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Targeted inhibition of phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) sensitizes pancreatic cancer cells to doxorubicin without exacerbating cardiac toxicity

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Running Title: BEZ235 augments doxorubicin without influencing cardiotoxicity

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Abbreviations: ABC transporters, AKT, protein kinase B; ATP-binding cassette transporters, BEZ, NVP-BEZ235; CDKN2A, cyclin-dependent kinase inhibitor 2A; CMC, carboxymethyl cellulose; DOX, doxorubicin; Gem, gemcitabine; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HRP, horse radish peroxidase; IP, intraperitoneal; IV, intravenous; KRAS, Kirsten rat sarcoma viral oncogene homolog; mTOR, mechanistic target of rapamycin; NMP, N-Methyl-2-pyrrolidone; PARP, poly ADP ribose polymerase; PDAC, pancreatic ductal adenocarcinoma; PDK1, pyruvate dehydrogenase kinase, isozyme 1; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; S6, ribosomal protein S6; SMAD4, SMAD family member 4; topo II, topoisomerase II; TP53, tumor protein 53; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

ABSTRACT

Pancreatic cancer has the lowest five-year survival rate of all major cancers despite decades of effort to design and implement novel, more effective treatment options. In this study, we tested whether the dual PI3K/mTOR inhibitor BEZ235 (BEZ) potentiates the anti-tumor effects of doxorubicin (DOX) against pancreatic cancer. Co-treatment of BEZ235 with DOX resulted in dose-dependent inhibition of the PI3K/mTOR survival pathway which corresponded with an increase in PARP cleavage. Moreover, BEZ co-treatment significantly improved the effects of DOX towards both cell viability and cell death in part through reduced Bcl-2 expression and increased expression of the shorter, more cytotoxic forms of BIM. BEZ also facilitated intracellular accumulation of DOX which led to enhanced DNA damage and ROS generation. Furthermore, BEZ in combination with gemcitabine (Gem) reduced MiaPaca2 cell proliferation but failed to increase ROS generation or BIM expression resulting in reduced necrosis and apoptosis. Treatment with BEZ and DOX in mice bearing tumor xenographs significantly repressed tumor growth as compared to BEZ, DOX or Gem. Additionally, in contrast to the enhanced expression seen in MiaPaca2 cells, BEZ and DOX co-treatment reduced BIM expression in H9C2 cardiomyocytes. Also, Bcl-2/Bax ratio was increased which was associated with reduction in cell death. In vivo echocardiography showed decreased cardiac function with DOX treatment, which was not improved by combination treatment with BEZ. Thus, we propose that combining BEZ with DOX would be a better option for patients than current standard of care by providing more effective tumor response without the associated increase in toxicity.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States and has the lowest five-year survival rate of the major cancers at less than 6% (Muniraj, et al., 2013; Hidalgo, 2010; Siegel, et al., 2015). It is a complex and heterogeneous disease that overwhelmingly harbors KRAS mutations (>95%) that initiate oncogenesis, usually followed by successive mutations of the tumor suppressor genes cyclin-dependent kinase inhibitor 2A (CDKN2A), tumor protein 53 (TP53), and SMAD4 (Bryant, et al., 2014; Hidalgo, 2010). 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. Surgical resection is the best option for a cure; however, only 15% of patients are eligible due to the late stage of diagnosis, and even with resection, overall survival remains poor (~20% 5 year survival) with many patients relapsing (Wormann and Algul, 2013; Hidalgo, 2010; Neesse, et al., 2013). If surgical resection is not an option, chemotherapy using gemcitabine (Gem), given either alone or in combination with other agents including nab-paclitaxel, remains the standard of care for PDAC (Neesse, et al., 2013; Von Hoff, et al., 2013). Alternatively, newer presumably more active regimens like FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan, oxaliplatin) are increasingly being incorporated into adjuvant trials (Tempero, 2015; Conroy, et al., 2011; Gourgou-Bourgade, et al., 2013). Unfortunately these measures provide only a minor benefit for patients as survival is increased by a paltry few months in large part because of associated resistance to the drugs given. Therefore, newer better treatment options that evade this resistance are greatly needed to combat this deadly disease.

The anthracycline antibiotic doxorubicin (DOX) remains a first-line anti-neoplastic drug for the treatment of a wide variety of cancers (Moretti, et al., 2009; Outomuro, et al., 2007). Its mechanism involves poisoning of topoisomerase II (topo II), intercalation into DNA, and generation of reactive oxygen species (ROS), all of which lead to DNA and/or cellular damage (Thorn, et al., 2011; Wang, et al., 2004; Hanusova, et al., 2011). However, DOX has low response rates in PDAC when it is used as a single agent and there is minimal survival advantage when DOX is used in combination with other

chemotherapeutics like 5-fluorouracil (Schwartz and Casper, 1995). Moreover, many cancers, including PDAC, acquire resistance to DOX due to increased survival signaling through the RAS and phosphoinositide 3-kinase (PI3K) pathways. Increased expression of the ATP-binding cassette (ABC) transporters is another means of resistance, leading to drug concentrations below the therapeutic threshold and treatment failure (Gottesman, et al., 2002; Schwartz and Casper, 1995). However, the use of higher concentrations of DOX is limited due to systemic toxicity, including cardiotoxicity (Hanusova, et al., 2011), which is widely believed to arise from iron-mediated ROS production leading to myocyte damage and loss (Ichikawa, et al., 2014). Therefore, combination therapy targeting one or more of these mechanisms of resistance is used to sensitize cancer cells and increase the effectiveness of DOX. This allows for better control of cancer in patients without increasing its cumulative dose (Slamon, et al., 2001; Myers and Cantley, 2010).

NVP-BEZ235 (BEZ) is a reversible dual PI3K/mTOR inhibitor which is active against the four PI3K paralogs as well as the most common PI3K mutants and mTOR (Maira, et al., 2008; Serra, et al., 2008). Inhibition with BEZ has effects on several downstream effectors including AKT, ribosomal protein S6, and the translation initiation factor 4E binding protein 1. It has previously been tested for use in hepatocellular carcinoma, multiple myeloma, and other cancers that commonly have mutations or increased gene expression that lead to overactivation of the PI3K pathway (Baumann, et al., 2009; Cebulla, et al., 2015; Kirstein, et al., 2013; McMillin, et al., 2009). BEZ has also been shown to have beneficial effects in combination with cytotoxic agents, including DOX (Westhoff, et al., 2013; Schult, et al., 2012; Manara, et al., 2010). However, the effect of combination therapy in pancreatic cancer and on cardiotoxicity has not been reported.

In the present study, we tested the hypothesis whether BEZ could enhance the efficacy of DOX in KRAS mutant PDAC and whether this combination would potentiate the cardiotoxic effects of DOX. Our results show that BEZ has a potent and selective sensitizing effect on DOX but not Gem in PDAC through enhancement of drug accumulation and increased expression of the smaller forms of BIM.

Importantly, the increase in cancer chemotherapeutic efficacy did not result in a deterioration of DOX-induced cardiac dysfunction.

Materials and Methods

Cell lines - MiaPaCa2 pancreatic cancer cells were obtained from American Type Culture Collection (MIA PaCa-2 ATCC CRL-1420). 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. All cells were grown in DMEM medium supplemented with 10%FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Compounds and reagents - BEZ235 (#NC9953953) and gemcitabine (#NC0063515) were purchased from Fisher Scientific and Doxorubicin (#D1515-10MG) was purchased from Sigma-Aldrich. 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-AKT308 (#13038), AKT (#9272), Phospho-ATM (#5883), Phospho-chk2 (#2661), Bcl-2 (#2870), Bax (#2772), BIM (#2933), 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-H_2DCFDA was purchased from Life Technologies (C400).

Cell viability - Cell viability was measured using the CellTiter 96 A$_{queous}$ 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% CO_2. 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 A$_{queous}$ 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 48hours. 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 permeablized 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 ^{o}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 4^oC 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). Densitometry was measured using Image J (National Institutes of Health, Bethesda, MD, USA)

Colony forming 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. Plates were then treated with the indicated concentrations for four hours after which cells were washed collected and replated in triplicate at a density of 500 cells/well in 6-well tissue culture plates. Plates were incubated for approximately two weeks to allow colony formation. Colonies were fixed in a 1:7 mixture of acetic acid:methanol for 5 minutes and then stained with 0.5% crystal violet for 20 minutes. After washing with water, plates were allowed to air dry and colonies counted. Colonies of 50 cells or more were counted.

DOX accumulation studies - DOX was measured using the debris pellet from protein lysate preparation. After centrifugation, pellet was re-suspended in 400 µl of acidified alcohol (50ml of 70% ethanol and 375 µl 12N HCl) then incubated at -20^0C 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. Slides were then blocked for 1 hour in blocking buffer (5% normal goat serum, 0.3% Triton X-100 in 1x PBS) and then incubated with primary antibody in blocking buffer overnight at 4 ^oC. After incubation, 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. Additional three washings in 1x PBS

were performed after which hard set mounting medium (Vectasheild #H1500) and cover slips were added. 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.

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 1×10^6 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 was composed of 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 IP once a week for three weeks. Control, DOX, and Gem groups were fed daily with solvent of the same mixture as for BEZ. Tumors were measured twice weekly by calipers using the formula $(L \times W^2)/2$ 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 (Das, et al., 2012), 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. $p < 0.05$ was considered to be

statistically significant.

RESULTS

Effect of Doxorubicin and BEZ235 on PI3/mTOR signaling and cell apoptosis. 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 1A). 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 in combination 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 all concentrations of BEZ treatment. This was observed even at the lowest concentration (50 nM) and remained inhibited in all treatment groups utilizing BEZ.

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 1A). 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 (Maira, et al., 2008). The results showed that the increased phosphorylation of AKT on T308 and S473 after treatment with BEZ (300 nM) did not reduce the effectiveness when combined with DOX. To prevent reactivation of AKT, we used siRNA to knockdown Rictor (mTORC2 activity) or PDK1 (PI3K activity). The results showed that there was little change in PARP cleavage in the siRNA target knockdown cells compared to the scrambled siRNA cells (Figure 1Band C).

Inhibition of PI3K and mTOR with BEZ235 improves doxorubicin induced cell killing. We further examined the effect of BEZ in potentiating 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 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 2A). 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 2B). BEZ caused marginal but significant increase in necrosis compared to control in MiaPaca2 cells while there was no significant increase in necrosis compared to control in Capan-1 or CD18 (Figure 2B). However, BEZ significantly increased the percentage of cell death in all cell lines when combined with DOX (Figure 2B).

BEZ had little effect on apoptosis as assessed by PARP cleavage (Figure 2C-E) and DNA fragmentation in MiaPaca2 cells (Figure 2F and G). Apoptosis was modest in cells treated with DOX alone but greatly enhanced when combined with BEZ (Figure 2C-G) which was associated with significant increase in reactive oxygen species (ROS) generation (Figure 2H).

We further assessed the modulation of mitochondrial associated pro-apoptotic protein expression and caspase activation following treatment with DOX and BEZ. The Bcl-2/Bax ratio was increased compared to control in cells treated with DOX indicating a pro-survival response. This was normalized to control levels when DOX was co-treated with BEZ (Figure 3A and D). 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 3B and E). The enhanced expression of BIM and activation of Bax leads to caspase dependent cell death (Wagner, 2005). The caspase inhibitor, Z-VAD, drastically reduced PARP cleavage after 24 hours of treatment with only a minor band remaining in the combination treated cells (Figure 3C). Caspase inhibition also resulted in a significant decrease in cell death in both the DOX as well as combination groups (Figure 3F) suggesting that increased expression of BIM and activation of pro-apoptotic proteins, including Bax, are required for enhanced killing of pancreatic cancer cells with the

combination treatment. Furthermore, 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 3G and H).

BEZ235 sensistizes doxorubicin killing of colon and lung cancer cells. PDAC is known for its unusual properties, including altered metabolism and powerful drug resistance which makes this cancer very difficult to treat. However, it shares the predisposition for activation mutations in KRAS and inactivation or loss of P53 with other tumor types. Therefore, we tested whether BEZ could sensitize lung and colon cancer cells to DOX in the same way as PDAC cells. Colon (HCT 116) and lung (H1299) cancer cells were treated for 24 hours with BEZ and DOX either alone or in combination. Similar to PDAC cells, the combination of BEZ and DOX also enhanced both PARP cleavage (apoptosis) (Figure 4A and C) and cell death (Figure 4B and D) in these cells.

BEZ235 does not enhance gemcitabine-induced cell killing. Gem is the standard of care for pancreatic cancer patients. We tested whether the combination of Gem with BEZ was also effective in MiaPaca2 cells similar to 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 in the Gem and BEZ combination (Figure 5A). However, BEZ did not enhance the cytotoxic effects of Gem. In fact, reduced trypan blue positive cells (Figure 5B) and PARP cleavage (Figure 5D and E) were observed with the combination treatment compared to Gem alone (Figure 5B, D, and E) while ROS generation was not significantly different in any of the treatment groups (Figure 5C).

BEZ235 increases intracellular DOX accumulation. To understand the mechanism of enhanced cell killing with BEZ and DOX, we considered the role of DOX-induced DNA damage. BEZ treatment caused 1.5 to 2 fold increase in the intracellular concentration of DOX as compared to DOX alone in MiaPaca2, HCT 116, and H1299 cells (Figure 6A-C), which was associated with a significant increase in activation of the DNA damage response protein chk2 in MiaPaca2 cells (Figure 6D-F). Conversely, ATM activation in the combination group was not significantly different from the DOX treated cells. This

finding is consistent with reports demonstrating that BEZ also inhibits ATM and DNA-PK (Gil Del Alcazar, et al., 2013; Mukherjee, et al., 2012).

BEZ235 potentiates DOX-induced inhibition of pancreatic tumor growth. We evaluated the effects of BEZ, DOX, and Gem in a mouse tumor model by implanting MiaPaca2 cells in female Athymic nude mice. Treatment with BEZ, DOX, or Gem alone reduced tumor growth as compared to the control. However, the combination of BEZ and DOX resulted in significant reduction of growth as shown by depressed tumor volume to below the beginning levels until day 35. Overall growth of the tumors remained significantly lower in the BEZ and DOX group as compared to DOX alone (Figure 7A). 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 either individually or in combination with with BEZ (Figure 7B).

DOX cytotoxicity is not enhanced with BEZ235 in cardiomyocytes . An undifferentiated rat myoblast cell line (H9C2) was used to evaluate *in vitro* effects of DOX and BEZ in causing cardiotoxicity. 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 resulted in a substantial increase in dead cells compared to control. However, in contrast to that observed effects in various cancer cells, combination treatment resulted in reduced amount of cell death (Figure 8A). The Bcl-2/Bax ratio was largely unaffected after treatment as shown by non-significant increases observed in all treatment groups (Figure 8B and C). Interestingly, the shorter forms of BIM had increased expression with DOX treatment while all splice variants were dramatically reduced after combination treatment (Figure 8B and D).

BEZ235 does not influence DOX-induced Cardiac Dysfunction. The major dose-limiting factor for the use of DOX clinically is cardiotoxicity leading to heart failure (Volkova and Russell, 2011; Vejpongsa and Yeh, 2014). We therefore evaluated the effects of combination therapy on cardiac function by echocardiography after completion of the treatment schedule (28 days) in the tumor-bearing mice. DOX treatment caused a significant decrease in systolic function as assessed by measuring fractional shortening

while BEZ did not potentiate the effect (Figure 9A and C). Diastolic function of the left ventricle, as

assessed by the E/A ratio, also remained unaffected in the BEZ and DOX combination group (Figure 9).

DISCUSSION

Pancreatic cancer is a devastating disease which has a death rate nearly as high as the incidence rate (Siegel, et al., 2015). Current treatment options for PDAC, which is the most common form of pancreatic cancer as well as the most deadly, have limited benefit to patients. Here, we show that inhibition of the PI3K/mTOR pathway with BEZ sensitizes pancreatic cancer cells to DOX. The enhanced sensitivity is in part due to down regulation of translational and survival signaling downstream of PI3K, including AKT. In addition, we observed increased intracellular accumulation of DOX in combinations with BEZ. This resulted in enhanced ROS formation, activation of DNA damage response proteins, and modulation of pro- and anti-apoptotic protein expression. Interestingly, similar enhancement in killing is observed in lung and colon cancer cell lines suggesting that this combination could be beneficial to multiple solid tumor types in addition to the pancreatic cancer. 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. These results suggest new possibilities of combining PI3K/mTOR inhibitors with traditional cytotoxic agents, including DOX, to achieve better control of PDAC.

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, as demonstrated by reduced cell death in H9C2 cells (Figure 8A), despite inhibition of the PI3K pathway, which is critical in protecting the heart from stress (Lin, et al., 2010).

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 (Neuzillet, et al., 2013). 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 (Edling, et al., 2010). Likewise, AKT2 amplification, which occurs in 10-20% of PDAC, can lead to constitutive activation of AKT and its downstream targets (Pettazzoni, et al., 2015; Ying, et al., 2011; Ruggeri, et al., 1998). In addition, activation of AKT has been associated with poor outcomes of PDAC patients (Yamamoto, et al., 2004), and there is also evidence that RAS mediates its

oncogenic initiation and maintenance through PI3K/PDK1 (Eser, et al., 2013). 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^{G12D} and PI3K (p110α^{H1047R}) driven PDAC cells (Diersch, et al., 2013). However, our current results and other studies demonstrate that BEZ reduces proliferation but does not induce a significant amount of cell death (Awasthi, et al., 2012; Venkannagari, et al., 2012), which may lead to earlier progression of tumors in patients. Therefore, combination therapy utilizing a cytotoxic agent, like DOX, would greatly enhance the clinical utilization of BEZ and possibly other PI3K pathway inhibitors. Along those lines, previous reports have shown that inhibiting the PI3K pathway significantly enhances DOX-induced cell killing in breast and ovarian cancer (Bezler, et al., 2012; Wallin, et al., 2010).

While there have been other reports demonstrating the benefits of combining BEZ with DOX and other cytotoxic agents (Manara, et al., 2010; Schult, et al., 2012; Westhoff, et al., 2013), we show for the first time that there are secondary effects of BEZ that can sensitize PDAC to DOX treatment. Our results demonstrate that BEZ co-treatment enhances DOX accumulation leading to increased ROS formation and DNA damage. We speculate that the increase in accumulation is due to inhibition of one or more ABC-type transporters, an effect that has been demonstrated with other PI3K pathway inhibitors, including LY294002 (PI3K) and rapamycin (mTORC1) (Arceci, et al., 1992; Hegedus, et al., 2012). In addition, BEZ has been shown to inhibit both ATM and DNA-PK mediated DNA damage responses, an affect shown to radiosensitize glioblastoma (Mukherjee, et al., 2012; Gil Del Alcazar, et al., 2013). Therefore, it is likely that along with BEZ's ability to increase DOX accumulation, it also induces a sensitizing effect towards DOX through inhibition of the DNA damage response.

BEZ treatment for extended periods in MiaPaCa2 cells leads to re-activation of the PI3K pathway and phosphorylation of AKT suggesting an enhancement of survival signaling that could result in treatment failure. Using siRNA targeted towards PDK1 and Rictor to specifically prevent respective phosphorylation of AKT downstream of PI3K and mTORC2, we demonstrate PI3K pathway reactivation

does not reduce the effectiveness of using BEZ in combination with DOX. These results suggest that the transient inhibition of AKT survival signaling, which occurs at earlier time points (Fokas, et al., 2012; Manara, et al., 2010), is enough to sensitize the cancer cells to DOX before loss of feedback inhibition through mTORC1 results in reactivation of the pathway. Similarly, others have shown that BEZ induces P53 independent apoptosis despite reactivation of AKT in MYC-driven lymphoma cells (Shortt, et al., 2013).

Our results demonstrate that PI3K/mTOR inhibition does not enhance the cell killing effect of Gem. Other reports have shown that BEZ and Gem have anti-proliferative effects, which is enhanced with combination treatment; although, high concentrations of BEZ (10 μM) failed to improve apoptosis (Awasthi, et al., 2012; Yi, et al., 2013). We also observe inhibition of proliferation with either BEZ or Gem in MiaPaca2 cells which is further reduced with combination treatment. Surprisingly, these results do not translate into increased cell death, and the combination is even less effective as compared to Gem alone. These results suggest 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 tumor studies, combination treatments with BEZ and DOX have significantly smaller tumor size compared to all other groups. This increase in efficacy could be due to a combination of reduced proliferation and survival signaling concomitant with increased DOX accumulation, ROS formation, and BIM expression. However, the increased anti-tumor efficacy of DOX and BEZ is not associated with a significant change in diastolic heart function in any group. Systolic function after DOX treatment is decreased but combination with BEZ does not cause further deterioration of contractile dysfunction. This unexpected finding could be because, unlike the increase in expression seen in MiaPaca2 cells, there is a profound loss of BIM expression and subsequent reduction of apoptosis in H9C2 cells. Likewise, the Bcl-2/Bax ratio, which is normalized in MiaPaca2 cells when BEZ is combined with DOX, remains elevated in H9C2 cells indicating a pro-survival response. Alternatively, the reduction in cell death could be a

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product of increased signaling through separate cell survival pathways. The ERK pathway parallels the PI3K/mTOR pathway which is important in cardiomyocytes survival during stress (Das, et al., 2009). ERK signaling is enhanced after treatment with BEZ (Moon du, et al., 2014), which essentially redirects signaling away from PI3K into RAS leading to cardiomyocyte survival. In addition, inhibition of mTORC1 with rapamycin protects the heart against ischemia-reperfusion injury through activation of JAK2/STAT3 (Das, et al., 2012). Likewise, inhibition of mTORC1 with BEZ could mediate a cardioprotective effect against DOX.

In summary, we provide compelling evidence that combining BEZ with DOX is highly effective in killing pancreatic cancer cells 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 offer better efficacy than the current standard of care used clinically. 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 new clinical option for patients suffering from PDAC.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Durrant, Das, Dent, Kukreja

Conducted experiments: Durrant, Dyer, Tavallai

Contributed new reagents or analytic tools: Dent, Kukreja

Performed data analysis: Durrant

Wrote or contributed to the writing of the manuscript: Durrant, Das, Kukreja

REFERENCES

Arceci RJ, Stieglitz K and Bierer BE (1992) Immunosuppressants FK506 and rapamycin function as reversal agents of the multidrug resistance phenotype. *Blood* **80:**1528-1536.

Awasthi N, Yen PL, Schwarz MA and Schwarz RE (2012) The efficacy of a novel, dual PI3K/mTOR inhibitor NVP-BEZ235 to enhance chemotherapy and antiangiogenic response in pancreatic cancer. *J Cell Biochem* **113:**784-791.

Baumann P, Mandl-Weber S, Oduncu F and Schmidmaier R (2009) The novel orally bioavailable inhibitor of phosphoinositol-3-kinase and mammalian target of rapamycin, NVP-BEZ235, inhibits growth and proliferation in multiple myeloma. *Exp Cell Res* **315:**485-497.

Bezler M, Hengstler JG and Ullrich A (2012) Inhibition of doxorubicin-induced HER3-PI3K-AKT signalling enhances apoptosis of ovarian cancer cells. *Mol Oncol* **6:**516-529.

Bryant KL, Mancias JD, Kimmelman AC and Der CJ (2014) KRAS: feeding pancreatic cancer proliferation. *Trends Biochem Sci* **39:**91-100.

Cebulla J, Huuse EM, Pettersen K, van der Veen A, Kim E, Andersen S, Prestvik WS, Bofin AM, Pathak AP, Bjorkoy G, Bathen TF and Moestue SA (2015) MRI reveals the in vivo cellular and vascular response to BEZ235 in ovarian cancer xenografts with different PI3-kinase pathway activity. *Br J Cancer* **112:**504-513.

Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardiere C, Bennouna J, Bachet JB, Khemissa-Akouz F, Pere-Verge D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M, Groupe Tumeurs Digestives of Unicancer and PRODIGE Intergroup (2011) FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* **364:**1817-1825.

Das A, Salloum FN, Durrant D, Ockaili R and Kukreja RC (2012) Rapamycin protects against myocardial ischemia-reperfusion injury through JAK2-STAT3 signaling pathway. *J Mol Cell Cardiol* **53:**858-869.

Das A, Salloum FN, Xi L, Rao YJ and Kukreja RC (2009) ERK phosphorylation mediates sildenafil-induced myocardial protection against ischemia-reperfusion injury in mice. *Am J Physiol Heart Circ Physiol* **296:**H1236-43.

Diersch S, Wenzel P, Szameitat M, Eser P, Paul MC, Seidler B, Eser S, Messer M, Reichert M, Pagel P, Esposito I, Schmid RM, Saur D and Schneider G (2013) Efemp1 and p27(Kip1) modulate responsiveness of pancreatic cancer cells towards a dual PI3K/mTOR inhibitor in preclinical models. *Oncotarget* **4:**277-288.

Edling CE, Selvaggi F, Buus R, Maffucci T, Di Sebastiano P, Friess H, Innocenti P, Kocher HM and Falasca M (2010) Key role of phosphoinositide 3-kinase class IB in pancreatic cancer. *Clin Cancer Res* **16:**4928-4937.

Eser S, Reiff N, Messer M, Seidler B, Gottschalk K, Dobler M, Hieber M, Arbeiter A, Klein S, Kong B, Michalski CW, Schlitter AM, Esposito I, Kind AJ, Rad L, Schnieke AE, Baccarini M, Alessi DR, Rad R, Schmid RM, Schneider G and Saur D (2013) Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer. *Cancer Cell* **23:**406-420.

Fokas E, Yoshimura M, Prevo R, Higgins G, Hackl W, Maira SM, Bernhard EJ, McKenna WG and Muschel RJ (2012) NVP-BEZ235 and NVP-BGT226, dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitors, enhance tumor and endothelial cell radiosensitivity. *Radiat Oncol* **7:**48-717X-7-48.

Gil Del Alcazar C, Gillam M, Mukherjee B, Tomimatsu N, Gao X, Yan J, Xie XJ, Bachoo R, Li L, Habib AA and Burma S (2013) Inhibition of DNA Double-Strand Break Repair by the Dual PI3K/mTOR Inhibitor NVP-BEZ235 as a Strategy for Radiosensitization of Glioblastoma. *Clin Cancer Res*

Gottesman MM, Fojo T and Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* **2:**48-58.

Gourgou-Bourgade S, Bascoul-Mollevi C, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, Adenis A, Raoul JL, Boige V, Berille J and Conroy T (2013) Impact of FOLFIRINOX compared with gemcitabine on quality of life in patients with metastatic pancreatic cancer: results from the PRODIGE 4/ACCORD 11 randomized trial. *J Clin Oncol* **31:**23-29.

Hanusova V, Bousova I and Skalova L (2011) Possibilities to increase the effectiveness of doxorubicin in cancer cells killing. *Drug Metab Rev* **43:**540-557.

Hegedus C, Truta-Feles K, Antalffy G, Brozik A, Kasza I, Nemet K, Orban TI, Ozvegy-Laczka C, Varadi A and Sarkadi B (2012) PI3-kinase and mTOR inhibitors differently modulate the function of the ABCG2 multidrug transporter. *Biochem Biophys Res Commun* **420:**869-874.

Hidalgo M (2010) Pancreatic cancer. *N Engl J Med* **362:**1605-1617.

Ichikawa Y, Ghanefar M, Bayeva M, Wu R, Khechaduri A, Naga Prasad SV, Mutharasan RK, Naik TJ and Ardehali H (2014) Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. *J Clin Invest* **124:**617-630.

Kirstein MM, Boukouris AE, Pothiraju D, Buitrago-Molina LE, Marhenke S, Schutt J, Orlik J, Kuhnel F, Hegermann J, Manns MP and Vogel A (2013) Activity of the mTOR inhibitor RAD001, the dual mTOR and PI3-kinase inhibitor BEZ235 and the PI3-kinase inhibitor BKM120 in hepatocellular carcinoma. *Liver Int* **33:**780-793.

Lin RC, Weeks KL, Gao XM, Williams RB, Bernardo BC, Kiriazis H, Matthews VB, Woodcock EA, Bouwman RD, Mollica JP, Speirs HJ, Dawes IW, Daly RJ, Shioi T, Izumo S, Febbraio MA, Du XJ and McMullen JR (2010) PI3K(p110 alpha) protects against myocardial infarction-induced heart failure: identification of PI3K-regulated miRNA and mRNA. *Arterioscler Thromb Vasc Biol* **30:**724-732.

Long J, Zhang Y, Yu X, Yang J, LeBrun DG, Chen C, Yao Q and Li M (2011) Overcoming drug resistance in pancreatic cancer. *Expert Opin Ther Targets* **15:**817-828.

Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chene P, De Pover A, Schoemaker K, Fabbro D, Gabriel D, Simonen M, Murphy L, Finan P, Sellers W and Garcia-Echeverria C (2008) Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol Cancer Ther* **7:**1851-1863.

Manara MC, Nicoletti G, Zambelli D, Ventura S, Guerzoni C, Landuzzi L, Lollini PL, Maira SM, Garcia-Echeverria C, Mercuri M, Picci P and Scotlandi K (2010) NVP-BEZ235 as a new therapeutic option for sarcomas. *Clin Cancer Res* **16:**530-540.

McMillin DW, Ooi M, Delmore J, Negri J, Hayden P, Mitsiades N, Jakubikova J, Maira SM, Garcia-Echeverria C, Schlossman R, Munshi NC, Richardson PG, Anderson KC and Mitsiades CS (2009) Antimyeloma activity of the orally bioavailable dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235. *Cancer Res* **69:**5835-5842.

Moon du G, Lee SE, Oh MM, Lee SC, Jeong SJ, Hong SK, Yoon CY, Byun SS, Park HS and Cheon J (2014) NVP-BEZ235, a dual PI3K/mTOR inhibitor synergistically potentiates the antitumor effects of cisplatin in bladder cancer cells. *Int J Oncol* **45:**1027-1035.

25

Moretti E, Oakman C and Di Leo A (2009) Predicting anthracycline benefit: have we made any progress? *Curr Opin Oncol* **21:**507-515.

Mukherjee B, Tomimatsu N, Amancherla K, Camacho CV, Pichamoorthy N and Burma S (2012) The dual PI3K/mTOR inhibitor NVP-BEZ235 is a potent inhibitor of ATM- and DNA-PKCs-mediated DNA damage responses. *Neoplasia* **14:**34-43.

Muniraj T, Jamidar PA and Aslanian HR (2013) Pancreatic cancer: a comprehensive review and update. *Dis Mon* **59:**368-402.

Myers AP and Cantley LC (2010) Targeting a common collaborator in cancer development. *Sci Transl Med* **2:**48ps45.

Neesse A, Krug S, Gress TM, Tuveson DA and Michl P (2013) Emerging concepts in pancreatic cancer medicine: targeting the tumor stroma. *Onco Targets Ther* **7:**33-43.

Neuzillet C, Hammel P, Tijeras-Raballand A, Couvelard A and Raymond E (2013) Targeting the Ras-ERK pathway in pancreatic adenocarcinoma. *Cancer Metastasis Rev* **32:**147-162.

Outomuro D, Grana DR, Azzato F and Milei J (2007) Adriamycin-induced myocardial toxicity: new solutions for an old problem? *Int J Cardiol* **117:**6-15.

Pettazzoni P, Viale A, Shah P, Carugo A, Ying H, Wang H, Genovese G, Seth S, Minelli R, Green T, Huang-Hobbs E, Corti D, Sanchez N, Nezi L, Marchesini M, Kapoor A, Yao W, Francesco ME, Petrocchi A, Deem AK, Scott K, Colla S, Mills GB, Fleming JB, Heffernan TP, Jones P, Toniatti C, DePinho RA and Draetta GF (2015) Genetic events that limit the efficacy of MEK and RTK inhibitor therapies in a mouse model of KRAS-driven pancreatic cancer. *Cancer Res* **75:**1091-1101.

Ruggeri BA, Huang L, Wood M, Cheng JQ and Testa JR (1998) Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas. *Mol Carcinog* **21:**81-86.

Schult C, Dahlhaus M, Glass A, Fischer K, Lange S, Freund M and Junghanss C (2012) The dual kinase inhibitor NVP-BEZ235 in combination with cytotoxic drugs exerts anti-proliferative activity towards acute lymphoblastic leukemia cells. *Anticancer Res* **32:**463-474.

Schwartz GK and Casper ES (1995) A phase II trial of doxorubicin HCl Liposome Injection in patients with advanced pancreatic adenocarcinoma. *Invest New Drugs* **13:**77-82.

Serra V, Markman B, Scaltriti M, Eichhorn PJ, Valero V, Guzman M, Botero ML, Llonch E, Atzori F, Di Cosimo S, Maira M, Garcia-Echeverria C, Parra JL, Arribas J and Baselga J (2008) NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. *Cancer Res* **68:**8022-8030.

Shortt J, Martin BP, Newbold A, Hannan KM, Devlin JR, Baker AJ, Ralli R, Cullinane C, Schmitt CA, Reimann M, Hall MN, Wall M, Hannan RD, Pearson RB, McArthur GA and Johnstone RW (2013) Combined inhibition of PI3K-related DNA damage response kinases and mTORC1 induces apoptosis in MYC-driven B-cell lymphomas. *Blood* **121:**2964-2974.

Siegel RL, Miller KD and Jemal A (2015) Cancer statistics, 2015. *CA Cancer J Clin* **65:**5-29.

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J and Norton L (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* **344:**783-792.

Tempero MA (2015) Multidisciplinary management of pancreatic cancer. *J Natl Compr Canc Netw* **13:**700-702.

Thorn CF, Oshiro C, Marsh S, Hernandez-Boussard T, McLeod H, Klein TE and Altman RB (2011) Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet Genomics* **21:**440-446.

Vejpongsa P and Yeh ET (2014) Prevention of anthracycline-induced cardiotoxicity: challenges and opportunities. *J Am Coll Cardiol* **64:**938-945.

Venkannagari S, Fiskus W, Peth K, Atadja P, Hidalgo M, Maitra A and Bhalla KN (2012) Superior efficacy of co-treatment with dual PI3K/mTOR inhibitor NVP-BEZ235 and pan-histone deacetylase inhibitor against human pancreatic cancer. *Oncotarget* **3:**1416-1427.

Volkova M and Russell R,3rd (2011) Anthracycline cardiotoxicity: prevalence, pathogenesis and treatment. *Curr Cardiol Rev* **7:**214-220.

Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J and Renschler MF (2013) Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* **369:**1691-1703.

Wagner G (2005) Ending the prolonged life of cancer cells. *Nat Chem Biol* **1:**8-9.

Wallin JJ, Guan J, Prior WW, Edgar KA, Kassees R, Sampath D, Belvin M and Friedman LS (2010) Nuclear phospho-Akt increase predicts synergy of PI3K inhibition and doxorubicin in breast and ovarian cancer. *Sci Transl Med* **2:**48ra66.

Wang S, Konorev EA, Kotamraju S, Joseph J, Kalivendi S and Kalyanaraman B (2004) Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms. intermediacy of H(2)O(2)- and p53-dependent pathways. *J Biol Chem* **279:**25535-25543.

Westhoff MA, Faham N, Marx D, Nonnenmacher L, Jennewein C, Enzenmuller S, Gonzalez P, Fulda S and Debatin KM (2013) Sequential Dosing in Chemosensitization: Targeting the PI3K/Akt/mTOR Pathway in Neuroblastoma. *PLoS One* **8:**e83128.

Wormann SM and Algul H (2013) Risk factors and therapeutic targets in pancreatic cancer. *Front Oncol* **3:**282.

Yamamoto S, Tomita Y, Hoshida Y, Morooka T, Nagano H, Dono K, Umeshita K, Sakon M, Ishikawa O, Ohigashi H, Nakamori S, Monden M and Aozasa K (2004) Prognostic significance of activated Akt expression in pancreatic ductal adenocarcinoma. *Clin Cancer Res* **10:**2846-2850.

Yi YW, Kang HJ, Kim HJ, Hwang JS, Wang A and Bae I (2013) Inhibition of constitutively activated phosphoinositide 3-kinase/AKT pathway enhances antitumor activity of chemotherapeutic agents in breast cancer susceptibility gene 1-defective breast cancer cells. *Mol Carcinog* **52:**667-675.

Ying H, Elpek KG, Vinjamoori A, Zimmerman SM, Chu GC, Yan H, Fletcher-Sananikone E, Zhang H, Liu Y, Wang W, Ren X, Zheng H, Kimmelman AC, Paik JH, Lim C, Perry SR, Jiang S, Malinn B, Protopopov A, Colla S, Xiao Y, Hezel AF, Bardeesy N, Turley SJ, Wang YA, Chin L, Thayer SP and DePinho RA (2011) PTEN is a major tumor suppressor in pancreatic ductal adenocarcinoma and regulates an NF-kappaB-cytokine network. *Cancer Discov* **1:**158-169.

FOOTNOTES

Meeting abstracts where work was previously presented:

Durrant D, Das A, Kukreja RC (2014). BEZ235, a selective PI3k/mTOR inhibitor, enhances the therapeutic efficacy of doxorubicin in pancreatic cancer. *Basic & Clinical Pharmacology &Toxicology*

Durrant D, Das A, Kukreja RC (2014). BEZ235, a selective PI3k/mTOR inhibitor, enhances the therapeutic efficacy of doxorubicin in pancreatic cancer. FASEB

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FIGURE LEGENDS

FIGURE 1. BEZ235 decreases PI3K/mTOR signaling and sensitizes cancer cells to doxorubicin. 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). (A) PI3K pathway signal transduction was assessed using western blot analysis to determine the inhibitory effects of BEZ. (B) siRNA knockdown of PDK1 and (C) Rictor was used to determine if feedback activation of AKT affected combination efficacy.

FIGURE 2. PI3K/mTOR inhibition enhances doxorubicin efficacy. (A) Cell viability was measured using the MTS assay after 48 hours treatment of MiaPaca2, Capan-1, and CD18 cells ($n \geq 4$). (B) Cell death (necrosis) was measured using the trypan blue exclusion assay after 48 hours treatment of MiaPaca2, Capan-1, and CD18 cells ($n = 3$). (C) Immunoblots of cl-PARP and actin in MiaPaca2 cell lysates after treatment for 24 hours (D) Densitometry analysis was used to get the ratio of cl-PARP to actin ($n = 3$). (E) Confocal microscopy of MiaPaca2 cells after treatment for 24 hours showing morphological changes between groups. cl-PARP (red), actin (green), and Nuclei (blue). (F) Apoptosis was Quantified using the TUNEL assay after treatment for 24 hours ($n \geq 4$). (G) Representative images of the TUNEL assay. (H) ROS production was assessed by measuring fluorescence (ex. 485, em. 538) of oxidized DCFDA after 6 hours of treatment using a 96-well plate reader ($n = 6$). [*]$p < 0.001$ vs DOX, [#]$p < 0.05$ vs DOX.

FIGURE 3. Pro- and anti-apoptotic protein expression is altered to enhance combination treatment in a caspase dependent manner. (A) Immunoblots of MiaPaca2 lysate after 24 hours of treatment showing expression of Bcl-2, Bax, and actin. (B) BIM EL, L, and S expression was assessed after 24 hours of treatment. (C) Immunoblots of MiaPaca2 lysates after 24 hours of treatment with DOX or BEZ+DOX with or without the caspase inhibitor Z-VAD. (D) Bcl-2/Bax densitometry quantification ($n = 3$). (E) BIM EL, L, and S densitometry quantification ($n = 3$) [α]$p < 0.01$ vs control, [#]$p < 0.05$ vs DOX. (F) Cell death (necrosis) was measured using the trypan blue exclusion assay after 48 hours treatment with DOX, BEZ,

or BEZ+DOX with or without Z-VAD of MiaPaca2 cells (n = 3) *p < 0.001, $^#$p < 0.05. (G) Representative images of the colony forming assay stained with crystal violet. (H) Quantification of colony forming assay (n = 3) *p < 0.001 vs DOX.

FIGURE 4. Enhancement of DOX efficacy with BEZ is not specific to PDAC. (A) Immunoblots of cl-PARP, p-AKT 473, AKT, p-S6, S6, and actin were visualized after treatment for 24 hours in HCT-116 and (C) H1299 cells. Cell death was measured using the trypan blue exclusion assay after 48 hours of treatment in (B) HCT-116 and (D) H1299 cells (n =3) *p < 0.001 vs DOX.

FIGURE 5. BEZ235 does not enhance Gemcitabine toxicity in MiaPaca2 cells. (A) Cell viability/proliferation was measured using the MTS assay after treatment for 48 hours (n = 6). (B) Cell death (necrosis) was measured using the trypan blue exclusion assay after treatment for 48 hours (n = 3). (C) ROS production was assessed by measuring fluorescence (ex. 485, em. 538) of oxidized DCFDA after 6 hours of treatment using a 96-well plate reader (n = 6). (D) Immunoblots of cl-PARP and actin in MiaPaca2 cell lysates after treatment for 24 hrs. (E) Densitometry analysis using Image J was used to get the ratio of cl-PARP to actin (n = 3). *p < 0.001 vs Gem alone, $^#$p < 0.05 vs Gem, $^\alpha$p < 0.01 vs BEZ+DOX.

FIGURE 6. BEZ235 enhanced doxorubicin accumulation leading to increased DNA damage. (A) DOX accumulation was measured after treatment for 48 hours showing increased accumulation in the combination group compared to DOX alone in MiaPaca2, (B) HCT 116, (C) and H1299 (n = 3) *p < 0.001 vs control. (D) Activation of the DNA damage proteins ATM and chk2 were visualizing by immunoblot analysis and showed an increase in phosphorylation with the combination treatment over treatment with DOX alone. (E) Densitometry analysis using Image J was used to get the ratio of p-ATM to actin *p < 0.001 vs control (F) and p-chk2 to actin. *p < 0.001 vs control, $^\alpha$p < 0.01 vs DOX.

FIGURE 7. PI3K/mTOR inhibition with BEZ235 potentiates doxorubicin-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, Gem 100 mg/kg (n = 9). (B) Mice were weighed twice a week to monitor for treatment related toxicities. $^{\alpha}p < 0.01$ and $^{\#}p < 0.05$ vs DOX.

FIGURE 8. Cell death is reduced in H9C2 cells through loss of pro-apoptotic protein expression. (A) Cell death (necrosis) was measured using the trypan blue exclusion assay after treatment for 48 hours in H9C2 cells (n = 3) $^{\#}p < 0.05$ vs DOX. (B) Immunoblots of H9C2 cell lysate after treatment for 24 hours looking at Bcl-2, Bax, BIM EL, L, and S. (C) Densitometry quantification measuring Bcl-2/Bax ratio (n = 3). (D) Densitometry quantification measuring BIM EL/actin ratio and BIM L,S/actin ratio. $^{\#}p < 0.05$ vs control.

FIGURE 9. Combining BEZ with DOX does not enhance cardiotoxicity. (A) Systolic and (B) diastolic heart function was assessed by echocardiography 28 days after the beginning of treatment in tumor bearing mice to study toxicity on the heart. (C) Representative echocardiography images of control, DOX treated, BEZ treated, and BEZ+DOX treated tumor bearing mice. $^{\#}p < 0.05$ vs control.

Figure 1.
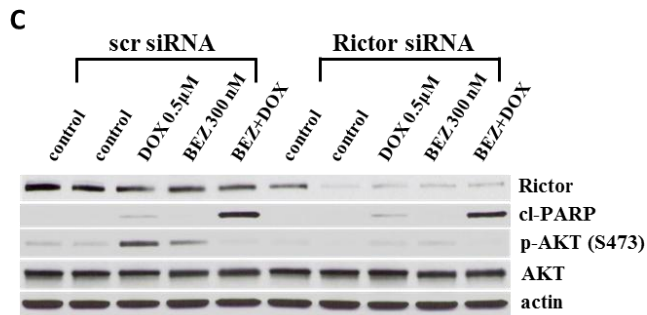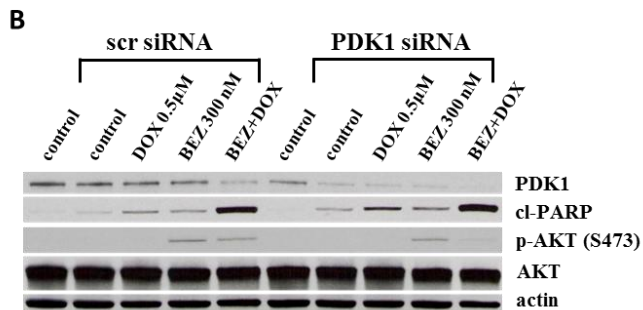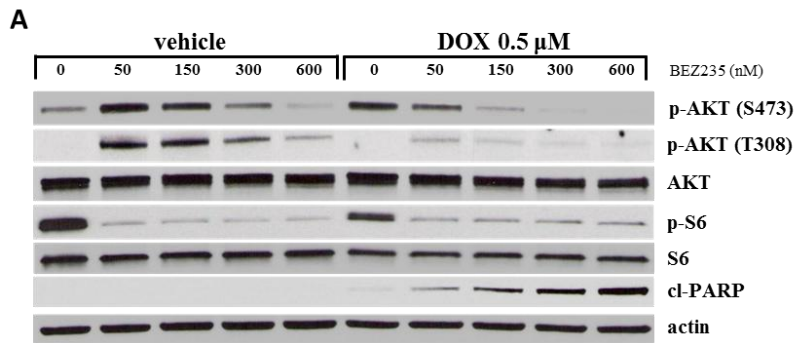
Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

A

B

Figure 8.

Figure 9.

A Fractional Shortening %

B E/A ratio

C

control

DOX

BEZ

BEZ+DOX