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ALTERATION OF AKT ACTIVITY INCREASES CHEMOTHERAPEUTIC DRUG AND HORMONAL RESISTANCE IN BREAST CANCER YET CONFERS AN ACHILLES HEEL BY SENSITIZATION TO TARGETED THERAPY

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Abstract

The PI3K/PTEN/Akt/mTOR pathway plays critical roles in the regulation of cell growth. The effects of this pathway on drug resistance and cellular senescence of breast cancer cells has been a focus of our laboratory. Introduction of activated Akt or mutant PTEN constructs which lack lipid phosphatase [PTEN(G129E)] or lipid and protein phosphatase [PTEN(C124S)] activity increased the resistance of the cells to the chemotherapeutic drug doxorubicin, and the hormonal drug tamoxifen. Activated Akt and PTEN genes also inhibited the induction of senescence after doxorubicin treatment; a phenomenon associated with unrestrained proliferation and tumorigenesis. Interference with the lipid phosphatase domain of PTEN was sufficient to activate Akt/mTOR/p70S6K as MCF-7 cells transfected with the mutant PTEN gene lacking the lipid phosphatase activity [PTEN(G129E)] displayed elevated levels of activated Akt and p70S6K compared to empty vector transfected cells. Cells transfected with mutant PTEN or Akt constructs were hypersensitive to mTOR inhibitors when compared with the parental or empty vector transfected cells. Akt-transfected cells were cultured for over two months in tamoxifen from which tamoxifen and doxorubicin resistant cells were isolated that were >10-fold more resistant to tamoxifen and doxorubicin than the original Akt-transfected cells. These cells had a decreased induction of both activated p53 and total $p21^{Cip1}$ upon doxorubicin treatment. Furthermore, these cells had an increased inactivation of GSK-3β and decreased expression of the estrogen receptor-α. In these drug resistant cells, there was an increased activation

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of ERK which is associated with proliferation. These drug resistant cells were hypersensitive to mTOR inhibitors and also sensitive to MEK inhibitors, indicating that the enhanced p70S6K and ERK expression was relevant to their drug and hormonal resistance. Given that Akt is overexpressed in greater than 50% of breast cancers, our results point to potential therapeutic targets, mTOR and MEK. These studies indicate that activation of the Akt kinase or disruption of the normal activity of the PTEN phosphatase can have dramatic effects on activity of p70S6K and other downstream substrates and thereby altering the therapeutic sensitivity of breast cancer cells. The effects of doxorubicin and tamoxifen on induction of the Raf/MEK/ERK and PI3K/Akt survival pathways were examined in unmodified MCF-7 breast cells. Doxorubicin was a potent inducer of activated ERK and to a lesser extent Akt. Tamoxifen also induced ERK. Thus a consequence of doxorubicin and tamoxifen therapy of breast cancer is the induction of a pro-survival pathway which may contribute to the development of drug resistance. Unmodified MCF-7 cells were also sensitive to MEK and mTOR inhibitors which synergized with both tamoxifen and doxorubicin to induce death. In summary, our results point to the key interactions between the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways in regulating chemotherapeutic drug resistance/sensitivity in breast cancer and indicate that targeting these pathways may prevent drug and hormonal resistance.

Keywords

Conventional therapy; targeted therapy; signal transduction inhibitors; breast cancer

Introduction

Breast cancer ranks as the second most common cause of cancer death among women in the United States. Only lung cancer, which results primarily from cigarette smoking, induces more cancer deaths in women in the USA. Approximately 1 in 7 women in the United States will be diagnosed with breast cancer during her lifetime (Jemal *et al*., 2004). Over 210,000 new cases of breast cancer are diagnosed in the United States each year (Centers for Disease Control and Prevention, 2006). Breast cancer is the cause of death of over 40,000 women in the United States each year. Many drugs have been demonstrated to extend survival of breast cancer patients. Anticancer agents frequently used to treat breast cancer include chemotherapeutic drugs such as methotrexate, 5-fluorouracil (5-FU), cyclophosphamide, anthracyclines, taxanes, monoclonal antibodies such as trastuzumab, hormonal based therapeutics such as tamoxifen and aromatase inhibitors. Mechanisms by which these agents inhibit breast cancer progression vary from drug to drug. While these drugs are the mainstay of chemo, immuno and hormonal therapy of breast cancer, a common problem with these treatments is the development of drug resistance. Breast cancer cells can become drug resistant by multiple mechanisms which include: increased expression of membrane transporters which transport the toxic drug out of the cell or modify/detoxify the drug, increased expression of signaling and anti-apoptotic pathways as well as other mechanisms which allow the cells to grow in the presence of the drug. This manuscript will discuss some of the mechanisms by which the altered expression of key signaling and apoptotic pathways may lead to breast cancer drug resistance and how targeting these pathways may result in the suppression of neoplastic growth.

Overview of the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways and their roles in breast cancer

Mutations may occur in breast and other cancer cells which result in elevated expression or constitutive activation of various growth factor receptors such as the epidermal growth factor receptor (EGFR or c-Erb-B1) or the related c-Erb-B2 receptor. The PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways are often activated by mutations/amplifications in these and other growth factor receptors. These pathways are also regulated by upstream Ras which is

mutated in 20 to 30% of human cancers. The PI3K and B-Raf kinases are also activated by mutation and in particular in breast cancer (Hollestelle *et al.*, 2007). Many of the events elicited by the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways have direct effects on survival and proliferative pathways. Aberrant regulation of these pathways can contribute to uncontrolled cell growth and lead to malignant transformation. Effective targeting of these pathways may result in suppression of cell growth and death of malignant cells. An overview of the effects of PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways on downstream signaling pathways leading to growth and the prevention of apoptosis in breast cancer is presented in Figure 1.

The PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways are also activated by many growth factors and cytokines which are important in driving proliferation and preventing apoptosis of breast cells (Blalock *et al.*, 1999, Lee *et al.*, 2002, Steelman *et al.*, 2004, McCubrey *et al.*, 2005, McCubrey *et al.*, 2007). After growth receptor ligation, Shc, a Src homology (SH)-2 (SH2)-domain containing protein, becomes associated with the c-terminus of the growth factor receptor (Matsuguchi *et al.*, 1994, Inhorn *et al.*, 1995, Okuda *et al.*, 1999). Shc recruits the GTP-exchange complex growth factor receptor bound-2 (Grb2)/son of sevenless exchange (Sos) proteins resulting in the loading of membrane bound Ras with GTP (Tauchi *et al.*, 1994, Lanfrancone *et al.*, 1995). Ras:GTP then recruits Raf to the membrane where it becomes activated, likely via a Src-family tyrosine (Y) kinase (Karin *et al.*, 1994, Lange-Carter *et al.*, 1994, Marais *et al.*, 1995). Raf is responsible for phosphorylation of the mitogen associated/ extracellular regulated kinase-1 (MEK1) (Xu *et al.*, 1995, Marais *et al.*, 1997, Mason *et al.*, 1999). MEK1 phosphorylates extracellular regulated kinases 1 and 2 (ERKs 1 and 2) on specific threonine (T) and Y residues (Xu *et al.*, 1995, Marais *et al.*, 1997, Mason *et al.*, 1999). Activated ERK1 and ERK2 serine (S)/T kinases phosphorylate and activate a variety of substrates. The number of potential substrates of the ERK kinases has been speculated to be more than several hundred. The 90 kDa ribosomal six kinase 1 $p90^{Rsk1}$ is one such substrate (Deng *et al.*, 1994, Davis *et al.*, 1995, Xing *et al.*, 1996, Cardone *et al.*, 1998, Coutant *et al.*, 2002, Iijima *et al.*, 2002, Allan *et al.*, 2003, Blalock *et al.*, 2003). $p90^{Rsk1}$ can activate the cyclic-AMP response element binding protein (CREB) transcription factor (Xing *et al.*, 1996). Moreover, ERK can translocate to the nucleus and phosphorylate additional transcription factors such as Elk1, CREB and Fos which bind promoters of many genes, including growth factor and cytokine genes important in stimulating the growth and survival of multiple cell types including breast cells (Deng *et al.*, 1994, Wang *et al.*, 1994, Davis., 1995, Thomas *et al.*, 1997, Robinson *et al.*, 1998, McCubrey *et al.*, 2000, Aplin *et al.*, 2001, Tresini *et al.*, 2001, Eblen *et al.*, 2001, Adachi *et al.*, 2002, Ponti *et al.*, 2002, Fry *et al.*, 2002). The Raf/MEK/ERK pathway can also modulate the activity of many proteins involved in apoptosis including: Bcl-2, Bad, Bim, myeloid cell leukemia-1 (Mcl-1), caspase 9, and survivin (Deng *et al.*, 2001, Carter *et al.*, 2003, Jia *et al.*, 2003, Troppmair *et al.*, 2003, Ley *et al.*, 2003, Weston *et al.*, 2003, Domina *et al.*, 2004, Harada *et al.*, 2004, Marani *et al.*, 2004, Gelinas *et al.*, 2006, McCubrey *et al.*, 2007).

Growth factor/cytokine receptor ligation also leads to rapid activation of phosphatidylinositol 3-kinase (PI3K) (Martelli *et al.*, 2007). Class IA PI3Ks are heterodimeric proteins which consist of 85-kDa regulatory and 110-kDa catalytic subunits. The p85 regulatory subunit contains: a SH2 domain, that recognizes phosphorylated (p) Y (pY) residues, an iSH2 domain (a rigid tether for p110), a conserved domain related to sequences present in the Break Point Cluster Region (BCR) gene and a SH3 domain which are found in proteins that interact with other proteins and mediate assembly of specific protein complexes via binding to proline-rich motifs (Yu *et al.*, 1996, Fu *et al.*, 2004). The 110-kDa PI3K Cass 1A catalytic subunit contains a p85 binding domain, a Ras binding domain, a kinase domain and a helical domain (Chang *et al.*, 2003, Steelman *et al.*, 2004). Cytokine stimulation often creates a PI3K binding site on the cytokine receptor. The p85 subunit SH2 domain associates with this site (Rao *et al.*, 1995,

Drexler *et al.*, 1996, Chang *et al.*, 2003). The p85 subunit is then phosphorylated which leads to activation of the p110 catalytic subunit. This often occurs at the inner leaflet of the cytoplasmic membrane, although there are important roles of PI3K in the nuclear membranes which have been reviewed recently (Martelli *et al.*, 2006, Evangelisti *et al.*, 2007, Cocco *et al.*, 2007).

The class IA regulatory subunits recruit the p110 catalytic subunit to pY residues in receptors, adaptor proteins and other molecules. This localizes the class IA p110 subunits in the membranes where their lipid substrates reside. The adaptor/regulatory subunits act to localize PI3K to the plasma membrane by the interaction of their SH2 domains with pY residues in activated receptors. They also serve to stabilize p110 and to limit its activity.

The preferred substrate for class I PI3K, which is the class of PI3K discussed in this manuscript, *in vivo* is phosphatidylinositol 4,5 bisphosphate [PtdIns(4,5)P_2] which is phosphorylated to yield phosphatidylinositol 3,4,5 trisphosphate [PtdIns(3,4,5)P_3]. PtdIns(3,4,5)P_3 serves as an anchor for pleckstrin homology (PH) domain-containing proteins, such as Akt or phosphoinositide-dependent protein kinase-1 (PDK-1). PDK1 then phosphorylates an Akt T regulatory residue. Akt is also a member of a multi-gene family (Akt-1, Akt-2 and Akt-3) and is also called protein kinase B (PKB). Depending on the Akt isoform, PDK1 can phosphorylate Akt on T 308/309/305. A second kinase phosphorylates Akt on a S regulatory residue (S 473/474/472) (Songyang *et al.*, 1997, Troussasrd *et al.*, 2003, Xu *et al.*, 2003, Persad *et al.*, 2003). The identity of the second kinase which phosphorylates Akt has remained elusive. Integrin linked kinase (ILK), PDK1, rictor-mTOR complex (see below) and Akt autophosphorylation have all been suggested to be responsible for phosphorylation of Akt at the second S phosphorylation site (Noguchi et *al.*, 2007). The PH domain leucine-rich repeat protein phosphatase (PHLPP) dephosphorylates S473 on Akt which induces apoptosis and inhibits tumor growth (Gao *et al.*, 2005)

Akt has been postulated to phosphorylate over nine thousand proteins (Lawlor *et al.*, 2001, Hay, 2005). Thus Akt is clearly a critical growth regulatory switch. Akt can transduce an anti-apoptotic signal by phosphorylating downstream target proteins involved in the regulation of cell growth [*e.g.*, glycogen synthase kinase-3β (GSK-3β), apoptotic signal kinase (ASK1), Bim, Bad, MDM-2, $p21^{Cip1}$, X-linked inhibitor of apoptosis (XIAP) and the Foxo3a transcription factor] (Songyang *et al.*, 1997, del Peso *et al.*, 1997, Scheid *et al.*, 1998, Nakae *et al.*, 1999, Brunet *et al.*, 1999, Mayo *et al.*, 2000, Medema *et al.*, 2000, Dijkers *et al.*, 2000, Mayo *et al.*, 2001, Gottlieb *et al.*, 2002, Zhou *et al.*, 2002, Dan *et al.*, 2004, Qi *et al.*, 2006). Phosphorylated Foxo3a loses its ability to induce Fas, $p27^{Kip1}$, Bim, Noxa, and Puma gene transcription (You *et al.*, 2006, Obexer *et al.*, 2007). Akt also phosphorylates I-κB kinase (I-κK), which subsequently phosphorylates inhibitory subunit (I-κB) which binds nuclear transcription factor kappa light chain in B cells (NF-κB) transcription factor, resulting in its ubiquitination and subsequent degradation in proteosomes (Ozes *et al.*, 1999, Romashkova *et al.*, 1999, Mayo *et al.*, 2000, Madrid *et al.*, 2000, Howe *et al.*, 2002, Howe *et al.*, 2004, Hu *et al.*, 2004, Shishodia *et al.*, 2004). Disassociation of I-κB from NF-κB enables NF-κB to translocate into the nucleus to promote gene expression. The PI3K/Akt pathway can also phosphorylate and activate CREB which regulates transcription of Mcl-1 and B cell leukemia-2 Bcl-2 (Du *et al.*, 1998, Wang *et al.*, 1999, Arcinas *et al.*, 2001). The PI3K/Akt pathway also regulates the mammalian target of rapamycin (mTOR) and ribosomal protein kinases such as p70 ribosomal six kinase (p70S6K) (Mahalingam *et al.*, 1996, Dufner *et al.*, 1999, Romanelli *et al.*, 1999, Harada *et al.*, 2001, Edinger *et al.*, 2004, Panwalker *et al.*, 2004, Jonassen *et al.*, 2004, Ma *et al.*, 2005) (see Below). It is worth noting that Akt can cause the activation of specific substrates (*e.g.*, IκKα and CREB) or may mediate the inactivation of other proteins (*e.g.*, Raf-1, B-Raf [by the Akt related kinase serum glucocorticoid kinase (SGK)], $p21^{Cip-1}$, Bim, Bad, procaspase-9, Foxo3a, and GSK-3β). An overview of the effects

of the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways on apoptotic and cell cycle cascades is presented in Figure 2.

mTOR is a 289-kDa S/T kinase. It regulates translation in response to nutrients/growth factors by phosphorylating components of the protein synthesis machinery, including p70S6K and eukaryotic initiation factor (eIF)-4E binding protein (4EBP-1), the latter resulting in release of the translation initiation factor eIF-4E, allowing eIF-4E to participate in the assembly of a translational initiation complex (Wendel *et al.*, 2004). p70S6K, which can also be directly activated by PDK-1, phosphorylates the 40S ribosomal protein, S6, leading to active translation of mRNAs (Giles *et al.*, 2005). Integration of a variety of signals (mitogens, growth factors, hormones) by mTOR assures cell cycle entry only if nutrients and energy are sufficient for cell duplication (Fingar *et al.*, 2004, Tokunaga *et al.*, 2004). Therefore, mTOR controls multiple steps involved in protein synthesis, but importantly enhances production of key molecules such as c-Myc, cyclin D1, $p27^{Kip1}$, and retinoblastoma protein (pRb) (Martin *et al.*, 2004). mTOR also controls the translation of hypoxia-inducible transcription factor-1α (HIF-1α) mRNA. HIF-1α upregulation leads to increased expression of angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (Witzig *et al.*, 2006). Moreover, HIF-1α regulates the glycolytic pathway by controlling the expression of glucose-sensing molecules including glucose transporter (Glut) 1 and Glut3 (Mobasheri *et al.*, 2005).

By regulating protein synthesis, p70S6K and 4E-BP1 also control cell growth and hypertrophy, which are important processes for neoplastic progression. Akt-mediated regulation of mTOR activity is a complex multi-step phenomenon. Akt inhibits tuberous sclerosis 2 (TSC2 or hamartin) function through direct phosphorylation (Manning *et al.*, 2002). TSC2 is a GTPase-activating protein (GAP) that functions in association with the putative tuberous sclerosis 1 (TSC1 or tuberin) to inactivate the small GTPase protein Rheb (Ras homolog enriched in brain) (Zhang *et al.*, 2003). TSC2 phosphorylation by Akt represses GAP activity of the TSC1/TSC2 complex, allowing Rheb to accumulate in a GTP-bound state. Rheb-GTP then activates, through a mechanism not yet elucidated, the protein kinase activity of mTOR when complexed with the Raptor (Regulatory associated protein of mTOR) adaptor protein and mLST8, a member of the Lethal-with-Sec-Thirteen gene family, first identified in yeast (Hay, 2005). The mTOR/Raptor/mLST8 complex (mTORC1) is sensitive to rapamycin and, importantly, inhibits Akt via a negative feedback loop which involves, at least in part, p70S6K (Hay, 2005). The relationship between Akt and mTOR is further complicated by the existence of the mTOR/Rictor (Rapamycin-insensitive companion of mTOR/mLST8 complex (mTORC2), which displays rapamycin-insensitive activity. The mTORC2 complex has been found to directly phosphorylate Akt on S473 *in vitro* and to facilitate T308 phosphorylation. Thus, the mTORC2 complex might be the elusive PDK-2 which phosphorylates Akt on S473 in response to growth factor stimulation (Hresko *et al.*, 2005). Akt and mTOR are linked to each other via ill-defined positive and negative regulatory circuits, which restrain their simultaneous hyperactivation through a mechanism involving p70S6K and PI3K. Assuming that equilibrium exists between these two complexes within the cell, when the mTORC1 complex is formed, it could antagonize the formation of the mTORC2 complex and reduce Akt activity (Hresko *et al.*, 2005). Thus, at least in principle, inhibition of the mTORC1 complex could result in Akt hyperactivation. This is one complication associated with rapamycin treatment (see below).

The PI3K/PTEN/Akt/mTOR pathway is negatively regulated by phosphatases. PTEN (phosphatase and tensin homologue deleted on chromosome ten) is primarily a lipid phosphatase that removes the 3-phosphate from the PI3K lipid product PtdIns $(3,4,5)P_3$ to produce PtdIns $(4,5)P_2$ which prevents Akt activation (Chang *et al.*, 2003, Li *et al.*, 1997., Steck *et al.*, 1997, Li *et al.*, 1997, Steelman *et al.*, 2004, Shaw *et al.*, 2006). PTEN is also a protein phosphatase (Steelman *et al.*, 2004, Mahimainathan *et al.*, 2004, Raftopoulou *et al.*,

2004). Two other phosphatases, SH2 domain-containing inositol 5′phosphatase (SHIP)-1 and SHIP-2, remove the 5-phosphate from PtdIns(3,4,5)P_3 to produce PtdIns(3,4)P_2 (Damen *et al.*, 1996, Kavanaugh *et al.*, 1996, Lioubin *et al.*, 1996, Muraille *et al.*, 1999, Taylor *et al.*, 2000). Mutations in these phosphatases, which eliminate their activity, can lead to tumor progression. Consequently, the genes encoding these phosphatases are referred to as anti-oncogenes or tumor suppressor genes.

Signaling pathways and breast cancer

Breast cancer originates from genetic causes. Mutated or amplified genes are either inherited or occur sporadically. Hereditary breast cancer accounts for only about 10% of all breast cancer cases and generally results from lack of a tumor suppressor gene as opposed to gain of an oncogene. Approximately 45% of hereditary breast cancer is attributable to mutations in breast cancer associated gene-1 (BRCA1) and an additional 45% is attributable to mutation in BRCA2 (Centers for Disease Control and Prevention, 2006). Other tumor suppressor genes implicated in hereditary breast cancer include p53 and PTEN (Steelman *et al.*, 2004). The p53 tumor suppressor is a transcription factor involved in cell cycle regulation and DNA damage repair. Germline p53 mutation is present in approximately 50% of patients with Li-Fraumeni syndrome (LFS), which is a multicancer familial syndrome that includes adrenocortical carcinoma, brain tumors, leukemia, and osteosarcomas in addition to early onset breast cancer. Breast cancer attributable to germline p53 mutation in the absence of LFS is rare. Germline PTEN mutation is present in approximately 80% of patients with Cowden syndrome (CS) (Navolanic *et al.*, 2003, Steelman *et al.*, 2004). This disease, which is also known as multiple hamartoma syndrome, is another familial syndrome that includes many different types of cancer conditions including early onset breast cancer. Mutations have been reported to occur at PTEN in breast cancer in varying frequencies (5-21%) (Rhei *et al.*, 1997, Singh *et al.*, 1998, Feilotter *et al.*, 1999, DeGraffenried *et al.*, 2004, Hollestelle *et al.*, 2007). Loss of heterozygosity (LOH) at PTEN is probably more common (30%) (Singh *et al.*, 1998). PTEN promoter methylation leads to low expression. In one study, 26% of primary breast cancers had low PTEN levels (Singh *et al.*, 1998). Low PTEN levels have been correlated with lymph node metastases and poor prognoses (Singh *et al.*, 1998, DeGraffenried *et al.*, 2004, Garcia *et al.*, 2004, Tsutsui *et al.*, 2005). Mutations at certain residues of PTEN, which are associated with CS, affect the ubiquitination of PTEN and prevent nuclear translocation. These mutations leave the phosphatase activity intact (Trotman *et al.*, 2007). Inhibition of PTEN activity leads to centromere breakage and chromosome instability (Shen *et al.*, 2007). PTEN expression may also be silenced in the absence of obvious genetic mutations. Disruption of PTEN activity by various genetic mechanisms could have vast effects on different processes affecting the sensitivity of breast cancers to diverse therapeutic approaches.

Sporadic breast cancer accounts for the remaining 90% of all breast cancer cases. The PI3K p110 catalytic subunit is mutated in approximately 25% of breast cancer specimens and these mutations frequently result in activation of its kinase activity (Bader *et al.*, 2005, Kang *et al.*, 2005, Engleman *et al.*, 2006, Vogt *et al.*, 2006, Tokunaga *et al.*, 2006, Hollestelle *et al.*, 2007) (See below). Somatic mutation of p53 is associated with many cancers and exists in approximately 20% of sporadic breast cancer cases. In contrast, somatic mutation of BCRA1 or BCRA2 is rare in breast cancer patients. Another important cause of sporadic breast cancer is amplification/overexpression of HER2 which occurs in approximately 25 to 30% of breast cancer. Expression and activity of downstream signal transduction cascades, such as the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways, change as a result of these mutations. Akt and ERK are frequently activated in breast cancer specimens (Hollestelle *et al.*, 2007, Greenman *et al.*, 2007). Thus the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways are therapeutic targets in breast cancer.

Association of genes that regulate signal transduction pathways with breast cancer implies an important role of these pathways in this disease. Perhaps the best example of this is association of HER2 gene amplification with breast cancer. While a normal breast cell possesses 20,000 to 50,000 HER2 molecules, amplification of this gene can increase levels of HER2 up to 2,000,000 molecules per cell (Chang *et al.*, 2003, Shelton *et al.*, 2005a). Overexpression of HER2 in breast cancers is linked to comedo forms of ductal carcinoma in situ (DCIS). DCIS is present in approximately 90% of the patients with HER2 amplification. HER2 overexpression will lead to increased expression of both the Raf/MEK/ERK and PI3K/Akt pathways. An overview of some of the mutations which can result in activation of the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK cascades is presented in Figure 3.

Effective targeting of signal transduction pathways activated by mutations and gene amplification may be an effective means to limit cancer growth and metastasis (See below). The PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK and PI3K/Akt pathways can be activated by mutations/amplifications of upstream growth factor receptors.

Mutations that lead to the expression of constitutively-active Ras proteins have been observed in approximately 20 to 30% of human cancers (Flotho *et al.*, 1999, Stirewalt *et al.*, 2001). These are often point mutations which alter key residues that affect Ras activity, although amplification of Ras is also detected in some tumors. Mutations that result in increased Ras activity also perturb the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK kinase cascades.

In a recent survey of 40 breast cancer lines, many were mutated at components of either the PI3K/PTEN/Akt/mTOR or Raf/MEK/ERK pathways (Hollestelle *et al.*, 2007). 36% were mutated at *PIK3CA* (PI3K p110 subunit gene), 21% at PTEN (with PTEN either mutation or no protein present), 13% were mutated at *K-RAS*, 5% at *H-RAS*, 3% at *N-RAS*, and 10% at *B-RAF*. In other studies it has been shown that the PI3K p110 catalytic subunit (*PIK3CA*) is mutated in: approximately 25% of breast, 32 % of colorectal, 27% of brain, 25% of gastric, 4% of lung cancers (Samuels *et al.*, 2004; Kang *et al.*, 2005, Vogt *et al.*, 2006). These mutations frequently result in activation of its kinase activity. A recent report indicated that Akt-1 is mutated in: 8% of breast, 6% of colorectal and 2% of ovarian cancers examined in that study (Carpten *et al.*, 2007). This mutation results in a lysine (K) substitution for glutamic acid (E) at amino acid 17 (E17K) in the PH domain. Cells with this Akt1 mutation have not been observed to have mutations at PI3K, a similar scenario is also frequently observed with Ras and B-Raf mutations (Greenman *et al.*, 2007). The Akt mutation alters the electrostatic interactions of Akt which allows it to form new hydrogen bonds with the natural phosphoinositol ligand (Carpten *et al.*, 2007). The PH domain mutation confers many different properties to the Akt-1 gene. Namely, the mutant Akt-1 gene: 1) has an altered PH domain conformation, 2) is constitutively active, 3) has an altered cellular distribution as it is constitutively associated with the cell membrane, 4) morphologically transforms Rat-1 tissue culture cells and 5) interacts with c-Myc to induce leukemia in Eμ-Myc mice (Eμ = enhancer of immunoglobulin M (μ) gene, Myc = Myc oncogene originally isolated in avian myelocytomatosis virus) (Carpten *et al.*, 2007). This PH domain mutated Akt-1 gene does not alter its sensitivity to ATP competitive inhibitors, but does change its sensitivity to allosteric kinase inhibitors (Carpten *et al.*, 2007). These results demonstrate that targeting the kinase domain of Akt may not be sufficient to suppress the activity of various Akt genes which have mutations in the PH domain (Carpten *et al.*, 2007). It will be important to determine if this Akt-1 mutation (E17K) is also present in other solid and non solid tumors as well as its overall mutation frequency in human cancer. In summary, mutations do occur at key components of the PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK pathways in breast cancer and targeting of these pathways may be important therapeutic approach, either by themselves as monotherapy or combination methods with various chemo-hormonal- and antibody treatments.

Furthermore as stated previously, components of these pathways may be activated either by upstream mutations or by other genetic/epigenetic mechanisms. Activated Akt is often upregulated in breast cancer cells and its overexpression has been associated with a poor prognosis (Clark *et al.*, 2002, DeGraffenried *et al.*, 2004, Tsutui *et al.*, 2005, Kirkegaard *et al.*, 2005, Tokunaga *et al.*, 2006). However, this may actually render the breast cancer cells sensitive to Akt as well as downstream mTOR inhibitors. The formation of the rapamycin sensitive mTORC1 complex in the drug resistant breast cancer cells which overexpress activated Akt may be different than in drug sensitive breast cells which do not overexpress activated Akt. In cells which express activated Akt, Akt should phosphorylate TSC-2 resulting in its inactivation. mTORC1 complex is then formed and downstream p70S6K and 4E-BP1 are phosphorylated, allowing the disassociation of eIF-4E, ribosome biogenesis and protein synthesis. In contrast, in the absence of constitutive Akt and ERK activation, which is the case in the drug sensitive cell, this complex should not be formed. Rapamycin targets this complex; hence the cells which constitutively-express activated Akt cells are more sensitive to rapamycin than the cells which do not. In the cells which do not constitutively-express activated Akt, this complex should be transiently assembled after growth factor treatment. In contrast, the assembly of the rapamycin-insensitive mTORC2 complex (Rictor, mTOR, mLST8) should be lower in the cells that constitutively express activated Akt than in those cells that do not as there is equilibrium between the mTORC1 and mTORC2 complexes. The significance of these complex biochemical signaling events is that breast cancer cells which overexpress activated Akt or lack PTEN expression have an Achilles heel with regards to therapeutic intervention as they are highly sensitive to rapamycin treatment. Breast cancer may also be sensitive to Raf/MEK inhibitors as ERK plays critical roles in the phosphorylation of TSC-2 and p70S6K (see below).

We have observed that ERK is activated after tamoxifen and doxorubicin treatment of the MCF-7 breast cancer cell line. Furthermore, drug resistant derivative cell lines have increased levels of constitutively active ERK. These results are important with regards to the sensitivity of breast cancer cells to MEK inhibitors. ERK can phosphorylate and contribute to the inactivation of TSC-2. Akt can also phosphorylate TSC-2, at a different residue, which leads to its inactivation. This also leads to mTOR activation. Inhibition of TSC-2 phosphorylation by PI3K/Akt and Raf/MEK inhibitors may make cells more sensitive to chemo-, hormonal-, and immunotherapies.

Aberrant regulation of apoptosis may contribute to breast cancer and subsequent drug resistance

Cell death following cytotoxic drug treatment is generally apoptotic as opposed to necrotic. Many chemotherapeutic drugs induce apoptosis by activating the intrinsic cell death pathway which involves cytochrome c release and activation of the apoptosome-catalyzed caspase cascade. During apoptosis, activation of caspase family cysteine proteases occurs. Although various cytotoxic drugs differ in their mechanisms of action, each ultimately relies upon built in apoptotic machinery to elicit cell death (Deng *et al.*, 2001, Harada *et al.*, 2001, Navolanic *et al.*, 2003, Chang *et al.*, 2003, Steelman *et al.*, 2004, Martelli *et al.*, 2007). Caspase family cysteine proteases are responsible for proteolytic cleavage of cellular proteins carboxyl terminal to aspartate residues.

In a study involving 46 breast cancer patients, 75% were determined to lack caspase-3 mRNA transcripts and protein expression (Devarajan *et al.*, 2002). The MCF-7 cell line has a mutation in caspase-3 and is deficient in certain aspects of apoptosis. In this respect, MCF-7 cells are a stringent model to investigate breast cancer apoptosis (Liang *et al.*, 2001). Caspase-9 can be activated in MCF-7 cells which can result in the sequential activation of caspases-7 and -6 (Twiddy *et al.*, 2006). Caspase-7 is activated by the apoptosome complex and forms a XIAP-

caspase-7 complex. This XIAP-caspase-7 complex is more stable in MCF-7 cells due to the absence of functional caspase-3. ERK activity maintains XIAP levels; however the mechanism by which this occurs is unknown. Resistance to chemotherapeutic drugs induced by the Raf/MEK/ERK pathway may be due in part to prolonged XIAP and caspase 9 expression which prevents caspase 7 from exerting its effects on apoptosis (Dan *et al.*, 2004, Gardai *et al.*, 2004). ERK phosphorylates caspase-9, which inhibits its activation. Negative regulation of caspases by ERK represents a mechanism by which Raf/MEK/ERK pathway activation prevents apoptosis. Raf/MEK inhibitors may affect caspase 9 activation and XIAP levels and promote apoptosis of breast cancer cells (see below).

Therapeutic targeting of the PI3K/Akt/PTEN/mTOR and Raf/MEK/ERK pathways

Small molecule inhibitors such as Imatinib have proven effective in the treatment of chronic myeloid leukemia (CML) and certain other cancers which proliferate in response to translocated BCR-ABL, mutant platelet derived growth factor receptor (PDGF-R) and c-Kit genes (Lee *et al.*, 2002, Hochhaus *et al.*, 2002, Navolanic *et al.*, 2003, Steelman *et al.*, 2004, Barnes *et al.*, 2005, Hollestelle *et al.*, 2007) such as gastro-intestinal stromal tumors (GIST). Non small cell lung carcinomas (NSCLC) which have mutations in EGFR are sensitive to EGFR inhibitors (Lynch *et al.*, 2004, Sordella *et al.*, 2004, Pao *et al.*, 2004, Shelton *et al.*, 2005, Pao *et al.*, 2005, Sequist *et al.*, 2005, McCubrey *et al.*, 2007). Currently, these cancers are the poster children of targeted therapies as their growth can be successfully inhibited and dramatic effects observed in cancer patients. Certain antibodies have been developed such as Herceptin which effectively target HER2 those breast cancer patients (25 to 30%) which overexpress HER2 due to genetic amplification and other mechanisms (Shelton *et al.*, 2005a).

PI3K, PDK, Akt, and mTOR inhibitors have been and are being developed. mTOR inhibitors have been used for many years as immunosuppressive drugs in kidney transplant patients. A side effect of mTOR inhibitors is the inhibition of a negative feed back pathway which can result in Akt activation (Weber et al., 2004). A diagram illustrating the sites of action and other inhibitors is presented in Figure 4.

PI3K can be inhibited with many inhibitors including: LY294002, Wortmannin, KN309, SF1124 and PX-866. Novel PI3K inhibitors have been developed. PWT-458 is a pegylated-17-hydroxywortmannin which inhibits PI3K and has been shown to suppress glioma, NSCLC and renal cell carcinoma in xenograft models (Yu *et al.*, 2005). PWT-458 augmented the anticancer effects of paclitaxel and pegylated rapamycin in certain xenograft models.

PDK1 can be inhibited with OSU-03012 and Celecoxib. Akt can be inhibited with many inhibitors including; A-443654, API-2, PX316, Curcumin, Rotenone (Deguelin), and Perifosine. Perifosine is an oral bioactive novel alkylphospholipid that inhibits Akt. mTOR can be inhibited by rapamycin and modified rapamycins (CCI-779, RAD001 and AP23573). Initially mTOR inhibitors showed much promise as PTEN is often deleted, mutated or silenced in various tumors by many different mechanisms. However, it has been recently determined that the mTOR pathway has a complicated feed-back loop which actually involves suppression of Akt, hence mTOR inhibitors would be predicted to activate Akt in some cells. Due to the complicated Akt/mTOR pathway, with many inhibitory phosphorylation events, an alternative therapeutic approach will be to develop strategies which simultaneously inhibit both Akt and mTOR activity. As pointed out previously, recently mutations in the PH domain of Akt have been detected in certain human cancers. Cells harboring these mutations may be refractory to Akt inhibitors which target the kinase domain but may be more sensitive to inhibitors which target the PH domain (Carpten *et al.*, 2007). The role of overexpression of the different Akt isoforms in cancer prognosis is controversial (Kornblau *et al.*, 2006, Tamburini *et al.*, 2007). Some studies have suggested that elevated Akt expression is associated with a poor prognosis

in breast (Kirkegaard *et al.*, 2005) and hematopoietic (Kornblau *et al.*, 2006) cancers. Controversially it has been observed recently that constitutive PI3K/Akt activation is a favorable prognostic factor in AML patients (Tamburini *et al.*, 2007) as Akt activation may keep a high percentage of the cells in S phase which renders them sensitive to chemotherapeutic drugs. These and other studies document the complexity of enhanced Akt expression which may make certain cells more sensitive or resistant to certain anti-cancer therapies.

Recent evidence has highlighted that mTOR can also be activated by Raf/MEK/ERK (Mukherjee *et al.*, 2005, Shimada *et al.*, 2006). This may well be another relevant cross-talk between the PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK pathways and offer a further rationale for treatments combining drugs which inhibit both signaling networks.

Raf and MEK inhibitors have been developed and some are in clinical trials (Hochhaus *et al.*, 2002, Navolanic *et al.*, 2003, Steelman *et al.*, 2004, Hollestelle *et al.*, 2007, Tortora *et al.*, 2007). We have determined that a consequence of doxorubicin and hormonal treatment of some breast, hematopoietic and prostate cancer cell lines is the induction of ERK (McCubrey *et al.*, 2007). Eliminating this deleterious side-effect of these compounds with Raf and MEK inhibitors may enhance the ability of chemo- and hormonal based therapies to kill drug resistant cancer.

Certain Raf inhibitors have been developed which are small molecule competitive inhibitors of the ATP-binding site of Raf protein. These inhibitors (*e.g.*, L-779,450, ZM 336372, Bay 43-9006, *a.k.a* Sorafenib) bind the Raf kinase domain and therefore prevent its activity. Some Raf inhibitors may affect a single Raf isoform (*e.g.*, Raf-1), others may affect Raf proteins, which are more similar (Raf-1 and A-Raf), while other pan Raf inhibitors may affect all three Raf proteins (Raf-1, A-Raf and B-Raf). We have observed that the L-779,450 inhibitor suppresses the effects of A-Raf and Raf-1 more than the effects of B-Raf (Shelton *et al.*, 2003). Like many Raf inhibitors, L-779,450 is not specific for Raf; it also inhibits the closely related $p38^{MAPK}$. Likewise, Sorafenib inhibits other kinases besides Raf (*e.g.*, VEGF-II Receptor, PDGF-R, Kit, Flt-3, and Fms) and is more appropriately referred to as a multi-kinase inhibitor. Knowledge of the particular Raf gene mutated or overexpressed in certain tumors may provide critical information regarding how to treat the patient as some cancers which overexpress a particular Raf gene may be more sensitive to inhibition by agents which target that particular Raf protein. Inhibition of certain Raf proteins might prove beneficial while suppression of other Raf proteins under certain circumstances might prove detrimental. Thus the development of unique and broad-spectrum Raf inhibitors may prove useful in cancer therapy. Some “Raf” inhibitors (Sorafenib) are approved for the treatment of certain cancers (*e.g.*, renal cell carcinoma) (Heimbrook *et al.*, 1998, Hall-Jackson *et al.*, 1999, Lyons *et al.*, 2001, Blagosklonny 2002, Shelton *et al.*, 2003, Tortora *et al.*, 2007).

Currently it is believed that MEK1 is not frequently mutated in human cancer. Recently, there was a report that MEK1 and MEK2, as well as B-Raf, are mutated in some patients with cardio-facio cutaneous syndrome (Rodriguez-Viciana *et al.*, 2006). Aberrant expression of MEK1 is observed in many different cancers due to the activation of the Raf/MEK/ERK pathway by upstream kinases (*e.g.*, BCR-ABL) and growth factor receptors (*e.g.*, EGFR, Fms, Flt-3, PDGFR) as well as other unknown mechanisms. Specific inhibitors to MEK have been developed (PD98059, U0126, PD184352 (*a.k.a.*, CI1040), PD-0325901, Array MEK inhibitors [ARRY-142886 and others]) (Tortora *et al.*, 2007). The successful development of MEK inhibitors may be due to the relatively few phosphorylation sites on MEK involved in activation/inactivation. MEK inhibitors differ from most other kinase inhibitors as they do not compete with ATP binding which confers a very high specificity (Delaney *et al.*, 2002). MEK inhibitors are very specific and do not inhibit many different protein kinases including $p38^{MAPK}$ and JNK (Davies *et al.*, 2000). The crystal structures of MEK1 and MEK2 have been

determined as ternary complexes with ATP and PD184352 and have revealed that both MEK1 and MEK2 have unique inhibitor binding sites located on a hydrophobic pocket adjacent to but not overlapping with the ATP binding site (Ohren *et al.*, 2004). Furthermore, effective targeting of MEK1,2 is highly specific as ERK1,2 are the only well-described downstream targets. An advantage of targeting the Raf/MEK/ERK cascade is that it can be targeted without knowledge of the precise genetic mutation, which results in its aberrant activation. This is important as the nature of the critical mutation(s), which leads to the drug resistant growth of at many breast cancers, is not currently known. An advantage of targeting MEK is that the Raf/MEK/ERK pathway is a convergence point where a number of upstream signaling pathways can be blocked with the inhibition of a single kinase (MEK). MEK inhibitors such as ARRY-142886 (AZD6244) are also being evaluated to treat hematopoietic malignancies such as multiple myeloma (Tai *et al.*, 2006, Wang *et al.*, 2006, Yeh *et al.*, 2007).

To our knowledge, no small molecular weight ERK inhibitors have been developed yet, however, inhibitors to ERK could prove very useful as ERK can phosphorylate many targets (Rsk, c-Myc, Elk, and at least 150 more). There are at least 2 ERK molecules regulated by the Raf/MEK/ERK cascade, ERK1 and ERK2. Little is known about the different *in vivo* targets of ERK1 and ERK2. However ERK2 has been postulated to have pro-proliferative effects while ERK1 may have anti-proliferative effects (Mazzucchelli *et al.*, 2002). Development of specific inhibitors to ERK1 and ERK2 might eventually prove useful in the treatment of certain diseases.

Combination therapies to enhance toxicity and effectiveness of breast cancer therapy

Classical chemotherapy often remains the most used anti-cancer therapy for many different types of cancers including breast cancer. Drugs such as doxorubicin and taxol are effective in the treatment of many cancers, even though in some cases drug resistance does develop after prolonged treatment. Doxorubicin and taxol target cellular events such as DNA replication and cell division which are downstream of the targets of signal transduction pathway inhibitors. Thus by combining classical chemotherapy with targeted therapy, it may be possible to enhance toxicity while lowering the effective concentrations of classical chemotherapeutics necessary for effective elimination of the particular tumor.

Recent studies have indicated that the effectiveness of certain antibody based therapies (*e.g.*, Herceptin, which targets HER2) may be greatly enhanced by inclusion of mTOR inhibitors. These observations were obtained in both preclinical studies performed in tissue culture and xenograft models and are being further evaluated in phase 2 clinical trials (Wang *et al.*, 2007). The cytotoxic effects of Herceptin can also be improved by addition of an inhibitor such as Lapatinib which targets both EGFR and HER2 (Konecny *et al.*, 2006). Many ongoing clinical trials are examining the effectiveness of targeting the PI3K/Akt/mTOR and other pathways. These and the other studies we have described document the promise of targeting the PI3K/PTEN/Akt/mTOR pathway in human health care. Furthermore, combining classical chemo and hormonal therapy with PI3K, Akt and mTOR inhibitors is also a very attractive therapeutic option under intense investigation.

We have investigated the effects of combining classical chemo- or hormonal therapies with signal transduction inhibitors in suppressing the growth of breast cancer cells. Treatment of breast cancer cells with MEK or mTOR inhibitors with doxorubicin, paclitaxel or tamoxifen resulted in a synergistic response documenting the effectiveness of classical chemo- hormonal with targeted therapies (see below).

Materials & 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 both estrogen receptor (ER)α and ERβ (Brooks *et al.*, 1973, Enmark *et al*, 1997, Fuqua *et al.*, 1999, Leygue *et al.*, 1999, Fasco *et al.*, 2000, Poola *et al.*, 2000, Yoshida *et al.*, 2000, Lespagnard *et al.*, 2001, Poola *et al.*, 2002) and (WT) PTEN (Li *et al.*, 1998, Lu *et al.*, 1999, Weng *et al.*, 1999, Clark *et al.*, 2002). 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 μg/ml streptomycin (Invitrogen), and 100 units/L penicillin G (Invitrogen) in Roswell Park Memorial Institute-1640 (RPMI 1640) medium (Invitrogen).

PTEN(WT), PTEN(C124S), and PTEN(G129E)Plasmids

A plasmid encoding human WT PTEN [PTEN(WT)] was generated by inserting the corresponding cDNA into the multiple cloning site of pEGFP-C2 (Leslie *et al.*, 2000). Mutated PTEN cDNAs were inserted into the multiple cloning site of pEGFP-C2 to generate plasmids encoding various PTEN mutants (Leslie *et al.*, 2000). These PTEN mutants include: PTEN (C124S) and PTEN(G129E). PTEN(C124S) and PTEN(G129E) each differ from PTEN(WT) by substitution of a single amino acid. PTEN(C124S) lacks both lipid and protein phosphatase activities (Myers *et al.*, 1997, Leslie *et al.*, 2000). PTEN(G129E) lacks lipid phosphatase activity yet retains protein phosphatase activity (Leslie *et al.*, 2000). These PTEN mutants were originally described in CS breast cancer patients and postulated to function as dominant negative (DN) mutations (Liaw *et al.*, 1997, Weng *et al.*, 2001).

Transfection of MCF-7 cells with PTEN 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 μg of DNA with Lipofectin (Invitrogen) as described by the manufacturer. 48 hrs after transfection, selection medium (RPMI + 10% FBS + 2 mg/ml G418) (Geneticin, Invitrogen, Carlsbad, CA) 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 μl of Tris-EDTA buffer. Mock transfected MCF-7 cells did not generate viable colonies in the presence of selection medium. The number of MCF-7 colonies present after incubation in selection medium for 3 weeks was determined. The mean number of MCF-7 colonies present and corresponding standard deviation was calculated from 3 replicate wells for each plasmid. The transfections were repeated 4 times and averaged together.

Analysis of Sensitivity to Doxorubicin and Rapamycin

Cells were seeded in 96-well cell culture plates (BD Biosciences) at a density of 5,000 cells/well in 100 μl/well of phenol red free RPMI-1640 containing 5% charcoal stripped FBS (CS-FBS). Cell culture plates were incubated for 1 day to permit cells to adhere to the bottom of each well. A 100 μl dose of treatment medium (doxorubicin or rapamycin) was added to each well the day after cells were initially seeded. Cell culture plates were incubated at 37 °C until extent of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma, St. Louis, MO) reduction in each well was quantified. Absorbance of each well was measured at 530 nm with a FL600 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT).

The mean and corresponding standard deviation of normalized adjusted absorbance was calculated from 8 replicate wells for each drug concentration and duration of incubation in order to investigate effects of the drug and ectopic gene expression on cell proliferation rate. Inhibitory concentration 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.

Apoptosis Analysis

The extent of apoptosis in the PTEN transfected cells after exposure to different concentrations of doxorubicin was estimated by determining the percentage of sub-G1 cells by flow cytometric analysis after PI staining of the cells. Results were analyzed by the ModFit LT 3.1 software program (Verity Software, Topsham, ME).

Clonogenic Assays

MCF-7 cells were collected and seeded in 6-well cell culture plates at densities of 1000 and 2000 cells/well (3 wells at each density). Cell culture plates were incubated for 1 day at 37 ° C in a cell culture incubator to permit cells to adhere to the bottom of each well. Cell culture plates were incubated for 3-4 weeks at 37 °C in a cell culture incubator. Cells were provided with fresh treatment containing 100 nM doxorubicin, 100 nM 4-hydroxy tamoxifen (4HT, Sigma), the combination of doxorubicin and 4HT or no supplement every 3 days. Cells were rinsed with PBS at the end of the 3 week treatment period. Rinsed cells were fixed in 100% (v/v) methanol (Sigma) for 10 minutes at room temperature then dried for 10 minutes at room temperature. Fixed cells were incubated in Giemsa stain (Sigma) for 5 minutes at room temperature. Stained cells were rinsed with water then dried. The number of colonies present in each well was counted. Colonies consisted of at least 50 cells. The mean number of colonies and corresponding standard deviation was calculated from 3 replicate wells for each condition.

Cellular Senescence Assay

When cells undergo senescence, they express senescence associated (SA) β-galactosidase (SA-β-gal). Senescent cells can be stained by a SA-β-gal senescence assay. 25,000 cells were seeded in each well of a 6 well plate containing an etched cover slip at different concentrations of doxorubicin and incubated for 4 days. SA-β-gal hydrolyzes X-gal (5-bromo-4-chloro-3-indolyl b-D-galactopyranoside) into a colorless galactose and 4-chloro-3-brom-indigo which forms an intense blue precipitate. SA-β-gal activity was detected at pH 6.0 as lysosomal β-gal has a pH optimum of 4.0. Senescent cells contained a dark blue stain in the perinuclear area, and displayed altered morphology. Fixed cells were photographed with a Nikon Eclipse E600 microscope under bright field settings.

Western blot analysis

MCF/EGFPc2 and MCF/PTEN(G129E) cells were collected after trypsination and plated in 100 mm tissue culture plates and cultured in RPMI + 10% FBS. After allowing the cells to adhere for 24 hrs, the monolayers were washed twice with PBS and then cultured with phenol red free RPMI 1640 containing 0.5% CS-FBS (to avoid endogenous estrogenic like compounds and reduce serum growth factors) for 24 hrs. Cells were then stimulated with 10% FBS for the indicated time intervals and protein lysates prepared as previously described (Shelton *et al.*, 2005b). Western blots were performed with antibodies specific for phospho and total Akt, Ask-1, p70S6K, ERK and Hsp70, as previous described (Shelton *et al.*, 2005b). Antibodies used in this study were purchased from Cell Signaling (Beverly, MA).

Results and discussion

Increased PTEN expression suppresses colony formation of MCF-7 cells

MCF-7 cells were transfected with PTEN plasmids encoding WT, C124S, G129E or the empty vector EGFP-C2 (Leslie *et al.*, 2000) to determine how signals transduced by this pathway affect breast cancer cell proliferation. The number of colonies present after transfection with plasmids encoding the PTEN mutants C124S or G129E or the empty vector EGFP-C2 were not statistically different. However, a decrease in the number of colonies was observed after transfection with a WT PTEN plasmid in comparison to transfection with the other plasmids. Thus increased expression of WT PTEN decreased the number of colonies isolated directly after selection in medium containing G418.

Effects of PTEN mutants on growth and induction of apoptosis in the presence of doxorubicin

The proliferation of pools of MCF-7 cells stably transfected with WT, C124S, G129E PTEN genes was compared with empty vector EGFP-C2 transfected cells, in the presence of different concentrations of doxorubicin over a 4 day time period in 96 well plates at 5000 cells/well. The growth of PTEN WT and EGFP-C2 transfected cells were similar in medium containing 0, 125 and 500 nM doxorubicin and no growth was observed at 2000 nM doxorubicin. Thus in stably transduced pools of PTEN (WT) and empty vector transfected cells, which had been isolated and expanded in culture for over one month, there was no difference in growth rates while there were differences in the initial colony formation efficiencies. In contrast, the growth PTEN mutant C124S and G129E cells differed from EGFP-C2 cells both in the absence and presence of doxorubicin. In the absence of doxorubicin, the mutant PTEN transfected cells reached lower saturation densities and proliferated at slower rates, while at higher doxorubicin concentrations, the mutant PTEN transfected cells proliferated to higher levels than either the EGFP-C2 or WT PTEN transfected cells. Thus, altering the levels of the PTEN mutant phosphatases changed the growth properties of MCF-7 cells both in the presence and absence of doxorubicin, but did not affect the colony formation efficiencies in the absence of doxorubicin.

The effects of the mutant and WT PTEN genes on the doxorubicin IC_{50}s were determined. While the IC_{50S} for the PTEN WT and empty vector transfected cells in the presence of doxorubicin were similar, the mutant PTEN transfected cells had higher IC_{50}s for doxorubicin. The IC_{50}s for doxorubicin in the C124S and G129E mutant PTEN transfected cells were 8.6 and 6.2-fold higher than empty vector or PTEN WT transfected cells. Statistical analysis revealed that the IC_{50} in the lipid and protein phosphatase deficient PTEN mutant transfected cells (C124S) was higher than in the PTEN mutant (G129E) transfected cells which just lacked PTEN phosphatase activity.

The effects of the PTEN mutants on the suppression of apoptosis were determined. MCF-7 cells transfected with WT PTEN, the various PTEN mutants or the empty EGFP-c2 vector were treated with different concentrations of doxorubicin and the extent of apoptosis measured by determining the percentage of sub-G1 cells after PI staining followed by flow cytometric analysis. Less apoptotic cells were observed in MCF-7 cells transfected with the PTEN mutants than in the PTEN WT or empty vector transfected cells. Furthermore less apoptotic cells were recovered from the MCF-7 cells transfected with the PTEN mutant lacking the lipid and protein phosphatase activity than the mutant lacking just lipid phosphatase activity. Thus altering the PTEN phosphatase activity decreased the induction of apoptosis by doxorubicin.

Mutant PTEN genes alter colony formation in medium containing doxorubicin or tamoxifen

The effects of inheritance of WT and mutant PTEN genes on colony formation in the presence and absence of chemotherapeutic (doxorubicin) and hormonal (tamoxifen [4HT]) based drugs were examined. The PTEN mutants had a higher cloning efficiency in medium containing 4HT than the PTEN (WT) or EGFP-C2 transfected cells. The lipid and protein phosphatase deficient mutant had a higher cloning efficiency in the presence of doxorubicin than the empty vector transfected cells. Thus the mutant PTEN gene altered the colony forming ability in the presence of drugs used to treat cancer patients.

Induction of cellular senescence in breast cancer cells after doxorubicin treatment

When certain cells are treated with chemotherapeutic drugs, they undergo cellular senescence. Drug resistance is often associated with an inhibition of cellular senescence (Ellmore et al., 2005, Lehmann *et al.*, 2007). When cells undergo senescence, they express SA-β-gal. Senescent cells can be visualized by a SA-β-gal senescence assay.

In the absence of doxorubicin, very little senescence was observed after a four day incubation period in the different PTEN transfected cells. In contrast in the MCF-7 cells transfected with the PTEN mutations resulting in loss of lipid or lipid and protein phosphatase activity, increased resistance to chemotherapeutic drug induced cellular senescence was observed. The C124S mutation deactivates both the lipid and protein phosphatase activity of PTEN, and conferred the strongest resistance to drug induced senescence. The G129E mutation disengaged only the lipid phosphatase activity, and also bestowed drug resistance, but still yielded more senescent cells upon doxorubicin treatment than the C124S mutation. Senescent cells flattened out and assumed a much wider shape in a 2-D plane. Photographs documenting the difference in the induction of cellular senescence in the empty vector and PTEN mutant C124S transfected cells after doxorubicin treatment are presented in Figure 5. In summary, both the protein phosphatase, as well as the lipid phosphatase activities of PTEN were important with regards to chemosensitivity in terms of both colony formation and inhibition of cellular senescence.

Effects of mutant PTEN expression on downstream signal transduction pathways important in breast cancer

The effects of inheritance of phosphatase deficient PTEN genes on signal transduction pathways were determined. Akt activation was monitored as decreased PTEN expression could result in Akt activation. Apoptotic signal kinase-1 (Ask-1) phosphorylation was also examined as it lies downstream of Akt. Phosphorylation of Ask-1 results in its ubiquitination and subsequent proteosomal degradation. p70S6K phosphorylation was monitored as it lies downstream of mTOR, is an important regulator of protein synthesis and its activation is a reflection of functional activation of this pathway. Activation of ERK was also examined as it is an important kinase implicated in proliferation and prevention of apoptosis and its activity can be regulated by Akt.

Upon serum stimulation of EGFP-C2 transfected cells, activated Akt was detected after one hr, however, its levels then decreased at later time points up to 24 hrs. In contrast, the levels of total Akt protein remained constant. Thus serum treatment, led to a transient increase in phosphorylated Akt. In contrast, activated Akt was detected after one hr of stimulation of mutant PTEN transfected cells and remained elevated throughout the time course. Thus the mutant PTEN transfected cells expressed activated Akt for prolonged time periods after serum treatment.

Higher levels of phosphorylated Ask-1 were detected in the mutant PTEN transfected than in empty vector transfected cells. Phosphorylated and ubiquitinated Ask-1 may contribute to the apoptotic resistance of the mutant PTEN transfected cells.

The levels of phosphorylated p70S6K increased in empty vector transfected cells 8 and 24 hrs after serum addition. Phosphorylated p70S6K was detected with phosphospecific antibodies which detect phosphorylation of p70S6K at either T389 or T421 and S424. In contrast, relatively equal levels of total p70S6K were detected. The levels of phosphorylated p70S6K were low after serum deprivation of mutant PTEN transfected cells but increased rapidly after serum addition (0.5 hr) and remained elevated over the time course. Thus the mutant PTEN transfected cells expressed activated p70S6K earlier than the empty vector transfected cells.

Phosphorylated ERK was detected 4 hrs after FBS stimulation of empty vector transfected cells and remained elevated for 24 hrs. In contrast, activated ERK was detected constitutively in the mutant PTEN transfected cells. Thus disruption of PTEN activity results in alterations in Akt, Ask-1, p70S6K and ERK activation. These results document the dramatic effects that altered PTEN expression can have on downstream anti-apoptotic and growth related signaling

Mutant PTEN genes confer an Achilles' heel to drug resistant breast cancer cells

It has been reported that altering Akt or PTEN levels will change the sensitivity of breast cancer cells to rapamycin (DeGraffenried *et al.*, 2004). However, the relationships between PTEN, rapamycin and chemotherapeutic drug resistance in breast cancer are not clearly elucidated. Thus we examined the effects of the mutant PTEN genes on the sensitivity of the breast cancer cells to the combination of doxorubicin and rapamycin treatment. The cells transfected with the mutant PTEN genes were more sensitive to rapamycin than the cells transfected with WT or the empty EGFPc2 vector. The effects of rapamycin were seen over a broad dose range (1 to 1000 nM). Rapamycin also synergistically suppressed (decreased) the IC_{50} for doxorubicin in the cells transfected with the PTEN mutants.

In this manuscript, the effects of introduction of WT and mutant PTEN genes on the clonogenicity and sensitivity of MCF-7 cells to doxorubicin, 4HT and rapamycin have been examined. Introduction of additional copies of WT PTEN into PTEN-positive MCF-7 cells, inhibited initial colony formation in the absence of doxorubicin or rapamycin when compared to MCF-7 cells transfected with mutant PTEN or the control EGFPc2 plasmid. While the WT PTEN gene reduced the colony forming ability in the absence of doxorubicin or rapamycin, it did not alter the growth rate or sensitivity to either doxorubicin or rapamycin in the pools of stably transfected PTEN WT cells which were isolated and expanded in the presence of G418. The two different assays differed in the time frames of the experiments; the initial colony formation assays were performed over a 3 to 4 week period while the growth assays were performed over a 5 day time period on the stably transfected cells. Furthermore, once the cells are selected to grow, they may be able to survive in the presence of higher levels of PTEN activity. Alternatively increased PTEN levels may be suppressed in the cells which survive and proliferate. These two possibilities are a common occurrence in gene transfer experiments and are under further investigation.

Introduction of PTEN mutants lacking lipid and protein or lipid phosphatase activity enhanced the growth of the breast cancer cells in the presence of chemotherapeutic drugs. Interfering with the phosphatase activity of PTEN also resulted in higher levels of activated Akt detected in the serum and hormone deprived samples and prolonged detection of activated Akt after FBS stimulation in the breast cancer cells transfected with PTEN mutant than in the empty vector transfected cells. Akt has been reported to slow the growth rate of certain cells as well as increase the cell size (Jin *et al.*, 2005). The phosphatase deficient PTEN transfected cells, which displayed elevated Akt signaling proliferated slower than the empty vector transfected cells. Phosphatase deficient PTEN mutants also decreased the sensitivity of MCF-7 cells to doxorubicin and increased their sensitivity to rapamycin when compared with the empty vector transfected cells.

Inheritance of a PTEN mutant lacking both lipid and protein phosphatase activity conferred more resistance to doxorubicin than the lipid phosphatase deficient PTEN mutant, indicating that interfering with both lipid and protein phosphatase activities of PTEN were important in terms of therapeutic resistance. Also rapamycin exerted a greater degree of inhibition of the doxorubicin IC_{50} in cells transfected with the lipid and protein phosphatase deficient PTEN gene than in the cells transfected with the lipid phosphatase PTEN mutant.

PTEN activity is critically involved in regulation of downstream Akt activity which is important in drug sensitivity. Altering normal PTEN activity by some PTEN mutations may lead to drug resistance. Augmented Akt activity may increase resistance to doxorubicin. In fact, published reports (Clark *et al.*, 2002, Tokunaga *et al.*, 2006) and our own preliminary data indicate that ectopic Akt expression increases the resistance of MCF-7 breast cancer cells to doxorubicin and tamoxifen.

These results are relevant to potential cancer therapies as the PTEN gene is frequently mutated in human cancer. Mutations occur which either delete the PTEN gene or alter its activity. Sometimes these mutations actually make the cells sensitive to Akt and mTOR inhibitors as the growth of the cells becomes dependent upon elevated Akt levels (DeGraffenried *et al.*, 2006). Furthermore, epigenetic mechanism may silence PTEN transcription and transcriptional mechanisms may alter the splicing of PTEN mRNA transcripts. Restoration of WT PTEN activity or inhibition of Akt activity are key strategies to prevent chemoresistance. PI3K, PDK, Akt and downstream mTOR inhibitors have been developed and evaluated in clinical trials. Unfortunately, PI3K (LY294002) and Akt (A-443654) inhibitors tend to have associated toxicity and specificity problems (Luo *et al.*, 2005., Martelli *et al.*, 2007). This may not be that surprising as this pathway regulates numerous pathways involved in cell cycle progression, growth and apoptosis as well as glucose homeostasis.

Some point mutations eliminating PTEN phosphatase activity such as the C124S and G129E, without gene deletion, may result in PTEN genes which serve as DN mutants suppressing the normal PTEN activity encoded by the remaining endogenous WT allele. These PTEN mutants may serve to stimulate chemo- and hormonal-resistance of certain tumors. Identification of such mutations may become important in breast cancer therapy as it has been shown that the loss of PTEN activity is associated with Herceptin resistance (Nagata *et al.*, 2007). Only one allele of PTEN may be mutated in many human cancers. Interesting both the C124S and G129E PTEN mutations were originally observed in CS patients and postulated to act as DN mutations in human cancer and other diseases (Lian *et al.*, 1997).

While inhibition of the lipid phosphatase activity of PTEN was sufficient to induce resistance of the cells to doxorubicin, inhibition of the protein phosphatase activity of PTEN further increased chemoresistance indicating that the protein targets of PTEN are also important in its effects as a tumor suppressor. Further elucidation of these targets of PTEN may aid in the identification of suitable targets for therapeutic intervention. Fak and Shc are known targets of the protein phosphatase activity of PTEN (Steelman *et al.*, Li *et al.*, 1997, Li *et al.*, 1997, Nimwegen *et al.*, 2006, Tamura *et al.*, 1998). Elucidating the protein substrates of the PTEN phosphatase could reveal important targets for therapeutic intervention.

The PTEN phosphatase deficient mutant transfected cells were also more resistant to the induction of cellular senescence induced by doxorubicin. While differences in the induction of cellular senescence in the PTEN WT and mutant transfected cells were not observed in the absence of doxorubicin, the PTEN mutant cells, which expressed higher levels of activated Akt than the PTEN WT cells were more resistant to the induction of cellular sensescence mediated by doxorubicin. Thus altering the PTEN genetic status in human cancer may inhibit

the ability of chemotherapeutic drugs to induce cellular senescence and allow the outgrowth of resistant clones which result in metastasis and death of the cancer patient.

Summary

Breast cancer is a common disease which affects women and men in the prime of their lives. Unfortunately there is no common magic bullet which can effectively cure all breast cancers. Breast cancer arises from many diverse genetic mechanisms. Frequently breast cancer becomes resistant to various therapies and residual, dormant, senescent, drug resistant breast cancer cells resume their growth and often metastize to other sites. At this point, therapeutic options are limited and we desperately need more effective treatments. This chapter has focused on the key roles that the PI3K/PTEN/Akt/mTOR pathway plays in breast cancer drug resistance and induction of cellular sensecence. Furthermore, breast cancer cells which proliferate in response to this pathway may have an Achilles heel and are highly sensitive to mTOR inhibitors.

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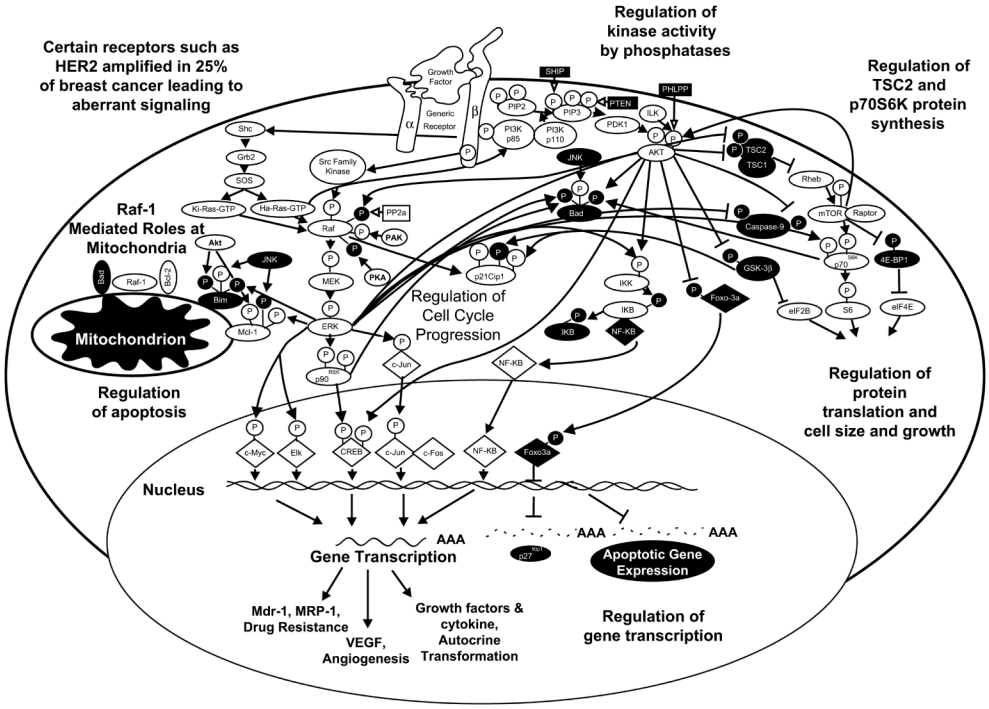

Figure 1. Overview of PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK Pathways

The PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK PI3K/PTEN/Akt/mTOR and pathways are regulated by upstream growth factor and mitogen receptors as well as Ras and various kinases. Many kinases serve to phosphorylate S/T and Y residues on Raf and S/T residues on Akt. Some of the Raf phosphorylation events serve to enhance its activity (shown by a black P in a white circle) whereas others serve to inhibit its activity (shown by a white P in a black circle. Moreover there are phosphatases such as PP2A, PTEN, SHIP and PHLPP, which remove phosphates on certain regulatory residues on Raf, MEK, ERK, PtdIns(3,4,5)P_3 and Akt as well as other signaling molecules. The PI3K/Akt pathway is also activated after receptor ligation. Akt and ERK have many downstream targets which serve to regulate cell growth and apoptosis. Some of the downstream transcription factors regulated by these pathways are indicated in diamond shaped outlines.

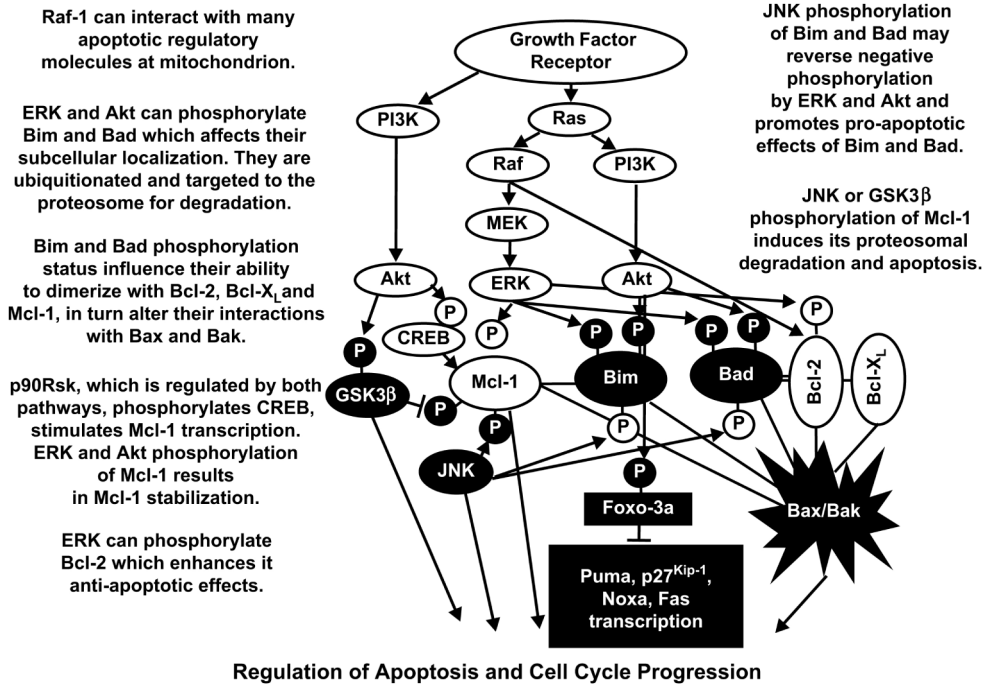

Figure 2. Effects of PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK Pathways on Apoptotic Circuity

Growth factors can induce multiple signal transduction pathways which can effect the expression of apoptotic molecules by transcriptional and post-transcriptional mechanisms. The effects of the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways are often counterbalanced by JNK and GSK-3β which can serve to promote apoptosis.

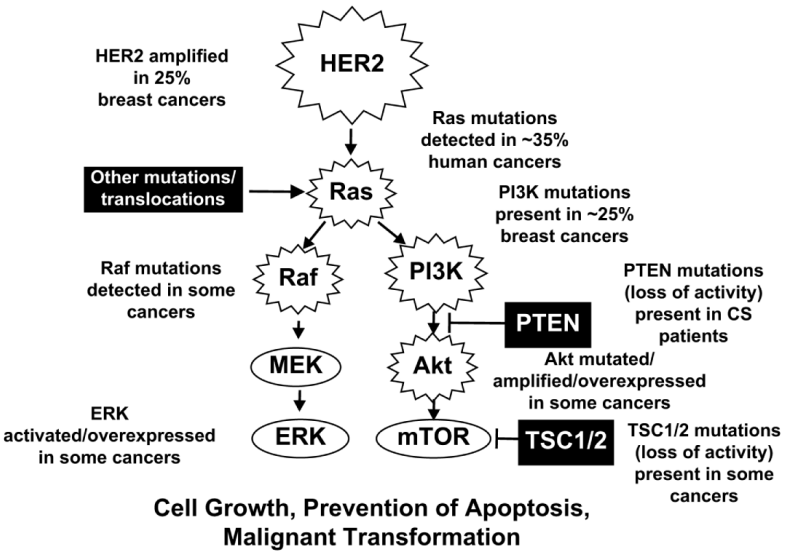

Figure 3. Sites of Gene Mutation/Amplification which Can Result in Activation of the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK Cascades

Amplification of HER2 is observed in 25 to 30% of breast cancer patients which can result in activation of both the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK cascades. Ras is mutated in approximately 35% of human cancers. B-Raf and Raf-1 are also mutated in certain human cancers. The PI3K/PTEN/Akt/mTOR pathway is also activated in breast cancer due to mutations at PI3K, PTEN and Akt as well as mutations at upstream receptors. Abnormal Akt activation is associated with a poorer prognosis for breast cancer and resistance to chemo-hormonal therapy.

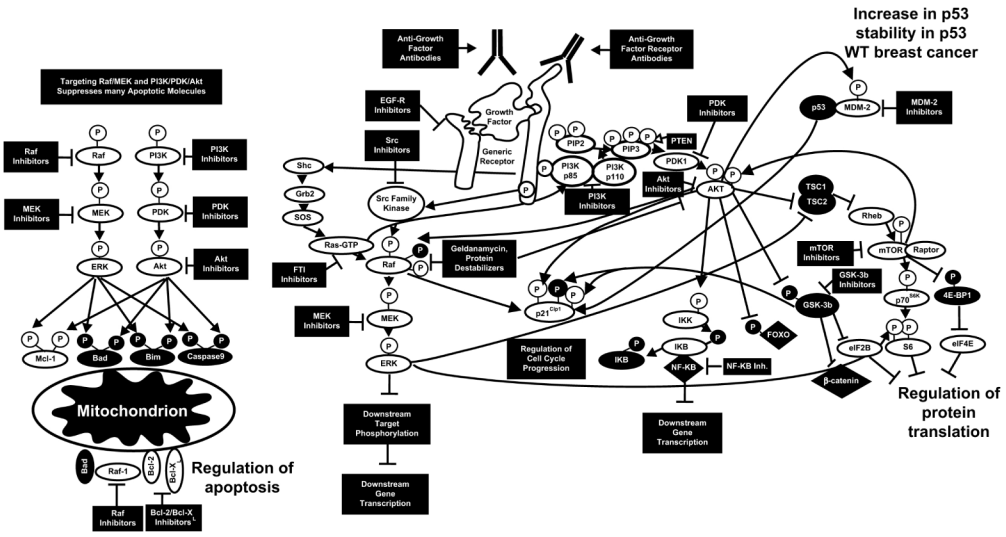

Figure 4. Targeting Signal Transduction Pathways in Breast Cancer

Potential sites of action of small molecular weight inhibitors and cytotoxic antibodies are indicated. In some cases inhibitors will suppress growth, apoptotic and cell cycle regulatory pathways. This diagram serves to illustrate the concept that targeting PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways can have dramatic effects on many growth regulatory molecules. Proteins inactivated by S/T phosphorylation induced by the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways are shown in black circles with white P in a black circle.

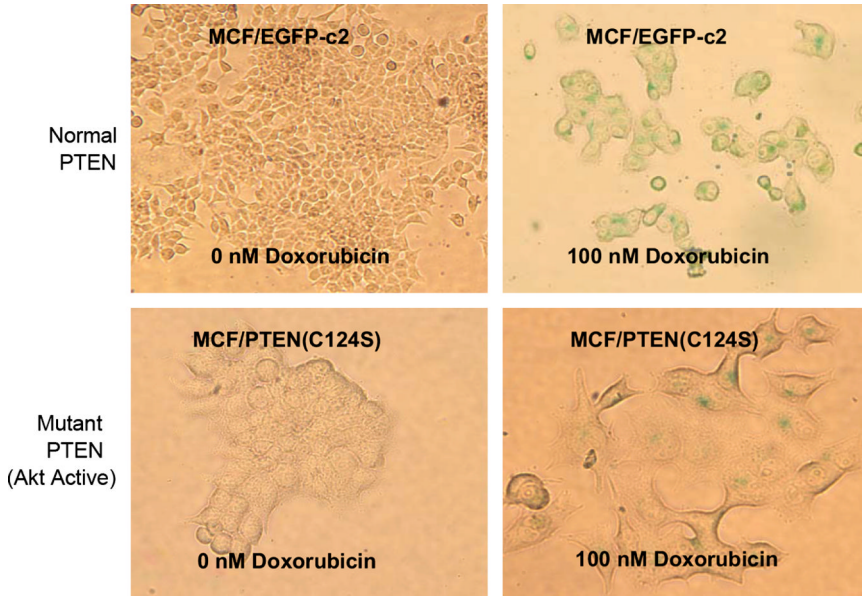

Figure 5. Induction of Cellular Senescence by Doxorubicin in the Presence and Absence of DN PTEN

MCF-7 cells transfected with the empty vector (EGFP-c2) as well as various PTEN constructs were seeded into 6 well plates containing cover slips and different concentrations of doxorubicin and cultured for 4 days. Induction of cellular senescence was determined by a β-galactosidase assay with X-gal as a substrate. In this figure, photographs of MCF/EGFP-c2 and MCF/PTEN(C124S) cells cultured in the presence and absence of 100 nM doxorubicin and subsequently stained for β-galactosidase activity are presented. Fixed cells were photographed under a Nikon Eclipse E600 microscope under bright field settings.