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THE CYTOTOXIC EFFECT OF THE BCL-2 FAMILY OF PROTEINS IN BREAST CANCER CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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LIST OF ABBREVIATIONS

ER: Estrogen receptor

PR: Progesterone receptor

HER2: Human epidermal growth factor receptor 2

TN: Triple negative

MCL-1: Myeloid cell leukemia-1

BCL-2: B-cell lymphoma-2

MOMP: Mitochondrial outer membrane permeabilization

PARP-1: Poly (ADP-ribose) polymerase-1

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Abstract

Breast cancer is the second leading cause of death amongst women ages 20 to 59. Despite advancements in cancer therapies, more research is necessary to improve the diagnoses and treatment of several types of breast cancer. Paclitaxel (Taxol) is a commonly utilized anti-cancer drug for various types of solid tumors. However, the molecular mechanism utilized by paclitaxel to induce cell death is still elusive. Previous studies in our laboratory have shown that the pro-apoptotic BCL-2 family protein, BAK (BCL-2 homologous antagonist/killer) plays an important role in paclitaxel-induced cell death. In untreated breast cancer cells, BAK is associated with the anti-apoptotic BCL-2 family protein MCL-1 (myeloid leukemia cell differentiation protein). BAK is activated with paclitaxel treatment in concert with loss of MCL-1 expression. In addition, it has been shown that the pro-apoptotic BH3-only BCL-2 family protein Noxa, specifically interacts with MCL-1 to inactivate MCL-1 function. Based on these observations, we hypothesized that modulation of Noxa/MCL-1 axis could mimic paclitaxel-induced cell death. Here, we found that down-regulation of MCL-1 induced cell death in all breast cancer cell lines that we tested, but not in a non-transformed breast epithelial cell line. In contrast, Noxa overexpression induced MCL-1

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degradation and cell death in some cell lines (Noxa-sensitive), while in others Noxa overexpression neither changed MCL-1 levels nor induced cell death (Noxa-resistant). Noxa strongly interacted with MCL-1 in the Noxa-sensitive cell line, but not in the Noxa-resistant cell line. Based on these findings, the overexpression of Noxa might have two different mechanistic effects on MCL-1 levels in the breast cancer cell lines (induction of MCL-1 degradation or no effect on MCL-1). In Noxa-sensitive cells, the finding could be used as a potential therapeutic strategy for the treatment of breast cancer.

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CHAPTER 1: INTRODUCTION

1.1 Cancer

Cancer is a leading cause of death worldwide. Lung, prostate, colorectal and breast cancers are responsible for the most cancer-related deaths each year (www.wcrf.org). In the United States, cancer is the second most common cause of death (exceeded only by heart disease) and in 2014, nearly 600,000 Americans are expected to die from this terrible disease (www.cancer.gov). Statistical data from the years 2005-2009, showed that in men cancer incidence rates were reduced by 0.6% and remain stable in women per year. Additionally, cancer death rates declined in men by 1.8% and by 1.5% in women each year (Siegel et al., 2013).

Cancer refers to a group of more than 100 diseases in which abnormal cells divide uncontrollably and are capable of invading other tissues. These cancerous cells can spread to different parts of the body through the blood and lymph systems. Cancer types can be grouped into broader categories. The main categories of cancer include:

- **Carcinoma** - cancer that arises from epithelial tissues that line or cover internal organs or the outside of the body (skin). There are two major carcinoma subtypes: adenocarcinoma - which are tumors that develop in the lining or inner surface of an organ or gland, and squamous cell carcinoma - which are tumors that arise in the squamous epithelium (epithelial cells that line and protect other cells) (www.cancer.gov).

  Carcinomas account for 80 to 90 percent of all cancer cases in the Western World (Weinberg, 2007)
- **Sarcoma** - cancer that originates in supporting and connective tissue including bone, cartilage, fat, muscle, and blood vessels (www.cancer.gov). Most commonly sarcomas

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- develop as a painful mass on the bone and occur predominantly in young adults. In oncology clinics, 1% of the tumors encountered are sarcomas (Weinberg, 2007).
- **Leukemia** - cancer that originates in blood-forming tissue such as the bone marrow and causes abnormal hematocytes to be produced and enter the blood stream. Leukemia is characterized as the overproduction of immature white blood cells that do not perform optimally. As a result, the patient is more prone to infections.
- **Lymphoma** – cancer that develops in the glands or nodes of the lymphatic system. Unlike leukemias, lymphomas are solid cancers.
- **Myeloma** - cancer that begins in the plasma cells of the bone marrow.
- **Central nervous system cancers** - cancers that originate in the tissues of the brain and spinal cord.
- Cancer can be caused by both external and internal factors such as tobacco, obesity, specific infections (Helicobacter pylori, Human papilloma virus, and Hepatitis B and C virus), reproductive behaviors, the use of exogenous hormones, consumption of red and processed meat, inherited or acquired genetic mutations, and sunlight. (www.cancer.org).

1.2 Breast Cancer

Breast cancer is the most common cause of cancer-related death among women and the most frequently diagnosed type of cancer among females worldwide (Jemal et al., 2011). In the United States alone, 1 out of every 8 women is estimated to be diagnosed with breast cancer annually (www.breastcancer.org). The World Health Organization states that since 2008, the incidences of breast cancer have increased by more than 20% while mortality rates have increased by 14%.

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Breast cancer can be classified into molecular subtypes using biological markers that includes the presence or absence of Estrogen receptors (ER+/ER-), Progesterone receptors (PR+/PR-), and Human epidermal growth factor receptor 2 (HER2+/HER2-). Therefore, breast cancer can be divided into 2 broad groups: **ER and/or PR-positive** and **ER and/or PR-negative** (Rakha et al., 2008).

In addition, the **ER/PR-positive** classification can be sub-divided into two groups referred to as Luminal A and Luminal B tumors depending on the levels of expression of HER2 (Schnitt, 2010). The Luminal A subtype makes up about 40% of breast cancers and they are defined by their lack of HER2. Luminal A tumors constitute the most common breast cancer subtype and they are usually slow growing and less aggressive with the most favorable short term prognosis. Luminal B tumors represent 10%~20% of breast cancers and are distinguished by their expression of HER2 or high cell proliferation rates. In general, luminal tumors often present a positive mean of prognosis due to the presence of various hormone receptors (ER, PR, and HER2) (Zhang et al., 2014).

Endocrine therapy is the first line of treatment of luminal type breast cancer and involves the inhibition or blocking of hormones. Normally, Luminal A tumors are associated with the most favorable short term prognosis versus Luminal B tumors. This association is in part due to the fact that the expression of hormone receptors is predictive of a favorable response to hormonal therapy. Nonetheless, about 30% of ER+ breast cancers are resistant to hormonal therapy, which suggests that other therapeutic approaches, are required for more effective results (Blows et al., 2010).

ER/PR-negative breast cancers can be sub-divided into two groups characterized by their expression of HER2. These include tumors which overexpress HER2 or tumors that are HER2-

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negative. HER2 overexpression is identified in about 15% of invasive breast cancers and is associated with low ER expression. HER2 is a transmembrane protein which is part of a family of receptors that include EGFR/HER1, HER2, HER3, and HER4. These receptors are responsible for regulating a complex signal transduction cascade that influences many cellular processes including cell cycle progression, cell survival, cell proliferation, and cell motility. HER2 overexpression results in the continual activation of a growth signaling pathway and contributes to the propagation of breast cancer cells (Slamon et al., 1987) Thus, HER2 positive breast tumors are likely to be aggressive, fast growing, and less receptive to hormonal treatment. In spite of these characteristics, pharmacological treatments that target HER2 have resulted in promising outcomes (Perou et al., 2000). One popular pharmacological treatment involves trastuzumab (Herceptin), a monoclonal antibody known to bind to the extracellular domain of HER2, which ultimately blocks the downstream signaling pathways in cancer cells but not in normal cells. Trastuzumab usage in combination with chemotherapy has proven to be an especially effective treatment for advanced-stage breast cancer that reduces the risk of relapse (Gajria and Chandarlapaty, 2011).

Between 12% and 24% of breast cancers are considered triple negative (TN) belonging to the basal-like subtype. Triple negative cells do not express the ER, PR, and HER2 (ER-/PR-/HER2-) (Sorlie et al., 2001; Bertucci et al., 2000; Bertucci et al., 2004). Therefore, TN breast cancer cells do not respond to hormonal therapy (such as tamoxifen or aromatase inhibitors) or therapies that target specifically HER2, such as trastuzumab.

Chemotherapy is typically the only therapeutic option to treat TN breast cancer due to their lack of hormonal receptors. Examples of chemotherapeutic agents are anthracycline and anthracycline/taxane which offer positive initial effects in the treatment of TN tumors, but a

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rapid relapse rate. Several emerging agents are currently under clinical trials for the treatment of TN breast cancer. Such agents include platinum chemotherapeutic drugs combined with poly (ADP-ribose) polymerase 1 (PARP1) inhibitors, which hinder DNA repair in cancer cells The majority of tumors with BRCA1 (tumor suppressor gene) dysfunctions are TN and have a deficient double-stranded DNA break repair. Thus, these tumors are more sensitive to PARP1 inhibitors. (Ovcaricek et al., 2011).

1.3 Paclitaxel Treatment

Paclitaxel (Taxol), a taxane plant product (Figure 1), is one of the most effective broad-spectrum anti-cancer agents that are recognized for the treatment of a variety of cancers including breast, lung, ovarian, head and neck as well as Kaposi's sarcoma. Paclitaxel was discovered in 1967 at the Research Triangle Institute (North Carolina, USA) when it was isolated from the bark and needles of the Pacific Yew evergreen tree ("*Taxus brevifolia*") (Jordan et al., 2004). Paclitaxel was approved by the FDA in 1992 and has since been used with high clinical frequency. Whereas other drugs like colchicine (a medication for the treatment of gout) cause the depolarization of microtubules, paclitaxel halts their function by having the opposite effect of hyper-stabilizing their structure. Particularly, paclitaxel binds to the β subunit of tubulin. Tubulin is the protein that polymerizes into long filaments that form microtubules- hollow fibers which serve as a "skeletal" system for a living cell. By binding to tubulin and causing the protein to lose its flexibility, paclitaxel prevents a cell from dividing (Figure 2). As a result, the microtubule/paclitaxel complex does not have the ability to disassemble. This complex harmfully affects cell function because the shortening and the lengthening of microtubules (called dynamic instability) is necessary for their normal transport of cellular material in the cell (Chu et al 2013). Also, during

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mitosis, microtubules are responsible for positioning the chromosomes during their replication following separation into the two daughter-cell nuclei. In addition to stabilizing microtubules, paclitaxel can sequester free tubulin resulting in the effective depletion. Normal cells are also affected adversely, but since cancer cells divide much faster than non-cancerous cells, they are far more susceptible to paclitaxel treatment (Hernandez et al., 2007)

The resistance of cancer cells to chemotherapeutic drugs represents a major problem in cancer treatment. Although Paclitaxel has proven effective for the treatment of cancer, patients often experience relapse after initially responding to paclitaxel. The mechanisms of paclitaxel resistance include point mutations in tubulin, altered expression of β tubulin isotypes and post-translational modifications (all of them except for acetylation) to tubulin. Molecular modifications to regulatory proteins involved directly in the function of microtubules (Stathmin, a microtubule destabilizer and MAP4, a microtubule stabilizer) can also affect paclitaxel sensitivity in a cell (Orr et al., 2003).

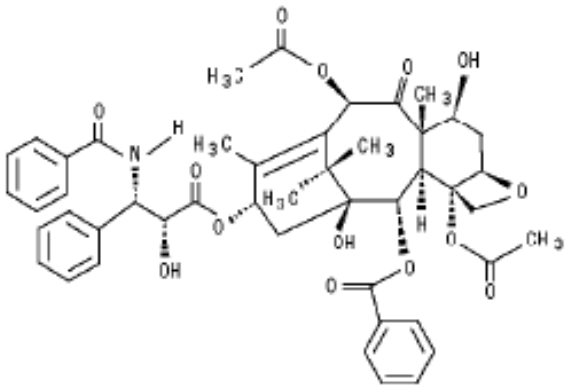

Figure 1: Structural formula of Paclitaxel. The chemical name for Paclitaxel is 5β, 20-Epoxy-l,2α,4,7β,10β,13α-hexahydroxytax-l l-en-9-one 4,10-diacetate 2-benzoate 13-ester with (2R,3S)-N-benzoyl-3-phenylisoserine (Orr et al., 2003).

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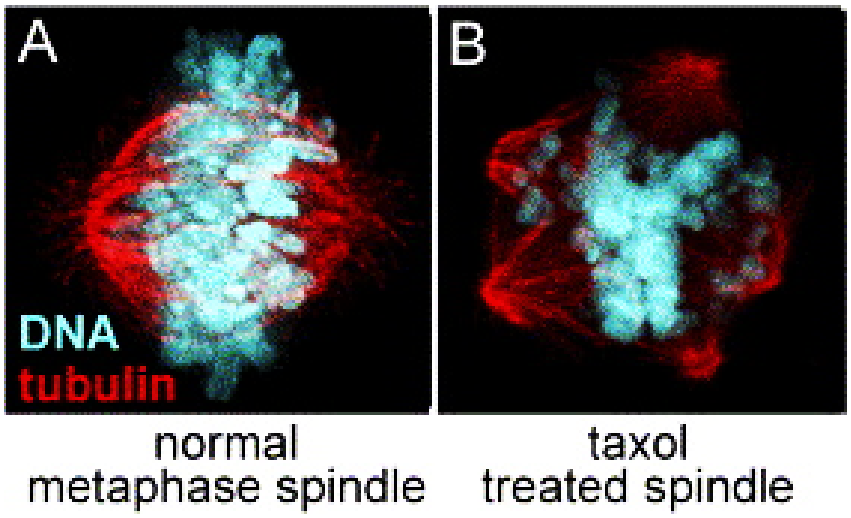

Figure 2. Paclitaxel as a mitotic stabilizer. (A) Normal metaphase spindle. Microtubules are shown in red and chromosomes in light blue. (B) Paclitaxel binds and stabilizes microtubules blocking cell division (modified from Jordan et al., 2004)

1.4 Apoptosis

Apoptosis (from Ancient Greek *apo* "away from" and *ptosis* "falling") is the process of programmed cell death that regulates cell proliferation in response to cell damage. Cell death is essential for proper homeostasis and adaptation to the changing environment. Dysregulation of apoptosis may result in the manifestation of cancer and autoimmunity (resistance to apoptosis). Meanwhile, excessive cell death gives rise to cardiovascular diseases and neurodegenerative disorders such as Alzheimer, Parkinson, and Huntington disease. Thus, apoptosis regulation is vital for the proper function of the body.

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Apoptosis is characterized by cell shrinkage, active membrane blebbing, chromatin condensation and fragmentation, cleavage of DNA and loss of adhesion to the surrounding environment (cells, tissues) (Nishida et al., 2008; Ouyang et al., 2012). Other types of cell death include autophagy and necrosis. Autophagy differs from apoptosis in that it involves a cellular mechanism of self-eating and often occurs when apoptosis is blocked. Necrosis is characterized by the swelling and bursting of cellular organelles eventually decomposing, and occurs when the plasma membrane is ruptured or when there is a drastic decrease of energy in the cell. Apoptosis occurs by two main pathways: the **extrinsic** or **cytoplasmic pathway**, and the **intrinsic** or **mitochondrial pathway** (Figure 3). Both pathways ultimately activate a cascade of Caspase proteases (Ghobrial et al., 2005). The **extrinsic pathway** (Figure 3) involves the death receptors on the cell surface. When these death receptors [such as FAS and TNFR1 (tumor necrosis factor receptor)] bind to their respective ligands (Fas-L and TNF, respectively), the intracellular regions of the receptors engage a specific adapter molecule that in turn will bind and activate an initiator caspase. The initiator caspase will later cleave and activate executioner caspases and apoptosis will follow. In the case of Fas-Fas-L, this complex recruits FAS-associated death domain protein (FADD) and pro-caspase-8 that in turn activates caspase-8, which proceeds to activate caspase-3 leading to apoptosis (Wajant, 2007)

The **intrinsic pathway** (Figure 3) engages a set of related proteins called the BCL-2 family proteins which regulate cell death through either induction or inhibition. This pathway of cell death is engaged by many factors including cell stresses, cytoskeletal disruption, DNA damage and many others. The intrinsic pathway involves the activation of effector proteins that make the holes in the outer mitochondrial membrane [Mitochondrial Outer Membrane Permeabilization (MOMP)] in order to release cytochrome c (Green, 2011). Ultimately, cytochrome c translocates

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from the mitochondrial intermembrane space to the cytosol and engages APAF1 (apoptotic protease activating factor 1), which induces apoptosome formation and then activates caspase-3 leading to apoptosis (Youle and Strasser, 2008).

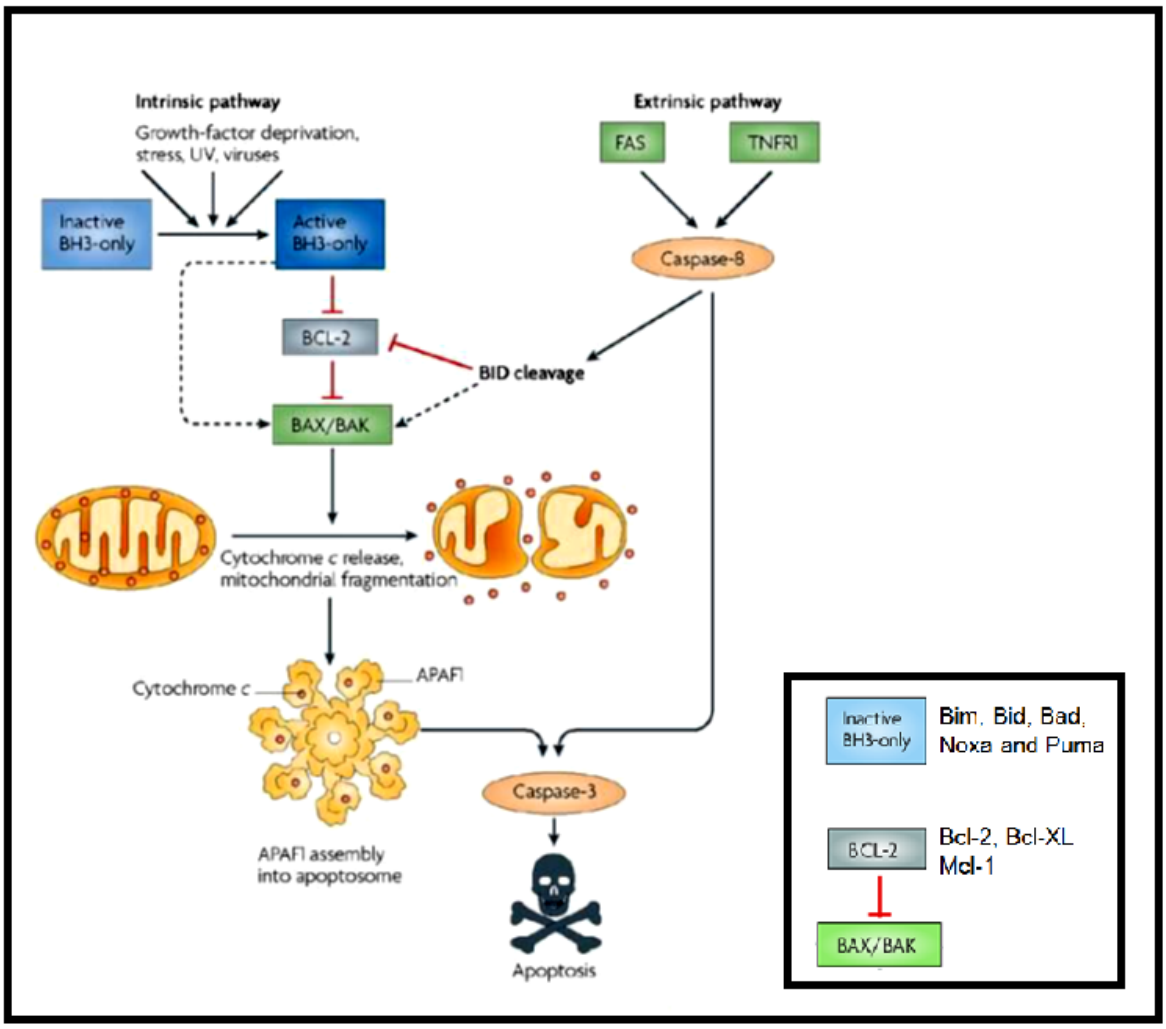

Figure 3. Schematic of the extrinsic and intrinsic pathways of apoptosis (modified from Youle and Strasser, 2008).

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1.5 BCL-2 Family of Proteins

The BCL-2 family proteins (B cell lymphoma-2) are an assortment of related molecules found not only in humans but in many other species including Caenorhabditis elegans, Drosophila, zebrafish, and mice. These species, utilized as study models, are of great importance in the investigation of BCL-2 family proteins and apoptosis. Although the sequences of most BCL-2 family proteins are quite dissimilar from one another, they all possess short regions known as BCL-2 homology (BH) domains (Figure 4). The number of BH domains can vary from molecule to molecule, with some having only one BH domain, and others having up to four (BH1, BH2, BH3, BH4). The BCL-2 family proteins can be described using three different classifications based on the structure and function (Figure 4). The first classification refers to the multidomain pro-apoptotic BCL-2 effectors, which stimulate apoptosis by triggering MOMP. This class includes BAK and BAX among others. The second classification refers to the anti-apoptotic BCL-2 proteins, which prevent apoptosis by stopping MOMP. Anti-apoptotic proteins include BCL-2, BCL-X_L, and MCL-1 among others. Lastly, a subfamily of molecules, the BH3-only proteins, shares only the BH3 domain in their sequence homology. This family functions by regulating the aforementioned pro-apoptotic or anti-apoptotic BCL-2 molecules by protein-protein interaction. The BH3-only members include BIM, BID, PUMA, and Noxa (Green, 2011).

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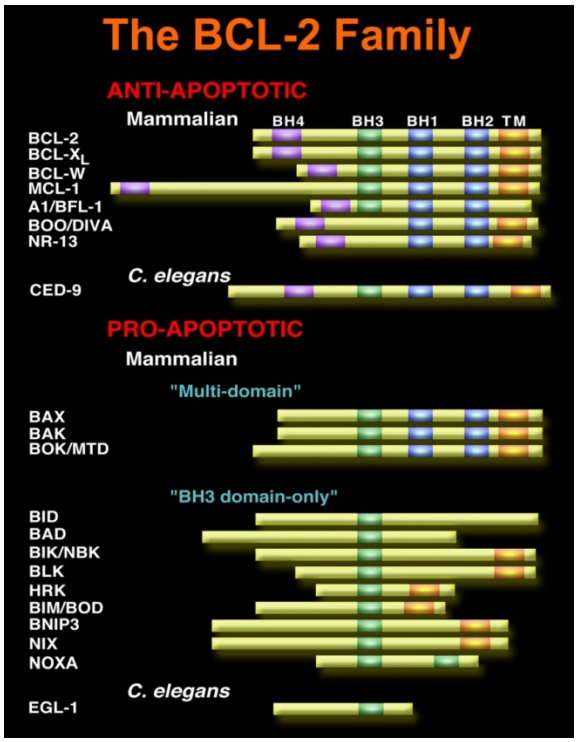

Figure 4: **Sequence homology of the BCL-2 family of proteins**. All of the BCL-2 family of proteins contains short BH (BCL-2 homology) domains in common.

Although BAK and BAX belong to the pro-apoptotic BCL-2 effectors, they are quite different. BAK is tethered to the mitochondria in the cell. In contrast, BAX is soluble in the cytosol but moves to the mitochondria when apoptosis is induced (Longuet et al., 2004) When both proteins are activated, they bury themselves in the mitochondrial outer membrane where they oligomerize and form holes. These holes allow the passage of large molecules (e.g. cytochrome c) through the membrane.

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Anti-apoptotic BCL-2 proteins prevent the oligomerization of BAK and BAX in order to block MOMP and stop apoptosis. To exert this function, the BH3 domain of BAK and BAX needs to bind to the BH groove of the anti-apoptotic BCL-2 proteins. This groove is present in all of the anti-apoptotic BCL-2 proteins and is critical for their function.

These BH3 regions are not exposed in the native proteins. They can only interact if they are embedded in a hydrophobic environment as that of the outer mitochondrial membrane. Furthermore, biochemical evidence shows that activation of BAK exposes its BH3 domain allowing another BAK molecule (with its exposed BH3 domain) to bind and form an oligomer. Instead, anti-apoptotic BCL-2 proteins bind to the exposed BH3 domain preventing this BAK-BAK interaction which is required for MOMP (Willis et al., 2005).

The BH3-only proteins also bind to the BH grooves of the anti-apoptotic BCL-2 proteins. Generally, the BH3-only proteins are characterized by their function because their BH3 region is not conserved in these molecules. They can interfere with the ability of an anti-apoptotic protein to bind to the BH3 domains of other proteins (ex. effector proteins BAK and BAX) and thus, neutralizing them. Furthermore, neutralization depends greatly on how well the anti-apoptotic protein binds to the BH3-only protein (binding specificities) and how much BH3-only protein is readily available for binding (Chipuk et al., 2010) .

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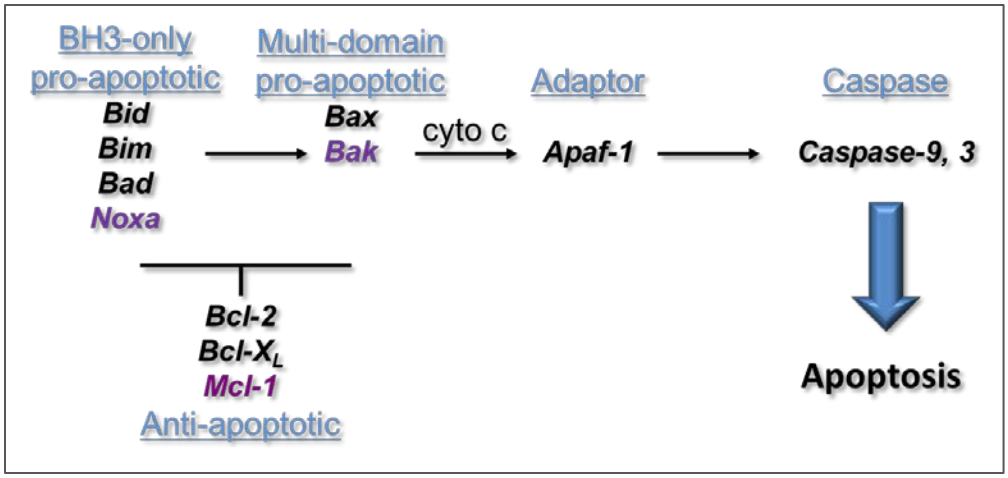

Figure 5: **The BCL-2 family proteins as the main players of the intrinsic pathway.** The BH3-only proteins promote MOMP and apoptosis through the activation of BAK and BAX. The anti-apoptotic BCL-2 proteins prevent by protein-protein interaction.

1.6 MCL-1 and NOXA

An anti-apoptotic BCL-2 family member, MCL-1 was originally identified in 1993 in the differentiating myeloid leukemia cell line (Kosopas et al., 1993). The human MCL-1 protein consists of 350 amino acid residues and is critical for the regulation of cell death and survival in both normal and cancer cells (Michels et al., 2005). Furthermore, MCL-1 deletion in mice results in an embryonic-lethal phenotype, suggesting a significant role in early development (Rickenberger et al. 2000). MCL-1 prevents cell death by sequestering pro-apoptotic family members such as the BH3-only group (Noxa, BID, BIM) and the multidomain pro-apoptotic effector proteins. (BAK, BAX) (Gelinas et al., 2005). MCL-1 protein has a high turnover rate in cells and its levels are actively regulated by phosphorylation/ubiquitination followed by

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proteasomal degradation. For example, GSK3 can phosphorylate MCL-1 to target it for degradation. MULE, an E3 ligase, ubiquitylates MCL-1 for degradation (Mojsa et al., 2014).

Noxa is a pro-apoptotic BH3-only molecule that consists of 54 amino acid residues in humans. The gene of Noxa was originally cloned as a p53 target gene. The tumor suppressor p53 induces apoptosis in cells exposed to lethal stresses. It has been shown that blocking endogenous Noxa suppresses p53-dependent apoptosis. Noxa is known to affect the function of MCL-1 by direct binding. Then, Noxa triggers the ubiquitin-proteasomal degradation of MCL-1 at the mitochondria, but the exact mechanism remains unclear. The degradation of MCL-1 in turn helps initiate the apoptotic cascade. MCl-1 degradation allows for the pro-apoptotic effector protein BAK to be unleashed (MCL-1 binds to BAK keeping it inactive) from the MCL-1-BAK complex promoting cell death (Oda et al., 2000).

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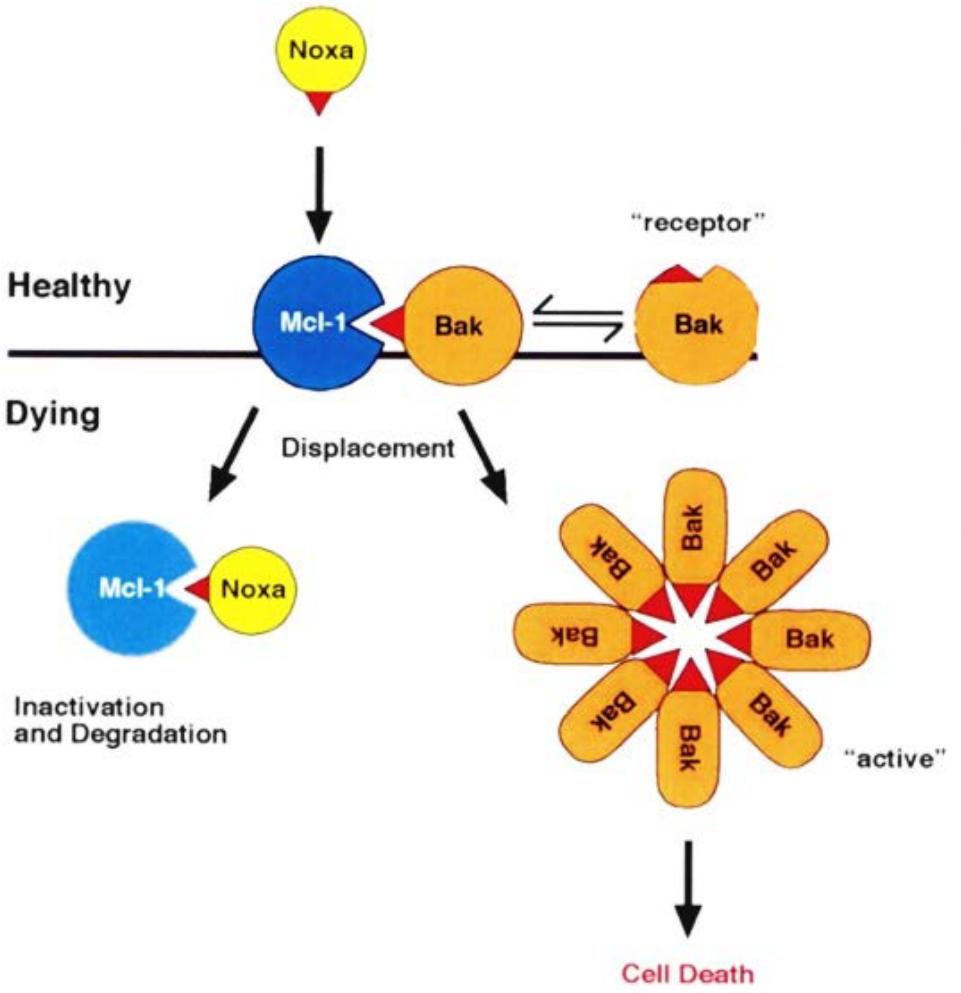

Figure 6: **Noxa- induced MCL-1 degradation and BAK activation.** In the presence of MCL-1, BAK is retained in its inactive form in the MCL-1/BAK complex. The apoptotic protein Noxa can sequester MCL-1, inducing BAK release and activation, oligomerization and eventually cell death (modified from Willis et al., 2005).

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1.7 Previous studies

When cells undergo paclitaxel-induced cell death, the BCL-2 family-dependent mitochondrial apoptotic pathway is activated. In the previous study, our lab has shown that inactivation of BAK by gene knockout in mouse embryonic fibroblasts or by shRNA-mediated knockdown in human breast cancer cells becomes more resistant to paclitaxel. In contrast, paclitaxel sensitivity is not affected by inactivation of BAX, suggesting that paclitaxel-induced apoptosis is BAK-dependent, but BAX-independent. In human breast cancer cells, paclitaxel treatment results in MCL-1 degradation which is prevented by a proteasome inhibitor. BAK is associated with MCL-1 in untreated cells and becomes activated in concert with loss of MCL-1 expression and its release from the complex. Thus, paclitaxel-induced apoptosis is mediated by the protein BAK through MCL-1 degradation (Miller et al., 2013).

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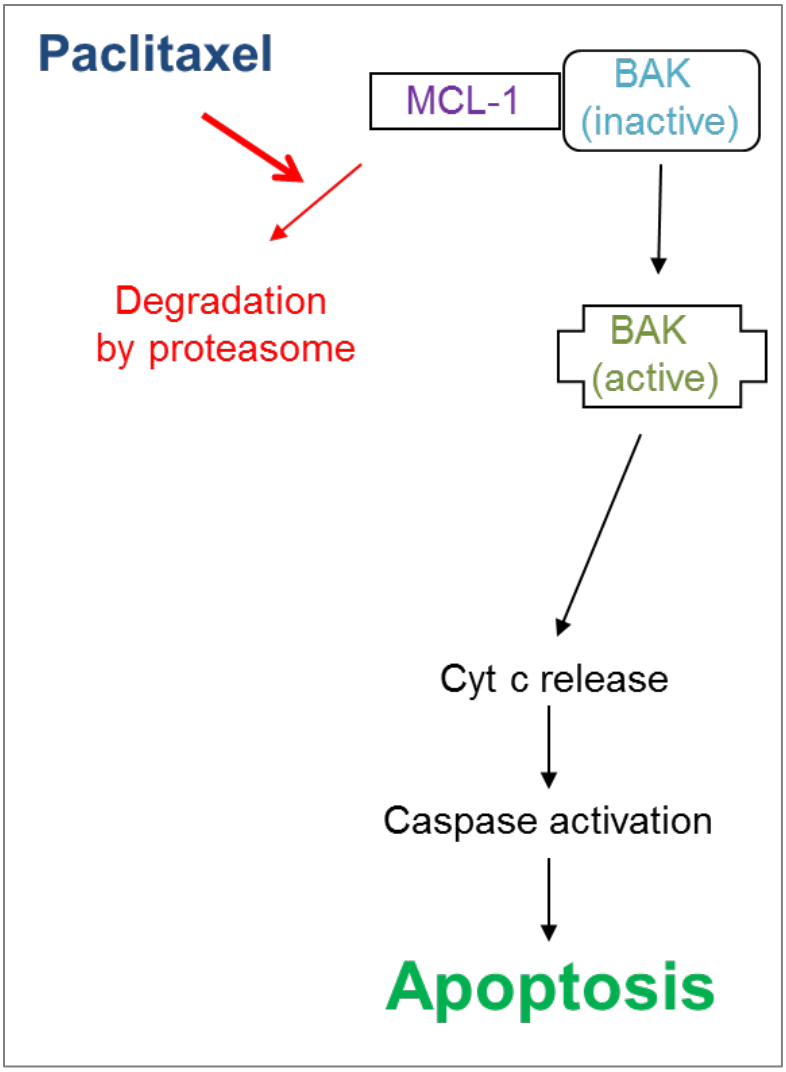

Figure 7: **Schematic of Paclitaxel-dependent MCL-1 degradation.** Paclitaxel-induced apoptosis involves MCL-1 degradation allowing the release and activation of BAK leading to cell death.

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1.8 Hypothesis

In addition to our previous studies, it has been demonstrated that Noxa specifically interacts with MCL-1 to inactivate MCL-1 function (Nakajima et al., 2014). Thus, our hypothesis is that modulation of the Noxa/MCL-1 axis could mimic paclitaxel-induced cell death in breast cancer cells. It can be surmised that either down regulation of MCL-1 or overexpression of Noxa in cancer cells could induce cell death.

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CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines and Cell Culture

MDA-MB-231, MDA-MB-468, Hs578T, BT-20, SKBR-3, MCF7, T47D, BT-474 breast tumor cell lines and 293T cells were purchased from the American Tissue Culture Collection (Manassas, VA). ZR-75 breast cancer cells were kindly provided by Dr. David Gewirtz (Virginia Commonwealth University, Richmond, VA). MDA-MB-231/shMCL-1, MDA-MB-468/shMCL-1, Hs578T/shMCL-1, BT-20/shMCL-1, SKBR-3/shMCL-1, MCF7/shMCL-1, T47D/shMCL-1, BT-474/shMCL-1, ZR-75/shMCL-1 and MCF-10A/shMCL-1 cells were maintained with the use of puromycin (Sigma, St. Louis, MO) at a 1 μg/mL concentration for selection. MDA-MB-231/Noxa WT, MDA-MB-468/Noxa WT, Hs578T/Noxa WT, BT-20/Noxa WT, SKBR-3/Noxa WT, MCF7/Noxa WT, T47D/Noxa WT, BT-474/Noxa WT, ZR-75/Noxa WT and MCF-10A Noxa WT cells were maintained using G418 (Sigma) at 0.4 mg/mL concentration for selection. All cells (except for MCF-10A) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA) and 5% 100 mg/ml penicillin G/streptomycin (Invitrogen) at 37°C in a humidified, 5% CO_2 incubator. MCF-10A cells were cultured using DMEM/F12 (Invitrogen) supplemented with 5% horse serum, mammary epithelial growth supplement (Invitrogen), 100 ng/ml cholera toxin, and 5% 100 mg/ml penicillin G/streptomycin at 37°C in a humidified, 5% CO_2 incubator.

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2.2 Chemicals and Antibodies

Paclitaxel was purchased from Sigma. Cell proliferation reagent WST-1 was purchased from Roche Diagnostics (Mannheim, Germany). Antibodies were purchased as follows: BIM (2933S), BCL-X_L (2764S), BID (2002), GAPDH (2118P), Anti-mouse IgG HRP-linked (7076S), and Anti-rabbit IgG HRP-linked (7074S) from Cell Signaling Technology (Beverly, MA); MCL-1(ADI-AAP-240-F) from Enzo Life Sciences (Farmingdale, NY); BCL-2 (2870S) from Sigma; Alpha-Tubulin (sc-8035), and BAX (sc-493) from Santa Cruz Biotechnology (Santa Cruz, CA); BAK (06-536) from EMD Millipore (Darmstadt, Germany); and Noxa (MA1-41000) from Thermo Fisher Scientific (Waltham, MA).

2.3 Plasmid Transfection and Lentivirus Infection

The lentiviral short-hairpin RNA (shRNA)-expressing construct were purchased from Sigma. Flag-tagged Noxa cDNA was cloned into pCDH-EF1-MCS-IRES-neo (System Biosciences, Mountain View, CA, USA). The constructs were transfected into 293T packaging cells along with the packaging plasmids (psPAX2 and pMD2.G) and the lentivirus-containing supernatants were used to transduce human breast cancer cells.

To generate recombinant lentivirus, the 293T cells (1 x 10^6 cells total) were plated on a 10cm cell culture dish. Transfection steps were carried out 2 days later as the 293T cells became approximately 70% confluence. First, 5 μg of DNA (including 2.5 μg of interested lentiviral expression constructs and 2.5 μg of lentiviral packaging plasmids) were mixed into 200 μL of (Optimum, Life Technologies, Grand Island, New York) medium (Invitrogen). In a separate tube, 15 μL of Endofectin (Gene Copoeia, Rockville, MD) was diluted into another 200 μL of medium.

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Next, the diluted Endofectin reagent was added drop wise into the DNA solution while gently vortexing the DNA-containing tube. The mixture was incubated for 15-20 minutes at room temperature. Finally, the DNA-Endofectine mixture was added gently into the 293T cells. The medium was replaced after 14-18 hours. After two days of incubation, the lentivirus shed into the medium was collected and used to infect the breast cancer cells of interest.

2.4 Cell Toxicity Assay

Breast cancer cells were seeded in triplicate in microtiter plates (96 wells) with a concentration of $1x10^4$ cells per well in 150 µL medium. Next day, cells were treated with different concentrations of paclitaxel and after 72 hours 4 µL of WST-1 reagent were added to the cells. Cytotoxicity of paclitaxel was determined by the WST-1 assay, which is based on the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulphonate), according to the manufacturer's instruction (Roche). The absorbance of the samples was measured using a microplate ELISA reader. The wavelength for measuring the absorbance for the reagent was 450 nm.

2.5 Western Blot Analysis and Immunoprecipitation

For Western blot analyses, whole cell lysates were prepared with CHAPS lysis buffer. CHAPS is a mild detergent which maintains the native structure of the protein in order to observe protein-protein interaction. CHAPS lysis buffer ([20 mM Tris (pH 7.4), 137 mM NaCl, 1 mM dithiothreitol (DTT), 1% CHAPS (3-[(3 Cholamidopropyl) dimethylammonio]-1-

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propanesulfonate), contains a 1:200 ratio of protease inhibitor (Sigma Aldrich), and a 1:100 ratio each of phosphatase inhibitor cocktails 2 and 3 (Sigma Aldrich). Protein concentrations were determined by spectrophotometric analysis using the Bradford method (Bio-rad, Hercules, CA). Equal amounts of each protein sample were loaded into an SDS-polyacrylamide gel, electrophoresed at 200 V for 1 hour, and transferred onto a Nitrocellulose membrane (Fischer Scientific, Pittsburgh PA). The membrane was blocked for 30 minutes in a blotting solution [5% skim milk in PBST (1 x PBS with 0.1% Tween-20)]. After the blocking step, the membrane was incubated with the respective antibodies overnight at 4 °C. The following day, after three separate 5 minutes washes with PBST, the membrane was incubated with the appropriate secondary antibody for 1 hour at room temperature, immediately followed by another three washes at 5 minutes apiece in PBST. The membrane was then developed using Pierce ECL 2 Western Blotting Substrate (Thermo Fisher Scientific). Primary antibodies for BIM, BCL-X_L, Cleaved PARP, GAPDH, BCL-2, BAX, BAK, and Alpha-Tubulin were used at a 1:1000 dilution. MCL-1 antibody was used at a 1:5000 dilution, while Noxa antibody was used at a 1:250 dilution. Secondary antibodies for HRP-linked anti-mouse- and anti-rabbit-IgG were used at a 1:2000 dilution.

For immunoprecipitation (IP), cell lysates were prepared with CHAPS lysis buffer. 500 µg of protein was incubated with MCL-1 or Noxa antibodies at 4°C for 2-3 hours. After the incubation period, the antibody-protein complex was captured with Protein A/G UltraLink Resin (Thermo Fisher Scientific) and the beads were washed with the CHAPS buffer three times. The beads were then suspended in 50 µL of the CHAPS buffer with 5x sample buffer. Samples were separated using SDS-PAGE.

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2.6 IC_{50} Calculations

IC_{50} (half maximal inhibitory concentration) for paclitaxel cytotoxicity was calculated for each cell line in this study. IC_{50} is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. For this study we used the Image J software to calculate the IC_{50} for each cell line using the measurements obtained from the microplate (ELISA) reader.

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CHAPTER 3: RESULTS

Section 3.1: Breast cancer cell lines and their molecular characteristics

Each of the following breast cancer cell lines was used in this study and their main characteristics have been outlined in Table 1.

Table 1. Molecular Classification of Breast Carcinoma

Subtype	Receptor Status	Responsiveness to Therapy	Cell Lines
Luminal A	ER+ PR+/- HER2-	endocrine and chemotherapy responsive	MCF-7, T47D
Luminal B	ER+ PR+/- HER2+	usually endocrine responsive, variable to chemotherapy	BT474, ZR75
Basal (TN)	ER- PR- HER2-	endocrine nonresponsive, often chemotherapy responsive	MDA-MB-468, MDA-MB-231, Hs578T, BT20
Her2	ER- PR- HER2+	trastusumab responsive, chemotherapy responsive	SKBR3

In addition to the various breast cancer cells in our study, we also used immortalized normal breast epithelial cells (non-cancerous) – MCF-10A.

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Section 3.2: Knockdown of MCL-1 induces cell death in breast cancer cells.

In order to examine whether down-regulation of MCL-1 in breast cancer cell lines could induce cell death, we introduced short-hairpin RNA (shRNA) for MCL-1to knock-down the MCL-1 expression and scrambled-shRNA as a control. Figure 8 shows representative cell lines in which MCL-1 was down-regulated by shMCL-1. All breast cancer cell lines examined in this study resulted in cell death upon MCL-1 knock-down (Table 2). In contrast, MCF-10A did not show cell death in the same condition. These results suggest that MCL-1 expression in breast cancer cells is critical to maintain cell survival.

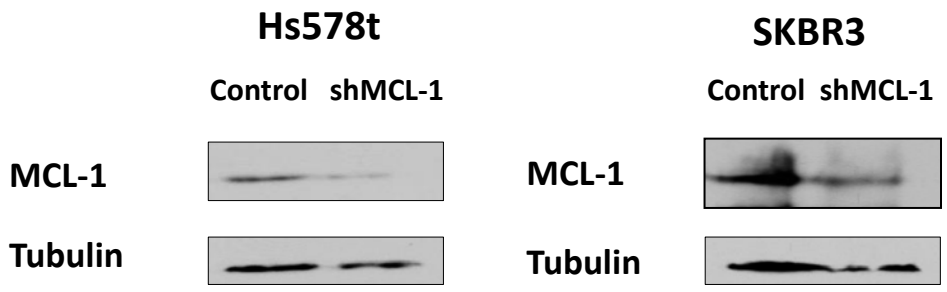

Figure 8. MCL-1 knock-down in cell lines Hs578t and SKBR3. Cells were infected with MCL-1 knock-down or non-targeting shRNA-containing lentivirus. MCL-1 expression levels were determined using Western blot analysis.

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Section 3.3: Overexpression of Noxa in breast cancer cells induces survival or cell death.

It has been proposed that Noxa sequesters MCL-1 allowing the effector protein BAK to be released, resulting in cell death induction. Therefore, we want to examine if up-regulation of Noxa in breast cancer cells can induce apoptosis. We introduced Noxa cDNA using lentivirus-mediated transfection. As a control, a lentiviral-vector alone was introduced. Noxa overexpression induced cell death in the following breast cancer cell lines: MDA-MB-468, BT-20, SKBR-3 and MCF-7 (Noxa-sensitive). In contrast, Noxa overexpression did not result in substantial amounts of cell death in the following breast cancer cell lines: MDA-MB-231, Hs578T, ZR75, T47D, BT-474, nor in the non-cancerous breast cell line MCF-10A (Noxa-resistant) (Table 2). Based on these results we speculate that the availability of Noxa is not the only factor when inducing cell death by MCL-1 degradation in some breast cancer cell lines. Furthermore, these results suggest that there are obvious molecular differences among the various breast cancer cell lines studied.

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Table 2. Results of MCL-1 knock-down and Noxa overexpression in breast cancer cells and MCF-10A.			
Breast Cancer Cell Lines	Molecular Subtype	Knock down of MCL-1	Enforced expression of Noxa
MDA-MB-231	Basal-like subtype	death	survive
MDA-MB-468	Basal-like subtype	death	death
Hs578 T	Basal-like subtype	death	survive
BT-20	Basal-like subtype	death	death
SKBR-3	HER2 subtype	death	death
MCF-7	Luminal A subtype	death	death
ZR-75-1	Luminal A subtype	death	survive
T47D	Luminal A subtype	death	survive
BT-474	Luminal B subtype	death	survive
Immortalised normal breast cells (non-cancerous)			
MCF-10A	Basal-like subtype	survive	survive

Table 2. Results of MCL-1 knock-down and Noxa overexpression in breast cancer cells and MCF-10A. None of the breast cancer cell lines tested survived the MCL-1 knock-down condition. In contrast, Noxa overexpression resulted in cell survival in MDA-MB-231, Hs578t, ZR-75-1, T47D, and BT474. Noxa overexpression also resulted in cell death in cell lines MDA-MB-468, BT-20, SKBR3, and MCF-7. Noxa overexpression in the triple negative breast cancer cell lines resulted in the survival of MDA-MB-231 and Hs578t; and cell death of cell lines MDA-MB-468 and BT-20 (highlighted in red above). MCF-10A (normal breast cells) survived both conditions.

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Section 3.4: The expression of the BCL-2 family proteins in TN breast cancer cells.

In order to further study the differences of Noxa-induced cell death among breast cancer cell lines in particular triple negative cells, we analyzed the endogenous expression levels of various BCL-2 family proteins. Protein expression levels were determined by Western blotting. GAPDH (a housekeeping protein in cells) was utilized as a control to demonstrate equal loading.

MCL-1 expression levels were relatively higher in MDA-MB-468 and Hs578T which appeared in comparison to MCF-10A, MDA-MB-231, and BT-20. Noxa expression levels were significantly higher in cell lines MDA-MB-231 and Hs578t. BAK expression levels were relatively the same for all cell lines except for BT20. BIM expression levels were significantly higher in MCF-10A, MDA-MB-468, and BT20 (Figure 9). The rest of the BCL-2 family proteins analyzed were similar in expression levels among the various cell lines.

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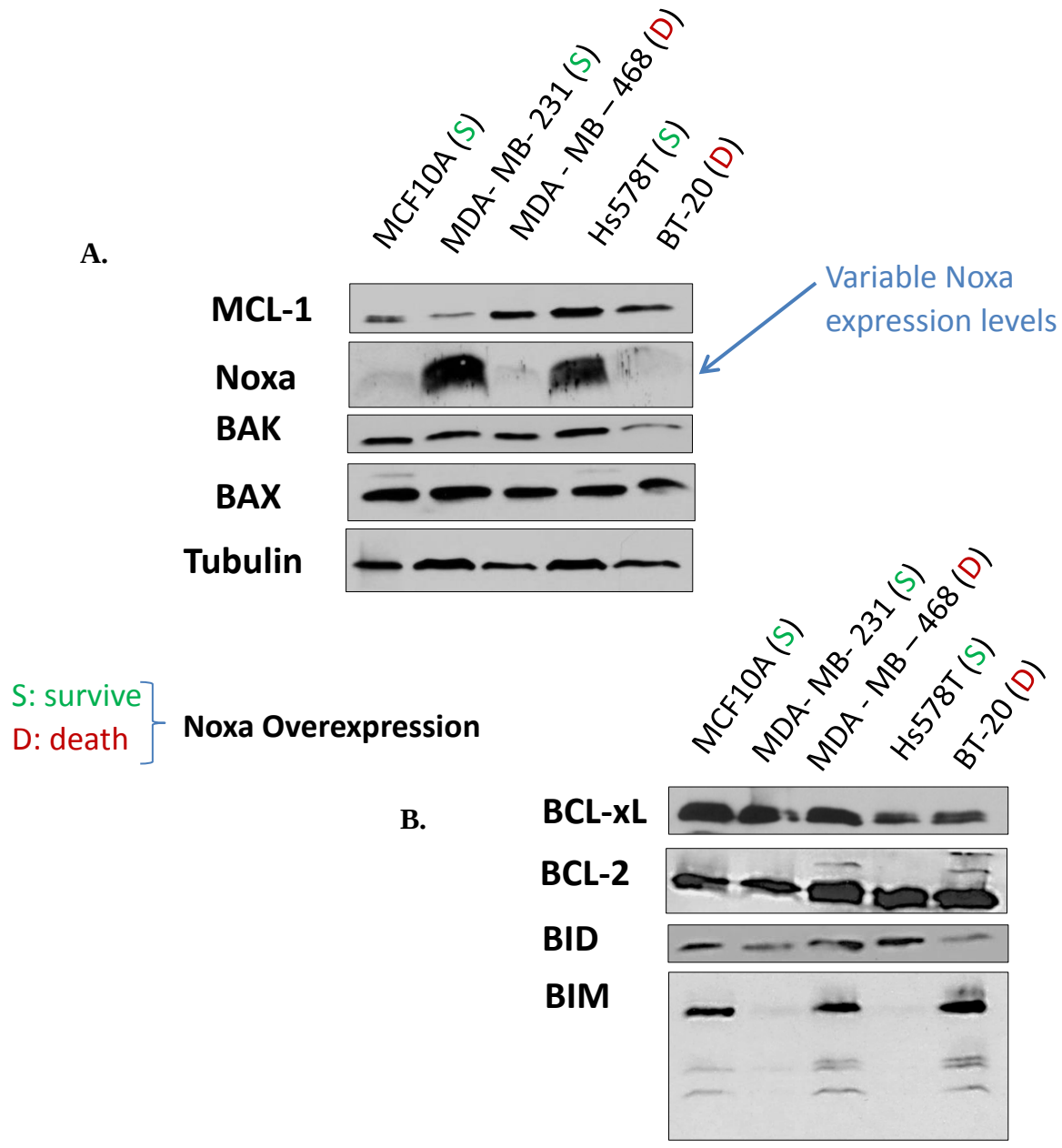

Figure 9: **Expression levels of the BCL-2 family of proteins in TN breast cancer cells**.

Different BCL-2 family proteins expression levels in TN breast cancer cells suggesting molecular differences among them.

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Section 3.5: The interaction of MCL-1 and Noxa is weak in Hs578 and strong in BT-20.

Overexpression of Noxa in the TN cells caused cell death in BT-20 cells but not in Hs578T cells. Furthermore, Western blot results (Figure 9) showed that the level of MCL-1 was decreased when Noxa was overexpressed in BT-20 cells, but not in Hs578T cells. Therefore, we wanted to further analyze the interaction of Noxa and MCL-1 in these two cell lines. We performed co-immunoprecipitation experiments to observe such interaction. Transfected Flag-tagged Noxa was equally immunoprecipitated with anti-Flag antibodies. However, the amount of co-immunoprecipitated MCL-1 was much less in Hs578T than in BT-20 (Figure 10). These results suggested that the interaction between Noxa and MCL-1 was much weaker in Hs578T resulting in cell survival. In contrast, Noxa and MCL-1 interaction was stronger upon Noxa overexpression in BT-20 cells, which caused MCL-1 degradation- cell death.

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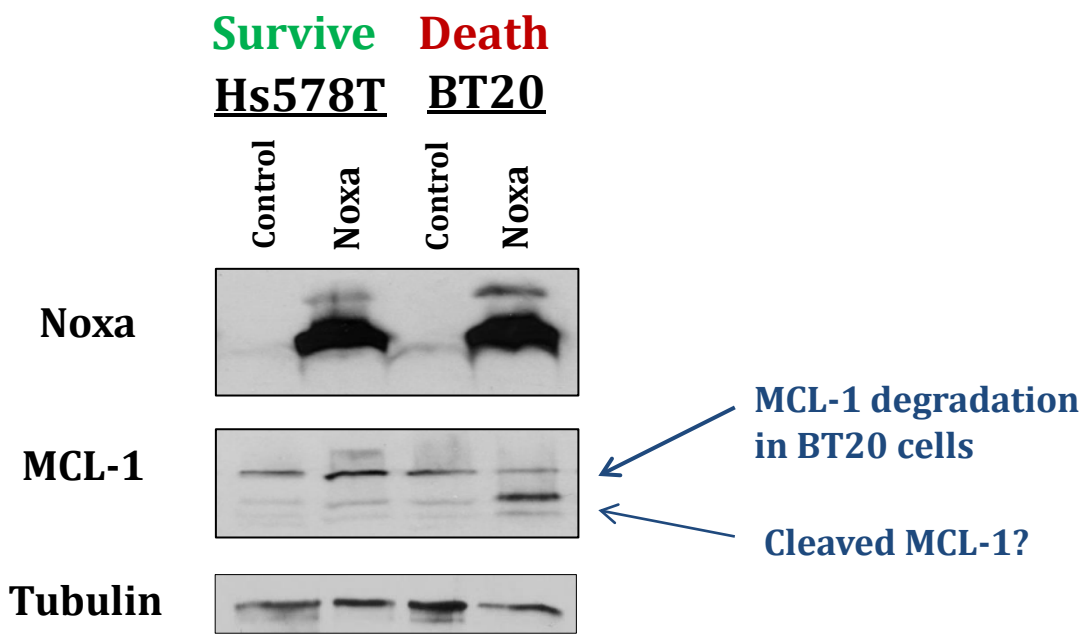

Figure 10. Noxa overexpression and its effect on MCL-1 protein levels in Hs578T and BT-20. Noxa up regulation induces MCL-1 degradation in BT-20 but not in Hs578T. We speculate that the band below MCL-1 might be the cleaved form of MCL-1 after caspases are activated.

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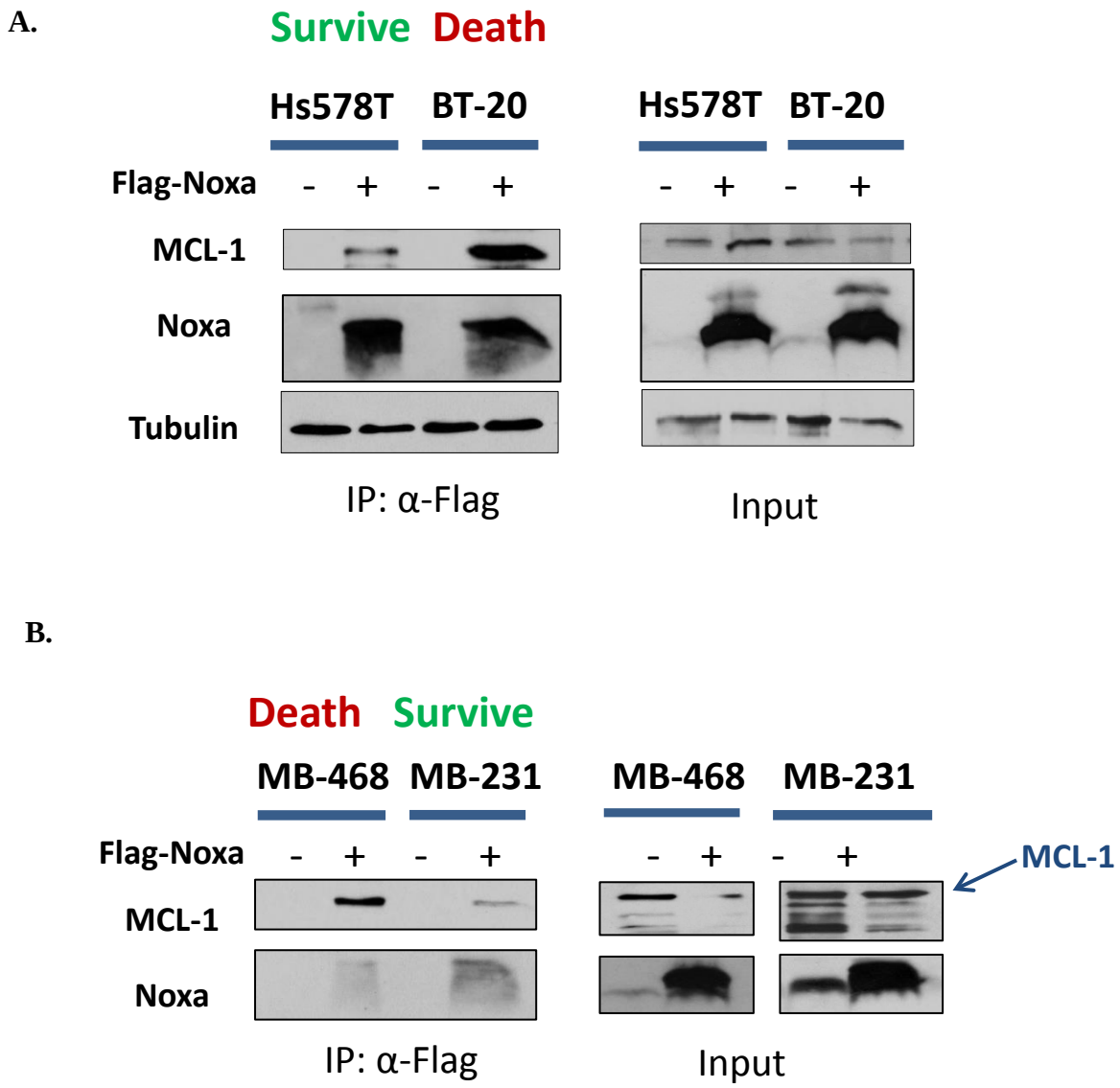

Figure 11. **IP results of Noxa overexpression and MCL-1 in TN cells**. Overexpression of Noxa resulted in Hs578T and MDA-MB-231 survival. In contrast in cell lines BT-20 and MDA-MB-468 Noxa overexpression resulted in cell death. Noxa-MCL-1 interaction shown here suggests weak binding in Hs578T and MDA-MB-231 but strong binding in BT-20 and MDA-MB-468 cell lines

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BT-20 and MDA-MB-468: **Hs578t and MDA-MB-231:**

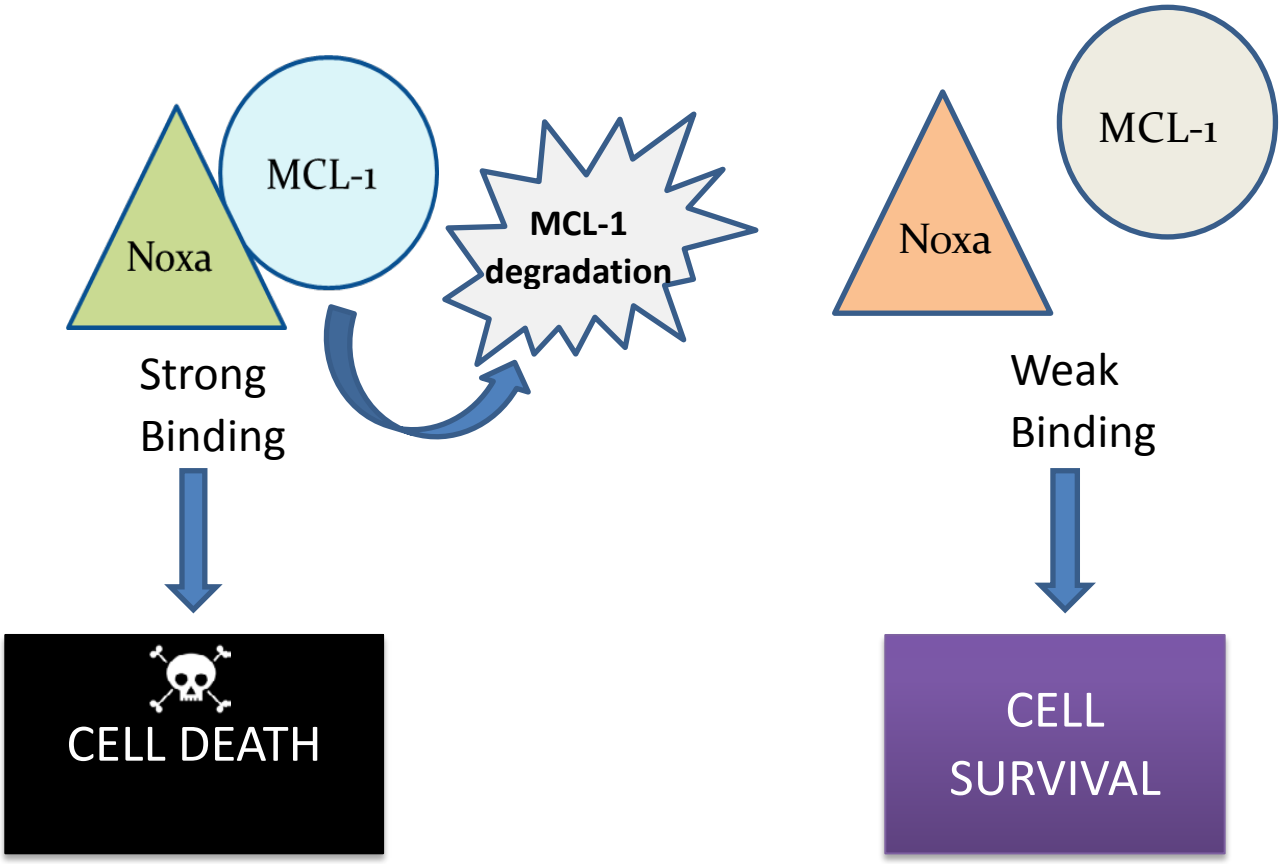

Figure 12. Schematic representation of Noxa and MCL-1 interaction in TN cells. In Hs578t and MDA-MB-231 cells weak binding of Noxa and MCL-1 (MCL-1 degradation does not occur) results in cell survival. In BT-20 and MDA-MB-468 cells, strong binding of Noxa and MCL-1 induces MCL-1 degradation resulting in cell death.

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Section 3.6: Paclitaxel sensitivity results of various breast cancer cell lines

In order to examine the correlation between cell death induced by Noxa overexpression and paclitaxel sensitivity, we treated breast cancer cells with different concentrations of paclitaxel (0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1 μM). Then we determined the IC50 for each cell line using WST-1 assay (Table 3). Non-cancerous MCF-10A was very resistant to paclitaxel. In contrast, breast cancer cell lines showed various sensitivities to paclitaxel. For example, ZR-75-1 was very sensitive, MDA-MB-468, T47D and BT-474 are sensitive, while Hs578Twere resistant. However, all of these cell lines did not show cell death induced by Noxa overexpression (Table 2 and 3). These results suggest that paclitaxel sensitivity may be regulated by more complex mechanisms.

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Table 3. IC_{50} values to Paclitaxel in breast cancer cells and MCF-10A.	
Breast Cancer Cell Lines	**Paclitaxel sensitivity IC_{50} (nM)**
MDA-MB-231	**57**
MDA-MB-468	**65**
Hs578 T	**401**
BT-20	**174**
SKBR-3	**11**
MCF-7	**263**
ZR-75-1	**0.58**
T47D	**98**
BT-474	**74**
Immortalised normal breast cells (non-cancerous)	
MCF-10A	**933**

Table 3. IC_{50} values to Paclitaxel in breast cancer cells and MCF-10A. IC_{50} values for various breast cancer cell lines were calculated by WST-1 assay. MCF-10A IC_{50} value to Paclitaxel was also calculated.

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CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Paclitaxel, a taxane family member, is widely used in the treatment of several cancers such as breast, lung, and ovarian. This chemotherapeutic agent causes mitotic arrest although its exact mechanism remains to be elucidated. Several reports have demonstrated that paclitaxel-induced cell death involves MCL-1 degradation and the presence of the effector protein BAK. Therefore, this study aims to mimic paclitaxel-induced cell death by the down regulation of MCL-1 and up regulation of Noxa in breast cancer cell lines. Results showed that MCL-1 knock-down was fatal for nine breast cancer cell lines examined (Table 2). Thus, MCL-1 is a critical pro-survival protein for breast cancer cells. Presumably, MCL-1 protein levels may be strictly controlled in normal cells. On the other hand, cancer cells may have a vast stock (of MCL-1) to ensure high levels of MCL-1 in order to escape apoptosis. Moreover, down regulation of the MCL-1 protein by shRNA knock-down may sensitize paclitaxel-resistant breast cancer cells allowing for better therapeutic methods.

Noxa overexpression has been shown to sensitize cancer cells to a particular drug treatment such as a BCL-2 inhibitor, ABT-737, in small cell lung cancer cells (by the induction of MCL-1 degradation leading to apoptosis). For that reason, we examined cell death in breast cancer cells upon Noxa overexpression. The results showed that the up-regulation of Noxa was lethal in MDA-MB-468, BT-20, SKBR-3, and MCF-7 but led to the survival of MDA-231, Hs578T, ZR-75-1, T47D, and BT-474 (Table 2). These results suggest that in breast cancer cells survived by Noxa overexpression, another factor (besides solely Noxa availability) is responsible for the induction of MCL-1 degradation. Perhaps the binding affinity between Noxa and MCL-1 plays a

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significant role in the induction of Noxa-dependent apoptosis. However, the reason for this difference in binding affinity remains to be elucidated.

Additionally, Noxa overexpression in the TN breast cancer cell Hs578T did not result in MCL-1 degradation. In contrast, we observed MCL-1 degradation in BT-20 cells when Noxa is up-regulated (Figure 9) between these cells to the effect of Noxa up-regulation prompted us to examine the protein-protein interaction of Noxa and MCL-1. We performed immunoprecipitation to examine such interaction in Hs578T and BT-20. Results obtained from Hs578T (Noxa resistant cell that did not show MCL-1 degradation) suggested a weak interaction between Noxa and MCL-1. Contrary, in BT-20 breast cancer cells (Noxa-sensitive cell that showed MCL-1 degradation) a strong protein-protein interaction was observed (Figure 10). These results suggest that the binding affinity between Noxa and MCL- 1 (either weak or strong) may be of importance to whether the cell is destined to survive or to perish.

We studied the endogenous expression levels of several of the BCL-2 family proteins in the TN breast cancer cell lines MDA-MB-231, Hs578T, MDA-MB -468, and BT-20. Although these cancer cells are similar in that they do not possess the ER, PR, and HER2, they differ in the expression levels of the BCL-2 family proteins (Figure 8). These differences in the endogenous levels of the BCL-2 family proteins among the TN breast cancer cells may be important for the development of more effective cancer treatments. Furthermore, we observed higher expression levels of the protein Bim in Noxa sensitive cells BT-20 and MDA-MB-468. In contrast, Noxa resistant cells MDA-MB-231 and Hs578T show low levels of Bim expression. We speculate that Bim (Bim can interact with all of the anti-apoptotic BCL-2 family proteins) may contribute alongside Noxa to induce MCL-1 degradation leading to apoptosis.

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These new findings have important implications for overcoming paclitaxel sensitivity and for the development of innovative and more effective therapeutic drugs. For future studies it will be significant to study MCL-1 cDNA for mutations, post-translational modifications, or any other factor that may be contributing to the lack of correlation in Noxa overexpression and MCL-1 degradation in some of the breast cancer cell lines we examined in this study.

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VITA

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