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# The Efficacy of a Novel, Dual PI3K/mTOR Inhibitor NVP-BEZ235 to Enhance Chemotherapy and Antiangiogenic Response in Pancreatic Cancer

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## ABSTRACT

Gemcitabine has limited clinical benefits for pancreatic ductal adenocarcinoma (PDAC). The phosphatidylinositol-3-kinase (PI3K)/AKT and mammalian target of rapamycin (mTOR) signaling pathways are frequently dysregulated in PDAC. We investigated the effects of NVP-BEZ235, a novel dual PI3K/mTOR inhibitor, in combination with gemcitabine and endothelial monocyte activating polypeptide II (EMAP) in experimental PDAC. Cell proliferation and protein expression were analyzed by WST-1 assay and Western blotting. Animal survival experiments were performed in murine xenografts. BEZ235 caused a decrease in phospho-AKT and phospho-mTOR expression in PDAC (AsPC-1), endothelial (HUVECs), and fibroblast (WI-38) cells. BEZ235 inhibited in vitro proliferation of all four PDAC cell lines tested. Additive effects on proliferation inhibition were observed in the BEZ235-gemcitabine combination in PDAC cells and in combination of BEZ235 or EMAP with gemcitabine in HUVECs and WI-38 cells. BEZ235, alone or in combination with gemcitabine and EMAP, induced apoptosis in AsPC-1, HUVECs, and WI-38 cells as observed by increased expression of cleaved poly (ADP-ribose) polymerase-1 (PARP-1) and caspase-3 proteins. Compared to controls (median survival: 16 days), animal survival increased after BEZ235 and EMAP therapy alone (both 21 days) and gemcitabine monotherapy (28 days). Further increases in survival occurred in combination therapy groups BEZ235 + gemcitabine (30 days, $P = 0.007$), BEZ235 + EMAP (27 days, $P = 0.02$), gemcitabine + EMAP (31 days, $P = 0.001$), and BEZ235 + gemcitabine + EMAP (33 days, $P = 0.004$). BEZ235 has experimental PDAC antitumor activity in vitro and in vivo that is further enhanced by combination of gemcitabine and EMAP. These findings demonstrate advantages of combination therapy strategies targeting multiple pathways in pancreatic cancer treatment. J. Cell. Biochem. 113: 784–791, 2012. © 2011 Wiley Periodicals, Inc.

**KEY WORDS:** PANCREATIC CANCER; NVP-BEZ235; GEMCITABINE; COMBINATION THERAPY

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human cancers and remains the fourth leading cause of cancer-related deaths in the United States. The prognosis of PDAC patients is very poor, with a 5-year survival rate <5%, mostly due to the late diagnosis, early metastasis, and high resistance to chemotherapy and radiation [Warshaw and Fernandez-del Castillo, 1992; Duffy et al., 2003; Jemal et al., 2009]. Despite advances in diagnostic techniques, drug development, and surgical procedures, the incidence of pancreatic cancer nearly equals its death rate [Jemal et al., 2009]. Complete resection is the most effective treatment for PDAC; however, due to locally extended and metastasized disease only about 10% of patients are eligible for resection, which is then often followed by local recurrences [Rosewicz and Wiedenmann, 1997; Brennan, 2004; Wilkowski et al., 2006]. Therefore, much attention has been placed on systemic treatment options for pancreatic cancer. PDAC is generally poorly responsive to cytotoxic chemotherapy [MacKenzie, 2004; Schneider et al., 2005]. Gemcitabine (Gem, G), a standard chemotherapeutic agent for PDAC, is a deoxycytidine nucleoside analog that requires intracellular phosphorylation to get converted into its active triphosphate form.

Additional Supporting Information may be found in the online version of this article.

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Gemcitabine exerts its cytotoxic effect mainly by being incorporated into the DNA strand and inhibit DNA synthesis. However, gemcitabine has been shown to be effective only in 20–30% of PDAC patients, leading to a median progression-free survival of approximately 6 months [Reni et al., 2005]. Therefore, new therapeutic strategies that overcome the obstacle of poor sensitivity and drug resistance are highly desirable for PDAC.

The phosphoinositide 3-kinase (PI3K)/AKT signaling pathway plays an important role in many biological processes including cell proliferation, differentiation, and survival [Vivanco and Sawyers, 2002; Cheng et al., 2005]. Deregulation of the PI3K/AKT pathway is a prominent characteristic of pancreatic cancer, and this appears to play an important role in the aggressive nature of this disease including resistance to chemotherapy [Bardeesy and DePinho, 2002; Agbunag and Bar-Sagi, 2004; Asano et al., 2004; Chadha et al., 2006]. PI3K is activated upon growth factor binding to their cognate receptors. Activated PI3K leads to the activation of AKT by phosphorylation at Ser473 and Thr308. AKT activates several downstream targets including mammalian target of rapamycin (mTOR) that plays a central role in cell proliferation through regulation of the cell cycle. Deregulation of mTOR signaling occurs in several human tumors including PDAC [Ito et al., 2006]. mTOR associates with Raptor (mTORC1 complex) to phosphorylate p70 S6 kinase, which in turn phosphorylates 4E-BP1, leading to increased cell proliferation. In addition, mTOR associates with Rictor (mTORC2 complex) and functions in a feedback loop to phosphorylate and activate AKT at Ser473. NVP-BEZ235 (BEZ235, B) is a novel small molecular mass compound that specifically and reversibly inhibits the catalytic activity of AKT, mTORC1, and mTORC2. This dual inhibition has been shown to have antitumor activity in several tumor types in vitro and in vivo by proapoptotic and antiproliferative activity [Maira et al., 2008; Serra et al., 2008; Konstantinidou et al., 2009; Manara et al., 2010; Santiskulvong et al., 2011]. BEZ235 treatment has demonstrated increased antitumor activity in combination with chemotherapy and radiation [Konstantinidou et al., 2009; Manara et al., 2010; Santiskulvong et al., 2011]. The antitumor effects of BEZ235 have been attributed to the induction of cell cycle arrest or apoptosis, and to its antiangiogenic properties [Maira et al., 2008; Serra et al., 2008; Konstantinidou et al., 2009; Manara et al., 2010; Herrera et al., 2011; Santiskulvong et al., 2011].

A complex interaction among tumor cells, stromal fibroblasts, extracellular matrix (ECM) components, and endothelial cells (ECs) is essential for tumor progression. Targeting ECs and fibroblasts for solid tumor treatment has demonstrated substantial benefit in an experimental model [Hayes et al., 2000; Kalluri and Zeisberg, 2006]. Endothelial monocyte activating polypeptide II (EMAP, E) is a proinflammatory cytokine with antiangiogenic and antiendothelial properties. In ECs, EMAP has been shown to induce apoptosis and inhibit proliferation, vascularization, and neovessel formation [Schwarz et al., 1999a; Berger et al., 2000]. EMAP inhibits primary and metastatic tumor growth due to its antiangiogenic activities [Schwarz et al., 1999a, 2010b; Schwarz and Schwarz, 2004]. The mechanism appears to involve its ability to bind to $\alpha5\beta1$ integrin and vascular endothelial growth factor (VEGF) receptors, leading to an inhibition of fibronectin and VEGF signaling [Schwarz et al., 2005; Awasthi et al., 2009]. Previous studies in our laboratory have shown that EMAP improves gemcitabine effects in experimental PDAC, and that this effect can be further enhanced when combined with gemcitabine and the anti-VEGF agent bevacizumab [Schwarz et al., 2009, 2010a]. In the present study, we tested the hypothesis that combination treatment of NVP-BEZ235 and EMAP with gemcitabine can enhance gemcitabine effects by blocking multiple pathways leading to progression of PDAC, as an option for future PDAC clinical applications.

## MATERIALS AND METHODS

### CELL CULTURE AND REAGENTS

Human PDAC cell lines AsPC-1, Panc-1, BxPC-3, and MIA PaCa-2 were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin solution (Sigma Chemical Co.) at 37°C in a humidified 5% $CO_2$ atmosphere. The normal human fetal lung fibroblast cell line WI-38 was grown in DMEM medium, supplemented with 10% FBS and 100 U/ml penicillin/streptomycin solution. Primary human umbilical vein endothelial cells (HUVECs) were grown in EndoGRO-LS medium containing EC growth supplements (Millipore Corp., Billerica, MA). Gemcitabine was purchased from Eli Lilly Corporation (Indianapolis, IN). BEZ235 was purchased from LC Laboratories (Woburn, MA). Recombinant human EMAP was self-prepared and purified as previously described [Schwarz et al., 2000]. The cell proliferation reagent WST-1 was purchased from Roche Diagnostic Corporation (Indianapolis, IN).

### CELL VIABILITY ASSAY

In vitro cell viability was measured by using the colorimetric WST-1 assay as previously described [Awasthi et al., 2009]. Briefly, 4,000 cells were plated in a 96-well plate in medium containing growth factors. After 24 h cells were treated with Gem, BEZ235 and EMAP, either alone or in combination. After an additional incubation of 72 h, 10 μl WST-1 reagent was added in each well followed by incubation for 2 h. The absorbance at 450 nm was measured using a microplate reader.

### WESTERN BLOT ANALYSIS

Cell monolayers were treated with 10 μM of Gem, BEZ235, or EMAP for 24 h. Total cell lysate was prepared, and equal amounts of protein were separated by SDS–PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% milk in TBS-T (10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20). The membranes were incubated overnight at 4°C with the following antibodies: AKT, phospho-AKT (Ser473), mTOR, phospho-mTOR (Ser2448), p70 S6K, phospho-p70 S6K (Thr389), 4E-BP1, phospho-4E-BP1 (Thr37/46), cleaved PARP-1, cleaved caspase-3 (all from Cell Signaling Technology, Beverly, MA), and α-tubulin (Sigma Chemical Co.). The membranes were then incubated with corresponding HRP-conjugated secondary antibodies (Pierce Biotechnologies, Santa Cruz, CA). Specific bands were detected using the enhanced chemiluminescence reagent (ECL, Perkin Elmer Life Sciences, Boston, MA) on autoradiographic film and quantitated by densitometry.

## ANIMAL STUDIES

Animal studies were performed in accordance with the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center, Dallas, TX. Female SCID mice (4–6 weeks old) were used for animal survival studies in a PDAC tumor xenograft model as previously described [Schwarz et al., 1999b]. Mice were injected intraperitoneally with AsPC-1 cells ($0.75 \times 10^6$), randomly grouped (n = 6–8 per group) and treated intraperitoneally for 14 days with PBS (control), gemcitabine (100 mg/kg in 100 μl PBS, twice weekly), BEZ235 (25 mg/kg in 100 μl PBS, 3× weekly), and EMAP (80 μg/kg in 100 μl PBS, 5× weekly), either alone or in combination. Animals were weighed two times per week and examined daily for signs of toxicity or distress. Moribund mice were euthanized in accordance with the local animal care committee protocol and examined for presence and extent of intra-abdominal tumor.

## STATISTICAL ANALYSIS

In vitro cell proliferation assay and Western blot densitometric analysis results are expressed as mean ± standard deviation (SD). Statistical significance was analyzed by the two-tailed Student's *t*-test using GraphPad Prism 4 Software (GraphPad Software, San

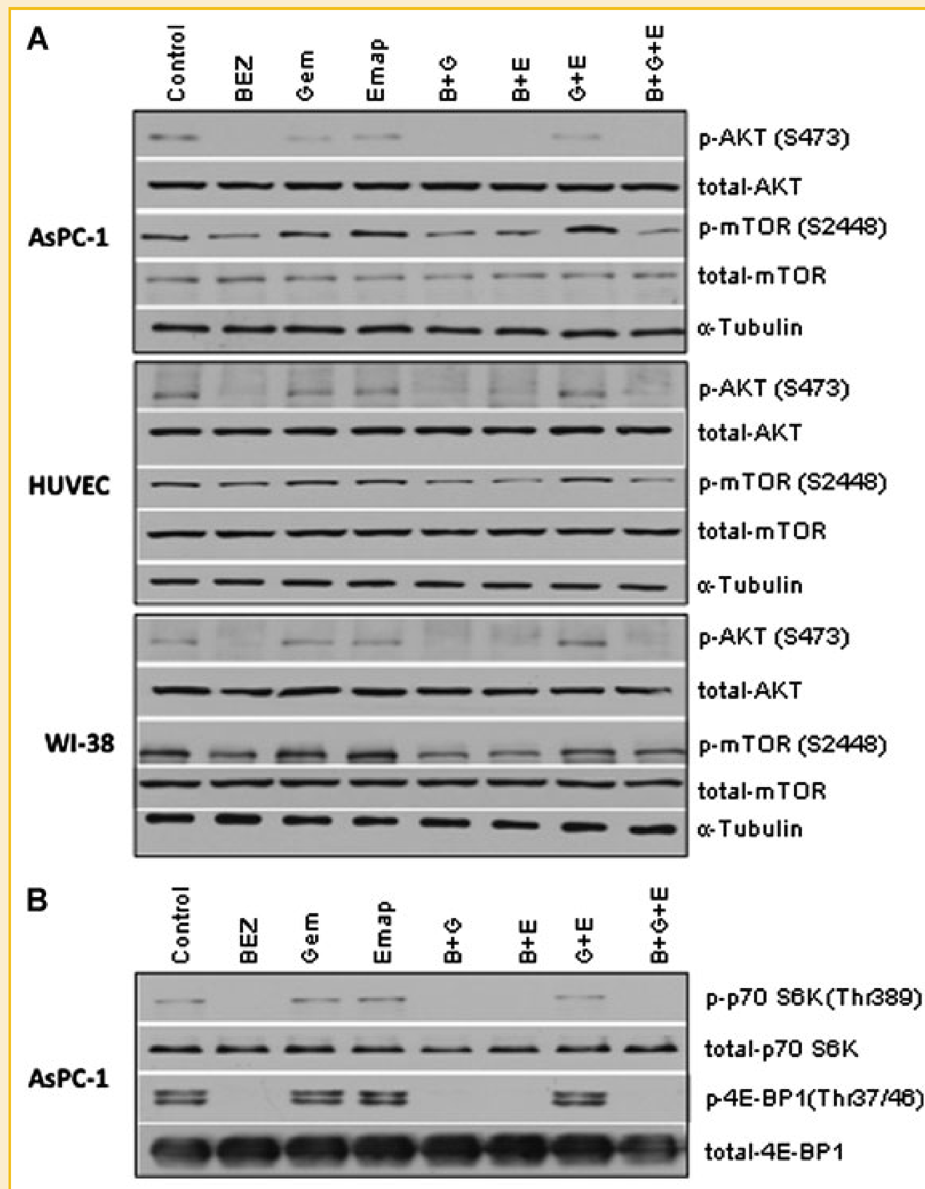


Fig. 1. BEZ235 inhibits the AKT/mTOR signaling pathway. A: Human PDAC (AsPC-1 cells), EC (HUVECs), and fibroblast cells (WI-38 cells) were treated with BEZ235 (10 μM), Gem (10 μM), and EMAP (10 μM), either alone or in combination for 16 h. Total cell extracts were analyzed by immunoblotting for p-AKT (S473), total-AKT, p-mTOR (S2448), total-mTOR, and α-tubulin (loading control). B: Human PDAC (AsPC-1) cells were treated with BEZ235 (10 μM), Gem (10 μM), and EMAP (10 μM), either alone or in combination for 16 h. Total cell extracts were analyzed by immunoblotting for p-p70 S6K (Thr389), total-p70 S6K, p-4E-BP1 (Thr37/46), and total-4E-BP1. Data are representative of two independent experiments with similar results.

Diego, CA). In survival studies, statistical differences were analyzed with StatView for Macintosh version 5.0.1 (SAS, Carey, NC) by nonparametric survival statistics and logrank testing. *P*-values of <0.05 were considered to represent statistically significant group differences.

## RESULTS

### EFFECT OF BEZ235 ON PI3K/AKT/mTOR SIGNALING PATHWAY

In human AsPC-1 PDACs, HUVEC ECs, and WI-38 fibroblasts, BEZ235 treatment caused a significant decrease in phospho-AKT (Ser473) and phospho-mTOR (Ser2448) protein expression. Treatment with Gem and EMAP caused no significant change in phospho-AKT (Ser473) and phospho-mTOR (Ser2448) protein expression at 10 μM treatment for 16 h. Combination of Gem and EMAP with BEZ235 had no additive effects on BEZ235-induced inhibition of phospho-AKT (Ser473) and phospho-mTOR (Ser2448) protein expression (Fig. 1A). mTORC1 downstream substrates p70 S6K (Thr389) and 4E-BP1 (Thr37/46) in AsPC-1 cells were also significantly dephosphorylated by BEZ235 treatment (Fig. 1B).

### EFFECT OF BEZ235 AND GEM ON PDAC CELL PROLIFERATION

BEZ235 and Gem both inhibited PDAC cell line proliferation but had differential inhibitory effects. At 10 μM concentration of BEZ235, percent inhibition in cell proliferation was 71, 54, 36, and 80 in AsPC-1, BxPC-3, Panc-1, and MIA PaCa-2. At 10 μM concentration of Gem, percent inhibition in cell proliferation was 40, 80, 49, and 70 in AsPC-1, BxPC-3, Panc-1, and MIA PaCa-2 cells. Interestingly, the combination of BEZ235 and Gem had additive effects on inhibition of proliferation of Panc-1 and MIA PaCa-2 cells but not in AsPC-1 or BxPC-3 cells (Fig. 2).

### EFFECT OF BEZ235, GEM, AND EMAP ON EC AND FIBROBLAST PROLIFERATION

Analysis of in vitro HUVEC and WI-38 cell proliferation in growth factor containing medium revealed that single agent BEZ235, Gem, and EMAP had significant dose-dependent inhibitory effect. Importantly, combination of these agents had additive effects on inhibition of cell proliferation of both cell lines. At an intermediate concentration of BEZ235 (1 μM), Gem (1 μM), and EMAP (5 μM), percent inhibition in HUVEC proliferation was 68, 59, 29, 93, 77, 63, and 84 in BEZ235, Gem, EMAP, B + G, B + E, G + E, and B + G + E groups; percent inhibition in WI-38 proliferation was 67, 54, 51, 91, 74, 79, and 89 in BEZ235, Gem, EMAP, B + G, B + E, G + E, and B + G + E groups (Fig. 3). In medium without growth factors, no growth was observed in HUVECs, and BEZ235 treatment had no effect on these resting HUVECs. However, in WI-38 cells some growth activity was elucidated, and BEZ235 had an inhibitory effect on this cell proliferation (data not shown).

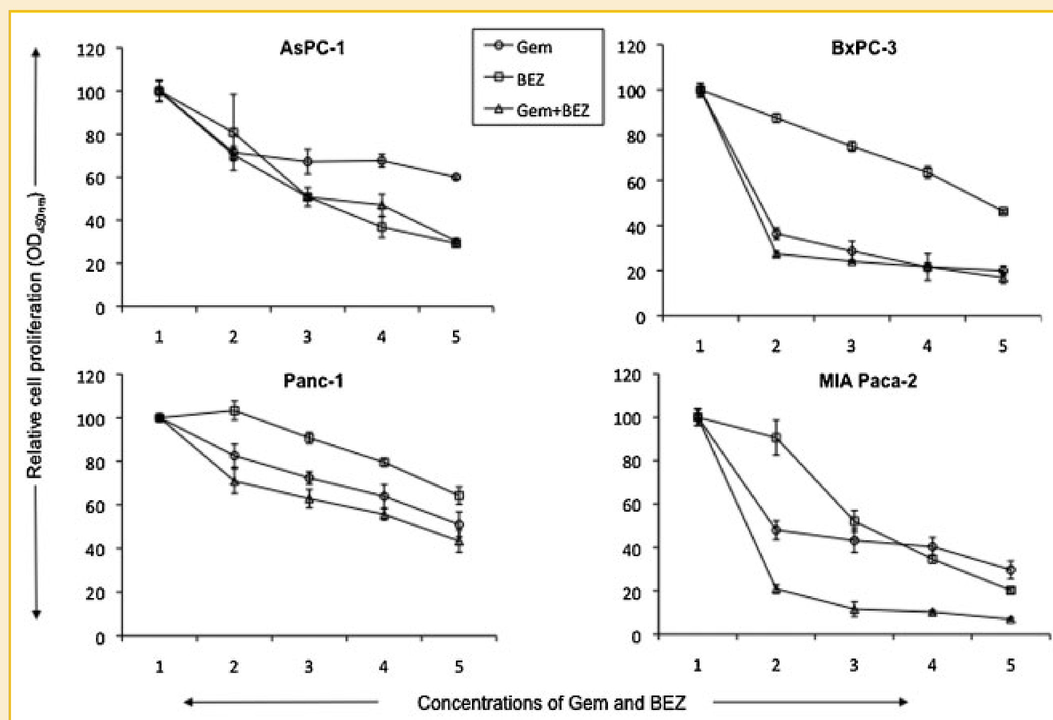


Fig. 2. BEZ235 and Gem inhibit in vitro cell proliferation of PDAC cells. AsPC-1, BxPC-3, Panc-1, and MIA PaCa-2 cells were plated on 96-well plates and treated with BEZ235 or Gem. After 72 h, 10 μl WST-1 reagent was added in each well and incubated for 2 additional hours. The absorbance at 450 nm was measured using a microplate reader. The resulting number of viable cells was calculated by measuring absorbance of color produced in each well. Numbers on *X*-axis represent treatment concentrations of BEZ235 and Gem. Number 1 represents control; numbers 2, 3, 4, and 5 represent BEZ235 and Gem concentrations of 100 nM, 500 nM, 1 μM, and 10 μM, respectively. Data are the mean ± SD of triplicate determinations.

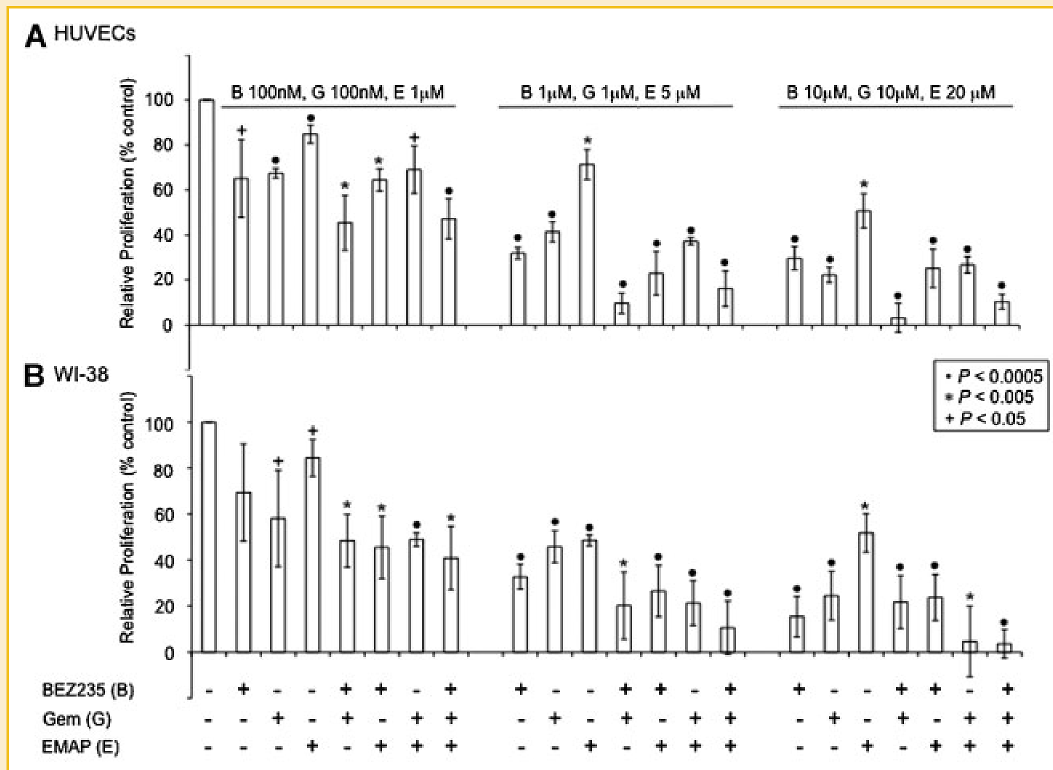


Fig. 3. BEZ235, Gem, and EMAP inhibit in vitro cell proliferation of EC (A) (HUVECs) and fibroblast cells (B) (WI-38). Cell were plated on 96-well plate and treated with BEZ235, Gem, and EMAP. After 72 h incubation, WST-1 reagent was added in each well and number of viable cells was calculated by measuring absorbance of color produced in each well. Data are representative of mean values ± SD of triplicate determinants. Symbols $^{+}$, $^{*}$, and $^{\bullet}$ represent $P$ values of <0.05, 0.005, and 0.0005 compared to controls.

### EFFECT OF BEZ235, GEM, AND EMAP ON APOPTOSIS MARKERS

Western blot analysis to evaluate if inhibition in cell proliferation was due to induction in apoptosis revealed that BEZ235 treatment either alone or in combination with Gem and EMAP induced apoptosis as observed by PARP-1 cleavage and caspase-3 cleavage (Fig. 4). BEZ235-induced expression of cleaved PARP-1 and cleaved caspase-3 was similar in AsPC-1, HUVECs, and WI-38 cells. Gem caused a significant increase in PARP-1 or caspase-3 cleavage in WI-38 fibroblast cells but no detectable change in AsPC-1 or HUVECs (Fig. 4). EMAP treatment caused no detectable change in these apoptosis marker protein.

### EFFECT OF BEZ235, GEM, AND EMAP ON ANIMAL SURVIVAL

In vivo animal survival studies in SCID-NOD mice resulted in a median survival (m.s.) of 16 days in the control group without treatment. Median animal survival was increased in the BEZ235 (21 days), EMAP (21 days), and Gem (28 days) therapy groups (Fig. 5). Further improvement in animal survival occurred in combination therapy groups B + G (m.s. 30 days, $P = 0.007$ vs. controls), B + E (m.s. 27 days, $P = 0.02$ vs. controls), G + E (m.s. 31 days, $P = 0.001$ vs. controls), and B + G + E (m.s. 33 days, $P = 0.004$ vs. controls) (Fig. 5). No sign of drug-related toxicity was observed in any of the treatment groups.

## DISCUSSION

PDAC shows limited susceptibility to almost all classes of cytotoxic drugs, with the mechanism for this phenomenon remaining unelucidated for the most part. Occurrence of several genetic abnormalities with very high frequency in PDAC includes K-Ras mutation, loss of p16, p53, and DPC4 (deleted in pancreatic cancer, locus 4) function, and over-expression of multiple receptor tyrosine kinases [Pellegata et al., 1994; Oikawa et al., 1995]. Some of these alterations up-regulate PI3K/AKT activity, a process which has been shown to stimulate proliferation, and to enhance survival response and drug resistance. Therefore, therapeutic targeting of the PI3K pathway with its downstream targets AKT and mTOR at multiple molecular levels may provide better antitumor effects than selective inhibition of only one component of the pathway. NVP-BEZ235 is a novel small molecular mass inhibitor that inhibits PI3K/AKT/mTOR signaling at the levels of both PI3K and mTOR. Combining conventional cytotoxic drugs such as gemcitabine with novel targeted agents that specifically interfere with key operational pathways responsible for PDAC progression has recently gained much attention in an effort to identify novel and effective treatments for PDAC. Our results demonstrate that a therapeutic combination of BEZ235 and gemcitabine can lead to significantly enhanced antitumor effects in vitro and in vivo, which makes such a

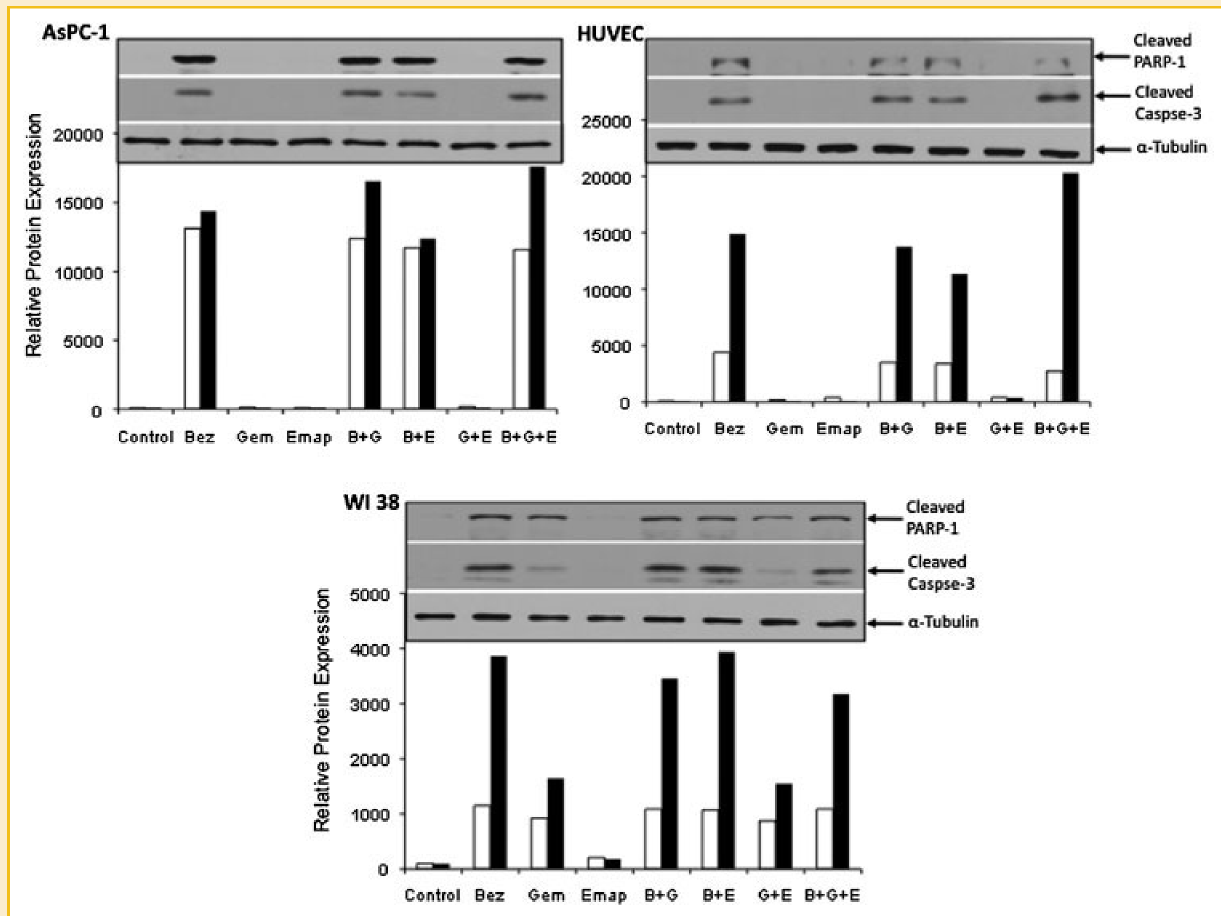


Fig. 4. Effects of BEZ235 (B), Gem (G), and EMAP (E) treatment on cleavage of PARP-1 and caspase-3 proteins. A subconfluent cell monolayer was treated with BEZ235 (10 μM), Gem (10 μM), and EMAP (10 μM). After 16 h of incubation, total cell lysate was prepared and analyzed by immunoblotting for cleaved PARP-1, caspase-3, and α-tubulin (loading control). The intensity of bands was quantitated by densitometry and is represented as the bar graph for cleaved PARP-1 (open bar) and cleaved caspase-3 (closed bar) after normalizing against α-tubulin expression. Data are representative of two independent experiments with similar results.

combination approach with these agents a plausible choice for future clinical applications.

Tumor angiogenesis plays an important role in PDAC progression that is highly dependent on the complex and extensive interaction of tumor cells, ECs, immune cells, and fibroblasts, all contributing to the multi-lineage, polydifferentiated nature of pancreatic cancer tumor components. Therefore, antiendothelial and antangiogenic agents may be beneficial in combination therapy approaches for PDAC treatment. The scope of this study was to evaluate the antitumor activity of BEZ235 and enhancement of Gem response by addition of BEZ235 and the antiangiogenic agent EMAP II in experimental pancreatic cancer. We demonstrate that BEZ235 treatment effectively blocked phosphorylation of AKT-Ser473 (a downstream target of PI3K activation and mTORC2 feedback loop function) and mTOR-Ser2448 (a downstream target of PI3K/AKT activation). Expression of downstream mTORC1 target proteins phospho-p70 S6K (Thr389) and phospho-4E-BP1 (Thr37/46) were also significantly decreased by BEZ235 treatment. These findings suggest that BEZ235 blocks functions of AKT, mTORC1, and mTORC2. BEZ235 treatment decreased cell proliferation and induced apoptosis in PDAC cell lines, ECs, and fibroblasts indicating that the in vivo antitumor effects of BEZ235 may be due to induction of apoptosis in various tumor cellular components, in addition to its antiangiogenic properties. The results show superior outcomes after combination therapy with all three agents, indicating a beneficial

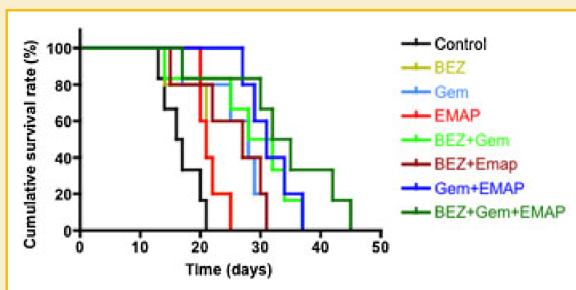


Fig. 5. Effects of BEZ235, Gem, and EMAP treatment of the overall survival of mice. AsPC-1 cells ($0.75 \times 10^6$) were injected intraperitoneally in SCID mice and treatment started after 2 weeks with BEZ235 (25 mg/kg, three times a week), Gem (100 mg/Kg, two times a week), and EMAP (80 μg/kg, five times a week) for 2 weeks. The curve represents the survival time from the beginning of therapy.

constellation of operational mechanisms. Interestingly, EMAP was able to enhance the survival results of BEZ235 therapy alone, which supports a possible use of BEZ235 in combination of antiangiogenic therapy approaches, too.

Pancreatic cancer cell lines display marked heterogeneity towards Gem and other chemotherapy agents. We also observed a similarly heterogeneous response of BEZ235 and Gem in inhibiting cell proliferation of four PDAC lines tested. Although both agents caused inhibition of cell proliferation to different extents, the addition of BEZ235 improved Gem effects in Panc-1 and MIA PaCa-2 cells not in AsPC-1 or BxPC-3 cells. Combination of EMAP with BEZ235 and Gem was tested in ECs and fibroblast cells, and a significant additive effect on inhibition of cell proliferation was achieved compared with single agent treatment. Based on the data obtained from in vitro combinations, we assume that the increased efficiency of BEZ235 regarding apoptosis induction can provide an effective additional antiproliferative component in the majority of cell types tested, sufficient for improved in vivo outcomes.

With the understanding that a high frequency of aberrant PI3K/AKT signaling occurs in pancreatic cancer, previous studies have reported that PI3K inhibition enhances Gem-induced apoptosis in human pancreatic cancer cells [Ng et al., 2000] and improves Gem activity in orthotopic human pancreatic cancer xenografts [Ng et al., 2001]. Later studies focused towards therapeutic targeting of the PI3K/AKT/mTOR network at multiple molecular levels rather than selective inhibition of only one component of the pathway to avoid PI3K/AKT pathway reactivation [Maira et al., 2008]. Recently, BEZ235 has been shown to have antitumor activity as single agent in a pancreatic cancer xenograft model [Cao et al., 2009]. Our results not only corroborate these findings, but also demonstrate the impact of BEZ235 and BEZ235-Gem combinations on several other, potentially relevant cell types and on experimental PDAC survival. In addition, we tested combination treatment benefits of BEZ235 with Gem and EMAP, based on previous studies in our lab that showed EMAP-derived improvements of Gem effects in vivo [Schwarz et al., 2000, 2009]. The observed advantages of combining these agents can indeed lend a strong rationale to a multi-agent clinical approach to PDAC that includes a cytotoxic agent, a targeted pathway blocker such as BEZ235, and an antiendothelial or antiangiogenic agent. Although optimal combination conditions and exact mechanisms are still not clear, these findings may provide a solid foundation for future evaluation of combination benefits of agents displaying these known effects.

In summary, our present study demonstrates that the dual PI3K/AKT/mTOR inhibitor BEZ235, either alone or in combination with Gem and EMAP, induced strong antiproliferative and proapoptotic effects in vitro. Combination of BEZ235 with Gem and EMAP significantly improved animal survival providing evidence that targeting multiple mechanisms of pancreatic cancer progression can be a promising therapeutic approach for PDAC treatment.

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