

Abstract

THE ROLE OF GSK-3 β IN MCF-7 BREAST CANCER
CELL SIGNALING AND DRUG RESISTANCE

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Glycogen synthase kinase- 3 β (GSK-3 β) is well documented to participate in a complex array of critical cellular processes. This versatile protein is involved in numerous signaling pathways that influence metabolism, embryogenesis, differentiation, migration, cell cycle progression, and survival. Aberrant activity of GSK-3 β has been implicated in pathologies such as type-2 diabetes, bipolar disorder, Alzheimer's, and cancer. GSK-3 β is normally active in the cytoplasm of resting cells in an unphosphorylated state where it suppresses an assortment of transcription factors implicated in oncogenesis. Lying downstream of the PI3K/PTEN/Akt pathway, GSK-3 β can be negatively regulated through phosphorylation by active Akt. Given this pathway's role in malignant transformation, prevention of apoptosis, drug resistance, and metastasis, this study was performed to elucidate the role of GSK-3 β in MCF-7 breast cancer cells. It was found that cells expressing a kinase dead (KD) form of GSK-3 β were more resistant to doxorubicin and tamoxifen, as well as highly clonogenic compared to cells harboring wild-type (WT) or constitutively active (A9) GSK-3 β . However, when treated with rapamycin, GSK-3 β KD cells show a marked decrease in proliferation as compared to WT or A9 cells. Additionally, resistance to doxorubicin and tamoxifen were alleviated in KD cells upon co-treatment with the Array MEK inhibitor. Taken together, these results suggest that the loss of GSK-3 β activity in MCF-7 breast cancer

cells promotes clonogenicity and drug resistance, but sensitizes the cells to signaling pathway blockade. Therefore, targeting aberrant activity of the PI3K/PTEN/Akt/GSK-3 β pathway may be a clinically relevant tool for both increasing efficacy of and avoiding resistance to conventional therapy.

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CHAPTER 1: INTRODUCTION

Breast Cancer Impact

Breast cancer is the leading cause of cancer-related death in women on a global scale. This disease was diagnosed in nearly 1.4 million women worldwide in 2008, and is responsible for more than 450,000 deaths every year. The reported incidence of breast cancer has nearly tripled since 1975, and these figures are predicted to continue to rise, as 2.1 million global cases are expected to occur in 2030. Given that sporadic cancer is a disease of industrialization, it is no surprise that North American, European, and Australian women have the highest rates of breast cancer in the world. In fact, the rate of breast cancer was nearly five times higher in Western Europe than in Eastern Africa in 2008 (World Cancer Research Fund, 2010). The greatest risk factor for the onset of breast cancer is age, but factors linked to lifestyle and diet can increase carcinogen exposure and limit vital nutrient intake, thereby promoting cancer formation (National Cancer Institute, 2010).

A woman's chance of developing breast cancer at some point in her life is one in seven (McCubrey *et al*, 2008). In the United States, breast cancer will be diagnosed in 262,000 women this year according to the American Cancer Society. Almost 208,000 of these cases will be advanced, invasive breast cancers that are more challenging to effectively treat and eliminate. Due to the life-threatening nature of advanced-stage breast cancers, it has been estimated that nearly 40,000 American women will succumb to this disease during 2010 (American Cancer Society, 2010). It should be noted that breast cancer is not gender specific. In 2010, the American Cancer Society estimated that

almost 2,000 men will be diagnosed with breast cancer and nearly 400 will die from this disease. In spite of these unfortunate statistics, breast cancer incidence and mortality in the United States have been declining since the 1990s. This can be attributed to heightened awareness, early detection through routine screening, and improvements in targeted treatments (American Cancer Society, 2010).

Breast cancer occurrence is attributed to both genetic and environmental factors. It has been observed that 5-10% of breast cancers are due to hereditary mutations, namely those involving BRCA1 and BRCA2 (National Cancer Institute, 2010; McCubrey *et al*, 2008). The remaining 90% of spontaneous breast cancers are caused by environmental exposures to carcinogens, and this fact is supported by recent findings of the President's Cancer Panel (Environmental Working Group, 2010). Exposure to carcinogens from a variety of sources can induce DNA damage at a rate beyond which the body can repair. Successive accumulations of mutations that inactivate tumor suppressors and activate oncogenes are sufficient for malignant transformation and cancer progression. These molecular changes modify the activity of proteins that are intimately involved in signaling pathways that control cellular proliferation, apoptosis, differentiation, and motility. One such protein is GSK-3 β , which plays an important role in PI3K/PTEN/Akt/mTOR signaling in both noncancerous and breast cancer cells (McCubrey *et al*, 2008). Alteration of GSK-3 β function can have far reaching effects on a diverse array of cellular processes including response to anticancer therapy and the development of drug resistance (Ougolkov and Billadeau, 2006).

Conventional Breast Cancer Therapy

The most commonly utilized treatment approach for breast cancer is surgical removal of the tumor coupled with adjuvant chemotherapy or radiation (National Cancer Institute, 2010). Radiation and chemotherapy are effective in killing or limiting the growth of actively dividing cancer cells through various mechanisms including the production of oxygen free radicals, DNA damage, and subsequent apoptosis. Common chemotherapeutic drugs used to treat breast cancer include the anthracyclines, taxanes, 5-fluorouracil, cyclophosphamide, and methotrexate (McCubrey *et al*, 2008). Doxorubicin, also known as adriamycin, is in the anthracycline class of antibiotic chemotherapeutic drugs. These compounds work by intercalating between adjacent DNA pairs and inhibiting topoisomerase II, thereby interfering with DNA, RNA, and protein synthesis (National Cancer Institute Drug Dictionary, 2010).

Given the systemic nature of these drugs and their activity in all dividing cells, chemotherapy has poor specificity for cancer cells and substantial toxicity for normal, healthy cells. Therefore, conventional therapy causes numerous debilitating side effects for a breast cancer patient. Cells that normally undergo constant renewal such as those of the epidermis, gastrointestinal epithelium, and bone marrow are severely damaged leading to changes in appearance, general health, and well-being. The destruction of the immune system, which is the body's first line of natural defense against cancer, is certainly counterproductive. Although some early stage cancers can be eliminated through surgery, chemotherapy and/or radiation, these therapies often fail to successfully treat advanced stage and metastatic disease (National Cancer Institute, 2010).

In addition to chemotherapy, another common treatment option for breast cancer involves a hormonal-based approach. Certain early stage breast cancers overexpress various isoforms of the estrogen receptor (ER), making their growth dependent on the presence of estrogen. The selective ER modulator, tamoxifen (4HT), can block estrogen signaling by competitively binding the ER and antagonizing its proliferative effects. Another way to block the effects of estrogen on ER+ breast cancer cells is by limiting estrogen synthesis in the body with aromatase inhibitors (National Cancer Institute, 2010). However, once a breast cancer has undergone additional molecular changes allowing it to overcome estrogen dependence, hormonal therapy is no longer effective.

Immunotherapy for breast cancer is also clinically available. Given that the human epidermal growth factor receptor (EGFR/HER2) is sometimes amplified in breast cancer cells, it can be used as a molecular target. Trastuzumab, or Herceptin, is a humanized monoclonal antibody specific for HER2 that binds to its extracellular domain. This binding limits the function of the receptor and induces a cytotoxic immune response against the tumor cell (National Cancer Institute, 2010). Based on a breast cancer's HER2 expression, therapy with Herceptin can be a vital component of adjuvant therapy by targeting and limiting the growth of HER2+ cells. However, like with hormonal therapy, breast cancers can eventually alter their expression of the targeted receptor, thereby bypassing the effects of immunotherapy.

Breast Cancer Drug Resistance

While conventional treatment approaches with radiation, chemotherapy, hormonal therapy, and immunotherapy are effective in limiting cancer growth for a period of time,

they often fail to elicit a lasting response. Even after a seemingly complete remission in response to therapy, less than 20% of patients with metastatic breast cancer will remain disease-free for more than five years. Many factors can influence how a cancer will respond to therapy, and cancers that initially respond can develop drug resistance, thereby leading to a poor prognosis and death. Drug resistance is a major clinical obstacle and many patients must endure several rounds of chemotherapy only to learn that their cancer has found a way to adapt to and overcome the intended effect of the drug (McCubrey *et al*, 2008).

Breast cancers can carry inherent drug resistance or develop an acquired resistance after exposure to the compound. Resistance is commonly developed in patients on anthracycline chemotherapy drugs, such as doxorubicin, daunorubicin, and epirubicin. Resistance can also occur in patients receiving hormonal therapy and immunotherapy once the cancer is no longer dependent on the targeted receptor to grow. Therefore, cancer cells are capable of undergoing calculated changes that confer survival advantages in otherwise nutrient-restricted or toxic environments (McCubrey *et al*, 2008).

These molecular changes involve multiple approaches such as pumping the compound out of the cell, modifying or detoxifying the drug, or turning on survival signaling pathways that prevent drug-induced apoptosis (McCubrey *et al*, 2008). ATP-binding cassette transporters such as multidrug resistance protein (MRP1) and the MDR1 product, P-glycoprotein (Pgp), actively expel chemotherapeutic drugs from the cell. These drug pumps have been detected in a large number of untreated breast cancers, and their expression increases upon chemotherapy exposure (Leonessa and Clarke, 2003).

Activation of cell survival pathways such as PI3K/Akt can prevent apoptosis in the presence of a drug by altering mitochondrial bioenergetics and inhibiting the release of cytochrome c (Aoudjit et al, 2009).

Therefore, breast cancer drug resistance is multifactorial and highly dependent on the molecular characteristics of the transformed cells. Instead of the trial and error approach to prescribing chemotherapy drugs, an oncologist can take cues from the unique genetic makeup of a patient's cancer when designing a course of therapy. Examining the expression of signaling molecules involved in promoting cell survival during chemotherapy can provide molecular markers for gauging treatment efficacy (McCubrey *et al*, 2008).

The Role of the PI3K/PTEN/Akt Signaling Pathway in Breast Cancer

The effects of hormones, growth factors, and cytokines on normal and malignant cells are orchestrated by complex interactions between signaling cascades. When an extracellular or intracellular receptor binds its specific ligand, the ligand's intended message is carried to the nucleus through protein kinase activity. It has been established that the PI3K/PTEN/Akt/mTOR pathway, shown in Figure 1, plays a critical role in normal cellular proliferation, survival, and apoptosis. Altered expression of this pathway is also implicated in carcinogenesis, and mutations leading to its activation have been identified to play a key role in breast cancer. Amplification of the growth factor receptor EGFR/HER2 is present in approximately 30% of breast cancers, leading to activation of PI3K/PTEN/Akt/mTOR. Additional growth factors that activate this pathway include

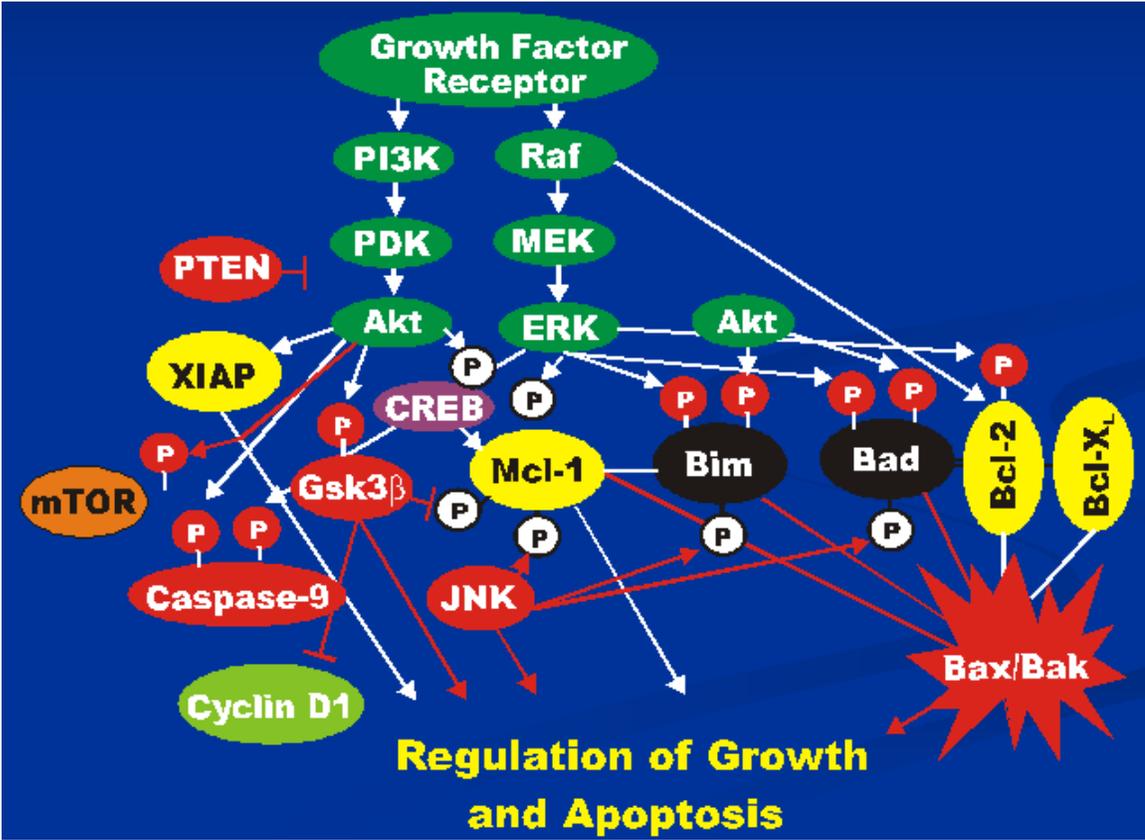


Figure 1: PI3K/Akt/mTOR and Raf/MEK/ERK Signaling

The effects of various growth factors on cellular growth and apoptosis are mediated through PI3K/Akt/mTOR and Raf/MEK/ERK in normal and malignant cells. These pathways are commonly mutated in breast cancer, leading to uncontrolled proliferation, prevention of apoptosis, drug resistance, and metastasis.

fibroblast growth factor (FGF), insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF) (McCubrey *et al*, 2008).

Growth factor/cytokine binding of its respective receptor induces a conformational change within the receptor creating a binding site for phosphatidylinositol 3-kinase (PI3K). PI3K contains an 85-kDa regulatory subunit responsible for binding and a 110-kDa catalytic region. The SH2 domain of the p85 subunit associates with the receptor, undergoes phosphorylation, and subsequently activates the p110 catalytic subunit. Alternatively, growth factor receptor ligation can result in PI3K activation from mutations in other signaling molecules such as the GTPase, Ras, which also activates the Raf/MEK/ERK pathway. Mutations in the PI3K gene itself can also lead to constitutive activation regardless of growth factor stimulation, and have been reported to be present in approximately 25% of breast cancers (McCubrey *et al*, 2008).

Regardless of the mode of PI3K activation, once the catalytic p110 subunit is recruited to the membrane, it begins phosphorylating its lipid substrate. Phosphatidylinositol 4,5 bisphosphate [PtdIns(4,5)P₂] is converted to phosphatidylinositol 3,4,5 trisphosphate [PtdIns(3,4,5)P₃], the latter of which attracts proteins containing pleckstrin-homology domains to the membrane. Two such proteins, Akt and phosphoinositide-dependent protein kinase (PDK1), anchor themselves to PtdIns(3,4,5)P₃, and Akt is subsequently phosphorylated by PDK1. This site of Akt phosphorylation (T 308/309/305) is dependent on its isoform (Akt-1, Akt-2, or Akt-3), which also determines the regulatory residue (S 473/474/472) to be phosphorylated by a second unknown kinase or through autophosphorylation (McCubrey *et al*, 2008).

Akt, also known as protein kinase B (PKB), is a major player in cell signaling and has been postulated to phosphorylate over 9,000 proteins. Over 50% of breast cancers display elevated levels of Akt (McCubrey *et al*, 2008). Akt activation leads to far reaching downstream cellular effects making it a pivotal regulatory switch for controlling proliferation, survival, migration, and response to a wide array of cancer therapies. Once PI3K/Akt signaling is initiated, this cascade of phosphorylations continues through other downstream signaling molecules such as mTOR, eIF4E, p70S6K, STAT3, GSK-3 β , eIF2B, NF- κ B, Bad, CREB and p21, resulting in their activation or deactivation in some cases (McCubrey *et al*, 2008).

Akt is negatively regulated by phosphatase and tensin homologue deleted on chromosome 10 (PTEN). PTEN is primarily a lipid phosphatase, but can also function as a protein phosphatase. PTEN prevents Akt activation by removing the 3-phosphate from PtdIns(3,4,5)P₃, the lipid product of PI3K. The disruption of PTEN's vital tumor suppressor activity is relatively common in breast cancer. PTEN mutations have been reported to occur in varying frequencies (5 – 21%), with loss of heterozygosity more common as it occurs in 30% of breast cancer cases. In one study, 26% of primary breast cancers had low PTEN levels, which correlated with lymph node metastases and poor prognosis (McCubrey *et al*, 2008). Therefore, loss of PTEN function within cancer cells promotes tumorigenicity and drug resistance by allowing for increased levels of active Akt.

One pivotal downstream signaling molecule regulated by Akt is mammalian target of rapamycin (mTOR), a 289-kDa serine/threonine kinase. By influencing protein

synthesis, mTOR serves a crucial role in promoting cell cycle entry only when nutrients and energy are available. Activated mTOR phosphorylates eukaryotic initiation factor (eIF)-4E binding protein (4EBP-1), which leads to the release of eIF4E and assembly of the translational initiation complex. Phosphorylation of p70S6K is also carried out by mTOR, as well as PDK-1. P70S6K initiates active translation by activating the 40S ribosomal protein, S6. Therefore, mTOR activity can lead to increased production of molecules such as c-Myc, cyclin D1, and p27. Hypoxia-inducible transcription factor-1 α (HIF-1 α) is also upregulated by mTOR and is involved in promoting angiogenesis and glucose uptake of cancer cells (McCubrey *et al*, 2008).

Phosphorylation by Akt also inhibits the activity of many signaling molecules. Activated Akt can promote carcinogenesis by inactivating proteins that normally function to limit cell growth. One such protein is GSK-3 β which has been shown to be downregulated in breast cancer cells in the presence of activated Akt (McCubrey *et al*, 2008). A study of patient tumor samples revealed a reduction or loss of cytoplasmic GSK-3 β in 53% of invasive ductal carcinomas (IDC) examined. This same study found that nuclear accumulation of GSK-3 β was present in 35% of IDC samples examined and was positively associated with tumor grade (Prasad *et al*, 2009). Therefore, increases in PI3K/Akt signaling and altered cellular localization of GSK-3 β may be among the regulatory factors that impinge upon the behavior of breast cancer cells.

Crosstalk with Raf/MEK/ERK

There is also interaction and crosstalk between the PI3K/PTEN/Akt pathway and the Raf/MEK/ERK pathway, further demonstrating the far reaching cellular effects of

Akt activation. GSK-3 β can be inactivated through phosphorylated p70S6K, which is downstream of both Akt and ERK. Collectively, these arrays of signals can lead to protein synthesis, cell cycle regulation, and anti-apoptotic gene expression in cancer cells thus allowing them to survive toxic therapies (McCubrey *et al*, 2008). Therefore, regulation of GSK-3 β can be influenced by complex interactions between multiple cell signaling pathways.

Targeting Aberrant Pathways with Small Molecule Inhibitors

Given the vital role of the PI3K/PTEN/Akt/ mTOR pathway in breast cancer, small molecule inhibitors (SMIs) that specifically target these proteins have been developed and tested for their efficacy as targeted treatments. SMIs such as Imatinib have proven effective in the treatment of CML and other cancers which proliferate in response to BCR-ABL, PDGFR, and c-Kit. Lung carcinomas which have mutations in EGFR are sensitive to EGFR inhibitors. PI3K, PDK, Akt, mTOR, Raf, MEK, and CaMK SMIs have been developed and some are in clinical trials. mTOR inhibitors have been used for many years as immunosuppressive drugs in kidney transplant patients (McCubrey *et al*, 2008). Therefore, SMIs of the PI3K/PTEN/Akt/mTOR pathway may serve as the basis for a targeted therapy that can be tailored to address the unique genetic alterations present in a breast cancer patient's tumor.

CHAPTER 2: STRUCTURE, FUNCTION, AND ROLES OF GSK-3 β

GSK-3 β is a 47-kDa serine/threonine (S/T) protein kinase found in all eukaryotes to be widely expressed in various organs with particularly high levels in the brain. It was originally discovered and named for its role in glycogen synthesis. This protein has since been shown to play a diverse array of cellular roles as a regulatory switch involved in many signaling pathways including PI3K/Akt, Raf/MEK/ERK, Wnt/ β -catenin, and Hedgehog. This allows GSK-3 β to regulate metabolism, protein synthesis, motility, and apoptosis in many different cell types under various conditions. Abnormal expression of GSK-3 β has been implicated in diseases such as bipolar disorder, Alzheimer's, diabetes, and cancer (Doble and Woodgett, 2003; Forde and Dale, 2007; Ougolkov and Billadeau, 2006).

GSK-3 β Structure

The human GSK-3 β gene contains 12 exons that code for the 7134 bp mRNA transcript shown in Figure 2. Also shown is the resulting protein, which is made up of 433 amino acids and consists of a kinase domain, including the substrate binding domains, flanked by an N-terminal and C-terminal domain (Atlas of Genetics and Cytogenetics in Oncology and Haematology, 2010). Unlike most kinases, GSK-3 β can undergo differential splicing in its kinase domain. A 13-amino acid insertion at residue 369 creates the GSK-3 β 2 variant, the activity of which is poorly defined (Forde and Dale, 2007).

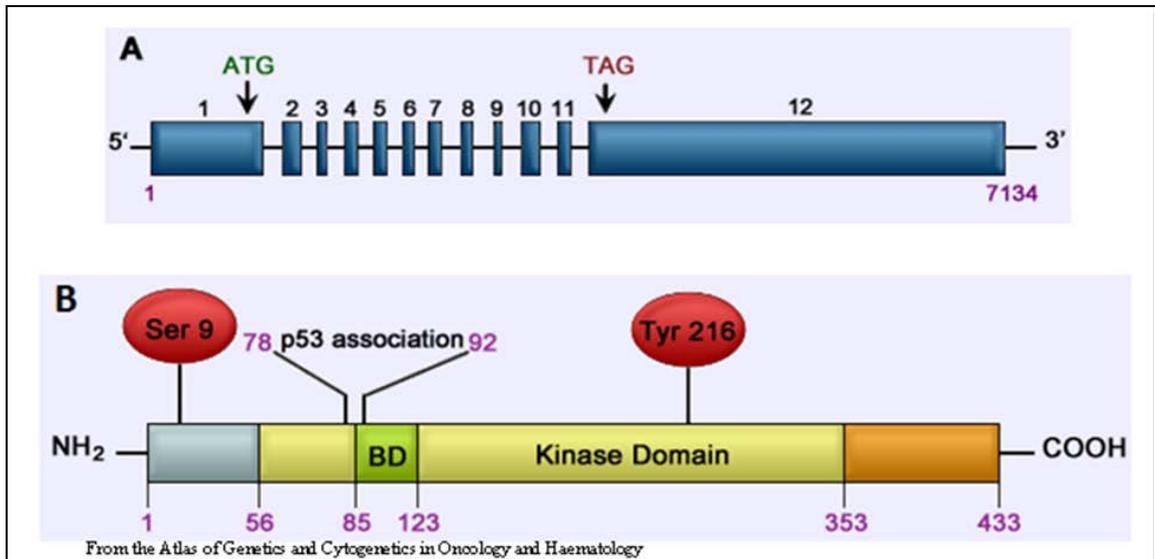


Figure 2: GSK-3 β mRNA and Protein Structure

A: GSK-3 β mRNA consists of 7134 base pairs that code for twelve exons.

B: The resulting GSK-3 β protein is 433 amino acids and contains a kinase domain flanked by an N-terminal and C-terminal region. Within the kinase domain, a basic domain is responsible for binding GSK-3 β substrates, such as p53.

GSK-3 β activity can be inhibited through phosphorylation at serine 9 (S9). This prevents substrate phosphorylation by occupying the GSK-3 β binding pocket and active site. Additionally, GSK-3 β can undergo T-loop phosphorylation at tyrosine 216 (Y216) which may serve to achieve maximum catalytic activity, but does not appear to be a prerequisite for kinase activity (Doble and Woodgett, 2003; Kockeritz et al, 2006). The other mammalian GSK isoform, GSK-3 α , is nearly identical within its kinase domain, but contains an additional N-terminal glycine-rich region, making it 51 kD (Doble and Woodgett, 2003; Forde and Dale, 2007; Kockeritz et al, 2006). The two isoforms differ widely in their C-terminal domains, with just 36% identity, and even though they share substrates, they are not functionally identical (Doble and Woodgett, 2003).

GSK-3 β Kinase Function

GSK-3 β shows specificity for substrates that have been primed, or previously phosphorylated by another kinase. As shown in Figure 3, the substrate binding region of GSK-3 β recognizes the consensus sequence S/T-X-X-X-Phospho-S/T, in which the last S/T is primed, and the first S/T is the residue targeted for phosphorylation. The crystal structure of GSK-3 β reveals that a region of positive charge created by the residues arginine 96 (R96), arginine (R180), and lysine 205 (K205) interacts with the primed S/T substrate residue in order for optimal alignment of the substrate and kinase domain to occur (Doble and Woodgett, 2003; Forde and Dale, 2007). Phosphorylation of GSK-3 β substrates can be selectively regulated through differential protein-binding domains present on GSK-3 β (Eom and Jope, 2009).

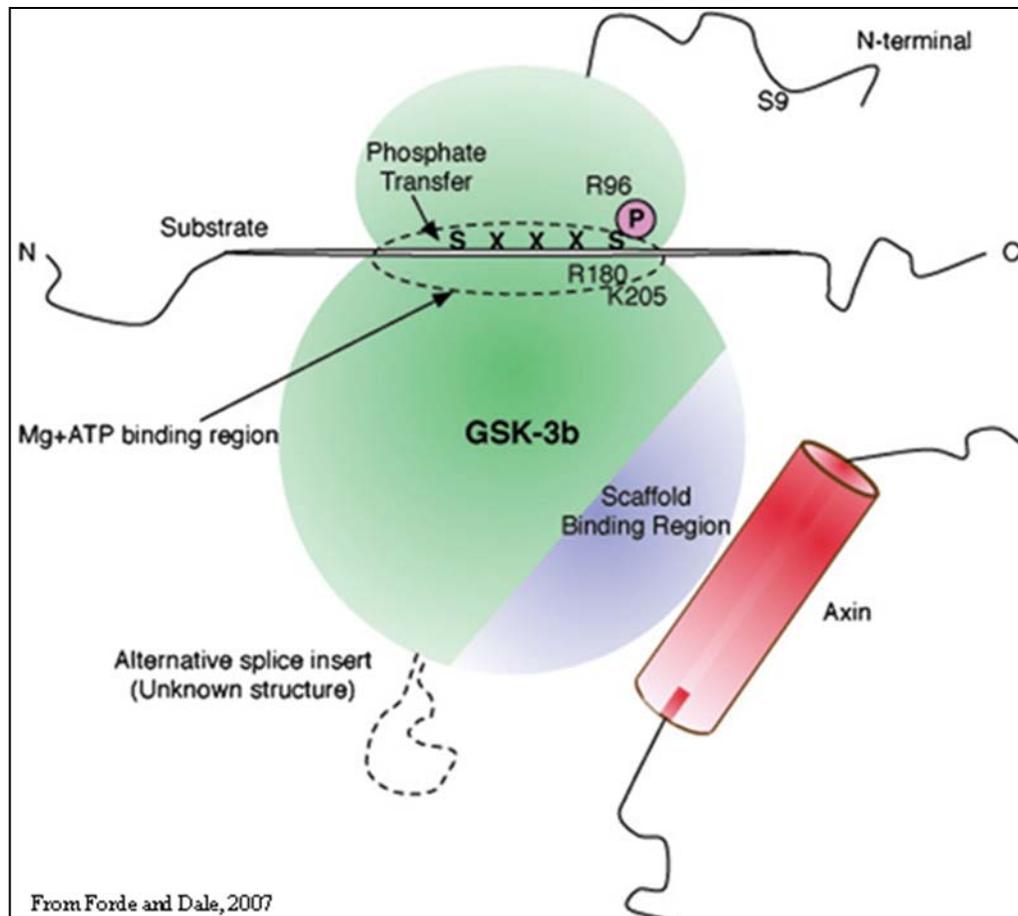


Figure 3: Interaction of GSK-3 β and its Substrates

GSK-3 β recognizes the consensus sequence S/T-X-X-X-Phospho-S/T on its substrates and binds them within its kinase domain at an area of positive charge created by the residues R96, R180, and K205. A phosphate is then transferred from ATP to the substrate. The location of an alternative splice insert and the scaffold binding region are also shown. GSK-3 β kinase activity is inhibited by phosphorylation at S9.

GSK-3 β is unlike most protein kinases in that it is normally constitutively active in resting cells, limiting them from undergoing the transcription, translation, and metabolic processes required to proliferate. Over 40 different proteins have been found to be phosphorylated by GSK-3 β (Eom and Jope, 2009). Reported GSK-3 β substrates include the transcription factors c-Myc, c-Jun, HSF-1, Notch, and β -catenin, the microtubule-associated protein Tau, the translation factor eIF2B, the cell cycle regulators cyclin D1 and cyclin E, and glycogen synthase. GSK-3 β regulates these and other oncogenic substrates by inhibiting their function and/or targeting them for degradation (Kockeritz *et al*, 2006, Nemoto *et al*, 2008). GSK-3 β can also upregulate activity of the tumor suppressor p53 by binding and acetylating it, thus promoting p53-induced apoptosis (Eom and Jope, 2009).

Regulation of GSK-3 β Activity

As mentioned, the kinase activity of GSK-3 β can be regulated through phosphorylation of S9. A number of protein kinases involved in many signaling pathways can perform this task in response to growth and survival promoting stimuli. When exposed to IGF, cells express elevated levels of Akt which leads to the inhibitory phosphorylation of GSK-3 β and the subsequent activation of glycogen synthase. Additional kinases that phosphorylate and inactivate GSK-3 β include PKC, p90^{RSK} in response to EGF or PDGF, PKA in response to elevated cAMP, and p70S6K in response to increased insulin and amino acid availability (Forde and Dale, 2007).

GSK-3 β can also be regulated through sequestration within cytosolic protein complexes. In unstimulated cells, GSK-3 β participates in the β -catenin destruction

complex in which it phosphorylates β -catenin, thereby targeting it for proteosomal degradation (Gosens *et al*, 2007; Yoeli-Lerner *et al*, 2009). In the presence of a molecule belonging to the Wnt family, GSK-3 β disassociates from the protein complex and β -catenin is instead free to translocate to the nucleus and serve as a transcriptional activator (Doble and Woodgett, 2003; Katoh and Katoh, 2006).

Like many molecules, GSK-3 β activity is also regulated by its localization within the cell. GSK-3 β is normally active in the cytoplasm of resting cells, but is rapidly translocated to the nucleus during cellular stress (Meares and Jope, 2007). Activity of nuclear GSK-3 β is also elevated as compared to cytosolic GSK-3 β (Li *et al*, 2008). Therefore, multiple modes of GSK-3 β regulation exist, many of which are implicated in tumorigenesis.

Role of GSK-3 β in the Brain

It should be noted that GSK-3 β is highly expressed in brain tissue where it performs numerous roles in cognitive functioning. Mood disorders such as depression and bipolar disorder, as well as Alzheimer's and Parkinson's disease, exhibit altered GSK-3 β levels. Various neuropsychiatric drugs, including lithium chloride, used to treat these conditions inhibit GSK-3 β activity, leading to a subsequent increase in β -catenin (Wada, 2009). Treatment with lithium was also shown to block ER α -mediated transcription in neuronal cells (Mendez and Garcia-Segura, 2006). GSK-3 β plays a role in neurological diseases by promoting neuronal apoptosis induced by oxidative stress (Nair and Olanow, 2007). In addition to neuronal cell apoptosis, endothelial, hepatocyte, fibroblast, and astrocyte apoptosis has been shown to be induced by overexpression of

GSK-3 β (Tan *et al*, 2006). Therefore, inhibition of GSK-3 β activity in the brain can have a mood stabilizing and neuroprotective effect. However, it is possible that interfering with GSK-3 β activity could also lead to cellular transformation, which should be a consideration when targeting GSK-3 β to treat neurological disorders.

Role of GSK-3 β in Cancer

Unlike PI3K and Akt, GSK-3 β has not been found to be commonly mutated in cancer (Wei *et al*, 2005). The Catalog of Somatic Mutations in Cancer (COSMIC) lists only two GSK-3 β mutations found out of 447 unique cancer samples from fifteen different primary tissues. Both mutations were detected in lung cancer, one involving a substitution at residue 25 and the other at residue 361, neither of which correspond to the GSK-3 β kinase domain (Sanger Institute, 2011). As mentioned, GSK-3 β can be alternatively spliced in its kinase domain, which may change its activity towards substrates or localization within the cell (Forde and Dale, 2007).

GSK-3 β regulation in cancer appears to be tissue specific in some respects, as certain forms of cancer have been shown to express high levels of active GSK-3 β while others harbor low levels. It was shown that high levels of nuclear GSK-3 β in pancreatic cancer cell lines was associated with dedifferentiation and NF- κ B mediated survival (Ougolkov *et al*, 2006). However, another study found that loss of GSK-3 β activity in pancreatic cancer cells resulted in resistance to radiotherapy due to the stabilization of β -catenin (Watson *et al*, 2010). GSK-3 β activity also appears to be important for leukemic cell growth, as inhibition of GSK-3 β led to an induction of apoptosis in leukemic cells (Holmes *et al*, 2008). However, it was found that GSK-3 β plays a pro-apoptotic role in

lung cancer by modulating survivin activity (Li *et al*, 2008). In gastric cancer cells, GSK-3 β inhibition promoted migration through activation of β -catenin (Mishra *et al*, 2010). GSK-3 β activity was also shown to be downregulated in cutaneous squamous cell and basal cell carcinomas, demonstrating that it plays a role in epidermal cell transformation (Ma *et al*, 2007).

In contrast to some forms of cancer, GSK-3 β activity levels in breast cancer have generally been reported to be low. A study of patient tumor samples revealed a reduction or loss of cytoplasmic GSK-3 β in 53% of invasive ductal carcinomas (IDCs) examined. This was associated with an increase in β -catenin activity and a decrease in expression of E-cadherin (Prasad *et al*, 2009). GSK-3 β and β -catenin also have been shown to regulate cadherin-11 in breast cancer cells, as inactivation of GSK-3 β leads to repression of cadherin-11 mRNA and protein levels (Farina *et al*, 2009). The results from these studies suggest that loss of cytoplasmic GSK-3 β can promote epithelial mesenchymal transition (EMT) in breast cancer.

Another study of clinical breast cancer specimens found that inactivation of GSK-3 β was associated with elevated levels of the prolactin receptor, which is implicated in tumorigenesis (Plotnikov *et al*, 2008). Expression of the breast cancer resistance protein (BCRP) was found to be downregulated in breast cancer cells when GSK-3 β was active, suggesting that GSK-3 β can prevent active drug efflux (Hartz *et al*, 2010). Inactivation of GSK-3 β by Akt was found to enrich for mammary stem cells in both normal and breast cancer cells through activation of β -catenin (Korkaya *et al*, 2009). Therefore,

GSK-3 β activity appears to limit proliferation and suppress the stem-like cell population in breast cancer.

The growth-limiting actions of GSK-3 β suggest that this kinase plays a role in tumor suppression, however, this does not appear to always be the case. GSK-3 β may actually participate in pro-inflammatory and anti-apoptotic processes by positively regulating NF- κ B in the nucleus, as demonstrated in pancreatic cancer (Ougolkov *et al*, 2006; Ougolkov and Billadeau, 2006). Therefore, the role of GSK-3 β in cancer progression remains to be controversial and extremely complex and may vary greatly among different cancer types. The cellular localization of GSK-3 β may be an important factor in switching between its growth-limiting and survival-promoting activities. It is likely that aberrant nuclear accumulation of GSK-3 β may be a hallmark of many cancers.

CHAPTER 3: METHODS AND RESEARCH AIMS

Materials and Methods

Cell Culture

MCF-7 cells were derived from a human breast adenocarcinoma (Soule *et al.*, 1973). MCF-7 cells have an epithelial morphology, are adherent, and form a monolayer in culture. These cells express ER α , ER β , and WT GSK-3 β (McCubrey *et al.*, 2008). MCF-7 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Cell culture medium for MCF-7 cells consisted of 10% (v/v) heat inactivated fetal bovine serum (FBS) (CellGrow-Mediatech, Herndon, VA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 100 μ g/ml streptomycin (Invitrogen), and 100 units/L penicillin G (Invitrogen) in Roswell Park Memorial Institute-1640 (RPMI 1640) medium (Invitrogen).

GSK-3 β Plasmids

A plasmid encoding human wild-type (WT) GSK-3 β [GSK-3 β (WT)] was generated by inserting the corresponding cDNA into the multiple cloning site of pEGFP-C2. Mutated GSK-3 β cDNAs were inserted into the multiple cloning site of pEGFP-C2 to generate plasmids encoding various GSK-3 β mutants. These GSK-3 β mutants include GSK-3 β (A9) and GSK-3 β kinase dead (KD). GSK-3 β (A9) differs from WT in that an alanine has been substituted for serine at residue 9, thereby rendering it constitutively active. GSK-3 β KD differs from WT through a substitution of methionine and alanine for lysine at positions 85 and 86, respectively. The suspected effects of these mutants on cell cycle and growth are shown in Figure 4.

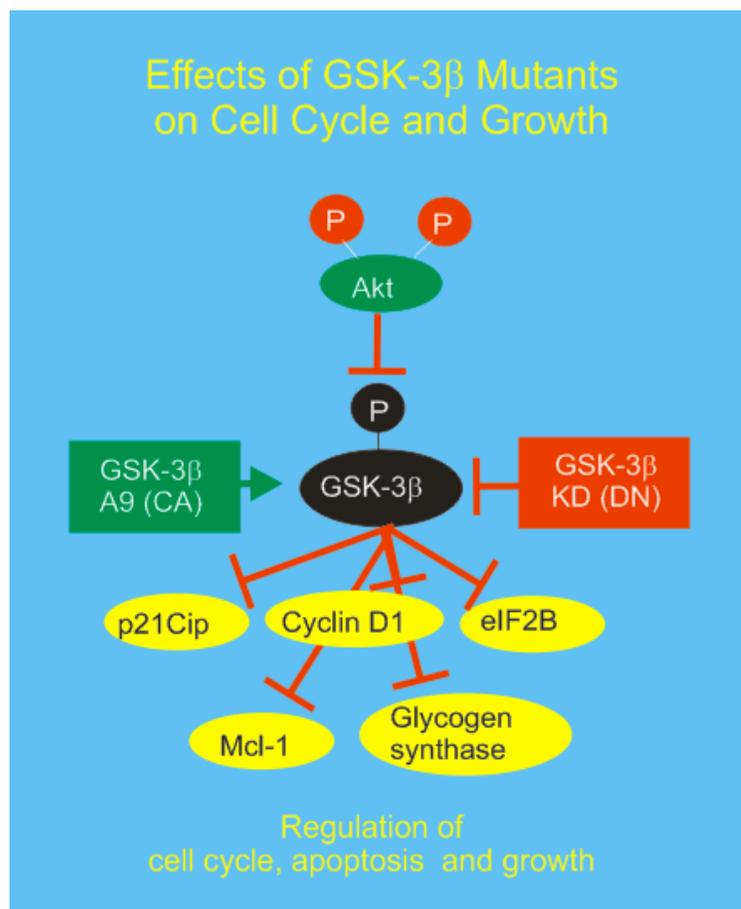


Figure 4: GSK-3 β Mutants

When active, GSK-3 β regulates cell cycle progression, apoptosis, and growth by downregulating the expression of p21CIP, cyclin D1, eIF2B, Mcl-1, and glycogen synthase. The mutants used in this study code for wild-type, constitutively active, or kinase dead GSK-3 β . The constitutively active mutant contains a substitution of alanine for serine at residue 9. The kinase dead mutant contains substitutions of methionine and alanine for lysine at positions 85 and 86, respectively.

Transfection of MCF-7 Cells with GSK-3 β Constructs

5×10^5 MCF-7 cells were plated into 6-well cell culture plates (BD Biosciences, Mountainview, CA) one day prior to transfection. Cells were rinsed with Opti-MEM medium (Invitrogen) to remove FBS. Cells were transfected with 10 μ g of DNA with Lipofectin (Invitrogen) as described by the manufacturer. 48 hours after transfection, selection medium (RPMI + 10% FBS + 2 mg/ml G418 (Geneticin)) (Invitrogen) was added to isolate stably transfected cells. Cells were provided with fresh selection medium every three days. Mock transfections were performed by replacing DNA with 10 μ l of Tris-EDTA buffer and did not generate viable colonies in the presence of selection medium.

Cell Proliferation in the Presence of Drugs and Inhibitors (MTT Analysis)

MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells were seeded in 96-well cell culture plate (BD Biosciences) at a density of 5,000 cells/well in 100 μ l of phenol red free RPMI-1640 containing 5% charcoal stripped FBS. Cell culture plates were incubated for one day to permit cells to adhere to the bottom of each well. Treatment medium was prepared by performing ten two-fold serial dilutions to create a range of eleven concentrations of doxorubicin (1.95-2,000 nM) (Sigma; St. Louis, MO), tamoxifen (0.98-1,000 nM) (Sigma), rapamycin (mTOR Inhibitor) (0.098-100 nM) (EMD Biosciences; Gibbstown, NJ), and Array MEK inhibitor (9.77-10,000 nM) (EMD Biosciences). Combination treatments consisting of doxorubicin or tamoxifen coupled with rapamycin or Array MEK were created by adding 1 nM of rapamycin or 1,000 nM of Array MEK to the serially diluted concentrations of doxorubicin or tamoxifen

described above. 100 μ l of treatment medium was added to each treatment well the day after cells were initially seeded, while the last well received non-treatment medium as a control. After 72 hours of treatment (four days after seeding), the amount of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma) reduction in each well was quantified by dissolving the formazan crystals in 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). Control plates were read on day one and day four after seeding to provide a base line for cell growth. The mean and corresponding standard deviation of normalized adjusted absorbance was calculated from eight replicate wells for each drug concentration. The inhibitory concentration of 50% (IC_{50}) is defined in this context as the concentration of the drug that causes MCF-7 cells to proliferate at a rate that is half as rapid as cells incubated in the absence of the drug.

Colony Formation Assays

MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells were collected and seeded in 6-well cell culture plates at a density of 1000 cells/well (three replicate wells for each condition). Cell culture plates were incubated for one day to permit cells to adhere to the bottom of each well. 24 hours after seeding, plates were then treated with 25 nM doxorubicin-containing media or media without the drug and incubated for three weeks at 37 $^{\circ}$ C. Cells were provided with fresh treatment-containing media every four days. Cells were rinsed with PBS at the end of the three week treatment period. Rinsed cells were then fixed in 100% (v/v) methanol (Sigma) for ten minutes at room temperature then dried for ten minutes at room temperature. Fixed cells were

incubated in Giemsa stain (Sigma) for five minutes at room temperature. Stained cells were rinsed with water then dried. Colonies consisted of at least 50 cells and the number of colonies present in each well was counted. The mean number of colonies and corresponding standard deviation was calculated from three replicate wells for each condition. Statistical significance was calculated using the GraphPad QuickCalcs software using an unpaired t test with a 95% confidence interval. Relationships that were extremely statistically significant had a P value less than or equal to 0.0005; those that were very statistically significant had a P value of less than or equal to 0.005; those that were statistically significant had a P value of less than or equal to 0.05.

Clonogenicity in Soft Agar

1.6% LMP agarose was prepared in deionized water, heated, and cooled. An equal part of agarose was mixed with 2X complete Dulbecco's Modified Eagle Medium (cDMEM). 2.5 ml of this solution was plated per well in 6 well plates (BD Biosciences) and allowed to solidify at room temperature for at least 30 minutes. This formed the bottom layer of soft agar. The top agar layer was formed by preparing 0.8% LMP agarose in deionized water. MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells were harvested, dissociated, and resuspended at 1.1×10^4 cells/ml in 2X cDMEM. Equal parts of 0.8% agarose and cell-containing media were mixed and 3.5 ml of this solution was carefully applied over the bottom layer of agarose. Once solidified at room temperature, plates were incubated at 37 °C for three weeks with an application of 0.1 ml of fresh media occurring every four days. At the end of three weeks, the agarose was stained with crystal violet (Sigma) for one hour. Colonies were

counted using a dissecting microscope and the mean and standard deviation was calculated based on three replicate wells for each condition. Statistical significance was calculated using the GraphPad QuickCalcs software using an unpaired t test with a 95% confidence interval. Relationships that were extremely statistically significant had a P value less than or equal to 0.0005; those that were very statistically significant had a P value of less than or equal to 0.005; those that were statistically significant had a P value of less than or equal to 0.05.

Protein Lysates

MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells were collected after trypsination and plated in 100 mm tissue culture plates in RPMI + 10% FBS for 24 hours to allow for attachment. Fresh media with or without 25 nM doxorubicin was then added to the plates and a 24 hour treatment was performed. Cells were lysed in ice cold 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 EGTA, 10 mM sodium fluoride, 1 mM tetrasodium PP_i, and 100 mM b-glycerophosphate]. Samples were spun, supernatants collected, and protein concentrations were quantified with BCA protein reagent (50:1 v/v) (Pierce; Rockford, IL) at an absorbency of 570 nm. Next, 10 μ l of loading dye was added and samples were boiled for three minutes.

Western Blot Analysis

10 μ l of each sample was 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 a gel cassette (Invitrogen; Carlsbad, CA) and the stacking gel [4% (w/v) acrylamide, 1.0M TRIS (pH 6.8), 10% (w/v) APS, 10% SDS, and TEMED] formed the upper 25% of the cassette.

The samples were then separated by electrophoresis in running buffer [200 mM glycine, 25 mM TRIS, and 1 mg/ml (10%) SDS] for approximately two hours alongside a protein ladder (Amersham Biosciences; Piscataway, NJ). Before transfer, membranes were briefly soaked in methanol and then placed in transfer buffer. Proteins were then transferred from gels to PVDF membranes (Pierce; Rockford, IL) by semidry electro-transfer for three hours in transfer buffer [200 mM glycine, 25 mM TRIS and 20% methanol] with a transfer apparatus (Bio-Rad; Richmond, CA). After transfer, the membranes were placed in blocking solution [10 µg/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 while rocking.

Western blots were performed with antibodies specific for phospho (P) and total Akt (P= S473), GSK-3β (P= S9), p70S6K (P=T389), S6 (P=S235/236), cyclin D1 (P=T286), and total ERα (Cell Signaling, Beverly, MA). ERα served as a loading control due to the repeated failure of the available β-actin antibody to produce results. 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 ten minutes each in 1X TBST to remove any residual primary antibody. Membranes were incubated for one hour on a shaker at room

temperature in secondary antibody solution [1:5000 (v/v) secondary antibody in 1X 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). Membranes were then developed in film cassettes on x-ray film (Research Products International; Mt. Prospect, IL) to visualize detected protein bands within sample lanes. Bands were normalized to the untreated control through densitometry with Image J software (obtained through the National Institutes of Health).

Research Objectives and Experimental Approaches

Objective 1: Determine if GSK-3 β Activity Affects MCF-7 Clonogenicity

To investigate the effect of GSK-3 β expression on MCF-7 clonogenicity, the colony formation ability (CFA) of MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells was determined under anchorage-dependent and anchorage-independent conditions. Clonogenicity was assessed in the presence or absence of doxorubicin to determine the effect of treatment with chemotherapy.

Objective 2: Determine if GSK-3 β Activity Alters MCF-7 Sensitivity to Therapy

To assess the effect of GSK-3 β expression on the sensitivity of MCF-7 cells to chemotherapy, hormonal therapy, and pathway inhibition, MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells were subjected to MTT analysis. Relative cell proliferation was calculated in the absence or presence of doxorubicin, tamoxifen, rapamycin, the Array MEK inhibitor, doxorubicin combined with rapamycin,

doxorubicin combined with the MEK inhibitor, tamoxifen combined with rapamycin, and tamoxifen combined with the MEK inhibitor.

Objective 3: Determine if GSK-3 β Activity Affects MCF-7 Cell Signaling

To study the effect of GSK-3 β expression on cell signaling in MCF-7 cells, MCF-7, MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells were left untreated or treated with doxorubicin. Western blot analysis was performed to determine the levels of phosphorylated and total Akt, GSK-3 β , p70S6K, S6K, and cyclin D1.

CHAPTER 4: RESULTS

Effect of GSK-3 β Expression on MCF-7 Clonogenicity

Colony Formation Ability was Enhanced in GSK-3 β KD Cells in the Presence of Doxorubicin

The colony-formation ability (CFA) of MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells was tested in the presence or absence of 25 nM doxorubicin over the course of three weeks to assess anchorage-dependent clonogenicity. As shown in Figure 5, the CFA of GSK-3 β WT cells was decreased by almost 90% when treated with doxorubicin. GSK-3 β A9 cells had an increased CFA as compared to WT cells, and a 90% reduction was also observed in their CFA in the presence of the drug. As for the GSK-3 β KD cells, CFA without doxorubicin was higher than in the WT cells, but lower than the A9 cells. However, when KD cells are tested in the presence of the drug, they exhibit only a 40% reduction in CFA. In the presence of doxorubicin, as compared to WT and A9 cells, KD cells showed a respective 4.5-fold and 2.5-fold enhancement in growth and CFA. These results demonstrate that loss of GSK-3 β kinase activity in MCF-7 cells enhances clonogenicity in the presence of chemotherapy.

Clonogenicity in Soft Agar was Enhanced in GSK-3 β KD Cells

To assess clonogenicity under anchorage-independent conditions, MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells were seeded as single cells in soft agar and allowed to expand for three weeks. Figure 6 shows the number of colonies that formed in the presence and absence of 25 nM doxorubicin. As expected, the CFA of all three cell lines was markedly decreased under anchorage-independent conditions as

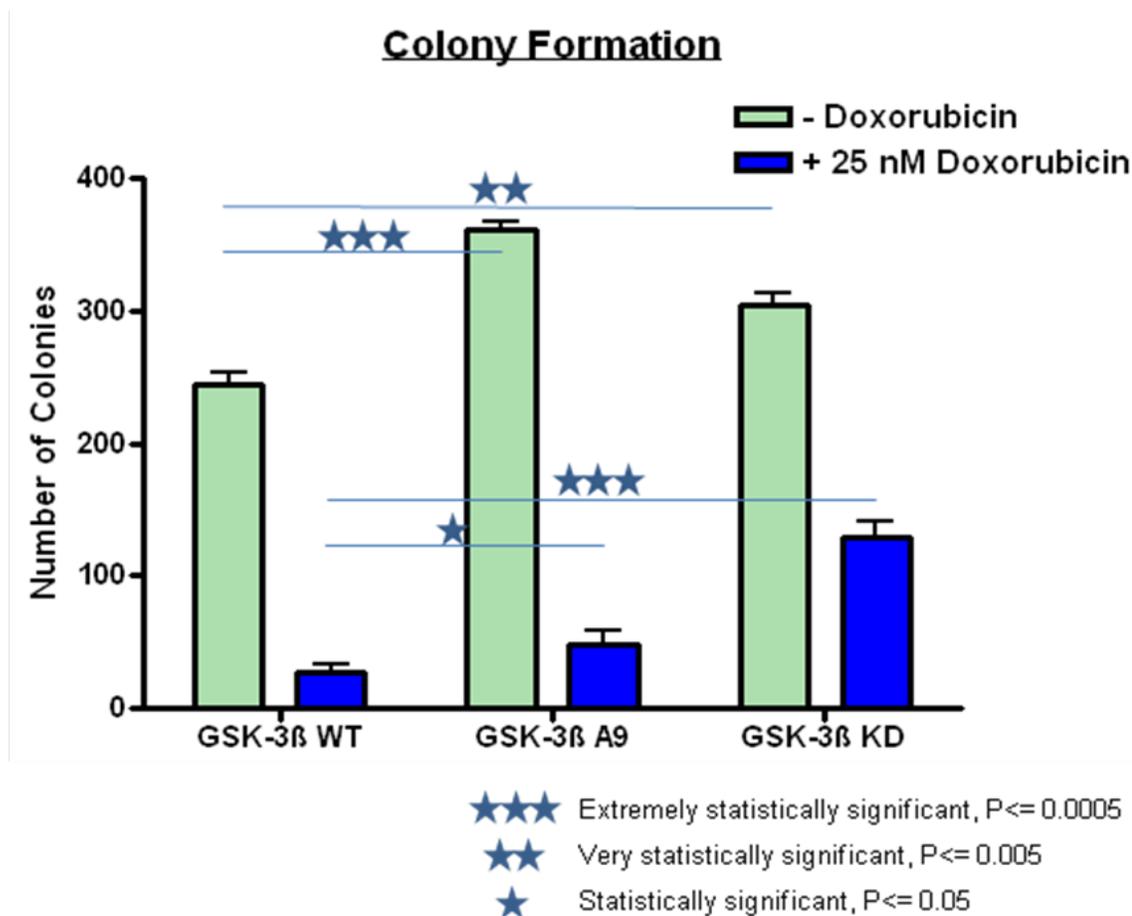


Figure 5: Anchorage-dependent Clonogenicity of GSK-3 β -transfected MCF-7 Cells
 GSK-3 β WT, A9, and KD cells were plated out in the absence or presence of 25 nM doxorubicin and grown for three weeks. Colonies were stained and counted, and the resulting means, standard deviations, and statistical significances were calculated.

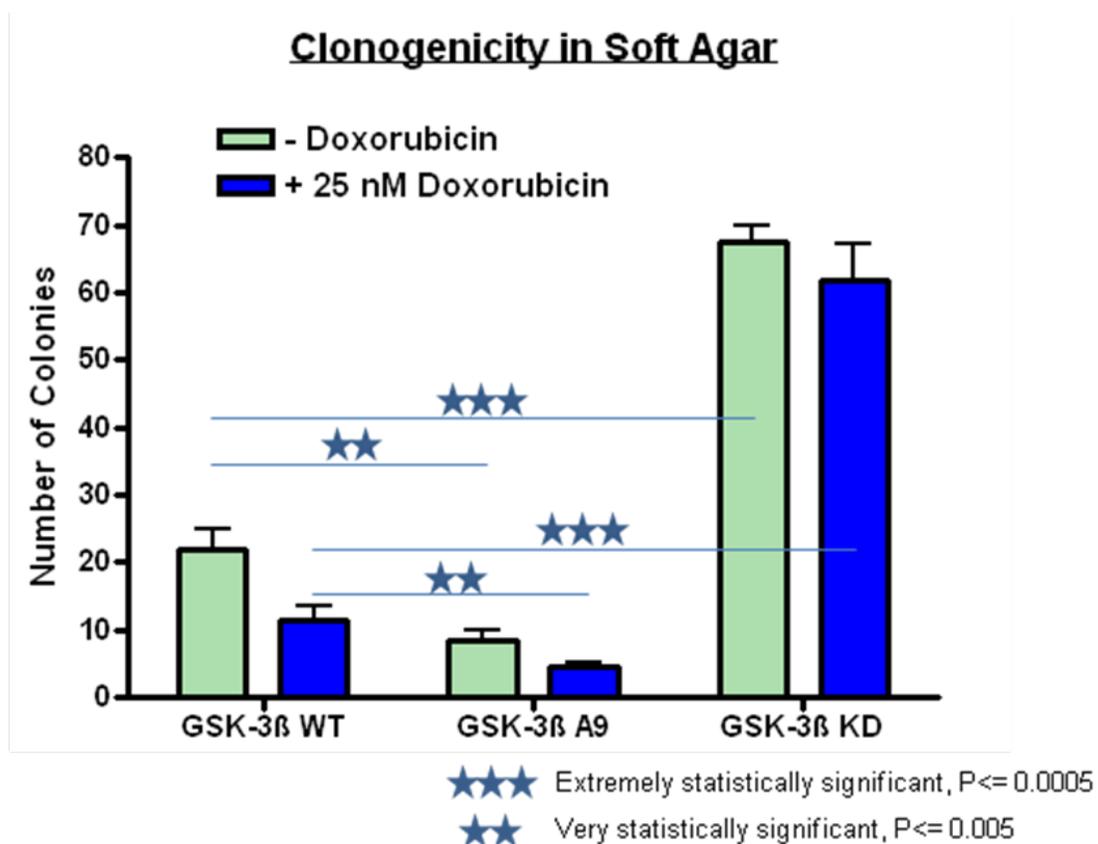


Figure 6: Anchorage-independent Clonogenicity of GSK-3 β -transfected MCF-7 Cells
 GSK-3 β WT, A9, and KD cells were seeded in soft agar in the absence or presence of 25 nM doxorubicin and grown for three weeks. Colonies were stained and counted, and the resulting means, standard deviations, and statistical significances were calculated.

compared to anchorage-dependent conditions. In the presence of doxorubicin, GSK-3 β WT cells experienced a 50% reduction in CFA. A 50% reduction in CFA was also observed for GSK-3 β A9 cells when treated with doxorubicin. However, this trend was not the case for GSK-3 β KD cells, which only revealed a 10% reduction in CFA in the presence of the drug. The CFA of GSK-3 β KD cells in doxorubicin was 5-fold greater than that for GSK-3 β WT cells and 13-fold greater than GSK-3 β A9 cells. These results show that loss of GSK-3 β kinase activity in MCF-7 cells confers elevated anchorage-independent clonogenicity in both the presence and absence of chemotherapy.

Effect of GSK-3 β Expression on MCF-7 Drug Resistance

GSK-3 β KD Cells had Increased Resistance to Doxorubicin

The sensitivity of MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells to doxorubicin was measured by MTT assay. Cells were grown for four days in the absence or presence of various concentrations of the drug ranging from 2 nM to 2000 nM. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 7 along with corresponding doxorubicin IC₅₀ values shown on a log scale. As demonstrated, GSK-3 β WT cells experienced the greatest sensitivity to the drug, with an IC₅₀ of 27 nM. A higher IC₅₀ of 46 nM doxorubicin was observed for GSK-3 β A9 cells. The greatest resistance to the drug was found in the GSK-3 β KD cells with a doxorubicin IC₅₀ of 68 nM, which is 2.5-fold higher than that of the GSK-3 β WT cells. Therefore, loss of GSK-3 β kinase activity in MCF-7 cells increases their resistance to chemotherapy.

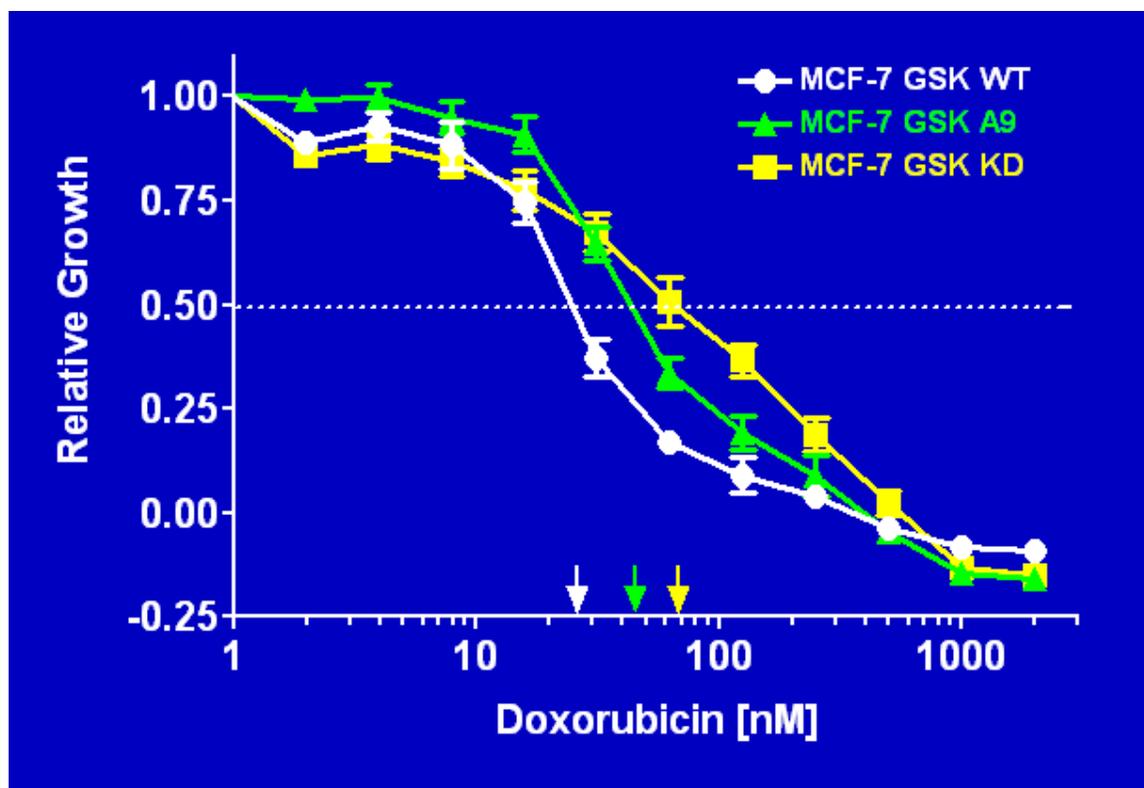


Figure 7: Sensitivity of GSK-3 β -transfected MCF-7 Cells to Doxorubicin

GSK-3 β WT, A9, and KD cells were treated with various concentrations of doxorubicin for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the doxorubicin IC₅₀ values, which are indicated with arrows.

GSK-3 β KD Cells had Increased Resistance to Tamoxifen

The sensitivity of MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells to tamoxifen was measured by MTT assay. Cells were grown for four days either untreated or in the presence of the drug ranging in concentration from 1 nM to 1000 nM. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 8 along with corresponding tamoxifen IC₅₀ values. As shown, GSK-3 β A9 cells experienced the greatest sensitivity to the drug, with an IC₅₀ of 31 nM. A higher IC₅₀ of 120 nM tamoxifen was observed for GSK-3 β WT cells. The greatest resistance to the drug was found in the GSK-3 β KD cells, the growth curve of which does not fall below 50% relative growth at the tamoxifen concentrations tested. An extension of the curve would likely identify the IC₅₀ of GSK-3 β KD cells to be around 2000 nM, which is 64-fold higher than that of the GSK-3 β WT cells. Therefore, loss of GSK-3 β kinase activity in MCF-7 cells increases their resistance to hormonal-based therapy.

GSK-3 β KD Cells had Increased Sensitivity to Rapamycin

The sensitivity of MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells to rapamycin was assessed through MTT analysis. Cells were grown in the absence or presence of various concentrations of the inhibitor ranging from 0.1 nM to 1 nM. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 9 along with corresponding rapamycin IC₅₀ values. As illustrated, GSK-3 β A9 cells experienced the least sensitivity to the inhibitor, with an IC₅₀ value outside of the concentration range tested. An IC₅₀ of 58 nM rapamycin was observed for GSK-3 β WT cells. In contrast to the results for doxorubicin and tamoxifen, the cell line most sensitive

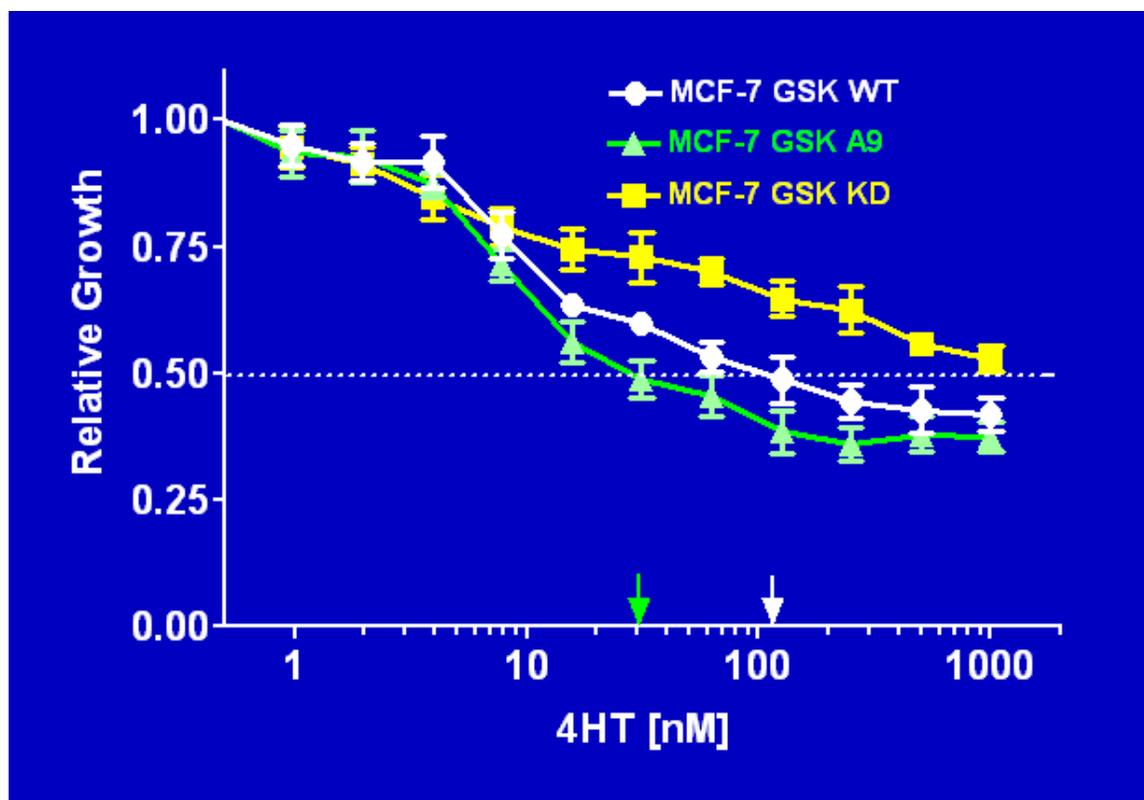


Figure 8: Sensitivity of GSK-3 β -transfected MCF-7 Cells to Tamoxifen

GSK-3 β WT, A9, and KD cells were treated with various concentrations of tamoxifen for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the tamoxifen IC₅₀ values, which are indicated with arrows.

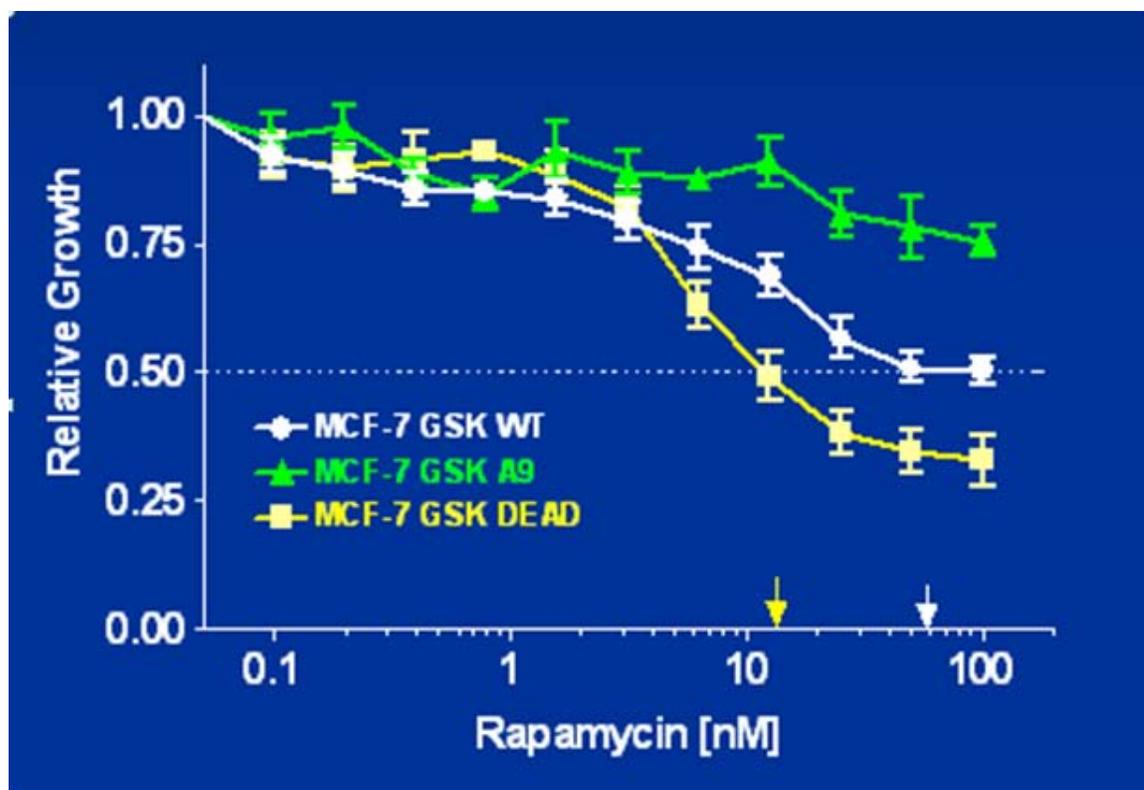


Figure 9: Sensitivity of GSK-3 β -transfected MCF-7 Cells to Rapamycin

GSK-3 β WT, A9, and KD cells were treated with various concentrations of rapamycin for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the rapamycin IC₅₀ values, which are indicated with arrows.

to treatment was GSK-3 β KD, in which a rapamycin IC₅₀ of 14 nM was observed. This is four times lower than the IC₅₀ observed for GSK-3 β WT cells. Therefore, although loss of GSK-3 β kinase activity in MCF-7 cells increases their resistance to conventional therapy, it appears to augment their sensitivity to mTOR inhibition through rapamycin.

GSK-3 β Cell Lines Responded Similarly to the Array MEK Inhibitor

The sensitivity of MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells to the Array MEK inhibitor was assessed by MTT assay. Cells were grown either untreated or treated with the inhibitor ranging in concentration from 10 nM to 10,000 nM. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 10 along with corresponding MEK inhibitor IC₅₀ values. As shown, the three cell lines did not vary greatly in their response. Proliferation maintained a plateau at lower concentrations of the inhibitor, but decreased and approached 50% of relative growth once treated with the highest inhibitor concentration tested. Therefore, GSK-3 β kinase activity in MCF-7 cells did not greatly influence their response to MEK inhibition.

Response to Doxorubicin of GSK-3 β WT Cells was not Altered by Inhibitors

The sensitivity of MCF-7/GSK-3 β WT cells to doxorubicin either alone or in combination with rapamycin or the Array MEK inhibitor was calculated through MTT analysis. Cells were grown either alone or in the presence of increasing concentrations of doxorubicin with the addition of either 1 nM rapamycin or 1000 nM Array MEK inhibitor. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 11 along with corresponding doxorubicin IC₅₀ values. As shown, the IC₅₀ was approximately 25 nM for all three treatment scenarios. Therefore, expression of

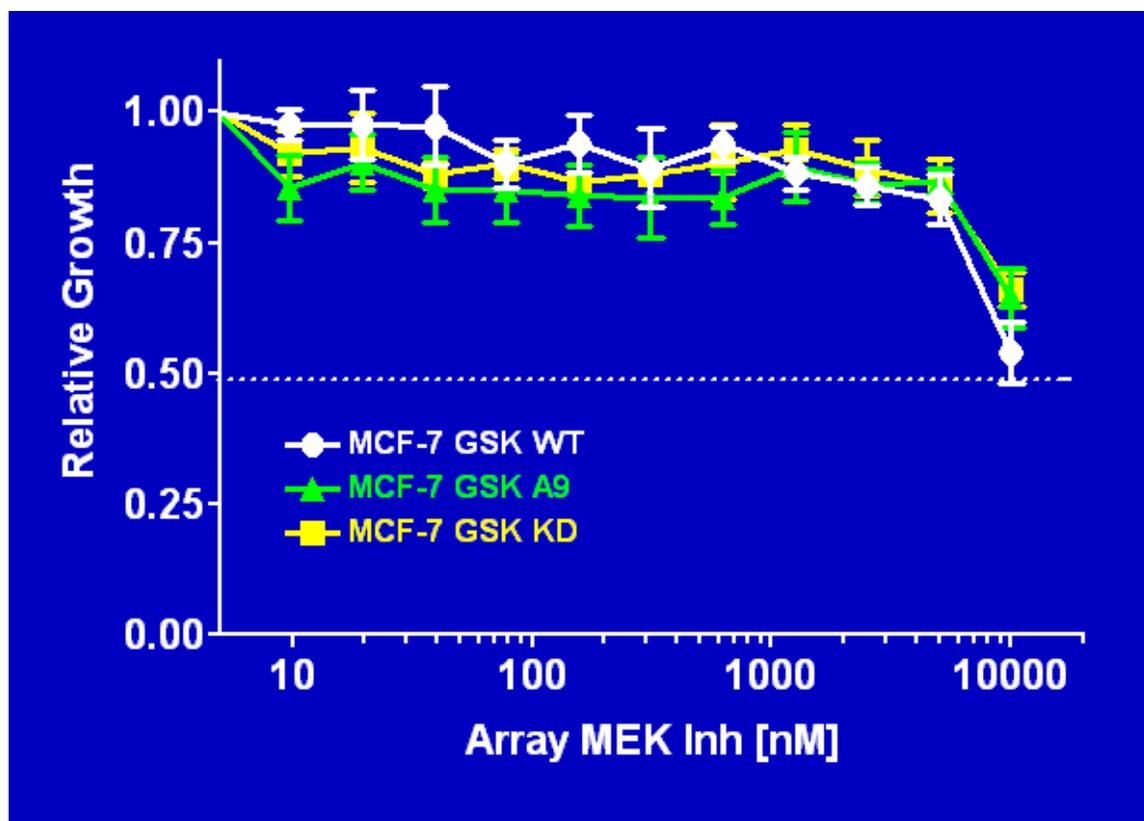


Figure 10: Sensitivity of GSK-3 β -transfected MCF-7 Cells to the Array MEK Inhibitor. GSK-3 β WT, A9, and KD cells were treated with various concentrations of the Array MEK inhibitor for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the inhibitor IC₅₀ values, which are indicated with arrows.

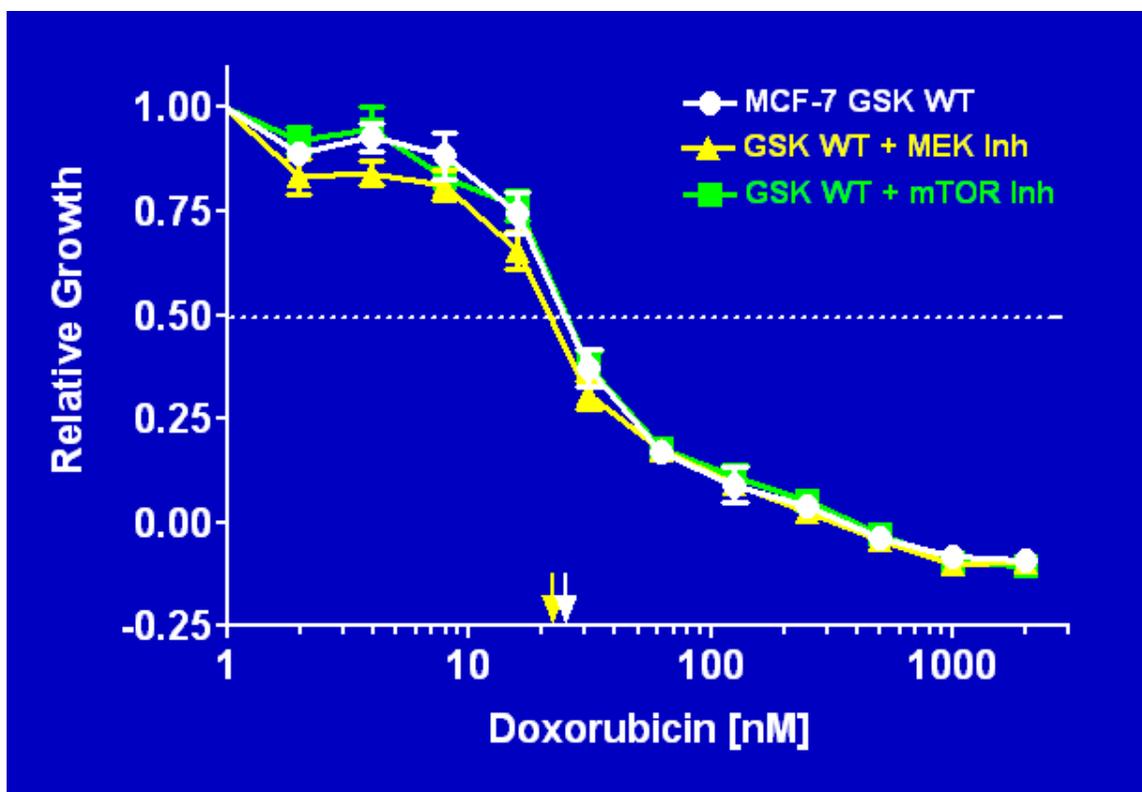


Figure 11: Sensitivity of GSK-3 β WT MCF-7 Cells to Doxorubicin

GSK-3 β WT cells were treated with various concentrations of doxorubicin in combination with 1000 nM Array MEK inhibitor or 1 nM rapamycin for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the doxorubicin IC₅₀ values, which are indicated with arrows.

wild-type GSK-3 β kinase activity in MCF-7 cells does not confer sensitivity to combination therapy as compared to chemotherapy alone.

Response to Doxorubicin of GSK-3 β A9 Cells was not Altered by Inhibitors

The sensitivity of MCF-7/GSK-3 β A9 cells to doxorubicin either alone or in combination with rapamycin or the Array MEK inhibitor was quantified by MTT assay. Cell proliferation was measured either alone or in the presence of increasing concentrations of doxorubicin with the addition of either 1 nM rapamycin or 1000 nM Array MEK inhibitor. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 12 along with corresponding doxorubicin IC₅₀ values. As shown, the IC₅₀ was approximately 44 nM for all three treatment types. Therefore, expression of constitutively active GSK-3 β kinase activity in MCF-7 cells does not render cells any more sensitive to combination therapy than chemotherapy alone.

Response to Doxorubicin of GSK-3 β KD Cells was Enhanced by MEK Inhibition

The sensitivity of MCF-7/GSK-3 β KD cells to doxorubicin either alone or in combination with rapamycin or the Array MEK inhibitor was assessed by MTT assay. Cells were grown untreated or in the presence of increasing concentrations of doxorubicin with the addition of either 1 nM rapamycin or 1000 nM Array MEK inhibitor. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 13 along with corresponding doxorubicin IC₅₀ values. As shown, the IC₅₀ was around 70 nM in the case of doxorubicin alone or when combined with rapamycin. Conversely, the co-treatment of doxorubicin and the Array MEK inhibitor was sufficient to lower the IC₅₀ doxorubicin to 24 nM, a 2.7-fold reduction. Therefore,

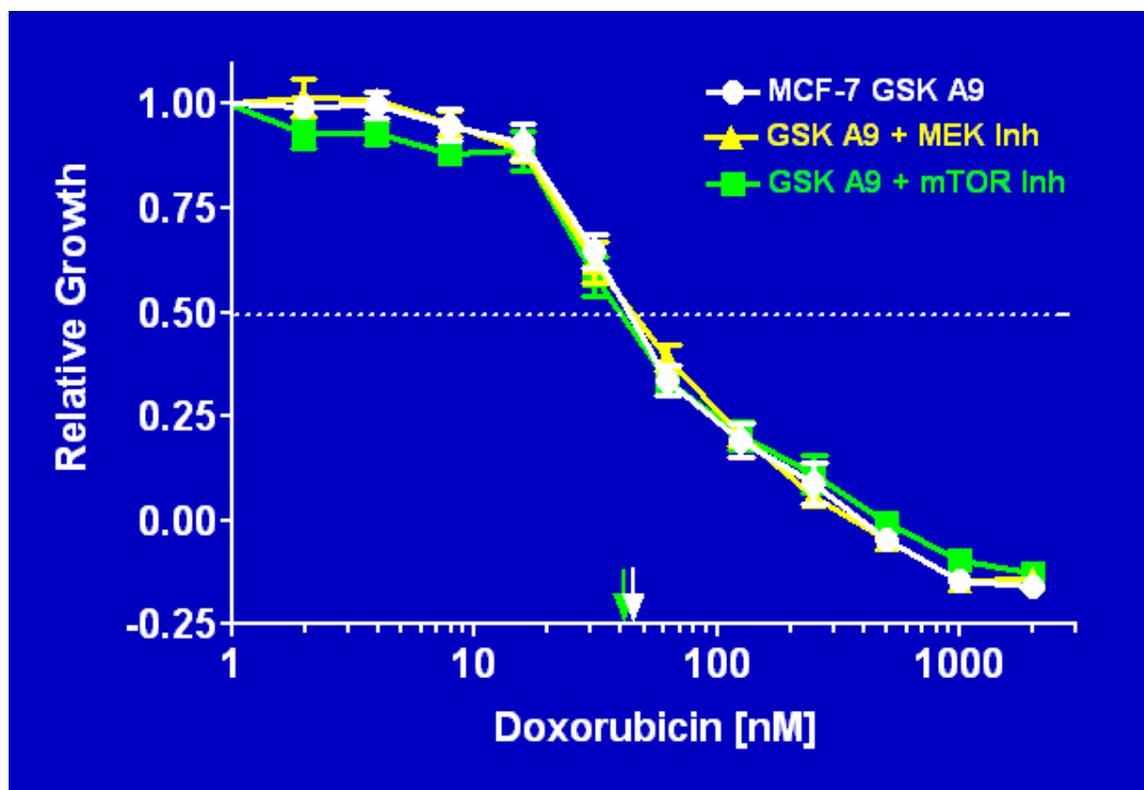


Figure 12: Sensitivity of GSK-3 β A9 MCF-7 Cells to Doxorubicin

GSK-3 β A9 cells were treated with various concentrations of doxorubicin in combination with 1000 nM Array MEK inhibitor or 1 nM rapamycin for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the doxorubicin IC₅₀ values, which are indicated with arrows.

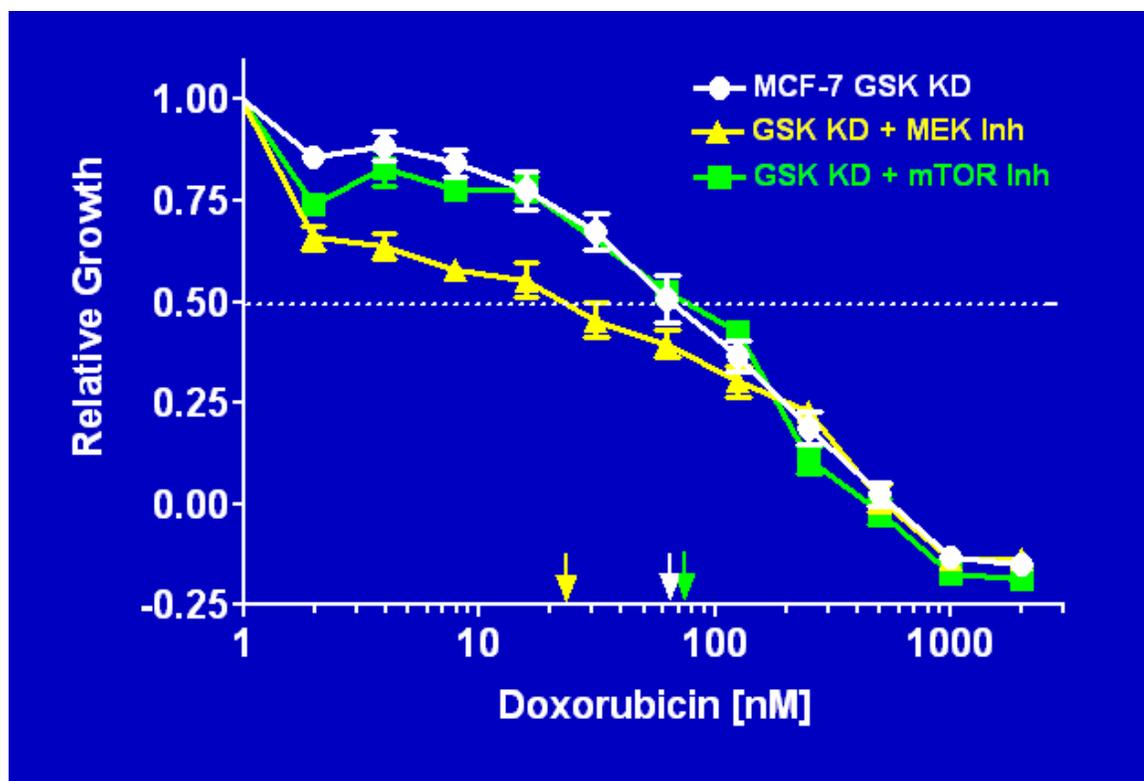


Figure 13: Sensitivity of GSK-3 β KD MCF-7 Cells to Doxorubicin

GSK-3 β KD cells were treated with various concentrations of doxorubicin in combination with 1000 nM Array MEK inhibitor or 1 nM rapamycin for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the doxorubicin IC₅₀ values, which are indicated with arrows.

expression of GSK-3 β that lacks kinase activity in MCF-7 cells confers sensitivity to combined chemotherapy and MEK inhibition.

Response to Tamoxifen of GSK-3 β WT Cells was Enhanced by Inhibitors

The sensitivity of MCF-7/GSK-3 β WT cells to tamoxifen either alone or in combination with rapamycin or the Array MEK inhibitor was determined with MTT analysis. Cells were grown either alone or in the presence of increasing concentrations of tamoxifen with the addition of either 1 nM rapamycin or 1000 nM Array MEK inhibitor. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 14 along with corresponding tamoxifen IC₅₀ values. As shown, the IC₅₀ was 120 nM when cells were treated with tamoxifen alone. A considerable reduction in tamoxifen IC₅₀ was observed when the cells were co-treated with rapamycin or the Array MEK inhibitor, with IC₅₀ values of 9.5 nM and 7 nM, respectively. This equates to approximately a 15-fold decrease. Therefore, expression of wild-type GSK-3 β kinase activity in MCF-7 cells grants sensitivity to a combination of hormonal therapy and pathway inhibition.

Response to Tamoxifen of GSK-3 β A9 Cells was Enhanced by Inhibitors

The sensitivity of MCF-7/GSK-3 β A9 cells to tamoxifen either alone or in combination with rapamycin or the Array MEK inhibitor was analyzed with MTT analysis. Proliferation was measured either alone or in the presence of increasing concentrations of tamoxifen with the addition of either 1 nM rapamycin or 1000 nM Array MEK inhibitor. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 15 along with corresponding tamoxifen IC₅₀ values.

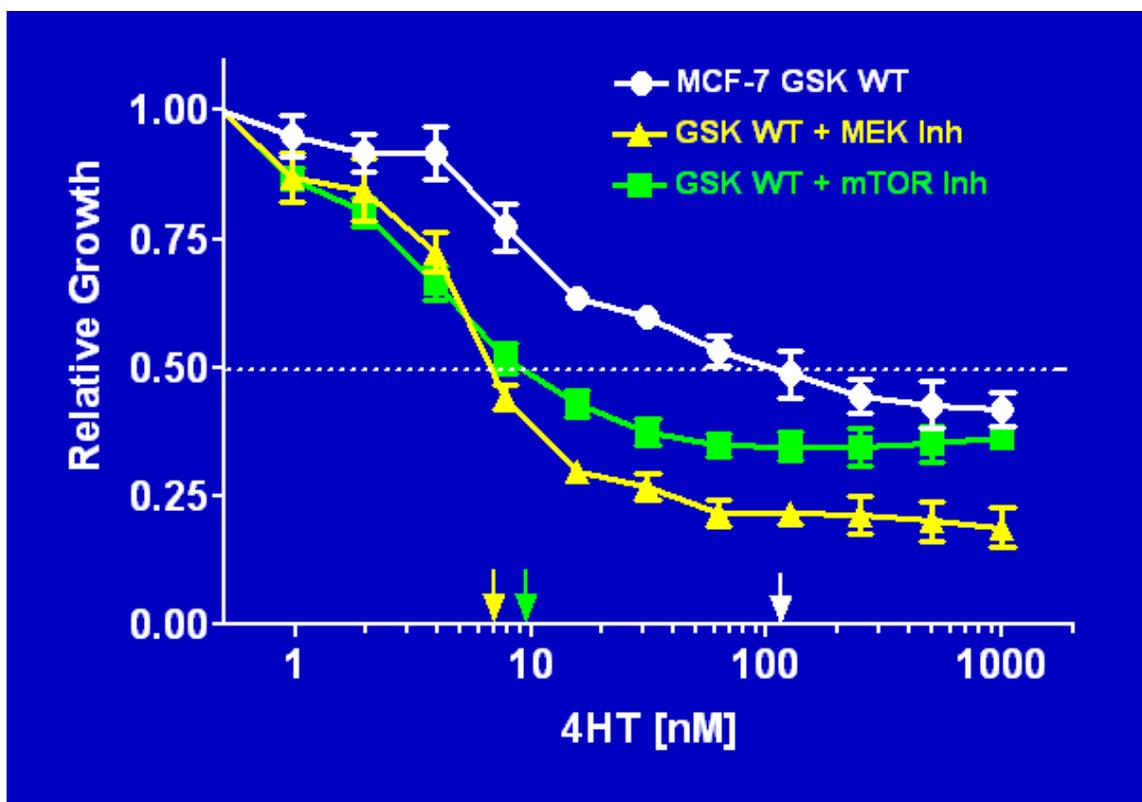


Figure 14: Sensitivity of GSK-3 β WT MCF-7 Cells to Tamoxifen

GSK-3 β WT cells were treated with various concentrations of tamoxifen in combination with 1000 nM Array MEK inhibitor or 1 nM rapamycin for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the doxorubicin IC₅₀ values, which are indicated with arrows.

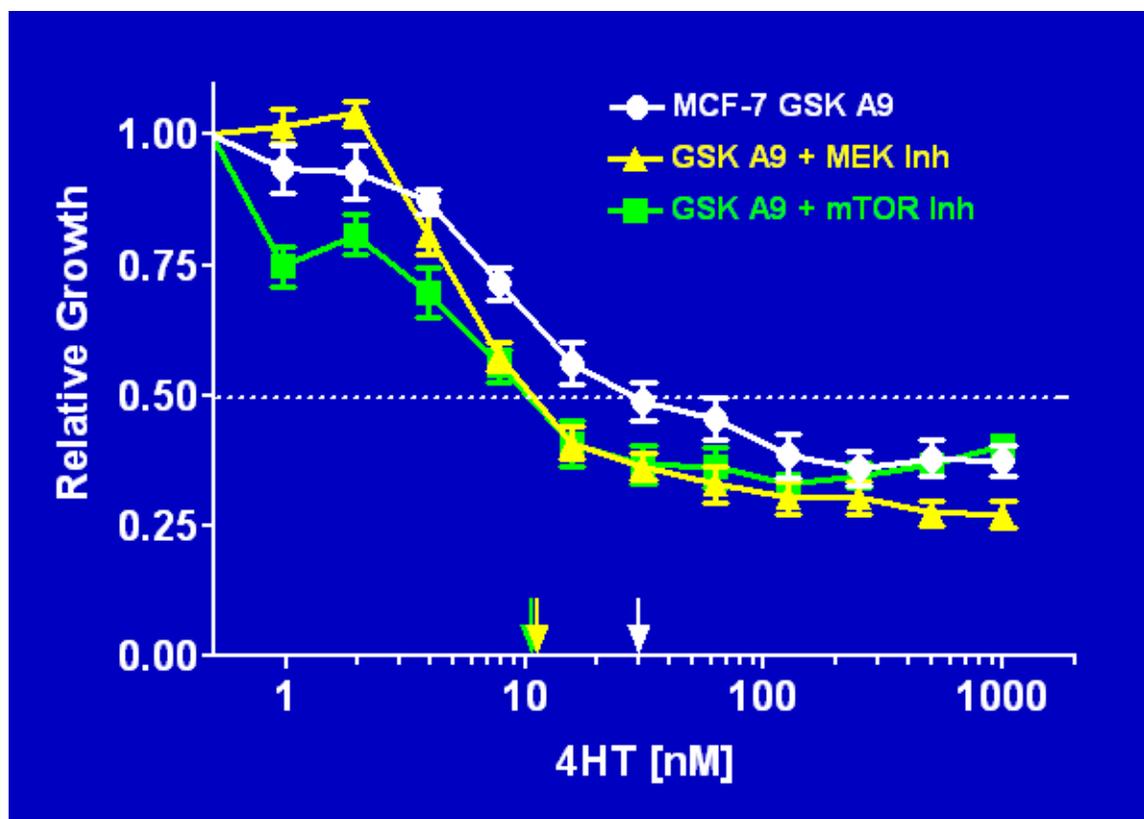


Figure 15: Sensitivity of GSK-3 β A9 MCF-7 Cells to Tamoxifen

GSK-3 β A9 cells were treated with various concentrations of tamoxifen in combination with 1000 nM Array MEK inhibitor or 1 nM rapamycin for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the doxorubicin IC₅₀ values, which are indicated with arrows.

As shown, the IC_{50} was 31 nM when cells were treated with tamoxifen alone. Tamoxifen IC_{50} was reduced by approximately 2.5-fold when the cells were co-treated with rapamycin or the Array MEK inhibitor, with IC_{50} values of 11 nM and 12 nM, respectively. Therefore, expression of constitutively active GSK-3 β kinase activity in MCF-7 cells confers sensitivity to a combination of hormonal therapy and pathway inhibition.

Response to Tamoxifen of GSK-3 β KD Cells was Enhanced by MEK Inhibition

The sensitivity of MCF-7/GSK-3 β KD cells to tamoxifen either alone or in combination with rapamycin or the Array MEK inhibitor was quantified with MTT analysis. Cells were grown either alone or in the presence of increasing concentrations of tamoxifen with the addition of either 1 nM rapamycin or 1000 nM Array MEK inhibitor. The relative growth curves of treated cells as compared to untreated cells are shown in Figure 16 along with corresponding tamoxifen IC_{50} values. As shown, the IC_{50} was not reached when cells were treated with tamoxifen alone at the concentrations tested, but could be approximated at 2000 nM. When co-treated with rapamycin, the growth curve is similar to that of tamoxifen alone, but deviates to below 50% growth at a concentration of 790 nM tamoxifen. However, the IC_{50} of tamoxifen was greatly reduced by 50-fold when the cells were co-treated the Array MEK inhibitor, in which an IC_{50} value of 40 nM was observed. Therefore, expression of GSK-3 β that lacks kinase activity in MCF-7 cells renders them sensitive to combined hormonal-based therapy and MEK inhibition.

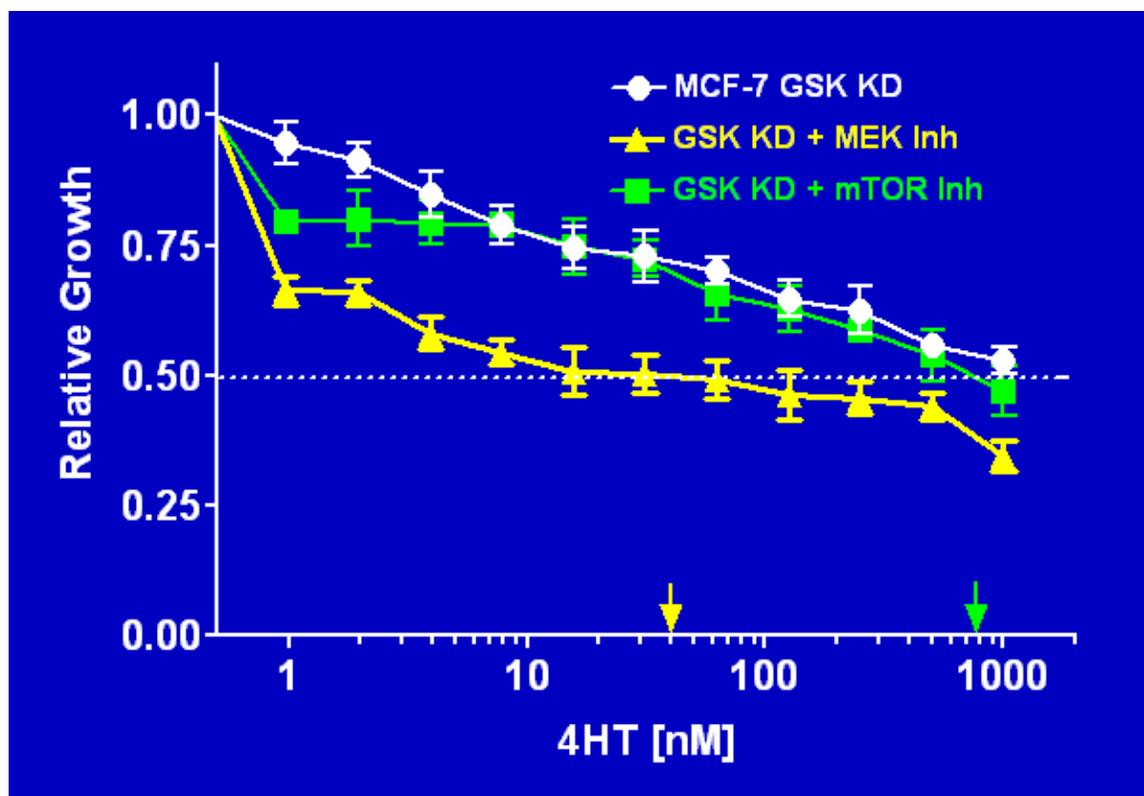


Figure 16: Sensitivity of GSK-3 β KD MCF-7 Cells to Tamoxifen

GSK-3 β KD cells were treated with various concentrations of tamoxifen in combination with 1000 nM Array MEK inhibitor or 1 nM rapamycin for four days to assess their relative growth as compared to untreated cells. The corresponding means and standard deviations were calculated, along with the doxorubicin IC₅₀ values, which are indicated with arrows.

Effect of GSK-3 β Expression on MCF-7 Cell Signaling

Doxorubicin Activated Akt in MCF-7 and MCF-7/GSK-3 β WT Cells

To examine the activity of proteins upstream and downstream of GSK-3 β , Western blot analysis was performed on MCF-7, MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells in the absence or presence of 25 nM doxorubicin. The expression levels of phosphorylated (P) and total (T) Akt, GSK-3 β , p70S6K, S6, and cyclin D1 were determined along with ER α as a loading control. As shown in Figure 17, P-Akt was expressed in all four cell lines when both untreated and treated with doxorubicin. As compared to the MCF-7 control cells, the three transfected cell lines showed a slight increase in P-Akt levels when untreated, with the highest level observed in GSK-3 β A9 cells. When control MCF-7 cells were subjected to the drug, a nearly 2-fold increase in P-Akt occurred. A slight elevation in active Akt was seen for GSK-3 β WT cells, while a small decrease was observed for GSK-3 β A9 cells in the presence of doxorubicin. No change was measured in P-Akt for GSK-3 β KD cells when treated as compared to untreated. Therefore, doxorubicin activates Akt in MCF-7 cells when WT GSK-3 β is expressed, but not when constitutively active or kinase dead GSK-3 β is present.

Doxorubicin Activated GSK-3 β in MCF-7 and MCF-7/GSK-3 β WT Cells

In the absence of doxorubicin, MCF-7 control cells expressed P-GSK-3 β as shown in Figure 17. Similar to P-Akt, there was a slight increase in P-GSK-3 β in the three GSK-3 β transfected cell lines. However, when treated with the drug, P-GSK-3 β

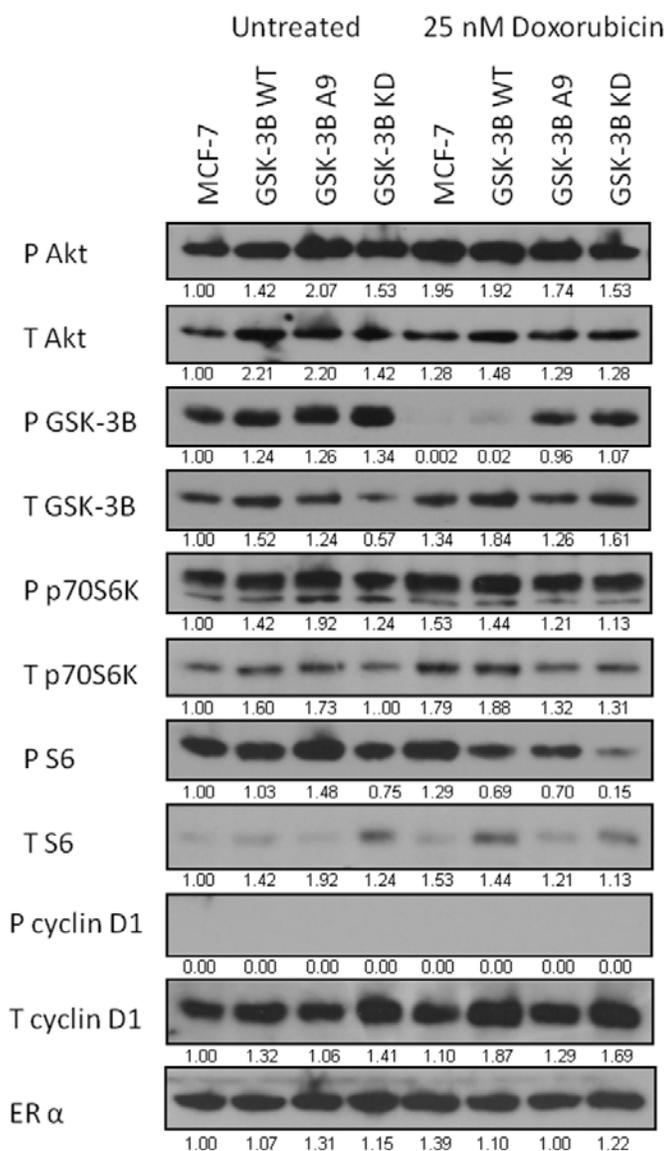


Figure 17: Western Blot Analysis of GSK-3 β -transfected MCF-7 Cells

MCF-7, GSK-3 β WT, A9, and KD cells were grown in the absence or presence of 25 nM doxorubicin for 24 hours, after which protein lysates were prepared and separated by SDS-PAGE. Blots were probed for antibodies specific for phosphorylated (P) and total (T) Akt, GSK-3 β , p70S6K, S6, and cyclin D1, along with ER α as a loading control. The band intensities were normalized to the untreated MCF-7 bands through densitometry.

levels were abrogated in MCF-7 and MCF-7/GSK-3 β WT cells, but not in MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells. Therefore, when WT GSK-3 β is expressed in MCF-7 cells, it can be activated upon treatment with doxorubicin even in the presence of activated Akt.

Doxorubicin Increased p70S6K in MCF-7 Cells, but not in MCF-7/GSK-3 β A9 Cells

As seen with P-Akt, P-p70S6K was expressed in all four cell lines in the absence or presence of doxorubicin, shown in Figure 17. When untreated, the highest level of P-p70S6K was observed in GSK-3 β A9 cells, which corresponded to a 2-fold increase in expression as compared to MCF-7 control cells. When treated with doxorubicin, P-p70S6K was elevated in MCF-7 cells, but decreased in GSK-3 β A9 cells, which was consistent with that seen for P-Akt levels. Changes in P-S6 were not consistent with P-Akt and P-p70S6K. P-S6 was expressed in all four cells lines in the absence of doxorubicin, but upon treatment, levels increased in MCF-7 control cells and decreased in MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells. Therefore, doxorubicin increases p70S6K and S6 activity in MCF-7 cells, but a consistent trend is not observed for the GSK-3 β transfected cell lines.

Cyclin D1 was Activate Regardless of GSK-3 β Expression or Doxorubicin Treatment

Figure 17 also shows that P-cyclin D1 was not detected in MCF-7, MCF-7/GSK-3 β WT, MCF-7/GSK-3 β A9, and MCF-7/GSK-3 β KD cells. This was observed in the absence or presence of 25 nM doxorubicin. Therefore, cyclin D1 is unphosphorylated and active in MCF-7 cells in spite of GSK-3 β expression or treatment with chemotherapy.

CHAPTER 5: DISCUSSION

Study Conclusions

Based on these findings, GSK-3 β plays a role in MCF-7 breast cancer clonogenicity, drug resistance, and cell signaling. When GSK-3 β lacking kinase activity was expressed in cells, a significant increase in both anchorage-dependent and anchorage-independent clonogenicity was observed as compared to GSK-3 β WT. This was the case whether doxorubicin was absent or present, and when treated, the drug had less of an inhibitory effect on GSK-3 β KD cells than WT or A9 cells. Interestingly, GSK-3 β A9 cells with constitutive kinase activity exhibited higher anchorage-dependent, but not anchorage-independent, clonogenicity than WT cells. It is possible that GSK-3 β performs dual roles by both limiting proliferation under certain conditions and allowing cell growth in others.

The anchorage-independent growth of GSK-3 β KD cells in doxorubicin was enhanced five times as compared to WT cells. This strongly suggests that altered GSK-3 β activity influences breast cancer proliferation, motility, and response to chemotherapy. The loss of normal GSK-3 β kinase activity may confer a survival advantage by upregulating factors involved in cell cycle progression, prevention of apoptosis, and anchorage-independence. These results are similar to those found for squamous cell and basal cell carcinomas, in which expression of kinase dead GSK-3 β enhanced anchorage-independent growth and tumorigenicity (Ma *et al*, 2007). It is likely that the mechanisms leading to loss of GSK-3 β activity also enrich for a subpopulation of stem-like cancer

initiating cells (CICs) that demonstrate enhanced motility, clonogenicity, and drug resistance.

Drug sensitivity assays revealed that resistance to doxorubicin and tamoxifen was greatly increased in GSK-3 β KD cells as compared to WT. However, drug resistant KD cells responded to mTOR inhibition with rapamycin. Additionally, a combination treatment of the Array MEK inhibitor and doxorubicin or tamoxifen was found to have a synergistic effect capable of alleviating drug resistance in GSK-3 β KD cells. This reveals that the same mechanisms involved in inhibiting GSK-3 β kinase activity create a cellular vulnerability to signaling blockades. Furthermore, targeting signaling molecules involved with the PI3K/Akt and Raf/MEK pathways may be sufficient to overcome resistance to chemo- and hormonal therapy. Therefore, genetic alterations leading to abnormal GSK-3 β activity should be taken into account when designing a course of breast cancer therapy. Combination drug/inhibitor treatments would be clinically advantageous by affording a lower drug IC₅₀ and a subsequent reduction in therapy-related side effects.

The role of GSK-3 β in PI3K/Akt signaling was assessed through Western blot, and some findings were contrary to what was expected. Given that GSK-3 β is negatively regulated by Akt and p70S6K, it was thought that GSK-3 β would be phosphorylated and inactivated in the presence of phosphorylated Akt and p70S6K. Doxorubicin treatment was shown to activate Akt in MCF-7 control and WT GSK-3 β cells, but a corresponding inhibition of GSK-3 β activity was not observed. Therefore, chemotherapy can induce GSK-3 β kinase activity in an Akt-independent fashion. Additionally, phosphorylated

GSK-3 β was detected in cells transfected with the constitutively active GSK-3 β mutant, which must be due to endogenous wild-type expression.

The influence of GSK-3 β expression on its downstream target, cyclin D1, did not reveal a change on cyclin D1 phosphorylation. Cyclin D1 was unphosphorylated and active in all cell lines, treated or untreated. This demonstrates that cyclin D1 can be regulated in a GSK-3 β - independent fashion and can remain unaffected by chemotherapy. Additional Western blot analysis of other downstream targets of GSK-3 β like Mcl-1 and p21CIP did not produce reliable results, likely because of the unavailability of fresh primary antibodies. Therefore, further study is required to provide a clearer picture of the effect of mutant GSK-3 β expression in MCF-7 cell signaling.

Akt-Independent Regulation of GSK-3 β

Given that GSK-3 β regulation is complex and multi-factorial, there are many alternate routes of GSK-3 β activation that are independent of Akt. Active GSK-3 β plays a role in the Wnt/ β -catenin pathway by participating in the formation of the β -catenin destruction complex. This sequestration within a protein complex would prevent Akt from accessing and phosphorylating GSK-3 β . Therefore, there may be different cellular pools of GSK-3 β that are under separate regulation by Wnt and Akt, in which the activation of one does not affect the other. Simultaneous Akt and GSK-3 β activity has also been demonstrated in pancreatic and colon cancer cell lines, showing that increased Akt expression does not always correlate with decreased GSK-3 β (Ougolkov and Billadeau, 2006).

Given that certain pools of GSK-3 β may remain active in cancer, the cellular localization of these pools may also be a factor involved in their regulation. GSK-3 β is predominantly found to be active in the cytoplasm, but can also translocate to the nucleus. The nuclear activities of GSK-3 β may seemingly conflict with its cytosolic roles, and aberrant nuclear accumulation of GSK-3 β has been found in other forms of cancer (Ougolkov *et al*, 2006). Recently, a nuclear localization signal (NLS) was identified in the basic domain of GSK-3 β , shown in Figure 18 (Meares and Jope, 2007). Therefore, modifications that unmask the NLS and allow for increased translocation of GSK-3 β to the nucleus may be involved with cancer progression.

Future Directions

Based on the trends identified in this study, future work should be performed to further elucidate the roles and regulation of GSK-3 β in breast cancer. Biochemical fractionation of cytosolic and nuclear components would reveal the cellular localization of GSK-3 β in MCF-7 cells when treated with chemotherapy, pathway inhibitors, and combinations thereof. Additional signaling molecules upstream and downstream of GSK-3 β could be examined through Western blot in cells subjected to these treatments. The effect of pathway blockade and combination treatments on clonogenicity in GSK-3 β transfected MCF-7 cells would also be a valid experiment. Lastly, although the role of GSK-3 β in stem-like cancer initiating cells was beyond the scope of this study, it would be an interesting avenue to explore.

In conclusion, these results demonstrate that GSK-3 β plays an important role in breast cancer. Loss of normal GSK-3 β kinase activity, whether through Akt or other

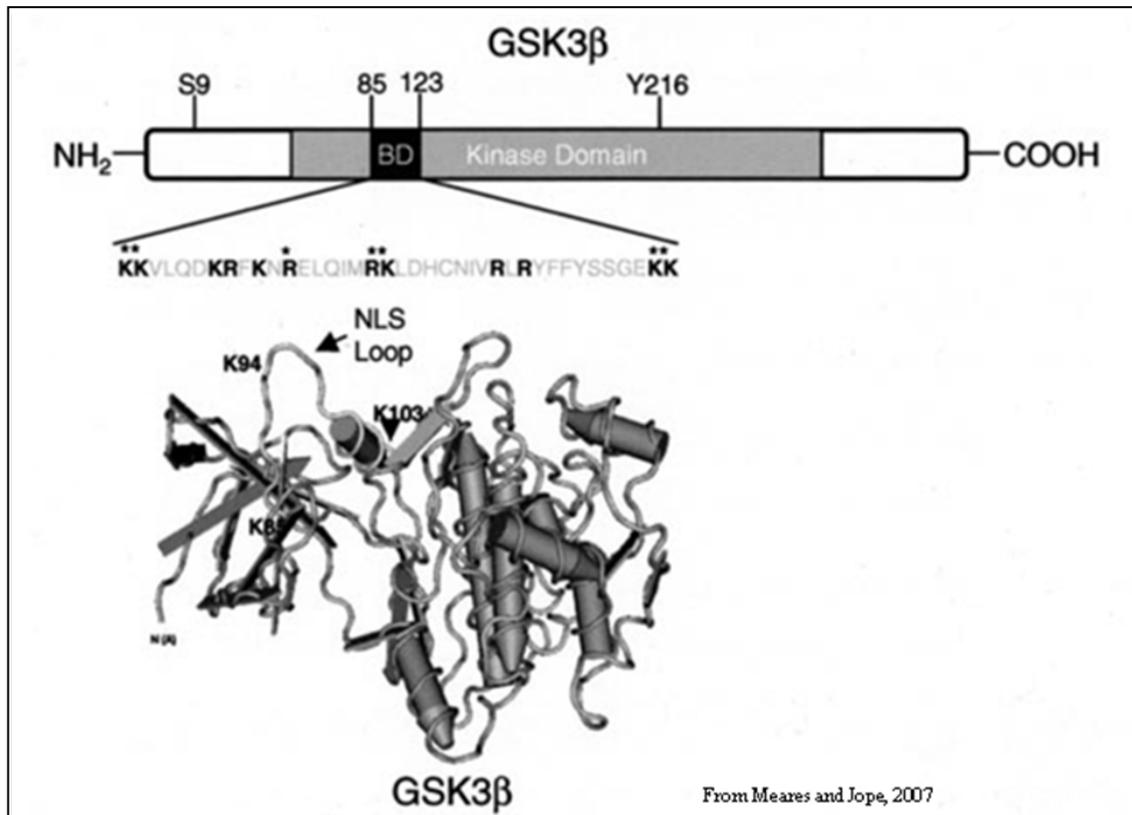


Figure 18: Nuclear Localization Sequence of GSK-3 β

An NLS has been identified within the basic domain, contained in the kinase domain, of GSK-3 β . This sequence includes residues 85-123 and contains 12 basic amino acids.

When the protein is folded, an accessible external NLS loop is created, as shown in the GSK-3 β crystal structure.

regulatory mechanisms, can drastically increase the clonogenicity and drug resistance of MCF-7 cells. However, this may confer an Achilles' heel by sensitizing these cells to targeted therapy with pathway inhibitors. By blocking signaling at various points, as shown in Figure 19, mTOR and MEK inhibitors may potentiate the effects of PI3K/Akt and MEK/ERK signaling on cell growth and apoptosis. Additionally, a synergistic effect can be achieved through combinations of chemotherapy and pathway inhibitors, thereby presenting an attractive treatment route for overcoming drug resistance and limiting side effects. As breast cancer therapy moves towards a targeted, individually tailored approach, the unique mutations and levels of protein kinases such as GSK-3 β in a patient's tumor should serve as valuable markers.

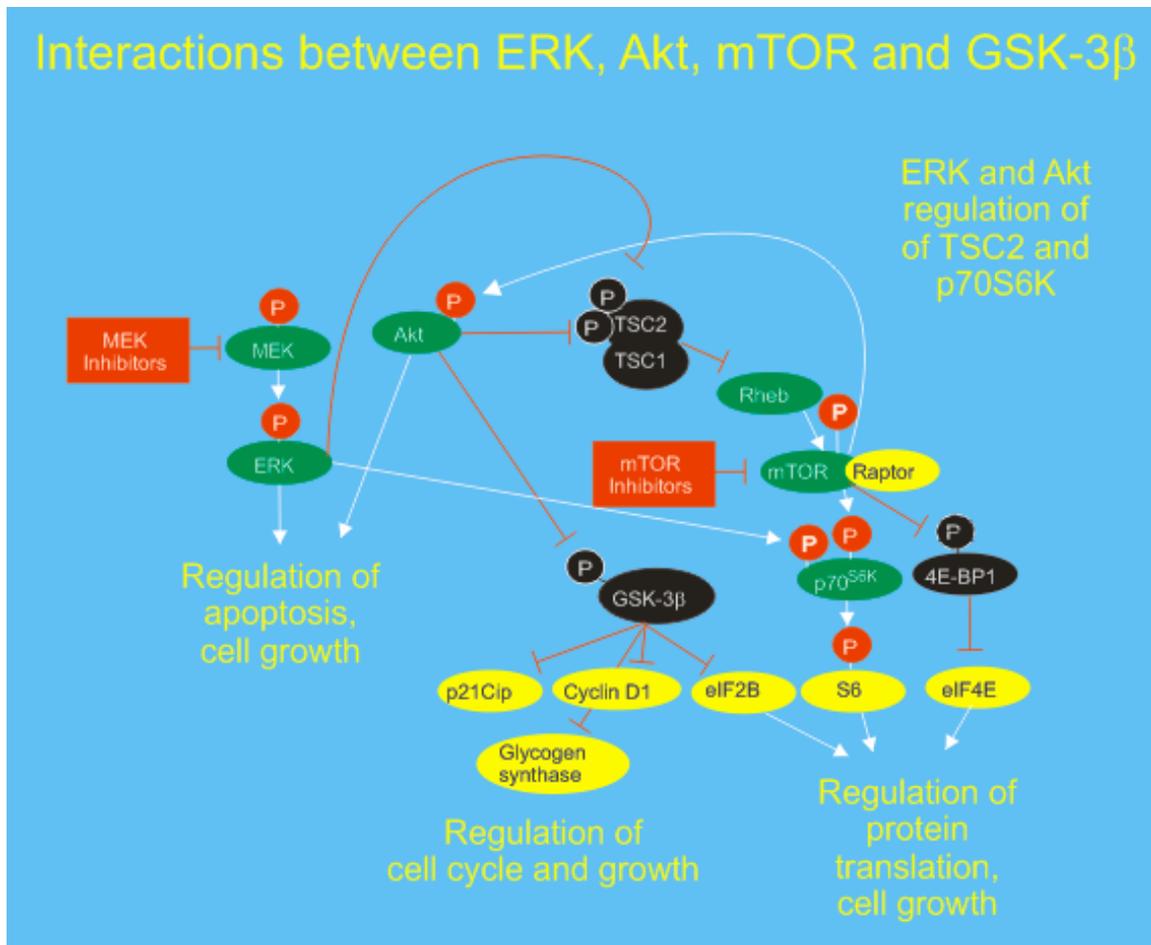


Figure 19: Effects of MEK and mTOR Inhibitors on Cell Signaling

Aberrant activity of the MEK/ERK and PI3K/Akt/mTOR pathways can be targeted through the use of small molecule inhibitors. Signaling can be blocked at various points to modulate the interactions between protein kinases and alter cell cycle progression, translation, and apoptosis

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