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Dual PI3K/mTOR Inhibition with BEZ235 Augments the Therapeutic Efficacy of Doxorubicin in Cancer without Influencing Cardiac Function

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DUAL PI3K/MTOR INHIBITION WITH BEZ235 AUGMENTS THE THERAPEUTIC EFFICACY OF DOXORUBICIN IN CANCER WITHOUT INFLUENCING CARDIAC FUNCTION

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Abstract

DUAL PI3K/MTOR INHIBITION WITH BEZ235 AUGMENTS THE THERAPEUTIC EFFICACY OF DOXORUBICIN IN CANCER WITHOUT INFLUENCING CARDIAC FUNCTION

By David Ellis Durrant, Ph.D.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2015

Major Director: Rakesh C Kukreja, Ph.D.
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Cancer continues to be a leading cause of death in the United States despite improved treatments. Cancerous lesions form after acquiring oncogenic driver mutations or losing tumor suppressor function in normal cells. Traditional therapies have included use of genotoxic substances that take advantage of the increased growth rate and loss of tumor suppressor function to cause cell death. One such drug is the anthracycline antibiotic doxorubicin (DOX). DOX intercalates into DNA and disrupts transcriptional machinery while also poisoning topoisomerase II. This results in single and double stranded DNA
breaks, which if severe enough leads to either necrotic or apoptotic cell death. DOX has been very effective at treating several different cancers and is still widely used today however its clinical use is limited due to cumulative dose dependent cardiotoxicity. Therefore, combination therapy targeting survival pathways is utilized to minimize the cumulative dose of DOX without ameliorating its anti-tumor effects.

We investigated the potential anti-cancer effects of combining the dual PI3K/mTOR inhibitor, BEZ235 (BEZ), with DOX in pancreatic, breast and other cancer cells lines as well as its associated effects on the heart. Our results showed that co-treatment of BEZ with DOX increased apoptosis in a manner that was dependent on inhibition of the AKT survival pathway. Moreover, BEZ co-treatment with DOX had additive effects towards cell viability while it significantly enhanced necrotic cell death compared to either drug alone. Furthermore, we observed that physiological concentrations of BEZ inhibited ABCB1 efflux resulting in increased intracellular accumulation of DOX, which led to increased DNA damage. In addition, BEZ in combination with gemcitabine (Gem) reduced cell proliferation but did not enhance necrosis or apoptosis. Treatment with BEZ and DOX in mice bearing tumor xenographs reduced tumor growth as compared to BEZ, DOX or Gem. Moreover, BEZ reduced DOX toxicity in rat myoblast cells and did not potentiate the effects of DOX in tumor-bearing mice. We propose that combining BEZ with DOX could be a novel therapeutic approach for the treatment of patients with cancer in the hope of improving the prognosis of this deadly disease.
CHAPTER 1: Introduction

The National Cancer Institute defines cancer as when “abnormal cells divide without control and can invade nearby tissues”. The change from normal to abnormal cells involves a complicated and heterogeneous set of events that includes mutations within the cancer genome and altered metabolism to give it a survival advantage over the surrounding normal cells (1). The issue of the direct cause of initiation of cancer remains controversial however. Mutations often occur within genes known to be tumor suppressors or oncogenes resulting in increased activation of pathways that control cell growth, proliferation, and survival. This change in growth rate enhances the requirement for metabolic intermediates which is achieved by a switch to aerobic glycolysis, also known as the “Warburg Effect” (2, 3). One theory is that genetic mutations lead to an increased proliferation and growth rate requiring the need for an altered metabolic state. On the other hand, there is increasing evidence that alterations to mitochondria first cause a change in metabolism which then results in genetic alterations to increase the efficiency of their metabolic requirements (2). Whichever the case, it is clear that both events are important to the survival and growth advantage of cancer cells and are therefore important targets for cancer treatment.

Cancer is the second leading cause of death in the United States behind heart disease but is expected to become the leading cause in the near future. The lifetime risk of
developing cancer is 43% for males and 38% for females (4). However, individually these risk percentages can be drastically altered dependent on several risk factors including lifestyle and genetics. In general, death rates are declining in both men and women with men seeing a large decrease in lung cancer mortality and steady decreases in colorectum and prostate cancer while women are seeing steady decreases in the mortality rates of colorectum and breast cancer. Much of this improvement comes from not only superior treatments but also improved diagnosis and preventative care.

Pancreatic cancer on the other hand, has a death rate that has remained unchanged for approximately forty years. It represents the fourth leading cause of cancer death in the United States and the lowest five year survival rate of all the major cancers (4-6). The reason is that symptoms are generally mild and often mistaken for other less severe maladies, thus allowing for continued growth and infiltration of the surrounding tissue leading to a more advanced stage at the time of diagnosis. Also, while surgery gives the best chance for a cure, only 15% of patients are eligible because of this late stage at diagnosis. These facts highlight the need for earlier detection and therefore an earlier stage at diagnosis, as well as considerably better therapies.

**HISTORICAL PERSPECTIVES**

**A brief history in cancer**

Early evidence from animal specimens seems to indicate that cancer had been around long before modern man appeared on the earth (7). The earliest known reference to cancer comes from the Edwin Smith Papyrus which is believed to date back to 3000 BC and describes a tumor like swelling of the breast which had was no cure (7, 8). It’s unclear
how prevalent cancer was in ancient times but there is evidence of trying to cure patients. Several cultures throughout the known world, including the Egyptians, Sumerians, Chinese, and Persians, all had their cultural “remedies” that used both herbal and toxic substances (7). The earliest accepted example of a neoplasm is from a skeleton dating to the Neolithic period (4000 BC) which resembles signs of multiple myeloma (8).

The term cancer was derived from the words carcinos and carcinoma, which were used by Hippocrates in ancient Greece because they reminded him of a moving crab. The humoral theory of disease, also formulated by Hippocrates, was based on the concept that there were four humors (blood, yellow bile, black bile, phlegm) which if kept in balance would result in good health. From this concept of health, the earliest known theory on cancer was formulated which suggested that cancer arose from an excess of black bile (9). Surprisingly, this theory lasted several hundred years until dissections revealed that black bile did not exist leading others to postulate theories, including that it is derived from coagulated lymph, and eventually leading to the modern theory that cancer is derived from cells, brought forth by Rudolf Virchow in 1863 with the help of microscopy (10). A Greek physician named Aulus Celsus, who lived when Greece was part of the Roman Empire, had great influence to modern times by making Latin the standard language of Medicine (7). He also wrote the text De Medicina, which described several varieties of cancer and even described breast cancer metastases to lymph nodes in the armpit. The genetic view of cancer was formulated by a German Biologist named Theodor Boveri. Great intuition, as well as his experience with doubly fertilized sea urchin eggs, which had chromosomes with four poles, led him to theorize that malignant cells also have alterations to their
chromosomes and that these alterations were the driving force behind their unlimited growth (11). Boveri also described the linear aspect of cellular information, which we now know are genes, and how if certain pieces of the chromosome are lost the cell can multiply without limitation, which is the basis for tumor suppressors and oncogenes.

**Cancer treatment**

Early treatments for cancer included cautery, knife, salts of lead, and salts of lead and sulfur or arsenic “Egyptian ointment”, the latter of which was in use until the 19th century (12). Hippocrates thought that those tumors that could not be killed by fire (cautery) were incurable and that deep seated tumor should not be treated because the patient would die quickly. The Roman Pliny (23-79 AD) described in his *Material Medica* remedies for advanced cancer which included a boiled mixture of ash of crab, egg white, honey, and powdered feces of falcons as being his most praised remedy (7). These early descriptions of the use of chemicals to treat cancer by the Egyptians and Greeks were fairly sporadic until the 16th century when Paracelsus initiated a systematic use of chemicals (13). He would travel around Europe promoting his chemical remedies and give ailing patients samples. He introduced several toxic substances as internal remedies, including Mercury, Lead, Sulfur, Arsenic, Iron, and Iodine but gave warning that their toxicity was dependent on the concentration. The beginning of chemotherapy use as we know it was not established until early in the twentieth century when nitrogen mustard, derived from World War 2 chemical weapons testing of mustard gas, was first used to treat a patient with non-Hodgkin’s lymphoma in 1943 (14). This was soon followed by the discovery of the anti-folate, methotrexate, in 1948 and the first “targeted” therapy, 5-fluorouracil (5-FU) in 1957.
These early studies laid the groundwork for the development of other chemotherapies, including the anthracyclines doxorubicin and daunorubicin, which were discovered in the 1960’s and remain some of the most effective anti-cancer drugs ever developed (15).

Surgery to remove cancerous tumors date back to at least 50AD, with the most commonly describes surgeries being mastectomies (7). In 1655, mastectomies followed by cauterization were performed, which no doubt improved safety of the surgery by preventing the severe loss of blood (13). The use of various concoctions, often times including poisons, date back to the earliest records of cancer. However, the use of chemicals prior to surgery began in 1712 (13). This was more or less the precursor to modern day neoadjuvant therapy and was used to cure the disease prior to resorting to surgery. Preceding the 18th century, many people including doctors thought of cancer as a contagious disease. A change in thinking occurred though, when an English surgeon named James Nooth inoculated himself with small pieces of breast tumor which ultimately failed to grow (16). Around this same time, surgeons began to emphasize the examination of wounds after tumors were removed to be sure that all pieces were sufficiently excised knowing that fragments left behind would result in recurrence. However, it wasn’t until 1846 that anesthesia was introduced and used in the first surgery, which was to remove a tumor. This was followed by a rapid increase in knowledge of surgery due to the advent of anesthesia leading the next one hundred years being termed “the century of the surgeon”.

In 1895, Wilhelm Conrad Roentgen discovered X-rays and marked the beginning of the field of radiology and radiation oncology. He took the first X-ray photograph of his wife’s hand on a photographic plate that same year and subsequently won the Nobel Prize
for his discovery. The following year, in 1896, radiotherapy was first used for the treatment of a patient with gastric carcinoma (17, 18). However, because of the low energies of X-rays, there was limited depth of penetration and therefore was mainly used for superficial cancers. After the discovery of radium and polonium, the field of radiation oncology grew rapidly. Radium was first used to treat a carcinoma in 1902 and was quickly followed up by implantation directly into tumors after which it gained traction as a wonder drug which found its way into several products (17).

These were the beginnings from which the knowledge of today was built. In the last few decades, advances in science and technology have led to exponential growth in our understanding of disease, including cancer. It’s from this past and the knowledge gained from it that we must continue to push the field forward and implement new therapies in the hopes that we can provide better outcomes to this and future generations.

**DOXORUBICIN USE IN CANCER**

The finding in 1943 that nitrogen mustard had anti-cancer effects spawned the discovery of numerous chemotherapy drugs. Anthracycline antibiotics were first discovered in the 1960’s by their isolation from the pigment producing bacteria *Streptomyces peucetius* and named doxorubicin (DOX) and daunorubicin (15). Remarkably, they are still in wide use and are some of the most successful anti-cancer drugs ever produced. DOX remains a first-line chemotherapy agent for several cancers including certain types of leukemia, lymphoma, and breast cancer. Despite its long history
of clinical and laboratory use, its mechanism of action still remains controversial and is likely a combination of several effects.

**Doxorubicin mechanism: topoisomerase poisoning**

What is believed to be the primary anti-cancer mechanism of DOX is through poisoning of topoisomerase II (topo II) and to a lesser extent topoisomerase I. Topo II are required in the nucleus to relieve under and over winding of the DNA strands as well as DNA knots and tangles (15, 19, 20). This occurs through the introduction of a double stranded break in the DNA that is protected through covalent interactions with tyrosine residues on the enzyme so as to not induce mutations, deletions, or a DNA damage response. The break in DNA allows for the passage of another DNA stand to relieve under or over coiling to allow continuation of DNA synthesis. In normal situations these breaks are extremely short-lived and reversible due to storage of energy in the covalent bonds between the DNA ends and tyrosine allowing the enzyme to religate the strands without the need for a high energy co-factor (20). However, DOX inhibits the religation step by binding to and stabilizing the DNA-enzyme intermediate complex. This itself causes serious effects but is amplified when DNA replication machinery or helicases traverse the DOX stabilized DNA-enzyme complex converting these breaks into permanent fractures no longer protected through tyrosine binding (19). The resulting fractures are prone to mutations and recombination leading to large insertions or deletions, which if severe enough leads to cell death. DOX also has similar effects towards topoisomerase I however this effect is much weaker at inducing cell death than that of topo II poisoning (15). Moreover, the deleterious actions of DOX as a topoisomerase poison are concentration
dependent so one can infer that better delivery of the drug to the cancer cell will result in enhanced effectiveness.

**Doxorubicin mechanism: ROS formation**

Whereas the primary anti-cancer effects of DOX are attributed to the poisoning of topo II, its secondary effect and what is believed to be the primary deleterious effects towards other cells, including cardiomyocytes, are the result of reactive oxygen species (ROS) generation (15, 21, 22). DOX can incur a one-electron reduction to produce a semiquinone that quickly regenerates its parent form (a futile cycle) by reducing oxygen to form superoxide anions which are then converted to hydrogen peroxide either spontaneously or by the enzymatic reaction of superoxide dismutase (15, 22). Hydrogen peroxide subsequently reacts with heavy metals, including iron (the Fenton reaction), to produce highly reactive hydroxyl radicals (22, 23). In addition, DOX can directly bind to iron in the presence of oxygen to produce ROS (24).

Low levels of ROS generation in the cell can induce proliferation and survival signaling but at higher levels can have several damaging effects. There are reports that DOX induced free radicals can directly oxidize DNA bases in a similar manner as that of ionizing radiation (15, 21, 25). The direct oxidation of bases increases the potency of DOX in tumor cells, although it is also implicated in the mutagenicity of the drug to normal cells and is thought to be involved in causing secondary malignancies (26). Also, polyunsaturated fatty acids in the membrane bilayers of both the plasma membrane and the mitochondrial membrane are susceptible to oxidation from hydroxyl radicals to form lipoperoxyl radical (27, 28). These lipid radicals can then react with other lipids to produce
another lipid radical and the unstable lipid hydroperoxide, including 4-hydroxy-2-nonenals (4-HNE) (29). This could have severe effects on the health of the cells by reducing the fluidity and breaking down of the membranes (30). Moreover, mitochondrial dysfunction through the oxidation of cardiolipin may be an important initiation step of apoptosis by mediating the release of cytochrome c (31).

The effects of DOX induced ROS formation on the cell amplifies the cytotoxic effects of topo II poisoning in cancer cells and is the primary culprit in treatment related cardiotoxicity. Thus novel therapies are needed that can amplify the anti-cancer effects of DOX without also incurring increased toxicity in the heart.

**DRUG RESISTANCE**

**ABC transporters**

DOX is an extremely successful therapeutic, however many cells are inherently resistant or develop acquired resistance to treatment resulting in cells which are unresponsive to multiple structurally unrelated drugs, an occurrence which has been termed multidrug resistance (MDR) (32, 33). There are several strategies cells use to evade drug toxicity and incur resistance including increased survival signaling and drug metabolism as well as decreased drug accessibility, but the vast majority of MDR in cancer is related to the overexpression of a family of protein transporters called the ATP Binding Cassette (ABC) transporters (32, 34, 35). The ABC transporter superfamily consists of 48 genes and 7 subfamilies (A-G) (36). In general, they are transmembrane proteins that use the energy from ATP hydrolysis for the transport of a wide variety of substrates ranging
from sizes of 330 daltons up to 4000 daltons and include cholesterol, lipids, cyclic nucleotides, and several anti-cancer agents including vinca alkaloids, taxanes, nucleoside analogs, and anthracyclines (33, 37). The proteins are either full or half transporters with an orientation of an inverted V with the membrane spanning the protein. Upon substrate binding, a conformational change occurs which requires binding of ATP and subsequent hydrolysis in the nucleotide binding sites allowing for release of the substrate across the membrane (38). When expressed at high levels, they actively pump cytotoxic agents out of the cell resulting in reduced intracellular concentrations (32, 33, 37). This effectively diminishes the damage incurred in the cell leading to a failure in cancer treatment.

The most commonly overexpressed transporters associated with cancer drug resistance are ABCB1 (MDR1, p-gp), ABCC1 (MRP1), and ABCG2 (BCRP1) (35). ABCB1, which has been shown to be overexpressed in up to 40-50% of examined cancer patients (39, 40), along with ABCG2 are localized to the apical surface of epithelial cells and are highly expressed in the blood brain barrier (BBB), placenta, liver, gut, and kidney (36). ABCC1 is expressed in most tissues with high expression in the lung, testis, and kidneys and is mainly localized to the basolateral surface (36). ABCB1 and ABCC1 are full transporters with both having 2 transmembrane domains and two nucleotide binding domains (35, 41). ABCG2 is a half transporter with one transmembrane domain and one nucleotide binding domain. It forms homo or heterodimers to become functionally active (41). There is also a high amount of redundancy among the gene substrates and studies have shown that knockout of one gene will result in increased of expression in other transporter genes to compensate for the loss (36).
Although three ABC transporters (ABCB1, ABCC1, ABCG2) are most commonly associated with cancer drug resistance, there are several others that are also known to confer resistance to anti-cancer agents. These include the ABCC subfamily members ABCC3, ABCC5, and ABCC10 (34, 42-44). Studies have shown that all three are involved in transport of the nucleoside analog gemcitabine (Gem) (45) and ABCC3 was shown to be upregulated in drug resistant patients as well as being a marker for poor clinical outcome (42). Along with Gem, ABCC5 also confers resistance to the nucleoside analogs 6-mercaptopurine and 6-thioguanine (34). ABCC10 transports several classes of anti-cancer drugs including the vinca alkaloids, anthracyclines, and the physiological substrate leukotriene (44). While less common in MDR, these transporters could still have serious implications to a drugs anti-cancer efficacy and needs to be kept in mind when considering drug treatment.

**Targeting ABC transporters**

Drug resistance was first described experimentally using a laboratory model of leukemia in 1950 (46). Daunorubicin was found to be actively transported outward in drug resistant cells that were cross resistant to vinca alkaloids and other anthracyclines in 1973 (46). Later it was found that this cross resistance was associated with a 170 kDa protein named p-glycoprotein (ABCB1). Since then, there have been several drugs developed to combat MDR specifically associated with overexpression of ABC transporters, but their effectiveness has been largely underwhelming. First generation inhibitors included verapamil, quinidine, and cyclosporine A and although there were cases that showed promise in improving overall survival, especially in p-gp positive tumors (34, 46, 47),
inhibitors have generally been unsuccessful and toxicity was common (32, 33). Second generation inhibitors had improved specificity but were still found to be ineffective clinically due to pharmacokinetic interactions which were sometimes unpredictable. Third generation inhibitors have better pharmacokinetics and further improved specificity. However, it remains to be seen whether they can be effective clinically. Some early data has shown promise resulting in phase II/III clinical trials but others have demonstrated similar toxicity issues as previous inhibitors and lack of overall effectiveness (48). For this reason, there is a need for alternative strategies into finding useful inhibitors that combat the active elimination of drugs from the cell, while at the same time minimizing toxicity.

One potential strategy is to find agents that aren’t specifically targeted towards ABC transporters, but still demonstrate the ability to inhibit efflux of cytotoxic drugs, either through direct inhibition or through competition with other substrates for access to the binding site.

There have been several reports of drugs that have been designed and studied to specifically inhibit various enzymes within the cell that have later been found to also inhibit ABC-type transporters. Sildenafil (Viagra), a potent inhibitor of phosphodiesterase 5, was first shown to potentiate the anti-cancer effects of DOX against prostate cancer when treated in combination (49). Later it was found that sildenafil inhibited the efflux activity of both ABCB1 and ABCG2 leading to increased drug accumulation (50). Similarly, several receptor tyrosine kinase inhibitors, including nilotinib, erlotinib, and sunitinib, have been shown to reverse MDR in cells associated with overexpression of ABC transporters (51-55). Rapamycin, a specific inhibitor of the mammalian target of
rapamycin (mTOR), has also been shown to modulate the intracellular accumulation of cytotoxic drugs through the inhibition of ABCB1 (56, 57). These drugs have dual roles in cancer treatment by inhibiting a pathway that is commonly over activated in cancer, as well as inhibiting the efflux capabilities of those cells, which are also commonly overexpressed. The strategy of using a dual role inhibitor has several potential benefits. These drugs would likely have fewer of the toxicities associated with pure ABC transporter inhibitors due to the weaker binding and specificity. Also, inhibition of their primary targets in combination with increasing accumulation of a secondary agent could provide a synergistic enhancement of drug effectiveness.
Figure 1. Mechanism of ABC transporter-mediated efflux of its substrate. ABC transporters are primarily transmembrane proteins that are composed of two transmembrane domains (TMD) and two nucleotide binding domains (NBD) for full transporters or one TMD and one NBD for half transporters. Half transporters subsequently require homo- or heterodimerization to become fully active transporters. The substrate binding pocket is located in the center of the TMD’s which have the shape of an inverted V. Upon substrate binding, ATP is hydrolyzed to ADP causing a conformational shift and allowing for release of the substrate across the membrane.
Survival Signaling Pathways

Phosphoinositide-3-kinase Signaling

Phosphoinositide-3-kinase (PI3K) and its downstream signaling partners are key regulators of cell proliferation, survival, and differentiation. They are also frequently mutated in cancer resulting in proteins that are stuck in a constitutively active state which has been identified as a key event for the initiation and development of precancerous lesions and tumor formation (58-60). PI3K is a heterodimer that is divided into three classes (class IA and B, class II, and class III) with mainly only class IA being associated with cancer (61). It plays an integral role in transmission of extracellular signals to intracellular targets. Signaling initiates with activation of one of several receptor tyrosine kinases, including human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), and insulin receptor that reside in the plasma membrane (62). Upon substrate binding, receptors homo- or heterodimerize leading to autophosphorylation in trans on the activation loop or juxtamembrane region causing a conformational shift that stabilizes the active state of the proteins (62). Phosphorylation of the tyrosine’s recruit PI3K, which is made up of a p110 catalytic subunit and a p85 regulatory subunit, to the receptors through binding of the p85 subunit to the phosphotyrosine motifs or through adaptor proteins associated with the receptors (including insulin substrate receptor 1 (IRS1)) (63). Alternatively, RAS, which is also activated through association with autophosphorylated receptor tyrosine kinases, can directly bind to the amino terminal RAS-binding domain and allosterically activate PI3K (64). Association with activated receptors or RAS on the membrane allows for a conformational change to occur leading to
activation of the catalytic subunit which then can phosphorylate its primary target
phosphatidylinositol 4,5 bisphosphate (PIP2) to create phosphatidylinositol 3,4,5
triphosphate (PIP3) (61, 63). Formation of PIP3 by PI3K is antagonized by the
phosphatase and tensin homologue (PTEN) through dephosphorylation of PIP3 to re-form
PIP2 (61, 63, 65). The proteins 3-phosphoinoside-dependent kinase 1 (PDK1) and AKT
are recruited to the plasma membrane through binding of their pleckstrin homology (PH)
domains to PIP3. Binding of AKT to PIP3 causes a conformational change which exposes
two residues important for full activation of the protein, threonine 308 (T308) and serine
473 (S473) (61). PDK1, which is activated through interaction of its PH domain with PIP3,
phosphorylates T308 on the activation loop of AKT. To achieve full activation, AKT is
phosphorylated at S473 by the mechanistic target of rapamycin (mTOR) in complex with
RICTOR (mTORC2), whose activation remains poorly understood but seems to be
dependent on growth factors and PI3K signaling (61, 66-68). Activated AKT is involved in
the regulation of several targets including some of which that are responsible for
controlling cell survival including inhibition of Bad, Bax, forkhead box 3A (FOXO3A),
and glycogen synthase kinase-3β (66, 67). Moreover, AKT, along with extracellular signal-
related kinase (ERK), has intimate control over processes such as protein translation and
 cellular growth through phosphorylation of the tuberous sclerosis complex 1 and 2
(TSC1/2) which acts to inhibit its GTPase activating protein (GAP) activity towards RAS
homolog enriched in brain (RHEB) (61, 67). RHEB bound to GTP can directly interact
with mTOR in complex with RAPTOR and PRAS40 (mTORC1) leading to activation of
its kinase activity and downstream phosphorylation of eukaryotic translation initiation
factor 4E (eif4E)-binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1) (69). Activation of mTORC1 and its downstream targets controls a vast array of important anabolic events. 5’ CAP dependent mRNA translation increases the translation capacity of the cell which regulates lipid and nucleic acid synthesis, transition to aerobic glycolysis, and pentose phosphate pathway proteins, all of which promote cell growth and proliferation (70). Therefore, activation of the PI3K pathway plays an integral role in cancer initiation and growth by increasing protein synthesis machinery allowing for sufficient supply of metabolic intermediates and switch to aerobic glycolysis. Moreover, evidence suggests that mTORC1 regulates autophagy through inhibition of a conserved protein complex containing ULK1 or ULK2 (71). Autophagy is thought to induce a prosurvival response when cells are undergoing metabolic or nutrient stress. It has also been shown to have tumor suppressor effects and therefore could promote tumor progression (71).

PI3K and its downstream effectors are predominantly regulated by upstream activation of one or more receptor tyrosine kinases. However, it can also be activated at several points through crosstalk with other pathways which can have a significant impact on its regulation. As mentioned previously, RAS directly binds to and activates PI3K thereby providing a direct link between the two important signaling pathways (64). Also, loss of signaling through MEK, a protein lying downstream of RAS and RAF, results in activation of AKT through reduced phosphorylation of GAB1, a receptor tyrosine kinase adaptor protein, by ERK (72). Similarly, AKT can phosphorylate RAF to negatively regulate its activity through binding to 14-3-3 resulting in sequestration in the cytosol (64). Moreover, ERK and RSK can cross activate mTORC1 through activation of RAPTOR and
inhibition of the TSC1/2 complex, albeit at different inhibitory sites than that of AKT (64). This is further supported by reports that RSK3/4 can mediate resistance to PI3K pathway inhibition through re-activation of mTORC1 signaling (73). ERK and AKT can also act on the same substrates to regulate cell survival. ERK and AKT both phosphorylate FOXO3A at several sites resulting in protein degradation and cytosolic sequestration, respectively (64, 68). In response to genotoxic insult, DNA damage response proteins are recruited to the site of damage and activate proteins to inhibit cell cycle progression (P53) and initiate DNA repair. DNA-PK is one of the many response proteins and is also in the same family of kinases as PI3K and mTOR. Studies have shown that DNA-PK phosphorylates AKT at S473 in response to genotoxic stress to induce a pro-survival response that is independent of insulin and growth factor signaling (74, 75).

In many cancers, activation of the PI3K/mTOR pathway is key to the oncogenic transformation of normal cells to cancerous lesions and ultimately progressing to tumor formation. Furthermore, through inhibition of key effectors of apoptosis, like Bad, Bax, and FOXO3A, overactivation of this pathway can lead to a drug resistant phenotype and treatment failure. For these reasons, therapies targeting proteins along this pathway, given as a monotherapy or in combination, could be a beneficial strategy to treat cancer by shutting down growth and survival signaling mediated through PI3K, AKT, and mTOR.
Figure 2. Signal transduction through PI3K. PI3K is activated through association with substrate bound receptor tyrosine kinases and mediates the transmission of extracellular signals to intracellular targets. Activity is negatively regulated by the phosphatase PTEN which in normal cells helps to control activity levels of the pathway. PI3K activation subsequently leads to activation of important downstream targets including AKT and mTOR which, along with ERK, are integral in transmitting signals for cell growth, survival, and proliferation.
Targeted therapy as we now know it was first described in 1996 with the development of imatinib (Gleevec), a drug that inhibits the kinase activity of the Bcr-Abl fusion protein which is known to be expressed in up to 95% of chronic myelogenous leukemia (76). Other targeted therapies were subsequently developed to inhibit key nodes in the cancer cell that drives tumorigenesis. These include inhibitors of several proteins along the PI3K/mTOR pathway, which is the most deregulated in cancer. However, therapies directed at individual proteins, (e.g. HER2, PI3K, and mTOR) have been met with resistance (63). Indeed, targeted inhibition of HER2 and EGFR can be overcome through acquired mutations to proteins downstream of the receptors, including PI3K, PTEN, and AKT, leading to reactivation of the pathway (61, 63, 67). Moreover, inhibition of mTORC1 causes a release in feedback inhibition through IRS1 mediating reactivation of PI3K and downstream signaling, ultimately resulting in treatment failure (61, 63, 67, 68, 77). As a result, dual kinase inhibitors which target both PI3K and mTOR have been developed to overcome the observed resistance of targeting a single kinase. One such inhibitor is NVP-BEZ235 (BEZ) a reversible dual PI3K/mTOR inhibitor which is active against the four PI3K paralogs as well as the most common PI3K mutants and mTOR (78, 79). Inhibition with BEZ has effects on several downstream effectors including AKT, ribosomal protein S6, and the translation initiation factor 4E binding protein 1. It inhibits PI3K and both complexes containing mTOR at nM concentrations with mTOR in complex 1 being slightly more sensitive than mTORC2 or PI3K (80). BEZ has been shown to have potent anti-proliferative qualities resulting in arrest at G0/G1 in the cell cycle that leads to
inhibition of tumor growth (78). Moreover, previous reports utilizing several different cell lines suggest that induction of apoptosis is dependent on the mutational status of the cancer with HER2 amplification and PI3K mutation dependent cancers being sensitive while those dependent on KRAS and PTEN mutations being insensitive due to resistance mediated by activation of ERK (78, 80-85). Utilization of BEZ as a monotherapy is likely to have a very narrow therapeutic range that is integrally dependent on the mutation status of the cells. Furthermore, being a reversible ATP competitive inhibitor, it is likely that removal of the drug will result in commencement of cell growth that may be more rapid than prior to treatment. As a result, combination treatments utilizing BEZ as a sensitizing agent towards other cytotoxic chemotherapeutics could provide much improved lasting efficacy over individual treatments with a much wider range of tumors that could see beneficial responses.

NEFARIOUS TOXICITIES

Doxorubicin induced cardiotoxicity

DOX has long been recognized for its beneficial effects towards tumors; however its use has been limited by potentially severe cardiotoxicity. These effects were recognized very early in the development of the drug with reports of cardiac dysfunction in some of the first clinical trials, as described in a Cancer Research article from 1970 (86). The most important risk factor in developing cardiac toxicity from DOX use is cumulative dose. A dose greater than 400 mg/m² results in an estimated risk of developing chronic heart failure at 3% to 5%, however the risk increases up to 26% with doses greater than 550 mg/m² and
up to 48% with doses greater than 700 mg/m² (87). In light of this, the maximum lifetime cumulative dose is set at 550 mg/m², which in a retrospective study found the incidence of heart failure at this dose to be 26% (88, 89). Nevertheless, cardiac events can develop at much lower cumulative doses if the patient presents with other risk factors like age, diabetes, hypertension, and prior treatment with radiation therapy. DOX-induced cardiotoxicity is grouped into three categories: acute, occurring during or immediately following infusion; early onset, occurring within the first year after treatment; and late onset, occurring at least one year after treatment and may not present until 10 to 20 years after the first dose (89).

As discussed previously, what is generally believed to be the main cause of DOX-induced cardiotoxicity is through generation of ROS via one electron reduction of the quinone moiety which then acts in a futile cycle with oxygen to produce free radicals leading to DNA, lipid, and protein oxidative damage as well as mitochondrial dysfunction. These cellular events induced by ROS formation contribute to cardiomyocyte cell death from both apoptotic and necrotic pathways (87). In addition, DOX can be converted to secondary alcohol metabolites which are less active at generating ROS but more active in deregulating calcium and iron homeostasis (88). Correspondingly, DOX has been shown to affect the mitochondria through increased Ca²⁺ levels which lead to mitochondrial swelling due to opening of the mitochondria permeability transition pore (mPTP) thereby triggering cell death (87).

While it is generally believed that DOX induces cardiotoxicity through increased production of ROS, there is still debate into cause of this increase. Topo IIα, one of two
isoforms of topo II and known to be a marker for proliferation, is overexpressed in cancer cells but is undetectable in non-proliferating cells, including cardiomyocytes. On the other hand, cardiomyocytes express topo IIβ but have undetectable levels of topo IIα. Studies have shown that topo IIβ knockout cardiomyocytes are resistant to DOX-induced cell death in part through the loss in repression of genes involved in mitochondrial biogenesis and oxidative phosphorylation pathways (90). This change in transcriptome by DOX affects oxidative phosphorylation and mitochondrial biogenesis pathways which is postulated to be the reason for the observed increase in ROS production. On the other hand, there is evidence that regulation of mitochondrial iron stores by a member of the ABC transporter superfamily (ABCB8) plays a major role in DOX-induced ROS production (22). Deletion of ABCB8 in mouse hearts resulted in mitochondrial iron accumulation and development of cardiomyopathy (91). Moreover, DOX treatment induced ROS production by increasing mitochondrial iron through decreased expression of ABCB8 (22). Conversely, overexpression of ABCB8 reduced mitochondrial iron stores preventing the damage incurred by DOX futile cycle-mediated ROS production. These results are further supported by data demonstrating that the only clinically approved cardioprotective agent for DOX-induced cardiomyopathy, dexrazoxane, works by migrating to the mitochondria where it chelates iron. It was demonstrated that specifically chelating iron in the mitochondria and not the cytosol was protective (22). Therefore, evidence suggests that classical inhibition of topo II as well as iron mediated ROS production both may be important in inducing cardiotoxicity and therefore should be considered when designing strategies for cardioprotection.
Combination therapy cardiotoxicity

The advent of targeted therapies brought forth the hope of preferentially killing cancer cells without the severe side effects that plague cytotoxic agents like DOX by shutting down signaling pathways key to cancer cell survival. Unfortunately, these have also been met with rapid resistance leading to continued growth of the tumors and recurrence. Moreover, the same signaling pathways that are important for cancer cell survival also regulate important processes in cardiomyocytes which can lead to deleterious effects on the heart (92). Indeed, clinical use of several kinase inhibitors has been reported to induce some form of cardiac dysfunction including hypertension, declines in left ventricular function, and congestive heart failure (87, 92, 93). Mechanistic reasons for the effects on the heart seem to point towards disruption of neuregulin-HER2 signaling, loss of survival signaling through ERK and PI3K, deregulation of calcium handling, and mitochondrial swelling (92).

Still, cardiac events using monotherapy of tyrosine kinase inhibitors are relatively rare and many times reversible. However, in the need to improve therapeutic efficacies against tumors, therapies combining targeted agents and cytotoxic agents are becoming tested with increasing frequency and have subsequently been found to have a high amount of toxicity, especially cardiotoxicity (94-96). HER2, for example, is highly expressed in certain forms of breast cancer so combining the anti-HER2 monoclonal antibody trastuzumab with a standard chemotherapy regimen of DOX and cyclophosphamide became an attractive strategy to gain better control over the tumor. While the combination did have significant improvement in objective response over DOX alone (50% vs 32%), it
also saw an increase in cardiac dysfunction which was present in 27% of the patients compared to 8% of those who received only DOX and cyclophosphamide (97). In addition, preclinical reports using the HER2/EGFR inhibitor lapatinib, have shown that it greatly potentiates the myocyte damaging effects of DOX (94). Similarly, neuregulin, an activator of erbB2 (HER2) and erbB3, has been demonstrated to protect cardiomyocytes against DOX-induced toxicity (98). These studies highlight the critical role that HER2 and other receptor tyrosine kinases play in the survival of cardiomyocytes when undergoing stress. Likewise, conditional knockouts of proteins along the PI3K/mTOR pathway in the heart develop cardiomyopathy and are more susceptible to damage from stress (95). On the other hand, lapatinib in combination with DOX reduced ERK phosphorylation but not AKT suggesting that ERK may be more important in protecting myocytes from DOX than AKT (94). In light of these studies, targeted inhibition of only one of the survival pathways (ERK or AKT) may provide a sufficient sensitizing effect to cancer cells without completely shutting down survival signaling in cardiomyocytes providing a muted toxicity profile compared to receptor tyrosine kinase inhibitors like trastuzumab.

CURRENT STUDY

The major goal was to investigate the effect of BEZ in enhancing the efficacy of DOX in KRAS or p53 mutant cancers particularly the pancreatic, breast, colon, and lung. We hypothesized that BEZ will sensitize cancer cells to DOX through inhibition of survival signaling and increased activation of pro-apoptotic proteins. Furthermore, based on previous clinical studies with trastuzumab and DOX showing increased risk of heart failure
when the drugs were treated in combination (97), we tested whether inhibition of PI3K/mTOR pathway signaling in the heart would potentiate the cardiotoxic effects of DOX. Accordingly, the specific goals of the study were as follows:

1) Interrogate the novel interaction of BEZ with ABC transporters and how this could affect cell sensitization to DOX.

2) Explore the mechanism by which BEZ sensitizes cancer cells to DOX through its inhibition of PI3K and mTOR and modulation of apoptosis-related proteins.

3) Determine whether combination treatment with BEZ and DOX has superior *in vivo* anti-tumor effects than individual drug treatments.

4) Examine the effects of the combination treatment with BEZ and DOX on mechanism of cell death in cardiomyocytes and cardiac function in tumor bearing mice.

We show that BEZ has a potent and selective sensitizing effect on DOX but not Gem in all KRAS or P53 mutant cancers tested. Furthermore, for the first time we show that physiological concentrations of BEZ treated in combination with DOX enhanced drug accumulation which resulted in increased DNA damage and ROS production. Moreover, expression of the anti-apoptotic protein Bcl-2 is reduced while the smaller, more cytotoxic forms of BIM are enhanced to favor apoptosis. Importantly, the increase in cancer chemotherapeutic efficacy did not result in a deterioration of DOX-induced cardiac
dysfunction. Based on these results, we propose that combining BEZ with DOX could be a potential therapeutic option for patients with pancreatic and other cancers. It is our hope that such a strategy may turn out to be significantly better than currently used regimens.
CHAPTER 2: Materials and Methods

Cell lines. MiaPaCa2 pancreatic cancer cells (MIA PaCa-2 ATCC CRL-1420), MDA-MB-231 (MDA-MB-231, ATCC HTB-26) and Mcf7 (MCF7 (ATCC HTB-22) breast cancer cells were obtained from American Type Culture Collection. Upon receiving the cells, they were thawed and expanded after which several stocks were frozen in liquid nitrogen. For experimental use, cells were thawed from a stock frozen in liquid nitrogen starting with passage number of 4 and discarded before passage number 20. Capan-1, and CD18 were obtained from Dr. Surinder Batra from the University of Nebraska Medical Center. HCT 116 and H1299 cells were obtained from Dr. Steven Grossman from Virginia Commonwealth University Medical Center. No cell line authentication was conducted by the authors. All cells were grown in DMEM medium supplemented with 10%FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Mia-dx drug resistant cells were derived from MiaPaCa2 cells through step wise treatment with increasing concentrations of DOX. In short, starting at 0.2 μM, cells were incubated with DOX for periods of 2 hours once every two to three weeks (depending on recovery time). Concentrations were increased by 0.1 μM at every treatment, up to a final concentration of 1 μM. After this point, cells were grown in DMEM medium supplemented with 10%FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.05 μM DOX. Mia-B1 cells were derived by transfecting MiaPaCa2
cells with the pHAMDRwt plasmid containing the full length ABCB1 gene. After transfection, ABCB1 overexpressed cells were selected by culturing in 0.5 μM DOX until colonies were formed and all non-transfected control cells were dead. Colonies were picked to obtain a single colony population. Cells were then grown in DMEM medium supplemented with 10%FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.05 μM DOX. A2780 ovarian cancer cells were grown in RPMI medium supplemented with 10%FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. A2780-dx drug resistant cells were derived from A2780 cells through stepwise treatment with DOX as previously described. After selection, cells were grown in RPMI medium supplemented with 10%FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.05 μM DOX.

pHAMDRwt plasmid (Addgene #10957) was obtained from Addgene and supplied by Michael Gottesman.

**Compounds and reagents.** BEZ235 (#NC9953953) and gemcitabine (#NC0063515) were purchased from Fisher Scientific and Doxorubicin (#D1515-10MG) was purchased from Sigma-Aldrich. Sildenafil was a gift from Pfizer. Antibody for actin-HRP (#sc1616) was purchased from Santa Cruz Biotechnology. Phospho-S6 ribosomal protein (#4858), S6 ribosomal protein (#2217), Phospho-AKT 473 (#4060), Phospho-AKT 308 (#13083), AKT (#9272), Phospho-ATM (#5883), Phospho-chk2 (#2661), Bcl-2 (#2870), Bax (#2772) and cleaved-PARP (#5625) were purchased from Cell Signaling Technology. Secondary antibody was purchased from VWR (#95017-556). Trypan blue dye (#T8154) was purchased from Sigma-Aldrich. TUNEL assay was purchased from Fisher Scientific (#NC9027080). Carboxy-H2DCFDA was purchased from Life Technologies (C400).
Cell viability. Cell viability was measured using the CellTiter 96 Aqueous One assay from Promega. Cell lines were plated at a density of 5000 cells/well in a 96 well plate for 48 hours in non-treated growth medium in an incubator set at 37°C and 5% CO2. Cells were then treated with drugs at the indicated concentrations for an additional 48 hours in the incubator. After treatment, medium was replaced with 100 μl of Aqueous One solution according to Promega’s protocol and incubated at 37°C for one hour. Viability was assessed by measuring the absorbance of each well using a 96 well plate spectrophotometer.

**Trypan Blue cell death assay.** Cells were plated into 6-well tissue culture plates at a density that would result in confluence of 70-80% after incubation for 48 hours. Cells were then treated in triplicate for the indicated time. Media was removed and placed into a centrifuge tube. 1 ml of 1x PBS was then added to the plates to remove any residual media after which it was placed into the corresponding centrifuge tube. Attached cells were trypsinized, collected and then added to its corresponding tube containing the media and PBS wash. Cells were pelleted by centrifugation and supernatant discarded. Cell pellet was re-suspended in normal media and 100 μl of cell suspension was mixed with 100 μl of Trypan Blue dye and placed onto a hemocytometer. Live (unstained) and dead (stained) were quantified under a microscope.

**TUNEL assay.** DNA fragmentation was measured using the ApoAlert DNA Fragmentation assay from Clontech and purchased from Fisher Scientific (#NC9027080). Slides were prepared using the protocol from Clontech. In short, cells were plated on 4-chamber microscope slides and allowed to attach for 48 hours. They were then treated for
an additional 24 hours with the indicated concentrations of drug and fixed in 4% paraformaldehyde for 15 minutes at room temperature. After fixation, they were washed with 1x PBS and incubated in 100% methanol for 10 minutes in -20°C. The slides were then washed with PBS and stored at -20°C in 70% ethanol. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes on ice after which they were washed in PBS. Slides were then incubated in equilibration buffer for 10 minutes after which they were incubated with TDT incubation buffer for 60 minutes at 37°C. Reaction was then terminated in SSC, washed, and coverslips added. TUNEL positive cells were visualized using the NIKON Eclipse Ti confocal microscope.

**Immunoblot analysis.** After treatment, cells were washed twice with 1x PBS then pelleted. Pellets were lysed with 1x lysis buffer (Cell Signaling #9803) plus 1 to 100 dilution protease inhibitor cocktail (Thermo Scientific #78410) and incubated on ice for 30 minutes after which samples were centrifuged at 12,000g for 10 minutes at 4°C to remove insoluble debris (debris pellet was used for DOX accumulation measurements described below). Supernatant was collected and protein measured using manufacturer’s protocol (Bio-Rad Protein Assay reagent #500-0006). Samples were combined with 2x Laemmli sample buffer (Bio-Rad #161-0737) and boiled for 5 minutes after which proteins were separated using SDS-PAGE on 4%-20% TGX gradient gel (Bio-Rad #567-1093) and transferred to nitrocellulose paper (Bio-Rad #162-0232). After blocking non-specific binding sites with 5% milk in 1x TBS-T (tris buffers saline with 0.05% tween), membranes were incubated with the primary antibodies at 4oC overnight, washed 4x for 10 minutes each with TBS-T and incubated an additional one hour with a secondary antibody. Membranes were then
washed 4 times with TBS-T for 10 minutes each and visualized using Western Lighting ECL plus (Perkin Elmer #NEL105001) and exposed on BioMax light film (Kodak #1788207).

**DOX accumulation studies.** Cells were plated in 6 well plates at a density of 20,000 cells/well and incubated at 37°C for 48 hours. After the 48 hour incubation, DOX (0.2 μM) was added in the presence or absence of the indicated concentrations of inhibitor. After 48 hours more, cells were trypsinized, washed twice with 1x PBS and re-suspended in 1 ml of 1x PBS. DOX was accumulation measured by flow cytometry using excitation wavelength of 488 nm.

**DOX efflux studies.** Cells were plated in 6 well plates at a density of 20,000 cells/well and incubated at 37°C for 48 hours. After the 48 hour incubation, DOX (5 μM) was added in the presence or absence of the indicated concentrations of inhibitor. After one hour, medium was replaced, without DOX, in the presence or absence of inhibitor for two hours. Cells were then trypsinized, washed twice with 1x PBS and re-suspended in 1 ml of 1x PBS. DOX accumulation was measured by flow cytometry using excitation wavelength of 488 nm.

**Intracellular DOX accumulation.** DOX was measured using the debris pellet from protein lysate preparation which contains the majority of the DOX. After centrifugation, pellet was re-suspended in 400 μl of acidified alcohol (50ml of 70% ethanol and 375 μl 12N HCl) then incubated at -200C overnight followed by centrifugation at 20,000 g for 10 minutes. Supernatant was aliquoted in triplicate into a black 96 well polystyrene microplates (VWR
# 82050-728) and fluorescence measured at excitation of 485 nm and emission of 595 nm using Molecular Devices Spectramax M5 plate reader.

**Immunofluorescence staining.** Cells were plated on 4-well glass chamber slides (World Wide Medical Products #354577) at a density of 10,000 cells/well and allowed to grow for 48 hours. Cells were then fixed in 4% paraformaldehyde for 15 minutes at room temperature and washed three times with 1x PBS for 5 minutes each. Cells were then incubated for 1 hour in blocking buffer (5% normal goat serum, 0.3% Triton X-100 in 1x PBS) followed by incubation with primary antibody in blocking buffer overnight at 4°C. Thereafter, slides were washed three times in 1x PBS for 5 minutes each and then incubated with secondary antibody in blocking buffer an additional 1 hour. The slides were subjected to three additional washings in 1x PBS were performed after which hard set mounting medium (Vectashield #H1500) was added and cover slips placed. Proteins were visualized using the NIKON Eclipse Ti confocal microscope.

**ROS measurement.** MiaPaca2 cells were seeded into a 96-well tissue culture plate at a density of 15,000 cells/well in phenol-free medium. After 24 hours, carboxy-DCFDA (Life Technologies #C-400) was added at a concentration of 20 μM in phenol-free medium and incubated at 37°C for 30 minutes. The wells were then washed one time with 1x PBS after which drugs (diluted in phenol-free medium) were added at a volume of 100 μl. DCFDA fluorescence was measured at 6 hours using the setting for excitation at 485nm and emission at 535 nm using Molecular Devices Spectramax M5 plate reader.

**ATPase assay.** ATPase activity was measured to test BEZ235’s capacity to be an ABCB1 substrate. Assay protocol (Promega #V3601) was followed according to manufacturers’
guidelines. Briefly, drugs were incubated with assay buffer, p-gp membranes, and ATP for 40 minutes at 37°C. Detection reagent was added then incubated for an additional 20 minutes at room temperature after which the luminescence was measured.

**Animal studies.** All mice were maintained in the vivarium at Virginia Commonwealth University and kept in accordance to a protocol approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Female 8-10 week old Athymic NCr nu/nu mice were inoculated with of 1 x 10^6 MiaPaca2 cells, in a 1:1 ratio of cells to Matrigel (BD #354234), subcutaneously into the right rear flank. Tumors were allowed to grow for two weeks prior to initiation of treatment. DOX was dissolved in saline and injected intravenously into the tail vein twice, two weeks apart, at a concentration of 10 mg/kg. BEZ was dissolved in 1-Methyl-2-pyrrolidinone (NMP) and then 0.5% sodium-carboxymethylcellulose (Na-CMC) was added so that the final volume contained 10% NMP and 90% of 0.5% Na-CMC. BEZ was fed by oral gavage daily for 28 days at a concentration of 40 mg/kg in a volume of 0.2 ml/mouse. Gem was dissolved in saline and injected intraperitoneally (IP) once a week for three weeks. Control, DOX, and Gem groups were fed daily with the vehicle of the same mixture as for BEZ. Tumors were measured twice weekly by calipers using the formula (L x W^2)/2 to get the tumor volume. Weight was measured twice weekly to monitor weight loss. Heart function was assessed using the Vevo770 imaging system (Visualsonics, Inc.), as previously reported (99), four weeks after start of treatment. Pentobarbital (30 mg/kg IP) was used for anesthesia.
**Statistical analysis.** Statistical analysis was performed with GraphPad Prism 4.0 (Graphpad Software Inc.). Data are presented as mean ± SEM. Statistical comparisons between 2 groups were performed with the unpaired Students t-test. One-way ANOVA was used when comparing more than two groups followed by Newman–Keuls post hoc test for pair-wise comparison. Two-way ANOVA was used when comparing the difference between normal vs drug resistant cell lines. p < 0.05 was considered to be statistically significant.
CHAPTER 3: Results

**BEZ235 and doxorubicin effects on ABC transporters**

**Doxorubicin resistant cells overexpress ABCB1**

Overexpression of ABC transporters leading to MDR is a major cause of treatment failure in cancer patients. This increase in expression results in improved drug efflux efficiency leading to sub-lethal intracellular levels. Therefore, it is of great interest to discover new inhibitors of these transporters that result in reversal of the MDR phenotype and re-sensitization of cancer cells to drug treatment. Since the early 1980’s, inhibitors that target ABC transporters have been investigated for their ability to reverse MDR. Recently, several inhibitors that were specifically designed to target enzymes in the signal transduction pathway, including those targeting PI3k and mTOR, have also been found to inhibit one or more of the ABC transporters leading to a reversal of MDR phenotypes (52, 100-102). Therefore, we set out to test the ability of the dual PI3k/mTOR inhibitor BEZ to re-sensitize cells overexpressing ABCB1 to DOX. To investigate this, it was necessary to create cells that overexpressed ABCB1. Two drug resistant cell lines (Mia-dx and A2780-dx) were created with sequential treatment of DOX over a six month period (Figure 3A). A third cell line (Mia-B1) was created by transfecting a plasmid carrying the MDR1 gene and selecting with DOX to obtain drug resistant colonies (Figure 3B). Each of the drug
resistant clones showed marked resistance to DOX in comparison to their respective parental cell lines when assessed over a range of concentrations starting at 0.05 µM and increasing up to 1.0 µM (Figure 4). Expression of ABCB1 was confirmed using western blot analysis and confocal microscopy. Of the drug resistant cell lines, two (Mia-B1 and A2780-dx) had an overexpression of ABCB1 protein, while the third (Mia-dx) had undetectable levels of ABCB1 (Figure 5). While ABCB1 is the best studied and most common ABC transporter related to DOX resistance, there are others that contribute to MDR in cancer and one or more of these may be involved in the resistance of Mia-dx cells.
Figure 3. Protocol for creating drug-resistant cell lines. (A) MiaPaca2 and A2780 parental cell lines were treated with increasing concentrations of DOX over a 6 month period to obtain drug resistance. The starting concentration of DOX was 0.2 μM, which was increased up to 1 μM with an incubation period of 2 hours. After treatments, cells were washed and allowed to recover for up to 2 weeks before starting the next DOX treatment protocol. This method created the Mia-dx and A2780-dx cell lines. (B) MiaPaca2 parental cells were transfected with a plasmid containing the MDR1 gene. Cells were selected using 1 μM DOX with resistant cell having overexpressed ABCB1 protein. This method created the Mia-B1 cell line.
Figure 4. Viability of doxorubicin (DOX)-resistant cancer cell lines.

Viability/proliferation was measured by MTS assay after challenging the cells to DOX concentrations ranging from 0.05 to 1 μM in the pancreatic cancer cell lines (A) Mia-dx and (B) Mia-B1, and the ovarian cancer cell line (C) A2780-dx. Cells viability was compared to their respective parental cell lines. 2-way ANOVA was used to measure significance. Data was plotted as mean ± SD of replicates of four. P < 0.0001 when comparing resistant cells vs their respective parental cells for all cell lines.
Figure 5. Doxorubicin (DOX)-resistance is mediated by ABCB1 overexpression.

Confocal microscope images of ABCB1 protein expression in (A) MiaPaca2, Mia-dx, and Mia-B1 cell lines as well as (B) A2780 and A2780-dx cell lines. (C) Western blot analysis showing expression of ABCB1 in all cell lines. Note that only Mia-B1 and A2780-dx showed increased expression of ABCB1.
BEZ235 treatment reverses drug-resistant phenotype

Overexpression of ABC transporters, including ABCB1, leads to increased drug efflux efficiency which in turn decreases intracellular drug accumulation (33, 34, 103). We wanted to test if BEZ treatment would increase accumulation of DOX in our drug resistant cell lines. Cells seeded into 6-well plates were treated with DOX, either alone or in combination with inhibitor for 48 hours. Taking advantage of DOX auto-fluorescence, cells were collected and drug accumulation directly measured by flow cytometry.

Sildenafil (SIL) inhibits ABCB1 mediated drug efflux (104) and therefore was used as a positive control. Control cells are shown as a blue peak and mark the baseline from which to compare the shift in fluorescence intensity. Parental cells treated with DOX are shown as a red peak which has shifted to the right indicating an increase in fluorescence intensity. Drug-resistant cells are shown as pink peaks and inhibitor treated cells are shown as green peaks.

SIL treatment caused a shift to the right in each drug-resistant cell line indicating its inhibition of ABC transporters leading to an increase in DOX accumulation (Figure 4). BEZ increased DOX accumulation to the same extent as SIL when using 50nM of drug and completely reversed the drug resistant phenotype at 150nM in the A2780-dx cell line (Figure 6A middle and right histogram). Similar results were seen in Mia-B1 cells, although with a more subdued response (Figure 6B). In these cells, SIL and 50nM BEZ showed only a minor increase in accumulation (Figure 6B left and middle histograms). While 150nM BEZ did enhance accumulation, it was not as complete a reversal as was observed in the A2780-dx cell line (Figure 6B right histogram). This difference is most
likely the result of the far greater expression of ABCB1 in Mia-B1 cells. Slightly different results were observed in the Mia-dx cell line. Mia-dx cells share the drug resistant phenotype with Mia-B1 and A2780-dx, but lack overexpression of ABCB1. In these cells, 50nM of BEZ had little effect on increasing DOX accumulation (Figure 6C middle histogram), and 150nM of BEZ only had minimal effects (Figure 6C right histogram). SIL increased accumulation in these cells to a greater extent than BEZ (Figure 6C left histogram), suggesting that BEZ may be more specific to ABCB1.
Figure 6. Effect of BEZ235 (BEZ) on doxorubicin (DOX) accumulation in resistant cell lines. Cells were treated for 48hr and accumulation was analyzed by flow cytometry. The light blue peak ▲ represents the untreated parental control, the red peak ▲ represents the DOX treated parental cells, the pink peak ▲ represents the DOX treated drug resistant cell line, and the green peak ▲ represents the DOX treated drug resistant cell line in combination with the indicated inhibitor. (A) A2780-dx, (B) Mia-B1, and (C) Mia-dx.
**BEZ235 directly inhibits ABCB1**

Our results show that BEZ is effective at increasing the accumulation of intracellular DOX after 48 hours of treatment. However, because BEZ inhibits key proteins involved in the regulation of signal transduction and protein expression, it is possible that the increased accumulation is not a result of direct inhibition of ABCB1 but because of a reduction in ABCB1 protein expression due to reduction in translation. To test this possibility, a drug efflux assay, which uses a much shorter treatment time of three hours, was performed. Cells that had been grown in 6-well plates were treated for one hour with either DOX alone or DOX in combination with the inhibitor. After one hour, the cells were washed and the media was replaced with DOX free media with or without inhibitor for an additional two hours to allow the cells to efflux the accumulated DOX. Cells were then collected and DOX fluorescence was measured using flow cytometry. Similar to the results in the 48 hour accumulation assay, BEZ and SIL were also able to inhibit DOX efflux in a dose dependent manner in both, A2780-dx and Mia-B1, cell lines (Figure 7A and B). On the other hand, neither SIL nor BEZ were able to inhibit efflux to a great extent in cells which have undetectable expression levels of ABCB1, Mia-dx (Figure 7C). These results demonstrate that BEZ is able to enhance the accumulation of DOX in ABCB1 overexpressing drug resistant cell lines through direct inhibition of the transporter.
Blue = A2780 control
Red = A2780 DOX 5µM
Pink = A2780-dx DOX 5µM
Green = A2780-dx DOX+SIL 10µM

Blue = A2780 control
Red = A2780 DOX 5µM
Pink = A2780-dx DOX 5µM
Green = A2780-dx DOX+Sild 20µM

Blue = A2780 control
Red = A2780 DOX 5µM
Pink = A2780-dx DOX 5µM
Green = A2780-dx DOX+BEZ 50nM

Blue = A2780 control
Red = A2780 DOX 5µM
Pink = A2780-dx DOX 5µM
Green = A2780-dx DOX+BEZ 150nM

Blue = A2780 control
Red = A2780 DOX 5µM
Pink = A2780-dx DOX 5µM
Green = A2780-dx DOX+BEZ 300nM
B

Blue = Mia control
Red = Mia DOX 5µM
Pink = Mia-B1 DOX 5µM
Green = Mia-B1 DOX+SIL 10µM

Blue = Mia control
Red = Mia DOX 5µM
Pink = Mia-B1 DOX 5µM
Green = Mia-B1 DOX+Sil 20µM

Blue = Mia control
Red = Mia DOX 5µM
Pink = Mia-B1 DOX 5µM
Green = Mia-B1 DOX+BEZ 50nM

Blue = Mia control
Red = Mia DOX 5µM
Pink = Mia-B1 DOX 5µM
Green = Mia-B1 DOX+BEZ 150nM

Blue = Mia control
Red = Mia DOX 5µM
Pink = Mia-B1 DOX 5µM
Green = Mia-B1 DOX+BEZ 300nM
Figure 7. Effect of BEZ235 (BEZ) on inhibition of ABCB1 mediated doxorubucin (DOX) efflux. Cells were treated for 1hr with DOX in combination with inhibitor (SIL or BEZ) followed by an additional two hours incubation with inhibitor treated DOX free media. The cells were analyzed by flow cytometry. In all histograms, the light blue peak represents the untreated parental control, the red peak represents the DOX treated parental cells, the pink peak represents the DOX treated drug resistant cell line, and the green peak represents the DOX treated drug resistant cell line in combination with the indicated inhibitor. (A) A2780-dx, (B) Mia-B1, and (C) Mia-dx.
**BEZ235 is a non-substrate inhibitor of ABCB1**

We have shown that BEZ can enhance accumulation of DOX in drug resistant cell lines through direct inhibition of ABCB1. Next, we wanted to determine if BEZ inhibits through competition of substrate (i.e. DOX) in the binding pocket or through inhibition of the ATPase domain. When a substrate interacts with ABCB1, it stimulates the rate of ATP hydrolysis of the transporter (38). To test the ability of BEZ to stimulate ATPase activity, different concentrations of BEZ, along with known ABCB1 substrates (verapamil, SIL and DOX), and an ATPase inhibitor (vanadate), were combined with isolated ABCB1 (p-gp) membranes in the presence of ATP. ATPase activity was quantified by the amount of inorganic phosphate released from ATP.

Each compound known to be a substrate of ABCB1 significantly stimulated ATP hydrolysis (Figure 8A). This included DOX, although it appeared to be a fairly weak substrate. On the other hand, in multiple experiments, BEZ either did not stimulate ATP hydrolysis or showed only a minor stimulation as compared to the no treatment group (Figure 8A and C).

To further examine this, parental and drug resistant cell lines were incubated with 50nM BEZ for 24 hours, and western blots were run to look at BEZ’s inhibition of downstream mTOR signaling. If BEZ were a substrate, then inhibition of mTOR would be diminished in ABCB1 overexpressing cell lines due to decreased accumulation. mTOR activity in all cell lines, including those overexpressing ABCB1, was dramatically reduced after treatment with BEZ (Figure 8B). These results suggest that accumulation of BEZ itself was not effected and supports our previous evidence that BEZ is a poor substrate of
ABCB1. Since BEZ inhibition of PI3k and mTOR occurs at the ATP binding site of these kinases, it is possible that BEZ also inhibits at the ATP binding site of ABCB1, thereby diminishing ATP hydrolysis and drug efflux efficiencies. If this were the case, BEZ should block the stimulation of ATPase activity when a substrate is combined with the isolated p-gp membranes. Our results demonstrate that vanadate is a potent inhibitor of ATP hydrolysis, even in the presence of verapamil (Figure 8C). However, BEZ is unable to alter the ATPase activity even at the higher concentration of 150nM indicating that it does not inhibit at the ATP binding site of ABCB1. These results provide strong evidence that BEZ is a poor substrate for ABCB1, and would be less likely to out-compete superior substrates like DOX for access to the binding site. BEZ is also not an inhibitor of ATPase activity indicating a separate inhibition profile. It is proposed BEZ is likely a non-substrate inhibitor that can bind at the substrate binding site and act as a competitive inhibitor of known substrates but is not a substrate itself as shown in the schematic (Figure 9).
Figure 8. BEZ235 (BEZ) is a weak substrate for ABCB1. (A and C) An ATPase activity assay using isolated p-gp membranes was used to determine if BEZ is a substrate of ABCB1. Verapamil was used as a positive control for ATPase stimulation. Vanadate inhibits ATPase activity by interacting with the ATP binding site. (B) Parental and drug-resistant cell lines were treated in the presence or absence of 50 nM BEZ for 24 hours after which cell lysates were collected to look at phosphorylation of ribosomal protein S6, a downstream marker of mTORC1. Data is plotted as mean ± SEM of triplicates.
Figure 9. Proposed mechanism for ABCB1 inhibition of BEZ235 (BEZ). (A)

Schematic showing the effect of ABCB1 on DOX accumulation. (B) Possible inhibitory mechanism of BEZ on ABCB1 by competitive inhibition leading to increased DOX accumulation.
BEZ235 re-sensitizes drug resistant cells to treatment with doxorubicin

Treatment failure in cancer patients is commonly associated with the overexpression of ABC transporters. One strategy to overcome this is to inhibit the transporters, thereby increasing accumulation of the cytotoxic agent within the cell. Therefore, we determined the ability of BEZ to re-sensitize ABCB1 overexpressing cells to DOX treatment. First, cell viability was used to determine the effects of combining BEZ with DOX after 48 hours of treatment. BEZ alone had a dramatic effect on the cell viability of both MiaPaca2 and Mia-B1 (Figure 10A and B). Treatment of 0.5 µM DOX decreased the number of viable cells in MiaPaca2 to 60% as compared to the control group (Figure 10A). Combination of DOX with BEZ caused significant reductions in cell viability compared to individual drug treatments, decreasing from 34% with 50 nM BEZ to 22% with 600 nM BEZ (Figure 10A). However, Mia-B1 cells responded quite differently when treated in the same manner as MiaPaca2 cells. As expected, due to overexpression of ABCB1 and similar to previous cell viability assays (Figure 2), cells treated with 0.5 µM of DOX alone had no decrease in viability as compared to the control group. Also, analogous to the results in Figure 5B which showed no increase in DOX accumulation, the use of DOX in combination with 50 nM BEZ did not increase the effects on viability compared to the cells treated with 50 nM BEZ alone (Figure 10B). Conversely, with increasing concentrations of BEZ (i.e. 150, 300, 600 nM) in combination with DOX, there was a corresponding significant decrease in the percentage of viable cells as compared to all other groups (Figure 10B). Similar results were seen in the ovarian cancer cell lines (Figure 10C and D). BEZ treatment alone decreased the percentage of viable cells
remaining in both A2780 and A2780-dx cells to below 50%. The A2780 parental cells were sensitive to DOX alone, with only 26% viable cells remaining after 48 hours of treatment. Combination with BEZ further reduced viability to approximately 15% in all groups, having shown little enhancement with increasing concentrations of BEZ (Figure 10C). In A2780-dx cells, similar to the results shown in Figure 2, 55% viable cells remained after treatment with DOX alone. Combinations with BEZ reduced viability in a dose dependent manner with approximately 15% viable cells remaining in the 600 nM BEZ group (Figure 10D).

Cell viability is merely a measure of how many cells are still alive after treatment and gives no information on whether a change is the result of reduced proliferation or increased cell death. To determine the role that apoptosis plays in the observed decrease in viability with combination treatment, cells were treated for 48 hours with BEZ and DOX, either alone or in combination for use in western blot analysis. Treatment with BEZ alone did not induce apoptosis (as measured by the extent of PARP cleavage) in any of the cell lines tested (Figure 11). Treatment of MiaPaca2 and A2780 cells with DOX resulted in PARP cleavage, which was enhanced with BEZ in a manner that mirrored the decrease in viability (Figure 11A and B). In both the Mia-B1 and A2780-dx cells, DOX treatment alone was unable to induce cleavage of PARP, while co-treatment with BEZ re-sensitized the cells in a dose dependent manner that also mirrored the decrease in viability (Figure 11A and B). These results demonstrate that BEZ’s ability to inhibit ABCB1 can re-sensitize drug-resistant cells to DOX leading to a dose-dependent decrease in cell viability and increase in apoptosis.
Figure 10. BEZ235 (BEZ) re-sensitizes drug-resistant cells to doxorubicin (DOX).

Cells seeded into 96-well plates were treated with the indicated drug concentrations. After 48 hours, cell viability was measured using the AQueous One cell viability assay kit from Promega. (A) MiaPaca2, (B) Mia-B1, (C) A2780, (D) A2780-dx. Data is plotted as mean ± SD of replicates of 4. #, *, and ** indicate significance of the combination treatment group to its respective BEZ only treatment group.
Figure 11. Effect of BEZ235 (BEZ) in enhancing doxorubicin (DOX)-induced cytotoxicity. (A) MiaPaca2 and Mia-B1 cells were treated with increasing concentrations of BEZ either alone or in combination for 48 hours. Cell lysates were collected and analyzed using western blot. Apoptosis was determined by the presence of cleaved PARP. (B) A2780 and A2780-dx were treated in a similar fashion and analyzed using western blot for the presence of cleaved PARP.
Effect of BEZ235 and doxorubicin on other ABC transporters

Several ABC transporters are overexpressed in cancer leading to multi-drug resistant phenotypes, including ABCB1 (32, 34, 105). However, others play important roles not only for xenobiotic clearance but also for transport of a wide range of substrates across membranes throughout the cell (22, 34, 46, 91). Modulation of some of these substrates by ABC transporters may influence the effectiveness of cancer drugs through direct or non-direct interactions (22, 91, 106). Also, transporter expression levels may be altered in response to drug treatment which could have an effect on treatment responses. Therefore, mRNA expression levels of several ABC family transporters were assessed using real-time quantitative PCR to test their responsiveness to treatment with BEZ and DOX either alone or in combination in MDA-MB-231 breast cancer cells.

ABC8 is a mitochondrial associated iron transporter which has been associated with iron-mediated ROS formation of DOX (22). mRNA expression was induced by nearly two-fold over control levels after and this effect was not enhanced when combined with BEZ (Figure 12A). This response may act as a protective mechanism to reduce the amount of ROS produced by the interaction of DOX with intra-mitochondrial iron.

The C branch of the ABC superfamily has the largest number of transporters known to confer resistance to clinically relevant chemotherapeutics, including DOX (42, 45, 107-109). ABCC1 along with ABCB1 (measured but not included due to low expression levels in these cells) and ABCG2 are the most common transporters associated with multi-drug resistance to cancer treatment. However, in these cells, ABCC1 had no response to DOX when treated alone and only a minimal, but significant, response was
observed when combined with BEZ (Figure 12B). On the other hand, ABCC3, ABCC5, and ABCC10 had small but significant increases in mRNA expression after treatment with DOX (Figure 12C - E). Interestingly, ABCC3 had a large response to BEZ treatment alone which was enhanced even more when combined with DOX (Figure 12C). ABCC5 and ABCC10 both had responses that mirrored the treatment-related accumulations of intracellular DOX. There was no change in expression in BEZ treated cells and significant increases in expression over DOX alone when BEZ was combined with DOX (Figure 12D and E).

As mentioned previously, ABCG2 is one of the three most commonly overexpressed transporters related to multi-drug resistance (41). Therefore it was important to also measure the mRNA expression levels of this protein. Similar to ABCC1, mRNA expression of ABCG2 was not significantly altered after treatment with DOX (Figure 12F). In addition, treatment with BEZ alone did not change mRNA expression. However, ABCG2 mRNA expression was significantly increased when BEZ was combined with DOX (Figure 12F).

These results suggest a differential response to BEZ and DOX treatment that is likely cell line dependent. Currently, it is unclear how the changes in expression of these transporters would affect the responsiveness of the cells to drug treatment or if BEZ would also inhibit efflux mediated by these transporters. It is postulated that these responses are protective mechanisms that would influence the effectiveness of drugs but further studies are needed to confirm this.
A

ABCB8

*p < 0.001 vs control and BEZ

relative expression

control  DOX 0.5 µM  BEZ 300 nM  BEZ+DOX
**ABCC1**

*p < 0.01 vs control and BEZ
*p < 0.05 vs DOX

**ABCC3**

*p < 0.001 vs all others
*p < 0.05 vs control

**ABCC10**

*p < 0.001 vs all others
*p < 0.01 vs control
*p < 0.05 vs BEZ

**ABCC5**

*p < 0.001 vs all others
Figure 12. Modulation of ABC transporter transcript levels after treatment with BEZ235 (BEZ) and doxorubicin (DOX). MDA-MB-231 cells were incubated for 24 hours in the presence of BEZ and DOX either alone or in combination. RNA was extracted and mRNA transcript levels were measured using quantitative real-time PCR looking at (A) ABCB8, (B) ABCC1, (C) ABCC3, (D) ABCC5, (E) ABCC10, AND (F) ABCG2.
Data is plotted as mean ± SEM of triplicates.
Pancreatic Cancer

Effects of BEZ235 (BEZ) on enhancing doxorubicin (DOX)-induced cell killing

Survival signaling in the cell is commonly mediated by two closely interconnected pathways which initiate at activated membrane bound receptors: the PI3K and RAS pathways (64). Mutations result in overactivation of these pathways leading to not only growth and survival advantages over the surrounding normal tissue but also reduced drug effectiveness (110). Therefore, an important treatment strategy is to target these pathways as a way of sensitizing the cells.

The dual kinase inhibitor, BEZ, targets the PI3K/AKT/mTOR pathway at multiple nodes, inhibiting PI3K along with both complexes of mTOR (Figure 13). This could lead to sensitization of cancer cells to cytotoxic agents like DOX. To examine this, MiaPaca2 cells were challenged with increasing concentrations of BEZ (50 nM to 600 nM) alone or in combination with 0.5 μM DOX for 24hr. An increase in phosphorylation of AKT on serine 473 (p-AKT 473) and threonine 308 (p-AKT 308) was observed at low concentrations of BEZ with maximum effect occurring at 50 nM (Figure 14A). This is most likely the result of a release of feedback inhibition of IRS1 through mTORC1 inhibition (Figure 13). At higher concentrations (150 nM - 600 nM), phosphorylation was sequentially reduced with increasing concentrations of BEZ. Treatment with DOX alone increased p-AKT 473 but not p-AKT 308, indicating a difference in the stress response to DOX between PI3K and mTORC2. However, at both sites, the inhibitory effect of BEZ was amplified when co-treated with DOX resulting in significant inhibition at the higher doses. Phosphorylation of ribosomal protein S6 (S6), a downstream marker of mTORC1
activity, was sensitive to BEZ treatment. Reduced phosphorylation was observed even at the lowest concentration (50 nM) and remained inhibited in all treatment groups utilizing BEZ (Figure 14).

**BEZ235 enhances doxorubicin-induced apoptosis**

Apoptosis was assessed using western blot analysis of the cleaved form of PARP (cl-PARP), a terminal step in the process involving the activation of caspase-3. BEZ alone had no effect on PARP cleavage at any of the combinations tested in MiaPaca2 cells; however, combination of BEZ with DOX induced a dose-dependent increase in apoptosis (Figure 14). The increase in cl-PARP corresponded to the level of AKT inhibition, which was maximally inhibited at higher concentrations. In subsequent experiments, 300 nM BEZ was used to achieve maximal inhibition of signaling while staying within the achievable mouse plasma concentrations (78).
**Figure 13. Schematic of BEZ235 (BEZ) inhibition.** BEZ inhibits at multiple nodes, including wild type and mutant forms of PI3K and both complexes containing mTOR. Inhibition at various nodal points with BEZ reduces survival and growth signaling downstream of PI3K and also prevents feedback activation by IRS1.
Figure 14. BEZ235 (BEZ) decreases PI3K/mTOR signaling and sensitizes cancer cells to doxorubicin (DOX). MiaPaca2 cells were treated for 24 hours with increasing doses of BEZ (0, 50, 150, 300, 600 nM) alone or in combination with DOX (0.5 μM). PI3K pathway signal transduction was assessed using western blot analysis to determine the inhibitory effects of BEZ.
Re-activation of AKT signaling does not reduce treatment efficacy

Our results indicated a release in feedback inhibition leading to activation of AKT signaling (Figure 14). This could potentially diminish the effectiveness of combination treatments with BEZ. Therefore, we used siRNA knockdown of PDK1 or Rictor to reduce activation of AKT by PI3K and mTORC2, respectively. Knockdown of PDK1 with siRNA resulted in reduced phosphorylation of AKT at threonine 308 compared to scrambled siRNA (Figure 15). However, reduced AKT phosphorylation resulted in only minor changes in apoptosis, as measured by PARP cleavage (Figure 15). Similarly, siRNA knockdown of Rictor reduced AKT phosphorylation at serine 473 to near untreated control levels (Figure 16). Again, very little change in apoptosis was observed between cells with reduced mTORC2 activity compared to those with normal activity (Figure 16). These results suggest that the transient inhibition of AKT survival signaling, which occurs at earlier time points, is enough to sensitize the cancer cells to DOX before loss of feedback inhibition through mTORC1 results in reactivation of the pathway.
Figure 15. Phosphorylation of AKT by PDK1 does not reduce combination effectiveness. MiaPaca2 cells were incubated with siRNA targeted towards PDK1 for 24 hours to knockdown protein expression. Cells were washed and treated as indicated with BEZ and DOX for an additional 24 hours after which cell lysates were analyzed by western blot.
Figure 16. Phosphorylation of AKT by mTORC2 does not reduce combination effectiveness. MiaPaca2 cells were incubated with siRNA targeted towards Rictor, a subunit required for activation of mTORC2, for 24 hours to knockdown protein expression. Cells were washed and treated as indicated with BEZ and DOX for an additional 24 hours after which cell lysates were analyzed by western blot.
BEZ235 enhances doxorubicin induced cell killing

We tested the hypothesis whether BEZ can potentiate the cell killing effects of DOX in MiaPaca2, Capan-1 and CD18 cells. Cells were treated for 48 hours with 300 nM BEZ and 0.5 μM DOX alone or in combination to measure the effects on proliferation/viability and cell death. Cell growth was reduced by more than 40% after treatment with DOX and nearly 70% following treatment with BEZ as compared to the control (Figure 17A). The combination had an additive effect on proliferation, with reduction in growth by 80% or more in all cell lines. Cell death (necrosis), as measured by the trypan blue exclusion assay, was significantly increased after treatment with DOX in all PDAC cell lines tested (Figure 17B). BEZ had marginal but significant effects compared to control in MiaPaca2 cells while there was no significant increase compared to control in Capan-1 or CD18 (Figure 17B). However, BEZ significantly increased the percentage of cell death in all cell lines when combined with DOX (Figure 17B).

BEZ had little effect on apoptosis as assessed by PARP cleavage (Figure 18A and B) in MiaPaca2 cells while it had modest effects in Capan-1 cells (Figure 18c and D). This may indicate a greater dependence on the PI3K pathway in Capan-1 cells than MiaPaca2 cells. In either cell line, there was little observed apoptosis in cells treated with DOX alone, showing only a slight cl-PARP band. However it was greatly enhanced when BEZ was combined with DOX (Figure 18). Likewise, the TUNEL assay was used to assess DNA fragmentation, another measure of apoptosis. Similar to PARP cleavage, apoptosis was found to be significantly increased in cells treated with BEZ and DOX in combination,
as shown by the representative images (Figure 19A) and corresponding quantification (Figure 19B).

There were also dramatic effects on MiaPaca2 cell morphology after treatment with BEZ and DOX. Using confocal microscopy and staining for actin (green), cleaved-PARP (red), and DAPI (nucleus, blue), cells were visualized after 24 hours of treatment. DOX treated cells are known to arrest mainly in the G2 phase of the cell cycle leading to an increase in cell and nuclear size before committing to cell death and shrinking (Figure 20, upper right panel). Intriguingly, despite causing very little cell death when treated alone, BEZ does show an obvious phenotype which resembles cells after serum starvation. It is known to arrest cells in the G1 phase leading to cells that are elongated and small (Figure 20, lower left panel). Cells treated with both BEZ and DOX were mostly small apoptotic cells with fragmented nuclei (Figure 20, lower right panel). These same effects can also be seen after 48 hours using phase contrast microscopy (Figure 21). There was a less obvious change in morphology in CD18 cells, but they do seem to be enlarged with DOX treatment and smaller with BEZ treatment similar to the changes seen in MiaPaca2 cells (Figure 22, upper right and lower left panels). However, there is a dramatic change when cells are treated with BEZ and DOX combined with most cells shriveled and dead (Figure 22, lower right panel).
Figure 17. PI3K/mTOR inhibition enhances doxorubicin (DOX) efficacy. (A) Cell viability was measured using the MTS assay after 48 hours treatment of MiaPaca2, Capan-1, and CD18 cells. Data is plotted as mean ± SEM of replicates of four. (B) Cell death (necrosis) was measured using the trypan blue exclusion assay after 48 hours treatment of MiaPaca2, Capan-1, and CD18 cells. Data is plotted as mean ± SEM of triplicates. *p < 0.001 vs DOX, #p < 0.05 vs DOX.
Figure 18. Combining BEZ235 (BEZ) with doxorubicin (DOX) enhances apoptosis in pancreatic cancer. (A) Immunoblots of cl-PARP and actin in MiaPaca2 and (B) Capan-1 cell lysates after treatment for 24 hours.
Figure 19. Doxorubicin (DOX) mediated DNA fragmentation is enhanced with 

**BEZ235 (BEZ).** (A) Apoptosis was assessed using the TUNEL assay after treatment for 24 hours. Representative images are shown with TUNEL positive nuclei stained green and 

DAPI stained nuclei blue. (B) Quantification of TUNEL positive cells. Data is plotted as 

mean ± SEM of replicates of four. *p < 0.05 vs DOX.
Figure 20. Changes in cell morphology following treatment with BEZ235 (BEZ) and doxorubicin (DOX). Representative confocal microscopy images of cell size and morphology after treatment with BEZ and DOX. Actin is stained green (alexafluor 488) and cl-PARP is stained red (alexafluor 555). Nuclei are stained with DAPI.
Figure 21. Changes in morphology of MiaPaCa2 cells after treatment with BEZ235 (BEZ) and doxorubicin (DOX). Phase contrast representative images showing altered cell morphology of MiaPaca2 cells after treatment.
**Figure 22.** Changes in morphology of CD18 cells after treatment with BEZ235 (BEZ) and doxorubicin (DOX). Phase contrast representative images showing altered cell morphology of CD18 cells after treatment.
Bcl-2 family proteins are modulated to induce caspase-dependent apoptosis following treatment with BEZ235 and doxorubicin

The Bcl-2 family of proteins plays a major role in the induction of apoptosis for both intrinsic and extrinsic pathways. Modulation of these proteins tips the balance to favor either survival or death and could influence the effectiveness of treatments. Our results showed that increases in mitochondrial associated pro-apoptotic protein expression and caspase activation are required in enhancement of DOX-induced cell death with BEZ. Apoptosis, measured by assessing PARP cleavage and DNA fragmentation, was enhanced in cells treated with BEZ and DOX (Figure 18 and 19). Correspondingly, the Bcl-2/Bax ratio was increased compared to control in cells treated with DOX indicating an activation of the cells pro-survival response. This was normalized to control levels when DOX was co-treated with BEZ (Figure 23A and B). BIM, an activator of Bax, has three splice variants (BIM EL, L, and S) with the shortest form being the most cytotoxic. Expression of the longest form of BIM (EL) was slightly enhanced with DOX treatment either alone or in combination; however, treatment with BEZ, either alone or in combination, resulted in robust expression of the smaller more cytotoxic forms of BIM (Figure 23C and D).

If enhanced expression of BIM and activation of Bax leads to caspase dependent cell death (111), then caspase inhibition should diminish the effects of our combination treatment. The caspase inhibitor, Z-VAD, was used to test the caspase dependence of combination treatment. Treatment with Z-VAD reduced PARP cleavage after 24 hours of treatment with only a minor band remaining in the combination treated cells (Figure 24A and B). Caspase inhibition also resulted in a significant decrease in cell death in both the
DOX and combination groups (Figure 24C), suggesting that increased expression of BIM and activation of pro-apoptotic proteins, including Bax, are required for BEZ’s enhancement of DOX-induced cell killing in pancreatic cancer cells.
Figure 23. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on pro- and anti-apoptotic proteins in pancreatic cancer cells. MiaPaca2 were treated with BEZ and DOX alone or in combination for 24 hours of treatment and cell lysates prepared for western blot analysis. (A) Immunoblots of Bcl-2, Bax, and actin. (B) Immunoblots of BIM EL, L, and S.
Figure 24. Role of Caspases in enhanced killing of pancreatic cancer cells with BEZ235 (BEZ) and doxorubicin (DOX). MiaPaca2 cells were treated with BEZ and/or DOX in the presence or absence of the caspase inhibitor Z-VAD. Cell lysates were prepared for western blot after 24 hours of treatment. Cell death was assessed by the trypan blue exclusion assay after 48 hours of treatment. (A) Western blot showing expression of cl-PARP and actin. (B) Percent of dead cells are plotted. Data are plotted as mean ± SEM of triplicates. *p < 0.001, #p < 0.05.
Effect of BEZ235 and doxorubicin on colony formation

The colony formation assay measures a cells ability to retain cell proliferation and growth potential, thereby escaping reproductive death after treatment with a cytotoxic agent. This is important in that cells that escape reproductive death within tumors can continue to grow leading to recurrences. MiaPaca2 cells, seeded in 10 cm plates, were treated with BEZ and DOX either alone or in combination for four hours. Following treatment, cells were washed, collected, and replated in 6-well plates at a density of 500 cells/well. After allowing colonies to form (~2 weeks), cells were stained and counted. The increase in caspase-dependent cell death observed in cells co-treated with BEZ and DOX corresponded with a significant reduction in colony formation as compared to cells treated with DOX alone (Figure 25A and B). These results suggest that combining BEZ with DOX could result in reduced recurrences due to loss of cell reproductive growth.
Figure 25. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on colony formation.

MiaPaca2 cells were treated with BEZ and/or DOX for 4 hours then re-plated to allow colony formation. After 2 weeks, colonies were fixed and stained with crystal violet. (A) Representative dishes showing MiaPaca2 colonies. (B) Quantification of results. p < 0.001 vs DOX.
**BEZ235 does not enhance gemcitabine-induced cell killing**

Gemcitabine (Gem) is the standard of care for pancreatic cancer patients and is used either alone or in combination but has very little improvement in survival. In order to obtain better clinical efficacy, new treatment strategies and combinations are needed. Therefore we tested the effect of Gem with BEZ in MiaPaca2 cells to show whether this combination was as effective as DOX and BEZ. Cell proliferation was assessed after 48 hours of treatment with BEZ either alone or in combination with 1 μM or 10 μM Gem. At both concentrations, Gem alone reduced proliferation compared to the control. Similar to DOX, the reduction in cell proliferation was enhanced when Gem was combined with BEZ (Figure 26A). These results suggested that combination treatment with BEZ and Gem was more effective than Gem alone. However, BEZ did not enhance the cytotoxic effects of Gem, which is in contrast to the enhanced cell killing effect of the BEZ and DOX combination as shown elsewhere. In fact, reduced trypan blue positive cells (Figure 26B) and PARP cleavage (Figure 27) were observed with the combination treatment compared to Gem alone. This was also evident visually, as the BEZ combination with Gem showed no change compared to the BEZ alone at 24 or 48 hour time points (Figure 28). This is in stark contrast to BEZ combination with DOX in MiaPaca2 and CD18 where the vast majority of cells were dead (Figures 21 and 22).
Figure 26. BEZ235 (BEZ) does not enhance Gemcitabine (Gem) toxicity in MiaPaca2 cells. (A) MiaPaca2 cell viability/proliferation was measured using the MTS assay after treatment for 48 hours. Data are plotted as mean ± SD of replicates of six. (B) Cell death (necrosis) was measured using the trypan blue exclusion assay after treatment for 48 hours. Data are plotted as mean ± SEM of triplicates. *p < 0.001 vs Gem alone, #p < 0.05 vs Gem.
Figure 27. Effect of BEZ235 (BEZ) and Gemcitabine (Gem) on apoptosis. MiaPaca2 cells were treated for 24 hours with BEZ and/or Gem and cell lysates analyzed with western blot. Note that cl-PARP is reduced when treated with the combination of BEZ and Gem.
Figure 28. Changes in cell morphology following treatment with Gemcitabine (Gem) and BEZ235 (BEZ). MiaPaca2 cells were treated with BEZ and/or Gem and phase contrast pictures were taken (A) 24 hours and (B) 48 hours later. Note that the morphology of cells treated with the combination of BEZ235 and Gem is nearly identical to cells treated with BEZ235 alone.
BIM is not induced by gemcitabine when combined with BEZ235

To explore the possible cause for the contrasting results observed between Gem and DOX combinations with BEZ, we determined the expression of the pro-apoptotic protein BIM in MiaPaca2 cells. As shown previously, BEZ and BEZ in combination with DOX induces robust expression of the smaller more cytotoxic splice variants of BIM (Figure 2C and D, Figure 29). However, Gem alone had no effect on the expression of any splice variant (Figure 29). Combinations of BEZ with Gem slightly reduced the expression of BIM EL, which was similar to the effect observed with BEZ alone. Unlike BEZ, the combination treatment induced very little expression of the smaller forms of BIM (Figure 29). This finding is similar to the phenotypic observation that the combination cells resembled the cells treated only with BEZ and further supports the hypothesis that BIM is a major mediator of apoptosis in cells treated with BEZ and DOX.
Figure 29. BIM expression following treatment with doxorubicin (DOX), BEZ235 (BEZ) and gemcitabine (Gem) in MiaPaca2 cells. MiaPaca2 cells were treated with BEZ and/or Gem/DOX for 24 hours and cell lysates were prepared for western blot analysis.

Expression of BIM EL and BIM L, S. Note that BIM expression is not enhanced following treatment with gemcitabine BEZ235 combination.
BEZ235 increases DOX accumulation

To further understand the mechanism of enhanced cell killing with BEZ and DOX, we considered the role of ABC transporters, which play a major role in the development of multi-drug resistance and treatment failure (32-34, 103). In cancer cells with high expression of ABC transporters, including ABCB1, intracellular drug accumulation is reduced below the therapeutic threshold due to increased efflux. Interestingly, BEZ enhanced accumulation of DOX, possibly through acting as a competitive inhibitor of the substrate binding site, thereby blocking efflux of DOX (Figure 9). However, ABCB1 expression in MiaPaca2 cells is very low leaving the possibility that an increase in DOX accumulation is not involved in the enhancement of efficacy when BEZ is combined with DOX. Therefore, cells were treated for 24 hours after which cells were collected and DOX accumulation measured. Interestingly, BEZ treatment nearly doubled the intracellular concentration of DOX as compared to DOX alone suggesting that BEZ has effects on not only ABCB1 but other transporters as well (Figure 30). We further investigated the impact of increased intracellular DOX accumulation on DNA damage. Activation of DNA damage response (DDR) proteins were visualized using western blot analysis. Cells treated with DOX in combination with BEZ were associated with increased phosphorylation of the DDR proteins ATM and chk2 compared to cells treated with DOX alone (Figure 31). These results suggest that even in the absence, or low expression, of ABCB1, BEZ can still cause increased accumulation of DOX likely through inhibition of one or more of the other transporters associated with drug resistance leading to increased DNA damage and cell death.
Figure 30. Effect of BEZ235 (BEZ) on accumulation of doxorubicin (DOX) in MiaPaca2 cells. MiaPaca2 cells were treated for 48 hours after which the cells were collected and analyzed for DOX accumulation using a 96 well fluorescence plate reader. Values are plotted as mean fluorescence over mg of total protein. Note that cellular accumulation of DOX is enhanced with BEZ treatment. Data are plotted as mean ± SEM of triplicates. *p < 0.001 vs DOX.
Figure 31. Effect of BEZ235 (BEZ) and doxorubicin (DOX) in activation of DNA damage response proteins. Western blots showing phosphorylation of DNA damage response proteins ATM and chk2.
BEZ235 potentiates DOX-induced inhibition of pancreatic tumor growth in vivo

We further evaluated the effects of BEZ, DOX, and Gem in a mouse tumor model. In these experiments MiaPaca2 cells were implanted into the back flanks of female Athymic nude mice and treating according to the protocol (Figure 32). DOX was injected into the tail vein at a concentration of 10 mg/kg on day 1 and 15. Gem was given as an intraperitoneal injection at a concentration of 100 mg/kg on day 1, 8, and 15. BEZ was given by oral gavage every day at a concentration of 40 mg/kg for 28 days. Tumor volumes were measured twice weekly throughout the study. Treatment with BEZ, DOX, or Gem alone reduced tumor volume as compared to the control, although the difference did not reach significance at any time point (Figure 33A). However, the combination of BEZ and DOX resulted in a significant reduction of tumor volume compared to all other treatment groups, as tumor volume was even depressed to below the initial volume until day 35 (Figure 33A).

With DOX treatment, it is important to also assess toxicity as this is one of the dose limiting factors for its use clinically. This is especially true when used in combination with drugs that are known to inhibit key survival pathways. Change in body weight can be used as a basic measure of overall toxicity in vivo because the amount of weight loss is related to the extent of toxicity. Our results show that there was no significant difference in body weights between the DOX and combination groups. Although there was some treatment related weight loss associated with DOX whether given alone or with BEZ these changes were similar throughout the study implying that the addition of BEZ did not enhance toxicity (Figure 33B).
Figure 32. Treatment protocol for mouse tumor studies. MiaPaca2 cells were injected into athymic nude mice. Two weeks later, mice were randomized into the 5 groups to receive the following drug treatments: 1. Control (no treatment); 2. Doxorubicin (DOX); 3. BEZ235 (BEZ); 4. DOX+BEZ and 5. Gemcitabine (GEM). The dose and treatment schedule for each drug is indicated in the protocol. On day 53, or when they reached predetermined tumor volume endpoint, mice were sacrificed and tumors collected.
Figure 3. BEZ235 (BEZ) potentiates doxorubicin (DOX)-induced inhibition of pancreatic xenographs. (A) Tumor growth curves of athymic nude mice with MiaPaca2 xenographs treated with vehicle, DOX 10 mg/kg, BEZ 40 mg/kg, and gemcitabine (Gem) 100 mg/kg (n = 9). (B) Average body weights during the course of treatment. Data are plotted as mean ± SEM from measurements of nine mice per group. *p < 0.01 and #p < 0.05 vs DOX.
Breast cancer

BEZ235 inhibits PI3K pathway signaling and sensitizes MDA-MB-231 breast cancer cells to doxorubicin

Breast cancer is the most diagnosed cancer and second leading cause of cancer death in women. It frequently harbors mutations/deletions within the PI3K pathway or hormone receptors that lead to overactivation of the PI3K pathway, helping to drive tumorigenesis and drug resistance. Breast cancer treatment commonly includes DOX in its regimens; however, it is frequently met with acquired or innate resistance leading to failure. Therefore, we further investigated the effectiveness of combining BEZ and DOX in the killing of breast cancer cells.

MDA-MB-231 cells were challenged with increasing concentrations of BEZ (50 nM to 600 nM) alone or in combination with 0.5 μM DOX. Cell viability was assessed 48 hours after treatment. All concentrations of BEZ along with the DOX treated cells resulted in a near 50% reduction in viability as compared to untreated control (Figure 3). Combination of BEZ with DOX led to a significant decrease in viability compared to the individual drug treatments (Figure 3).

Cell lysates were prepared after 24 hours of treatment with BEZ and DOX for analysis of PI3K and mTOR signaling with western blot. There was an initial increase in activation of AKT, as shown by phosphorylation of serine 473, at the lowest dose of 50 nM (Figure 35), an effect likely resulting from the release of a feedback inhibition loop through mTORC1. At the higher concentrations tested (150, 300, 600 nM), AKT activation was reduced to below control levels. At all concentrations, mTORC1 activity was greatly
reduced as compared to the untreated control. This decrease in signaling corresponded to a
dose-dependent increase in apoptosis, measured by assessing PARP cleavage (Figure 35).
Figure 34. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on viability of MDA-MB-231 breast cancer cells. Cells seeded into 96-well plates were treated with the indicated concentrations of BEZ and DOX alone or in combination. After 48 hours, cell viability was measured using the AQueous One cell viability assay kit from Promega. Note that cell viability is significantly reduced with BEZ and DOX combination. Data are plotted as mean ± SEM of replicates of six. *p < 0.001.
Figure 35. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on PI3K/mTOR signaling and PARP cleavage in breast cancer cells. MDA-MB-231 cells were treated with increasing doses of BEZ (0, 50, 150, 300, 600 nM) alone or in combination with DOX (0.5 μM) for 24 hours. PI3K/mTOR signaling proteins (AKT, S6) and apoptosis were assessed using western blot analysis. Note that BEZ decreases PI3K/mTOR signaling and sensitizes breast cancer cells to DOX.
BEZ235 inhibition is rapid and sustained in MDA-MB-231

There are several regulators of signaling along the PI3K pathway with some leading to an active state allowing transmission of the signal while others promote an inactive state to halt the transmission. We asked whether inhibition of PI3K and mTOR signaling was an early event and how long its inhibition would be sustained in an *in vitro* cell culture model. MDA-MB-231 cells were treated with BEZ and DOX, alone or in combination, for 4, 8, 16, 24, and 48 hours. Cell lysates were prepared and analyzed using western blot analysis. AKT inhibition occurred as early as 4 hours and was sustained for up to 48 hours after initiation of treatment with BEZ either alone or in combination. However, there is some reactivation of the pathway at the later time points, particularly 48 hours. Similarly, mTORC1 signaling was inhibited as early as 4 hours and sustained for up to 48 hours (Figure 36).

These results demonstrate that the effect of BEZ on PI3K/mTOR signaling is rapid and sustained for up to 48 hours in MDA-MB-231 cell culture. This allows for continuous depletion of survival signaling and increased sensitivity of cells to DOX.
Figure 36. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on PI3K/mTOR signaling. MDA-MB-231 cells were treated with BEZ and/or DOX and collected at various time. Cell lysates were analyzed using western blot for AKT and S6 activation after 4 to 48 hours of treatment. Note that PI3K pathway inhibition is rapid and sustained.
BEZ235 increases doxorubicin accumulation in breast cancer cells

Previous results have shown that BEZ inhibits ABCB1 leading to increased accumulation of DOX (Figures 6 and 7). Also, in MiaPaca2 cells, which have a low expression of ABCB1, BEZ co-treatment with DOX leads to a two-fold increase in accumulation as compared to DOX alone suggesting that BEZ inhibits other transporters implicated in drug resistance. Here we test whether BEZ treatment in MDA-MB-231, which also has low expression of ABCB1, will enhance accumulation over time. Cells treated with DOX alone had an initial increase in accumulation at 4 and 8 hours which quickly plateau showing little change from 16 to 48 hours (Figure 37, red bars). However, cells treated with both BEZ and DOX had a steady increase in accumulation at each time point (Figure 37, green bars). The 24 hour time points both show reduced accumulation for unknown reasons.

In the absence of BEZ, the cells quickly reach equilibrium and are able to pump out DOX as fast as it comes in. In the presence of BEZ however, DOX transport out of the cell is reduced, never reaching equilibrium, allowing accumulation of the drug throughout.

Necrotic cell death was interrogated after 48 hours of treatment to determine its association with DOX accumulation. BEZ and DOX individually had mild effects on cell death (Figure 38A). However, there was a greater than additive increase in cell death (Figure 38A) with combination treatment that also mirrored the increase in DOX accumulation (Figure 38B).

These results strongly suggest that BEZ enhances the effects of DOX in MDA-MB-231 cells by not only inhibition of PI3K survival signaling, but also through a time
dependent increase in DOX accumulation. Moreover, since MDA-MB-231 cells express little ABCB1, BEZ likely acts as an inhibitor to one or more of the other ABC transporters involved in drug resistance indicating that it may be useful in a wide range of cancer types.
Figure 37. Effect of BEZ235 (BEZ) on doxorubicin (DOX) accumulation in breast cancer cells. DOX fluorescence was measured at various time points to see the effect of BEZ on accumulation over time using a 96 well fluorescence plate reader (ex. 485, em. 595). Note that BEZ enhances time-dependent accumulation of DOX in MDA-MB-231 cells. Data are plotted as a single measurement to demonstrate the time-dependent nature of DOX accumulation.
Figure 38. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on cell death in MDA-MB-231 cells. Cells were treated with BEZ and/or DOX for 48 hours and cell death was quantified using the trypan blue exclusion assay. DOX accumulation was quantified by directly measuring DOX fluorescence in a 96 well fluorescence plate reader (em. 485, ex. 595). (A). Percent of dead cells; (B). DOX accumulation. Note that BEZ enhances cell death and also causes increased accumulation of DOX in MDA-MB-231 cells. Data are plotted as mean ± SEM of triplicates. #p < 0.05, *p < 0.001.
Colony formation in MDA-MB-231 cells is depleted with combination treatment

Our results have shown that combination treatment significantly reduces colony formation in pancreatic cancer (Figure 25). Here we interrogate the effect of combination treatment with BEZ and DOX on reducing colony formation in breast cancer cells. MDA-MB-231 cells were treated with BEZ and DOX either alone or in combination for four hours. Following treatment, cells were washed, collected, and re-plated in 6-well plates at a density of 500 cells/well. The colonies were allowed to form for ~2 weeks after which they were stained and counted. Colony formation was significantly reduced to less than 10 colonies per well in cells treated with the combination treatment of BEZ and DOX as compared to DOX alone (Figure 39). Individual DOX treatments did reduce colony formation but still allowed approximately 40 colonies per well to growth which could potentially lead to faster time to recurrences clinically. BEZ treatment alone had no effect on colony formation as compared to control. This supports the need for the addition of a cytotoxic agent like DOX to treatment regimens based on targeted agents, such as BEZ’s use in KRAS mutant MiaPaca2 and MDA-MB-231 cells.
Figure 39. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on colony formation in breast cancer cells. MDA-MB-231 breast cancer cells were treated with BEZ, DOX or the combination of both drugs for 4 hours and then re-plated to allow colony formation. After 2 weeks, colonies were fixed, stained with crystal violet, and counted. Note that the colony formation is significantly reduced with combination treatment. Quantification of results. Data are plotted as mean ± SEM of triplicates. $^a$p < 0.01 vs DOX.
Effect of combination treatment with BEZ235 (BEZ) and doxorubicin (DOX) in cells with PI3K activating mutations

Mutations to PI3K and PTEN (or deletions to PTEN) are some of the most common events in cancer which lead to pathway over-activation. There have been several agents which target proteins along this pathway developed for use as anti-cancer drugs, including BEZ. Our results have shown that BEZ has dramatic effects in enhancing DOX efficacy in KRAS mutant cancers. Here we examined whether BEZ can enhance the effects of DOX in PI3K mutant breast cancer cells. Mcf-7 cells were treated with BEZ and/or DOX for 24 or 48 hours. The results show that DOX itself had minimal effects on cell viability whereas BEZ reduced cell viability whether it was treated alone or in combination with DOX (Figure 40A). It is noteworthy that DOX did not enhance the effects of BEZ and there were a slightly higher number of viable cells compared to BEZ alone. Furthermore, necrotic cell death was unaffected by DOX treatment while BEZ itself resulted in a significant increase (Figure 40B). Again, while there was a significant increase in cell death with the combination treatment over BEZ alone, the effects were minimal (Figure 40B) despite the fact that there is nearly double the amount of DOX accumulated within the cells (Figure 40C). There was a slight increase in AKT phosphorylation on serine 473 with DOX treatment. On the other hand, AKT activation was completely inhibited with BEZ treatment whether treated alone or in combination with DOX (Figure 41). Similar results were observed with inhibition of mTORC1 activity as S6 phosphorylation was dramatically reduced with BEZ treatment. However, while there was little effect on
apoptosis with DOX, BEZ strongly induced PARP cleavage that was enhanced very little with the addition of DOX (Figure 41).

These results suggest that cell lines which have an activating mutation within the PI3K/mTOR pathway would not have a significant benefit from the addition of DOX to BEZ.
Figure 40. Effect of BEZ235 (BEZ) and doxorubicin (DOX) in PI3K mutant breast cancer cells. PI3K mutant Mcf7 cells were treated with BEZ and/or DOX for 48 hours after which cell viability was measured using the MTS assay. Cell death was measured using the trypan blue exclusion assay. DOX accumulation was quantified by directly measuring DOX fluorescence in a 96 well fluorescence plate reader (em. 485, ex. 595). (A) Cell proliferation. Data are plotted as mean ± SEM of replicates of four. (B) Cell death and (C) DOX accumulation. Data are plotted as mean ± SEM of triplicates. Note that Mcf7 cells did not have significant benefit from the addition of DOX to BEZ. *p < 0.001, #p < 0.05.
Figure 41. Effect of doxorubicin (DOX) and BEZ235 (BEZ) on survival signaling in Mcf7 cancer cells. Mcf7 cells were treated with BEZ and/or DOX for 24 hours and cell lysates analyzed using western blot for PARP cleavage, AKT and S6 activation. Note that survival signaling is depleted with BEZ235 but had little effect in apoptosis when treated alone or in combination.
Other solid tumors

**BEZ235 enhances the effectiveness of doxorubicin against colon and lung cancer**

Our results have shown that combining BEZ with DOX can significantly increase treatment effectiveness compared to the single agents in pancreatic cancer and KRAS driven breast cancer. KRAS mutations, along with mutations or deletions in P53, are also common in other cancers, including those of the colon and lung. Therefore, we sought to test the combination effectiveness in these cancer types as well.

Colon (HCT 116) and lung (H1299) cancer cells were grown in 10 cm plates and treated with BEZ and DOX either alone or in combination for 24 or 48 hours. Cell lysates were assessed for apoptosis (cl-PARP) and PI3K pathway activation (p-AKT and p-S6) using western blot analysis. The phosphorylation at serine 473 of AKT was reduced following treatment with BEZ regardless of its use in combination with DOX in HCT 116 cells (Figure 42A). S6 phosphorylation was also considerably reduced with BEZ treatment, although when combined with DOX it is slightly less efficient (Figure 42A). Combination with BEZ enhanced PARP cleavage (apoptosis) and a significant increase in necrosis as compared to DOX alone (Figure 42A and B). Furthermore, similar to pancreatic cancer, the increase in cell death following treatment with BEZ and DOX paralleled a significant increase in DOX accumulation with BEZ suggesting an important role for ABC transporter inhibition in the enhancement of DOX efficacy (Figure 42C).

Similar results were observed in the H1299 cells. Apoptosis and necrosis were both enhanced in the combination treated cells compared to those treated with BEZ or DOX alone. This corresponded to a reduction in PI3K pathway signaling with BEZ treatment.
and a significant increase in DOX accumulation when BEZ was combined with DOX (Figure 43).

Pancreatic cancer is predominantly driven by KRAS mutations with mutations or deletions in P53 also common. These results demonstrate that other cancer types which harbor mutations in KRAS (HCT 116) and P53 (H1299) can also be sensitized to DOX when combined with BEZ indicating the potential for benefits to a wide range of cancer types.
Figure 42. Effect of BEZ235 (BEZ) and doxorubicin (DOX) in KRAS mutant colon cancer. HCT-116 cells were treated with BEZ, DOX or in combination for 24 hours. Cell death was measured using trypan blue exclusion assay after 48 hours of treatment. DOX fluorescence was measured after 24 hours of treatment using a 96 well fluorescence plate reader. (A) Immunoblots of cl-PARP, p-AKT 473, AKT, p-S6, S6, and actin. (B) Percent cell death (C) Dox accumulation. Note that BEZ enhanced the effects of doxorubicin in KRAS mutant colon cancer cells. For B and C, Data are plotted as mean ± SEM of triplicates. *p < 0.001 vs DOX.
Figure 43. Effect of BEZ235 (BEZ) and doxorubicin (DOX) in P53 mutant lung cancer cells. H1299 lung cancer cells were treated with BEZ, DOX or in combination for 24 hours. Cell death was measured using trypan blue exclusion assay after 48 hours of treatment. DOX fluorescence was measured after 24 hours of treatment using a 96 well fluorescence plate reader. (A) Immunoblots of cl-PARP, p-AKT 473, AKT, p-S6, S6, and actin (B) Percent cell death (C) Dox accumulation. Note that BEZ enhanced the effects of doxorubicin in p53 mutant lung cancer cells. For B and C, Data are plotted as mean ± SEM of triplicates. *p < 0.001 vs DOX.
Cardiotoxicity

Effects of BEZ235 combination with doxorubicin on rat myoblast cytotoxicity

Cardiotoxicity, which may ultimately lead to heart failure, is the main dose limiting factor for clinical use of DOX clinically (86, 112). Moreover, combination therapies using inhibitors of growth factor signaling with DOX show increased cardiac dysfunction (95, 113-115). One major factor of this could be because the inhibitors, like the monoclonal HER2 antibody trastuzumab, block at the initiating node upstream of both RAS and PI3K (Figure 44). This effectively stops all survival signaling downstream of these receptors making the cells, including cardiac cells, susceptible to treatment with cytotoxic agents, including DOX. Therefore, we examined the effects of combining BEZ with DOX in an undifferentiated rat myoblast cell line (H9C2) for in vitro studies of DOX cytotoxicity. Cells were treated for 48 hours with DOX and BEZ either alone or in combination after which expression of apoptotic proteins and cell death were assessed. DOX treatment significantly increased dead cells compared to control. However, combination treatment did not enhance cell death and in fact, resulted in reduced cell death (Figure 45).

The ratio of the pro-survival protein Bcl-2 over the pro-apoptotic protein Bax is used as a measure of apoptosis in cells, with an increase over control favoring cell survival. The Bcl-2/Bax ratio was increased nearly threefold with BEZ treatment, alone or in combination, an effect which was not seen with DOX treatment alone (Figure 46). Interestingly, the shorter forms of BIM had increased expression with DOX treatment while all splice variants were dramatically reduced after combination treatment (Figure 47). This is in stark contrast to the increased expression observed in the pancreatic cancer
cells and supports the previous results in H9C2 cells showing reduced cell death compared to DOX treated cells.
Figure 44. Schematic of trastuzumab effects on cell signaling. Trastuzumab is a monoclonal antibody against HER2. Binding of this antibody to HER2 receptor reduces signaling through both RAS and PI3K to cause reduced survival and growth in the cancer cell. Combinations using trastuzumab and DOX have been reported to cause increased cardiac toxicity (95, 113-115).
Figure 45. Cell death is reduced in H9C2 cells with co-treatment of BEZ235 (BEZ) with doxorubicin (DOX). Cell death (necrosis) was measured in using the trypan blue exclusion assay after treatment either alone or in combination with the indicated concentrations of BEZ and DOX for 48 hours. Data are plotted as mean ± SEM of triplicates. #p < 0.05 vs DOX.
Figure 46. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on Bcl2/Bax expression in H9C2 cells. Immunoblots of Bcl2, Bax and actin from H9C2 cell lysate after treatment with BEZ and DOX either alone or in combination with the indicated concentrations for 24 hours. Note that the Bcl-2/Bax ratio is significantly higher with BEZ and BEZ+DOX as compared to control and DOX treatment groups.
Figure 47. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on BIM expression in H9C2 cells. Immunoblots of BIM EL, BIM L, S, and actin from H9C2 cell lysate after treatment with BEZ and DOX either alone or in combination with the indicated concentrations for 24 hours. Note that BIM expression is lost when BEZ is combined with DOX.
Effect of BEZ235 on doxorubicin-induced Cardiac Dysfunction

The major dose-limiting factor for the use of DOX clinically is cardiotoxicity leading to heart failure (116, 117). We evaluated the effects of combination therapy on cardiac function by echocardiography after completion of the treatment schedule (28 days) in the tumor-bearing mice (Figure 48). DOX treatment caused a significant decrease in systolic function as assessed by measuring fractional shortening. Systolic function in mice treated with BEZ alone or in combination with DOX was not significantly different than untreated controls (Figure 49A and C). In addition, left ventricular diastolic function as assessed by the E/A ratio was not significantly different among the 4 groups (Figure 49B).

To further interrogate why BEZ does not enhance the cardiotoxic effects of DOX, mice were treated with 5 mg/kg DOX and 40 mg/kg BEZ either alone or in combination. Twenty four hours later, whole heart lysates were for western blot analysis. There was increased activation of both AKT and ERK in the hearts with combination treatment which was not observed in the DOX or BEZ treated mice (Figure 50). With BEZ treatment, there is a loss of feedback inhibition through IRS1 leading to reactivation of PI3K. More importantly, transient inhibition of PI3K re-directs signaling into the RAS pathway leading to increased survival signaling through ERK, which includes phosphorylation and degradation of BIM (Figure 51).
Figure 48. Treatment protocol for mouse cardiotoxicity studies. MiaPaca2 cells were injected into athymic nude mice. Two weeks later, mice were randomized into the following 4 groups to receive the following drug treatments: 1. Control (no treatment); 2. Doxorubicin (DOX); 3. BEZ235 (BEZ); and 4. DOX+BEZ. The dose and treatment schedule for each drug is indicated in the protocol. On day 28, echocardiography was performed to measure systolic and diastolic heart function using VEVO700 echocardiography system.
A

Frational Shortening

\[ n = 9 \]

\[ ^p < 0.05 \text{ vs control} \]

B

E/A ratio

\[ n = 9 \]
Figure 49. Cardiac function following treatment with BEZ235 (BEZ) and Doxorubicin (DOX) treatment in tumor bearing mice. Cardiac function was assessed by echocardiography 28 days after treatment in tumor bearing mice. (A) Systolic function, (B) diastolic function, and (C) Representative M-mode images from Control, DOX, BEZ and BEZ+DOX. *p < 0.05 vs control. Note that combining BEZ with DOX does not enhance cardiotoxicity. Data are plotted as mean ± SEM from measurements of nine mice.
Figure 50. Effect of BEZ235 (BEZ) and doxorubicin (DOX) on survival signaling in the heart. c57 mice were treated with DOX and BEZ alone or in combination for 24 hours. The mice were sacrificed, hearts collected, and tissue lysates prepared. Western blots were performed for expression of AKT and ERK.
Figure 51. Possible mechanisms of cardioprotection with BEZ235. BEZ inhibits both AKT and mTOR leading to reduced downstream survival and growth signaling. Inhibition of mTORC1 releases a feedback inhibition loop through IRS1 leading to re-activation of PI3K and AKT. Signaling through the RAS pathway is also enhanced leading to ERK activation. ERK is an important mediator of survival signaling in the heart and may also influence the expression levels of BIM, a downstream target.
CHAPTER 4: Discussion

ABC transporters

BEZ235 inhibition of ABCB1

A major cause of treatment failure in cancer patients is overexpression of one or more of the ABC-type transporters which lead to a multi-drug resistance phenotype by reducing intracellular concentrations of drug to below their therapeutic threshold (32, 33). The most commonly overexpressed transporter in cancer is ABCB1, which is known to confer resistance against several clinically used drugs, including etoposide, paclitaxel, vincristine, and doxorubicin (105). Since the early 1980’s, there have been studies investigating the use of ABCB1 inhibitors clinically, but results so far have been underwhelming (32-34). Our data demonstrates that BEZ increases accumulation of DOX in ABCB1 overexpressed drug resistant cells, but has a less dramatic effect on accumulation in drug resistant cells with undetectable levels of ABCB1. Moreover, at the nanomolar concentrations used in this study, BEZ does not inhibit the ATPase activity of ABCB1 and is, at most, a very weak substrate. Additionally, this data demonstrates that BEZ is able to re-sensitize ABCB1 overexpressing cells to DOX treatment in a
concentration dependent manner. For the first time, our results suggest that BEZ has the potential to reverse the MDR phenotype in cancer cells overexpressing ABCB1.

Cancer cells with overexpression of one or more of the ABC transporters have reduced intracellular accumulation of cytotoxic agents to levels below the effective dose due to their increase in efflux capacity (32-34). Inhibitors of these transporters prevent cellular efflux, resulting in enhanced intracellular concentrations of the cytotoxic agent and improved efficacy. Our results show that treatment of ABCB1 overexpressing cells, A2780-dx and Mia-B1, with BEZ in combination with DOX for 48 hours increase the intracellular concentration of DOX to levels nearing those of their respective parental cell lines. However, the PI3k/mTOR pathway plays an important role in signaling and protein translation, so it is conceivable that BEZ increases accumulation of DOX by other means besides direct inhibition of the transporter. Along those lines, rapamycin, an inhibitor of mTOR complex 1, increases accumulation of vincristine and DOX in drug resistant lymphoma cell lines, at least in part through the down regulation of ABCB1 protein expression (101). To address the possibility of expression change of ABCB1, we demonstrate that BEZ can inhibit the efflux capacity of ABCB1 overexpressing cells using a shorter treatment time of three hours (one hour co-treatment of BEZ and DOX), to a similar extent as that previously shown with 48 hours treatment. This makes it unlikely that there would be any meaningful reduction in the levels of ABCB1 expression over the time frame of the experiment. Similar results are described for the PI3k inhibitor LY294 002, where treatment for 0.5 hours and 24 hours did not change the resulting effect on drug accumulation, which was concluded to be indicative of a substance that did not change
ABCB1 expression (118). These studies are more in line with our results, which indicate that BEZ inhibits ABCB1 function without decreasing its expression.

Several kinase inhibitors, including sildenafil and sunitinib, reverse the MDR phenotype of cells overexpressing ABC transporters through interaction at the substrate binding site (50, 54). These compounds not only bind, but are substrates themselves, as shown by their capacity to enhance ATPase activity in isolated membranes overexpressing ATP transporters. BEZ, on the other hand, has very little effect on ATPase stimulation in isolated p-gp membranes whereas the positive controls verapamil and sildenafil significantly enhance activity. Furthermore, BEZ is still able to inhibit downstream mTOR signaling in all drug resistant cell lines to the same extent as their respective parental cell lines. This would not be expected if BEZ were a favorable substrate because there would be reduced accumulation within the cell leading to a muted response.

BEZ inhibits PI3k and mTOR through binding at the ATP binding cleft suggesting the possibility that it inhibits ABCB1 in a similar fashion (78). However, our results indicate that BEZ does not inhibit ABCB1 function through inhibition of ATP hydrolysis since it is unable to prevent verapamil mediated stimulation of ATPase activity. These data suggest that in the nanomolar range, BEZ mediated inhibition of ABCB1 efflux is not through competitive inhibition at the ATP binding site and is not likely to be a competitive substrate inhibitor. Our results are similar to those of LY294 002, which also does not involve direct interaction with the substrate binding site (118). Moreover, a high-throughput screening assay measuring ABCB1 mediated calcein AM efflux was used to identify BEZ as a substrate inhibitor of ABCB1 (119). In contrast to our results, the
concentrations of BEZ used to inhibit calcein AM efflux were in the micromolar range, with several of the studies using 20 µM. It has been shown that plasma levels of BEZ in mice peak at 1.68 µmol/L 30 minutes after an oral gavage feeding of 50 mg/kg and rapidly decrease from there (78). We believe that in vitro concentrations below 1 µM would be more translatable to a clinical setting making our finding that BEZ inhibits ABCB1 in the nanomolar range important.

Over expression of ABC transporters in cancer cells can lead to a decrease in drug accumulation and treatment failure. Therefore, it is essential for an inhibitor to be able to re-sensitize overexpressed cells to treatment with a cytotoxic agent like DOX. Our results show that BEZ reduces the viability of ABCB1 overexpressing cells when co-treated with DOX in a dose dependent manner (Figure 10). We also show that this reduction in viability is the result of a corresponding increase in apoptosis, shown by increases in the cleaved form of PARP (Figure 11). Other reports have shown that BEZ can enhance the cytotoxicity of a drug, even when treatment with BEZ alone only had minimal effects on cell death (120-124). It has been said that this enhancement of cytotoxicity is due to inhibition of survival signaling within the PI3k and mTOR pathways. While to a degree this is correct, especially in cells with amplified signaling within these pathways, our data suggests that this enhancement of toxicity is also the result of increased accumulation of the drug due to inhibited ABCB1 mediated efflux. Further studies are necessary to re-examine to what extent increased accumulation of these cytotoxic agents in combination with BEZ plays in the enhancement of cytotoxicity. Phosphorylation of ABCB1 also plays a role in the efficiency of substrate transport (125). It cannot be ruled out that a change in
phosphorylation state of ABCB1 is occurring after treatment with BEZ since we are also manipulating an important signaling pathway involved in the activation of several kinases. It is conceivable that a decrease in efflux efficiency could occur without a decrease in total protein levels through inhibition of a kinase upstream ABCB1.

Somewhat surprising was that DOX appears to be a weak substrate for ABCB1 despite it being widely known that DOX resistance in tumors is, in part, mediated by overexpression of ABC transporters (109). In pancreatic cancer, which is known to be resistant to drug treatment, even patients without preoperative chemotherapy were shown to have ABCB1 expression rates of more than 50% (40). Also, treatment with DOX selects for drug resistant clones which overexpress ABCB1 indicating that these transporters may be the best option for protection against this drug. In spite of this, our results indicate that DOX is not an ideal substrate for ABCB1. This may explain the discrepancy between our studies with DOX, a weak substrate, and the previous report with calcein AM (119), likely a much better substrate for ABCB1. This highlights the importance of selecting the correct cytotoxic agent when seeking to enhance its efficacy through inhibition of ABCB1 transport.

Traditional ABCB1 inhibitors are known to have associated toxicities due to decreased systemic clearance of anti-cancer agents, which needs to be considered when using ABC transporter inhibitors (33). There are currently more powerful inhibitors being tested that have greater specificity to particular ABC transporters, however there remains the possibility that the clinical benefit will not be great even with this increase in specificity (33). This is because substrate redundancy makes the therapeutic window
extremely narrow for inhibitors specific to one transporter. We propose another alternative to circumvent toxicity associated with co-treatment of ABC transporter inhibitors with anti-cancer drugs. For those drugs which are weak substrates, like DOX, it may be more beneficial to combine them with drugs that themselves have anti-cancer activity along with being weak or non-substrate inhibitor, like BEZ. This may prove to be a beneficial strategy at attacking both ABCB1 negative as well as ABCB1 overexpressing cancer cells leading to improved outcomes for wide range of patients.

**Treatment related induction of ABC transporter mRNA**

While ABCB1 was the first discovered and is one of the best studied ABC transporters, it is not alone in conferring multi-drug resistance. There is a significant amount of redundancy between the transporters of which several are implicated in resistance to a wide range of cancer drugs (32, 34). Moreover, ABC transporters which do not have a direct role in cancer drug efflux may still mediate their efficacy and/or toxicity. Change in efflux transporter expression could also have major implications on a drug’s effectiveness over time. Therefore, it is important to address the effects of a drug on expression of multiple multi-drug resistance related and unrelated transporters.

Along those lines, ABCB8 does not directly transport DOX but still could have implications on DOX anti-cancer effects as well as cardiotoxicity (22, 106). In MDA-MB-231 cells, ABCB8 expression is induced with DOX treatment. This is important because ABCB8 confers resistance that is specific to DOX in melanoma cells by protecting the mitochondrial genome (106). Other studies using genetic deletion of ABCB8 in mice demonstrate that it is a mitochondrial iron transporter, and loss of expression causes
mitochondrial iron accumulation, increased ROS production, and cell death (22). These studies suggest that ABCB8 expression confers resistance to DOX through export of mitochondrial iron leading to a reduction in ROS production and mitochondrial genome stability while its inhibition results in enhanced cytotoxicity not only in cancer cells but normal tissues as well. It remains to be seen what impact the increase in expression observed in MDA-MB-231 cells would have on DOX. Mitochondrial iron is important in DOX-mediated ROS production therefore the increase in transcription is likely a protective response of the cell. In addition, since combination with BEZ does not increase ABCB8 mRNA expression compared to DOX, and based on our results that BEZ inhibits ABCB1 to induce intracellular DOX accumulation, it does not appear that DOX concentration alone determines transcript levels. On the other hand, this gene may have a low rate of transcription such that DOX alone causes the cell to reach its max rate of transcription so that the addition of more DOX has no effect. Further studies are needed to elucidate this as well as determine whether the increased expression observed in breast cancer cells is ubiquitous or tissue specific.

There are several ABC transporters that illicit drug resistance to cancer therapies. ABCC1 is involved in resistance to drugs as well as inflammatory responses mediated by leukotrienes (126, 127). However, ABCC1 has low expression in MDA-MB-231 cells and DOX treatment did not affect ABCC1 mRNA expression in these cells. BEZ co-treatment with DOX did cause a significant increase in transcript levels however the increase was minor and not likely to have an effect on protein levels or drug efficacy.
ABCC3 is another member of the ABCC subfamily known to confer resistance to anti-cancer drugs. Gene expression is induced after 24 hours of DOX treatment in MDA-MB-231 cells, although the response was minimal and not likely to alter protein expression. On the other hand, BEZ alone stimulates a nearly four-fold increase in ABCC3 transcript levels while combinations with DOX increase expression nearly six-fold. This dramatic induction of the gene with BEZ implies a regulatory role of the PI3K pathway on ABCC3. In neuroblastoma cells, ABCC3 expression is directly negatively regulated by MYCN through associating with the core promoter region and repressing transcription (126). It has also been shown that active GSK3 reduces MYC protein expression through phosphorylation of threonine 58 which leads to protein degradation (128, 129). Therefore, BEZ could reduce MYC protein expression by its inhibition of AKT activity which frees repression of GSK3. The reduction in MYC protein would then release its inhibitory function on ABCC3 resulting in an increase in expression.

The impact of such an increase in expression remains unclear. ABCC3 is important in bile acid transport along with chemotherapeutic agents (126). It is upregulated in non-small cell lung cancer and used as a marker for multi-drug resistance (42). Interestingly, the ABCC subfamily has been shown to confer resistance to Gem but ABCB1 and ABCG2 had little involvement when tested with differing ABC transporter inhibitors (45). Correspondingly, our results demonstrate that BEZ enhances the effects of DOX but not Gem in pancreatic cancer cells. Increased expression of ABCC3 due to BEZ treatment could play a major role in these conflicting results. Furthermore, whereas it may have a negative impact on cancer treatment if overexpression in cancer cells reduces intracellular
concentrations of the drug, we speculate that BEZ treatment could have beneficial effects in other diseases that rely on elimination of bile acids, such as cholestasis which use the export of bile acids and glucuronides by ABCC3 as an alternative route of export (127). In depth expression analysis after treatment with BEZ is needed to confirm a change in transcript levels in other normal tissues, like the liver.

Two other members of the ABCC subfamily implicated in chemoresistance are ABCC5 and ABCC10. Expression of both transporters after treatment with DOX is significantly increased while they are increased even more in combinations with BEZ. These results parallel the increase in intracellular concentration of DOX observed breast, pancreatic, colon and lung cancer cells. In other studies, ABCC5 transfected cells were shown to confer increased resistance to DOX and nucleoside analogs like 5-fluorouracil (109) while ABCC10 is a broad specificity transporter of xenobiotics, including taxanes and vinca alkaloids (130). Interestingly, ABCC5 is a cGMP transporter which is expressed in the heart and could, along with phosphodiesterase 5 (PDE5), be involved in the regulation of intracellular levels of cGMP and its possible paracrine effects (131). In support of this, PDE5 and ABCC5 have similar affinities for cGMP, and PDE5 inhibitors have been shown to also inhibit ABCC5 (132). In cancer, ABCC5 is increased in cervical cancers during growth as well as breast cancer metastasis to the bone (107, 133). The reason behind the upregulation remains unknown but it is speculated that ABCC5 reduces intracellular levels of cGMP thereby relieving its inhibitory effects on cell growth. However, this remains inconclusive as one study found no effect on cell proliferation when ABCC5 was knocked down in breast cancer cells (107).
Unlike ABCC5, ABCC10 does not transport cGMP or cAMP but does have a physiological role in the transport of leukotrienes (44, 130). In cancer, ABCC10 is implicated in resistance to vinca alkaloids and is the only other ABC transporter besides ABCB1 that has been shown to confer resistance to taxanes (102). Our results show a responsiveness of ABCC5 and ABCC10 to DOX at the level of transcription but it is unlikely that this will have any effects on efficacy since DOX is a poor substrate for the ABCC subfamily. Moreover, it is unknown what effect BEZ might have on the efflux capacity of these transporters. Since BEZ is an ATP-binding pocket inhibitor it would be easy to surmise that it would at least be a weak substrate for ABCC5 and/or ABCC10.

ABCG2, along with ABCB1 and ABCC1, is among the most frequently upregulated genes associated with cancer drug resistance. Similar to the results for the ABCC subfamily, ABCG2 seems to be responsive to the intracellular concentrations of DOX. This suggests a protective response of the cell to mitigate the damage brought on by DOX exposure. ABCG2 is known to mediate resistance to several anti-cancer therapies including DOX (41, 100, 134-137). However, a mutation at arginine 482 is necessary for transport of anthracyclines (41, 135) therefore protein overexpression does not necessarily translate into a reduction in DOX efficacy. Furthermore, although there is no evidence of inhibition of ABCG2 efflux by BEZ, several other inhibitors of PI3K pathway kinases do interact with ABCG2 to modulate its function (100, 136). Therefore, considering that BEZ seems to modulate one or more ABC transporters other than ABCB1, we speculate that ABCG2 inhibition is involved in the enhancement of DOX accumulation.
ABC transporters have an important role in cancer multi-drug resistance and anticipating changes in expression with cancer therapy can help clinicians develop better treatment regimens to maximize the effects of a given therapy. Post-treatment expression profiles can also open the door to novel uses for a drug that would not have otherwise been known. Extensive research is needed to determine if the changes in expression observed in our studies can be realized in other cancer and disease models.

Cancer

Pancreatic cancer

Pancreatic cancer is a devastating disease which has a death rate nearly as high as the incidence rate (138). Current treatment options for pancreatic ductal adenocarcinoma (PDAC), which is the most common form of pancreatic cancer as well as the most deadly, have an extremely limited benefit to patients. In the present study, we show that inhibition of the PI3K/mTOR pathway with BEZ sensitizes pancreatic cancer cells to DOX, but not Gem, both in vitro as well as in an in vivo mouse tumor model. The enhanced sensitivity to DOX-induced killing of pancreatic cancer cells is in part due to down regulation of translational and survival signaling downstream of PI3K, including AKT. In addition, we observe increased ROS formation and intracellular accumulation of DOX, which likely occurs through inhibition of one or more of the ABC-type transporters, a family of proteins necessary for the elimination of xenobiotics, including DOX (32, 139). As a result, combinations with BEZ cause enhanced activation of DNA damage response proteins and modulate the levels of pro- and anti-apoptotic proteins to favor induction of apoptosis.
(Figure 30 and 31). Interestingly, similar enhancement in killing was observed in breast, lung, and colon cancer cell lines (Figures 38, 42, and 43) providing evidence that this combination could be beneficial to multiple solid tumor types. Also, our results demonstrate that BEZ does not enhance the cell killing effect of Gem, which is the standard of care for pancreatic cancer patients. Clinically, cardiac dysfunction is the major dose-limiting factor for the use of DOX in patients with cancer. Our results show that treatment of DOX along with BEZ does not increase the cardiotoxic effects of DOX both in vitro and in vivo despite inhibition of the PI3K pathway, which is critical in protecting the heart from stress (140). These confounding results may involve the severe reduction in expression of the pro-apoptotic protein BIM when combining BEZ and DOX in the in vitro cardiomyocyte studies. These results suggest new possibilities of combining PI3K/mTOR inhibitors with traditional cytotoxic agents, including DOX, to achieve better control of PDAC.

The classical RAS pathway, which transmits signals through RAS to the extracellular-signal related kinase (ERK), demonstrates an important target for treatment of PDAC; however, there have been few successes to date (141). Due to the high degree of crosstalk, as well as overexpression of one or more receptor tyrosine kinases (RTKs), there is also a high prevalence of enhanced activation of the PI3K/mTOR pathway (142). Moreover, activation of AKT has been associated with poor outcomes of PDAC patients (143), and there is also evidence that RAS mediates its oncogenic initiation and maintenance through PI3K/PDK1 (58). Therefore, targeting the PI3K/mTOR pathway also represents a potential treatment strategy in PDAC.
Supporting the evidence that RAS drives tumorigenesis in PDAC through PI3K, a previous report has shown that dual inhibition of PI3K and mTOR with BEZ is equally sensitive in both Kras<sup>G12D</sup> and PI3K (p110α<sup>H1047R</sup>) driven PDAC cells (144). However, our current results and other studies demonstrate that BEZ reduces proliferation but does not induce a significant amount of cell death (145, 146), which may lead to earlier progression of tumors in patients. Therefore, combination therapy utilizing a cytotoxic agent, like DOX, could greatly enhance the clinical utilization of BEZ and possibly other PI3K pathway inhibitors. Previous studies in breast and ovarian cancer have shown that inhibiting the PI3K pathway significantly enhances DOX cell killing (110, 147). Studies utilizing similar combinations in PDAC are limited because PDAC is not a primary indication for DOX. This is because of resistance mechanisms toward DOX which include not only activation of survival signaling but also enhanced drug elimination via the ABC transporters and reduced drug delivery (148) leading to intracellular concentrations below the therapeutic threshold. However, our results show that DOX could be a very effective therapeutic for treatment of PDAC when combined with the correct secondary agent to overcome resistance. Therefore, this strategy needs to be evaluated in the future.

BEZ treatment for extended periods in MiaPaCa2 cells leads to re-activation of the PI3K pathway and phosphorylation of AKT, likely through inhibition of mTORC1 causing a release of feedback inhibition through IRS1, suggesting an enhancement of survival signaling that could result in treatment failure. However, siRNA knockdown of PDK1 and Rictor to specifically prevent phosphorylation of AKT downstream of PI3K and mTORC2 respectively, demonstrates that reactivation of AKT does not reduce the effectiveness of
using BEZ in combination with DOX (Figure 15 and 16). This suggests that the transient inhibition of AKT survival signaling, which occurs at earlier time points as previously described (120, 149), is enough to sensitize the cancer cells to DOX before loss of feedback inhibition through mTORC1 results in reactivation of the pathway. This has also been shown by others who demonstrate that BEZ induces P53 independent apoptosis despite reactivation of AKT in MYC-driven lymphoma cells (150). Furthermore, the dose required for BEZ to prevent reactivation of the AKT pathway seems to be cell line dependent as other cell lines failed to show such an effect.

Our results demonstrate that PI3K/mTOR inhibition does not enhance the cell killing effect of Gem. Other studies report that BEZ and Gem have anti-proliferative effects, which is enhanced with combination treatment (145, 151); although, high concentrations of BEZ (10 μM) fail to improve apoptosis (145, 151). We also observe inhibition of proliferation with either BEZ or Gem in MiaPaca2 cells which is further reduced with combination treatment (Figure 26). Surprisingly, these results do not translate into increased cell death, and the combination is even less effective as compared to Gem alone (Figure 26). As previously discussed, BEZ treatment causes a significant increase in expression of the ABC transporter ABCC3, which is known to confer resistance to Gem (45). Increased expression of this protein could lead to reduced intracellular concentrations, and thus effectiveness, of Gem but not DOX explaining the discrepancy. This suggests that the type of cytotoxic agent employed can play a major role in its effectiveness when combined with PI3K pathway inhibitors. It is also likely that not all
PI3K or dual PI3K/mTOR inhibitors will have similar effects on DOX enhancement in PDAC and other cells due to the unique properties of BEZ.

In mouse studies, combination treatment with BEZ and DOX significantly reduces tumor size compared to all other groups (Figure 33). This increased efficacy could be due to combination of reduced proliferation and survival signaling concomitant with increased DOX accumulation, ROS formation, and BIM expression. PDAC tumors have a unique tumor microenvironment known for their extensive buildup of stroma called desmoplasia that results in tumors with reduced vasculature and intratumoral hypoxia which makes tumors extremely resistant to drug treatment (6, 148). Our tumor model using MiaPaca2 xenographs in immunocompromised nude mice likely does not resemble the tumor microenvironment of PDAC patients clinically for several reasons, but most glaringly, because of a lack of immune response cells infiltrating the tumor. Therefore, it is unknown whether accumulation of drugs clinically would be sufficiently enhanced to reach levels needed for a therapeutic response. However, by targeting multiple resistance mechanisms that reduce survival signaling through PI3K and inhibit ABC efflux transporters, we are able to maximize the effects of the drug combination that we believe would be more effective than the current standard of care.

In summary, our results provide compelling evidence that combining BEZ with DOX is highly effective in killing pancreatic cancer cells in vitro and reducing tumor size in vivo. In contrast, BEZ does not enhance the effects of Gem which is the standard of care for PDAC suggesting that combinations with DOX would be more effective than current treatment standards. Moreover, BEZ treatment does not exacerbate DOX induced cell
death in vitro or contractile dysfunction in tumor bearing mice signifying there would be minimal increase in the cardiotoxic effects with combination therapy. Mechanistic investigations reveal that BEZ enhances the effects of DOX in cancer cells through down regulation of PI3K signaling, increased ROS generation, altered expression of BIM, and improved DOX accumulation. Based on these results, we propose that combining BEZ with DOX could be an attractive clinical option for patients suffering from PDAC.

**Breast cancer**

Breast cancer is the most common malignancy in American women, with an estimated 231,840 new cases diagnosed in 2015 (4). Treatments include surgery, radiation, and anti-cancer drugs, including cytotoxic agents and targeted therapies. Since its discovery more than 30 years ago DOX has remained a first-line anti-neoplastic drug for the treatment breast cancer. However, treatment related toxicities and acquired drug resistance limit the effectiveness of this drug and others. Therefore, combination therapies which target resistance mechanisms in the tumor and allow for reduced cumulative doses are greatly needed. Here we provide evidence that BEZ enhances the effects of DOX in a manner that is dependent on the mutation type of the cell. In KRAS mutant MDA-MB-231 cells, combinations with BEZ enhance the efficacy of DOX in a time- and dose-dependent manner increasing apoptosis and necrosis in a manner that parallels the increase in intracellular DOX accumulation (Figures 35-38). Moreover, colony formation is significantly reduced with combination treatment of MDA-MB-231 cells. On the other hand, PI3K mutant Mcf7 cells do not benefit to the same extent from combining BEZ with DOX. Cell viability, cell death, and apoptosis are only marginally affected by combination
treatment (Figures 40 and 41). The reason for this discrepancy is likely due to the reliance of the cells to a particular pathway, and in this case, the PI3K pathway.

Activating mutations along the PI3K/AKT/mTOR pathway are the most frequently occurring mutations in breast cancer and help to drive tumor initiation and maintenance (152, 153). They also provide a promising target in the treatment of breast cancer, as demonstrated by the number of inhibitors being developed which target this pathway (153). However, beneficial responses to these drugs are cell line dependent and there is acquired resistance associated with treatment due to upregulation of alternative survival pathways, including ERK, MYC, and RSK (73). Likewise, our results and others show that PI3K wild-type and KRAS mutant MDA-MB-231 cells are resistant to BEZ treatment while PI3K mutant Mcf-7 cells are sensitive (154-156). This demonstrates the dependencies of these cells on the PI3K pathway for survival highlighted by the observation that MDA-MB-231 cells are resistant despite a similar level of reduction in PI3K pathway activation. Also in MDA-MB-231, inhibition of both AKT and S6 occurs as early as four hours after treatment and is sustained for at least 48 hours (Figure 36). Therefore, the effects on the PI3K pathway are not the cause of resistance to BEZ. For that reason, combination therapies, including those using cytotoxic agents like DOX, are looked upon to provide better treatment responses over a wide range of tumors and mutation types. Our results demonstrate that combining BEZ with DOX improves the outcome over single agent treatments in MDA-MB-231 cells. These results are similar to our observations in PDAC cells, an outcome that may be correlated to an activating mutation in KRAS. Conversely, while DOX alone had minimal effects in Mcf-7 cells, BEZ
is extremely effective on its own and the addition of DOX does not have a profound effect on cell death and apoptosis. In light of this, we speculate that combination therapy using BEZ and DOX may be most beneficial in cells which do not have an activating mutation along the PI3K pathway.

Our data demonstrates the anti-proliferative effects of BEZ on breast cancer cells irrespective of the mutation status of the cells. This is similar to previous reports in breast and other cancer cell lines which show that BEZ induces a strong G₀/G₁ arrest leading to reduced proliferation (78, 145, 146, 156). However, the reduction in proliferation does not always correlate with increased cell death. Induction of cell death by BEZ is dependent on the mutation and/or expression status of the cell, with those harboring activating mutations to PI3K or overexpressing HER2 being the most sensitive to treatment while KRAS mutant and PTEN deleted cells being resistant (156). Despite the relative resistance of KRAS cells to BEZ, there is still dependence on this pathway, especially in times of stress as shown with our combination with DOX. Similar to PDAC, induction of BIM may be important in this sensitization. BEZ is known to increase mRNA and protein expression of BIM in ovarian cancer cells (80) as well as transcriptionally through a mechanism involving FOXO3A, which is inhibited by AKT (146).

Resistance to chemotherapy is often mediated by the expression of one or more ABC-type transporters (32, 34, 105). Likewise, our results indicate that BEZ inhibits ABCB1 which can re-sensitize drug resistant cells to DOX. However, increased accumulation of DOX is observed in cells which do not express a high amount of ABCB1 protein, like MiaPaCa2. Similarly, neither MDA-MB-231 nor Mcf-7 cells express ABCB1
to a great extent (157), but still show enhanced accumulation of DOX when combined with BEZ. This reiterates the fact that BEZ likely inhibits other ABC transporters besides ABCB1. Surprisingly, unlike what is observed in MDA-MB-231 cells, the increase in DOX accumulation only results in a small enhancement of cell death in Mcf-7 cells. It’s possible that BEZ alone is effective enough in cells addicted to the PI3K pathway that the addition of another cytotoxic agent is not needed. On the other hand, in vivo results would provide a better understanding of how well BEZ works as a single agent. The addition of a cytotoxic agent like DOX may provide a better overall response in these cells by inducing a longer lasting effect, rather than the transient effects that would be expected due to the metabolism and elimination of the drug.

**Colon and lung cancer**

Lung and colon cancer represents the first and third leading cause of cancer death in the United States, respectively (4). Both types commonly express mutations or deletions to KRAS, EGFR, and P53 which help to drive tumorigenesis as well as resistance to therapeutics (158, 159). The PI3K pathway is also frequently deregulated leading to several inhibitors of this pathway, including BEZ, being tested for use as monotherapy or in combination (158-164). Our results demonstrate that BEZ can enhance the effects of DOX in HCT 116 colon cancer and H1299 lung cancer cells resulting in more apoptosis and cell death (Figures 42 and 43). Similar to pancreatic and breast cancers, DOX accumulation was also increased in these cell lines. These results reveal the wide ranging potential for combining BEZ with DOX in the treatment of cancer.
Similar to our results in breast cancer, BEZ reduces PI3K and mTOR signaling to below control levels in both colon and lung cancer cells (Figures 42 and 43). However, BEZ alone induces little cell death indicating these cells can easily bypass this pathway to induce alternate survival mechanisms. This is not unexpected considering HCT 116 harbor KRAS activating mutations along with its mutations in PI3K while H1299 has wild type KRAS and PI3K. Our results in breast and pancreatic cancer and other reports using cell lines without activating mutations in PI3K have shown a similar resistance to BEZ (154-156). Interestingly, BEZ does have anti-proliferative effects against HCT 116 cells which can lead to a significant reduction in tumor growth in a mouse xenograph tumor model (165, 166). This is likely the result of BEZ’s ability to arrest the cell cycle and not from an increase in cell death which could potentially lead to an early time to recurrence. However, BEZ combinations with DOX significantly increase cell death which corresponds to increased cleavage of PARP, a marker for apoptosis. Enhancement of cell death along with reduced proliferation offers a better therapeutic impact than either drug treated alone.

Drug resistance mediated by the overexpression of one or more ABC transporters is a major clinical problem (32, 33). At least one transporter is overexpressed in most if not all resistant cancer cells, including HCT 116 and H1299 (167, 168). Our results demonstrate that BEZ can enhance DOX accumulation by approximately two fold over DOX alone in both cell lines which corresponds to the observed increase in cell death (Figure 42 and 43). It is likely that this sensitization is dependent on both decreased survival signaling along with increased drug delivery, which in turn results in an enhancement of DNA damage and cell death. BEZ has previously been shown to increase
irenotecan effectiveness in HCT 116 cells through its inhibition of the PI3K pathway (166). Likewise, BEZ overcomes gefitinib-acquired resistance through down-regulation of the PI3K/mTOR pathway in lung cancer (169). We hypothesize that in addition to its inhibitory effects on the PI3K pathway, BEZ may also enhance drug accumulation of irenotecan and gefitinib to further enhance the combinations effects. Previous reports have shown that sorafinib, a platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR) inhibitor, can inhibit ABCG2 and overcome resistance to irenotecan in colorectal cancer (168), demonstrating that irenotecan is a substrate for at least one ABC transporter. We have shown that BEZ inhibits ABCB1 but that there are likely other targets. It’s not hard to presume from BEZ’s enhancement of irenotecan and sorafinib overcoming ABCG2-induced resistance to irenotecan that one of the other transporters inhibited by BEZ is ABCG2. Further studies in cell lines overexpressing ABCG2 are needed to confirm this but the overall implications of these results along with our previous studies in other cancer types could be substantial.

**Cardiotoxicity**

Cancer patient survival is continuing to improve with the advent of more efficacious therapies that lead to better overall survival. According to the SEER cancer statistics review, with breast cancer alone, the NCI estimates that approximately 2.6 million women diagnosed with cancer were alive in 2008. However, several therapies used in the treatment of cancer can cause serious health risks, including cardiovascular events leading to heart failure which many times do not become apparent until years later
(170). This is especially true in cases where combination therapies utilizing anthracyclines and receptor tyrosine kinases are used. In HER2 positive breast cancer, the humanized monoclonal antibody trastuzumab inhibits HER2 signaling resulting in clinical responses that are enhanced with the addition of DOX, as shown in a pivotal phase III clinical trial (97). However, combination treatment in these patients was associated with unacceptably high rates of New York Heart Association class III/IV cardiotoxicity resulting in all large adjuvant trials only evaluating sequential dosing strategies despite the improved anti-cancer effects of co-treatment (113). In light of this, we assessed the cardiotoxic effects of combining BEZ, an inhibitor that targets a pathway downstream of HER2, with DOX in both in vitro and in vivo models. Our results show that cell death is reduced with combination treatment with BEZ and DOX compared to treatment with DOX alone in H9C2 rat myoblast cells. The reduction in cell death is associated with an increase in the Bcl-2/Bax ratio as a result of decreased Bax expression, as well as loss of BIM expression. Both of these events signal a pro-survival response in the cell that helps mitigate the cytotoxic effects of DOX. Moreover, the effect on cardiac function in tumor bearing mice treated with DOX is not potentiated with the addition of BEZ (Figure 49). Also, our results show that mouse hearts treated with a single dose of DOX and BEZ display increased survival signaling through AKT and especially ERK (Figure 50). These results suggest that combining an inhibitor of the PI3K pathway with DOX will not cause an increase in cardiac toxicity leading to heart failure.

This observation is potentially significant given the negative effects associated with trastuzumab and anthracycline co-administration. There are some key differences in
signaling between uses of trastuzumab that could explain this. It targets an initiating node (HER2) that transmits extracellular signals into the cell causing a host of events that play key roles in cell proliferation, growth, and survival among others (171). This reduces signaling through the PI3K and RAS pathways, both of which are important for survival signaling in the heart through inhibition of several pro-apoptotic proteins, including Bax, BIM, and BAD (172). Inhibition using trastuzumab releases the inhibitory effects on these pro-apoptotic proteins leaving cardiomyocytes susceptible to damage after treatment with DOX. HER2 also has important roles in heart development and function. This is demonstrated by the report that HER2 knockout mice develop cardiomyopathy consistent with dilated cardiomyopathy (173). Moreover, inhibition of HER2 with trastuzumab itself causes cardiotoxicity in 4-7% of patients highlighting its necessary role in normal cardiac function (113, 170).

Our results also show that treatment with BEZ alone displayed no significant changes in H9C2 cell death or cardiac function which is in line with our biochemical assessment of pro-apoptotic protein expression (Figures 45-47 and 49). Interestingly, when combined with DOX in vitro, there is reduced cell death as compared to DOX alone consistent with the decrease in BIM protein expression (Figures 45 and 47). This is opposite to the increased expression observed with combination treatment in pancreatic cancer. As yet, the mechanism underlying the differential regulation of BIM protein expression is unknown, although both AKT and ERK play important roles in both its transcriptional and post-translational regulation (174-177). BIM is transcriptionally regulated by the nuclear localization of FOXO3A. FOXO3A in turn is regulated by
inhibitory phosphorylation by AKT, which mediates its exclusion from the nucleus and interaction with the protein 14-3-3 ultimately leading to degradation (176, 177).

Theoretically, treatment with BEZ should relieve the inhibitory phosphorylation on FOXO3A promoting its nuclear localization and transcription of BIM. This anticipated result is observed in our cancer cells which help to promote cancer cell death. However, the decrease in BIM expression after treatment with BEZ in H9C2 cells is likely related to the alternate regulatory pathway through ERK. Whereas BEZ treatment inhibits PI3K/mTOR pathway activation, it causes an increase in activation of the ERK pathway (178) resulting from redirection in signaling away from PI3K. BIM is a direct target of ERK and its phosphorylation promotes ubiquitination and degradation of the protein (174, 175). Therefore, it is likely that in H9C2 cells, the redirection of signaling into the RAS pathway leads to increased activation of ERK and consequently, a reduction in BIM protein.

Other contradictory reports have shown a role for ERK in DOX-induced cell death and cardiomyopathy (179-181). The reason for this discrepancy may be due to the difference in DOX concentration and route of administration. In H9C2 and HK-2 (kidney) cells, 1 μM or higher concentrations of DOX were used to show an increase in ERK phosphorylation and that this increase was in part responsible for apoptosis induction (179, 180). Although these plasma concentrations are achievable, it is at the higher end of a steep bell curve that likely is not sustained for long periods of time. An alternate explanation for the increased activation of ERK in these situations is that at higher concentrations, or more cytotoxic stress, the protection incurred by ERK gives way and switches to a pro-death
cascade that is not observed at lower concentrations of DOX. Also, the mouse
cardiotoxicity studies used intraperitoneal (IP) injections of high concentrations of DOX
(181). In our experience, IP injection of DOX causes a host of gastrointestinal issues that
more closely resemble a model of inflammation. It is possible that the reduced function
observed in the hearts of these mice was a result of septic shock and organ failure.

In tumor bearing mice, DOX results in a significant reduction in systolic function
four weeks after the initialization of the treatment. However, unlike with combinations
containing trastuzumab, the addition of BEZ did not augment the cardiotoxicity. Similar to
the studies with H9C2 cells, the reason for this may be due to increased survival signaling
through ERK. Heart cell lysates, collected 24 hours after treatment, displayed increased
phosphorylation of both AKT and ERK. Although it needs to be confirmed, we
hypothesize that BIM expression in this situation is also decreased leading to a protective
effect on the heart. In addition, inhibition of mTORC1 with rapamycin protects the heart
against ischemia-reperfusion injury through activation of JAK2/STAT3 (99). Likewise,
inhibition of mTORC1 with BEZ could mediate a cardioprotective effect against DOX in
conjunction with the enhancement of other survival pathways.

Our results suggest that BEZ induces a cardioprotective effect against DOX in the
heart through enhanced activation of the ERK survival signaling pathway. This results in a
corresponding ERK-dependent decrease in BIM expression that mediates the decreased
cardiotoxicity. Therefore, we believe that this could have major implications on the use of
this combination treatment in the clinic.
CHAPTER 5: Study Limitations

Our results provide evidence of the beneficial effects of combining BEZ with DOX to treat cancer which does not also enhance the cardiotoxic effects of DOX. However, there are several aspects of inhibiting the PI3K/mTOR pathway in combination with DOX that could not be addressed in this study that remain important in the effects of this combination. Many of these processes relate to the activation level of the PI3K pathway with several being specific to mTOR. Autophagy is a process that utilizes cellular catabolic degradation of intracellular organelles, cytoplasm, and proteins to maintain metabolic rates when undergoing stress (182). It is also predominantly under the control of mTORC1 which down-regulates its activity through phosphorylation of unc-51-like autophagy activating kinase 1 (ULK1) (183). Under nutrient rich conditions, the PI3K/mTOR pathway is activated resulting in inhibition of autophagy. Conversely, when the cell is deprived of nutrients or cellular energy levels are low, autophagy is activated in order to maintain metabolites to the cytoplasm where they are reused as a source of energy or to provide building blocks for the synthesis of new molecules (183). Autophagy is initiated through a multi-protein complex containing ULK1, FIP200 (RB1-inducible coiled-coil 1), autophagy-related 13 (ATG13), and ATG1 which is under the control of mTORC1 (184, 185). Furthermore, nucleation of the phagophore requires a second complex containing vacuolar protein sorting 34 (VPS34 or PIK3C3) and Beclin1 which can recruit various different proteins that regulate autophagy (185). This includes covalent attachments of
ATG5 to ATG12 and phosphatidylethanolamine to microtubule-associated protein 1 light chain 3 (LC3) (184). This process, along with the recruitment of several other ATG proteins, allows for growing of the membrane to enclose the cellular contents in the completed autophagosome. The autophagosomes then fuse with lysosomes with the help of lysosomal-associated membrane protein 2 (LAMP2) and RAB7A to form autolysosomes which degrade the engulfed components (184-186).

Autophagy not only has important regulatory functions in normal cells, but also has important functions in tumor initiation and promotion (187). However, there are conflicting roles for autophagy in cancer with evidence of it also acting as a tumor suppressor. Similarly, autophagy is thought to act as a pro-survival mechanism for cancer cells which can lead to drug resistance (185). In light of this, BEZ could result in enhanced autophagic response that could delay commitment to cell death. Paradoxically, others have shown that autophagy is an important pathway that is necessary for the pro-death mechanisms of rapamycin (185). Studies which utilize knockdown of key autophagy proteins, including Beclin1, ATG5, and ATG7, would be beneficial to elucidate the role that autophagy has in the enhancement of cell death when BEZ is combined with DOX. In addition, autophagosome formation utilizing LC3-GFP constructs transfected into cells would help determine the extent of autophagy induction after short and long term drug treatments. These important studies could help elucidate the opposite effects towards combination treatment that was observed in cancer cells as compared to H9C2 cells. Moreover, BIM has been shown to inhibit autophagy through its anchoring of Beclin1 to microtubules (188). Therefore, since BEZ inhibits mTOR activity and BIM expression is
induced (at least in cancer cells) it seems as though autophagy is being acted on through opposing signals. These aforementioned studies would interrogate the role that autophagy plays with the combination of BEZ and DOX in cancer and heart cells.
Figure 52. **Autophagy initiation and maturation.** Autophagy is primarily controlled by mTORC1 through its regulation of a complex containing ULK1. Upon loss of signaling through mTORC1, inhibition of ULK1 is relieved allowing for nucleation and initiation of autophagy. Maturation requires the recruitment of several ATG protein complexes and many other proteins including Beclin1 and LC3 which allows for elongation of the autophagosome and engulfment of organelles and other cytoplasmic components.
In addition to down-regulating autophagy, increased BIM expression is likely to mediate an apoptotic response through its inhibition of pro-survival proteins. Our results suggested a correlation of such an effect with enhanced cell death observed in cancer cells while loss of BIM expression corresponded to reduced cell death in H9C2 cells. However, further studies are needed to confirm the role of BIM in mediating the effects of BEZ and DOX. Apoptosis is initiated through two separate but interconnected pathways termed the intrinsic and extrinsic pathways that are dependent on activation of caspases (189-191). The extrinsic pathway involves the activation of death receptors and formation of death-inducing signaling complexes (DISC) which include pro-caspase 8 that is subsequently cleaved into its active form (189). Active caspase 8 can then either act on downstream caspases to directly induce an apoptotic response or can cleave another Bcl-2 family pro-apoptotic protein, Bid, to form the active truncated form of Bid (t-Bid) which translocates to the mitochondria and acts as an amplifier of the apoptotic signal (189). The intrinsic pathway is characterized by activation of pro-apoptotic Bcl-2 family proteins Bax and Bak which induce mitochondrial membrane permeabilization and release of cytochrome-c, Smac/DIABLO, apoptosis inducible factor (AIF), and endonuclease G (192). Bax and Bak are activated by BH3 only members of the Bcl-2 family, including BIM, Bid, Bad, PUMA, and NOXA, and inhibited by the anti-apoptotic members, Bcl-2, Bcl-xL, and Mcl-1 (193). Cytochrome-c release initiates the formation of the apoptosome and subsequent activation of caspases 9 and 3 to trigger execution of cell death (192).

Our results in cancer imply the activation of Bax and Bak through increased expression of BIM. However, confirmatory experiments are needed. If BIM is important in
the enhancement of cell death mediated by treatment with BEZ and DOX, then knockdown should significantly reduce the effects of combination treatment. Likewise, BIM induces its apoptotic response through activation of Bax and Bak, therefore knockdown of these proteins individually or together should dramatically reduce effectiveness of treatment. Moreover, the extrinsic pathway of apoptosis could be involved in the induction of cell death. Therefore, experiments utilizing inhibition of this pathway, either through the use of caspase 8 inhibitors or through overexpression of FADD-like interleukin-1 β-converting enzyme-like protease (FLICE)-inhibitory proteins (FLIP) should be performed to determine the extent of extrinsic pathway involvement.

Our results showed that Z-VAD nearly completely abrogated PARP cleavage. However, while there was a significant decrease in cell death, there remained a large percentage of cells that were not protected. This suggests an alternate mode of cell death induction. AIF is released after mitochondrial membrane permeabilization along with cytochrome-c and induces caspase independent cell death (194, 195). Likewise, cell death induced by treatment of BEZ and DOX may depend on caspase dependent and independent mechanisms, including AIF release, toxic autophagy, and necrosis/necroptosis.
Figure 53. **Extrinsic and Intrinsic apoptosis pathways.** (A) Extrinsic apoptosis initiates through activation of death receptors and processing of pro-caspase 8 within the DISC to active caspase 8 through a process which is inhibited by FLIP. Caspase 8 can directly activate downstream caspases or cleave the pro-apoptotic protein BID to form tBID which translocates to the mitochondria to amplify the apoptotic signal. (B) Intrinsic apoptosis is characterized by activation of pro-apoptotic proteins Bax and Bak through a process mediated by relieving of the inhibitory functions of one or more anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1) by BH3 only proteins, including BIM. Activation of Bax and Bak allows for dimerization and mitochondria permeability allowing for release of cytochrome-c and AIF into the cytoplasm mediating both caspase dependent and independent apoptosis.
CHAPTER 6: Conclusions

Our results suggest that BEZ in combination with DOX could potentially be an attractive clinical option for patients with a range of cancer types. This conclusion is based on our results showing enhanced anti-cancer efficacy which does not augment the cardiotoxic effects observed with combination therapies utilizing receptor tyrosine kinase inhibitors and DOX. The largest benefit was seen in cancers that had mutations to proteins outside of the PI3K pathway and was especially effective in KRAS mutant pancreatic cancer. The reason for this is still largely unknown but seems to rely on a combination of factors, at least one of which may be unique to BEZ. KRAS mutant cancers traditionally are thought to signal through RAF, MEK, and ERK to transmit its oncogenic signaling in a process that is independent of PI3K. However, it is now know that the RAS proteins, including KRAS, directly activate PI3K leading to downstream signaling and pancreatic cancer has been shown to be dependent on PI3K signaling. With that being said, inhibition of PI3K and its downstream targets itself does not kill KRAS mutant cells and at most arrests cell proliferation, which is in contrast to PI3K mutant cells which were sensitive to BEZ treatment. While tumor growth may arrest for a short time, BEZ likely would not be an effective monotherapy in cancers which do not have PI3K mutations or HER2 overexpression, as evidenced by the clinical trial data. For that reason, the addition of a cytotoxic agent like DOX is critical for effective treatment of the cancer. With combination
treatment, BEZ acts to sensitize the cells so that DOX can efficiently kill the cancer cell. The sensitizing effect of BEZ may also be the result of decreased metabolic efficiency through loss of glycolytic enzymes which are under the translational control of AKT and mTOR. Evidence for this can be seen in microscopy pictures of BEZ treated cells which seem to have a phenotype resembling nutrient starvation. Future studies are needed to confirm this but unpublished data from this lab demonstrates that glucose deprivation sensitizes cells to DOX which seems to support the hypothesis.

BEZ is an inhibitor of ABC transporter mediated efflux of DOX. We describe BEZ’s direct inhibition of ABCB1 which can overcome the multi-drug resistant phenotype. However, as evidenced by the increase in DOX accumulation in cells that do not express a high amount of ABCB1 (MiaPaCa2 and MDA-MB-231), it is highly likely that BEZ also inhibits other efflux transporters. Studies using overexpression of these other transporters will be needed to further expand on this interesting observation. Clinically there are tremendous implications for drugs that can increase tumor drug delivery, especially considering pure efflux inhibitors have been met with toxicity due to the loss of endogenous functions of the transporters. BEZ is essentially a dual-role inhibitor, its primary function being a PI3K/mTOR inhibitor and a novel secondary function of inhibiting ABC transporter efflux of DOX. Our results suggest that DOX is not a great substrate for ABCB1 and possibly other transporters. Therefore, BEZ, which we believe to be a weak inhibitor of ABC efflux proteins, can sufficiently outcompete DOX for the substrate binding site allowing for nuclear accumulation. However, it would not outcompete many endogenous substrates needed for biological function of normal cells.
thereby reducing ABC transporter related toxicities. Furthermore, the increased accumulation of drugs when co-treated with BEZ may be specific to DOX and its related derivatives as evidenced by the reduction in efficacy when combined with Gem in pancreatic cancer cells. Moreover, BEZ mediates an increase in expression of efflux transporters that efficiently transport Gem so the type of drug could have a major impact on drug efficacy in the clinic.

Another significant finding of this study is that BEZ does not augment the cardiotoxic effects of DOX. Cardiotoxicity can be severe with DOX if cumulative doses get too high and clinical trials in combination with trastuzumab had to be discontinued because it potentiated those effects. Therefore, the finding that BEZ can reduce the cardiotoxic effects is extremely important. Damage to the heart is generally ascribed to DOX-induced production of ROS leading to oxidative damage and cell death but may at least partially be dependent on isoform specific topo II inhibition. Our studies in cancer cells indicate that DOX induces ABCB8 expression which could have a protective effect by reducing intramitochondrial iron levels. However, in these cells at least, BEZ had no effect on expression of this transporter. In the heart, it has been shown that ABCB8 expression is decreased after DOX exposure which may mediate the increase in ROS production and leading to cell death. It would be interesting to see if BEZ had any effects on expression of ABCB8 in the heart which could explain the observed protective effects. Also, expression levels of other transporters known to confer resistance to DOX may be modulated by BEZ to induce protection. Of course inter-species differences can’t be ruled out as the effects of BEZ on ABC transporter expression could be drastically different in
humans as that observed in mice. It would be extremely informative to obtain patient
samples from clinical trials using BEZ to assess the change in expression of ABC
transporters to corroborate our pre-clinical studies.

In summary, we propose that combining BEZ with DOX would provide a
beneficial therapeutic option for patients with pancreatic and other cancers. It is our hope
that such a strategy would be significantly better than currently used regimens.
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HONORS AND AWARDS

- 2014 - Late-breaking abstract accepted for oral presentation at the American heart Association’s (AHA) scientific sessions annual meeting in Chicago
- 2014 - Served as the student representative on an Assistant professor to Associate professor promotion committee
- 2014 - Travel award from the American Society for Pharmacology and Experimental Therapeutics (ASPET) for the 17th World Congress of Basic and Clinical Pharmacology in Cape Town, South Africa
- 2014 - Travel award from the American Society for Pharmacology and Experimental Therapeutics (ASPET) for the Experimental Biology meeting in San Diego
- 2014 - Abstract for the Experimental Biology annual meeting selected for a press release
- 2013 - Awarded Predoctoral Fellowship from the American Heart Association
- 2012 - Abstract from the American Heart Association selected for national press release, news conference, and oral presentation for the scientific sessions annual meeting
- 1997 - Academic Scholarship at Snow College

Undergraduate Students Trained

- Samya Dyer, BS, 2013-2015

Press releases

- [http://www.spectrum.vcu.edu/profiles/doctoral-student-crosses-disciplines-to-develop-a-new-therapy-for-pancreatic-cancer/#.VD1X2fk7uSo](http://www.spectrum.vcu.edu/profiles/doctoral-student-crosses-disciplines-to-develop-a-new-therapy-for-pancreatic-cancer/#.VD1X2fk7uSo)
Professional Organizations

- American Society for Pharmacology and Experimental Therapeutics (ASPET)
- American Heart Association (AHA)
- American Society for Biochemistry and Molecular Biology (ASBMB)
- American Association for the Advancement of Science (AAAS)

Patents

- New hemiasterlins that inhibit tubulin polymerization and synergize colchicine site tubulin inhibitor for cancer therapy, provisional patent filed through VCU, April, 2008

- Composition and application of stilbenes in cancer therapy. Full patent filed through VCU, April, 2007

- Methods and compositions involving protein kinase c-δ, filed through University of Utah, 2003

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**Technical Skills and Expertise**

- Animal handling: IP, IV, PO drug treatment
- Tumor xenographs
- Mouse echocardiography
- Cell culture: maintenance, transfection, drug treatment
- Protein analysis: western blotting, coomassie stain, silver stain, immunoprecipitation
- Cell proliferation assays (MTS)
- Real-time qPCR
- DNA isolation, cloning, and plasmid construction
- Confocal microscopy: histological and immunofluorescence staining
- Flow cytometry
- Drug accumulation assay
- Mitochondria isolation
- Bacteria protein expression and extraction