

ABSTRACT

ACTIVATION OF THE PI3K/PTEN/AKT AND RAS/RAF/MEK/ERK SIGNAL TRANSDUCTION PATHWAYS DECREASES SENSITIVITY OF HUMAN BREAST CANCER CELLS TO CONVENTIONAL CHEMOTHERAPY

By

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The PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK pathways have an essential role in the regulation of cell growth. The activity in the PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK pathways can be a factor in resistance to chemotherapeutic and hormonal drug treatments and are linked to a poor prognosis in cancer patients. The PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK pathways are related to mutations and overexpression in cancer, both in downstream and upstream components. Our laboratory has isolated drug resistant breast cancer cells which exhibit sensitivity to inhibitors and are directed at the PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK pathways. It has been hypothesized that drug resistant cells may be tumor-initiating cells because of their stem cell-like characteristics and altered growth properties. An effect of drug therapy is the induction of endurance leading to drug resistances by permitting these cancer cells with altered signaling pathways to expand and grow under conditions which normally would lead to cell death. Distortion by PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK

pathway expression leads to deliberate induction of resistance to doxorubicin and tamoxifen, but sensitivity to mTOR inhibitors. Some inhibitors such as MEK and AKT have increased the ability of doxorubicin and tamoxifen to induce cell death. Therefore I performed the following experimental aims: 1) Observe AKT expression in MCF-7, T47D, and MDA-MB-231 breast cancer cell lines; 2) Observe MEK expression in MCF-7, T47D, and MDA-MB-231 breast cancer cell lines; 3) Observe PTEN expression in MCF-7 breast cancer cell lines; 4) Determine the effects of inhibiting PI3K/PTEN/AKT/mTOR and Ras/Raf/MEK/ERK on chemotherapeutic drug sensitivity; and 5) Document the level of NGAL in these cell lines, which is a marker for a poor prognosis.

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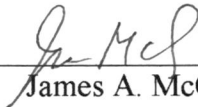
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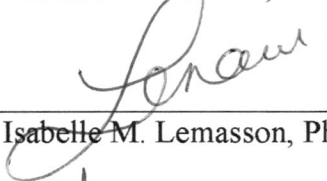
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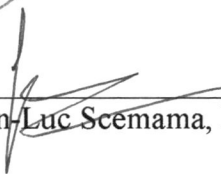
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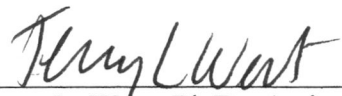
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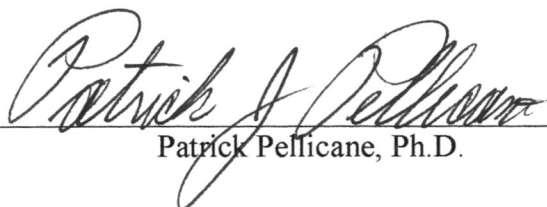
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LIST OF ABBREVIATIONS

AKT	AKR mouse thymoma
APS	Ammonium persulfate
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma-2
BCR	Breakpoint cluster region
BMD	Bone mineral density
BSA	Bovine serum albumin
CS FBS	Charcoal stripped fetal bovine serum
CS	Cowden's Syndrome
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ER	Estrogen receptor
ER-	Estrogen receptor negative
ER+	Estrogen receptor positive
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GLB	Gold lysis buffer
GSK-3 β	Glycogen synthase kinase 3 beta
HER	Human epidermal growth factor receptor
HER-2	Human epidermal growth factor receptor-2
4-HT	4-Hydroxytamoxifen
IDC	Invasive ductal carcinoma
IGF	Insulin-like growth factor
IgG ₁	Immunoglobulin G ₁
IHC	Immunohistochemistry
MAPK	Mitogen activated protein kinase
MCF-7	Michigan Cancer Foundation-7
MEK	MAPK/ERK kinase
MMP-9	Matrix metalloproteinase-9
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2 <i>H</i> -tetrazolium bromide
NF- κ B	Nuclear factor immunoglobulin κ chain enhancer-B cell

NGAL	Neutrophil gelatinase associated lipocalin
p38MAPK	p38 Mitogen-activated protein kinase
p53	Tumor protein 53
PBS	Phosphate buffered saline
PKB	Protein kinase B
PDK1	Phosphoinositide dependent kinase-1
PDZ	PSD95/Dlg/ZO-1
PI3K	Phosphatidylinositol 3-kinase
PI(3,4,5)P ₃	Phosphatidylinositol 3,4,5-trisphosphate
PI(4,5)P ₂	Phosphatidylinositol 4,5-bisphosphate
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PVDF	Polyvinylidene fluoride
Ras	Rat sarcoma
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640
RTKs	Receptor protein tyrosine kinases
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Ser	Serine
Src	Sarcoma
TBS	Tris buffered saline
TBST	TBS Tween
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
Thr	Threonine
TOR	Target of rapamycin
Tris	Tris(hydroxymethyl)aminomethane
VEGF	Vascular endothelial growth factor
WT	Wild-type

CHAPTER 1: INTRODUCTION

Breast cancer is among the world's most frequent types of cancers, with women being primarily affected. It is estimated that there will be 184,450 new cases of breast cancer diagnosed in the United States this year, and 40,930 individuals will succumb to the disease (National Cancer Institute, 2008). From these numbers, approximately 1,990 of these new cases will be in men, making breast cancer the most diagnosed, and the second most common cause of cancer deaths in women in the United States (American Cancer Society, 2008). Approximately 13% of women will be diagnosed with some form of breast cancer during their lifetime (Pandolfi, 2004). Early detection of breast cancer through self-exams and mammography screenings is the most effective means of an early diagnosis. Prognosis correlates with treatment, and the earlier the cancer is detected and treated, the better one's chances are of remission and survival.

There are many pharmacological agents which can be attributed to prolonging the survival of patients suffering from breast cancer. While there are a variety of therapies that can be used to treat breast cancer, drug resistance is among the highest concerns and is the basis of much scientific investigation. There are numerous oncogenes that are responsible for causing breast cancer, all with different functions and chemotherapeutic sensitivity. These genes exist within various signaling pathways that are responsible for controlling a variety of mechanisms, including cell cycle progression and apoptosis. Research suggests that the activation and inhibition of these pathways are key regulators in resistance to anticancer agents. Breast cancer development and drug resistance has also been attributed to signal transduction cascades which exist downstream of epidermal

growth factor (EGF) receptor (EGFR) isoforms; however there are a multitude of other mechanisms that can result in the activation of signaling pathways other than EGFRs (Steelman *et al.*, 2008).

1.1 The function of PTEN and its role in the PI3K/PTEN/AKT pathway

The phosphatidylinositol 3' kinase (PI3K)/PTEN/AKT signaling pathway plays an important role in the regulation of cell growth. One protein in this pathway, the lipid phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a key gene in tumor suppression, and also regulates the PI3K/AKT pathway. PTEN is a tumor suppressor protein as it normally functions to limit cell growth.

Tumors may occur in a variety of tissues and are present in many carcinomas, such as those of the breast. Loss of PTEN or its function as the result of mutation is both common and important in breast cancer. PTEN is not only responsible for antagonizing tumorigenesis, but also sensitizing cancer cells to therapy (Pandolfi, 2004). It is widely documented that PTEN acts as a tumor suppressor by inhibiting cell proliferation and also by promoting apoptosis (Wan *et al.*, 2007). In addition to acting as a tumor suppressor, PTEN is a central player in the PI3K/AKT signaling pathway. The primary function of PTEN is to oppose the activation of this pathway by dephosphorylating the D3 position of membrane phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3), thus prohibiting the activation of PI3K-dependent kinase (PDK1). When PTEN function is lost, activated PI3K is responsible for phosphorylating phosphatidylinositol (4,5)-bisphosphate (PIP2), which is a membrane lipid, to phosphatidylinositol (3, 4, 5)-

trisphosphate (PIP3), which causes the activation of PDK1. As a result, PDK1 phosphorylates AKT at threonine 308, while a second kinase phosphorylates AKT on serine 473 (McCubrey *et al.*, 2008).

1.2 Isoforms and functions of AKT

There are three isoforms of AKT that exist, including AKT-1, -2, and -3. All of these isoforms are not catalytically active within the cytosol, but through translocation to the membrane by their pleckstrin homology (PH) domain and phosphorylation activation at both threonine 308 and serine 473 by PI3K, they are sequestered to the plasma membrane. The expression of AKT-1 is predominant in most tissues, whereas the presence of AKT-2 is associated with insulin-responsive tissues and AKT-3 is associated with breast tumors that are estrogen receptor (ER) negative. Breast tumor studies *in vivo* indicate a strong correlation between activated AKT (pAKT) and levels of both AKT-1 and -2, and also illustrate an increased expression of both proteins when compared with normal breast epithelial cells. One recent study suggests that pAKT is expressed throughout the cytoplasm in 81% of invasive ductal carcinomas (IDCs), with 19% of tumors showing no activation. It is therefore implied that pAKT is associated with larger breast tumors as well as increasing levels of cytoplasmic p53. This association stresses the importance of the PI3K/AKT signaling pathway in fostering cell growth and survival, in addition to establishing resistance to apoptosis (Vestey *et al.*, 2005).

1.3 The role of AKT activation on the PI3K/PTEN/AKT pathway

Since phosphorylation is critical for the activation of the AKT pathway, then dephosphorylation of PIP3 to PIP2 which controls the PH domain mediated translocation of AKT and PDK to the membrane must occur. PTEN dephosphorylates PIP3 upstream of AKT, and is currently the best known inhibitor of the AKT pathway. The PKB/AKT pathway is crucial for protein synthesis, control of cell cycle, cellular metabolism, and survival of the cell, making it a target for studies surrounding chemotherapeutic resistance and malignant transformation of various tissues. There is a wide array of factors that cause the activation of AKT, including but not limited to phosphatase inhibitors, growth factors, and cellular stress. Initial activation of AKT occurs after phosphorylation at Thr 308 by PDK1, however full activation does not occur until the critical phosphorylation at Ser 473. For years it was unknown which kinase was responsible for causing phosphorylation at Ser 473, but it is now believed that the rapamycin-insensitive mammalian Target of Rapamycin (mTOR) is the kinase that is responsible for phosphorylating AKT at Ser 473 (Modak and Bryant, 2008).

1.4 HER-2 over-expression in breast cancer

HER-2 is an epidermal growth factor receptor (EGFR) from the receptor tyrosine kinase family that is normally present on epithelial cells, and is amplified and over-expressed in approximately 30% of human breast cancers. This overexpression is typically associated with a poor prognosis. Activation of AKT has been demonstrated as a cell survival signal. PTEN is responsible for blocking the activation of AKT through

dephosphorylation of PIP3 to PIP2, and blocking the PI3K signaling pathway. As seen in many cancers, the loss of PTEN promotes the activation of AKT. EGFR is responsible for a cascade of signals that are responsible for activating AKT. Therefore, the loss of PTEN in the presence of over-expressed EGFR causes activation of AKT; the only way activation of AKT can be blocked is by the inactivation of EGFR or AKT (Diehl *et al.*, 2007).

A recent study suggests a novel pathway for AKT activation and cell survival. In HER-2 over-expressing breast carcinoma cells, EGF independence was associated with AKT activation by p38MAPK. While this mechanism is unknown, data suggests that the p38MAPK-dependent activation of AKT may provide an additional survival factor responsible for enhancement of the aggressive nature of human breast cancer cells that over-express HER-2. Studies indicate that AKT-1 may block cell motility and invasion, but it increases proliferation. AKT-1 overexpression in HER-2 transgenic mice therefore increases proliferation of mammary tumors, resulting in fewer metastases. Additional data suggests that blocking AKT activation leads to an indirect activation of apoptotic signals through the GSK-3 β pathway, which is downstream of AKT. Thus, when treating tumor proliferation, survival signals that result from HER-2 overexpression need to be taken into account (Diehl *et al.*, 2007).

1.5 Activation of the PI3K/PTEN/AKT pathway

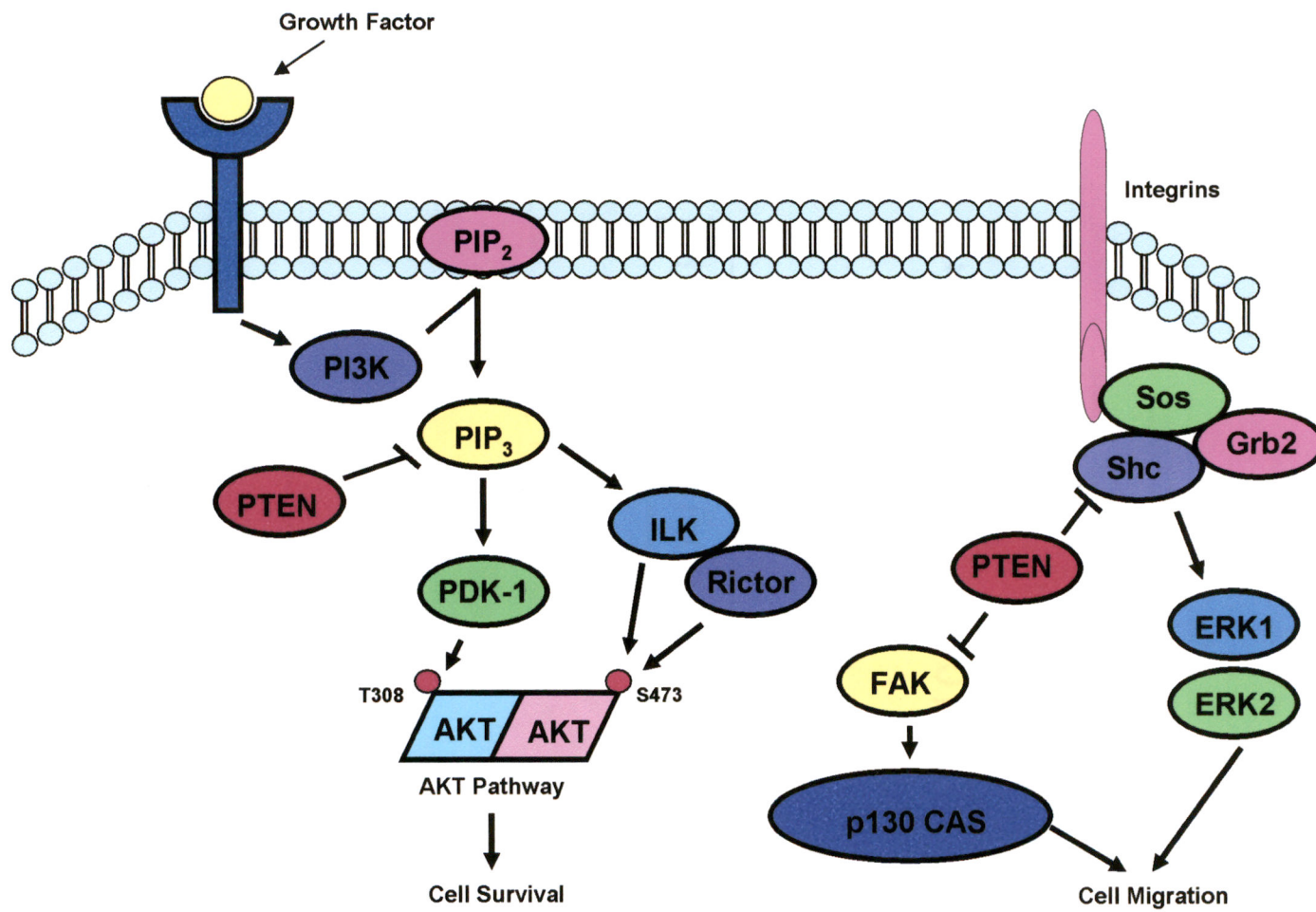
AKT is responsible for regulating cell cycle progression, cell survival, proliferation, migration, metabolism, tumor growth, and angiogenesis. A variety of

cytokines and growth factors are responsible for activating AKT, including epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF). PI3K can be activated by receptor protein tyrosine kinases (RTKs) in response to growth factors, as well as non-receptor protein tyrosine kinases. RTKs interact with a regulatory subunit of PI3K known as p85. In addition to PTEN which activates the AKT pathway, oncogenes such as Ras and Src may also play a role. The activation of AKT has been found to correlate with resistance to radiation therapy and chemotherapy; therefore, in order to sensitize cancer cells to drug treatment, combination therapies including AKT inhibitors and antitumor drugs may be employed.

There are a variety of factors that are responsible for the activation of the PI3K signaling cascade (Figure 1), including the activation of RTKs, PI3K mutations and amplification, and lost function of PTEN. Since AKT activation by PDK1 is essential in tumor formation and growth as well as angiogenesis, the PI3K/PTEN/AKT signaling pathway can be attributed to play a key role in cancer progression. Therapies which target signaling molecules have demonstrated a profound effect on inhibiting angiogenesis and tumor growth in animals. mTOR and p70^{S6K} are both downstream molecules activated by AKT which regulate tumor growth. Evidence suggests that PI3K, AKT, mTOR, and p70^{S6K} inhibitors would have a detrimental effect on the activation of the PI3K/AKT signaling pathway. Because of this, PI3K, AKT, mTOR, and p70^{S6K} inhibitors are being developed for use in cancer therapy. It is also believed that mTOR and PI3K inhibitors may have synergistic effects that prevent tumor development when

Figure 1. Activation of the PI3K/PTEN/AKT signal transduction pathway.

Epidermal growth factors are responsible for binding membrane bound receptors and activating PI3K. PI3K phosphorylates $PI(3,4)P_2$ to $PI(3,4,5)P_3$. When PTEN is active, $PI(3,4,5)P_3$ is dephosphorylated, and the signaling cascade is halted at $PI(3,4,5)P_3$. When PTEN mutants exist, $PI(3,4,5)P_3$ activates PDK-1 and ILK. PDK-1 is responsible for phosphorylating the Thr 308 residue of PKB, while ILK or Rictor is responsible for phosphorylating the Ser 473 residue of AKT. The complete activation of AKT does not occur until the crucial phosphorylation of AKT at Thr 308. AKT activation occurs following this double phosphorylation, activating a cascade of additional downstream signals which lead to cell survival. This figure illustrates examples of both lipid (PI3K cascade) and protein (Sos/Shc/Grb2 cascade) phosphatase activity as indicated in PTEN(C124S) and PTEN(G129E) mutants.



used in combination with other chemotherapeutic drugs. While the PI3K/AKT/mTOR signaling pathway is required in normally functioning cells to regulate proliferation, lowered levels of these particular kinase activities are believed to be sufficient for the survival of normal cells (Jiang and Lu, 2008).

1.6 PTEN mutants expressed in human breast carcinomas

Several common mutant forms of PTEN exist, demonstrating amino acid substitutions within the phosphatase domain which affect its activity. PTEN(C124S) lacks lipid and protein phosphatase activity, whereas another mutant, PTEN(G129E) retains protein phosphatase activity while lacking lipid phosphatase activity. The PTEN(399stop) construct lacks the PDZ domain-binding motif (Steelman *et al.*, 2008), which is important in mediating protein-protein interactions, resulting in the formation of localized protein complexes. These interactions can be responsible for sequestering various components of signaling pathways to their desired membranes (Leslie *et al.*, 2000). Patients with Cowden's syndrome (CS) show germline PTEN mutations, and therefore tend to have a high incidence (25-50%) of breast cancer. Originally, PTEN(C124S) and PTEN(G129E) mutations were identified in Cowden's syndrome patients, and it is believed that they function as natural dominant-negative (DN) mutations (Steelman *et al.*, 2008). In terms of chemotherapeutic resistance, inheritance of a PTEN mutant which lacks both lipid and protein phosphatase activity (PTEN(C124S)) demonstrates more resistance to doxorubicin than a mutant which lacks only lipid phosphatase activity (PTEN(G129E)). This implies that a mutation resulting in

the loss of lipid and protein phosphatase activity is a determining factor in chemotherapeutic resistance. Additionally, it has been suggested that PTEN mutants lacking only lipid phosphatase activity demonstrate earlier activation of p70^{S6K} and increased levels of activated AKT. This indicates that altering lipid phosphatase activity is a key factor in the expression of AKT and downstream p70^{S6K} (Steelman *et al.*, 2008).

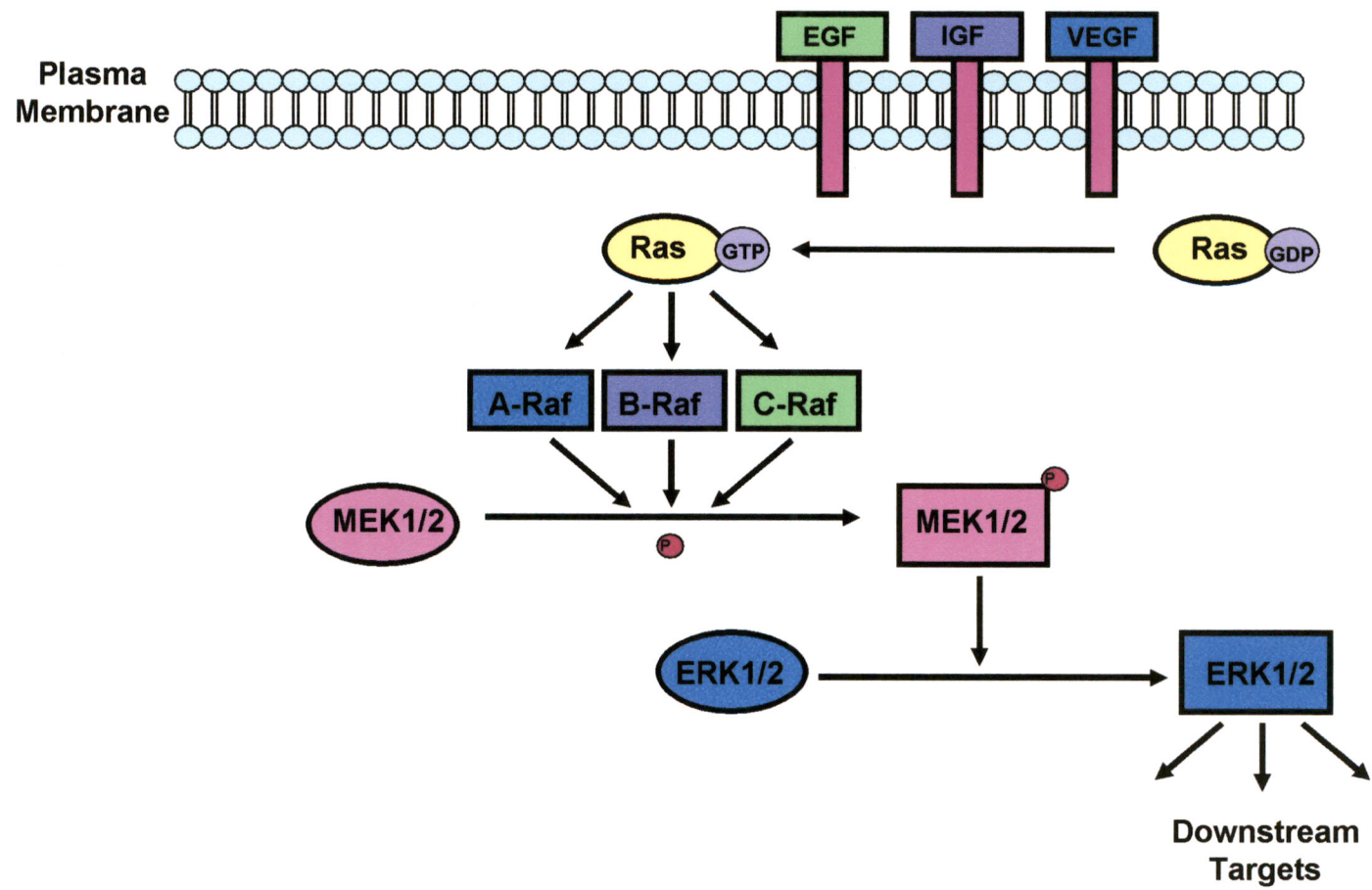
1.7 Activation and function of proteins within the Ras/Raf/MEK/ERK pathway

There are numerous interactions that occur between signaling pathways in the cancer cell. One such interaction is the signaling between the PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK pathways. The Ras/Raf/MEK/ERK pathway is another crucial pathway involved in signal transduction from membrane receptors and non-receptor tyrosine kinases to the nucleus, all of which allow the regulation of gene expression. The Ras/Raf/MEK/ERK pathway is known to alter the prevention of apoptosis, as well as the regulation of gene expression resulting from the transmission of signals from growth factor receptors. The Ras/Raf/MEK/ERK pathway has several different functional locations, including the mitochondrial membrane, nucleus, and cytoplasmic membrane. In the mitochondrial membrane, different anti-apoptotic molecules interact with Raf-1, whereas in the nucleus transcription factors and other substrates are phosphorylated by ERK (McCubrey *et al.*, 2008).

The Ras/Raf/MEK/ERK signaling cascade transmits signals from cell surface receptors to transcription factors resulting in the regulation of gene expression (Figure 2). Often, this pathway is activated by over-expression or mutations in upstream molecules

Figure 2. Activation of the Ras/Raf/MEK/ERK signal transduction pathway.

Various growth factors including epidermal growth factor, vascular endothelial growth factor, and insulin-like growth factor are responsible for binding to receptors in the plasma membrane of cells and causing conversion of GDP to GTP which bind to Ras. When Ras is activated, Raf is activated. Raf is a member of a multi-gene family including A-Raf, B-Raf, and C-Raf. The activation of Raf results in the phosphorylation of MEK1/2. MEK1/2 phosphorylation results in ERK1/2 phosphorylation which activates downstream targets leading to cellular proliferation and survival. When cells are grown in the presence of MEK inhibitors, phosphorylation of ERK1/2 does not occur, leading to a decrease in cellular proliferation and cell death.



such as BCR-ABL and EGFR. Raf plays a role in generating phosphorylation of proteins that control apoptosis; this can happen both downstream of MEK and ERK, or independently of MEK and ERK. Raf activity is regulated by Ras and B-Raf, as well as PI3K, PTEN, and AKT. Abnormal activation of the Ras/Raf/MEK/ERK pathway is a result of mutations in any or all of the aforementioned genes which regulate Raf. The subsequent activation of Raf plays a role in chemotherapeutic drug resistance, as it stimulates resistance to paclitaxel and doxorubicin in breast cancer cells, thus making the Ras/Raf/MEK/ERK pathway an important target for therapeutic intervention (McCubrey *et al.*, 2006).

The activation of Raf results in downstream activation of mitogen-activated protein kinase/ERK kinase (MEK1), thus activating extracellular-signal-regulated kinases 1,2 (ERK1,2) (McCubrey *et al.*, 2006). Raf phosphorylation positively regulates MEK1. ERK is a prime target of MEK1, however downstream ERK has numerous targets. Because of this, MEK1 therapeutic targeting is fairly specific. The activities of ERK are regulated by MEK1 and MEK2. ERK is responsible for phosphorylating a variety of transcription factors directly, but can activate the nuclear factor immunoglobulin κ chain enhancer-B cell (NF- κ B) transcription factor through an indirect mechanism. Many transcription factors within the nucleus can be phosphorylated by ERK, and it can also phosphorylate numerous proteins involved in the regulation of the cell cycle. More specifically, ERK2 has been associated with cell proliferation while ERK1 has been shown to inhibit the effects of ERK2 in certain cells (McCubrey *et al.*, 2007).

1.8 Pharmacological treatment of breast cancer

Chemotherapeutic agents have been shown in numerous studies to benefit the treatment and prognosis of breast cancer patients. These agents, including but not limited to anthracyclines, taxanes, trastuzumab, tamoxifen, and rapamycin, are typically administered to patients in varying combinations, as the mechanisms by which these agents reduce cancer invasion and progression vary from drug to drug.

1.8.1 Anthracyclines

Frequently used anthracyclines in the treatment of breast cancer include both epirubicin and doxorubicin (Adriamycin). These agents are responsible for disrupting DNA structure by intercalating between adjacent DNA base pairs. As a result of this intercalation, synthesis of DNA and RNA is disrupted, and DNA cleavage mediated by isoforms of topoisomerase II is also induced (Goodman *et al.*, 1974, 1977; Tewey *et al.*, 1984; Capranico *et al.*, 1990). Several isoforms of topoisomerase II exist, including topoisomerase II α , topoisomerase II β -1, and topoisomerase II β -2 (Tsai-Pflugfelder *et al.*, 1988; Chung *et al.*, 1989; Jenkins *et al.*, 1992; Davies *et al.*, 1993). Decreased expression of topoisomerase II α is associated with decreased anthracycline sensitivity, making it an important contribution to the cytotoxicity of anthracyclines (Jarvinen *et al.*, 2000).

1.8.2 Taxanes

During cell division, the mitotic spindle distributes chromatids to each daughter cell. These mitotic spindles are comprised of microtubules which are assembled from heterodimers. Polymerization and depolymerization of these microtubules is an essential component in proper spindle formation. Taxanes, including paclitaxel and docetaxel, disrupt mitotic spindle formation by stabilizing microtubules (Schiff and Horwitz, 1980; Kumar, 1981; Rowinsky *et al.*, 1988). Tubulin heterodimers are composed of α - and β -tubulin. Taxanes function by binding to β -tubulin and stabilizing microtubules, and this disruption in mitotic spindle function prevents cell division (McCubrey *et al.*, 2008).

1.8.3 Trastuzumab

Trastuzumab (Herceptin) is a humanized mouse monoclonal immunoglobulin G₁ (IgG₁) κ antibody that binds to the extracellular domain of HER-2 (Hudziak *et al.*, 1989; Carter *et al.*, 1992). Binding trastuzumab results in accelerated endocytic degradation of HER-2. This degradation decreases signal transduction cascades in the PI3K/PTEN/AKT and Ras/Raf/MEK/ERK pathways downstream of HER-2 that are responsible for promoting cell cycle progression and inhibiting apoptosis, resulting in the prevention of cellular proliferation (McCubrey *et al.* 2008). Trastuzumab treatment is most effective for breast cancers where HER-2 overexpression exists, or where the gene encoding HER-2 is amplified (Vogel *et al.*, 2002).

1.8.4 Tamoxifen

Tamoxifen acts primarily on breast carcinomas that are estrogen receptor (ER) positive (ER+). Cellular proliferation is inhibited by tamoxifen as a result of its binding to estrogen receptors (ERs) (Borgna and Rocheford, 1981). Two isoforms of ERs exist, including ER α and ER β (Watler *et al.*, 1985; Green *et al.*, 1986; Mosselman *et al.*, 1996; Moore *et al.* 1998; Ogawa *et al.*, 1998a; Shoda *et al.*, 2002), which bind together in homodimeric and heterodimeric combinations (Cowley *et al.*, 1997; Pace *et al.*, 1997; Pettersson *et al.*, 1997, Ogawa *et al.*, 1998b). ERs are a group of transcription factors inducing protein expression which are responsible for promoting cell cycle progression (cyclin D1) and inhibiting apoptosis (Bcl-2) (Dong *et al.*, 1999; Perillo *et al.*, 2000). Transcription of these genes is stimulated when ERs bind to estrogens. There is competition for the same ER α and ER β binding sites between Tamoxifen and its metabolites. Therefore, binding of tamoxifen or its metabolites to ERs prevents estrogens from promoting cell proliferation (McCubrey *et al.*, 2008).

The transactivation potential of ERs bound to tamoxifen or its metabolites depends on whether ER α or ER β isoforms are present. Tamoxifen is a partial agonist for ER α homodimers and pure antagonist for ER β homodimers (Barkhem *et al.*, 1998). Tamoxifen is converted to 4-hydroxytamoxifen (4-HT) by hepatic metabolism, which is similar to tamoxifen in that it is a partial agonist for ER α homodimers; however, it is a pure antagonist for ER β homodimers (McCubrey *et al.*, 2008). Tamoxifen displays a variety of estrogenic effects, including the stabilization of bone mineral density (BMD),

increased frequencies of endometrial cancer, and thromboembolic disease (Bernstein *et al.*, 1999; Bergman *et al.*, 2000; Yoneda *et al.*, 2002).

In order to determine if treatment with tamoxifen is appropriate, tumor biopsy specimens from breast cancer patients are analyzed for ER α expression using immunohistochemistry (IHC) (Harvey, 1999). Only breast cancer patients with ER α positive (ER+) tumors are administered tamoxifen, since the therapeutic benefit of treatment is significantly higher in breast cancer patients with ER+ tumors than those with ER negative (ER-) tumors (Chang *et al.*, 1999; Chang *et al.*, 2000). For treatment of breast cancer patients with ER+ tumors, the administration of tamoxifen along with additional chemotherapeutic drugs is more effective than administration of the chemotherapeutic drugs alone; however this is not the case for breast cancer patients with ER- tumors (Fisher *et al.*, 2001).

1.8.5 Rapamycin

Rapamycin is a bacterial macrolide which was originally identified as an antifungal agent, and has found to possess both immunosuppressive and antitumor effects (Sehgal, 2003). Clinically it is used as an immunosuppressant for recipients of solid-organ transplants as well as in cardiovascular stents to prevent postangioplasty restenosis (Ponticelli, 2004; van der Hoeven *et al.*, 2005). Recently, the National Cancer Institute has performed screens and studies testing rapamycin's potential for treating cancer. These studies and screenings indicated that these drugs are highly effective against a

variety of tumors both in cell lines and in phase I/II studies (Monks *et al.*, 1991; Dancey, 2002).

Rapamycin functions by indirectly inhibiting the target of rapamycin (TOR) proteins which exist downstream of the PI3K/AKT pathway. These proteins are a highly conserved group of serine/threonine kinases (Jacinto *et al.*, 2003; Hidalgo *et al.*, 2000). The mammalian TOR (mTOR) regulates phosphorylation of at least two proteins that are important for translation: one which controls ribosomal biogenesis (S6K1) and the other, which regulates translation of mRNAs with complex cap structures, including those encoding c-myc and cyclin-D1 (4E-BP1) (Inoki *et al.*, 2005). This demonstrates mTOR's central role in accumulation of cell mass as well as increases in cell number (Chang *et al.*, 2007). Additionally, mTOR is activated by at least two different classes of stimuli, including nutrient and energy availability and mitogens that activate the PI3K/AKT pathway (Inoki *et al.*, 2005).

Rapamycin, as well as its analogs, have shown great promise in the treatment of sporadic breast cancer tumors. Studies indicate that tumors that respond to rapamycin and its analogues are ER+ with PTEN mutations, and also over-expressed erbB2 (Yu *et al.*, 2001). PTEN mutations and over-expression of erbB2 are responsible for activation of the PI3K/AKT pathway, essentially increasing their dependence on the functioning of the pathway and also increasing sensitivity to rapamycin (Chang *et al.*, 2007).

1.9 Neutrophil gelatinase associated lipocalin (NGAL)

Neutrophil gelatinase associated lipocalin (NGAL) is a lipocalin that is noted for its ability to sequester iron and siderophores, which is an important component of innate immunity to bacterial infections. Lipocalins comprise a family of approximately 20 small secreted proteins which are classified based on their three-dimensional structure (Devarajan, 2007). More specifically, they are capable of binding to a large variety of molecules due to a single eight-stranded anti-parallel β -barrel which forms an enclosing calyx (Flower *et al.*, 2000). Due to their structure, lipocalins are noted to be extremely effective in shuttling diverse substances, such as arachidonic acid, prostaglandins, pheromones, retinoids, fatty acids, steroids, and iron (Goetz *et al.*, 2000). Initially, NGAL was isolated as a 25kDa protein that was covalently bound with matrix metalloproteinase-9 (MMP-9) in human neutrophils. Studies in NGAL deficient mice displayed increased sensitivity to Gram-negative bacterial infections as well as increased susceptibility to death from sepsis (Flo *et al.*, 2004; Berger *et al.*, 2006).

More recently, NGAL has been investigated for its presence in a variety of human cancers. In initial findings, a heterogeneous expression of NGAL was found in primary breast cancers at both the mRNA and protein levels (Stoesz *et al.*, 1998). It was found within breast carcinoma cells, but not in normal ductal epithelium, with the largest quantity being found in the lumen of normal ducts in the vicinity of tumors which expressed NGAL. These findings have been confirmed in a large study using 207 invasive breast cancer samples, with approximately one-third of the samples being interpreted as positive for NGAL staining (Bauer *et al.*, 2007). The function of NGAL in

breast cancers is not fully understood, however preliminary evidence suggests that it may be associated with a poor prognosis. Some of these markers include estrogen and progesterone-receptor negative status as well as high proliferation rates. The correlation between NGAL expression and ER status has been confirmed in a series of studies profiling gene expression (Gruvberger *et al.*, 2001; van de Vijyer *et al.*, 2002; Van't Veer *et al.*, 2002; Wang *et al.*, 2005). Additional findings dealing with negative steroid receptor status, poor histologic grade, and presence of lymph node metastasis indicate that tissue NGAL expression may provide information for prognosis based on risk assessment and potential identification in a subset of patients that may benefit from more aggressive adjuvant therapy (Devarajan, 2007).

Studies *in vitro* have illustrated the role of NGAL over-expression on MCF-7 human breast cancer cells, demonstrating increased growth rates, proliferation, angiogenesis, and increased levels of MMP-9. Since NGAL is known to bind MMP-9, its degradation is reduced and MMP-9 enzyme activity is increased *in vitro* (Yan *et al.*, 2001). Unfortunately, MMP-9 activity has been shown to increase the progression of cancer in patients due to its role in degradation of basement membranes and extracellular matrix. As a result, vascular endothelial growth factor (VEGF) is liberated and angiogenesis is enabled, leading to increased invasion and metastasis (Lochner *et al.*, 1998; Lee *et al.*, 2005).

1.10 Experimental objectives

All of the previously stated oncogenes play a central role in tumorigenesis and chemotherapeutic drug resistance. Therefore, it is important to understand the role that each gene plays, and also the effect of manipulating these genes. Oncogenes can be over-expressed or inhibited, causing a cascade of signals that alter the normal survival of the cell. In hopes of understanding the roles of various oncogenes, it is necessary to observe the effects of inhibiting certain oncogenes and how this plays a role in signaling pathways. Specifically the roles of PI3K, AKT, and PTEN on the PI3K/AKT signaling pathway should be investigated. It is also of value to note how cell lines transfected with specific genes, such as mutant PTEN and AKT, compare with wild-type constructs when oncogenes are over-expressed or inhibited.

In order to observe the effect of oncogenic expression and sensitivity to chemotherapeutics, MTT cell-proliferation assays and Western Blot Analyses were performed. From here, cell viability and protein expression, both which are indicative of signal transduction were assessed. To test levels of protein expression, probing with a series of antibodies to detect the presence of specific oncogenes, and the levels to which they are phosphorylated in response to exposure to a variety of drugs and inhibitors was employed. The results of these experiments revealed more about the individual roles of oncogenes, as well as how cells adapt to the inhibition of signal transduction pathways.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Cell culture

MCF-7 cells were obtained from the American Type Culture Collection (ATCC), and were derived from a human breast adenocarcinoma. These cells have an epithelial morphology and form a monolayer in culture. They have a modal chromosome number of 69. MCF-7 cells are adherent, estrogen receptor (ER) positive (ER+) and express wild type (WT) PTEN (Steelman *et al.*, 2008).

T47D cells were obtained from the ATCC and were derived from an invasive ductal carcinoma. These cells have epithelial morphology and a modal chromosome number of 65. T47D cells are adherent, ER+, and express WT PTEN.

Roswell Park Memorial Institute 1640 (RPMI 1640) cell culture medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (MediaTech-Cellgro; Manassas, VA), [2nM L-glutamine, 100µg/ml streptomycin, and 100 units/L penicillin G (Invitrogen; Carlsbad, CA)] was used to allow MCF-7 and T47D cells to adhere to culture flasks (Steelman *et al.*, 2008).

MDA-MB-231 cells were obtained from the ATCC and were derived from a human breast adenocarcinoma. These cells have an epithelial morphology and a modal chromosome number of 57. MDA-MB-231 cells are adherent, ER-, and express WT PTEN.

Dulbecco's Modified Eagle's Medium (DMEM) cell culture medium containing 10% (w/v) heat-inactivated FBS was used to allow MDA-MB-231 cells to adhere to

culture flasks. All cells were cultured in a humidified 37°C incubator with a 5% CO₂ atmosphere.

2.2 PTEN(WT), PTEN(C124S), PTEN(G129E), and PTEN(399stop) plasmids

A plasmid which encodes human wild type PTEN [PTEN(WT)] was produced by inserting the aforementioned gene into the multiple cloning site of pEGFP-C2. A variety of mutant PTEN genes were inserted at multiple cloning sites of pEGFP-C2; these mutants included PTEN(C124S), PTEN(G129E), and PTEN(399stop) (Steelman *et al.*, 2008). Each mutant differs from PTEN(WT) by the substitution of a single amino acid; C124S is replaced by serine in PTEN(C124S), while G129E is replaced with glutamic acid in PTEN(G129E). PTEN(C124S) lacks lipid and protein phosphatase activity, whereas PTEN(G129E) lacks only lipid phosphatase activity. PTEN(399stop) is missing the final 5 carboxyl terminal amino acids of PTEN(WT), and this deletion is responsible for preventing interactions between the carboxyl terminal protein binding motif of PTEN, and PDZ domains of other proteins (Steelman *et al.*, 2008).

2.3 Transfection of MCF-7, T47D, and MDA-MB-231 cells with PTEN constructs

Cells were seeded at a density of 5×10^5 cells in each well of a six-well cell culture plate (Becton Dickinson Labware; Franklin Lakes, NJ) one day prior to transfection (Steelman *et al.*, 2008). To remove residual FBS, cells were washed with Opti-MEM medium (Invitrogen; Carlsbad, CA). After 24 hours, cells were transfected with 10 µg of DNA with Lipofectin (Invitrogen; Carlsbad, CA) as suggested by the

manufacturer. 48 hours post-transfection, selection medium (RPMI + 10% FBS + 2mg/ml G418) (Invitrogen; Carlsbad, CA) was added to establish stably transfected cells. The cells were provided fresh selection media every three days (Steelman *et al.*, 2008, 2005)

2.4 Protein lysates

MCF-7, T47D, and MDA-MB-231 transfected cell lines were collected after being trypsinized, washed twice with 1X phosphate-buffered saline (1X PBS) [110 mM NaCl, 6.7 mM Na₂HPO₄, 2.1 mM KCl, and 1.1 mM KH₂PO₄] and were plated in 100 mM tissue culture plates in the presence of clear RPMI 1640 containing 5% charcoal stripped (CS) FBS for 24 hours. The cells were treated with 4-HT, doxorubicin, rapamycin and/or paclitaxel for various time intervals. In the inhibitor experiments, the cells were serum-starved for 24 hours with clear RPMI containing 0.5% CS FBS, and then incubated with inhibitors for 1 hour, followed by treatments described above (McCubrey *et al.*, 2006).

Cells were then lysed on ice in Gold Lysis Buffer (GLB) [20 mM Tris (pH 7.9), 137 mM NaCl, 5 mM Na₂EDTA, 10% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 1 mM sodium orthovanadate, 1 mM ethylene glycos-bis (β -aminoethyl ether)-N, N, N',N'tetraacetic acid (EGTA), 10 mM sodium fluoride, 1 mM tetrasodium PP_i, and 100 mM b-glycerophosphate]. Insoluble material was removed through centrifugation at 12,000 rpm while cellular protein was quantified at an absorbency of 570 nM (McCubrey *et al.*, 2000). Western blot analysis was then

performed with antibodies specific for phospho and total AKT, PTEN, MEK, ERK, GSK-3 β , and p70^{S6K}.

2.5 Western blot analysis

After quantification of protein lysates by BCA protein reagent (50:1 v/v) (Pierce; Rockford, IL), 10 μ l of loading dye was added to each sample and boiled at 90°C for 3 minutes. Samples were then loaded into 10% SDS-PAGE separating and stacking gels. The separating gel [10% (w/v) of 37.5:1 (w/w) acrylamide:bisacrylamide, 1.5M TRIS (pH 8.8), 10% (w/v) ammonium persulfate (APS), 10% sodium dodecylsulfate (SDS), and TEMED] comprised the lower 75% of the cassette (Invitrogen; Carlsbad, CA). After the separating gel polymerized, the stacking gel [4% (w/v) acrylamide, 1.0M TRIS (pH 6.8), 10% (w/v) APS, 10% SDS, and TEMED] was added, comprising the upper 25% of the cassette.

The samples were then electrophoresed in running buffer [200 mM glycine, 25 mM TRIS, and 1 mg/ml (10%) SDS] at 77 volts for approximately 120 minutes and run alongside a protein ladder (Amersham Biosciences; Piscataway, NJ). Proteins were then transferred from gels to PVDF transfer membranes (Pierce; Rockford, IL) by semidry electro transfer at 20 volts for three hours in transfer buffer [200 mM glycine, 25 mM TRIS and 20% methanol] with a Bio-Rad[®] gel transfer apparatus (Bio-Rad; Richmond, CA). Membranes were briefly soaked in methanol and then soaked in transfer buffer for 30 minutes prior to electro transfer. After transfer, the membranes were placed in blocking solution [10 μ g/ml BSA in 137 mM NaCl, 20 mM TRIS, 1g sodium azide, and

0.5% (v/v) Tween[®] 20 (1X TBST)] overnight at 4°C or 4 hours at room temperature while shaking.

After blocking, the membranes were incubated overnight at 4°C in primary antibody solution [1:1000 (v/v) primary antibody and 10 mg/ml BSA in 1X TBST]. The membranes were then washed three times for five minutes in 1X TBST to remove any residual primary antibody, and were incubated for one hour on a shaker at room temperature in secondary antibody solution [1:5000 (v/v) secondary antibody in TBST]. Following incubation, the membranes were washed three times for ten minutes in 1X TBST to remove residual antibody solution. Membranes were then incubated at room temperature for one minute in ECL Western Blotting Detection Substrate (1:1 v/v) (Pierce; Rockford, IL). Luminol oxidation results in light emission which can be detected on x-ray films. Membranes were then developed in film cassettes with exposure to x-ray film (Research Products International; Mt. Prospect, IL) to determine activated protein levels within samples.

2.6 Analysis of MCF-7, T47D, and MDA-MB-231 cell sensitivity to anticancer agents (MTT Analysis)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a tetrazolium salt used to measure cellular proliferation *in vitro*. Metabolically active cells take up the MTT, while mitochondrial enzymes reduce the MTT to insoluble formazan crystals. To measure cell proliferation and sensitivity to anticancer agents, cells were plated in 96-well culture plates (Corning; Corning, NY) at a density of approximately

5×10^3 cells per well in 100 μ l/well of clear RPMI-1640 containing 10% FBS. Plates were incubated for 24 hours to allow cells to attach to the bottom of each well. A 100 μ l dose of treatment medium was added to each well 24 hours after cells were initially seeded (Steelman *et al.*, 2008). Cells were treated with two-fold serial dilutions of various chemotherapeutic drugs, including doxorubicin (Sigma; Milwaukee, WI), rapamycin (EMD Biosciences; Gibbstown, NJ), paclitaxel (Sigma; Milwaukee, WI), and tamoxifen (4HT) (Sigma; Milwaukee, WI) and/or inhibitors [MEK (U0126 and PD0329501) and PI3K (LY294002)] (EMD Biosciences; Gibbstown, NJ), bringing the total volume of each well to 200 μ l. Plates with controls were read on the first day after seeding to provide a base line for cell growth. After 72 hours (4 days from seeding), the amount of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma; St. Louis, MO) reduction in each well was quantified by dissolving the insoluble formazan crystals in 100-200 μ l of dimethyl sulfoxide (DMSO), and reading the absorbance at 570 nm with a FL600 microplate fluorescence reader (Bio-Tek Instruments; Winooski, VT) (Steelman *et al.*, 2008).

2.7 NGAL secreted protein collection and analysis

MCF-7, T47D, and MDA-MB-231 sensitive and drug resistant cells were seeded at 1×10^6 cells per well in 6-well plates and were allowed to adhere and proliferate for 72 hours. Cells were plated out in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) containing 0 nM, 10 nM, or 25 nM doxorubicin.

Seventy-two hours post incubation the supernatant was collected from the wells and placed into labeled microcentrifuge tubes at a volume of 1mL/tube for NGAL extraction. For each 1 mL sample, 250 μ l of trichloroacetic acid (TCA) was added and the sample was vortexed to precipitate NGAL out of solution. The samples were placed in a centrifuge at 4°C, incubated for ten minutes, and were then spun at 14,000 rpm for 15 minutes. The supernatant was then removed and discarded, and the pellet was washed with 200 μ l acetone and re-suspended. The sample was re-spun at 14,000 rpm for 10 minutes, and the supernatant was discarded. This step was repeated a second time again discarding the supernatant. After the final spin, the remaining pellet was re-suspended in blue loading dye for Western Blot Analysis, mixed, and boiled for 3 minutes at 90°C. Samples were then loaded into 12% polyacrylamide gels and run as previously described for Western Blot Analysis.

In addition to Western Blot Analysis, separate membranes were removed from the transfer apparatus and stained with Coomassie Blue to observe total protein levels. Each membrane was first stained with Coomassie Blue [0.1% v/v Coomassie Brilliant Blue R, 50% Methanol, 7% Acetic Acid, 42.9% Deionized H₂O]. After approximately 2-3 minutes, the dye was removed and membranes were placed in a primary wash [50% methanol, 7% acetic acid, 43% Deionized H₂O] until saturated with Coomassie Blue dye. The wash was then changed, and a secondary wash [90% methanol, 10% acetic acid] was added to remove any residual dye from the background.

CHAPTER 3: RESULTS

3.1 Analysis of MCF-7 cells

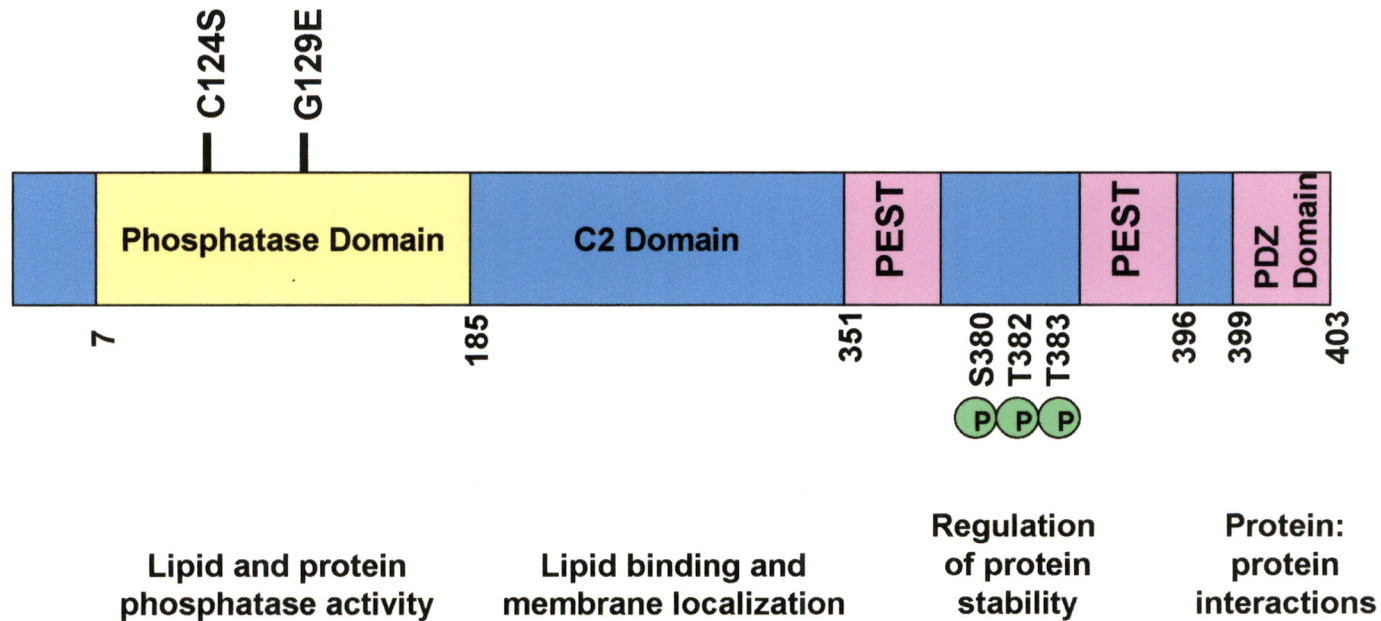
3.1.1 PTEN domains and functions

In order to alter the activity of the PI3K/PTEN/AKT cascade, plasmids encoding various PTEN constructs were transfected into MCF-7 cells. The plasmids used included PTEN(WT), PTEN(C124S), PTEN(G129E), and PTEN(399stop), and were manipulated to test the effect of signals within the pathway on anti-cancer drugs that are frequently used for breast cancer treatment.

PTEN is a gene composed of 403 amino acids divided into various residues (Figure 3). The phosphatase domain represents amino acids 7 through 185, and is the site of lipid and protein phosphatase activity. Two common mutant genes, PTEN(C124S) and PTEN(G129E) are represented in this region, where PTEN(C124S) lacks both lipid and protein phosphatase activity, and PTEN(G129E) lacks lipid phosphatase activity and retains protein phosphatase activity. Amino acid residues 186 through 351 make up the C2 domain, where lipid binding and membrane localization occur. Residues 400-403 comprise the PDZ binding motif, a region at the end of the gene segment that is responsible for mediating interaction with other proteins which contain PDZ domains. The PTEN(399stop) gene lacks a PDZ binding motif, thus preventing interaction of PTEN(399stop) with the PDZ domains of other proteins.

Figure 3. PTEN structure and function. The PTEN gene is composed of 403 amino acids. Residues encoding the phosphatase domain, C2 domain, region of protein stability regulation, and PDZ domain binding motif are indicated. The phosphatase domain contains regions that code for lipid and protein phosphatase activity, with the C124S gene representing a loss in both lipid and protein phosphatase activity, and the G129E gene representing a loss in only lipid phosphatase activity. The C2 domain may foster membrane localization as a result of lipid binding. The PDZ domain binding motif is responsible for mediating association with other proteins containing PDZ domains.

PTEN Protein Domains

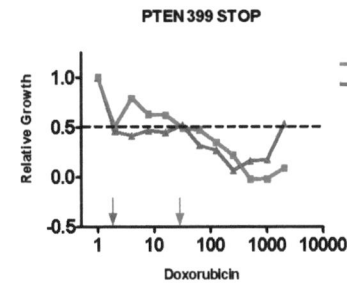
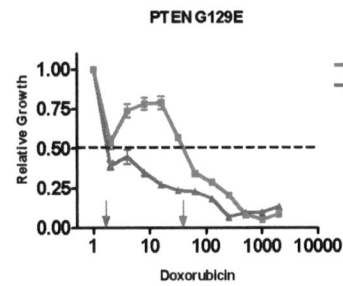
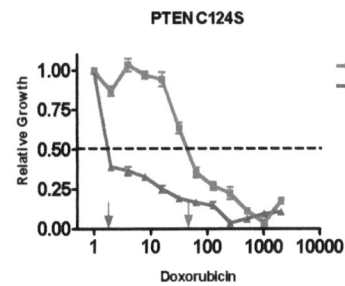
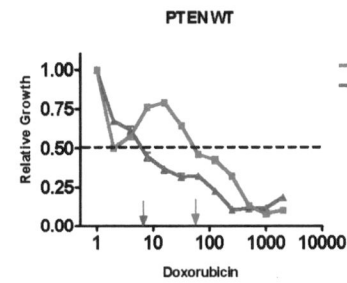
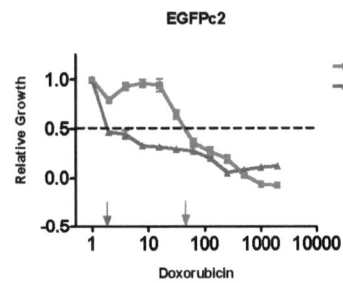
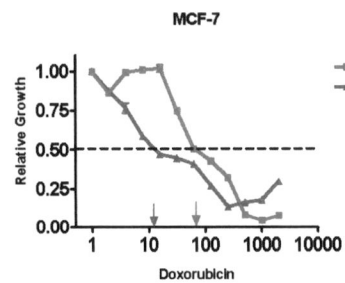


3.1.2 Cellular proliferation of MCF-7 cells transfected with PTEN mutants in the presence of doxorubicin.

Proliferation rates of MCF-7 PTEN mutants in the presence of doxorubicin and doxorubicin with 4-HT were measured using MTT analysis (Figure 4). MCF-7 cell line mutants that were tested included MCF-7 sensitive cells, EGFPc2, PTEN WT, PTEN C124S, PTEN G129E, and PTEN 399stop. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well in 100 μ l clear RPMI and allowed to adhere to the well for 24 hours. Doxorubicin and/or doxorubicin with 4-HT was added in a series of serial two-fold dilutions, with the first column of the 96-well plate containing the highest concentration of doxorubicin, and the twelfth column of the plate containing only media. The 4-HT was kept constant at a concentration of 200 nM.

Doxorubicin inhibitory concentration 50% (IC_{50}) indicates the concentration of doxorubicin that causes cells to proliferate at a rate that is half as rapid as cells incubated in media without doxorubicin. Relative growth of cells in all MCF-7 PTEN mutant cell lines was determined by calculating the increase in the number of cells after 4 days of treatment with each doxorubicin and/or doxorubicin and 4-HT concentration divided by the increase in the number of cells after 4 days of treatment in the presence of no doxorubicin and/or doxorubicin and 4-HT. The mean relative growth rate and corresponding standard deviation for each condition was calculated from absorbance values of 8 replicate wells. Arrows illustrate where the cell lines cross the IC_{50} , and results indicate that the MCF-7 sensitive, EGFPc2, PTEN WT, PTEN C124S, PTEN

Figure 4. Relative growth of MCF-7 PTEN mutants in presence of doxorubicin and doxorubicin combined with 4-HT. MTT was used to measure proliferation of various PTEN mutants in MCF-7 cells. Cell lines tested include MCF-7 cells sensitive to doxorubicin, MCF-7 cells transfected with an empty vector (EGFPc2) (control), MCF-7 cells expressing wild type (WT) PTEN, MCF-7 cells expressing C124S (lacking lipid and protein phosphatase activity), MCF-7 cells expressing G129E (lacking lipid phosphatase activity but retaining protein phosphatase activity), and MCF-7 cells expressing 399Stop (lacking a PDZ domain binding motif). Doxorubicin inhibitory concentration 50% (IC_{50}) indicates the concentration of doxorubicin that causes cells to proliferate at a rate that is half as rapid as cells incubated in media without doxorubicin. Arrows indicate where the growth curve crosses the IC_{50} . Relative growth was determined by calculating the increase in the number of cells after 4 days of treatment with each doxorubicin and/or doxorubicin and 4-HT concentration divided by the increase in the number of cells after 4 days of treatment in the presence of no doxorubicin and/or doxorubicin and 4-HT. The mean relative growth rate and corresponding standard deviation for each condition was calculated from absorbance values of 8 replicate wells. Standard deviation is indicated by error bars.



G129E, and PTEN 399stop cell lines all cross the 50% proliferation threshold when exposed to doxorubicin and 4-HT sooner than cell lines exposed only to doxorubicin.

3.1.3 Cellular proliferation of MCF-7, T47D, and MDA-MB-231 cells in the presence of doxorubicin

MTT assays were performed in doxorubicin without the presence of 4-HT on MCF-7, T47D, and MDA-MB-231 cell lines that were sensitive to doxorubicin and cell lines that were 10 nM doxorubicin resistant (Figure 5). Cells that were sensitive to doxorubicin crossed the IC_{50} threshold before the doxorubicin resistant cells in the MCF-7, T47D, and MDA-MB-231 cell lines. These results were expected, as cell lines resistant to doxorubicin grow better in the presence of the drug than cells not resistant to doxorubicin. In the MCF-7 and MDA-MB-231 cell lines, the doxorubicin resistant cell lines were able to withstand a much higher concentration of doxorubicin before crossing the IC_{50} , whereas in the T47D cell line, the resistant cell lines withstood a higher concentration, but not by a margin as large as the other cell lines.

3.1.4 MCF-7 basal MEK protein levels

Western blot analysis was performed on MCF-7 sensitive, 10 nM doxorubicin resistant, and 25 nM doxorubicin resistant cell lines (Figure 6). Each cell line was plated out at 1×10^6 cells per plate in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours supernatant was removed, cells were rinsed twice with 1X PBS, and each

Figure 5. Relative Growth of MCF-7, T47D, and MDA-MB-231 in Doxorubicin.

MTT was utilized to measure cell proliferation in MCF-7, T47D and MDA-MB-231 sensitive and 10 nM doxorubicin resistant cell lines in the presence of the indicated concentrations of doxorubicin. Relative growth was determined by calculating the increase in the number of cells after 4 days of treatment with each doxorubicin concentration divided by the increase in the number of cells after 4 days of treatment in the presence of doxorubicin. The mean relative growth rate and corresponding standard deviation for each condition was calculated from absorbance values of 8 replicate wells. Standard deviation is indicated by error bars, while arrows indicate where the growth curve crosses the IC_{50} . Asterisks (*) represent statistical differences where $P < 0.005$ as determined by student T-Test.

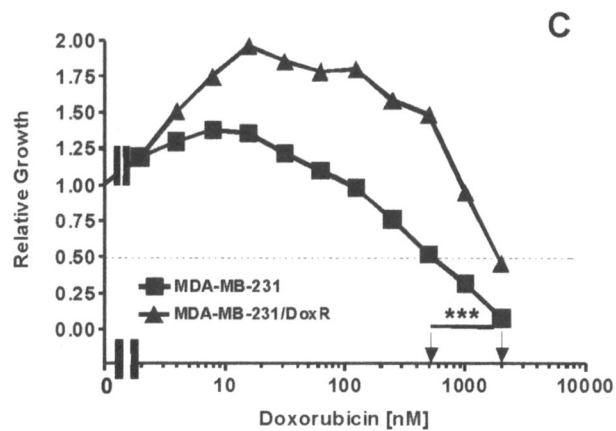
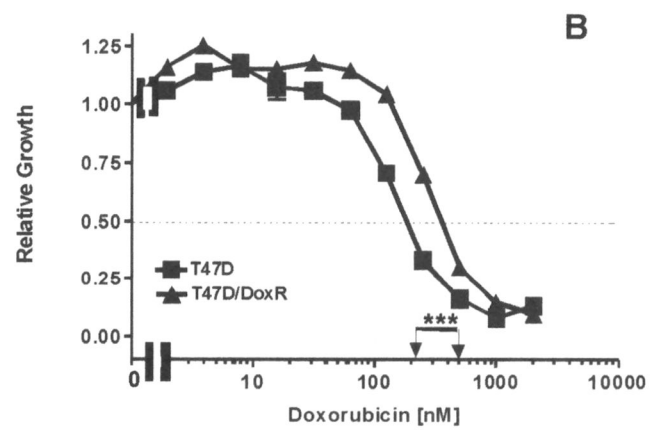
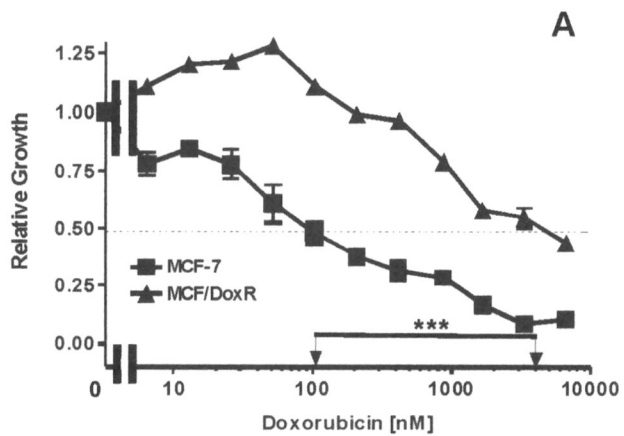
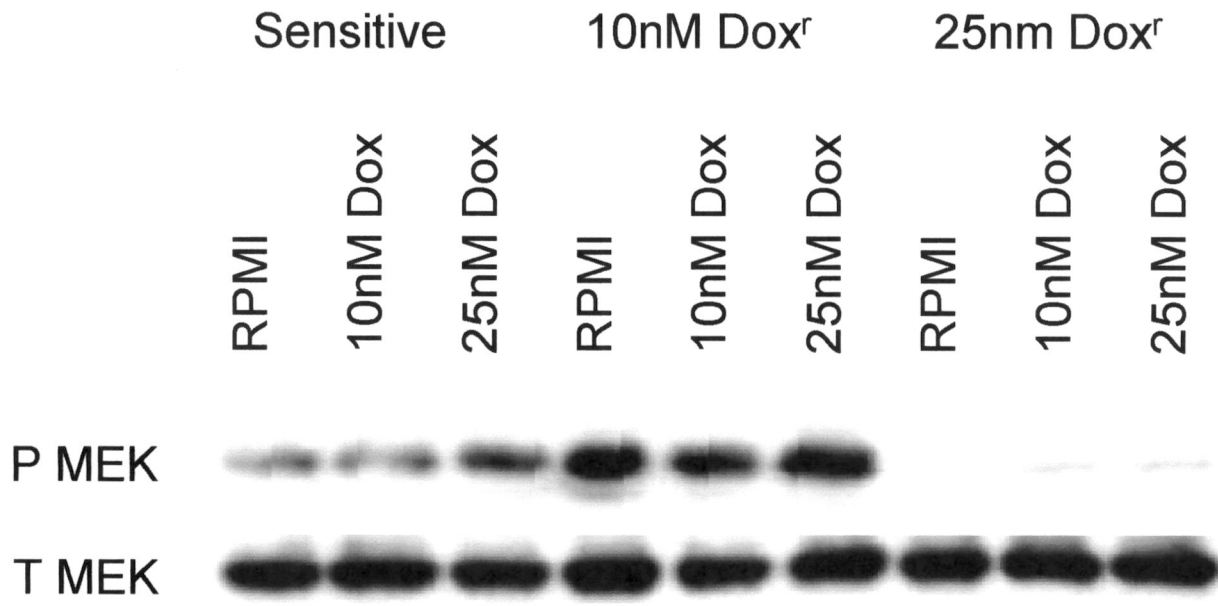


Figure 6. Western Blot Analysis of Phospho MEK and Total MEK basal protein levels in MCF-7 cells sensitive to doxorubicin, MCF-7 cells resistant to 10 nM doxorubicin, and MCF-7 cells resistant to 25 nM doxorubicin. Cells were seeded at a density of 1×10^6 cells per plate and each cell line was cultured overnight in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours, supernatant was removed, cells were rinsed twice with 1X PBS, and each cell line was cultured for 24 additional hours in red RPMI with 10% FBS, RPMI with 10% FBS + 10 nM doxorubicin, and RPMI with 10% FBS + 25 nM doxorubicin. Proteins harvested were subjected to western blot analysis. SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g protein. PVDF membranes were probed with 0.1% (v/v) Phospho and Total MEK antibody in primary blocking buffer. Primary blocking buffer consisted of 0.05% (v/v) Tween 20 and 10mg/ml BSA in TBS. TBS consisted of 150 mM NaCl and 20 mM Tris at a pH of 7.5.



cell line was cultured for 24 additional hours in RPMI with 10% FBS, RPMI with 10% FBS + 10 nM doxorubicin, and RPMI with 10% FBS + 25 nM doxorubicin. Protein lysates were collected and quantified, and SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v) Phospho and Total MEK antibody in primary blocking buffer.

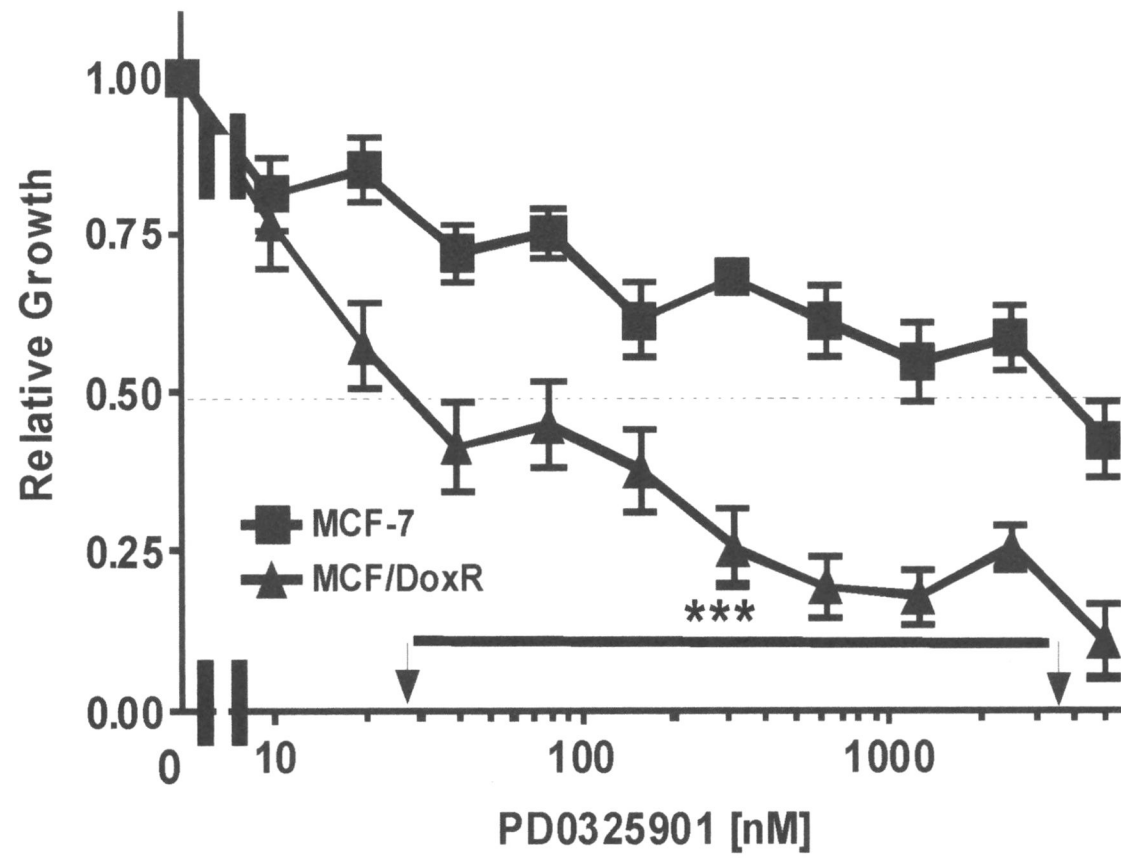
Total MEK was used as a loading control, while the MCF-7 results were indicated in the Phospho MEK samples. MCF-7 cells sensitive to doxorubicin had the lowest expression of MEK when cultured in media containing no doxorubicin and had the highest expression of MEK when cultured in media containing 25 nM doxorubicin. MCF-7 cells that were 10 nM doxorubicin resistant contained higher levels of MEK in all media, and 25 nM doxorubicin resistant cells contained very low levels, though levels were higher in media containing 25 nM doxorubicin.

3.1.5 Proliferation of MCF-7 cells in the presence of MEK inhibitor PD0325901

MTT assays were performed in MCF-7 cell lines that were sensitive to doxorubicin and resistant to 10 nM doxorubicin (Figure 7). The MCF-7 sensitive cell line is not as sensitive to the MEK inhibitor when compared with the doxorubicin resistant cell line that is affected by the MEK inhibitor. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well in 100 μ l clear RPMI and allowed to adhere to the well for 24 hours. PD0325901, a MEK inhibitor, was added in a series of serial two-fold dilutions, with the first column of the 96-well plate containing the highest concentration of inhibitor, and the twelfth column of the plate containing only media.

Figure 7. Relative Growth of MCF-7 in presence of MEK inhibitor PD0325901.

MTT was utilized to measure cell proliferation in MCF-7 sensitive and 10 nM doxorubicin resistant cell lines in the presence of the indicated concentrations of MEK inhibitor. Relative growth was determined by calculating the increase in the number of cells after 4 days of treatment with each doxorubicin concentration divided by the increase in the number of cells after 4 days of treatment in the presence of doxorubicin. The mean relative growth rate and corresponding standard deviation for each condition was calculated from absorbance values of 8 replicate wells. Standard deviation is indicated by error bars, while arrows indicate where the growth curve crosses the IC_{50} .



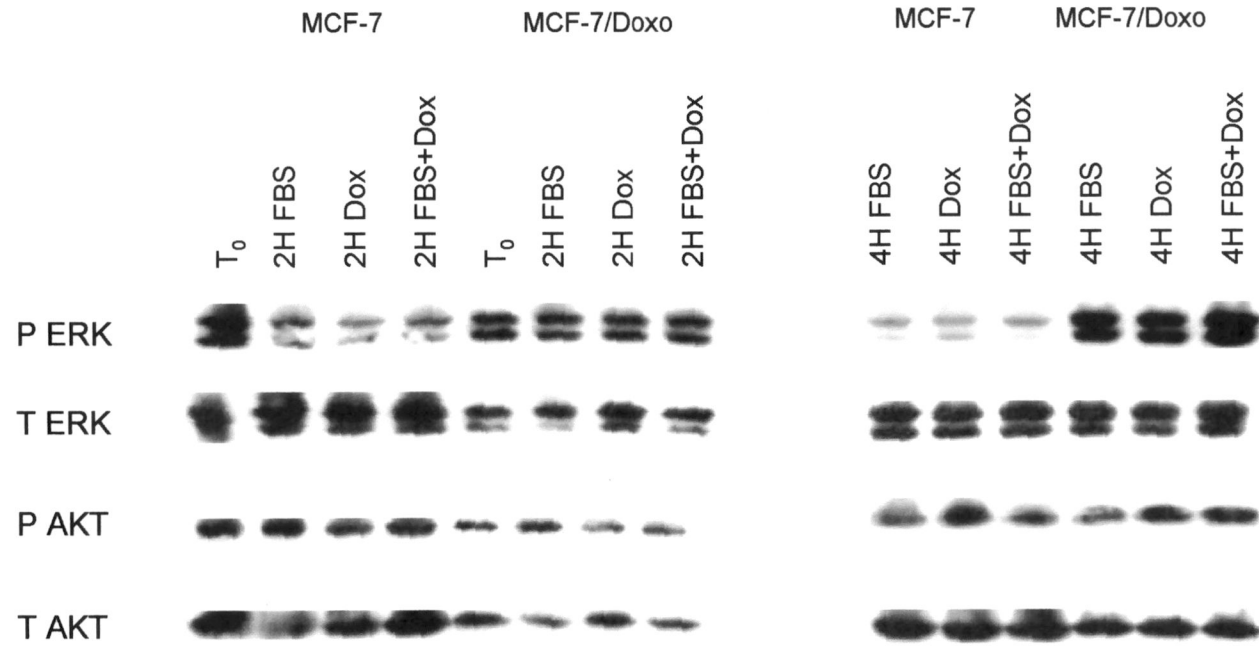
Relative growth rate was determined as previously described. IC_{50} levels demonstrate the effect of a MEK inhibitor on MCF-7 cells, indicating that resistant cells require MEK to control downstream ERK signaling to continue cell growth and proliferation. With a MEK inhibitor, the signaling cascade is stopped and ERK is not phosphorylated, causing a reduction in cell viability.

3.1.6 Effect of FBS and Doxorubicin on ERK and AKT levels in MCF-7 sensitive, 10 nM doxorubicin resistant, and 25 nM doxorubicin resistant cells.

Western blot analysis was performed on MCF-7 cells sensitive to doxorubicin and resistant to 10 nM doxorubicin (Figure 8). Cells were seeded at a density of 1×10^6 cells per plate and each cell line was cultured overnight in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours, the cells were stimulated with 10% FBS, 10 nM doxorubicin, and 10% FBS + 10 nM doxorubicin. Samples were harvested prior to addition of FBS and/or doxorubicin representing T_0 , and again at two and four hours post-treatment. After protein quantification, SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v) Phospho ERK, Total ERK, Phospho AKT, and Total AKT antibody in primary blocking buffer.

Figure 8 shows elevated levels of ERK in the MCF-7 10 nM doxorubicin resistant cell lines when compared with the doxorubicin sensitive cell lines, while at 4 hours, FBS and doxorubicin appear to have a synergistic effect. This evidence does not exist at the 2 hour time point; however this may be explained by a threshold in doxorubicin resistant

Figure 8. Western Blot Analysis of Phospho ERK, Total ERK, Phospho AKT, and Total AKT protein levels in MCF-7 cells sensitive to doxorubicin and MCF-7 cells resistant to 25 nM doxorubicin after treatment with FBS and doxorubicin. Cells were seeded at a density of 1×10^6 cells per plate and each cell line was cultured overnight in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours, the cells were stimulated with 10% FBS, 10 nM doxorubicin, and 10% FBS + 10 nM doxorubicin. Samples were harvested prior to addition of FBS and/or doxorubicin representing T_0 , and again at 2 hours and 4 hours post-treatment. Proteins harvested were subjected to western blot analysis. SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v) Phospho ERK, Total ERK, Phospho AKT, and Total AKT antibody in primary blocking buffer. Primary blocking buffer consisted of 0.05% (v/v) Tween 20 and 10mg/ml BSA in TBS. TBS consisted of 150 mM NaCl and 20 mM Tris at a pH of 7.5.



cells. The 4 hour time point allows for a longer activation period of ERK signaling, which can be explained by the 4 hour FBS and doxorubicin combination sample when compared to the FBS and doxorubicin samples alone.

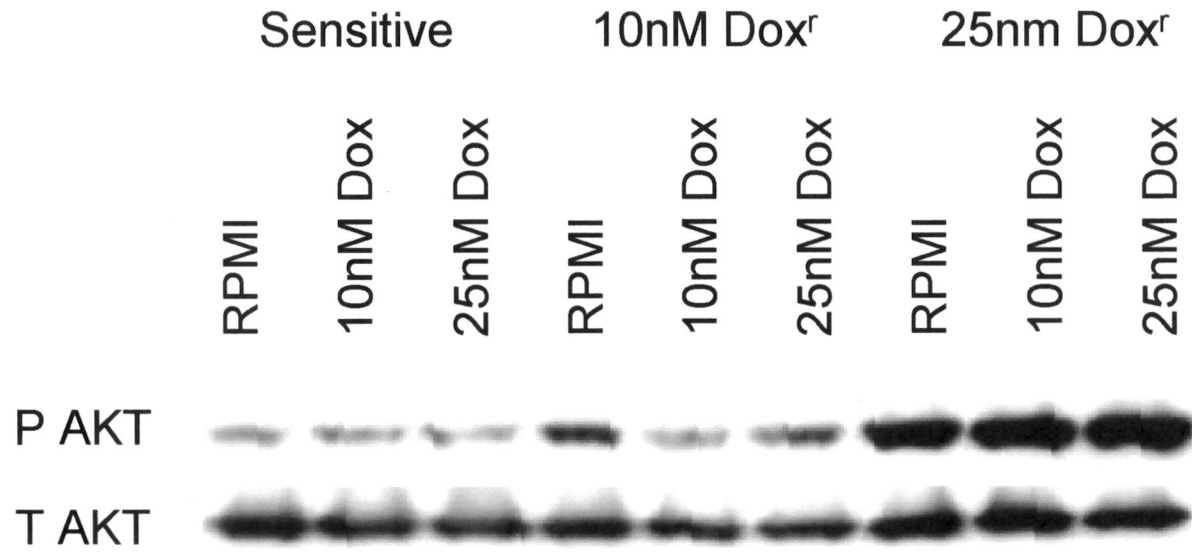
While the AKT levels seem to be higher in the 2 hour doxorubicin sensitive samples, the 4 hour time point indicates higher levels for doxorubicin resistant cell lines in the presence of doxorubicin alone and FBS and doxorubicin in combination. This could be indicated by an activation threshold, where there are higher levels of activated AKT at the 4 hour time point in the doxorubicin resistant cell lines. It appears that there is no increase in activation of AKT in the doxorubicin sensitive cell lines at the 4 hour time point when compared with the 2 hour time point, indicating that there is no additional activation over time. Since ERK and AKT proteins exist in separate signaling cascades, it should be noted that the difference in results between the two proteins is likely.

3.2 Analysis of T47D cells

3.2.1 T47D basal AKT protein levels

Western blot analysis was performed on T47D sensitive, 10 nM doxorubicin resistant, and 25 nM doxorubicin resistant cell lines (Figure 9). Each cell line was plated out at 1×10^6 cells per plate in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours, supernatant was removed, cells were rinsed twice with 1X PBS, and each cell line was cultured for 24 additional hours in RPMI with 10% FBS, RPMI with 10%

Figure 9. Western Blot Analysis of Phospho AKT and Total AKT basal protein levels in T47D cells sensitive to doxorubicin, T47D cells resistant to 10 nM doxorubicin, and T47D cells resistant to 25 nM doxorubicin. Cells were seeded at a density of 1×10^6 cells per plate and each cell line was cultured overnight in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours, supernatant was removed, cells were rinsed twice with 1X PBS, and each cell line was cultured for 24 additional hours in red RPMI with 10% FBS, RPMI with 10% FBS + 10 nM doxorubicin, and RPMI with 10% FBS + 25 nM doxorubicin. Proteins harvested were subjected to western blot analysis. SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g protein. PVDF membranes were probed with 0.1% (v/v) Phospho and Total AKT antibody in primary blocking buffer. Primary blocking buffer consisted of 0.05% (v/v) Tween 20 and 10mg/ml BSA in TBS. TBS consisted of 150 mM NaCl and 20 mM Tris at a pH of 7.5.



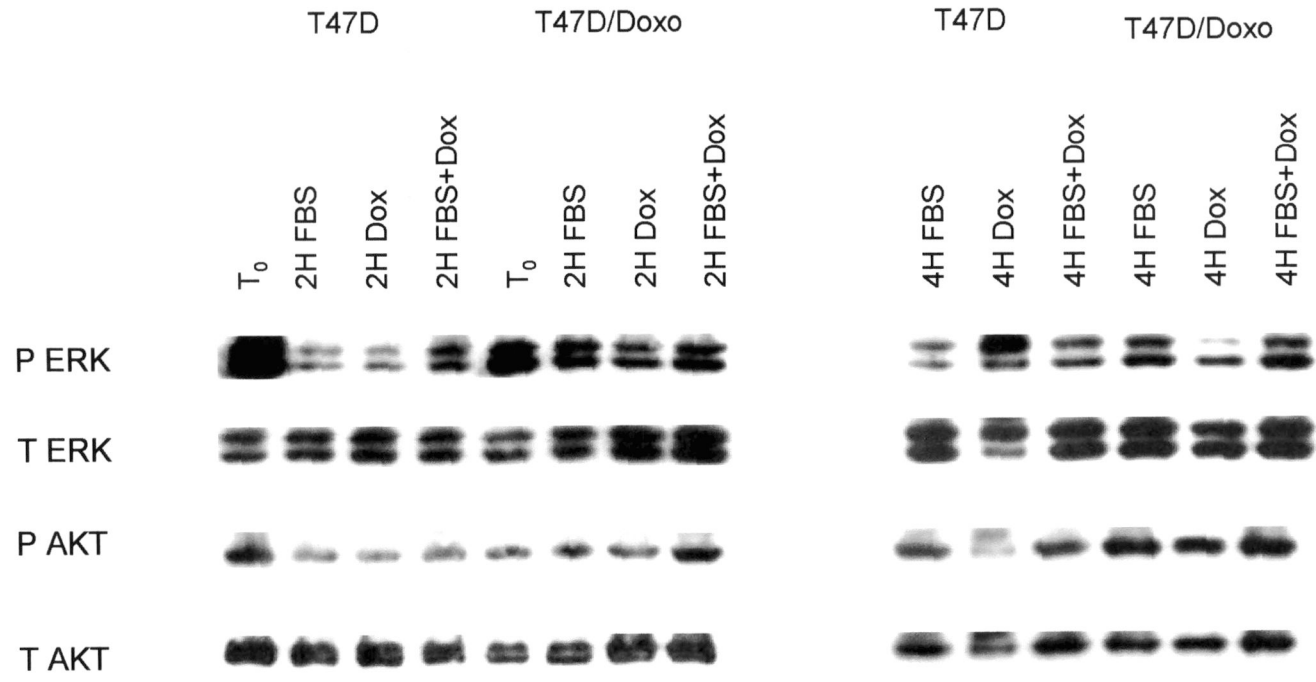
FBS + 10 nM doxorubicin, and RPMI with 10% FBS + 25 nM doxorubicin. Protein lysates were collected and quantified, and SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g protein. PVDF membranes were probed with 0.1% (v/v) Phospho and Total AKT antibody in primary blocking buffer.

Total AKT was used as a loading control, while the T47D results were indicated in the Phospho AKT samples. T47D cells sensitive to doxorubicin had the lowest expression of AKT when cultured in all media. T47D cells that were 10 nM doxorubicin resistant contained higher levels of AKT in all media than the sensitive cell lines, while 25 nM doxorubicin resistant cells contained the highest levels of activated AKT in all media. Results indicate that in the 25 nM resistant cell line, AKT levels were equal and type of media did not have an effect on AKT activation and expression.

3.2.2 Effect of FBS and Doxorubicin on ERK and AKT levels in T47D sensitive, 10 nM doxorubicin resistant, and 25 nM doxorubicin resistant cells

Western blot analysis was performed on T47D cells sensitive to doxorubicin and resistant to 10 nM doxorubicin (Figure 10). Cells were seeded at a density of 1×10^6 cells per plate and each cell line was cultured overnight in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours, the cells were treated with 10% FBS, 10 nM doxorubicin, and 10% FBS + 10 nM doxorubicin. Samples were harvested prior to addition of FBS and/or doxorubicin representing T_0 , and again at two and four hours post-treatment. After protein quantification, SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v)

Figure 10. Western Blot Analysis of Phospho ERK, Total ERK, Phospho AKT, and Total AKT protein levels in T47D cells sensitive to doxorubicin and T47D cells resistant to 25 nM doxorubicin after treatment with FBS and doxorubicin. Cells were seeded at a density of 1×10^6 cells per plate and each cell line was cultured overnight in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours, the cells were treated with 10% FBS, 10 nM doxorubicin, and 10% FBS + 10 nM doxorubicin. Samples were harvested prior to addition of FBS and/or doxorubicin representing T_0 , and again at 2 hours and 4 hours post-treatment. Proteins harvested were subjected to western blot analysis. SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v) Phospho ERK, Total ERK, Phospho AKT, and Total AKT antibody in primary blocking buffer. Primary blocking buffer consisted of 0.05% (v/v) Tween 20 and 10mg/ml BSA in TBS. TBS consisted of 150 mM NaCl and 20 mM Tris at a pH of 7.5.



Phospho ERK, Total ERK, Phospho AKT, and Total AKT antibody in primary blocking buffer.

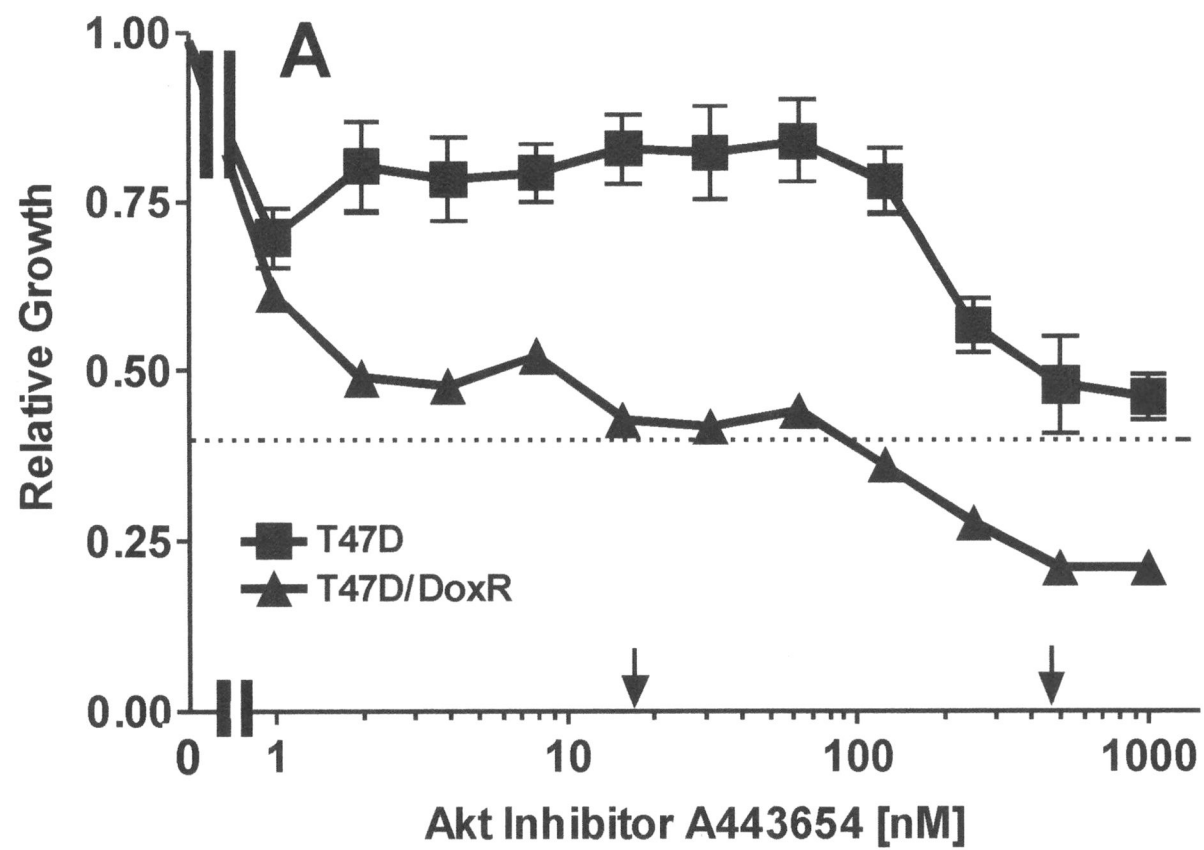
Total ERK was used as a loading control, while elevated levels of ERK at the 2 hour time point in the T47D 10 nM doxorubicin resistant cell lines are indicated in Figure 10. The 4 hour time point shows a more sporadic pattern of activation, indicating that maximum activation may be reached at 2 hours. In the doxorubicin sensitive cell line appears to show a synergistic effect between the FBS and doxorubicin at 2 hours, while at 4 hours doxorubicin seems to upregulate the activation of ERK.

Levels of activated AKT were also measured using Total AKT as a loading control. Figure 10 indicates that the doxorubicin resistant cell line contains higher overall levels of activated AKT, with the highest levels occurring with the 4 hour time point. In these samples, FBS and doxorubicin appear to have a synergistic effect, while in the doxorubicin sensitive lines, this effect does not appear to exist.

3.2.3 Proliferation of T47D cells in the presence of AKT inhibitor A443654

MTT was utilized to measure cell proliferation in T47D sensitive and 10 nM doxorubicin resistant cell lines in the presence of the indicated concentrations of AKT inhibitor (Figure 11). The T47D sensitive cell line has signaling cascades which are not driven by AKT as a downstream target, whereas the doxorubicin resistant cell lines are affected by the AKT inhibitor. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well in 100 μ l clear RPMI and allowed to adhere to the well for 24 hours. A443654, an AKT inhibitor, was added in a series of serial two-fold dilutions, with the

Figure 11. Relative Growth of T47D in presence of AKT inhibitor A443654. MTT was utilized to measure cell proliferation in T47D sensitive and 10 nM doxorubicin resistant cell lines in the presence of the indicated concentrations of AKT inhibitor. Relative growth was determined by calculating the increase in the number of cells after 4 days of treatment with each doxorubicin concentration divided by the increase in the number of cells after 4 days of treatment in the presence of doxorubicin. The mean relative growth rate and corresponding standard deviation for each condition was calculated from absorbance values of 8 replicate wells. Standard deviation is indicated by error bars, while arrows indicate where the growth curve crosses the IC_{50} .



first column of the 96-well plate containing the highest concentration of inhibitor, and the twelfth column of the plate containing only media. Relative growth rate was determined as previously described. IC_{50} levels demonstrate the effect of an AKT inhibitor on T47D cells, indicating that resistant cells require AKT to control downstream mTOR signaling to continue cell growth and proliferation. With an AKT inhibitor, the signaling cascade is stopped and mTOR is not phosphorylated, causing a reduction in cell viability.

3.2.4 Proliferation of T47D cells in the presence of MEK inhibitor PD0325901

MTT assays were performed in T47D cell lines that were sensitive to doxorubicin and resistant to 10 nM doxorubicin (Figure 12). The T47D sensitive cell line has signaling cascades which are not driven by MEK as a downstream target, whereas the doxorubicin resistant cell lines are affected by the MEK inhibitor. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well in 100 μ l clear RPMI and allowed to adhere to the well for 24 hours. PD0325901, a MEK inhibitor, was added in a series of serial two-fold dilutions, with the first column of the 96-well plate containing the highest concentration of inhibitor, and the twelfth column of the plate containing only media. Relative growth rate was determined as previously described. IC_{50} levels demonstrate the effect of a MEK inhibitor on T47D cells, indicating that resistant cells require MEK to control downstream ERK signaling to continue cell growth and proliferation. With a MEK inhibitor, the signaling cascade is stopped and ERK is not phosphorylated, causing a reduction in cell viability.

Figure 12. Relative Growth of T47D in presence of MEK inhibitor PD0325901.

MTT was utilized to measure cell proliferation in T47D sensitive and 10 nM doxorubicin resistant cell lines in the presence of the indicated concentrations of MEK inhibitor. Relative growth was determined by calculating the increase in the number of cells after 4 days of treatment with each doxorubicin concentration divided by the increase in the number of cells after 4 days of treatment in the presence of doxorubicin. The mean relative growth rate and corresponding standard deviation for each condition was calculated from absorbance values of 8 replicate wells. Standard deviation is indicated by error bars, while arrows indicate where the growth curve crosses the IC_{50} .

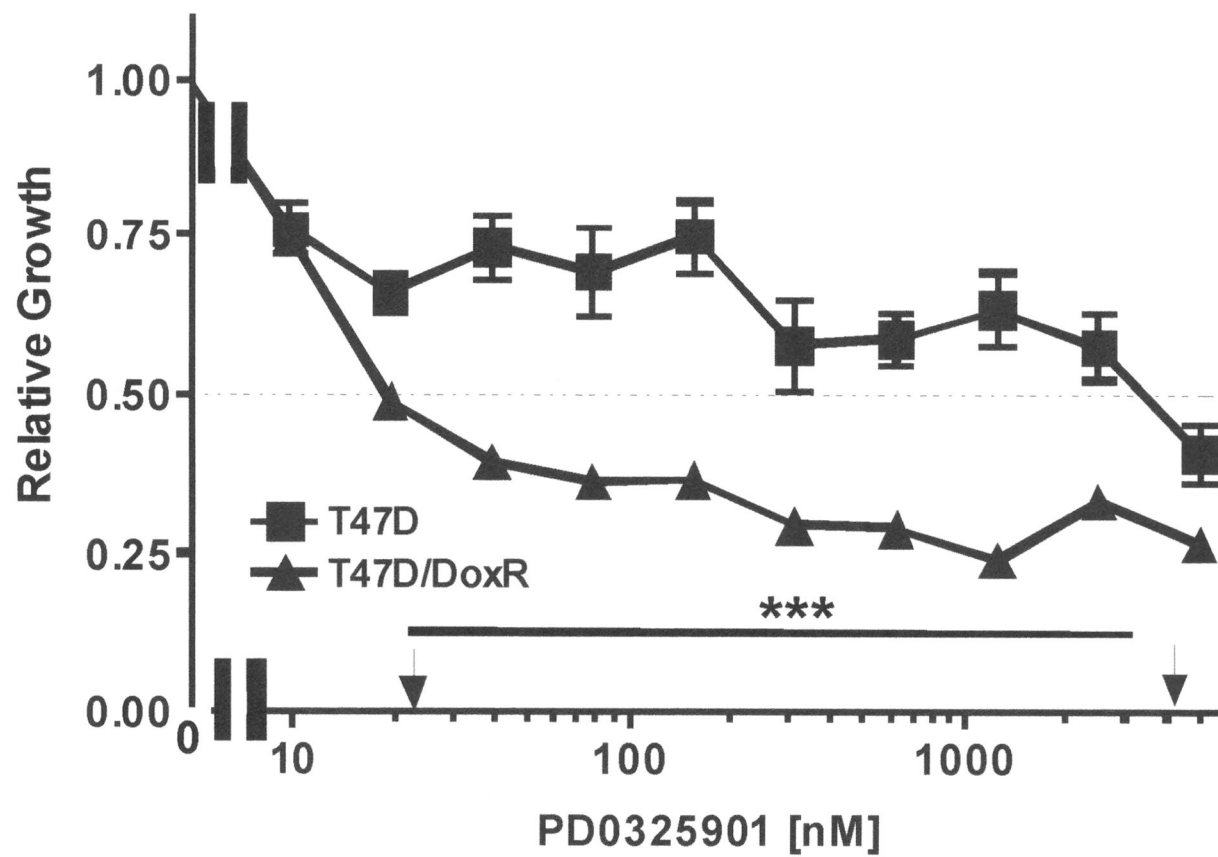
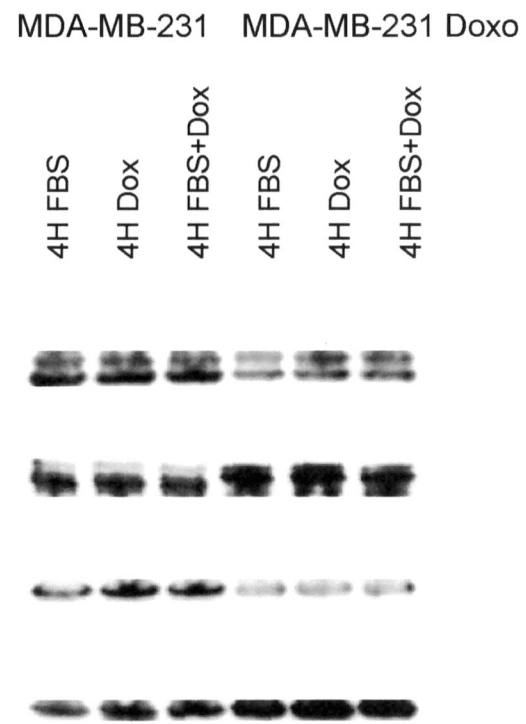
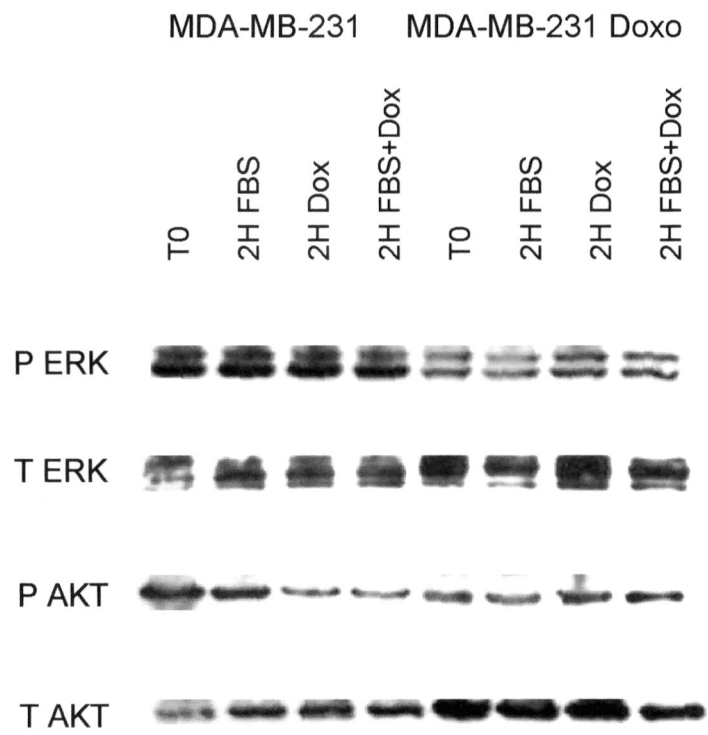


Figure 13. Western Blot Analysis of Phospho ERK, Total ERK, Phospho AKT, and Total AKT protein levels in MDA-MB-231 cells sensitive to doxorubicin and MDA-MB-231 cells resistant to 25 nM doxorubicin after treatment with FBS and doxorubicin. Cells were seeded at a density of 1×10^6 cells per plate and each cell line was cultured overnight in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours, the cells were treated with 10% FBS, 10 nM doxorubicin, and 10% FBS + 10 nM doxorubicin. Samples were harvested prior to addition of FBS and/or doxorubicin representing T_0 , and again at 2 hours and 4 hours post-treatment. Proteins harvested were subjected to western blot analysis. SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v) Phospho ERK, Total ERK, Phospho AKT, and Total AKT antibody in primary blocking buffer. Primary blocking buffer consisted of 0.05% (v/v) Tween 20 and 10mg/ml BSA in TBS. TBS consisted of 150 mM NaCl and 20 mM Tris at a pH of 7.5.



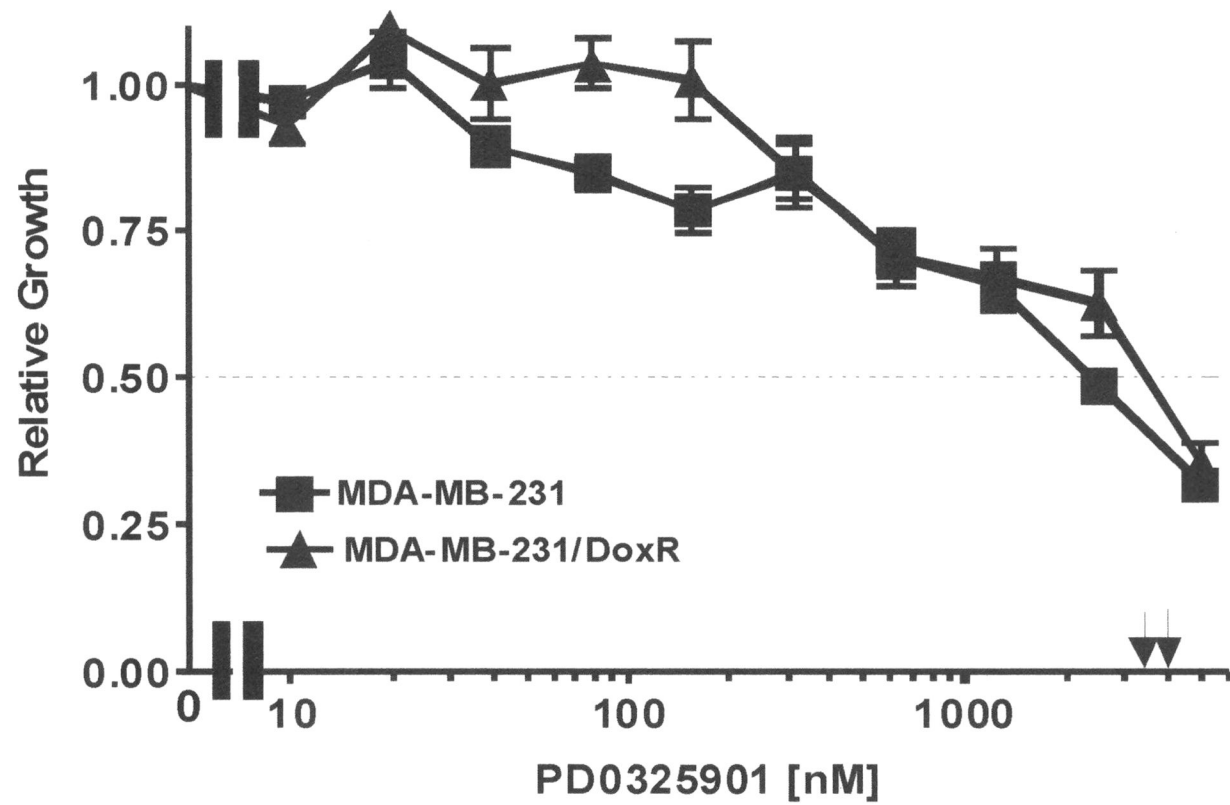
3.3 Analysis of MDA-MB-231 cells

3.3.1 Effect of FBS and Doxorubicin on ERK and AKT levels in MDA-MB-231 sensitive, 10 nM doxorubicin resistant, and 25 nM doxorubicin resistant cells

Western blot analysis was performed on MDA-MB-231 cells sensitive to doxorubicin and resistant to 10 nM doxorubicin (Figure 13). Cells were seeded at a density of 1×10^6 cells per plate and each cell line was cultured overnight in clear RPMI containing 10% FBS to allow cells to adhere. After 24 hours, the cells were treated with 10% FBS, 10 nM doxorubicin, and 10% FBS + 10 nM doxorubicin. Samples were harvested prior to addition of FBS and/or doxorubicin representing T_0 , and again at 2 hours and 4 hours post-treatment. Proteins harvested were subjected to western blot analysis. SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v) Phospho ERK, Total ERK, Phospho AKT, and Total AKT antibody in primary blocking buffer.

Figure 13 shows elevated levels in ERK in the MDA-MB-231 doxorubicin sensitive cell lines at both 2 and 4 hours. There does not appear to be a synergistic effect, however lower levels of ERK present in the doxorubicin resistant cell lines could indicate that there is an upstream mutation in the Ras/Raf/MEK/ERK pathway resulting in decreased cell viability. This, taken with the data presented in Figure 14, could confirm this upstream mutation. Elevated AKT levels are also present in the doxorubicin sensitive cell lines, indicating active signaling in the PI3K pathway. There does not appear to be a synergistic effect between FBS and doxorubicin in this cell line at 2 or 4 hours; however activation of AKT in the sensitive cell line could also indicate an

Figure 14. Relative Growth of MDA-MB-231 in presence of MEK inhibitor PD0325901. MTT was utilized to measure cell proliferation in MDA-MB-231 sensitive and 10 nM doxorubicin resistant cell lines in the presence of the indicated concentrations of MEK inhibitor. Relative growth was determined by calculating the increase in the number of cells after 4 days of treatment with each doxorubicin concentration divided by the increase in the number of cells after 4 days of treatment in the presence of doxorubicin. The mean relative growth rate and corresponding standard deviation for each condition was calculated from absorbance values of 8 replicate wells. Standard deviation is indicated by error bars, while arrows indicate where the growth curve crosses the IC_{50} .



upstream mutation in the PI3K/AKT pathway that is causing a decrease in cell proliferation.

3.3.2 Proliferation of MDA-MB-231 cells in the presence of MEK inhibitor PD0325901

MTT assays were performed in MDA-MB-231 cell lines that were sensitive to doxorubicin and resistant to 10 nM doxorubicin (Figure 14). Cells were seeded in 96-well plates at a density of 5×10^3 cells per well in 100 μ l clear RPMI and allowed to adhere to the well for 24 hours. PD0325901, a MEK inhibitor, was added in a series of serial two-fold dilutions, with the first column of the 96-well plate containing the highest concentration of inhibitor, and the twelfth column of the plate containing only media. Relative growth rate was determined as previously described. IC₅₀ levels demonstrate the effect of a MEK inhibitor on MDA-MB-231 cells, indicating that while resistant cells in other cell lines require MEK to control downstream ERK signaling to continue cell growth and proliferation, in the MDA-MB-231 cells they are able to proliferate at higher concentrations of MEK inhibitor than MDA-MB-231 resistant cells. This may suggest an upstream mutation which leads to the lower proliferation level (Ripple *et al.*, 2005). In the other cell lines a MEK inhibitor causes a cessation in the signaling cascade and ERK is not phosphorylated, resulting in a reduction in cell viability. However in this case, the doxorubicin cell lines do not exhibit this same mutation, making it appear as if the sensitive cell line is more affected by the presence of an inhibitor.

3.4 Analysis of NGAL secreted protein levels in MCF-7, T47D, and MDA-MB-231 cells sensitive to doxorubicin, resistant to 10 nM doxorubicin, and resistant to 25 nM doxorubicin

Western blot analysis was performed on MCF-7, T47D, and MDA-MB-231 cells sensitive to doxorubicin, resistant to 10 nM doxorubicin, and resistant to 25 nM doxorubicin. Cells were seeded at a concentration of 1×10^6 cells per well, while MCF-7 and T47D cell lines were cultured in red RPMI with 10% FBS, RPMI with 10% FBS + 10 nM Doxorubicin, and RPMI with 10% FBS + 25 nM doxorubicin. MDA-MB-231 cell lines were cultured in DMEM with 10% FBS, DMEM with 10% FBS + 10 nM Doxorubicin, and DMEM with 10% FBS + 25 nM doxorubicin. The cells were allowed to adhere to the well, proliferate, and secrete the NGAL protein for 72 hours. After 72 hours, the supernatant was collected from each well and subjected to NGAL protein precipitation as previously described. Proteins harvested from supernatant were subjected to western blot analysis. SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v) NGAL antibody in primary blocking buffer.

Figure 15 represents western blot analysis of MCF-7, T47D, and MDA-MB-231 cell lines sensitive to doxorubicin, resistant to 10 nM doxorubicin, and resistant to 25 nM doxorubicin when plated out in each of the aforementioned medias. NGAL protein expression can be observed in the 25 nM doxorubicin resistant cell lines, indicating that the highest levels of NGAL protein expression occur in cell lines with resistance to doxorubicin. Studies indicate that NGAL expression in human breast cancers is

associated with a poor prognosis (Devarajan, 2007), suggesting that cell lines with higher doxorubicin resistance would express higher levels of said protein. The level of protein expression is also increased in these cell lines depending on the concentration of doxorubicin in their respective media. The presence of 25 nM doxorubicin seems to increase the level of NGAL expression when compared with the 10 nM doxorubicin, and also the RPMI with no doxorubicin.

Samples were also organized according to sensitivity to doxorubicin (Figure 16). The cell lines that are sensitive to doxorubicin displayed no NGAL expression, while the cell lines with the highest resistance to doxorubicin displayed high levels of NGAL expression. The MCF-7 cell line had no NGAL expression in the presence of RPMI, suggesting that this particular cell line may require the presence of doxorubicin to stimulate NGAL production. Overall, this evidence is consistent with findings that breast cancer cells expressing NGAL correlate with other markers of poor prognosis, such as estrogen and progesterone receptor-negative status and high proliferation fraction (Stoesz *et al.*, 1998; Gruvberger *et al.*, 2001; Van de Vijver *et al.*, 2002; Van't Veer *et al.*, 2002; Wang *et al.*, 2005).

Figure 15. Western Blot Analysis of NGAL secreted protein levels in MCF-7, T47D, and MDA-MB-231 cells sensitive to doxorubicin, resistant to 10 nM doxorubicin, and resistant to 25 nM doxorubicin. Cells were seeded at a density of 1×10^6 cells per well and MCF-7 and T47D cell lines were cultured in red RPMI with 10% FBS, RPMI with 10% FBS + 10 nM Doxorubicin, and RPMI with 10% FBS + 25 nM doxorubicin. MDA-MB-231 cell lines were cultured in DMEM with 10% FBS, DMEM with 10% FBS + 10 nM Doxorubicin, and DMEM with 10% FBS + 25 nM doxorubicin. Proteins harvested from supernatant were subjected to western blot analysis. SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v) NGAL antibody in primary blocking buffer. Primary blocking buffer consisted of 0.05% (v/v) Tween 20 and 10mg/ml BSA in TBS. TBS consisted of 150 mM NaCl and 20 mM Tris at a pH of 7.5.

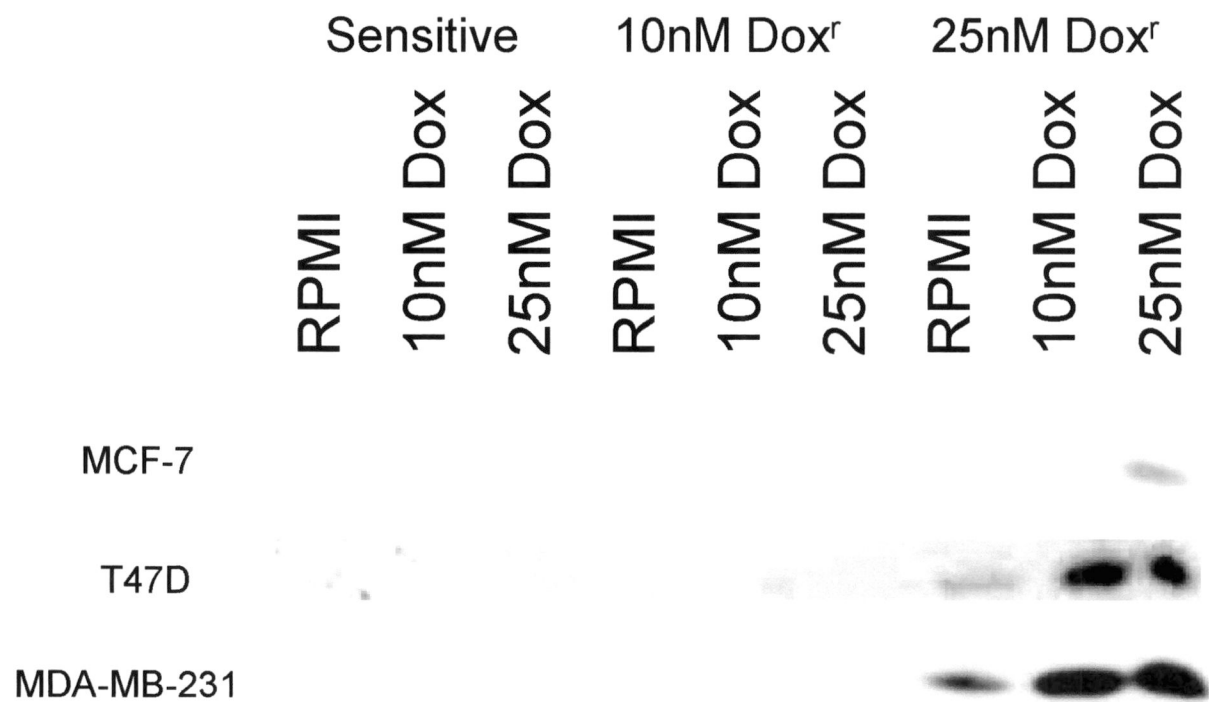
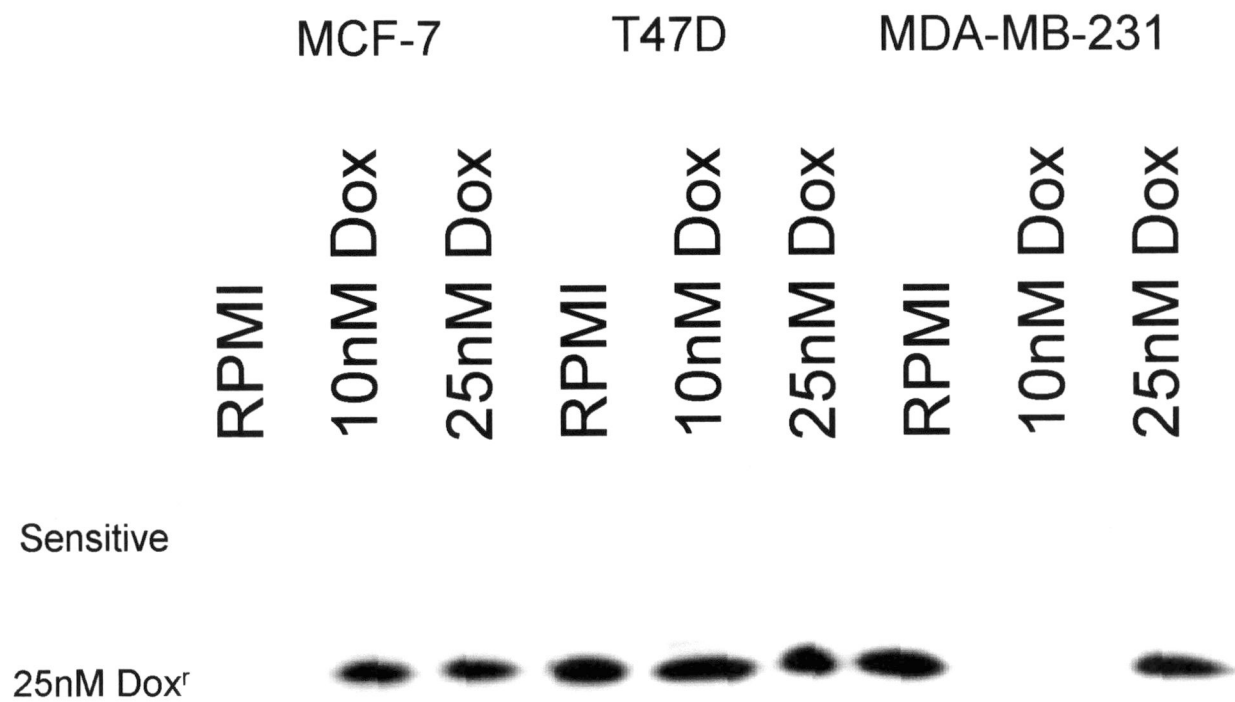


Figure 16. Western Blot Analysis of NGAL secreted protein levels in MCF-7, T47D, and MDA-MB-231 cells sensitive to doxorubicin and resistant to 25 nM doxorubicin. Cells were seeded at a density of 1×10^6 cells per well and MCF-7 and T47D cell lines were cultured in red RPMI with 10% FBS, RPMI with 10% FBS + 10 nM Doxorubicin, and RPMI with 10% FBS + 25 nM doxorubicin. MDA-MB-231 cell lines were cultured in DMEM with 10% FBS, DMEM with 10% FBS + 10 nM Doxorubicin, and DMEM with 10% FBS + 25 nM doxorubicin. Proteins harvested from supernatant were subjected to western blot analysis. SDS-PAGE gels were loaded with 10 μ l samples containing 10 μ g of protein. PVDF membranes were probed with 0.1% (v/v) NGAL antibody in primary blocking buffer. Primary blocking buffer consisted of 0.05% (v/v) Tween 20 and 10mg/ml BSA in TBS. TBS consisted of 150 *mM* NaCl and 20 *mM* Tris at a pH of 7.5.



CHAPTER 4: DISCUSSION

4.1 Analysis of MCF-7 cells

Activation of the PI3K/PTEN/AKT and the Ras/Raf/MEK/ERK signaling pathways has been identified as a major contributor of breast cancer development (Yoell-Lerner *et al.*, 2005). The activation of these pathways has also been associated with decreased sensitivity of breast cancer cells to chemotherapeutic anti-cancer drugs (Campbell *et al.*, 2001; Clark *et al.*, 2002). It was of interest to investigate the individual roles of each of these pathways and to determine if cells used both pathways as a means of survival. Activity of the PI3K/PTEN/AKT pathway was measured using PTEN mutants in MCF-7 cells expressing PTEN(WT), PTEN(C124S), PTEN(G129E), and PTEN(399stop).

4.1.1 Effect of PTEN(WT) and mutant PTEN on cellular proliferation in doxorubicin

Effects of doxorubicin and doxorubicin with 4-HT on the proliferation rates of MCF-7 cells transfected with PTEN(WT), PTEN(C124S), PTEN(G129E), and PTEN(399stop) were observed to test the effect of these mutations on sensitivity to chemotherapeutic treatment. (Figure 4). With all of the tested cell lines, the addition of 4-HT to doxorubicin had a greater effect on the proliferation rate of cells when compared with doxorubicin alone. This indicates that doxorubicin alone is not sufficient for significant reduction of cell proliferation activity in these mutants, and that in patients

where these mutants are noted, combination therapy would likely produce the most desirable results.

4.1.2 Effect of doxorubicin on MCF-7, T47D, and MDA-MB-231 cells sensitive to doxorubicin and resistant to 10 nM doxorubicin.

In these cell lines, sensitivity to doxorubicin was observed in cell lines known to be sensitive to doxorubicin as well as cell lines known to be resistant to the drug. As previously stated, doxorubicin is responsible for disrupting DNA structure by intercalating between adjacent DNA base pairs. As a result of this intercalation, synthesis of DNA and RNA is disrupted, and DNA cleavage mediated by isoforms of topoisomerase II is also induced (Goodman *et al.*, 1974, 1977; Tewey *et al.*, 1984; Capranico *et al.*, 1990). All three cell lines show decreased cellular proliferation in the presence of doxorubicin in sensitive cell lines (Figure 5), indicating that doxorubicin is an appropriate treatment for cells that fit into these categories. The importance of further tests to indicate specific characteristics of breast cancers should be employed to ensure that the most effective treatment is being utilized.

4.1.3 Expression of MEK in MCF-7 cells

Basal protein levels indicate that MEK is over-expressed in cells with resistance to 10 nM doxorubicin when compared with sensitive cells (Figure 6). This is not the case for 25 nM doxorubicin however, indicating a threshold for MEK activation in the Ras/Raf/MEK/ERK pathway. The presence of media with doxorubicin appears to increase

the level of activated MEK. When compared with an MTT cell proliferation assay (Figure 7), evidence suggests that through the use of a MEK inhibitor, doxorubicin resistant cells have a lower proliferation rate. This is due to the role of MEK in the Ras/Raf/MEK/ERK signaling cascade, where MEK is necessary to phosphorylate ERK, causing an increase in cell proliferation and survival. This pathway is not activated in cells sensitive to doxorubicin, and thus the presence of a MEK inhibitor does not have an effect.

4.1.4 Effect of FBS and doxorubicin on MCF-7 cells

Figure 8 indicates the result of FBS, doxorubicin, or a combination of FBS and doxorubicin on the activation of ERK and AKT proteins in MCF-7 cells. These results shows elevated levels of ERK in the MCF-7 10 nM doxorubicin resistant cell lines when compared with the doxorubicin sensitive cell lines, where at 4 hours, FBS and doxorubicin appear to have a synergistic effect. Since this evidence does not exist at the 2 hour time point, this may be an indication of activation threshold in doxorubicin resistant cells. The 4 hour time point allows for a longer activation period of ERK signaling, which can be explained by the 4 hour FBS and doxorubicin combination sample when compared to the FBS and doxorubicin samples alone.

AKT levels seem to be higher in the 2 hour doxorubicin sensitive cell lines, however it appears to be elevated in the resistant cell lines in the presence of doxorubicin and doxorubicin with FBS in the 4 hour time point. This could be indicated by an activation threshold, where there are higher levels of activated AKT at the 4 hour time

point in the doxorubicin resistant cell lines. It appears that there is no increase in activation of AKT in the doxorubicin sensitive cell lines at the 4 hour time point when compared with the 2 hour time point, indicating that there is no additional activation over time. Since ERK and AKT proteins exist in separate signaling cascades, it should be noted that the difference in results between the two proteins is likely.

4.2 Analysis of T47D cells

4.2.1 Western blot analysis of activated protein levels in T47D cells

Basal AKT levels in T47D cells indicate increased activation in cells with resistance to doxorubicin (Figure 9). The most profound effect can be noted in cells with a 25 nM resistance to the drug, indicating that AKT is over-expressed in these cells, thus contributing to chemotherapeutic resistance and cell survival. Results indicate that in the 25 nM resistant cell line, AKT levels were equal and type of media did not have an effect on AKT activation and expression.

Figure 10 demonstrates the effect of FBS, doxorubicin, and FBS with doxorubicin on ERK and AKT activation in sensitive and 10 nM doxorubicin resistant cell lines. Both of these proteins function in separate pathways, and both cell lines show an increased activation of these proteins in the doxorubicin resistant cell lines. The doxorubicin sensitive cell line appears to show a synergistic effect between the FBS and doxorubicin at 2 hours, while at 4 hours doxorubicin seems to upregulate the activation of ERK. In the doxorubicin resistant samples showing activated AKT, FBS and doxorubicin appear

to have a synergistic effect, while in the doxorubicin sensitive lines this effect does not appear to exist.

4.2.2 Effect on cell proliferation of T47D cells grown in the presence of inhibitors

The effect of AKT and MEK inhibitors was tested on T47D cells sensitive to doxorubicin and resistant to 10 nM doxorubicin. Figure 11 illustrates the effect of the AKT inhibitor A443654 on the cell lines. In the presence of an AKT inhibitor, doxorubicin resistant cell lines have decreased proliferation due to their dependence on the activation of AKT. AKT activates downstream mTOR which contributes cell survival, and when AKT is inhibited the signaling cascade is unable to activate mTOR.

The MEK inhibitor PD0325901 was also used to observe the effect of MEK on T47D cell proliferation (Figure 12). In the presence of a MEK inhibitor, doxorubicin resistant cell lines were also unable to proliferate at a level equal to sensitive cells that do not have activated MEK. While MEK and AKT operate in different signaling pathways (Ras/Raf/MEK/ERK and PI3K/PTEN/AKT respectively), these results confirm the importance of these proteins in their signaling cascades. Without the ability to activate downstream proteins, cells are unable to proliferate and survive unless signals from other pathways are activated.

4.3 Analysis of MDA-MB-231 cells

4.3.1 Effect of FBS and Doxorubicin on activated ERK and AKT levels

The data in Figure 13 demonstrates the effect of FBS, doxorubicin, and a combination of FBS and doxorubicin on activated levels of ERK and AKT. In this particular cell line, doxorubicin resistant cells do not express elevated levels of ERK and AKT, indicating the possibility of upstream mutations in their signaling cascades. Levels of AKT and ERK are higher in the sensitive cell lines than the resistant lines, which is variably different than the other cell lines. MDA-MB-231 cells are estrogen-receptor negative (Ripple *et al.*, 2005), and more exploratory future experiments could determine if this has an effect on AKT and ERK activation in these particular conditions.

4.3.2 Effect on cell proliferation of MDA-MB-231 cells grown in the presence of a MEK inhibitor

The MEK inhibitor PD0325901 was used to determine effect on proliferation of MDA-MB-231 sensitive and 10 nM doxorubicin resistant cell lines (Figure 14). Results indicate that the presence of a MEK inhibitor does not have the same effect on doxorubicin resistant cell lines of MCF-7 and T47D cells, indicating the possibility of an upstream mutation. Doxorubicin resistant cells should have decreased proliferation rates in the presence of a MEK inhibitor, but cross-talk between the Ras/Raf/MEK/ERK and PI3K/PTEN/AKT pathways is one explanation for the shift in data. Future experiments could look at the effect of additional inhibitors on this cell line, as well as whether estrogen-receptor status plays a role.

4.4 Effect of cell sensitivity and doxorubicin concentration on NGAL secreted protein production

NGAL production was measured in MCF-7, T47D, and MDA-MB-231 cell lines sensitive to doxorubicin, resistant to 10 nM doxorubicin, and resistant to 25 nM doxorubicin. Results in figures 15 and 16 illustrate the levels of NGAL expressed by each of the aforementioned cell lines. NGAL is associated with a poor prognosis in breast cancer, and high levels of protein expression in the doxorubicin resistant cell lines support this claim. There does not appear to be a difference in NGAL expression level in these cell lines. The level of NGAL expression over time is also unknown, and future research should indicate NGAL expression over a series of time points to determine of a threshold in protein secretion is reached.

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