copy number in each cell. Similarly, knockdown of XIAP was not able to fully sensitize SUM190 cells, indicating that other factors may be involved. This may be due to compensation by other IAP family members, which has been shown to occur when XIAP is knocked down (Harlin et al., 2001; Verhagen et al., 2001). Further studies need to be done to determine whether compensation of other IAPs like cIAP1 and cIAP2 are overcoming the complete knockdown of XIAP.

Taken together, data in this chapter demonstrate that IBC cells have differential sensitivity to therapeutic agents, but both SUM149 and SUM190 IBC cells require XIAP expression for survival. This strengthens the idea that XIAP is a potential therapeutic target for IBC patients.
4 Examination of Apoptotic Dysregulation in IBC Cells Resistant to ErbB2 Targeting Agents

Introduction

As discussed in Section 1.2.4, increased overexpression of ErbB2 in IBC patients has made it an attractive therapeutic target. ErbB receptor signaling is largely regulated by a family of peptide ligands (epidermal growth factor family members) that bind to their cognate ErbB receptors (ErbB1, ErbB3, ErbB4), with ErbB2 lacking an exogenous ligand (Hynes and Lane, 2005). Binding of ligand to its cognate ErbB receptor(s) triggers the formation of receptor homo- or heterodimers (with ErbB2 the preferred heterodimeric partner) and induces autophosphorylation of tyrosine residues within the cytoplasmic tail of the receptor. Lacking an exogenous ligand, ErbB2 is autophosphorylated and transactivated by its heterodimeric partner. ErbB2 contains six tyrosine autophosphorylation sites that serve as docking sites for phosphotyrosine-binding domain and/or Src homology 2 (SH2) containing proteins, which in turn activate downstream MAPK-Erk1/2 and PI3K-Akt proliferation and survival signaling pathways (Hynes and Lane, 2005). Trastuzumab (Herceptin\textsuperscript{R}) is a FDA approved humanized anti-ErbB2 monoclonal antibody for treating patients with ErbB2-overexpressing breast cancers (Cobleigh et al., 1999) and is used in various combinations, with objective response rates between 15 and 26% (Cobleigh et al., 1999; Vogel et al., 2002). However, subsets of women with ErbB2 overexpressing tumors do not respond and resistance is common (Nahta and Esteva, 2006; Nahta et al., 2006). Lapatinib (GW583340/GW572016/Tykerb\textsuperscript{R}), a dual inhibitor of the oncogenic ErbB2 and ErbB1
receptor tyrosine kinases, blocks ErbB2 tyrosine autophosphorylation with consequential downstream inhibitory effects on MAPK-Erk1/2 and PI3K-Akt growth/survival signaling in tumor cell lines, tumor xenografts, and in patients, notably those with ErbB2-overexpressing breast cancers where lapatinib induces tumor cell apoptosis (Burris et al., 2005; Konecny et al., 2006; Spector et al., 2005). In a recent clinical trial, lapatinib showed a 50% clinical response rate in ErbB2-overexpressing IBC patients (Spector et al., 2006); however, acquired resistance is a common outcome even in those patients who show an initial clinical response.

It is clear that deregulation of most growth promoting factors triggers apoptosis in a ‘normal cell’ and this fundamental characteristic is lost in cancers (Evan and Vousden, 2001; Hanahan and Weinberg, 2000). In part, this failure is due to defects in caspase activation, the execution phase of apoptosis (discussed in Section 1.1.1). X-linked inhibitor of apoptosis protein (XIAP) is one of the most potent and versatile caspase inhibitors and is part of a family of proteins called IAP proteins (Devi, 2004). This family consists of proteins with 1-3 baculovirus IAP repeats (BIR), which are highly conserved domains necessary for function. IAP homologs have been found across many species, including Caenorhabditis elegans, yeast, insects and other non-primate mammals.

Recent studies (Asanuma et al., 2005; Milella et al., 2004; Nguyen et al., 2006; Xia et al., 2006a) in ErbB2 overexpressing non-IBC cell lines and tumor samples have demonstrated dysregulation in the apoptotic pathway in response to trastuzumab and lapatinib. Inhibition of ErbB2 phosphorylation and downstream PI3K-Akt and MAPK-Erk1/2 signaling, although perhaps necessary for lapatinib anti-tumor activity, is not
sufficient (Xia et al., 2006a; Xia et al., 2006b). Instead, this led to a unique observation that ErbB2 regulates a member of the IAP protein family, survivin, and that the anti-tumor effects of lapatinib were more closely related to lapatinib-induced downregulation of survivin.

In this chapter, we evaluated the immune-mediated function and signaling effects of trastuzumab on survival signaling and expression of anti-apoptotic proteins XIAP and survivin in ErbB2 overexpressing IBC and non-IBC cells. We identified that response to trastuzumab inversely and distinctly correlates with expression of XIAP and survivin in the breast cancer cell lines. Further, downregulation of XIAP expression using siRNA in combination with trastuzumab sensitized the ErbB2 overexpressing IBC cells by abrogating the dual function of XIAP on caspase inactivation and increased p-AKT survival signaling.

**Sensitivity of IBC Cells to ErbB-Targeting Agents**

First, we determined the sensitivity of the two IBC cell lines to the two ErbB2-targeting agents trastuzumab and a lapatinib analog (GW583340). Results in Table 5 show that SUM190 cells are resistant to the anti-proliferative effects of trastuzumab but are sensitive to GW583340. SUM149 cells, which do not express high levels of ErbB2, are resistant to trastuzumab, which was expected. However, these cells are sensitive to GW583340, probably because they have an activated ErbB1 receptor (Willmarth and Ethier, 2006). In contrast, the non-IBC ErbB2 overexpressing SKBR3 and BT474 cells are both sensitive to trastuzumab (Brockhoff et al., 2007; Longva et al., 2005) and lapatinib (Rusnak et al., 2001).
Table 6: Sensitivity (IC$_{50}$) of a panel of LABC cell lines to trastuzumab and lapatinib.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Type</th>
<th>Cell Line</th>
<th>Trastuzumab</th>
<th>Lapatinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>ErbB2 Overexpressing</td>
<td>IBC</td>
<td>SUM190</td>
<td>Resistant (72 h)</td>
<td>15 µM (24 h)</td>
</tr>
<tr>
<td></td>
<td>Non-IBC</td>
<td>SKBR3</td>
<td>60% viability 15 µg/ml (24 h)</td>
<td>0.032 +/- 0.005 µM (72 h) (Hegde et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BT-474</td>
<td>0.2 µg/ml (120 h) (Yakes et al., 2002)</td>
<td>0.1 +/- 0.003 µM (72 h) (Rusnak et al., 2001)</td>
</tr>
<tr>
<td>Triple Negative</td>
<td>IBC</td>
<td>SUM149</td>
<td>Resistant (72 h)</td>
<td>4.5-5 µM (24 h)</td>
</tr>
<tr>
<td></td>
<td>Non-IBC</td>
<td>MDA-MB-231</td>
<td>Resistant (72 h)</td>
<td>18.6 µM (7 days) (Konecny et al., 2006)</td>
</tr>
</tbody>
</table>

Using MTT assay, the sensitivity of the above ErbB2 targeting agents was determined in ErbB2 overexpressing IBC cells (SUM190) and ErbB1 activated IBC cells (SUM149) and compared to LABC cells with similar molecular characteristics. Numbers represent IC$_{50}$ (time).
Resistance of SUM190 Cells to Signaling Effects of Trastuzumab

4.1.1 Trastuzumab Binds to ErbB2 of SUM190 IBC Cells and Elicits an ADCC Response

The ability of trastuzumab to bind cell surface ErbB2 and elicit antibody-dependent cell-mediated cytotoxicity (ADCC) were tested in an ErbB2 overexpressing IBC cell line (SUM190) and compared to the ErbB2 overexpressing non-IBC cell line SKBR3 (Fig. 4.1). Incubation of the breast cancer cells with trastuzumab reveals specific binding to cell surface ErbB2 in both SKBR3 and SUM190 cells as shown in Figure 4.1B (mean fluorescent intensity = 897.32 and 1197.09, respectively) compared to insignificant cell surface binding to the non-ErbB2 overexpressing non-IBC (MDA-MB-231 and SUM159PT) cells. ADCC has been reported as a prominent immune-mediated effect of trastuzumab in ErbB2 overexpressing breast cancer cells (Nahta and Esteva, 2006). Data in Figure 4.1C show that trastuzumab caused a strong ADCC response in the SUM190 cells (80% cell lysis), which is comparable to the non-IBC SKBR3 cells (70% cell lysis).
Figure 4.1: Binding of trastuzumab and induction of ADCC in IBC and non-IBC cells.

A) ErbB2 western blot analysis of a panel of IBC and non-IBC cells, showing that SUM190 IBC cells have as much or more ErbB2 expression as SKBR3 and BT474 cells. B) Binding of trastuzumab to IBC and non-IBC cells using flow cytometry, which shows that trastuzumab can bind to the ErbB2 receptor on SUM190 cells. C) ADCC analysis of
ErbB2 overexpressing SKBR3 non-IBC cells and SUM190 IBC cells, which indicates that trastuzumab is able to induce an ADCC effect on SUM190 cells.

4.1.2 Trastuzumab Does Not Inhibit SUM190 IBC Cell Proliferation

In cell proliferation assays (Figure 4.2), SUM190 was insensitive to trastuzumab (80-90% proliferation at 50 µg/ml up to 72 h; Table 5), which was similar to the IBC cell line SUM149, isolated from an aggressive primary IBC tumor which are estrogen receptor-negative and express constitutively active ErbB1 but do not express any other active ErbB family members (Rao et al., 2000). Trastuzumab caused a dose and time-dependent decrease in cell proliferation in ErbB2 overexpressing non-IBC SKBR3 cells (20-25% at 48 h, 35-40% at 72 h), which was consistent with previously published studies (Longva et al., 2005; Lu et al., 2001).
Figure 4.2: Proliferation of IBC and non-IBC cells post-trastuzumab treatment.

SUM149, SUM190, and SKBR3 cells were treated with indicated concentrations of trastuzumab for 72 h. Cell proliferation was monitored by MTT assay and represented as means of triplicate values±SEM (n = 3); **, p < .01 for SKBR3 50 µg/ml vs. untreated control. These data show that SUM190 cells [which have ErbB2 overexpression (Fig. 4.1A) and can bind trastuzumab (Fig. 4.1B)] are de novo resistant to the anti-proliferative affect of trastuzumab whereas SKBR3 cells are sensitive.
4.1.3 Trastuzumab Does Not Inhibit Downstream Signaling in SUM190 IBC cells

The effect of trastuzumab on ErbB2 downstream signaling has been reported in various breast cancer cells including SKBR3 and was compared here with the SUM190 IBC cells. Incubation of cells for 1 h with 15 µg/ml trastuzumab slightly decreased p-ErbB2 expression in SKBR3 cells, but did not alter ErbB2 phosphorylation or total ErbB2 expression in the SUM190 cells. A significant downregulation of p-AKT post-trastuzumab treatment was observed in SKBR3 cells consistent with that reported by Longva, et al. (Longva et al., 2005) and Asanuma, et al (Asanuma et al., 2005). In contrast, trastuzumab did not alter p-AKT in SUM190 cells (Figure 4.3). This inability to decrease p-AKT may in part be due to high constitutive expression of activated AKT and MAPK and relatively high XIAP and survivin mRNA expression in SUM190 cells as shown in Figure 4.4.
Western blot analysis of p-ErbB2 and p-AKT in SUM190 and SKBR3 cells treated for 1 h with 0-15 µg/ml trastuzumab. Phospho blots were stripped and reprobed with total protein. Numbers represent densitometric analysis of phospho protein to total protein expression. These data indicate that trastuzumab can inhibit downstream signaling in sensitive SKBR3 cells but not in resistant SUM190 IBC cells.
Figure 4.4: Evaluation of activated AKT and MAPK and XIAP and survivin mRNA expression in a panel of IBC and non-IBC cells.

A) Basal expression of p-AKT and p-MAPK in a panel of IBC and non-IBC cells. Actin was used as a loading control. Phospho blots were stripped and reprobed with corresponding total protein. B) RT-PCR analysis of basal XIAP and survivin mRNA expression in a panel of IBC and non-IBC cells. SKBR3 expression was set to 1. These data indicate that SUM190 cells have constitutively active pAKT and very high levels of the anti-apoptotic proteins XIAP and survivin, which may be why trastuzumab cannot inhibit downstream signaling in these cells.
4.1.4 XIAP Expression is Increased in SUM190 IBC Cells Post-Trastuzumab Treatment

Evaluation of the effect of trastuzumab on the anti-apoptotic signaling pathway revealed a distinct mechanism of upregulation of XIAP and survivin expression in trastuzumab-resistant SUM190 cells as shown in Figure 4.5. In contrast, a decrease in XIAP and survivin expression was observed in SKBR3 cells post-trastuzumab treatment.
Figure 4.5: XIAP and survivin protein expression are upregulated in resistant SUM190 cells but downregulated in sensitive SKBR3 cells post-trastuzumab treatment.

A) XIAP immunoblot analysis of SUM190 and SKBR3 cells treated for 48 h with 0-15 µg/ml trastuzumab. GAPDH was used as a loading control. B) Survivin immunoblot analysis of SUM190 and SKBR3 cells treated for 48 h with 15 µg/ml. GAPDH was used as a loading control. Numbers represent densitometric analysis of protein expression normalized to GAPDH. These data show that in sensitive SKBR3 cells, XIAP and survivin expression decrease. However, in resistant SUM190 cells, expression of both XIAP and survivin increase, which may be why these cells are resistant to trastuzumab.
4.1.5 Inhibition of XIAP in Combination with Trastuzumab Decreases Viability of SUM190 IBC cells in a Caspase-Dependent Process

The effect of XIAP siRNA, survivin siRNA, and Embelin, a non-peptidomimetic XIAP small molecule inhibitor which inhibits the formation of XIAP complex with procaspase 9, causing release of active caspase 9 (Ahn et al., 2007; Nikolovska-Coleska et al., 2004) were evaluated as single agents in a panel of IBC cell lines. siRNA targeting survivin and XIAP (Fig. 4.6B) decreased target proteins in the IBC cells tested. Representative immunoblots of SUM190 cell lysates treated for 48 h with various siRNAs are shown in Figure 4.6, panel A and B. Similarly, representative immunoblots (Fig. 4.6C) show that Embelin decreased procaspase 9 levels (Fig. 4.6C) and caused 40-50% decrease in cell viability in the SUM190 (Fig. 4.6D, p < 0.025) cells. In contrast, inhibition of XIAP and survivin protein expression alone by respective siRNA did not have a significant change in SUM190 cell viability (Fig. 4.6D).
Figure 4.6: Knockdown of XIAP or survivin using siRNA alone has no effect on viability of SUM190 cells.

Survivin (A), XIAP (B), and procaspase 9 (C) western blot analysis of cells transfected with survivin siRNA, XIAP siRNA, or treated with embelin, respectively. Numbers represent densitometric analysis of protein expression normalized to GAPDH. D) Cells were transfected for 48 h with 100 nM survivin or XIAP siRNA alone or 20 µM Embelin. Cell viability was monitored by trypan blue exclusion assay and represented as mean±SEM (n=3). * represents p < 0.05 for Embelin vs. control. These data indicate that inhibition of survivin of XIAP alone using siRNA down not cause SUM190 cell death.
Inhibition of XIAP function does increase cell death, indicating that the apoptotic pathway is intact in this cell line.

Since our data in Figure 4.5 show that trastuzumab treatment caused an increase in XIAP and survivin protein expression in SUM190 cells, we evaluated the combination of trastuzumab with inhibition of XIAP or survivin using XIAP siRNA, Embelin or survivin siRNA (Fig. 4.7). Treatment of cells with (trastuzumab + XIAP siRNA) in combination caused a significant decrease (p < 0.025) in cell viability (45-50% decrease compared to controls). This inhibitory effect on cell viability was not observed in (trastuzumab + control siRNA) treatment or (trastuzumab + survivin siRNA) treated cells. Further, although embelin as a single agent decreased SUM190 cell viability, Embelin in combination with trastuzumab was similar to trastuzumab alone suggesting that increased XIAP expression post-trastuzumab treatment (Fig. 4.5) overrides the effect of Embelin, which unlike siRNA has no effect on XIAP translation, but rather inhibits XIAP interaction with procaspase 9, a downstream event. Moreover, in SKBR3 cells wherein trastuzumab treatment causes a decrease in XIAP expression (Fig. 4.5), further inhibition of XIAP using siRNA in combination with trastuzumab had no significant potentiation of decrease in cell viability (Appendix, Fig. 9.1) over that seen with trastuzumab alone, suggesting a need for treatment-dependent specific increase in XIAP for a combination strategy involving specific XIAP inhibition to be effective. Data in Figure 4.7 show that the decrease in viability seen in the (trastuzumab + XIAP siRNA) combination corresponded with decreased procaspase 9. Further, a significant decrease in
p-AKT levels (Fig. 4.7C) was seen only in the combination of XIAP inhibition along with trastuzumab treatment in the SUM190 cells, which have constitutively activated p-AKT (Fig. 4.3B) that is unaffected by trastuzumab treatment alone.
Figure 4.7: Inhibition of XIAP using siRNA in combination with trastuzumab sensitizes resistant SUM190 cells through a decrease in p-AKT and an increase in caspase activity.

A) Cell viability at 48 h post treatment with various combinations was monitored by trypan blue exclusion assay and represented as mean±SEM (n=3). *p < 0.05 for (XIAP siRNA + trastuzumab) versus trastuzumab alone, showing that XIAP is necessary for the survival of SUM190 cells in response to trastuzumab. Immunoblot analysis of cells transfected for 48 h with 100nM XIAP siRNA or treated for 48 h with Embelin alone or in combination with 15 µg/ml trastuzumab (T) with antibodies against p-AKT and p-MAPK (B) and procaspase 9 (C). Phospho blots were stripped and reprobed for corresponding total protein. Numbers represent densitometric analysis of protein expression normalized to total protein or GAPDH. The western immunoblot data show that the XIAP + trastuzumab combination is able to decrease pro-survival signaling and increase apoptotic signaling via decrease in inactive procaspase 9 (and increase in active caspase 9).
**Discussion**

In this chapter, XIAP, an anti-apoptotic protein that can inhibit both mitochondrial and extrinsic pathways of apoptosis, was shown to play a key role in therapeutic resistance of inflammatory breast cancer cells to trastuzumab. A distinct mechanism of increased expression of XIAP and survivin, key members of the IAP family post-trastuzumab treatment was observed in ErbB2 overexpressing SUM190 IBC cells, an established ErbB2 overexpressing IBC line isolated from a primary tumor of an IBC patient (Forozan et al., 1999). The model of sensitivity and resistance to trastuzumab is shown in Figure 4.8.

Our data reveal that trastuzumab can bind effectively to cell surface ErbB2 and elicit a potent ADCC response in both sensitive SKBR3 non-IBC and resistant SUM190 IBC cells (Fig. 4.8 A,B 1). Whether this immune-mediated mechanism plays a role in the anti-tumor activity of trastuzumab in IBC tumors needs to be elucidated in tumor models since insignificant levels of pro-inflammatory cytokines and thereby fewer host inflammatory cells are typically identified in the tumor stroma (Kleer et al., 2000; Rosen, 1996). Further, Gennari et al. (Gennari et al., 2004) have postulated that since ADCC does not change ErbB2 levels or alter downstream ErbB2 signaling, it may not be the key mechanism of trastuzumab antitumor activity.

In this chapter, SUM190 cells were shown to be insensitive to the signaling effects of Trastuzumab mediated via its direct inhibitory effect on ErbB2 dimerization and downstream survival signaling (Fig. 4.8B 2) through inhibition of PI3K activity and...
subsequent AKT phosphorylation, a signaling pathway typically inhibited in trastuzumab-sensitive breast cancer cells (Asanuma et al., 2005; Longva et al., 2005; Yakes et al., 2002). In addition, SUM190 cells have high constitutively activated p-AKT (Fig. 4.8B 3). Further, trastuzumab treatment was observed to induce overexpression of XIAP and survivin in the trastuzumab-resistant SUM190 IBC cells compared to the sensitive SKBR3 cells wherein a corresponding decrease in XIAP and survivin was observed (Fig. 4.8A, B 5, 6). In addition, XIAP and survivin levels were decreased in response to GW583340 (Fig. 4.8A 2, 5, 6), which is a more potent inhibitor of ErbB2 signaling compared to trastuzumab. These data reveal a functional link between ErbB2 signaling and anti-apoptotic pathways.

Specific inhibition of XIAP mRNA using an siRNA strategy (Devi, 2006) in the absence of trastuzumab treatment decreased XIAP protein expression; however, this did not lead to a significant change in viability or apoptosis. This is not surprising as previous reports from our lab and others have shown that in many cell lines, inhibiting XIAP by itself does not increase spontaneous apoptosis (Amantana et al., 2004; McManus et al., 2004), but there is a need for an initial insult or stress to the cells like treatment with therapeutic agents. In contrast, a combination of XIAP inhibition along with trastuzumab sensitized the IBC cells to trastuzumab. This suggests the potential mechanism of action of the combination to be able to counteract the effect of trastuzumab to increase XIAP expression thereby alleviating the inhibitory effect of XIAP on caspase activation and also decreasing AKT phosphorylation. The observed interplay between activated AKT and XIAP (Fig. 4.8 A, B 3) is consistent with earlier reports (Asselin et al., 2001b), which
have demonstrated that XIAP overexpression can induce phosphorylation of AKT, thereby stabilizing its activity. A similar mechanism has also been reported in prostate cancer cells in our previous study (Amantana et al., 2004) and in melanoma cells (Zhang et al., 2001) in response to TRAIL, a natural ligand for death receptors that is regulated by the cross-talk between XIAP and p-AKT in potentiating cancer cell survival.

Compared to XIAP siRNA, which works at the level of mRNA translation, direct activation of caspase 9 function by using a small molecule inhibitor, embelin, which abrogates the binding of XIAP to procaspase 9, significantly decreased IBC cell viability revealing the inhibitory effect of XIAP on caspase-dependent cell death. However, embelin failed to have any cell killing effect in combination with trastuzumab because embelin has no effect on XIAP translation or stability. XIAP is one of the few eukaryotic cellular mRNAs with an internal ribosomal entry sequence (IRES), which allows for XIAP translation during periods of cellular stress (Holcik et al., 2000b). The increase in XIAP protein levels that we see after treatment of cells with trastuzumab may be in part due to a cellular stress response increasing translation of XIAP from the IRES, although this needs to be tested in our experimental model.

Similar to XIAP, the role of another key IAP member, survivin, has been identified as a potential prognostic marker in breast cancer (Ryan et al., 2006). An inverse correlation of survivin expression and sensitivity to lapatinib and trastuzumab has been observed in non-IBC ErbB2 overexpressing BT474 and SKBR3 cells respectively (Asanuma et al., 2005; Xia et al., 2006b). In this chapter, upregulation of survivin expression was observed in the SUM190 IBC cells within 1 h of trastuzumab treatment.
Although trastuzumab increased survivin expression in SUM190 cells, inhibition of survivin by siRNA alone or in combination with trastuzumab did not have any significant effect on cell viability compared to trastuzumab alone. This is most likely since survivin is not a direct caspase inhibitor, and reveals the dominant role of XIAP in resistance to apoptotic response to trastuzumab in ErbB2 overexpressing IBC cells.

Although trastuzumab is widely used for ErbB2 overexpressing breast cancers, resistance and cardiotoxicity remain major hurdles. To our knowledge, this is the first evidence that trastuzumab treatment causes XIAP upregulation in trastuzumab-resistant ErbB2 overexpressing breast cancer cells. Resistance mechanisms that have been previously reported include reduced phosphatase and tensin homologue (PTEN) expression, overexpression of the insulin-like growth factor-1 receptor (IGF-1R), and downregulation of p27kip1 (Nahta and Esteva, 2006). Furthermore, it has been shown that constitutively active AKT can block the cell cycle arrest and apoptotic-inducing abilities of trastuzumab (Yakes et al., 2002). These resistance mechanisms could account for the observation that a large majority of patients with ErbB2 overexpressing metastatic breast cancer rarely respond to trastuzumab as a single agent (Cobleigh et al., 1999). The ErbB2 overexpressing IBC cell line (SUM190) described in this study mimics this phenomenon, since it has low PTEN expression (Appendix, Fig. 9.2), activated AKT, and relatively high expression of XIAP, all of which were observed to correlate with resistance. In addition, a previous report has demonstrated the ability of trastuzumab to decrease Bcl-2 protein levels in trastuzumab-sensitive non-inflammatory breast cancer cells (Milella et
al., 2004) further supporting the role of anti-apoptotic proteins in sensitivity to trastuzumab.

Despite advances in understanding the underlying molecular mechanisms of therapeutic resistance in inflammatory breast cancer, minimal treatment options exist for these patients. The results in this chapter establish the feasibility of development of a targeted therapy that potentiates apoptosis in combination with ErbB2 targeting strategies for inflammatory breast cancer therapy.
Figure 4.8: Schematic of mechanism of XIAP in trastuzumab-sensitive (A) and trastuzumab-resistant (B) breast cancer cell lines.

A) Potential mechanisms of trastuzumab response in SKBR3 and B) Resistance to trastuzumab-mediated signaling in ErbB2 overexpressing SUM190 IBC cells. (1) Trastuzumab increases antibody-dependent cell mediated cytotoxicity (ADCC) in both panels. (2) However, trastuzumab binding suppresses ErbB2 receptor signaling by inhibiting ErbB2 receptor dimerization and subsequent phosphorylation, which in turn inhibits PI3K activity in panel A whereas no decrease in p-ErbB2 is seen in SUM190 cells (panel B). Downregulation of PI3K activity by trastuzumab in the sensitive cells inhibits AKT activation leading to inhibition of downstream survival signaling including survivin abundance (4,5). Trastuzumab response in the sensitive breast cancer cells correlates with a significant downregulation of XIAP (6). Overall (A), XIAP decrease causes apoptosis (7). In contrast in panel B(5,6), trastuzumab treatment is observed to further increase survivin and XIAP expression in SUM190 cells. Overall in B, increased XIAP inhibits release of active caspases from the corresponding procaspases leading to a potent suppression of apoptosis (7). The action sites of siRNA targeting XIAP or survivin and embelin are shown.
Examination of Apoptotic Dysregulation in IBC Cells with Acquired Resistance to a Lapatinib-Analog (GW583340)

Introduction

As discussed in Section 1.1.2, apoptotic dysregulation is a fundamental characteristic of cancer that allows transformed cells to survive and proliferate (Evan and Vousden, 2001; Hanahan and Weinberg, 2000). In part, this is due to defects in caspase activity, the execution phase of apoptosis. The inhibitor of apoptosis proteins (IAPs; discussed in Section 1.1.4) are one of the major protein families that regulate caspase activation and programmed cell death (Hunter et al., 2007). The family currently consists of eight members that are characterized by the presence of one or more baculoviral IAP repeat (BIR) domains and are highly conserved among mammalian and non-mammalian species (Deveraux and Reed, 1999).

In particular, one of the IAP proteins, X-linked inhibitor of apoptosis protein (XIAP; discussed in Section 1.1.5), has been identified as the most potent caspase inhibitor to date (Deveraux and Reed, 1999). XIAP can bind and inhibit activation of procaspases 9, 7, and 3 (Liston et al., 2003). This leads to inhibition of both intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways of apoptosis (Hunter et al., 2007), which is not evident with another prominent anti-apoptotic protein Bcl-2, which inhibits cytochrome c release from the mitochondria but does not directly bind to caspases (Cory and Adams, 2002). In addition, XIAP mRNA has an internal ribosomal entry sequence (IRES) (Holcik et al., 2000a), which has been identified to be upregulated during cellular stress (Holcik et al., 1999; Holcik et al., 2000b; Yamagiwa et al., 2004).
XIAP is expressed in almost all tissues and cell types (Duckett et al., 1996); however, it is often overexpressed in tumors versus normal tissue (LaCasse et al., 1998), including breast cancer (Jaffer et al., 2007), and has been strongly linked to therapeutic resistance in cervical, ovarian, and prostate cancers (Amantana et al., 2004; Nachmias et al., 2004). In addition to its caspase-binding function, XIAP has been observed to regulate the activity of key survival factors like AKT, nuclear factor kappa B (NF-κB), and another IAP family member, survivin (Dubrez-Daloz et al., 2008). Therefore, there is a growing interest in targeting XIAP, and inhibitors of XIAP are currently being developed to help overcome resistance to mainstay therapies (Schimmer et al., 2006).

In Chapter 4, we reported a novel functional link between the epidermal growth factor receptor 2 (ErbB2) signaling pathway and XIAP in SUM190 cells, an ErbB2 overexpressing inflammatory breast cancer (IBC) cell line resistant to trastuzumab (an ErbB2 targeting monoclonal antibody) (Aird et al., 2008). IBC is an aggressive, fast-growing, and highly invasive cancer that is clinicopathologically distinct from a neglected locally advanced breast cancer (LABC) (Anderson et al., 2003). IBC tumors are often resistant to chemo- and radio-therapy and therefore disease-free survival is poor (Chu et al., 1980; Rouesse et al., 1986). ErbB2 is commonly overexpressed in IBC tumors (Van den Eynden et al., 2004); however, the development of acquired resistance to FDA approved agents, trastuzumab (humanized ErbB2 monoclonal antibody) and lapatinib (a dual ErbB1/2 tyrosine kinase inhibitor), limits the clinical efficacy of these anti-ErbB2 therapeutic strategies (Nahta and Esteva, 2006; Nahta et al., 2006; Xia et al., 2006a). Clinical trials using lapatinib as a monotherapy have shown that it is effective in
patients with ErbB2 overexpressing breast cancer that have been heavily pre-treated with other therapeutics including trastuzumab (Geyer et al., 2006; Ryan et al., 2008) with response rates ranging from 7-35% (Medina and Goodin, 2008). Interestingly, in IBC patients lapatinib has a greater efficacy with response rates ranging from 50-100% (Cristofanilli et al., 2006; Johnston et al., 2008). However, clinical studies with lapatinib as a monotherapy also indicate that clinical responses are generally short-lived in breast cancer patients (Burris et al., 2005), and acquired resistance is common. Previously reported mechanisms of lapatinib resistance include activation of estrogen receptor (ER) signaling (Xia et al., 2006a), upregulation of the anti-apoptotic protein MCL-1 (Martin et al., 2008), and potentially the modulation of cancer cell metabolism (Spector et al., 2007). In the present chapter, we evaluated XIAP action in a model of acquired resistance to a lapatinib analog (GW583340) in both ErbB2 overexpressing and ErbB1 activated IBC cell lines wherein cells were chronically exposed to GW583340, similar to patients receiving daily doses of lapatinib when given as a monotherapy. Continuous exposure to GW583340 for more than 3 months converted the parental GW583340-sensitive IBC cells to being resistant to the apoptotic-inducing and growth inhibitory effects of the inhibitor. We identified XIAP overexpression to be the key difference between the parental GW583340-sensitive and GW583340-resistant IBC cell lines studied here. Further, XIAP downregulation using embelin (a small molecule inhibitor that interrupts the interaction between XIAP and procaspase 9) (Nikolovska-Coleska et al., 2004) caused reversal of GW583340-resistance in the acquired resistant IBC cellular
models. Results in the present chapter support the observation that XIAP is required for the survival of IBC cells with acquired GW583340 resistance.

**ErbB2 Overexpressing and ErbB1 Activated IBC Cell Lines are Sensitive to a Lapatinib-Analog (GW583340)**

First, the effects of GW583340, which has similar composition as the clinical compound lapatinib/Tykerb, were examined. Data in Table 6 indicate that both SUM190 and SUM149 cells are sensitive to GW593340. Immunoblot analysis of SUM190 cell lysates at 48 h post-GW583340 treatment revealed a significant inhibition of p-AKT expression at the lowest concentration of 1 µM with complete inhibition at higher concentrations (Fig. 5.1). However, analysis of cell proliferation by MTT and viability by trypan blue exclusion assays (Fig. 5.3) revealed that >50% cell killing response to GW583340 treatment occurred only at the higher 20 µM concentration, which correlated with downregulation of XIAP expression. XIAP downregulation was also seen in SUM149 cells treated with GW583340 (Fig. 5.2). Prolonged treatment up to 7 days showed a similar trend in effects of GW583340 on signaling and viability (Fig. 5.3). This is consistent with prior reports in breast cancer patients and BT474, a non-IBC cell line (Xia et al., 2006a; Xia et al., 2006b) that inhibition of ErbB2 phosphorylation and downstream PI3K-Akt, MAPK-Erk1/2 signaling, although perhaps necessary for lapatinib anti-tumor activity, is not sufficient and requires downregulation of the anti-apoptotic proteins. The model of IBC cell sensitivity to GW583340 is shown in Figure 5.3.
Figure 5.1: XIAP, pro-caspase 9, p-AKT, and survivin protein expression levels demonstrate that SUM190 cells are sensitive to GW583340.

Western blot analysis of SUM190 cells treated with indicated concentrations of GW58340 for 24 h. GAPDH was used as a loading control. Numbers represent densitometric analysis of protein expression normalized to GAPDH. These data show that GW583340 can decrease pro-survival signaling in SUM190 cells, which are sensitive to the agent (Table 5).
Figure 5.2: XIAP protein expression inversely correlates with sensitivity to GW583340.

XIAP western blot analysis of SUM190 and SUM149 cells treated with indicated concentrations of GW583340 for 24 h. GAPDH was used as a loading control. Numbers represent densitometric analysis of XIAP protein expression normalized to GAPDH. The XIAP western immunoblot data show that in both sensitive cell lines, XIAP is markedly decreased upon treatment, indicating it is necessary for the continued survival of these cells.
Figure 5.3: Schematic of lapatinib analog (GW583340) sensitivity in the IBC cellular model.

In sensitive cells, GW583340 binds to the receptor, which disallows its phosphorylation. This decreases downstream signaling, including p-AKT and XIAP, which leads to death.
Development of a Model of Acquired Resistance of IBC Cells to an ErbB1/2 Tyrosine Kinase Inhibitor

Because resistance to lapatinib monotherapy in patients treated with daily doses of lapatinib is commonly seen (Burris et al., 2005), GW583340-resistant lines (referred to here as rSUM190 and rSUM149) were established by chronic exposure of the parental SUM190 and SUM149 cells to increasing concentrations of GW583340 for greater than 3 months (see Materials and Methods). GW583340 treatment at concentrations in which the rSUM149 and rSUM190 cells were established for acquired resistance to this agent caused significant growth inhibition in the parental SUM149 (Fig. 5.3A; p<0.005) and SUM190 (Fig. 5.3B; p<0.05) cells. However, the growth curves indicate that rSUM149 and rSUM190 cells have similar doubling times to their parental counterparts (Table 7; SUM149 14.1 h vs. rSUM149 14.9 h; SUM190 39.6 h vs. rSUM190 40.1 h). Analysis of cell death as measured by Annexin/PI staining shows an increase in total dead cells 24 h post treatment in the parental cells compared to rSUM149 (Fig. 5.3C, left panel; p<0.05) and rSUM190 (Fig. 5.3D, left panel; p<0.05) cells. In addition, exposing rSUM149 and rSUM190 cells (which are maintained in 7.5 µM and 2.5 µM GW583340, respectively) to increasing concentrations of GW583340 (up to 20 µM for 24 h) caused only a modest increase (10-20%) in cell death as measured by 7-AAD viability stain (Fig. 5.3C, D, right panels, left y-axis) and no decrease in cell proliferation (MTT assay, Fig. 5.3C, D; right panels, right y-axis) compared to 70-80% cell death and decrease in proliferation in the parental SUM149 and SUM190 cells. It should be noted that the effect of GW583340 on
decreasing cell proliferation in parental SUM149 and SUM190 cells saturates to 50-60% at 10-20 µM at 24 h and only increase in time of treatment causes further significant growth inhibition. These data support the establishment of two IBC cell models (rSUM149 and rSUM190) with acquired resistance to GW583340.
Table 7: Doubling times of parental and resistant IBC cells.

<table>
<thead>
<tr>
<th></th>
<th>ErbB1 Activated IBC Cells</th>
<th>ErbB2 Overexpressing IBC Cells</th>
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<tr>
<td>SUM149</td>
<td>rSUM149</td>
<td>SUM190</td>
</tr>
<tr>
<td>14.1 hrs</td>
<td>14.9 hrs</td>
<td>39.6 hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rSUM190</td>
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<td>40.1 hrs</td>
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To determine whether the resistant cells had similar growth patterns as their parental counterparts, the doubling times of all four cell lines were determined. The data indicate that the resistant cells have a similar growth pattern as the parental cells, showing that these cells are no longer sensitive to the growth-inhibitor effects of GW583340.
Figure 5.4: Generation of IBC cells with acquired resistance to GW583340.

SUM149 and rSUM149 cells treated with 7.5 µM GW583340 (A) and SUM190 and rSUM190 cells treated with 2.5 µM GW583340 (B). Cell growth was assessed at the indicated time points by trypan blue exclusion assay. [**p<0.005, rSUM149 vs. SUM149 treated with 7.5 µM GW583340 (n=2-3); *p<0.05, rSUM190 vs. SUM190 treated with 2.5 µM GW583340 (n=2)]. Cell death and proliferation of rSUM149 cells and SUM149 cells (C) and rSUM190 and SUM190 cells (D) treated for 24 h with GW583340. Cell death was assessed by Annexin V/PI (left panel) or 7-AAD (right panel, left y-axis) staining and proliferation was determined by MTT assay (right panel, right y-axis). Bars represent mean ± SEM of the total dead cell count relative to the DMSO control or percentage proliferation relative to the DMSO control. [*p<0.05, **p<0.005, n=2] These data indicate...
that resistant SUM149 and SUM190 cells are no longer sensitive to the growth-inhibitory effects of GW583340.

**Mechanism of Lapatinib-Analog is Intact in IBC Cells with Acquired Resistance**

Evaluation of the effect of GW583340 on the ErbB signaling pathways in SUM149 and SUM190 IBC cells revealed that treatment with GW583340 caused a marked and comparable downregulation of p-ErbB1 in both parental SUM149 and rSUM149 cells compared to untreated parental cells (Fig. 5.4A). Similarly, expression of p-ErbB2, p-AKT, and p-MAPK were inhibited in the GW583340-treated SUM190 and rSUM190 cells. However, an increase in total MAPK expression was observed in the rSUM190 cells (Fig. 5.4B).
Figure 5.5: The primary mechanism of action (decrease in p-ErbB1/2) of GW583340 remains intact in the resistant IBC cells.

A) Immunoblot analysis of SUM149 and rSUM149 cells (growing in GW583340) with an antibody against p-ErbB1. The p-ErbB1 blot was stripped and reprobed for ErbB1 total protein. B) Immunoblot analysis of SUM190 and rSUM190 cells (growing in GW583340) with antibodies against p-ErbB2, p-MAPK, and p-AKT. Phospho blots were stripped and reprobed for corresponding total protein. In addition, GAPDH was used as a loading control for MAPK. Numbers represent densitometric analysis of protein expression normalized to total protein. These western immunoblot data show that GW583340 can still inhibit ErbB1 and ErbB2 phosphorylation in the resistant cells, indicating there are other mechanisms of resistance involved.
In order to evaluate the effect of direct inhibition on PI3K (downstream effector in ErbB signaling), SUM190 and rSUM190 cells were treated with a PI3K inhibitor (LY294002). LY294002 treatment inhibited p-AKT as per its mechanism of action and increased cell death of SUM190 cells (Fig. 5.5; p<0.05). In contrast, the rSUM190 cells were not affected by direct inhibition of PI3K signaling using LY294002. This insensitivity potentially indicates that in ErbB2 overexpressing rSUM190 cells, GW583340 retains the ability to inhibit p-ErbB2, but the cells no longer rely on signaling downstream of the ErbB2 receptor. These data suggest that the primary mechanism of GW583340 action as a dual ErbB1/2 kinase inhibitor (Xia et al., 2002) is not compromised in the rSUM149 and rSUM190 cells and thereby does not explain the decreased sensitivity to GW583340-induced cell death observed in the rSUM149 and rSUM190 cells.
Figure 5.6: Parental SUM190 cells are sensitive to inhibition of PI3K whereas rSUM190 cells are insensitive.

Immunoblot analysis (left panel) of SUM190 cells treated with 40 µM LY294002 with an antibody against p-AKT. The phospho blot was stripped and reprobed for total AKT. Numbers represent densitometric analysis of p-AKT normalized to total AKT. This shows the mechanism of LY294002. Cell death (right panel) was assessed by Annexin V/PI staining of rSUM190 cells and SUM190 cells treated for 48 h with 40 µM LY294002. DMSO was used as a vehicle control. Bars represent the mean ± SEM of the percentage of total dead cells. [*p<0.05, n=2] These data indicate that resistant SUM190 cells are no longer sensitive to inhibition of PI3K, demonstrating that another pro-survival signaling pathway has taken over in the resistant cells.
XIAP Expression is Necessary and Sufficient for Acquired Resistance of IBC Cells to a Lapatinib-Analog

5.1.1 Increased XIAP Expression Correlates with Acquired Resistance of IBC Cells to a Lapatinib-Analog

The effect of GW583340 on apoptotic signaling was thereby interrogated in these cell lines. Western immunoblot analysis of key anti-apoptotic proteins revealed sustained survivin (Fig. 5.6A) and Bcl-2 (Fig. 5.6B) expression in rSUM149 and rSUM190 cells. Further, immunoblot analysis of Forkhead box O3 (FOXO3a), a transcription factor known to promote pro-apoptotic gene transcription, revealed a decrease in FOXO3a expression in rSUM149 and rSUM190 cells (Fig. 5.6C); however, the difference was more marked in the rSUM190 cells.
Figure 5.7: Effect of acquired resistance to GW583340 on apoptotic signaling in IBC cells.

A) Survivin immunoblot analysis of parental and resistant IBC cells.  B) Bcl-2 immunoblot analysis of parental and resistant IBC cells.  C) FOXO3a immunoblot analysis of parental and resistant IBC cells. GADPH was used as a loading control for all immunoblots.  These western immunoblots show that the anti-apoptotic proteins survivin and Bcl-2 are maintained in the resistant cells and that FOXO3a, which has been previously shown to be a resistance mechanism in non-IBC cells, is decreased in resistant cells.
XIAP, one of the most potent caspase inhibitors that can modulate both the mitochondrial and extrinsic apoptotic signaling cascades, was characterized (Fig. 5.7) in the IBC cells described herein. A 2-3 fold overexpression of XIAP protein levels was observed by immunoblot analysis in rSUM149 and rSUM190 cells compared to untreated parental cells (Fig. 5.7A). In addition, inhibition of XIAP expression in both SUM190 and SUM149 cells was observed post-GW583340 treatment. XIAP immunofluorescence (Fig. 5.7B) and further quantitative analysis of the mean intensity of XIAP staining per pixel supports the XIAP overexpression in rSUM149 and rSUM190 cells (Fig. 4C; SUM149 vs. rSUM149, p<7x10^{-7}; SUM190 vs. rSUM190, p<5x10^{-6}). These data identify a mechanism of apoptotic dysregulation associated with overexpression of XIAP in rSUM149 and rSUM190 cells maintained in GW583340.
Figure 5.8: XIAP expression is upregulated in IBC cells with acquired resistant to GW583340.

A) XIAP immunoblot analysis of rSUM190 and rSUM149 cells (growing in GW583340). Numbers represent densitometric analysis of XIAP expression normalized to GAPDH. B) Representative fluorescent microscopy images of parental and resistant SUM149 and SUM190 cells probed with an XIAP antibody and counterstained with Hoechst. C, Mean XIAP staining intensity per pixel in parental and resistant IBC cells. Bars represent the average mean XIAP staining intensity per pixel ± SEM in over 20 single cells taken from 10 different fields. These data show that resistant cells (in the constant presence of GW583340) have increased XIAP expression.
5.1.2 Exogenous Overexpression of XIAP in Parental IBC Cells Reverses Sensitivity to a Lapatinib-Analog

To determine whether XIAP overexpression is a potential factor in acquired resistance to GW583340, parental SUM149 cells were stably transfected using a lentiviral expression system with exogenous wildtype XIAP and a corresponding vector control (Fig. 5.8, left panel). Data in Figure 5.8 show that exogenous overexpression of XIAP in parental IBC cells reverses sensitivity of parental SUM149 cells to GW583340-induced cell death, which demonstrates that XIAP expression is sufficient to mediate resistance of these cells to the ErbB1/2 tyrosine kinase inhibitor.
Figure 5.9: Exogenous overexpression of XIAP in parental IBC cells reverses sensitivity to GW583340.

Wildtype XIAP (wtXIAP) or vector control (Vector cont.) were stably overexpressed in parental SUM149 cells (left panel, immunoblot analysis). Actin was used as a loading control, and numbers represent densitometric analysis of XIAP normalized to actin. Cells were treated for 24 h with GW583340 and viability was assessed via trypan blue exclusion (left panel). Bars represent mean +/- SEM. (*p<0.05, n=2). These data indicate that XIAP is sufficient to cause resistance to GW583340.
5.1.3 Inhibition of Increases Apoptosis of IBC cells with Acquired Resistance to a Lapatinib-Analog

Upregulation of XIAP seems to correlate with acquired resistance to GW583340-induced apoptotic response when cells are chronically exposed to GW583340 (Fig. 5.7). Additionally, exogenous overexpression of XIAP caused parental IBC cells to be resistant to the cell death induced by GW583340 (Fig. 5.8), which is similar to that seen is the acquired resistance model (rSUM149) with endogenously high levels of XIAP (Fig. 5.7). Therefore, we evaluated the effect of inhibition of XIAP action. For this purpose, embelin (a small molecular inhibitor that has been shown to prevent binding of XIAP to procaspase 9 and thereby increase caspase 9 activity) was employed (Nikolovska-Coleska et al., 2004). The mechanism of embelin is demonstrated in Figure 5.9A wherein decreased levels of procaspase 9 were observed with increasing concentrations in parental and rSUM149 and rSUM190 cells. This decrease in procaspase 9 post-embelin treatment correlated with increased caspase 9 activity (Fig. 5.9A, p<0.005) and apoptosis as measured by nucleosome enrichment (Fig. 5.9A; SUM149 and rSUM149, p<0.005; rSUM149, p<0.05). Treatment of another IBC-like cell line (SUM44) with embelin did not decrease procaspase 9 expression (Fig. 5.9A), and these cells have been observed to be resistant to the apoptotic-inducing effects of the XIAP inhibitor (Appendix, Fig. 9.3), which demonstrates the specificity of embelin.

Since rSUM149 and rSUM190 cells maintained in GW583340 show XIAP overexpression and are resistant to GW583340-mediated apoptosis, experiments were conducted to determine if inhibition of XIAP action using embelin would sensitize the
resistant cells to GW583340. Addition of embelin to the rSUM149 and rSUM190 cells growing in GW583340 for 48 h caused significant cell death compared to GW583340 alone in the absence of embelin (p<0.005). Additionally, treatment of parental cells with both GW583340 (Fig. 5.3, 5.9B) and embelin (Fig. 5.9A-B) alone or in combination (Fig. 5.9B) significantly increased cell death compared to vehicle control cells (SUM149, p<0.005; SUM190, p<0.005). Data from our previous study (Aird et al., 2008) showed that treatment of sensitive parental cells with GW583340 decreases XIAP expression. Taken together, these data indicate that XIAP is a point of failure in both parental and GW583340-resistant IBC cells. Moreover, no synergy was observed between GW583340 and embelin treatment, which is most likely because these drugs affect the same pathway (i.e., decrease in XIAP). In summary, these data demonstrate that inhibition of XIAP binding to procaspase 9 using embelin and resultant increase in caspase activity causes apoptosis and potentially overcomes the acquired resistance to cell death in rSUM149 and rSUM190 cells.
Figure 5.10: Embelin sensitizes GW583340-resistant IBC cells to apoptosis via increase in caspase 9 activity.

A) Effect of embelin on procaspase 9 protein expression levels (left panels), activity (right, upper panel), and apoptosis (right, lower panel). Numbers on the western immunoblots represent densitometric analysis of procaspase 9 normalized to GAPDH. These data indicate that embelin decreases procaspase 9 expression, increases caspase 9 activity, and causes apoptosis in IBC cells. B) Effect of GW583340 and embelin treatments as shown by + and - on total cell death in SUM149 and rSUM149 (growing in
GW583340) (left panel) and SUM190 and rSUM190 (growing in GW583340) (right panel) cells. Bars represent mean +/- SEM of triplicate values. [resistant cells treated with GW583340 vs. embelin, p<0.05, n=2-3] These data show that inhibition of XIAP function in combination with GW583340 can sensitize resistant IBC cells.

**Discussion**

We report in this chapter that apoptotic dysregulation correlating with XIAP overexpression in two IBC cell models of acquired resistance to a lapatinib analog (GW583340). The parental cells, SUM190 (ErbB2 overexpressing) and SUM149 (ErbB1 activated) derived from primary tumors of IBC patients (Forozan et al., 1999), were sensitive to GW583340-mediated cell death. A marked decrease in p-ErbB2 or p-ErbB1 and corresponding inhibition of downstream signaling were evident in cells with acquired resistance to GW583340 (rSUM190 and rSUM149, respectively), similar to the parental counterparts treated with the drug, suggesting that the primary mechanism of action of GW583340, a dual ErbB1/2 tyrosine kinase inhibitor, was not compromised in the resistant cells. The model of acquired resistance in the IBC cellular model is shown in Figure 5.11.

Lapatinib is a dual tyrosine kinase inhibitor and is therefore effective in tumors with either ErbB2 expression or ErbB1 expression. Both ErbB2 overexpressing and ErbB1 activated IBC cells were sensitive to the growth-inhibitory and apoptotic-inducing effects of GW583340 (a lapatinib analog). Evidence from the clinic has shown that IBC tumors are relatively more responsive to lapatinib than other breast cancer types (RR = 50% in IBC vs. <10% in non-IBC; (Cristofanilli et al., 2006; Johnston et al., 2008)); however, the response to lapatinib is often short-lived and resistance is common (Burris...
et al., 2005). Two recent studies (Martin et al., 2008; Xia et al., 2006a) have shown that apoptotic signaling is an important mechanism of lapatinib resistance, and the apoptotic pathways have been characterized to be dysregulated in IBC vs. other LABC types (Bertucci et al., 2004; Boersma et al., 2008; Nguyen et al., 2006; Van Laere et al., 2005). Xia et al. (Xia et al., 2006a) reported that acquired resistance to lapatinib in the estrogen receptor (ER)-dependent non-IBC BT474 cells is due to increased activity of the transcription factor FOXO3a, which regulates ER downstream anti-apoptotic proteins such as survivin and Bcl-2. The other report demonstrated that MCL-1 (an anti-apoptotic member of the Bcl-2 family) is increased in colon cancer cells resistant to lapatinib (Martin et al., 2008). These studies support the idea that dysregulation of the apoptotic signaling pathway plays a key role in the resistance of cancer cells to lapatinib. In addition, a previous study in our lab has shown that XIAP expression correlates with resistance to trastuzumab in the ErbB2 overexpressing SUM190 IBC cells (Aird et al., 2008) further supporting the hypothesis that the anti-apoptotic signaling pathway is dysregulated in response to ErbB2 targeting agents.

In the present chapter, a model of acquired resistance to a dual ErB1/2 tyrosine kinase inhibitor (lapatinib analog, GW583340) was generated because resistance to lapatinib monotherapy in patients treated with daily doses of lapatinib is commonly seen (Burris et al., 2005). The GW583340-resistant lines (rSUM190 and rSUM149) were established by chronic exposure of the parental SUM190 and SUM149 cells to the drug for greater than 3 months. It was shown that the primary mechanism of action of the tyrosine kinase inhibitor remained intact in the GW583340-resistant cellular model, and
therefore we hypothesized that the apoptotic pathway was dysregulated. A significant overexpression of XIAP was observed to be mediated by IRES-dependent translation in the acquired resistant cell models studied here. In addition, it has been reported that FOXO3a, a transcription factor that promotes pro-apoptotic gene transcription (Lee et al., 2008b), and XIAP expression show an inverse correlation (Lee et al., 2008b). FOXO3a is upregulated by c-jun N-terminal kinase (JNK) (Lee et al., 2008b), which is negatively regulated by XIAP (Kaur et al., 2005). It is therefore not surprising that FOXO3a expression was decreased in the GW583340-resistant IBC cells wherein XIAP was dramatically upregulated. In addition, rSUM149 and rSUM190 cells had sustained Bcl-2 and survivin levels similar to a previous report in a non-IBC cell line resistant to lapatinib (Xia et al., 2006a).

The present data show that XIAP was specifically overexpressed in the acquired resistance IBC model (rSUM149 and rSUM190). Alternatively, exogenous overexpression of XIAP in parental cells reversed the sensitivity to GW583340-mediated apoptosis, revealing the critical role of XIAP in therapeutic resistance. Interestingly, we observed that in contrast to targeting XIAP, siRNA-mediated inhibition of another IAP (survivin) implicated in therapeutic resistance in breast cancer in these cells had no significant effect on viability or apoptosis (Appendix, Fig. 9.4), similar to our previous report in trastuzumab resistance in IBC (Aird et al., 2008). This is consistent with the role of survivin as a non-traditional inhibitor of apoptosis as it has not been effectively shown to functionally inhibit caspases (Altieri, 2003) but is rather a mitotic regulator (Lens et al., 2006). Interestingly, XIAP has been previously shown to bind and regulate the
function of survivin (Dohi et al., 2004), and therefore it is appealing to speculate that inhibition of both XIAP and survivin may be even more potent than inhibition of these molecules separately.

Embelin, an inhibitor of XIAP’s primary role of a caspase inhibitor (Nikolovska-Coleska et al., 2004), was used as a proof of principle agent to cause specific abrogation of the inhibitory interaction between XIAP and procaspase 9; treatment of rSUM190 and rSUM149 cells with embelin decreased cell viability and increased apoptosis. This indicates that XIAP is critical for survival of cells with acquired resistant to GW583340.

It is clear that apoptotic dysregulation is a critical factor in acquired lapatinib resistance in breast cancer. The results in this chapter establish the feasibility of targeting XIAP in combination with lapatinib to enhance tumor apoptosis in IBC therapy.
In cells with acquired GW583340 resistance, GW583340 can still bind to and inhibit the phosphorylation of ErbB1 and ErbB2 and subsequent downstream signaling. However, there is a marked increase in XIAP expression in these cells, which allows for their survival. Inhibition of XIAP function using embelin is able to sensitize the resistant cells, leading to apoptosis. The mechanism of XIAP upregulation is not yet known but will be evaluated in the next chapter.
6 Internal Ribosomal Entry Site-Mediated Translation of XIAP Correlates with Increased XIAP Expression in IBC Cells with Acquired Resistance to a Lapatinib-Analog

Introduction

There are many cellular processes that can cause upregulation of XIAP at both the transcriptional and translational level in addition to increased stability of either the mRNA or the protein (discussed in Sections 1.1.5.3 and 1.1.5.4, respectively). The aim of studies in this chapter is to elucidate the mechanism of XIAP upregulation in the GW583340-resistant IBC cells.

6.1.1 XIAP IRES

XIAP is one of the only cellular mRNAs with an internal ribosomal entry sequence (IRES) (Bert et al., 2006; Holcik et al., 1999; Lewis and Holcik, 2005; Stoneley and Willis, 2004) (discussed further in Section 1.1.5.4). IRES elements are generally thought of as viral elements (Balvay et al., 2009; Sarnow, 2003). An IRES is a part of the 5’ UTR with a high degree of secondary structure (Fig. 1.1) that allows translation of that mRNA during times of cellular stress when other parts of the canonical translation machinery have been shut down. This allows for translation of cellular mRNAs during times of stress, including heat shock, growth arrest, radiation, and apoptosis. It has been previously shown (Holcik et al., 1999; Holcik et al., 2000b) that XIAP is specifically translated off the IRES in cancer cells that have been subjected to low dose radiation and are resistant to the effects of radiotherapy. Just as radiotherapy causes stress to a cell, so
does introducing an agent that essentially causes the same effect as growth factor withdrawal like trastuzumab or lapatinib. Two studies have shown that GW2974 (a lapatinib analog) causes phosphorylation of AMP kinase (Shell et al., 2008; Spector et al., 2007), indicating that this drug also causes an increase in cellular stress. We therefore aimed to look at whether the upregulation of XIAP protein expression post-trastuzumab and post-lapatinib analog treatment is due to translation off of the IRES due to cellular stress.

6.1.2 XIAP Stability

There is some evidence that XIAP can be phosphorylated (discussed in Section 1.1.5.5), which stabilizes the protein by protecting it from ubiquitination and subsequent proteasomal degradation. Previously, it had been shown that AKT can phosphorylate XIAP on serine residue 87, a step that protects XIAP from autoubiquitination and from degradation due to cisplatin induced apoptosis (Dan et al., 2004). However, we cannot account for this in lapatinib analog resistant cells as they have undetectable levels of active phosphorylated AKT (Fig. 8). However, a recent study has now shown that XIAP is also a substrate of Raf-1 (Tian et al., 2006). Raf-1 is known to be an important player in the MEK kinase pathway and has been shown to be an important protein in conferring the IBC molecular phenotype (Kleer et al., 2005; Kleer et al., 2004; van Golen et al., 2000; Van Laere et al., 2005). Although it has been traditionally associated with the activation of MEK and MAPK, studies have also shown that Raf-1 has functions independent of this pathway (Huser et al., 2001; Mikula et al., 2001). Interestingly, Raf-1 has been shown to activate NFκB by dissociating the IκB inhibitory complex. Data from
Neil Spector’s lab has indicated that an ER-independent mechanism of lapatinib acquired resistance may be due to activation of the NFκB pathway (Xia et al., 2006a). Therefore it is interesting to speculate that Raf-I may be the cause of upregulation of NFκB activity and upregulation of XIAP by stabilizing the protein to allow accumulation. We therefore propose to look at the levels of phosphorylated XIAP pre- and post-trastuzumab and lapatinib analog treatment to determine if this is regulating XIAP expression.

**XIAP mRNA Expression and Protein Stability Remains Unchanged in Resistant IBC Cells**

To address the mechanism of XIAP upregulation in the GW583340-resistant IBC cells, XIAP transcription and translation of XIAP protein were characterized in the IBC cells. Real time RT-PCR analysis (Fig. 6.1) showed no significant change in XIAP mRNA levels (SUM149 vs. rSUM149, p=0.467; SUM190 vs. rSUM190, p=0.233).

Moreover, the protein stability of XIAP remained unchanged in cycloheximide-based chase experiments (Fig. 6.2).
Figure 6.1: XIAP mRNA expression is similar in parental and resistant IBC cell lines.

RT-PCR analysis of XIAP mRNA expression in parental and resistant IBC cells. β-actin was used as an internal control, and XIAP was normalized to actin using the ΔΔCT method [p= ns (not significant), n=2]. These data indicate that increase in XIAP mRNA expression is not the cause of increased XIAP protein expression in resistant cells.
Figure 6.2: XIAP protein stability is similar in parental and resistant IBC cell lines.

XIAP immunoblot analysis of parental and resistant SUM149 IBC cells treated with cycloheximide and harvested at the indicated time points. Numbers represent densitometric analysis of XIAP normalized to GAPDH. These data indicate that increase in XIAP stability is not the cause of increased XIAP protein expression in resistant cells.
No Change in XIAP Stability Increased IRES Activity in IBC Cells with Acquired Resistance to a Lapatinib-Analog

XIAP has been identified to have an IRES (internal ribosomal entry sequence) element in its 5’UTR that can be used as a non-canonical translational start site in times of cellular stress (Holcik et al., 1999; Holcik et al., 2000b; Yamagiwa et al., 2004). In order to characterize the IRES-mediated translation of XIAP in the IBC cells, a luciferase reporter construct was generated wherein the 5’UTR of XIAP, which contains the IRES, was cloned immediately upstream of the firefly luciferase gene (pGL3-XIAP.IRES). Data in Figure 6.3 reveal that both rSUM149 and rSUM190 cells had higher luciferase activity than their parental counterparts when firefly luciferase expression was normalized to the co-transfected renilla luciferase plasmid (SUM149 vs. rSUM149, p<0.005; SUM190 vs. rSUM190, p<0.005). Additionally, transfection of cells with truncated forms of the XIAP IRES [pGL3-XIAP.IRES (cont 1) and pGL3-XIAP.IRES (cont 2)], which acted as negative controls, did not elicit any luciferase activity. These data demonstrate that the upregulation of XIAP in GW583340-resistant cells is predominantly driven by IRES-mediated translation of XIAP and not increase in XIAP mRNA.
Luciferase activity was quantitated in SUM149, rSUM149, SUM190, and rSUM190 cells co-transfected with pGL3-Basic, pGL3-XIAP.IRES, pGL3-XIAP.IRES(cont 1), or pGL3-XIAP.IRES(cont 2) and a renilla plasmid (pRL-TK). Numbers represent the ratio of firefly luciferase activity to renilla luciferase activity taken as a percentage of their respective untreated readout. [** p<0.005, n=2] These data indicate that increase in XIAP IRES activity may be the reason for the increased XIAP protein expression seen in resistant IBC cells.


**Discussion**

This chapter demonstrates that the increase in XIAP observed in cells with acquired GW583340 resistance (Chapter 5) is due to IRES-mediated translation of XIAP mRNA. The model of this is shown in Figure 6.4.

The results herein show that XIAP upregulation was not mediated by increased mRNA translation or protein stability. Previous reports have shown that XIAP can be upregulated at the transcription level, mostly due to increased NFκB activity (Stehlik et al., 1998), which can lead to apoptotic resistance. Interestingly, the resistant rSUM149 and rSUM190 cells had decreased activated NFκB (Appendix, Fig. 9.9); therefore, it is not surprising that no change in mRNA expression was observed in these cells. Further, a number of reports have demonstrated that XIAP can be stabilized either through phosphorylation (Dan et al., 2004; Tian et al., 2006) or complex formation (Arora et al., 2007; Dohi et al., 2004). Our data indicate that there was no change in XIAP stability, although more detailed studies need to be performed using pulse-chase analysis to prove this.

First identified by Holcik et. al in 1999 (Holcik et al., 1999), the XIAP IRES has been shown to be active during periods of cellular stress, including irradiation (Gu et al., 2009; Holcik et al., 1999; Holcik et al., 2000b) and serum starvation (Holcik et al., 1999; Riley et al.). To our knowledge, this is the first report of increased XIAP IRES activity in response to a therapeutic drug. Recent reports indicate that XIAP has two distinct 5’ UTRs (one long and one short) (Gu et al., 2009; Riley et al.), and it would be interesting
to determine whether there is a shift in expression of these two UTRs in GW583340-resistant IBC cells. Moreover, many RNA-binding proteins have been shown to affect XIAP IRES activity (discussed in Section 1.1.5.4), and further studies are needed to test whether these are upregulated in the IBC cells with acquired GW583340 resistance.

The results in this chapter identify XIAP as a stress-related target for therapeutic intervention and establish the feasibility of targeting the XIAP IRES in combination with lapatinib to enhance tumor apoptosis in IBC therapy.
Figure 6.4: Schematic of lapatinib analog (GW583340) acquired resistance model demonstrating an increase in XIAP IRES activity.

In cells with acquired GW583340 resistance, GW583340 can still bind to and inhibit the phosphorylation of ErbB1 and ErbB2 and subsequent downstream signaling. However, there is a marked increase in XIAP expression, which was shown to be due to increased IRES activity due to a possible stress mechanism.
7 **Oxidative Stress is the Potential Stress-Related Mechanism in Resistant IBC Cells**

**Introduction**

Inflammatory breast cancer (IBC) is an aggressive, highly invasive tumor affecting younger women with racial disparity and with one of the worst clinical outcomes among breast cancers (Anderson et al., 2005; Woodward and Cristofanilli, 2009). The majority of IBC tumors are negative for estrogen receptor (ER) and have either overexpression or activation of epidermal growth factor receptors 1 and/or 2 (ErbB1, ErbB2) (Van den Eynden et al., 2004); therefore, ErbB1/2 targeting agents, including lapatinib (a dual ErbB1/2 tyrosine kinase inhibitor) are approved for treatment of IBC patients. The primary mechanism of action of lapatinib lies in its ability to downregulate ErbB1 and ErbB2 receptor phosphorylation, which leads to inhibition of downstream survival signaling via the PI3K-Akt and MAPK pathways (Xi et al., 2002). However, recent data suggest that lapatinib’s mechanism of action is more multifactorial than was first appreciated. Studies have demonstrated that lapatinib affects survivin stability (Xia et al., 2006b), multidrug resistance proteins (Dai et al., 2008), efflux and uptake transporters (Polli et al., 2008), and cell metabolism (Spector et al., 2007). Although all these mechanisms can potentially lead to cell death and the clinical response (CR) rate of patients receiving lapatinib monotherapy has been remarkable [50% CR rate in ErbB2-overexpressing IBC patients (Johnston et al., 2008)], a significant number of patients do not respond to lapatinib monotherapy and acquired resistance is frequent (Burris et al., 2005; Chen et al., 2008). Therefore, studies elucidating mechanisms of
lapatinib resistance are clinically significant. Acquired resistance to lapatinib occurs despite the decrease in ErbB1/2 phosphorylation and downstream AKT activation (Aird et al., 2010; Xia et al., 2006a). Additionally, resistance does not seem to be due to receptor mutation or increased downstream signaling as seen with another ErbB2 targeting agent (Trastuzumab- a humanized ErbB2 monoclonal antibody), but rather observed to occur via maintenance or upregulation of key survival/anti-apoptotic proteins such as survivin (Xia et al., 2006a), Mcl-1 (Martin et al., 2008), and XIAP (Aird et al., 2010), and NFkB (Xia et al., 2010).

Chapter 5 identified that XIAP expression is critical for survival of these resistant cells (Aird et al., 2010). Upregulation of XIAP in the lapatinib-analog-resistant cells was shown to be due to non-canonical protein translation via its IRES (internal ribosomal entry site) element, indicating activation of a stress response pathway (Aird et al., 2010; Holcik et al., 1999). Additionally, activation of AMPK, a kinase that responds to stresses that cause ATP depletion (Hardie, 2004), corresponds with lapatinib treatment in AU565 breast cancer cells and cardiomyocytes (Shell et al., 2008; Spector et al., 2007). These studies indicate that a stress response may lead to lapatinib-mediated apoptosis. Alternatively, this stress response may eventually lead to the ability of cancer cells to overcome death-inducing stress-response signaling, thereby leading to therapeutic resistance.

Oxidative stress, where there is an imbalance between reactive oxygen species (ROS) and the cell’s antioxidant capacity (Ott et al., 2007) (Fig. 7.1), is one of the classical stress response mechanisms that modulate apoptosis (Agostinelli and Seiler,
and a critical path of therapeutic-agent induced stress in cancer cells (Agostinelli and Seiler, 2006). Increase in p-AMPK, which has been observed post-lapatinib treatment (Shell et al., 2008; Spector et al., 2007), can be due to increased oxidative stress (Choi et al., 2001). Moreover, previous studies have shown that in cardiomyocytes, blockade of the ErbB2 receptor leads to cell death via ROS (Gordon et al., 2009). Therefore, the present chapter aimed to understand the effects of a lapatinib-analog (GW583340) on oxidative stress-induced apoptosis in IBC cells.

The results from this chapter show that the lapatinib-analog GW583340 induces generation of reactive oxygen species (ROS), including hydrogen peroxide radicals, cytoplasmic and mitochondrial superoxide, in IBC cells responding to GW583340-mediated apoptosis. Sensitivity to GW583340 and generation of ROS was reversed in the presence of a potent antioxidant (SOD mimic, MnTnHex-2-PyP). ROS levels were minimal in GW583340-resistant IBC cells (rSUM149 and rSUM190), corresponding to high levels of SOD1, SOD2, and glutathione content. Moreover, cells with acquired resistance to GW53340 had low levels of ROS and resistance to classical ROS mediators, which was potentially due to high basal levels of antioxidants. Overcoming the high levels of antioxidants in the resistant cells using SOD modulators was able to induce apoptosis in the presence of ROS generators. These data reveal a novel mechanism of lapatinib-analog-induced apoptosis and acquired resistance, whereby cells gain antioxidant capacity, making them cross-resistant to other oxidative stress inducers.
Therapeutic agents can induce oxidative stress by increase reactive oxygen species (ROS). If the cell’s antioxidant capability and the levels of ROS are imbalanced, the cell can potentially undergo apoptosis.
Lapatinib Analog Mediates Reactive Oxygen Species-Induced Apoptosis in IBC Cells

We hypothesized that the cell death effect of ErbB1/2 kinase inhibition is due to generation of ROS. In order to evaluate this, an ErbB1/2 dual kinase small molecule inhibitor GW583340 (a research grade lapatinib analog) was tested for oxidative stress response in two sets of isogenic IBC models of GW583340-sensitivity and acquired resistance. These are: 1. ErbB1 activated, triple negative, basal-like cell pairs (SUM149, rSUM149); and ErbB2 overexpressing, ER-ve, PR-ve cell pairs (SUM190, rSUM190). The GW583340-resistant clonal population of rSUM149 and rSUM190 were generated in the laboratory as previously reported (Aird et al., 2010) and maintained in 7.5 µM and 2.5 µM GW583340, respectively. The cells were treated with GW583340 for 24 h at the indicated concentrations that induce apoptosis in the sensitive cells (Aird et al., 2010), and accumulation of reactive species (summarized in Table 8) was observed using flow cytometry.
Table 8: Types of reactive species, cellular production, antioxidants, and the dye/indicator that can be used to determine levels.

<table>
<thead>
<tr>
<th>Type of Reactive Species</th>
<th>Produced By</th>
<th>Antioxidant</th>
<th>Dye/Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide (O$_2^-$)</td>
<td>Mitochondrial respiration, NADPH oxidase, Flavoenzymes</td>
<td>Superoxide dismutase</td>
<td>Dihydroethidium, MitoSox Red</td>
</tr>
<tr>
<td>Hydrogen Peroxide (H$_2$O$_2$)</td>
<td>From O$_2^-$ via SOD, Glucose oxidase, Zanthine oxidase</td>
<td>Catalases, Glutathione peroxidase, Peroxiredoxin</td>
<td>Carboxy-H$_2$DCFDA, Dihydrorhodamine 123, Dihydrocalcein AM</td>
</tr>
<tr>
<td>Hydroxyl Radical (OH)</td>
<td>From O$_2^-$ and H$_2$O$_2$ via Fe or Cu</td>
<td>Very difficult to monitor</td>
<td></td>
</tr>
<tr>
<td>Nitric Oxide (NO)</td>
<td>Nitric oxide synthase</td>
<td>Glutathione, Thioredoxin</td>
<td>DAF-FM, DAF-FM diacetate, Griess Reagent, 1,2-diaminoanthraquinone sulfate</td>
</tr>
<tr>
<td>Singlet Oxygen (¹O$_2$)</td>
<td></td>
<td></td>
<td>Singlet Oxygen Sensor Green Reagent, trans-1-(2'- Methoxyvinyl)pyrene</td>
</tr>
</tbody>
</table>
Results demonstrate that GW583340 treatment caused a significant increase in ROS accumulation in both SUM149 and SUM190 cells: Fig. 7.2A: H$_2$O$_2$–derived radicals; Fig. 7.3A: cytoplasmic superoxide anions (O$_2^-$); and Fig. 7.3B: mitochondrial O$_2^-$, which were measured by flow cytometry using carboxy-H$_2$DCFDA, dihydroethidium (DHE), and MitoSOX Red, respectively. These levels increased in a dose-dependent manner (Appendix, Fig. 9.5, 9.6). The levels of ROS accumulation were similar to treatment with classical ROS inducing agents- H$_2$O$_2$ (Fig. 7.2B) (Eruslanov and Kusmartsev) and paraquat (Fig. 7.3C), a well known O$_2^-$-generating agent (Elorza et al., 2008).

In contrast, the isogenic GW583340-acquired resistance models (rSUM149, rSUM190) of each of these parental IBC lines (Aird et al., 2010) showed minimal increase or basal levels of ROS accumulation in the presence of similar GW583340 concentrations, which indicates that GW583340 sensitivity correlates with ROS generation.

Further, GW583340 treatment in the parental cells caused p-AMPK activation (Fig. 7.3D), which can be a marker of oxidative stress (Choi et al., 2001) similar to what has been previously reported in lapatinib-sensitive AU565 breast cancer cells (Shell et al., 2008; Spector et al., 2007). In contrast, no change in p-AMPK expression was observed in the rSUM149 and rSUM190 cells that are maintained in GW583340, indicating the loss of a stress response to GW583340 in the IBC cells with acquired resistance.

Additionally, treatment of an ErbB2 negative/ErbB2 low breast cancer cell line (SUM44), which is not sensitive to the death-inducing effects of GW583340, did not increase ROS in this cell line (Appendix, Fig. 9.7), demonstrating the specificity of this
phenomenon. Taken together, these data demonstrate that GW583340 increases oxidative stress by increasing accumulation of ROS in sensitive IBC cells.
Figure 7.2: GW583340 and H$_2$O$_2$ increase accumulation of H$_2$O$_2$–derived radical in sensitive but not resistant IBC cells.

A, SUM149 (left panel) and SUM190 (right panel) cells were treated with 2.5 µM and GW583340, respectively, for 24 h, and H$_2$O$_2$–derived radical accumulation was compared to rSUM149 and rSUM190 cells growing in GW583340. B, SUM149 and rSUM149 cells were treated with 500 µM H$_2$O$_2$ (left panel) and SUM190 and rSUM190 cells were treated with 100 µM H$_2$O$_2$ (right panel) and H$_2$O$_2$–derived radical accumulation was assessed. Bars represent mean±SEM of the percentage of cells with high carboxy-H$_2$DCFDA fluorescence (n=3; *p<0.05, **p<0.01, #<0.005). These data indicate that GW583340 causes an increase in H$_2$O$_2$–derived radical accumulation in sensitive cells similar to that on H$_2$O$_2$ itself. However, resistant cells have no increase in H$_2$O$_2$–derived radical accumulation.
Figure 7.3: GW583340 and paraquat increase superoxide accumulation in sensitive but not resistant IBC cells.

A) SUM149 (left panel) and SUM190 (right panel) cells were treated with 2.5 μM and GW583340, respectively, for 24 h, and cytoplasmic superoxide accumulation was compared to rSUM149 and rSUM190 cells growing in GW583340. Bars represent
mean±SEM of the percentage of cells with high DHE fluorescence (n=3; *p<0.05). SUM149 (left panel) and SUM190 (right panel) cells were treated with 2.5 µM and GW583340, respectively, for 24 h, and mitochondrial superoxide accumulation was compared to rSUM149 and rSUM190 cells growing in GW583340. Bars represent mean±SEM MitoSOX Red fluorescence normalized to DMSO (n=3; *p<0.05, #p<0.005). Data indicate that GW583340 increases superoxide accumulation in sensitive, but not resistant, IBC cells. C, SUM149 and rSUM149 cells were treated with 5 mM paraquat (left panel) and SUM190 and rSUM190 cells were treated with 0.5 mM paraquat (right panel) and superoxide accumulation was assessed. Bars represent mean±SEM MitoSOX Red fluorescence normalized to DMSO (n=3; *p<0.05, #p<0.005). D, p-AMPK expression in SUM149 and SUM190 cells treated for 1 h with 7.5 µM and 2.5 µM GW583340, respectively (left panel). p-AMPK expression was assessed in rSUM149 and rSUM190 cells (right panel) and compared to their parental counterparts. Blots were stripped and reprobed for total AMPK. These western blots show that GW583340 increases acute stress in sensitive cells and yet this is no longer seen in resistant cells with persistent GW583340 expression.

**ErbB1/2 Kinase Inhibitor-Mediated Cell Death is Regulated by Reactive Oxygen Species**

Since GW583340 treatment causes apoptosis in the parental SUM149 and SUM190 cells (Aird et al., 2008; Aird et al., 2010) and data in Figure 7.2 and 7.3 show decreased ROS levels in the resistant counterparts, cell death/apoptosis was assessed in these cells by measuring mitochondrial membrane potential using TMRE, a dye that is sequestered by active mitochondria, and cytoplasmic nucleosomes. ROS generation in the cytoplasm and mitochondria of sensitive IBC cells treated with GW583340 or paraquat correlated with a significant loss of mitochondrial membrane potential (Fig. 7.4A). A corresponding significant increase in cytosolic nucleosome enrichment was observed in the sensitive cells with high ROS levels (Fig. 7.4B), which was similar to cytosolic nucleosome enrichment mediated by the positive control staurosporine [shown previously in (Aird et al., 2010)]. In contrast, the clonal population of GW583340-resistant cells (rSUM149, rSUM190), which had minimal increase in the levels of ROS,
displayed intact mitochondrial integrity and basal or near basal levels of cytosolic nucleosomes.
Figure 7.4: GW583340 and paraquat change mitochondrial membrane potential and increase apoptosis of sensitive but not resistant IBC cells.

Parental cells treated with GW583340 (SUM149: 7.5 µM GW583340; SUM190 : 2.5 µM GW583340) were compared to rSUM149 and rSUM190 cells growing in the same concentration of GW583340. Mitochondrial membrane potential (A) and apoptosis (B) were assessed by TMRE staining and nucleosome enrichment, respectively (n=2, *p<0.05, #p<0.005). These data show that ROS induction by GW583340 and paraquat lead to mitochondrial injury and apoptosis.
**Lapatinib Analog-resistant IBC Cells Have Increased Antioxidant Expression and Potential**

Because data in Figures 7.2 and 7.3 indicate that cytoplasmic and mitochondrial ROS are not induced by GW583340 treatment in the acquired resistant IBC cells (rSUM149 and rSUM190), the levels of well known cellular antioxidants (SOD1/Cu,Zn SOD, SOD2/MnSOD, and reduced glutathione) were characterized in the rSUM149 and rSUM190 cells. SODs catalyze dismutation of superoxide into hydrogen peroxide and oxygen: SOD1 is located in the cytoplasm, nucleus and mitochondrial intermembrane space, whereas SOD2 is located in the mitochondrial matrix (Miao and St Clair, 2009). Additionally, reduced glutathione is the major cellular antioxidant that is critical for cellular detoxification of $\text{H}_2\text{O}_2$ (Balendiran et al., 2004). Data in Figure 7.5A demonstrate that compared to parental SUM149 cells, rSUM149 cells maintained in GW583340 have increased basal levels of SOD1 and SOD2 protein expression as measured by immunoblot analysis. Further, reduced glutathione content, as measured by the GSH-Glo™ detection kit, was significantly increased in rSUM149 cells compared to parental SUM149 cells ($p<0.05$). Similarly, rSUM190 cells displayed increased SOD1, SOD2, and reduced glutathione expression compared to SUM190 cells (Fig. 7.5B). These data show that cells with acquired GW583340 resistance have increased antioxidant expression.
Figure 7.5: Antioxidant expression is increased in parental and GW583340-resistant IBC cells.

A, Left panel: immunoblot analysis of SOD1 and SOD2 in rSUM149 vs. SUM149 cells. Actin was used as a loading control. Numbers represent densitometric analysis of SOD1 or SOD2 normalized to actin. Right panel: reduced glutathione content in SUM149 and rSUM149 cells (right panel). Bars represent mean±SEM luciferase RLU normalized to SUM149 (n=2; *p<0.05). B, Left panel: immunoblot analysis of SOD1 and SOD2 in rSUM190 vs. SUM190 cells. Actin was used as a loading control. Numbers represent densitometric analysis of SOD1 or SOD2 normalized to actin. Right panel: reduced glutathione content in SUM190 and rSUM190 cells (right panel). Bars represent mean±SEM luciferase RLU normalized to SUM190 (n=2; *p<0.05). These data indicate that antioxidant expression is increased in cells resistant to GW583340, which may be why they are able to overcome ROS accumulation.
Treatment of IBC Cells with a Potent Antioxidant Reverses ROS-Mediated Apoptosis

In order to directly correlate ROS increase with sensitivity to GW583340 in the parental IBC cells, we tested the effect of a SOD mimic (MnTnHex-2-PyP) on GW583340-mediated apoptosis. The SOD mimic (MnTnHex-2-PyP) has been previously shown to be a potent antioxidant both in vitro and vivo (Batinic-Haberle, 2002; Fernandes et al.; Saba et al., 2007) and can reverse the effects of the oxidizing agent paraquat in SUM149 cells (Appendix, Fig. 9.8). Data in Figure 7.6A (SUM149) and 7.6B (SUM190) show that treatment for 24 h with 30 µM SOD mimic alone had no effect on basal ROS levels in the mitochondria, cell viability, or apoptosis. Further, treatment for 24 h with 20 µM GW583340 increases mitochondrial ROS (Fig. 7.6A, B, left panel) and correlated with decreased cell death, as observed by TMRE staining, and apoptosis quantitated by nucleosome enrichment. In contrast, combination treatment of 20 µM GW583340 and 30 µM SOD mimic for 24 h significantly reversed the generation of ROS levels compared to GW583340 alone. Further, the combination (GW583340+SOD mimic) displayed 50-70% viability compared to 15-30% viability in cell treated with GW583340 alone. Taken together, these data indicate the specificity of GW583340-mediated ROS-induced apoptosis in sensitive cells and demonstrate that this observation can be reversed using an SOD mimic.
Figure 7.6: SOD mimic/potent antioxidant reverses ROS accumulation and cell injury/apoptosis induced by GW583340 in parental IBC cells.

SUM149 (A) and SUM190 (B) cells were treated with 30 µM SOD mimic and 20 µM GW583340 alone or in combination for 24 h, and mitochondrial superoxide generation or mitochondrial integrity were assessed using MitoSOX Red and TMRE, respectively. Bars represent mean ± SEM MitoSOX Red or TMRE staining normalized to untreated or DMSO (n=2; *p<0.05, **p<0.005). These data show that the cell death observed post-GW583340 treatment is specific to ROS induction and accumulation.
Redox Modulators Reverse Resistance to the Lapatinib Analog

Because GW583340-resistant cells show less ROS accumulation after treatment with H$_2$O$_2$ and paraquat (Fig. 7.2B&7.3C) and have high expression of antioxidants (Fig. 7.5), we next determined the effect of modulation of the redox pathway on the viability and apoptosis of rSUM149 and rSUM190 cells. For this, two different agents were used: 2-methoxyestradiol (2-ME) and sodium diethyldithiocarbamate trihydrate (DETC). 2-ME has been shown in increase superoxide accumulation (Huang et al., 2000) and is in clinical trials for a variety of cancers (Trachootham et al., 2009), and DETC is a copper chelator with a similar mechanism of action of tetrathiomolybdate, which is currently in clinical trials for breast cancer (Trachootham et al., 2009). Data in Figure 7.7A&B show the effect of various agents on cell viability of rSUM149 and rSUM190 cells, respectively, as measured by trypan blue exclusion. The rSUM149 and rSUM190 cells are maintained in GW58330 with no inhibitory effect on viability; however, addition of 2-ME (1 µM) and 100 µM DETC (10-100 µM dose range tested) showed a decrease in viability, which was significant in the rSUM149 cells. In addition, when paraquat was added along with a suboptimal concentration of DETC (10 µM), a significant decrease in viability was observed (black bar) compared to paraquat alone (checked bar) in the rSUM149 and rSUM190 cells maintained in GW583340. These data indicate that redox modulators can reverse the resistance of rSUM149 and rSUM190 cells to ROS-mediated apoptosis.
Figure 7.7: Modulation of the redox pathway decreases the viability of GW583340-resistant IBC cells.

rSUM149 (A) and rSUM190 (B) cells growing in GW583340 (white bars) were treated with 1 μM 2-ME (dotted bars), 10-100 μM DETC (striped bars), paraquat (checked bars), and paraquat and 10 μM DETC in combination, and viability was assessed using trypan blue exclusion. Bars represent mean ± SEM % viable cells normalized to control cells.
(n=2; *p<0.05, **p<0.01). These data indicate that modulation of the redox pathway can sensitize resistant cells to ROS-inducing agents.

**Embelin Mediates Reactive Oxygen Species-Induced Cell Death in Resistant IBC Cells**

Data in Chapter 5 showed that endogenous XIAP is a critical factor in resistance of IBC cells to GW583340 resistance because GW583340-resistant cells were highly sensitive to an XIAP small molecule inhibitor (embelin). To determine whether XIAP plays a role in ROS-mediated apoptosis of these cells, rSUM149 cells were treated with embelin for 48 h. Inhibition of the XIAP-procaspase 9 interaction using embelin, which our previous study shows mediates apoptosis of these cells (Aird et al., 2010), increased mitochondrial superoxide (Fig. 7.8A, left panel), which correlated with decreased mitochondrial integrity (Fig. 7.8A, right panel).

To determine the mechanism of embelin-induced ROS, we examined the levels of antioxidants that data in Figure 7.5 indicate are high in rSUM149 cells. SOD1 (Fig. 7.8B), but not SOD2 (Fig. 7.8C), expression was markedly decreased post-embelin treatment. Additionally, glutathione levels were significantly decreased by embelin (Fig. 7.8D; p<0.05), indicating that embelin overcame the high antioxidant potential of the GW583340-resistant cells to cause apoptosis.
Figure 7.8: Embelin increases ROS generation and decreases antioxidant levels in GW583340-resistant rSUM149 cells.

A) rSUM149 cells were treated with 50 μM embelin for 48 h and mitochondrial superoxide (left panel) and mitochondrial integrity (right panel) were assessed using MitoSOX Red and TMRE, respectively. Bars represent mean ± SEM fluorescence intensity normalized to DMSO (n=2). *p<0.05  
B) rSUM149 cells were treated with 50 μM embelin for 48 h and glutathione content was assessed. Bars represent mean ± SEM luminescence normalized to DMSO (n=2). *p<0.05  
These data show that embelin can modulation the redox pathway by increasing ROS and decreasing...
**Discussion**

We report in this chapter a novel mechanism of apoptosis induced by ErbB1/2 targeting via ROS production in two cellular models of inflammatory breast cancer. Treatment of IBC cells that are sensitive to the apoptotic-inducing effects of an ErbB1/2 dual kinase small molecule inhibitor, GW583340, caused a marked increase in ROS, which included H$_2$O$_2$–derived radicals, cytosolic and mitochondrial superoxide. Further, this increase in ROS and apoptosis in the presence of GW583340 was reversible in the presence of a potent antioxidant/SOD mimic (MnTnHex-2-PyP). In contrast, isogenic cells with acquired resistance to GW583340 (rSUM149 and rSUM190) showed only minimal ROS accumulation, which corresponded with high antioxidant protein expression. Modulation of SOD activity using clinically relevant copper chelators/SOD inhibitors was able to sensitize the resistant cells to ROS-induced cell death.

Lapatinib is a fairly selective dual ErbB1/ErbB2 tyrosine kinase inhibitor (Karaman et al., 2008), which binds to the ATP-binding pocket on the receptors to disallow phosphorylation and downstream signaling (Fig. 7.9 [1]) (Bose and Zhang, 2009; Xia et al., 2002). However, over the past several years, different labs have determined that lapatinib has additional mechanisms of action, including mediating a stress-response (Aird et al., 2010; Shell et al., 2008; Spector et al., 2007). Spector et al. and Shell et al. identified that activation of AMPK is one of these stress response mechanisms (Shell et al., 2008; Spector et al., 2007). AMPK is activated due to changes in the AMP:ATP ratio, and oxidative stress is one of the stressors that causes a shift in
this ratio (Fig. 7.9 [2]) (Hardie, 2004). In the IBC cell lines studied here, we observed an increase in p-AMPK 1 h post-GW583340 treatment (Fig. 7.2&7.3A). Interestingly, both SUM149 and SUM190 cells have mutated p53 (Chinnaiyan et al., 2000), and therefore changes in AMPK may not change the metabolic status of these cells (Jones and Thompson, 2009) like AU565 cells, which have wildtype p53 (Neve et al., 2006). Genotyping studies are currently ongoing in our lab to assess changes in gene expression of important metabolic parameters in the parental and isogenic GW583340-acquired resistant IBC cell models. Nevertheless, these data indicate that acute exposure (Fig. 7.9 [3]) may be a mechanism of lapatinib-induced apoptosis in sensitive cellular models, whereby a significant increase in ROS leads to phosphorylation and activation of AMPK, both of which can lead to apoptosis (Fig. 7.9 [4] [5]) (Agostinelli and Seiler, 2006; Hardie, 2007).

Studies in Chapter 6 also demonstrated a stress-related mechanism, whereby translation of the anti-apoptotic protein XIAP was induced via its internal ribosomal entry site (IRES) (Aird et al., 2010), which is known to be more active during periods of cellular stress (Holcik et al., 1999). Interestingly, in brain models of ischemia/reperfusion, XIAP has been shown to modulate SOD expression levels (Resch et al., 2008; Zhu et al., 2007), potentially through upregulation of NFκB signaling (Kairisalo et al., 2007). Although a recent report has shown that activation of Rel A (a part of the NFκB complex) is important in mediating acquired resistance to lapatinib in non-IBC breast cancer models (Xia et al., 2010), the rSUM149 and rSUM190 IBC cells have lower activated Rel A compared to their parental counterparts (Appendix, Fig. 9.9).
This suggests that there may be other potential mechanisms of XIAP regulation of antioxidants, such as changes in Nrf2, c-Jun, and HIF-1 (Trachootham et al., 2009). A very recent study has shown that XIAP can modulate SOD1 activity through ubiquitination of copper chaperone of superoxide dismutases (CCS) (Brady et al.). It is intriguing to speculate that XIAP, which is markedly overexpressed in rSUM149 and rSUM190 cells, is mediating the changes in SOD1 expression seen in this study (potential mechanism illustrated in Figure 7.9), although further studies must be undertaken to conclusively prove this hypothesis.

To provide sufficient evidence that oxidative stress is indeed in question, an SOD mimic, Mn(III) meso-tetrakis(N-n-hexylpyridinium-2yl)porphyrin was employed (Batinic-Haberle, 2002; Batinic-Haberle et al., 2010). Relative to other SOD mimics reported thus far, this compound is identified as a potent compound that mimic the ability to catalyze O$_2^-$ dismutation (Batinic-Haberle, 2002) and has enhanced efficacy both in in vitro and in vivo models due to its enhanced lipophilicity. It is efficient in suppressing oxidative stress in vivo at low concentrations (0.05 mg/kg) single or multiple injections (Batinic-Haberle et al., 2010). Its appropriate lipophilicity along with cationic charge allows its accumulation in mitochondria and the nucleus where it acts presumably in a MnSOD fashion (Batinic-Haberle et al.; Zhao et al., 2005b). The data herein demonstrate that GW583340 increases superoxide, and treatment of cells with GW583340 in combination with the SOD mimic can significantly reverse increase in mitochondrial superoxide, correlating with a decrease in cell death and apoptosis (Fig. 7.9 [6]). The combination was not fully able to return ROS levels to basal levels, which may be due to
either cytosolic superoxide or H$_2$O$_2$-derived radicals that are not decreased by treatment with the SOD mimic. Interestingly, treatment of cells with the combination of GW583340 and a different SOD mimic that preferentially accumulates in the cytosol did not have as significant an effect on ROS levels or viability. This suggests that the accumulation of superoxide in the mitochondria due to GW583340 treatment is the insult that pushes the cell over the threshold (Kong et al., 2000) to undergo apoptosis, which is similar to the study by Gordon et al. who showed that ErbB2 blockade in cardiomyocytes leads to cell death specifically via the mitochondria (Gordon et al., 2009). ROS in the mitochondria could leak into the cytoplasm, which would increase damage to the cell. Moreover, because lapatinib has been shown to inhibit efflux transporters (Dai et al., 2008; Polli et al., 2008), the ROS accumulation in the cell cannot be efficiently detoxified, which potentially increases cell death. Therefore, is interesting to speculate whether treatment of cells with GW583340 in combination with both mitochondrial and cytosolic SOD mimics would have an even greater affect on GW583340 efficacy.

This report also demonstrated that IBC cells with acquired resistance have increased antioxidant expression and are resistant to other ROS-generating agents, including H$_2$O$_2$ and paraquat. Although there is still debate in the field as to whether increased antioxidants are harmful or beneficial to tumor cells and therapeutic sensitivity (Ladas et al., 2004), our data demonstrate that in terms of acquired resistance to GW583340, increased antioxidant expression allows for the continued survival and evasion of apoptosis of these cells (Fig. 7.9 [7]). Increased SOD1/2 expression can detoxify cells from mitochondrial and cytoplasmic superoxide (Miao and St Clair, 2009),
and increased reduced glutathione is particularly important for detoxification of H$_2$O$_2$ (Halliwell and Gutteridge, 1985). When both are overexpressed, such as in the case of both GW583340-resistant IBC cell lines, this can lead increased survival (Li et al., 2000), which is potentially why the GW583340-resistant cells are also insensitive to H$_2$O$_2$ and paraquat.

Importantly, previous studies have shown that increased antioxidant expression can lead to resistance to therapeutic agents whose mechanism of action is in part due to increase in ROS (Agostinelli and Seiler, 2006), including cisplatin (Balendiranan et al., 2004; Brown et al., 2009), doxorubicin (Cho et al., 2008; Kalinina et al., 2001; Kalinina et al., 2006), arsenic trioxide (Hour et al., 2004), and others (Benlloch et al., 2006; Choi et al., 2000; Li and Oberley, 1997; Mantymaa et al., 2000; Schiff et al., 2000). Therefore, we can speculate that our GW583340-resistant IBC cellular models are potentially cross-resistant to these ROS generators, and preliminary data (Appendix, Fig. 9.10) shows that they are indeed cross-resistant to the effects of cisplatin. Because increase in antioxidant expression can decrease the efficacy of some therapeutic agents, a number of inhibitors of the antioxidant system are currently being tested in Phase II trials for a variety of solid tumors (Trachootham et al., 2009). By modulating redox pathways in this study, we were able to decrease viability and increase apoptosis of cells with acquired resistance to GW583340. Both 2-ME, which can increase O$_2^\cdot$ levels (Huang et al., 2000), and DETC, a copper chelator similar to tetrathiomolybdate (Khan and Merajver, 2009) that eliminates the activity of Cu,ZnSOD1 (Heikkila et al., 1976), were able to induce cell death in the GW583340-resistant rSUM149 and rSUM190 cells (Fig. 7.9 [8]). Moreover,
suboptimal concentrations of DETC were also able to sensitize the cells to paraquat, demonstrating that modulation of the redox pathway in GW583340 cells is a potential therapeutic strategy for sensitization of these cells to ROS-generating agents.

Taken together, the data in this study demonstrate a novel mechanism of lapatinib-analog-induced apoptosis and suggest a novel therapeutic strategy using modulation of the redox pathway for IBC patients with acquired resistance to ErbB1/2 kinase inhibitors.
Figure 7.9: Schematic of possible mechanism of XIAP in resistance of oxidative stress-induced apoptosis mediated by the lapatinib analog (GW583340).

Schematic representation of mechanism of action of GW583340 in sensitive (A) and acquired resistance (B) IBC models. [1] GW583340 binds to and inhibits phosphorylation of ErbB1 and ErbB2 and subsequently decreases p-Akt expression in both sensitive and acquired resistance models (Aird et al., 2010; Xia et al., 2006a; Xia et al., 2002). However, in sensitive cells, GW583340 treatment causes an acute stress by increasing ROS [3], which in turn can increase AMPK activation [2] (Choi et al., 2001). Both a substantial increase in ROS (Kong et al., 2000) and activation of AMPK (Shell et al., 2008; Spector et al., 2007) and changes in metabolism (Spector et al., 2007) can independently lead to apoptosis of sensitive cells [4-5]. Treatment of sensitive cells in the presence of a SOD mimic can reverse this ROS-induced apoptosis [6]. In contrast, cells with acquired GW583340 resistance have higher antioxidant expression [7] corresponding with no change in ROS accumulation or p-AMPK activation [9] in the presence of GW583340 and cell survival. Modulation of the redox pathway using 2-ME or DETC can sensitize these cells to ROS-generating agents [8].
Conclusions, Implications, and Future Directions

In conclusion, the data presented in this dissertation demonstrate novel mechanisms of trastuzumab and ErbB1/2 kinase inhibitor resistance and add to our understanding of the apoptotic pathway downstream of these agents. Moreover, these studies add to the knowledge of the molecular characteristics of the two only commercially available IBC cell lines. This chapter will briefly summarize our findings in the context of the current literature on IBC and XIAP and discuss implications of this research and future research that has arisen due to the results reported herein.

The study of correlating apoptosis with therapeutic resistance of IBC cancer cells was motivated by two observations: 1) IBC cells have potentially increased apoptotic dysregulation, and 2) IBC patients have more aggressive disease that is generally more resistant to therapeutic intervention that non-IBC patients. In this study, we sought to address the mechanism of apoptotic dysregulation that potentially leads to the therapeutic resistance of IBC cells to currently used therapeutic agents, which we found to be mediated by XIAP expression. At the inception of this study, no reports had been published to show resistance mechanisms of IBC cells to trastuzumab, and no studies had been done at all in the realm of lapatinib resistance, although a number of reports have now been published using non-IBC breast cancer cell lines (Liu et al., 2009b; Martin et al., 2008; Xia et al., 2006a; Xia et al., 2010). To our knowledge, these are still the only studies demonstrating resistance mechanisms to trastuzumab and an ErbB1/2 kinase inhibitor in IBC cell lines.
**Clinical Implications**

The clinical implications of these data, although still untested, may be important. These include:

1) **XIAP may be a biomarker of response to therapeutic agents in IBC patients.**

Studies in Chapters 3-7 all demonstrate that XIAP expression corresponds to sensitivity or resistance of the IBC cells to various therapeutic agents with different mechanisms of action (TRAIL, cisplatin, trastuzumab, and an ErbB1/2 tyrosine kinase inhibitor). This suggests that examining XIAP levels post-treatment may serve as a valuable biomarker to confirm treatment efficacy. However, XIAP levels are not affected, patients should be changed to a different regimen.

A few studies have used XIAP as a biomarker of response to chemotherapy with inconclusive results (Ferreira et al., 2001a; Muris et al., 2005; Parton et al., 2002; Tamm et al., 2004b). One study was performed in breast cancer patients (Parton et al., 2002), and the authors concluded that XIAP was not a predictive marker of response in their sample, although it was very small (only 35 patients). These studies indicate that XIAP may be an unreliable marker of therapeutic response. This will need to be investigated further in IBC patients treated with ErbB targeting therapies and other therapeutics used in this dissertation to gain conclusive results.

2) **Reactive oxygen species accumulation may be a biomarker of response to lapatinib in IBC patients.**

Chapter 7 indicates that the lapatinib analog GW583340 increases ROS accumulation in sensitive, but not resistant, IBC cells, which leads to apoptosis.
Therefore, evaluation of ROS accumulation in the tumor post-lapatinib treatment may be a novel biomarker to determine efficacy of the drug. If there is no increase in ROS, patients should be changed to a different regimen. Alternatively, the antioxidant levels pre-treatment could be assessed to determine whether there is a certain amount of antioxidants that may lead to resistance to lapatinib. This would need to be assessed in a large number of patients to determine a cut-off value for high and low levels and which antioxidants are most important.

3) Diet/supplement intake may affect lapatinib efficacy.

To date, this is also the first study to show that a lapatinib analog mediates ROS-induced apoptosis. These data could potentially have huge implications on the diet and supplement intake of patients prescribed lapatinib. Although there is still debate about whether antioxidants are good or bad during cancer therapy (Ladas et al., 2004), these studies show that increased intake of antioxidants during treatment with lapatinib could potentially reduce its efficacy. Of course, further studies are needed in preclinical mouse models to determine whether dietary intake of antioxidants is able to decrease the efficacy of lapatinib on tumor apoptosis. Another strategy could be to assess epidemiological data, one study has already been completed to look at the affect of diet on bioavailability of lapatinib (Koch et al., 2009), to determine whether there is a correlation between supplement intake and lapatinib efficacy.
4) Patients with lapatinib resistance may be cross-resistant to other ROS-generating therapeutic agents.

Data shown in Chapter 7 demonstrate that GW583340-resistant cells are also resistant to the apoptotic-inducing effects of paraquat and H2O2, two well-known oxidative stress-inducing agents. This suggests that these cells may be resistant to a variety of other agents whose mechanism of action is fully or in part due to an increase in ROS (cisplatin, doxorubicin, and 5-FU, to name a few). This is significant and may lead to different treatment regimens for patients with lapatinib resistance. To date, no research has been done to determine cross-resistance patterns in lapatinib-resistant patients, but analysis of microarray data that has already been generated may be beneficial for this purpose. Further studies need to be performed to determine what agents can be used once cells/patients become resistant to lapatinib.

5) Modulation of XIAP function/expression may be a novel therapeutic strategy for IBC patients with de novo or acquired resistance to therapeutic agents.

It is clear that knockdown of XIAP expression and/or function in vitro can reverse resistance of IBC cells to various therapeutic agents. Although more work needs to be done in preclinical mouse models, this is potentially a novel therapeutic strategy down the road for women with IBC.

Currently, there is one anti-XIAP strategy (AEG35156, Aegera Therapeutics, Montreal, Quebec) in clinical trials for breast cancer and other advanced solid and hematological tumors (Dean et al., 2009; Lacasse et al., 2005; Schimmer et al., 2009; Tamm, 2008) (www.clinicaltrials.gov). To date, only one Phase II trial has reported its
results, which were very promising. In refractory acute myeloid leukemia (AML) patients, treatment with 350 mg/m$^2$ AEG35156 in combination with idarubicin and cytarabine (the standard line treatment) decreased XIAP mRNA to a median of 90% and 91% of patients (10 of 11) had a complete response (CR) (Schimmer et al., 2009). Moreover, this dose was well tolerated. These results indicate that knockdown of XIAP using antisense technology may be a promising therapeutic strategy for other cancers, including inflammatory breast cancer.
Unanswered Questions

Although there are potentially significant clinical implications, there are still a number of questions that arise from the research set forth in this dissertation that remain to be answered. These include:

1) IBC vs. non-IBC: Does this data translate?

Interestingly, we found that resistance of these IBC cells to the ErbB1/2 kinase inhibitor (lapatinib, GW583340) was markedly different than resistance of non-IBC cell line models, such as BT474 and SKBR3 (Xia et al., 2006a; Xia et al., 2010). It is unclear whether this is due to slight differences in the lapatinib analogs used, differences in estrogen receptor status, or truly a difference in the molecular pathogenesis of the IBC cell lines. One necessary question that comes out of these studies is: *Will inhibition of XIAP in combination with ErbB-targeting agents have a similar effect on non-IBC cell lines?* Asked another way: *Is XIAP specific to the pathogenesis and acquired resistance of IBC cells?* Although these questions were not addressed in this study, we can speculate that because XIAP has been implicated as a therapeutic target in numerous cancer models (A D Schimmer, 2006; Devi, 2004; Schimmer et al., 2006), including breast cancer and recently aggressive triple negative breast cancer (Wang et al., 2010), conclusions from this study can be translated into other cancer models. What is potentially more important than the broader impact these studies have on different cancer models is that these studies conclusively indicate that XIAP could be a novel therapeutic target for IBC, which because of its other unique molecular features makes it highly difficult to treat. Although we believe this is very exciting, further studies must be
carried out to determine whether the results seen in IBC cell lines are similar to what is truly happening in vivo. It is imperative that patient biopsies, both pre- and post-treatment, be examined for XIAP expression, and due to the IRES-mediated translation of XIAP and not the increase in XIAP mRNA, this needs to be done directly in tissue samples, not microarray studies. Moreover, the scientific world would greatly benefit from an increased number of IBC cell line models.

2) Will inhibition of XIAP in combination with therapeutic agents work?

The studies in this dissertation demonstrated that downregulation of XIAP in combination with therapeutic agents can sensitize IBC cells in culture. However, it is well known that cells in culture and tumors in vivo are two very different things. Although it is clear that embelin is safe (it has been used for centuries in China as an herbal remedy), its effects on inflammation (Chitra et al., 1994), hormone regulation (Githui et al., 1991), and antioxidant pathways (Joshi et al., 2007) in vivo may confound or counteract the effects of the ErbB-targeting agent itself. Therefore, preclinical mouse models need to be developed to test embelin in combination with the ErbB-targeting agents in vivo. These studies will determine whether embelin is a candidate for use as an XIAP inhibitor in combination with other therapeutic agents in the clinic.

If embelin, either due to its off-target effects or bioavailability, is not efficacious in combination with therapeutic agents in xenograft mouse models, there are a variety of other XIAP inhibitors (Cheung et al., 2006), although only one (AEG35156) is in clinical trials (Dean et al., 2009; Lacasse et al., 2005; Schimmer et al., 2009; Tamm, 2008), that
could be investigated in these models. AEG35156, discussed above, has had promising results in clinical trials (Schimmer et al., 2009), and further trials in breast cancer or IBC may be warranted. Other XIAP inhibitors are being developed, although they are not as far along as AEG35156 (Cheung et al., 2006; Danson et al., 2007; Gimenez-Bonafe et al., 2009; Jana and Paliwal, 2007; Rajapakse, 2007). Because they have different inhibitory properties, these all need to be tested in this model to determine which is the best to move forward with. In addition, novel XIAP inhibitors still need to be screened for, and one method to do this will be discussed further in future directions.

Moreover, although XIAP can be knocked out in normal cells without issue and XIAP knockout mice are phenotypically normal (Harlin et al., 2001), these and other studies indicate that because XIAP is not only a caspase inhibitor, signaling in normal cells may be dysregulated due to XIAP inhibition. Therefore, more studies need to be performed in normal cells to determine whether XIAP inhibition leads to changes in normal cell signaling beyond just the apoptotic pathways. These studies would determine whether inhibition of XIAP in combination with ErbB targeting agents is safe for patients.

Additionally, although it has been known for some time that XIAP is involved in cellular signaling pathways, the literature on the different pathways XIAP is involved in has recently exploded. This does have implications because inhibition of XIAP in combination with therapeutic agent could lead to up or down regulation of other pathways that may synergize or antagonize the mechanism of action of the drug. This would need to be investigated on a drug-by-drug basis.
3) Does XIAP play a role in mediating resistance to oxidative-stress induced apoptosis?

A few studies have demonstrated a role for XIAP in mediating antioxidant expression and capacity (Brady et al.; Kairisalo et al., 2007; Resch et al., 2008; Zhu et al., 2007), but to our knowledge none of these studies have been done in a cancer model. The results in Chapter 7 add to the knowledge of the role of XIAP in cellular signaling and further demonstrate that XIAP is more than just a caspase inhibitor and may be involved in mediating resistance to ROS generation. The studies outlined herein were preliminary, and further studies need to be undertaken to determine whether XIAP expression or function truly modulates antioxidant expression or ROS accumulation. To this end, the lab now has stable XIAP overexpression and knockdown cells with the appropriate vector controls (all plasmids were kindly provided by Dr. Colin Duckett, University of Michigan). Moreover, various mutants have been generated to further study the more basic mechanisms of XIAP function in the realm of redox pathways (Fig 8.1). These cells will be useful in determining the role of XIAP in oxidative stress and which domain(s) is important for that particular function.
Figure 8.1: XIAP mutants that have been stably transfected into IBC cell lines.
**Future Directions**

There are a number of scientific questions and projects that have been generated by the research described in this dissertation. The future studies that come out of this project include:

1) Validation of findings in xenograft mouse models.

The optically imageable model developed in SCID mice in collaboration with Dr. Mark Dewhirst’s group will be used to study the efficacy of the XIAP PMOs alone or in combination with trastuzumab and lapatinib as a proof of concept. Ten-week-old female athymic nude mice will be orthotopically injected with stably transfected luciferase expressing sensitive and resistant IBC cells (1 x 10^6 cells) into the upper left mammary fat pad.

**Experimental Design:**

1. Characterization of the bioavailability and optimal drug doses of the XIAP in a murine model. We need to first demonstrate that XIAP is being affected in vivo tumors when the chosen XIAP inhibitor is administered. To do this, we will treat xenograft animals (6 mice/group) for inhibition of XIAP in the tumor after treatment with a single dose of embelin or antisense molecules. The tumor will be lysed 24 h after treatment and analyzed for XIAP levels as previously described (Uchida et al., 2004).

2. Efficacy Studies: The optimal dose of embelin and/or XIAP antisense will then be tested in combination with trastuzumab (10 mg/kg twice weekly, i.p.)
(Nagata et al., 2004) and lapatinib (orally-100 mg/kg daily) (Xia et al., 2004). Tumor growth delay will be observed after the daily administration of therapies beginning at day 1 when tumor reaches the volume of ~200 mm$^3$ and will be stopped (mice will be sacrificed if signs of morbidity are observed) after five-fold increase in tumor volume is reached. Tumor growth will be followed by measuring tumor volumes using formula $V = \frac{a \times b^2}{2}$, where $a$ is the longer and $b$ is the shorter diameter. Mice will be sacrificed and tumors will be collected and tumor lysis will be made for protein expression and analysis. In addition tumors will be processed for histology, which will include H&E staining (for cell proliferation) and trichrome-Masson staining (for neovascularization). Tunel assay will be done for apoptosis detection.

These studies will validate whether inhibition of XIAP in sensitive and resistant cells increase tumor apoptosis, thereby decreasing tumor progression.

2) Validation of findings in patient samples.

Experimental Design:
To address the clinical relevance of our overall findings and hypotheses, XIAP protein expression will be examined in fresh tumor biopsy obtained prior to treatment, at day 28 of treatment, and another tumor biopsy at the time of disease progression. Pretreatment biopsies will serve as controls and each patient will serve as his/her control. Total RNA and protein will be isolated from tumor tissue and processed using standard protocols,
and microarray and IHC analysis will be performed, respectively, to determine XIAP expression levels. This will be correlated with clinical response of patients.

3) Development for an assay to test for XIAP inhibition in a high throughput fashion and development of novel XIAP/pan-IAP inhibitors.

Currently, we are using western immunoblot to study XIAP expression. Although this is a tried and true method to observed XIAP levels, there needs to be an assay to observe XIAP in a high throughput manner. To date, no such assay exists. This will aid in the development of novel XIAP inhibitors that are potentially more specific that embelin or that only affect the IRES of XIAP, which may be safer to normal cells.

Although the human inhibitor of apoptosis proteins are highly conserved and have some redundant biological function, they are not homologous at the nucleotide sequence level. Therefore a pan-IAP inhibitor cannot be based on a genomic strategy like siRNA or antisense oligonucleotides. In my opinion, the best way to try to develop a pan-IAP inhibitor would be to try and target the functional proteins. There are cellular proteins that naturally do this (Smac, Omi/HtrA2, XAF-1, etc…) by binding of an IAP binding motif (IBM) on these proteins to the BIR domains of IAPs (including XIAP, survivin, and livin). The IBM is a tetrapeptide on the far N-terminus of the mature forms of these proteins and is composed of small, hydrophobic amino acids with alanine always in the first position. These tetrapeptides have been synthesized and used in vitro with some success, however targeting peptides to cancer cells in vivo would be a huge hurdle this technology would have to get over.
A more recent paper has identified copper to be a negative regulator of XIAP (Mufti et al., 2006; Mufti et al., 2007) as copper binding to a certain sequence on XIAP (CXXC- which occurs in all 3 BIR domains in the RING domain) can cause a conformational change that no longer allows the protein to inhibit procaspase 3. Although to date other IAPs have not been shown to bind copper, they all possess copper binding motifs in their BIR domains. I therefore hypothesize that copper can bind to and change the function of IAPs other than XIAP.

However, injecting copper into a tumor is probably not the way to go and although it might be a pan-IAP inhibitor, there will be numerous other pathways affected. I propose that we screen a compound library for small molecules that competitively inhibit the binding of copper to the IAPs. The endpoint of this screen would be to find molecules that can bind to the copper binding motifs of the IAPs and potentially regulate their function in a similar way as copper itself. I am choosing to go with small molecules because they are 1) easy to screen for, 2) easy to deliver in cells and animals, and 3) have a history of low toxicity and high efficacy.

There are numerous potential limitations with this hypothesis. First, copper may not negatively regulate the other IAPs as it does XIAP. In vitro studies could be performed to access the functional regulation of the other IAPs by copper binding. Second, even if copper can bind to and negatively regulate all the IAPs, the binding motif of this site may not be specific enough to only pull out small molecules that bind IAPs. There is a possibility that this motif is similar enough in sequence and structure that if a small molecule is found, it will have an effect on other proteins.
Screening for Pan-IAP Inhibitors

To do this screen, we would need to develop a high throughput way to measure copper in solution. I suggest an ELISA method where a polyclonal XIAP antibody is coated onto 96 well plates, purified XIAP is added and incubated, plates are washed, and then the compounds from the library added to the plates. After another wash, a buffer with a known concentration of free copper ions is added and incubated. This solution is then taken off, put into another 96 well plate and accessed for free copper ion concentration. The compounds identified in this screen would go on to be screened against survivin, then livin, then the other IAPs in consecutive screens.

Again, there are potential limitations in this part of the project as well. Even though I propose to use polyclonal antibodies to coat the plates, it is possible that the copper binding motif will be hidden by the antibody-protein interaction. In addition, there may not be one small molecule that is able to bind to all of the IAP’s copper binding motifs. Finally, this is an in vitro assay and tells us nothing about functionality of the protein once bound to the putative small molecule and if the small molecule would be able to bind in the more complicated environment of the cell.

4) Determination of the role of XIAP in oxidative stress.

It is clear for the data in Chapter 7 that resistant cells (which have increased XIAP-Chapter 5) have increased antioxidants. Moreover, modulation of XIAP function using embelin changed the expression of antioxidants and increased the accumulation of ROS. Taken together, these data potentially indicate that XIAP plays a role in resistance
to oxidative stress-induced apoptosis. However, further work needs to be done to prove this hypothesis.

**Experimental Design:**

1. **Determine whether stable overexpression or knockdown of XIAP changes the expression levels of redox signaling pathway proteins.** To do this, we will use a PCR array from SABiosciences to look at the changes in >80 different transcripts that are known to play a role in the redox pathway. IBC cells with stable overexpression or knockdown of XIAP will be compared to vector control cells. The data from these experiments will determine whether changes in XIAP expression cause changes in redox pathways.

2. **Determine whether stable overexpression or knockdown of XIAP changes the response of IBC cells to ROS-inducing agents.** To do this, we will use the PCR array in IBC cells with stable overexpression or knockdown of XIAP that are treated with ROS-inducing agents paraquat and H$_2$O$_2$. The results will be compared to treated vector control cells. Additionally, apoptosis and viability assays (caspase activity, nucleosome enrichment, trypan blue) will be performed to determine differences in cell death post-treatment. The data from these experiments will determine whether changes in XIAP expression mediate cellular responses to oxidative stress.
Summary

In conclusion, we found that XIAP expression inversely correlated with sensitivity of IBC cells to therapeutic agents, and XIAP expression was specifically and markedly increased in IBC cells with resistance to both trastuzumab and a lapatinib analog. This was shown to potentially be due to oxidative stress-mediated activation of the XIAP IRES, leading to increased translation of XIAP. Increased XIAP expression not only inhibited caspase-mediated apoptosis, but also changed cellular signaling parameters so that the cells were able to survive under persistent stress. Inhibition of XIAP in these resistant cells was able to sensitize the IBC cells to both agents, demonstrating the feasibility of targeting XIAP in combination with either trastuzumab or lapatinib. While we are encouraged by these results, in vivo studies still need to be done to determine whether this phenotype is indeed demonstrated by IBC patients being treated with these agents and whether inhibition of XIAP could indeed lead to changes in tumor growth and apoptosis without affecting normal cells. Our complete understanding of the molecular characteristics of IBC could greatly benefit patients with this devastating disease. Moreover, understanding the signaling functions of XIAP could be of great benefit to a variety of cancers and other diseases where this signaling pathway is dysregulated.
Figure 9.1: Effect of XIAP siRNA alone and in combination with trastuzumab in SKBR3 cells.

Viability of SKBR3 cells transfected with XIAP siRNA alone and in combination with 15 µg/ml trastuzumab. Viability was assessed using trypan blue exclusion.
Figure 9.2: PTEN expression in a panel of breast cancer cell line.

PTEN western immunoblot expression in a panel of breast cancer cell lines. Actin was used as a loading control.
Figure 9.3: Effect of embelin on SUM44 cells.

SUM44 cells were treated with the indicated concentrations of embelin for 48 h and proliferation was assessed using MTT.
Figure 9.4: Effect of survivin siRNA on viability of GW583340-resistant IBC cells.

rSUM190 (A) and rSUM149 (B) cells were transfected with 100 nM survivin siRNA for 48 h and survivin expression (left panels) and viability (right panels) were assessed using western immunoblot and trypan blue analysis, respectively. Bars represent mean±SEM viability normalized to untreated (n=2).
Figure 9.5: Effect of 20 μM GW583340 on ROS generation in SUM149 cells.

Cells were treated with 20 μM GW583340 for 24 h, and hydrogen peroxide radical generation was assessed using carboxy-H$_2$DCFDA staining (upper left panel). Bars represent mean±SEM of the percentage of cells with high carboxy-H$_2$DCFDA (n=3). To determine superoxide generation and mitochondrial integrity, cells were treated with 20 μM GW583340 for 24 h, and cytosolic superoxide generation (upper right panel) mitochondrial superoxide generation (lower left panel), and mitochondrial integrity (lower right panel) were assessed using DHE, MitoSOX Red, and TMRE, respectively. Bars represent mean±SEM of the percentage of cells with high DHE staining or MitoSOX Red or TMRE staining normalized to DMSO (n=3).
Figure 9.6: Effect of 20 μM GW583340 on ROS generation in SUM190 cells.

Cells were treated with 20 μM GW583340 for 24 h, and hydrogen peroxide radical generation was assessed using carboxy-H$_2$DCFDA staining (upper left panel). Bars represent mean±SEM of the percentage of cells with high carboxy-H$_2$DCFDA (n=3). To determine superoxide generation and mitochondrial integrity, cells were treated with 20 μM GW583340 for 24 h, and cytosolic superoxide generation (upper right panel) mitochondrial superoxide generation (lower left panel), and mitochondrial integrity (lower right panel) were assessed using DHE, MitoSOX Red, and TMRE, respectively. Bars represent mean±SEM of the percentage of cells with high DHE staining or MitoSOX Red or TMRE staining normalized to DMSO (n=3).
Figure 9.7: Effect of GW583340 on ROS generation in SUM44 cells.

Cells were treated with the indicated concentrations of GW583340 for 24 h, and proliferation (A) and viability (B) were assessed via MTT and trypan blue assays, receptively. C) Hydrogen peroxide radical and superoxide generation were assessed using carboxy-H$_2$DCFDA (white bars) staining or DHE (black bars), respectively. Bars represent mean±SEM of the percentage of cells with high fluorescence (n=2).
Figure 9.8: Effect of SOD mimic in combination with paraquat in SUM149 IBC cells.

Cells were mitochondrial superoxide generation was assessed using MitoSOX Red. Bars represent intensity of MitoSOX Red staining normalized to untreated.
Figure 9.9: Basal p-NFκB expression in parental and resistant IBC cells.

p-NFκB immunoblot analysis of rSUM149, SUM149, rSUM190, and SUM190 cells. GAPDH was used as a loading control.
Cells were treated with the indicated concentrations of cisplatin for 24 h, and viability (left panel) and signaling (right panel) were assessed via trypan blue exclusion and western immunoblot analysis, receptively. Bars represent mean ± SEM of the percentage of viable cells normalized to DMSO (n=2).
References


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Biography

Katherine Marie Aird was born to Dr. Charles L. Aird and Donna M. Aird in Riyadh, Saudi Arabia on July 27th, 1983. She subsequently moved with her family to Jeddah, Saudi Arabia; Huddleston, VA; Springboro, OH; New Delhi, India; and finally Singapore, where she graduated from Singapore American School. After high school, she attended Johns Hopkins University in Baltimore, MD, where she majored in Biology and did tuberculosis research with Dr. Yukari Manabe. After graduating from Johns Hopkins University in 3 years, Katherine went to London where she continued researching tuberculosis with Dr. Ruth McNerney at the London School of Hygiene and Tropical Medicine. Upon returning to the US, she enrolled as a PhD student at Duke University and started her rotations in May 2005. Upon completing her rotations, Katherine chose the lab of Dr. Gayathri Devi to perform her thesis work on apoptotic dysregulation and therapeutic resistance of inflammatory breast cancer models.

EDUCATION

Duke University, Durham, NC (December 2005-present)

Doctor of Philosophy

Department of Pathology

Johns Hopkins University, Baltimore, MD (September 2001-May 2004)

Bachelor of Arts in Biology, graduated with honors

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HONORS, AWARDS, and FELLOWSHIPS

1. Women in Endocrinology Young Investigator Award (2008)
2. Duke Comprehensive Cancer Center Outstanding Poster Award (2008)
3. Department of Defense Predoctoral Traineeship Award Recipient (2008-present)
4. Duke Comprehensive Cancer Center Young Investigator Award (2006)

PUBLICATIONS and PRESENTATIONS

Peer Reviewed Publications


Conference Presentations and Proceedings

8. Aird KM, Ghanayem RB, Peplinski S, Lyerly HK, Devi GR, “Functional Link between X-linked Inhibitor of Apoptosis Protein and Therapeutic Resistance in


