HHS Public Access

Author manuscript

Biochim Biophys Acta Gen Subj. Author manuscript; available in PMC 2024 February 06.

Published in final edited form as:

Biochim Biophys Acta Gen Subj. 2020 June ; 1864(6): 129556. doi:10.1016/j.bbagen.2020.129556.

A Dual PI3 Kinase/mTOR Inhibitor BEZ235 reverses doxorubicin resistance in ABCB1 overexpressing ovarian and pancreatic cancer cell lines

David E Durrant,

Anindita Das,

Samya Dyer,

Rakesh C Kukreja

VCU Pauley Heart Center, Division of Cardiology, Virginia Commonwealth University Richmond, VA 23298, United States

Abstract

Background: Multi-drug resistance (MDR) develops because cancer cells evade toxicity of several structurally unrelated drugs. Besides other mechanisms, MDR is linked to the overexpression of ATP Binding Cassette (ABC), transporters, among which ABCB1 is the best characterized one. Since overactivation of PI3K/Akt/mTOR plays a pivotal role in the growth of human cancers, we hypothesized whether dual PI3K and mTOR inhibitor, BEZ235 (BEZ, dactolisib) reverses resistance to doxorubicin (DOX).

Methods: Ovarian (A2780) and pancreatic (MiaPaca2) cancer cells were used to generate DOX-resistant clones by overexpressing ABCB1 or stepwise treatment of DOX. Intracellular accumulation of DOX was measured by flow cytometry after treatment with BEZ.

Results: BEZ treatment caused increase in intracellular levels of DOX which was almost identical to the naïve parental cell lines. BEZ was found to be weak substrate for ABCB1 as demonstrated by minimal increase in ATPase activity. BEZ treatment caused dose-dependent decrease in cell viability in combination with DOX which was associated with increase in cleaved PARP expression in the drug resistant clones.

Address for Correspondence: Rakesh C. Kukreja, PhD, Eric Lipman Professor, Division of Cardiology, Pauley Heart Center, Box 980281, Virginia Commonwealth University Medical Center, 1101 East Marshall Street, Room 7020d, Richmond, Virginia 23298-0281, Tel: 1-804-828-5521; Fax: 1-804-828-8700, rakesh.kukreja@vcuhealth.org.

Authors Contributions

D.E.D was responsible for the study concept and contributed to the acquisition, analysis, interpretation of data and preparation of the manuscript. A.D. contributed to the study concept and design of the experiments; S.D. contributed to the acquisition and analysis of data; R.C.K. contributed to the study concept and critically editing the manuscript. All authors approved the final version of the manuscript. The corresponding author is responsible for ensuring that the descriptions are accurate and agreed by all authors.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conclusions: These results suggest that BEZ is a non-substrate inhibitor of ABCB1 and is able to effectively re-sensitize cells overexpressing ABCB1 to the effects of DOX.

General Significance: Dual PI3 Kinase/mTOR inhibitor, BEZ has the potential to reverse MDR in cancer patients.

Introduction

Cancer results from acquired mutations in normal cells that confer a growth or survival advantage over the surrounding cells (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). Commonly, cancer is treated with cytotoxic agents like the anthracycline antibiotic doxorubicin (DOX), which inhibits type II topoisomerase by preventing re-ligation of the cleaved DNA intermediate resulting in single and double stranded breaks in DNA (Froelich-Ammon and Osheroff, 1995). Even though DOX and other cytotoxic agents have been utilized in treating cancer, their effectiveness has been limited because many cells evade the toxicity of several structurally unrelated drugs, a phenomenon termed as multidrug resistance (MDR) (Gottesman et al., 2002, Fletcher et al., 2010). MDR occurs due to increased drug metabolism and decreased drug accessibility, but the vast majority of MDR in cancer is related to the overexpression of a family of proteins known as the ATP Binding Cassette (ABC) transporters (Gottesman et al., 2002, Szakács et al., 2006). These transporters use energy from ATP hydrolysis for the transport of a wide variety of substrates ranging from cholesterol and lipids, to anti-cancer agents (Fletcher et al., 2010). The most commonly overexpressed transporters associated with cancer drug resistance are ABCB1 (MDR1, p-gp), ABCC1 (MRP1), and ABCG2 (BCRP1) (Wesołowska, 2011). Both ABCB1 and ABCC1 are full transporters having 12 transmembrane domains and two nucleotide binding domains (Wesołowska, 2011, Mo and Zhang, 2012). ABCG2 is a half transporter with six transmembrane domains and one nucleotide binding site. It forms homo or heterodimers to become functionally active (Mo and Zhang, 2012). The increased expression of ABC transporters results in improved drug efflux efficiency leading to sub-lethal intracellular levels while their inhibition causes reversal of the MDR phenotype and re-sensitization of cancer cells to drug treatment.

Since the early 1980’s, inhibitors targeting ABC transporters have been investigated for their ability to reverse MDR (Gottesman et al., 2002). Several drugs designed to specifically inhibit various enzymes within signal transduction pathways result in the inhibition of ABC-type transporters leading to a reversal of MDR phenotypes. It has been reported that overexpression of ABCB1 is found in 40–50% of examined cancer patients with poor clinical outcome (Ding et al., 2011, Lee et al., 2012). Sildenafil, which is a potent inhibitor of phosphodiesterase, also inhibited the efflux activity of both ABCB1 and ABCG2 leading to increased drug accumulation (Shi et al., 2011). We previously demonstrated that sildenafil potentiated the anti-cancer effects of DOX against prostate cancer when treated in combination (Das et al., 2010). Several of the receptor tyrosine kinase inhibitors, including nilotinib, erlotinib, and sunitinib, have been shown to reverse MDR in cells associated with overexpression of ABC transporters (Shi et al., 2007, Tiwari et al., 2009, Tiwari et al., 2013, Shukla et al., 2009, Villar et al., 2012).

Overactivation of PI3K/Akt/mTOR plays a pivotal role in many human cancers. Rapamycin, a specific inhibitor of the mammalian target of rapamycin (mTOR), has been shown to modulate the intracellular accumulation of cytotoxic drugs through the inhibition of ABCB1 (Arceci et al., 1992, Pawarode et al., 2007). NVP-BEZ235 (BEZ) is an imidazo[4,5-c]quinoline derivative that inhibits PI3K and mTOR kinase activity by binding to the ATP-binding cleft of these enzymes. BEZ235 has shown beneficial effects on a variety of tumors *in vivo* and *in vitro* (Maira et al., 2008, McMillin et al., 2009, Serra et al., 2008, Baumann et al., 2009), including lymphoid malignancies (Shull et al., 2015, Wong et al., 2014). Moreover, BEZ235 reversed chemoresistance and overcame radioresistance (Park et al., 2015, Chang et al., 2014). We recently demonstrated that BEZ potentiates the antitumor effects of DOX against pancreatic cancer (Durrant et al., 2015). Cotreatment of BEZ235 with DOX resulted in dose-dependent inhibition of the phosphoinositide 3-kinase/mechanistic target of rapamycin survival pathway, which also corresponded with increase in apoptosis.

In the present study, we postulated that BEZ may be able to re-sensitize cells overexpressing ABCB1 to the chemotherapeutic drug, DOX. We show the secondary effect of BEZ in enhancing the accumulation of DOX within cells when treated in combination with DOX. In addition, BEZ enhanced the accumulation of DOX in drug resistant ovarian and pancreatic cancer cells overexpressing ABCB1.

Materials and Methods

Cell lines.

MiaPaCa2 pancreatic cancer cells were obtained from American Type Culture Collection (ATCC) and grown in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Mia-dx drug resistant cells were generated through step-wise treatment of MiaPaCa2 cells with increasing concentrations of DOX. In brief, starting at 0.2 μM, the cells were incubated with DOX for periods of 2 hours once every two to three weeks (depending on recovery time). DOX 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 (Addgene #10957) containing the full length ABCB1 gene. After transfection, cells overexpressing ABCB1 were selected by culturing in 0.5 μM DOX until colonies were formed and all non-transfected control cells were dead. 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 described above for MiaPaCa2 cells. 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.

Compounds and reagents.

BEZ235 and DOX were purchased from Fisher Scientific. Rhodamine-123 was purchased from Invitrogen (Life Technologies). pHAMDRwt plasmid was purchased from Addgene. Sildenafil was a gift from Pfizer. Antibody for actin-HRP (#sc1616) were purchased from Santa Cruz Biotechnology. Phospho-S6 ribosomal protein (#4858), S6 ribosomal protein (#2217), and cleaved-PARP (#5625) were purchased from Cell Signaling Technology. Secondary antibody was purchased from VWR (rabbit #95017–556).

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.

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. Thereafter, DOX (0.2 μM) was added in the presence or absence of the indicated concentrations of the inhibitor. After 48 hours, cells were 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.

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. Forty-eight hours later, DOX (5 μM) was added in the presence or absence of the indicated concentrations of the inhibitor. After one hour, medium was replaced with fresh medium (minus DOX) in the presence or absence of inhibitor and efflux was allowed to continue 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.

Immunoblot analysis.

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. 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°C overnight, washed 4x for 10 minutes each with TBS-T and incubated for an additional 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 exposing on BioMax light film (Kodak #1788207).

Immunofluorescence staining.

Cells were plated on 4-well glass chamber slides (Lab-tek #177399) 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 immersed in blocking buffer (5% normal goat serum, 0.3% Triton X-100 in 1x PBS) for 1 hour and then incubated 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. Slides were washed an additional three times in 1x PBS, after which hard set mounting medium (Vectasheild #H1500) and cover slips were added. Proteins were visualized using the NIKON Eclipse *Ti* confocal microscope.

ATPase assay.

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

Results

BEZ sensitizes drug resistant cells to DOX

As stated in the Methods, two drug resistant cell lines (Mia-dx and A2780-dx) were generated with sequential treatment of DOX over a six-month period. A third cell line (Mia-B1) was generated by transfecting with a plasmid carrying the MDR1 gene and treated with DOX to obtain drug resistant colonies. 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 from 0.05 μM to 1.0 μM (Fig 1A-C). Expression of ABCB1 was confirmed using western blot analysis and confocal microscopy. Two of the drug resistant cell lines, Mia-B1 and A2780-dx had an overexpression of ABCB1 protein, while the third (Mia-dx) had undetectable levels of ABCB1 (Fig. 1D-F).

BEZ increases intracellular accumulation of DOX

Overexpression of ABC transporters, including ABCB1, leads to increased drug efflux efficiency, which in turn, decreases intracellular drug accumulation. We tested if BEZ treatment would increase accumulation of DOX in the drug resistant cell lines. Cells were treated with DOX, either alone or in combination with BEZ or Sildenafil for 48 hours. Taking advantage of the DOX auto-fluorescence, cells were collected, and then drug accumulation was directly measured by flow cytometry. Sildenafil (10 μM) was used as a positive control because it has been previously shown to inhibit ABCB1 mediated drug

efflux (Das et al., 2010). BEZ increased DOX accumulation to the same extent as sildenafil (50 nM) (Fig. 2A left and middle histograms), and completely reversed the drug resistant phenotype with BEZ (150 nM) in the A2780-dx cell line (Fig. 2A right histogram). Similar results were seen in Mia-B1 cells (Fig. 2B), although with a more subdued response. In these cells, sildenafil and 50 nM BEZ showed only a minor increase in DOX accumulation (Fig. 2B left and middle histograms). While BEZ (150 nM) enhanced DOX accumulation, it was not as complete a reversal as seen in the A2780-dx cell line (Fig 2B right histogram). This difference is most likely the result of the far higher expression of ABCB1 in Mia-B1 cells similar to the A2780-dx cells. Slightly different results were seen 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 this cell line, BEZ (50 nM) had little effect on increasing DOX accumulation (Fig 2C middle histogram), and BEZ (150 nM) had minimal effects (Fig 2C right histogram). Sildenafil was able to increase accumulation in these cells to a greater extent than BEZ (Fig 2A left histogram), suggesting that BEZ may be more specific to ABCB1.

BEZ inhibits ABCB1 mediated DOX efflux

Since BEZ inhibits key proteins involved in the regulation of signal transduction and protein expression, it is possible that the increased accumulation of DOX could be unrelated to the direct inhibition of ABCB1. Therefore, to minimize the potential changes in protein expression, a drug efflux assay with much shorter treatment time of three hours was performed. Cells were treated for one hour with either DOX alone or in combination with the inhibitor. Subsequently, the cells were washed and the media was replaced with DOX free media, either 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. BEZ and sildenafil inhibited DOX efflux in a dose dependent manner in both A2780-dx and Mia-B1 cell lines (Fig 3A-B). On the other hand, neither sildenafil nor BEZ inhibited efflux to a greater extent in cells with undetectable expression levels of ABCB1 i.e., Mia-dx (Fig. 3C). These results show that BEZ enhanced accumulation of DOX in ABCB1 overexpressing drug resistant cell lines through direct inhibition of the transporter.

BEZ interaction with ABCB1

Since BEZ can enhance accumulation of DOX in drug resistant cell lines through direct inhibition of ABCB1, we further wanted to determine if BEZ inhibits this transporter 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 ATP hydrolysis of the transporter. To test the ability of BEZ to stimulate ATPase activity, we used ABCB1 substrates (verapamil, sildenafil and DOX), and an ATPase inhibitor (vanadate), which were combined with isolated pgp membranes in the presence of ATP. ATPase activity was quantitated by the amount of inorganic phosphate released from ATP. As shown in figure 4A, all known ABCB1 substrates significantly stimulated ATP hydrolysis, including DOX. However, DOX was found to be a fairly weak substrate. Low concentration of BEZ (50 nM) did not stimulate ATP hydrolysis and even at the higher dose showed only a minor

stimulation as compared to the no treatment group. This indicates that BEZ is either not a substrate, or at most is a very weak substrate of ABCB1.

Since BEZ is a dual inhibitor of PI3/mTOR signaling, we determined the role of this pathway in the inhibition of ABCB1 transporter. The parental and drug resistant cell lines were treated with 50 nM BEZ for 24 hours. The results showed that mTOR activity (S6 phosphorylation) was decreased after treatment with BEZ in all cell lines, including those overexpressing ABCB1 (Fig 4C). These results indicate that accumulation of BEZ itself was not affected and further suggesting that BEZ is a very poor substrate of ABCB1. BEZ inhibition of PI3k and mTOR occurs at the ATP binding site of these kinases, therefore 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 be able to block the stimulation of ATPase activity when a true substrate is combined with the isolated pgp membranes. Our results show that vanadate is a potent inhibitor of ATP hydrolysis, even in the presence of verapamil (Fig 4B). However, BEZ is unable to alter the ATPase activity even at the higher concentration (150nM) indicating that it does not inhibit the ATP binding site of ABCB1. These results provide strong evidence that BEZ is a poor substrate for ABCB1, and when taken solely as a substrate, would be far less likely to outcompete superior substrates like DOX for access to the binding site. BEZ is also not an inhibitor of ATPase activity indicating a separate inhibition profile, likely a non-substrate inhibitor.

BEZ re-sensitizes drug resistant cells to DOX

Treatment failure in cancer patients is commonly associated with the overexpression of ABC transporters. One strategy to overcome this is to inhibit the transporters which would increase accumulation of the cytotoxic agent within the cell. Our results have shown that BEZ inhibited ABCB1 mediated DOX efflux resulting in increased accumulation (Fig 3). To show if BEZ could re-sensitize ABCB1 overexpressing cells to DOX treatment, we examined cell viability following combination treatment with BEZ and DOX. BEZ alone had a dramatic effect on the cell viability of both MiaPaca2 and Mia-B1 due to its ability to arrest cells at G1 (Fig 5). Treatment of MiaPaca2 cells with DOX (0.5 μM) decreased cell viability to 60% as compared to the control group (Fig 5A). Cell viability was further reduced following combination treatment with DOX and BEZ, decreasing from 34% to 22% with increasing concentrations of BEZ, from 50 nM to 600 nM respectively. DOX with BEZ (150 nM) exerted maximum effect in reducing the cell viability. Mia-B1 cells responded quite differently when treated in the same manner as MiaPaca2 cells. As expected, due to overexpression of ABCB1 and what was seen in a previous cell viability assay, the group treated with DOX (0.5 μM) alone had no effect on viability as compared to the control group (Fig 1B). Also, the combination of DOX with 50 nM BEZ had no effect on viability compared to the cells treated with 50 nM BEZ alone (Fig. 5B). Conversely, with increasing concentrations of BEZ (i.e. 150, 300, 600 nM) in combination with DOX, there is a significant decrease in the percentage of viable cells as compared to all other groups (Fig. 5B). Similar results were obtained in the ovarian cancer cell lines (Fig. 5C,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 are sensitive to DOX alone, with only 26% viable cells remaining after 48 hours of treatment. Combination treatment further

reduced viability down to 15%, with little or no further effect on viability with increasing concentrations of BEZ (Fig. 5C). In A2780-dx cells, 55% cells remained viable after treatment with DOX alone. Combination with BEZ significantly reduced the percentage of viable cells in a dose dependent manner with approximately 15% remaining in the 600 nM BEZ group (Fig. 5D).

To further confirm cell viability results, we determined the expression of apoptosis proteins in the cell lysates following treatment with DOX, BEZ and DOX with BEZ. Cells treated with BEZ alone showed no effect on expression of cleaved form of PARP (an indicator of apoptosis) in any of the cell lines (Fig. 6). Treatment of MiaPaca2 and A2780 cells with DOX alone induced PARP cleavage, and BEZ treatment enhanced the expression of cleaved form of PARP in a manner that mirrored the decrease in viability (Fig. 6A and B top rows). In both Mia-B1 and A2780-dx cells, DOX treatment alone did not induce cleavage of PARP, while co-treatment with BEZ re-sensitized these cells in a dose-dependent manner thus mirroring the decrease in viability (Fig. 6 A and B second rows).

Discussion

A major cause of treatment failure in cancer patients is the overexpression of ABC transporters, which leads to MDR phenotype (Liao et al., 2019, Gottesman et al., 2002, Fletcher et al., 2010). ABCB1 is the most commonly overexpressed transporter in cancer cells, which has been shown to confer resistance against several clinically used drugs, including etoposide, paclitaxel, vincristine, and DOX (Ambudkar et al., 1999). Since the early 1980's, a number of studies have investigated various ABCB1 inhibitors, but no modulators with clinical efficacy have been approved (Gottesman et al., 2002, Szakács et al., 2006, Fletcher et al., 2010). In the present investigation, we have shown that BEZ significantly increased accumulation of DOX in ABCB1 overexpressing drug resistant cells, but had little effect in drug resistant cells with undetectable levels of ABCB1. Moreover, the nanomolar concentrations of BEZ used in this study did not cause significant inhibition of the ATPase activity of ABCB1 transporter. Additionally, our data shows that BEZ re-sensitized ABCB1 overexpressing cells to DOX treatment in a dose-dependent fashion. Taken together, these results demonstrate that BEZ has the potential to reverse the MDR phenotype in cancer cells overexpressing ABCB1.

Cancer cells with an overexpression of one or more of the ABC transporters show reduced intracellular accumulation of cytotoxic agents to levels below the effective dose due to their increased efflux capacity (Fletcher et al., 2010, Gottesman et al., 2002, Szakács et al., 2006). Inhibitors of these transporters are thought to prevent this cellular efflux, resulting in intracellular concentrations of the cytotoxic agent above the effective threshold. Our results show that treatment of ABCB1 overexpressing A2780-dx and Mia-B1 cells with BEZ in combination with DOX caused increase in the intracellular concentration of DOX to levels close to their respective parental cell lines (Fig 1). Since the PI3k/mTOR pathway plays an important role in signaling and protein translation, it is quite possible that BEZ increases DOX accumulation by other means besides direct inhibition of the transporter. Along those lines, rapamycin, an inhibitor of mTOR complex 1, was shown to increase the accumulation of vincristine and DOX in drug resistant lymphoma cell lines, at least in part

through the down regulation of ABCB1 protein expression (Pop et al., 2009). To address the possibility of change in the expression of ABCB1, our results suggest that BEZ can inhibit the efflux capacity of ABCB1 overexpressing cells following brief exposure time, essentially to a similar extent as shown for the 48 hour treatment (Fig. 3). Since the total duration of the experiment was three hours (including one hour co-treatment of BEZ and DOX), it is unlikely that there would be any meaningful reduction in the levels of ABCB1 expression in such a short time frame. Similar results were reported for the PI3k inhibitor, LY294 002, where treatment for 0.5 hours and 24 hours did not change the ABCB1 expression (Barancík et al., 2006). These studies are more in line with the results in the present study, which indicate that BEZ inhibits ABCB1 function without decreasing its expression.

Several inhibitors, including those targeting kinases (sunitinib) and phosphodiesterase 5 (sildenafil), reverse the MDR phenotype of cells overexpressing ABC transporters through interaction at the substrate binding site (Shukla et al., 2009, Shi et al., 2011). 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, had very little effect on ATPase stimulation in isolated pgp membranes whereas verapamil and sildenafil (the positive controls), significantly enhanced activity (Fig. 4 A,B). In addition, BEZ was 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 have been expected if BEZ were a favorable substrate because there would be reduced accumulation within the cell leading to a muted response (Fig 4C). It is known that BEZ inhibits PI3k and mTOR through binding at the ATP binding cleft suggesting the possibility that it inhibits ABCB1 in a similar fashion [20]. However, our data suggests that BEZ does not inhibit ABCB1 function through inhibition of ATP hydrolysis since it is unable to prevent verapamil mediated stimulation of ATPase activity (Fig 4B). These results indicate that BEZ mediated inhibition of ABCB1 efflux in the nanomolar range is not through competitive inhibition at the ATP binding site (and thus is not likely to be a competitive substrate inhibitor). Our results are similar to those seen in LY294 002 which does not interact directly with the substrate binding site (Barancík et al., 2006). A high-throughput screening assay measuring ABCB1 mediated calcein AM efflux demonatrated that BEZ as a substrate inhibitor of ABCB1 (Ansbro et al., 2013). In contrast to the present study, 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 previously that plasma levels of BEZ in mice peaked at 1.68 μmol/L 30 minutes after an oral gavage feeding of 50 mg/kg and rapidly decreased from there (Maira et al., 2008). We believe that concentrations below 1 μM would be more translatable in a clinical setting making our results of ABCB1 inhibition with nanomolar concentrations of BEZ even more attractive.

Over expression of ABC transporters in cancer cells can lead to decreased 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 reduced the viability of ABCB1 overexpressing cells when co-treated with DOX in a dose dependent manner (Fig. 5 B,D). Our data also shows that reduction in viability is associated with corresponding increase in apoptosis, as shown by increased cleaved form of PARP (Fig. 6 A,B). Other studies have shown that BEZ can enhance the

cytotoxicity of a drug, even when treatment with BEZ alone had only minimal effects on cell death (Manara et al., 2010, Lin et al., 2012, Schult et al., 2012, Yi et al., 2013, Yang et al., 2013). It has been suggested that the increase in cytotoxicity is due to the inhibition of the PI3k and mTOR survival signaling pathways. While this may be the case, especially in cells with amplified signaling from mutations within these pathways, the enhancement is also the result of increased accumulation of the drug due to inhibited ABCB1 mediated efflux. Further studies are required to reexamine the extent to which the increased accumulation of these cytotoxic agents in combination with BEZ play a role in in enhancing the cytotoxicity. Phosphorylation of ABCB1 also plays a role in the efficiency of substrate transport (Germann et al., 1996). We cannot rule out a change in phosphorylation state of ABCB1 following treatment with BEZ which could cause decrease in efflux efficiency without decrease in the total protein levels through inhibition of signaling upstream of ABCB1.

Another interesting observation in the current study was that DOX appeared to be the weak substrate for ABCB1 (Fig. 4A) although it is believed that DOX resistance in tumors is, in part, mediated by overexpression of ABC transporters. 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% (Lee et al., 2012). Also, DOX treatment can result in drug resistant clones that overexpress ABCB1 indicating that these transporters may be the best option for cells to export this cytotoxic agent. In spite of this, our results indicate that DOX is not an ideal substrate for ABCB1. 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 (Fletcher et al., 2010). 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 (Fletcher et al., 2010). Another way to circumvent toxicity associated with co-treatment of ABC transporter inhibitors with anti-cancer drugs is to find non-substrate alternatives for cancer treatment (Polgar and Bates, 2005). We speculate that 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 inhibitors, 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.

Clinical trials testing the effectiveness of using BEZ for the treatment of cancer revealed high toxicity profiles (Fazio et al., 2016, Carlo et al., 2016). In both studies, the vast majority of patients had to prematurely discontinue treatment due to adverse events that included diarrhea, nausea, hyperglycemia, and others. While these reports are disappointing, there remains the possibility of using BEZ in combination therapies that use lower doses than what would be required for use as monotherapies. This could especially be important when combined with compounds that are substrates of ABCB1 such as DOX, as lower dosing would be required to achieve intracellular concentrations required for therapeutic efficacy.

In summary, our results demonstrate that BEZ at clinically relevant concentrations not only causes accumulation of DOX in ABCB1 overexpressing drug resistant cells, but

also re-sensitized cells to DOX treatment. We previously demonstrated that the therapeutic efficacy of DOX could be enhanced in pancreatic cancer when combined with BEZ, while the standard of care drug, gemcitabine, showed reduced potency. The present study reveals the need to re-evaluate the use of DOX and other chemotherapeutic agents when used in appropriate combinations to enhance their efficacy. In addition, while DOX was used to validate the ABCB1 inhibitory effects of BEZ, similar combinations with other ABCB1 substrates like paclitaxel and vincristine could potentially result in a similar re-sensitization of drug resistant cells through the same mechanism. This suggests that BEZ has the potential to reverse the MDR phenotype in multiple cancer types overexpressing ABCB1. These results suggest that BEZ has the potential to reverse the MDR phenotype in cancer cells overexpressing ABCB1.

Acknowledgements

This work was supported by the National Institute of Health [RO1HL134366 (to A.D. and R.C.K., R37-HL51045 and R01-CA 221813 to RCK)] and the American Heart Association [Grant PT109079 to D.E.D].

References

AMBUDKAR SV, DEY S, HRYCYNA CA, RAMACHANDRA M, PASTAN I & GOTTESMAN MM. 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol, 39, 361–98. [PubMed: 10331089]

ANSBRO MR, SHUKLA S, AMBUDKAR SV, YUSPA SH & LI L. 2013. Screening compounds with a novel high-throughput ABCB1-mediated efflux assay identifies drugs with known therapeutic targets at risk for multidrug resistance interference. PLoS One, 8, e60334.

ARCECI RJ, STIEGLITZ K & BIERER BE. 1992. Immunosuppressants FK506 and rapamycin function as reversal agents of the multidrug resistance phenotype. Blood, 80, 1528–36. [PubMed: 1381629]

BARANCÍK M, BOHÁCOVÁ V, SEDLÁK J, SULOVÁ Z & BREIER A. 2006. LY294,002, a specific inhibitor of PI3K/Akt kinase pathway, antagonizes P-glycoprotein-mediated multidrug resistance. Eur J Pharm Sci, 29, 426–34. [PubMed: 17010577]

BAUMANN P, MANDL-WEBER S, ODUNCU F & 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, 485–97. [PubMed: 19071109]

CARLO MI, MOLINA AM, LAKHMAN Y, PATIL S, WOO K, DELUCA J, LEE CH, HSIEH JJ, FELDMAN DR, MOTZER RJ & VOSS MH. 2016. A Phase Ib Study of BEZ235, a Dual Inhibitor of Phosphatidylinositol 3-Kinase (PI3K) and Mammalian Target of Rapamycin (mTOR), in Patients With Advanced Renal Cell Carcinoma. Oncologist, 21, 787–8. [PubMed: 27286790]

CHANG L, GRAHAM PH, HAO J, NI J, BUCCI J, COZZI PJ, KEARSLEY JH & LI Y. 2014. PI3K/Akt/mTOR pathway inhibitors enhance radiosensitivity in radioresistant prostate cancer cells through inducing apoptosis, reducing autophagy, suppressing NHEJ and HR repair pathways. Cell Death Dis, 5, e1437. [PubMed: 25275598]

DAS A, DURRANT D, MITCHELL C, MAYTON E, HOKE NN, SALLOUM FN, PARK MA, QURESHI I, LEE R, DENT P & KUKREJA RC. 2010. Sildenafil increases chemotherapeutic efficacy of doxorubicin in prostate cancer and ameliorates cardiac dysfunction. Proc Natl Acad Sci U S A, 107, 18202–7. [PubMed: 20884855]

DING PR, TIWARI AK, OHNUMA S, LEE JW, AN X, DAI CL, LU QS, SINGH S, YANG DH, TALELE TT, AMBUDKAR SV & CHEN ZS. 2011. The phosphodiesterase-5 inhibitor vardenafil is a potent inhibitor of ABCB1/P-glycoprotein transporter. PLoS One, 6, e19329.

DURRANT DE, DAS A, DYER S, TAVALLAI S, DENT P & KUKREJA RC. 2015. Targeted Inhibition of Phosphoinositide 3-Kinase/Mammalian Target of Rapamycin Sensitizes Pancreatic

Cancer Cells to Doxorubicin without Exacerbating Cardiac Toxicity. Mol Pharmacol, 88, 512–23. [PubMed: 26101222]

FAZIO N, BUZZONI R, BAUDIN E, ANTONUZZO L, HUBNER RA, LAHNER H, DE HERDER WW, RADERER M, TEULÉ A, CAPDEVILA J, LIBUTTI SK, KULKE MH, SHAH M, DEY D, TURRI S, AIMONE P, MASSACESI C & VERSLYPE C. 2016. A Phase II Study of BEZ235 in Patients with Everolimus-resistant, Advanced Pancreatic Neuroendocrine Tumours. Anticancer Res, 36, 713–9. [PubMed: 26851029]

FLETCHER JI, HABER M, HENDERSON MJ & NORRIS MD. 2010. ABC transporters in cancer: more than just drug efflux pumps. Nat Rev Cancer, 10, 147–56. [PubMed: 20075923]

FROELICH-AMMON SJ & OSHEROFF N. 1995. Topoisomerase poisons: harnessing the dark side of enzyme mechanism. J Biol Chem, 270, 21429–32. [PubMed: 7665550]

GERMANN UA, CHAMBERS TC, AMBUDKAR SV, LICHT T, CARDARELLI CO, PASTAN I & GOTTESMAN MM. 1996. Characterization of phosphorylation-defective mutants of human P-glycoprotein expressed in mammalian cells. J Biol Chem, 271, 1708–16. [PubMed: 8576173]

GOTTESMAN MM, FOJO T & BATES SE. 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer, 2, 48–58. [PubMed: 11902585]

HANAHAN D & WEINBERG RA. 2000. The hallmarks of cancer. Cell, 100, 57–70. [PubMed: 10647931]

HANAHAN D & WEINBERG RA. 2011. Hallmarks of cancer: the next generation. Cell, 144, 646–74. [PubMed: 21376230]

LEE SH, KIM H, HWANG JH, LEE HS, CHO JY, YOON YS & HAN HS. 2012. Breast cancer resistance protein expression is associated with early recurrence and decreased survival in resectable pancreatic cancer patients. Pathol Int, 62, 167–75. [PubMed: 22360504]

LIAO D, ZHANG W, GUPTA P, LEI ZN, WANG JQ, CAI CY, VERA AA, ZHANG L, CHEN ZS & YANG DH. 2019. Tetrandrine Interaction with ABCB1 Reverses Multidrug Resistance in Cancer Cells Through Competition with Anti-Cancer Drugs Followed by Downregulation of ABCB1 Expression. Molecules, 24.

LIN SF, HUANG YY, LIN JD, CHOU TC, HSUEH C & WONG RJ. 2012. Utility of a PI3K/mTOR inhibitor (NVP-BEZ235) for thyroid cancer therapy. PLoS One, 7, e46726.

MAIRA SM, STAUFFER F, BRUEGGEN J, FURET P, SCHNELL C, FRITSCH C, BRACHMANN S, CHÈNE P, DE POVER A, SCHOEMAKER K, FABBRO D, GABRIEL D, SIMONEN M, MURPHY L, FINAN P, SELLERS W & GARCÍA-ECHEVERRÍA 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, 1851–63. [PubMed: 18606717]

MANARA MC, NICOLETTI G, ZAMBELLI D, VENTURA S, GUERZONI C, LANDUZZI L, LOLLINI PL, MAIRA SM, GARCÍA-ECHEVERRÍA C, MERCURI M, PICCI P & SCOTLANDI K. 2010. NVP-BEZ235 as a new therapeutic option for sarcomas. Clin Cancer Res, 16, 530–40. [PubMed: 20068094]

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 & MITSIADES CS. 2009. Antimyeloma activity of the orally bioavailable dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235. Cancer Res, 69, 5835–42. [PubMed: 19584292]

MO W & ZHANG JT. 2012. Human ABCG2: structure, function, and its role in multidrug resistance. Int J Biochem Mol Biol, 3, 1–27. [PubMed: 22509477]

PARK H, KIM Y, SUL JW, JEONG IG, YI HJ, AHN JB, KANG JS, YUN J, HWANG JJ & KIM CS. 2015. Synergistic anticancer efficacy of MEK inhibition and dual PI3K/mTOR inhibition in castration-resistant prostate cancer. Prostate, 75, 1747–59. [PubMed: 26250606]

PAWARODE A, SHUKLA S, MINDERMAN H, FRICKE SM, PINDER EM, O'LOUGHLIN KL, AMBUDKAR SV & BAER MR. 2007. Differential effects of the immunosuppressive agents cyclosporin A, tacrolimus and sirolimus on drug transport by multidrug resistance proteins. Cancer Chemother Pharmacol, 60, 179–88. [PubMed: 17031644]

POLGAR O & BATES SE. 2005. ABC transporters in the balance: is there a role in multidrug resistance? Biochem Soc Trans, 33, 241–5. [PubMed: 15667317]

POP IV, POP LM, GHETIE MA & VITETTA ES. 2009. Targeting mammalian target of rapamycin to both downregulate and disable the P-glycoprotein pump in multidrug-resistant B-cell lymphoma cell lines. Leuk Lymphoma, 50, 1155–62. [PubMed: 19557637]

SCHULT C, DAHLHAUS M, GLASS A, FISCHER K, LANGE S, FREUND M & 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, 463–74. [PubMed: 22287733]

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 & 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, 8022–30. [PubMed: 18829560]

SHI Z, PENG XX, KIM IW, SHUKLA S, SI QS, ROBEY RW, BATES SE, SHEN T, ASHBY CR, FU LW, AMBUDKAR SV & CHEN ZS. 2007. Erlotinib (Tarceva, OSI-774) antagonizes ATP-binding cassette subfamily B member 1 and ATP-binding cassette subfamily G member 2-mediated drug resistance. Cancer Res, 67, 11012–20. [PubMed: 18006847]

SHI Z, TIWARI AK, SHUKLA S, ROBEY RW, SINGH S, KIM IW, BATES SE, PENG X, ABRAHAM I, AMBUDKAR SV, TALELE TT, FU LW & CHEN ZS. 2011. Sildenafil reverses ABCB1- and ABCG2-mediated chemotherapeutic drug resistance. Cancer Res, 71, 3029–41. [PubMed: 21402712]

SHUKLA S, ROBEY RW, BATES SE & AMBUDKAR SV. 2009. Sunitinib (Sutent, SU11248), a small-molecule receptor tyrosine kinase inhibitor, blocks function of the ATP-binding cassette (ABC) transporters P-glycoprotein (ABCB1) and ABCG2. Drug Metab Dispos, 37, 359–65. [PubMed: 18971320]

SHULL AY, NOONEPALLE SK, AWAN FT, LIU J, PEI L, BOLLAG RJ, SALMAN H, DING Z & SHI H. 2015. RPPA-based protein profiling reveals eIF4G overexpression and 4E-BP1 serine 65 phosphorylation as molecular events that correspond with a pro-survival phenotype in chronic lymphocytic leukemia. Oncotarget, 6, 14632–45. [PubMed: 25999352]

SZAKÁCS G, PATERSON JK, LUDWIG JA, BOOTH-GENTHE C & GOTTESMAN MM. 2006. Targeting multidrug resistance in cancer. Nat Rev Drug Discov, 5, 219–34. [PubMed: 16518375]

TIWARI AK, SODANI K, DAI CL, ABUZNAIT AH, SINGH S, XIAO ZJ, PATEL A, TALELE TT, FU L, KADDOUMI A, GALLO JM & CHEN ZS. 2013. Nilotinib potentiates anticancer drug sensitivity in murine ABCB1-, ABCG2-, and ABCC10-multidrug resistance xenograft models. Cancer Lett, 328, 307–17. [PubMed: 23063650]

TIWARI AK, SODANI K, WANG SR, KUANG YH, ASHBY CR, CHEN X & CHEN ZS. 2009. Nilotinib (AMN107, Tasigna) reverses multidrug resistance by inhibiting the activity of the ABCB1/Pgp and ABCG2/BCRP/MXR transporters. Biochem Pharmacol, 78, 153–61. [PubMed: 19427995]

VILLAR VH, VÖGLER O, MARTÍNEZ-SERRA J, RAMOS R, CALABUIG-FARIÑAS S, GUTIÉRREZ A, BARCELÓ F, MARTÍN-BROTO J & ALEMANY R. 2012. Nilotinib counteracts P-glycoprotein-mediated multidrug resistance and synergizes the antitumoral effect of doxorubicin in soft tissue sarcomas. PLoS One, 7, e37735.

WESOŁOWSKA O. 2011. Interaction of phenothiazines, stilbenes and flavonoids with multidrug resistance-associated transporters, P-glycoprotein and MRP1. Acta Biochim Pol, 58, 433–48. [PubMed: 22187677]

WONG J, WELSCHINGER R, HEWSON J, BRADSTOCK KF & BENDALL LJ. 2014. Efficacy of dual PI-3K and mTOR inhibitors in vitro and in vivo in acute lymphoblastic leukemia. Oncotarget, 5, 10460–72. [PubMed: 25361005]

YANG F, QIAN XJ, QIN W, DENG R, WU XQ, QIN J, FENG GK & ZHU XF. 2013. Dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 has a therapeutic potential and sensitizes cisplatin in nasopharyngeal carcinoma. PLoS One, 8, e59879.

YI YW, KANG HJ, KIM HJ, HWANG JS, WANG A & 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, 667–75. [PubMed: 22488590]

Review Highlights

- Multidrug Resistance (MDR) decreases effectiveness of cancer drugs
- Targeting ATP Binding Cassette (ABC) transporters is known to reverse MDR
- Dual PI3Kinase/mTOR Inhibitor BEZ235 increased doxorubicin accumulation in drug-resistant cancer cells
- BEZ235 improved doxorubicin-induced cancer cell killing
- MDR in cancer cells can be reversed by BEZ235.

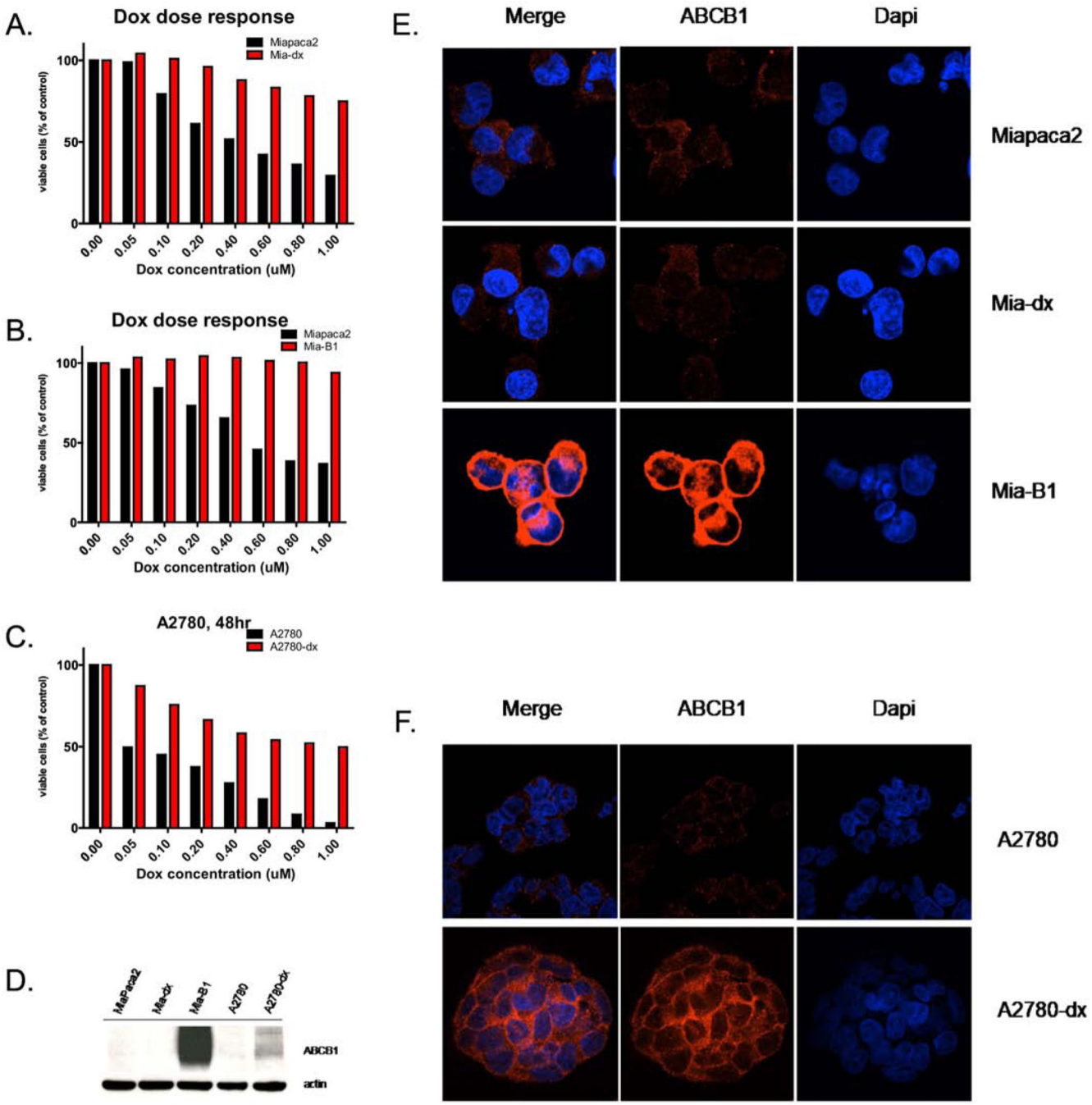


Figure 1. BEZ235 (BEZ) sensitizes drug resistant pancreatic and ovarian cancer cells to doxorubicin (DOX).

Cell viability was measured after 48hr of treatment with increasing concentrations of DOX ranging from 0.05 μM to 1 μM using the AQueous One cell viability assay from Promega. (A) Mia-dx and (B) Mia-B1 were plotted against their parental cell line, MiaPaca2, and (C) A2780-dx was plotted against its parental cell line, A2780. Protein expression of ABCB1 was visualized in each cell line after western blot analysis (D) or confocal microscopy (E-F) using an antibody against ABCB1.

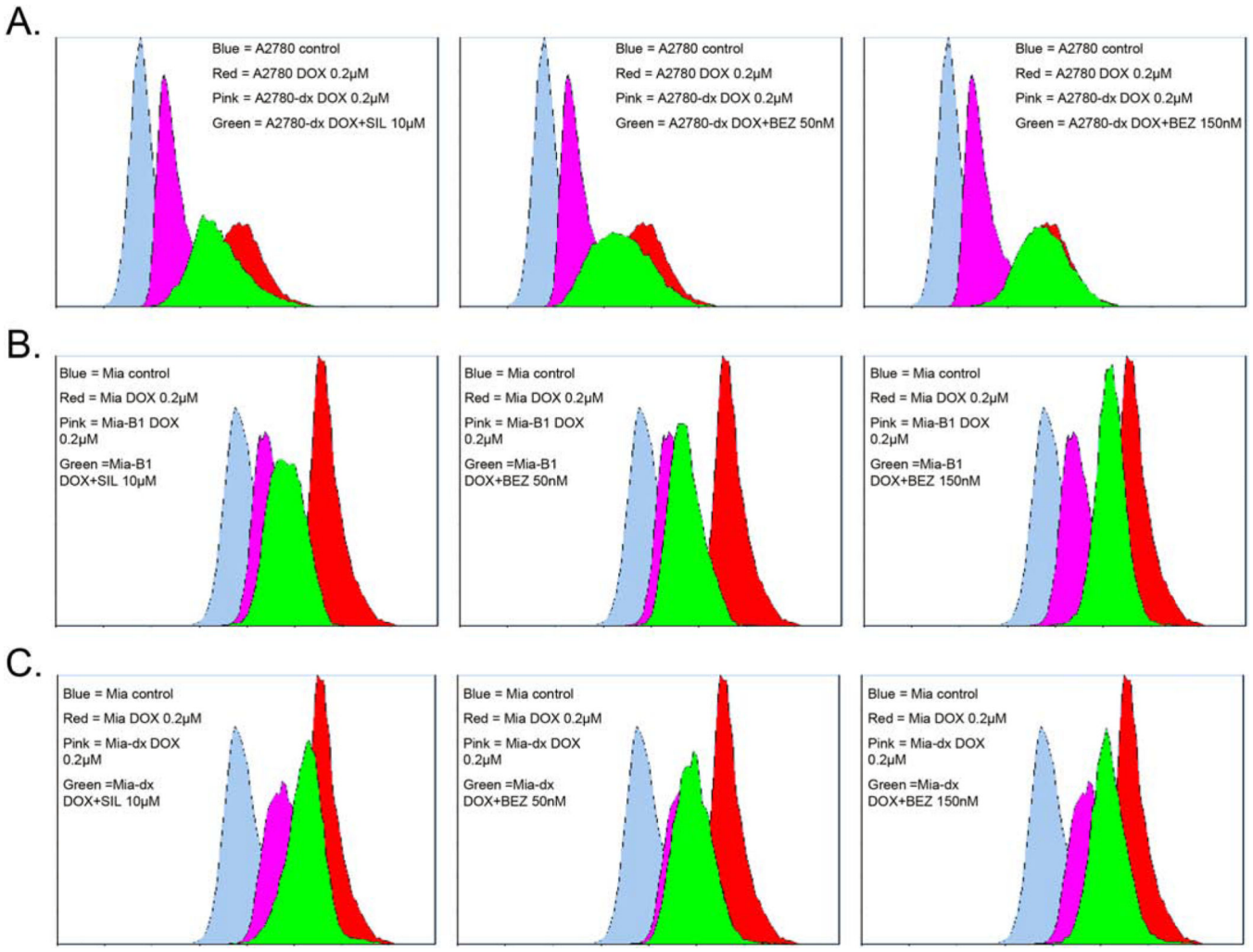
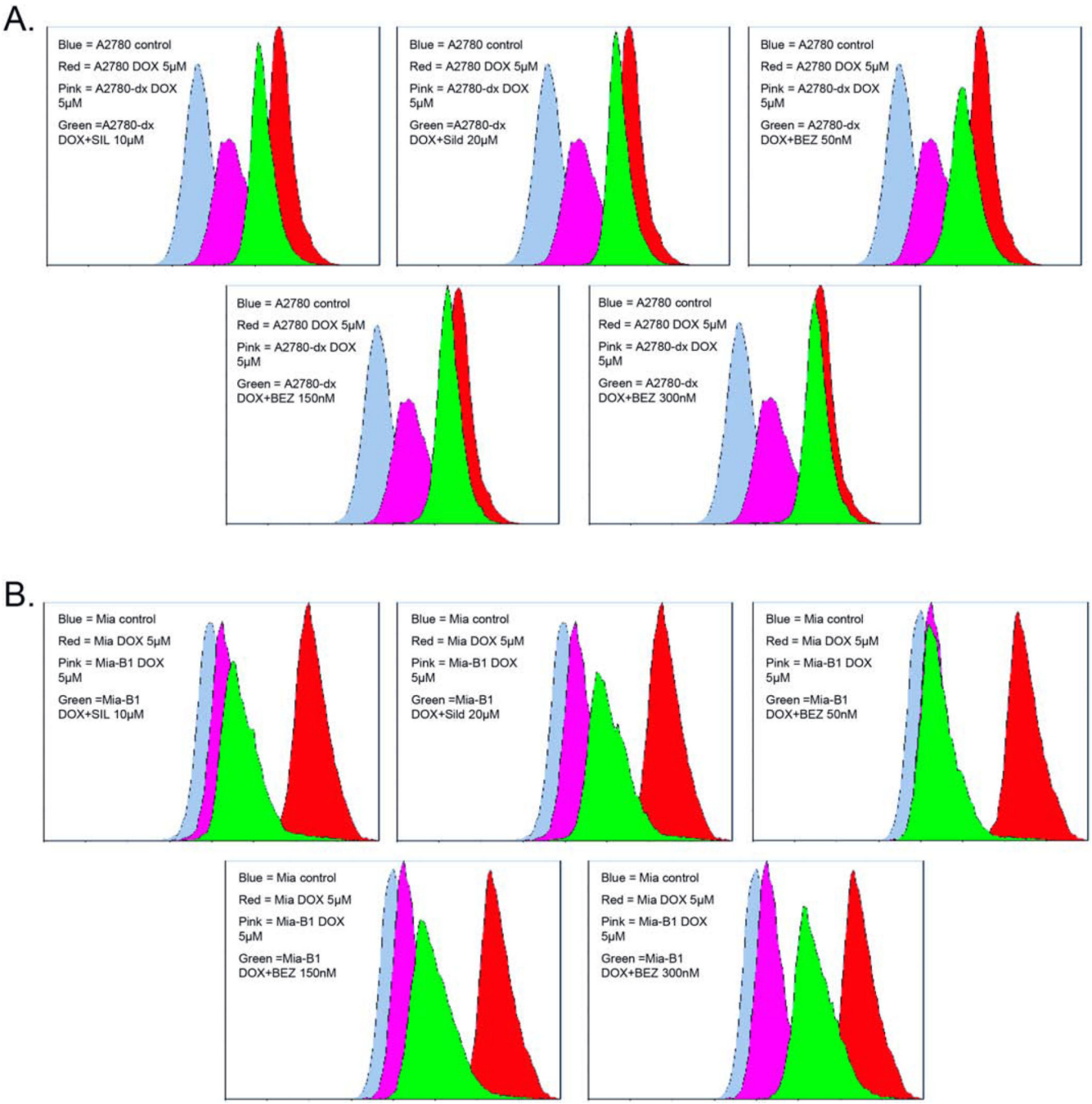


Figure 2. BEZ235 increases intracellular accumulation of doxorubicin.

Cells were treated for 48hr with 0.2 μM DOX in combination with either sildenafil (SIL) or BEZ after which they were collected. DOX accumulation was analyzed by flow cytometry taking advantage of DOX auto fluorescence. 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 DOX treated drug resistant cell line in combination with the indicated inhibitor. (A) A2780-dx, (B) Mia-B1, and (C) Mia-dx.



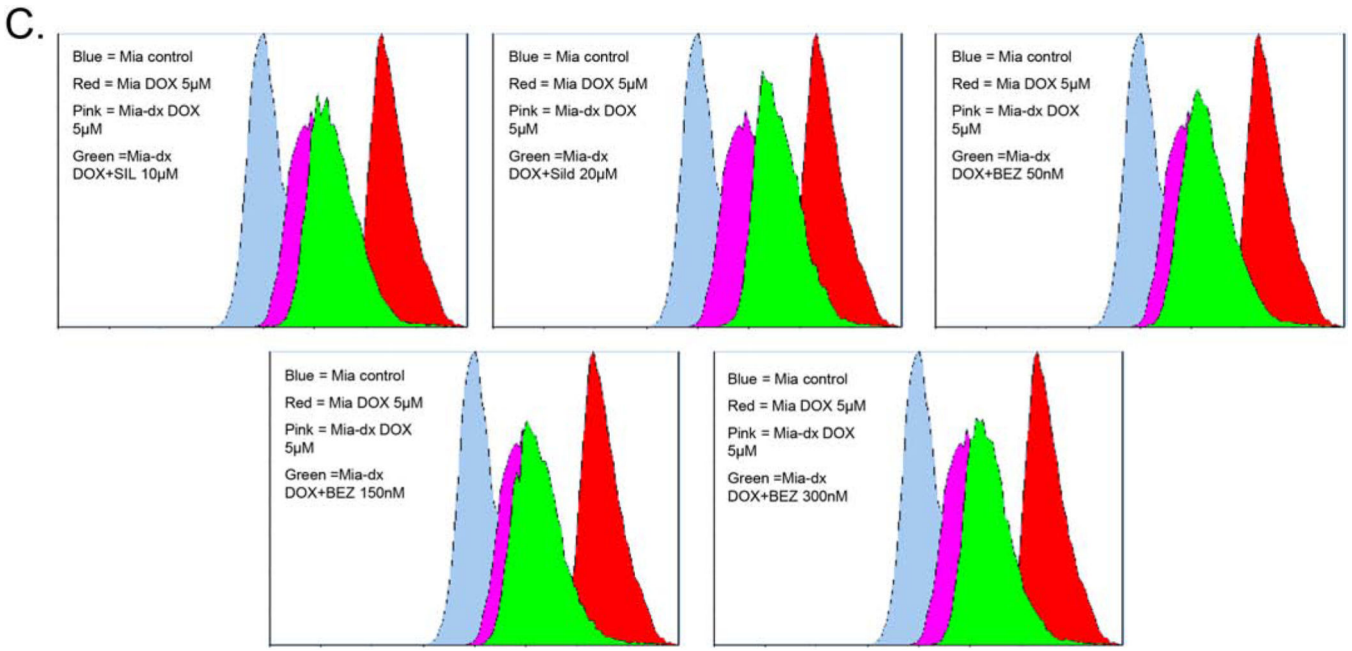


Figure 3. BEZ235 inhibits ABCB1 mediated efflux of doxorubicin

Cells were treated for 1hr with 5 μM DOX in combination with either sildenafil (SIL) or BEZ235 (BEZ) followed by incubation for two hours in DOX free media treated with SIL or BEZ. The cells were then collected and DOX accumulation was analyzed by flow cytometry. Similar to figure 3, in all histograms, the light blue peak represents untreated parental control, red peak represents DOX treated parental cells, pink peak represents DOX treated drug resistant cell line, and green peak represents DOX treated drug resistant cell line in combination with the indicated inhibitor. (A) A2780-dx, (B) Mia-B1, and (C) Mia-dx.

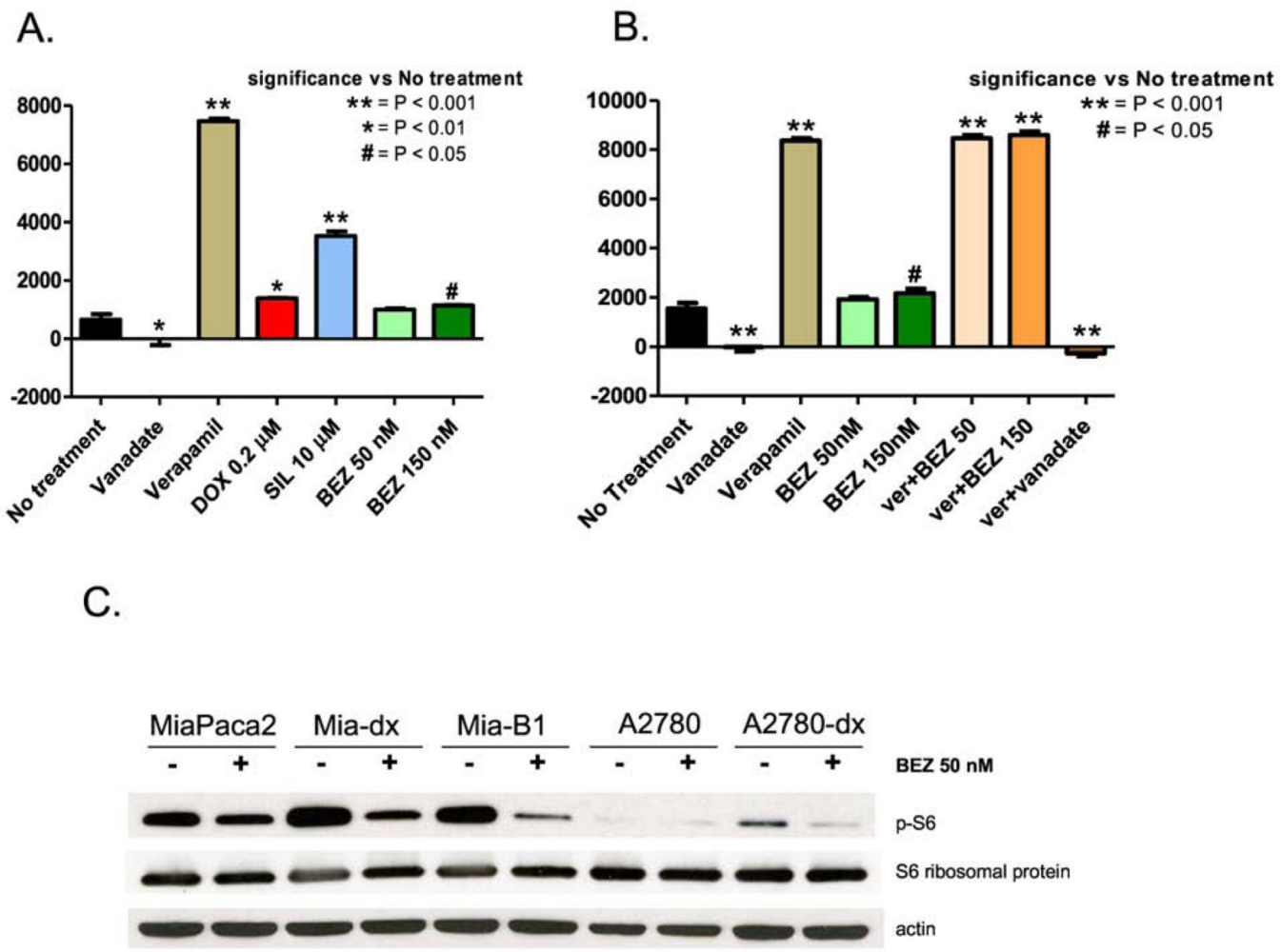


Figure 4. BEZ is a weak substrate for ABCB1 transporter.

(A-B) ATPase activity assay (Promega) using isolated pgp membranes was used to determine if BEZ is a substrate of ABCB1. Verapamil, a known substrate of ABCB1, was used as a positive control for ATPase stimulation. Vanadate inhibits ATPase activity even in the presence of substrate through interaction at the ATP binding site and preventing ATP hydrolysis. (C) The different cell lines were treated in the presence or absence of 50 nM BEZ for 24 hours. Cell lysates were collected and Western blots were performed to determine the phosphorylation of ribosomal protein S6, a downstream marker of mTORC1.

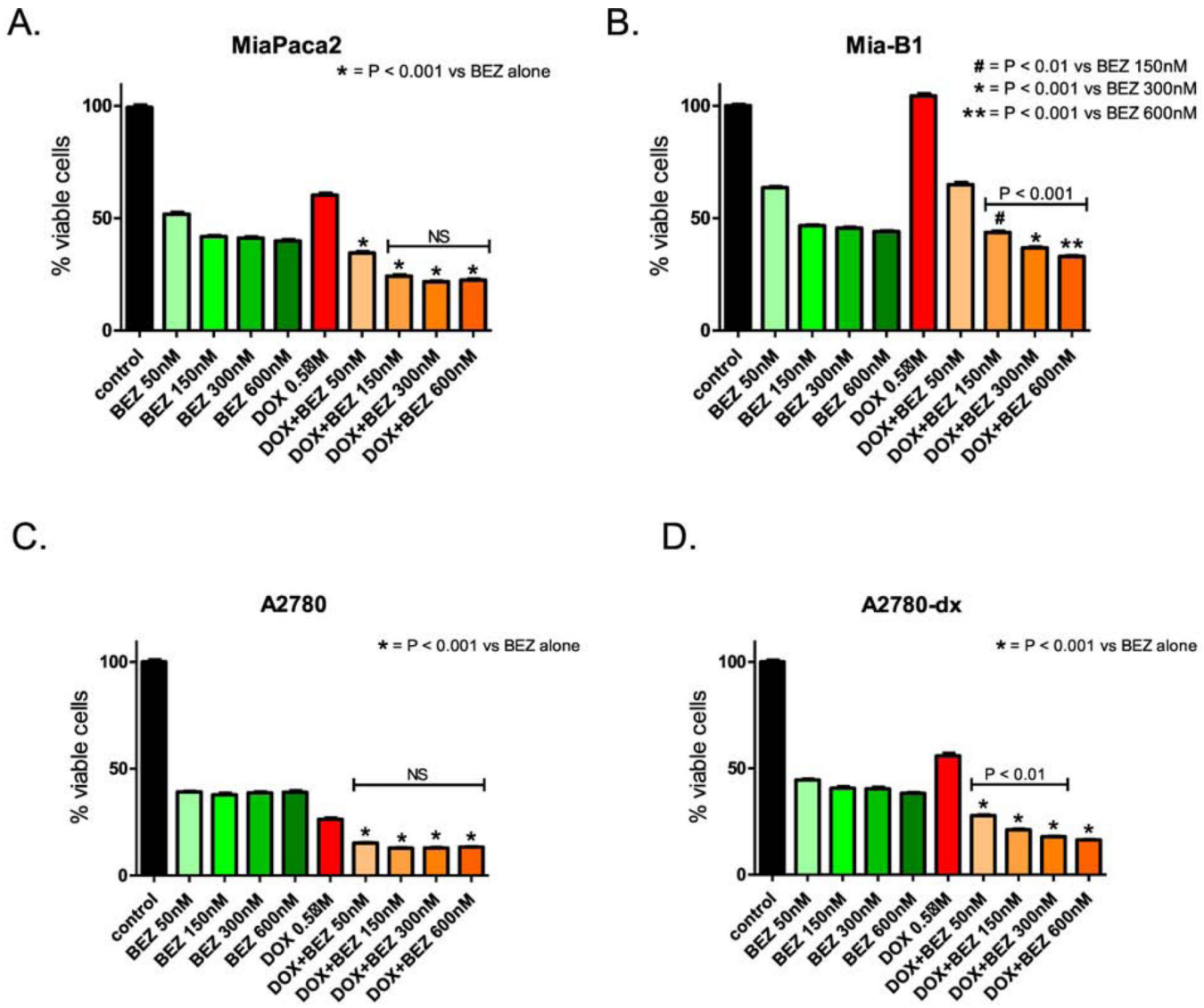


Figure 5. BEZ re-sensitizes drug-resistant cells to DOX.

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. #, *, and ** indicates significance of the combination treatment group to its respective BEZ only treatment group.

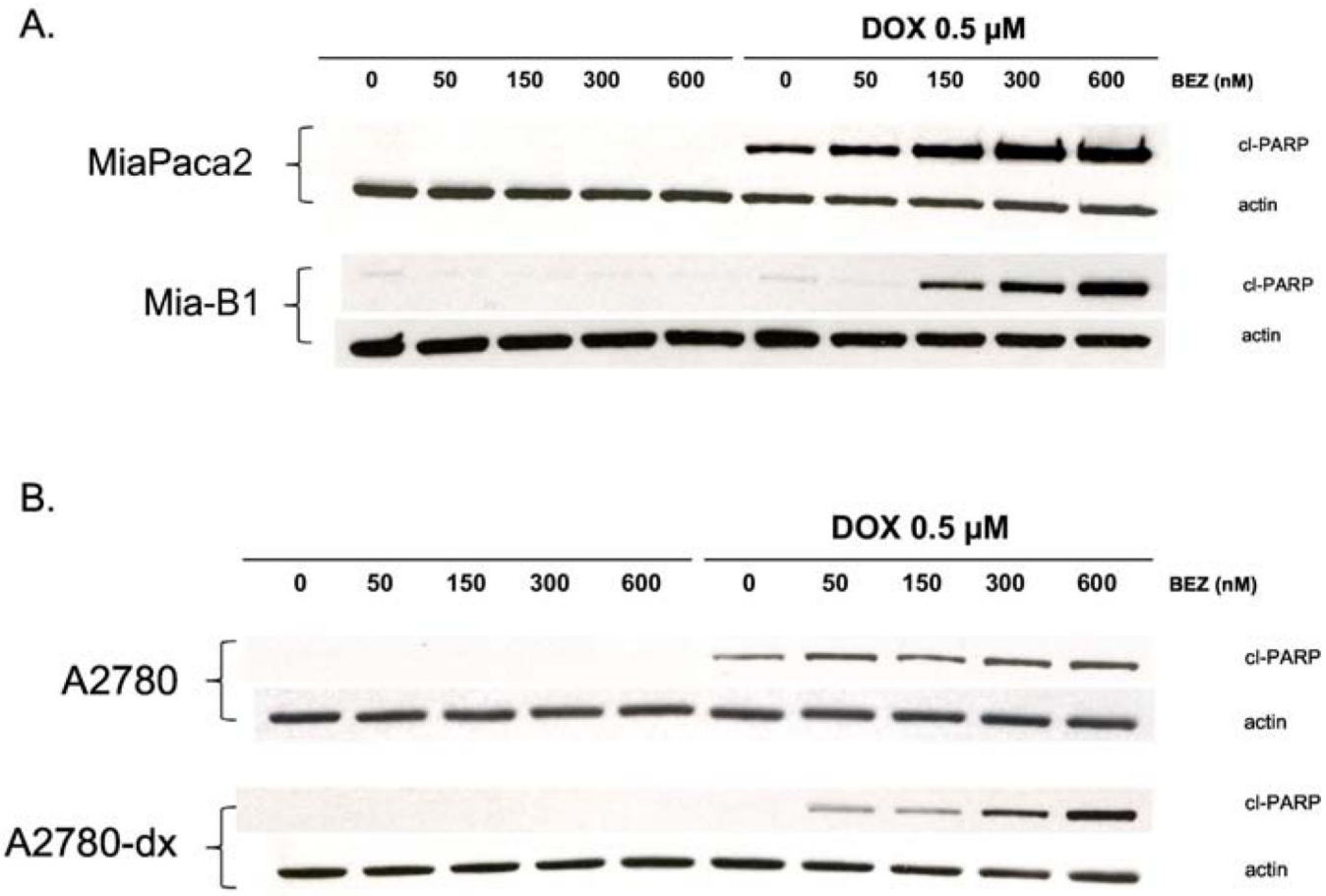


Figure 6. BEZ treatment in combination with DOX increases expression of cleaved PARP.
Cells were treated with the indicated drug combinations in 75cm^2 flasks for 48 hours. Cell lysates were collected and analyzed using western blot. Apoptosis was measured by the amount of cleaved PARP present.