

Antimyeloma Activity of the Orally Bioavailable Dual Phosphatidylinositol 3-Kinase/Mammalian Target of Rapamycin Inhibitor NVP-BEZ235

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Abstract

The phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway mediates proliferation, survival, and drug resistance in multiple myeloma (MM) cells. Here, we tested the anti-MM activity of NVP-BEZ235 (BEZ235), which inhibits PI3K/Akt/mTOR signaling at the levels of PI3K and mTOR. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric survival assays showed that MM cell lines exhibited dose- and time-dependent decreased viability after exposure to BEZ235 (IC₅₀, 25–800 nmol/L for 48 hours). MM cells highly sensitive (IC₅₀, <25 nmol/L) to BEZ235 (e.g., MM.1S, MM.1R, Dox40, and KMS-12-PE) included both lines sensitive and resistant to conventional (dexamethasone, cytotoxic chemotherapeutics) agents. Pharmacologically relevant BEZ235 concentrations (25–400 nmol/L) induced rapid commitment to and induction of MM.1S and OPM-2 cell death. Furthermore, normal donor peripheral blood mononuclear cells were less sensitive (IC₅₀, >800 nmol/L) than the majority of MM cell lines tested, suggesting a favorable therapeutic index. In addition, BEZ235 was able to target MM cells in the presence of exogenous interleukin-6, insulin-like growth factor-1, stromal cells, or osteoclasts, which are known to protect against various anti-MM agents. Molecular profiling revealed that BEZ235 treatment decreased the amplitude of transcriptional signatures previously associated with *myc*, ribosome, and proteasome function, as well as high-risk MM and undifferentiated human embryonic stem cells. *In vivo* xenograft studies revealed significant reduction in tumor burden ($P = 0.011$) and survival ($P = 0.028$) in BEZ235-treated human MM tumor-bearing mice. Combinations of BEZ235 with conventional (e.g., dexamethasone and doxorubicin) or novel (e.g., bortezomib) anti-MM agents showed lack of antagonism. These results indicate that BEZ235 merits clinical testing, alone and in combination with other agents, in MM. [Cancer Res 2009;69(14):5835–42]

Introduction

Multiple myeloma (MM) has pathophysiologic features distinct from other cancers (1); however, many of the molecular pathways that result in growth and survival of MM cells are also implicated in many other cancers (2). Targeting one such pathway, the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway, may provide profound antitumor effects both in MM and other tumor types. This cascade represents an important target in MM because major growth factors, such as insulin-like growth factor-1 (IGF-1) and interleukin-6 (IL-6), activate PI3K and its downstream targets Akt and mTOR to stimulate proliferation, survival, and drug resistance of MM cells (3–5). Because of its complexity and extensive cross-talk with other cascades, therapeutic targeting of the PI3K-Akt-mTOR pathway at multiple molecular levels may provide better antitumor effects than selective inhibition of only one component of the pathway. Targeting the PI3K pathway at multiple levels may prevent the development of drug resistance.

In this study, we examined the effects of the small molecular mass inhibitor NVP-BEZ235 (BEZ235), which inhibits the PI3K/Akt/mTOR signaling axis at the levels of both PI3K and mTOR. This dual inhibitor has been shown to have activity in multiple tumor types. It was first documented to be an orally bioavailable inhibitor active in PC3M prostate cancer cells *in vitro* and *in vivo*, which targets the p110 isoforms at low nanomolar levels (6). Further studies showed that BEZ235 inhibited vascular endothelial growth factor-induced endothelial cell proliferation both *in vitro* and *in vivo* and has activity in BT474 breast cancer cells harboring HER2 amplification (7). In addition, cell lines harboring K-Ras and B-Raf mutations tend to be less sensitive to BEZ235 but remain responsive to nanomolar levels of the drug (8).

Due to the activity of BEZ235 in solid tumors and the dependence on the PI3K signaling in MM, we explored the potential anti-MM activity of BEZ235 in our preclinical models. We observed a distinct pattern of activity of BEZ235 compared with other anti-MM agents. Specifically, we observed a dose- and time-dependent decrease of MM cell viability *in vitro* on exposure to pharmacologically relevant concentrations of BEZ235, with activity against MM cell lines sensitive and resistant to conventional or novel anti-MM agents. Rapid commitment of MM.1S and OPM-2 cells to death was triggered by BEZ235. Importantly, normal donor peripheral blood mononuclear cells (PBMC) were less sensitive than the overwhelming majority of MM cell lines tested, suggesting a favorable therapeutic index. Combinations of BEZ235 with conventional (e.g., dexamethasone and doxorubicin) or novel (e.g.,

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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bortezomib) anti-MM agents showed additive activity, indicating that these BEZ235-based combinations can be feasible in clinical settings. These results provide the framework for clinical trials of the dual PI3K/mTOR inhibitor BEZ235 to improve patient outcome in MM.

Materials and Methods

Cell lines. We evaluated a panel of human MM cell lines (ARK, Delta 47, Dox40, JJN3, KMS-5, KMS-11, KMS-12-BM, KMS-12-PE, KMS-28-BM, KMS-28-PE, KMS-34, MM.1R, MM.1S, MR20, NCI-H929, OCI-MY5, OPM-2, OPM-6, S6B45, U266, and XG1) as well as the human immortalized bone marrow stromal cell (BMSC) line HS-5 and the immortalized hepatocyte cell line THLE-3. In addition, MM.1S-myrAkt and MM.1S-Bcl-2 cells were established by stable transfection of MM.1S cells with constructs encoding for constitutively active myristoylated Akt and for Bcl-2, respectively (9). Cells were grown in RPMI 1640 (BioWhittaker) with 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% FCS (Life Technologies).

Compounds and reagents. BEZ235 (CAS 915019-65-7, C₃₀H₂₃N₅O, molecular weight 469.55) was synthesized and provided by Novartis AG. Dexamethasone and doxorubicin were purchased from Sigma-Aldrich. PS-341 (bortezomib) was synthesized and provided by Millennium Pharmaceuticals. LY294002 was purchased from Sigma and used at concentrations indicated. Rapamycin was purchased from Calbiochem and used at concentrations indicated. Recombinant human IL-6 and IGF (R&D Systems) were used at 10 and 50 ng/mL, respectively, in cultures of MM.1S cells.

Cell viability assessment. For the panel of MM cell lines (details described in Supplementary Data), THLE-3 hepatocytes, and HS-5 stromal cells, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International) colorimetric survival assay, as previously published (10). Cell lines were treated in 48-well plates at a density of 20,000 per well for 48 h in a 37°C incubator with 5% CO₂. Peripheral blood samples from healthy donors were collected using heparinized tubes and processed by Ficoll (Amersham Biosciences) density gradient centrifugation to isolate PBMCs. Cells were plated at 150,000 per well and stimulated with 5 µg/mL phytohemagglutinin (PHA; Sigma-Aldrich) for 24 h before exposure to BEZ235 for 48 h. Viability was assessed by MTT assays, as described above. For patient samples, bone marrow aspirates from MM patients were collected, and PBMCs were separated by Ficoll gradient centrifugation and selected using CD138 microbeads (Miltenyi Biotec). Purified CD138⁺ patient cells were plated at a density of 10,000 per well and treated for 48 h, and viability was measured using the CellTiter-Glo assay (Promega). Combination studies were performed in cell lines and doses described in the figure legend, and viability was measured using the CellTiter-Glo assay.

Cell death commitment assay. The minimum exposure of MM cells to BEZ235 that is required to commit them to death was evaluated by incubating cells in 24-well plates with BEZ235 (100 nmol/L) for 0 to 24 h. Following incubation, the cells were washed with drug-free medium to remove any residual drug and then incubated in drug-free medium for an additional 3 d, resulting in equal length of incubation for all experimental conditions. MM cell survival was quantified by MTT and expressed as percentage of the value obtained from respective controls.

Stromal cell coculture. The luciferase (luc)-expressing MM.1S-GFP/luc and OPM2-GFP/luc cell lines were generated by retroviral transduction with the pGC-gfp/luc vector (kind gift of C.G. Fathman, Stanford University, Stanford, CA) and used for coculture experiments with the luc-negative human stromal line HS-5 (11). Using the compartment-specific bioluminescence imaging (CS-BLI) technique (11), HS-5 stromal cells were plated in 96-well plates and allowed to attach overnight. MM cells were then added and treated with BEZ235 for 48 h at the doses indicated. Following incubation, luciferin substrate (Xenogen Corp.) was added to the culture, and the resulting bioluminescence signal was measured using a Luminoskan luminometer (Labsystems).

Osteoclast differentiation/coculture. PBMCs were isolated as described above and cultured in α-MEM containing 10% fetal bovine serum

(FBS) and penicillin/streptomycin for 16 h to allow attachment. Nonadherent cells were removed, fresh media supplemented with MCSF (25 ng/mL; R&D Systems) and RANKL (50 ng/mL; PeproTech) were added, and cells were cultured for 3 to 4 wk to differentiate mature osteoclasts. Using the CS-BLI technique (11), mature osteoclasts were plated in 96-well plates, allowed to attach overnight, and used in coculture experiments with MM cells.

Flow cytometric analysis of apoptosis and cell cycle. MM.1S cells were treated with 100 nmol/L BEZ235 for 0 to 48 h. For evaluation of apoptosis, cells were processed using an Annexin V/propidium iodide (PI) kit (Becton Dickinson Biosciences). For cell cycle analyses, cells were stained using a solution of PI (Sigma) and RNase A (Sigma) following 70% ethanol fixation. Data were collected using a Beckman Coulter flow cytometer and analyzed using FlowJo analysis software (TreeStar).

Immunoblotting analysis. For immunoblotting analyses, parental MM.1S, MM.1S-myrAkt, and MM.1S-Bcl-2 cells (10 × 10⁶ per condition) were plated in RPMI 1640 with 10% FBS, penicillin, and streptomycin. BEZ235 (100 nmol/L) was added for 0 to 48 h and immunoblotting was performed with primary antibodies purchased from Santa Cruz Biotechnology, Upstate Biotechnology, or Cell Signaling. Secondary antibodies were purchased from Jackson ImmunoResearch.

Immunofluorescence staining procedures. U20S cells were treated in the presence and absence of 250 nmol/L BEZ235 for 0, 0.5, 1, and 2 h. Cells were fixed and stained for expression of FITC-Foxo3a (Becton Dickinson) and Hoechst (Invitrogen). Staining was imaged using Eclipse E800 microscope (Nikon) and MetaVue (Molecular Devices) software and analyzed using Design Standard CS3 (Adobe) software.

Gene expression profiling of cells treated with BEZ235. Total RNA extraction and purification, cDNA synthesis and cRNA labeling, as well as Affymetrix chip (human HT-U133A and HT-U133B) hybridization and data analysis were performed as previously described (9, 12). Briefly, MM.1S cells were treated with 100 nmol/L BEZ235 or with DMSO for 2, 4, 8, 16, and 24 h. Total RNA was extracted and purified, cDNA synthesized, and cRNA labeled before hybridization to the HT-U133A and HT-U133B arrays. For each time point of the analysis, gene expression profile of BEZ235-treated MM.1S cells was compared with profile of MM.1S cells cultured in the absence of the drug; filtering of up-regulated or down-regulated transcripts was based on conventional criteria for statistical significance incorporated in the software (9, 12–14). Transcripts previously shown to reflect the activation state of pathways pertinent to tumor cell proliferation, survival, drug resistance, and other functional end points of tumor cell biology were evaluated in respect to their differences in expression between BEZ235-treated versus control MM.1S cells for each of the time points of the analysis. For this analysis, we focused on a compendium of signatures previously determined to reflect: ribosomal and proteasome pathways [as included in the Gene Set Enrichment Analysis (GSEA) database⁴ and refs. 15, 16]; signatures of self-renewal implicated in normal and/or cancer stem cell biology, including myc (17, 18), hTERT (19), Hedgehog (20), and Notch (21), a transcriptional signature of genes up-regulated in undifferentiated human embryonic stem cells (22), and a signature of genes enriched in mouse embryonic neural and hematopoietic stem cells and considered to define a genetic program for stem cells (as included in the GSEA database; ref. 23); and the HT-U133A and HT-U133B probes corresponding to the IRF4 target genes identified by Shaffer and colleagues (24). We also studied a transcriptional signature of genes down-regulated by p53 function (as identified with the use of Ingenuity Pathway Analysis; ref. 25); a transcriptional signature previously reported to correlate with unfavorable clinical prognosis of MM patients (26); and a signature of genes overexpressed in angiogenic versus non-angiogenic tumors, as identified by Hu and colleagues (27). The probes corresponding to each of these signatures were used to filter the data set (either using the Ingenuity Pathway Analysis software or Microsoft Excel) and the outputs were processed to calculate the average value (±95% confidence

⁴ <http://www.broad.mit.edu/GSEA>

limit) of the difference in absolute expression signal in MM cells cultured in the presence versus absence of BEZ235.

In vivo antitumor activity of BEZ235. MM.1S-GFP/luc cells (10^6 per mouse) were injected s.c. into γ -irradiated (150 rads) nude mice. After tumor engraftment, mice were randomly assigned to receive 30 mg/kg BEZ235 or vehicle orally. Mice were monitored regularly for changes in tumor size (by calipers), changes in body weight, and signs of infection or paralysis. Mice were treated, monitored, and sacrificed in accordance with approved protocol of the Dana-Farber Cancer Institute Animal Care and Use Committee. Overall survival (defined as time between initiation of treatment and sacrifice or death) was compared in control versus BEZ235-treated mice by Kaplan-Meier method.

Results

Combined inhibition of PI3K and mTOR in MM cells *in vitro*.

The synergistic activity of the PI3K inhibitor LY294002 in combination with the mTOR inhibitor rapamycin (Supplementary Fig. S1) supports the notion that dual PI3K/mTOR inhibitors, such as BEZ235, may have potent activity in MM. We therefore tested a panel of MM tumor cell lines (Fig. 1A) with BEZ235 (0–800 nmol/L) for 48 hours and evaluated tumor cell viability using MTT

colorimetric survival assays. The majority of MM cell lines had IC_{50} values <50 nmol/L (Fig. 1A), which were lower than in a select panel of non-MM cell lines (data not shown). MM.1S cells exhibited the lowest IC_{50} value (<25 nmol/L), with comparably high sensitivity with BEZ235 in JJN3, MM.1R, ARK, and Dox40 cell lines. MM patient tumor cells were collected from bone marrow aspirates and isolated using CD138 selection by Miltenyi microbeads and their viability was assessed. BEZ235 induced cytotoxicity in three of five MM patient samples, with IC_{50} values <200 nmol/L (Fig. 1B).

We next evaluated the effect of BEZ235 on PBMCs, the stromal cell line HS-5, and the immortalized hepatocyte cell line THLE-3. HS-5 BMSCs were insensitive to BEZ235 at concentrations up to 800 nmol/L (Fig. 1C), with IC_{50} higher than the majority of MM cell lines. Similarly, THLE-3 cells had an IC_{50} >800 nmol/L (Fig. 1C). PBMCs from healthy donors also remained viable when exposed to BEZ235 for 48 hours at concentrations as high as 800 nmol/L (Fig. 1D). Furthermore, PBMCs stimulated with PHA (5 ng/mL) for 24 hours and treated for an additional 48 hours with BEZ235 remained $\geq 50\%$ viable even at concentrations of BEZ235 as high as 800 nmol/L (Fig. 1D), suggesting a favorable therapeutic index.

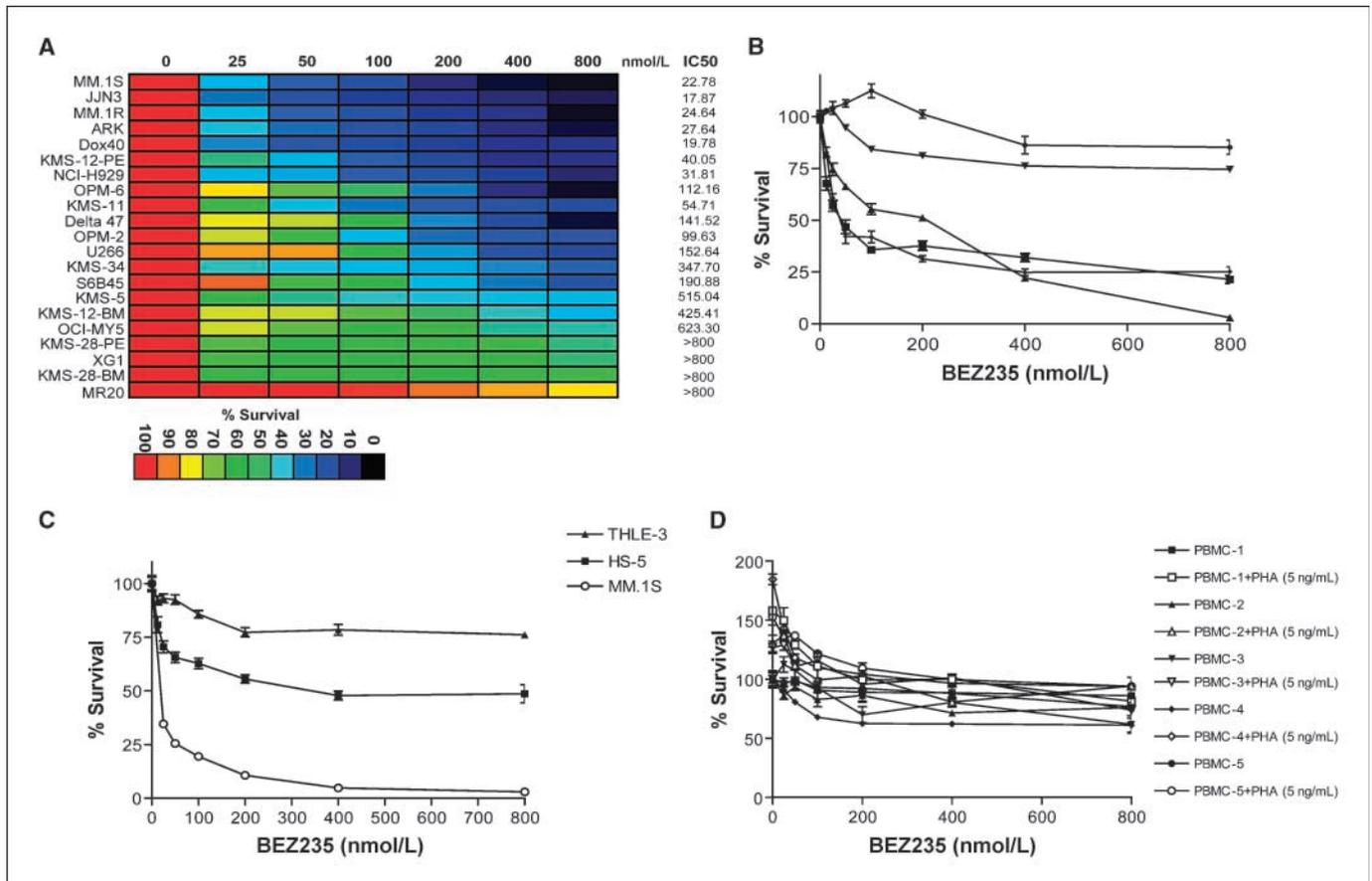


Figure 1. Activity of BEZ235 on MM cells. **A**, a panel of MM cell lines was treated with BEZ235 (at 0–800 nmol/L) for a period of 48 h, and viability was assessed by MTT assay. The most sensitive cell lines (MM.1S, JJN3, and MM.1R) have IC_{50} values <25 nmol/L, whereas the less sensitive MM cell lines (MR20, KMS-28-BM, XG1, and KMS-28-PE) have IC_{50} values >800 nmol/L. **B**, bone marrow aspirates from MM patients were processed using Miltenyi anti-CD138 microbeads for purification of MM cells. Viability of MM cells treated with or without increasing doses of BEZ235 for 48 h was assessed. **C**, nonmalignant cells were also treated with BEZ235, and their viability was assessed by MTT assay. The BMSC line HS-5 and the immortalized hepatocyte cell line THLE-3 were exposed to BEZ235 (0–800 nmol/L) for 48 h and compared with the MM cell line MM.1S. IC_{50} values for HS-5 and THLE-3 were >800 nmol/L. **D**, in addition, both unstimulated and PHA (5 ng/mL)-stimulated PBMCs were exposed to BEZ235 (0–800 nmol/L) for 48 h, and their viability was assessed by MTT. Due to cell stimulation, there was an increase in viable PBMCs following 48 h in the absence of the drug; cells remained $>50\%$ viable at drug concentrations up to 800 nmol/L. Values were normalized to the nonstimulated drug-free control.

MM cells were analyzed for their commitment to death following exposure to BEZ235. After exposure to 100 nmol/L BEZ235 for 2 to 24 hours, cells were washed and cultured in drug-free medium for an additional 72 hours. Cell viability was measured by MTT assay and compared with control cells without drug. MM.1S cells require less than 24 hours of exposure to commit themselves to >50% decrease in viable cell numbers (Supplementary Fig. S2). In contrast, one of the most resistant cell lines (MR20) and an intermediately sensitive cell line (OPM2) require more than 24 hours of exposure to commit cells to death (Supplementary Fig. S2).

BEZ235 overcomes stromal-derived, osteoclast-derived, and cytokine protection of MM cells. To assess the effect of stromal cells on the activity of BEZ235, MM.1S-GFP/luc MM cells were treated with BEZ235 in the presence or absence of HS-5 stromal cells (Supplementary Fig. S3A and B) or differentiated osteoclasts (Supplementary Fig. S3C and D), and the viability was assessed by CS-BLI. The sensitivity of MM.1S (Supplementary Fig. S3A) or OPM-2 (Supplementary Fig. S3B) cells was unaffected by coculture with BMSCs. Mature osteoclasts also provided no protection against the effects of BEZ235 on MM.1S (Supplementary Fig. S3C) and OPM2 (Supplementary Fig. S3D) cell lines; MM.1S cells

supplemented with exogenous IL-6 (10 ng/mL) or IGF-1 (50 ng/mL) also provided no survival benefit to MM.1S cells treated with BEZ235 (data not shown).

BEZ235 overcomes the effects of Akt but not Bcl-2 overexpression. *In vitro* anti-MM activity of BEZ235 against MM.1S cells was compared with its activity against isogenic myristoylated Akt-expressing or Bcl-2-overexpressing MM.1S cells. MM.1S cells engineered to express myristoylated Akt or Bcl-2 were treated with 100 nmol/L BEZ235 for 48 hours and compared with parental cells. MM.1S cells stably expressing myristoylated Akt had comparable sensitivity as parental MM.1S cells (Fig. 2A), whereas cells overexpressing Bcl-2 were less sensitive to BEZ235 (Fig. 2B). Immunoblotting analysis of parental MM.1S cells treated with 100 nmol/L BEZ235 revealed decreased phosphorylation of Akt at Ser⁴⁷³ and Thr³⁰⁸, with stable total Akt levels (Fig. 2C). Comparable changes in Ser⁴⁷³ and Thr³⁰⁸ phosphorylation were observed with BEZ235 treatment in Akt-overexpressing MM.1S cells (Fig. 2C). In parental MM.1S cells, levels of the antiapoptotic Bcl-2 family members Bad and Bcl-XL remained constant during BEZ235 exposure, but a compensatory increase in Bcl-2 levels was observed (Fig. 2D). In Bcl-2-overexpressing MM.1S cells, stable high levels of Bcl-2, Bad, and Bcl-XL were observed during BEZ235 treatment

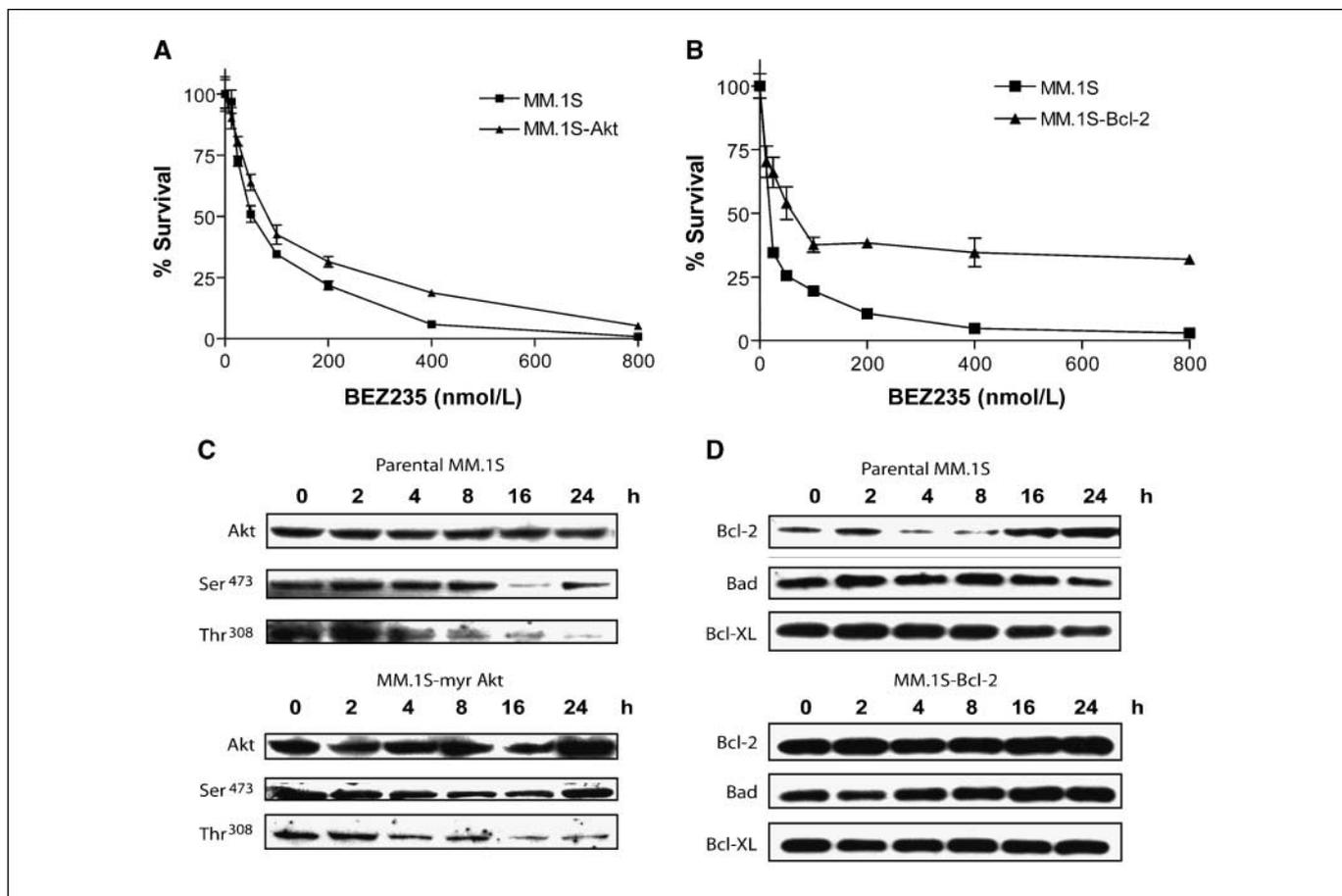


Figure 2. Overexpression of Bcl-2, but not myristoylated Akt, provides protection against BEZ235. MM.1S cells engineered to overexpress Akt or Bcl-2 were treated with BEZ235 for 48 h and compared with parental controls. MM.1S-Akt cells were equally active compared with parental MM.1S cells (A), whereas MM.1S-Bcl-2 cells were more resistant to BEZ235 than controls (B). C, immunoblotting analysis of parental MM.1S cells treated with 100 nmol/L BEZ235 revealed decreased phosphorylation of Akt at Ser⁴⁷³ and Thr³⁰⁸. Comparable results were obtained with BEZ235 treatment of the myristoylated Akt-expressing MM.1S-Akt cells. D, during BEZ235 exposure, Bcl-2 levels increased in the parental MM.1S cells where Bad and Bcl-XL levels remained constant. Constitutively high levels of Bcl-2, as well as stable levels of Bad, and Bcl-XL were observed in the Bcl-2-overexpressing MM.1S-Bcl-2 cells.

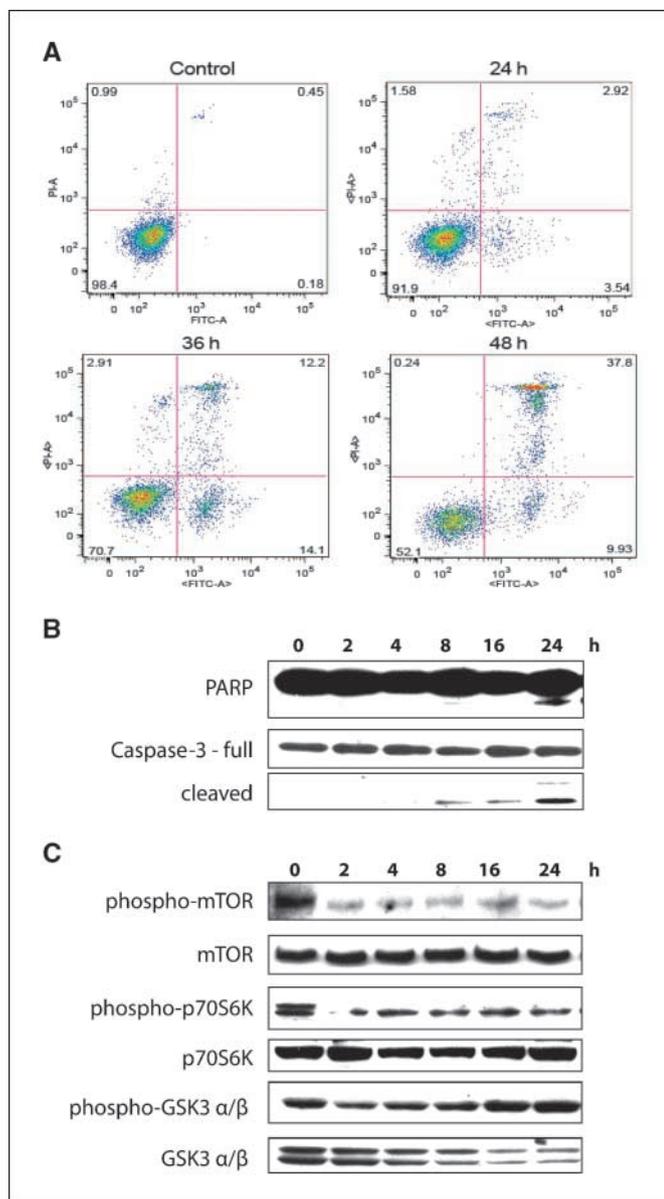


Figure 3. Treatment with BEZ235 induces apoptotic cell death in MM cells. **A**, MM.1S cells were exposed to 100 nmol/L BEZ235 for 0 to 48 h. Cells were collected, washed, stained for Annexin V and PI, and analyzed using flow cytometry. Exposure to BEZ235 resulted in time-dependent increases in Annexin V⁺ events, with increased Annexin V⁺PI⁺ events at later time points indicating apoptotic cell death of MM.1S cells. **B**, immunoblotting analysis of MM.1S cells treated with 100 nmol/L BEZ235 revealed cleavage of caspase-3 and PARP. **C**, in addition, decrease in downstream targets phospho-mTOR, phospho-p70S6K, and phospho-GSK3 (with late rebound for phospho-GSK3) was observed in response to drug treatment.

(Fig. 2D), supporting the notion that high levels of Bcl-2 may be associated with decreased sensitivity to BEZ235.

Cell cycle profiles of BEZ235-treated MM cells. The cell cycle profile of BEZ235-treated MM cell lines was evaluated by PI staining and flow cytometric analysis. Treatment of MM.1S cells with BEZ235 (100 nmol/L for up to 48 hours) increased the percentage of cells in G₀-G₁ phase followed by an increase in sub-G₁ population (Supplementary Fig. S4).

BEZ235 causes apoptotic cell death in MM.1S cells. Analysis of MM cells exposed to BEZ235 was performed by Annexin V/PI

staining. MM.1S cells treated with 100 nmol/L BEZ235 up to 48 hours were collected and analyzed by flow cytometry. Annexin V⁺ and Annexin V⁺PI⁺ staining increased in response to BEZ235 treatment at 48 hours (Fig. 3A).

Western blot analysis of MM.1S cells treated with BEZ235 revealed a pattern of molecular signaling events consistent with inhibition of cell proliferation and initiation of cell death. During the first 8–24 hours of BEZ235 treatment, apoptosis was associated with cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP; Fig. 3B). In addition, downstream targets of PI3K signaling were affected, with phospho-mTOR, phospho-p70S6K, and phospho-glycogen synthase kinase (GSK3) levels decreased in response to BEZ235 treatment, although phospho-GSK3 levels increased at later time points (Fig. 3C). In addition, nuclear translocation of Fox3a was observed in U2OS cells in response to BEZ235 treatment (Supplementary Fig. S5).

Pathway activation status analysis of MM cells treated with BEZ235. Overall activity of signaling pathways can be revealed by changes in select groups of genes previously shown to reflect the pathway activation state. We specifically evaluated multiple transcripts that collectively identify phenotypic changes induced by BEZ235 treatment. We observed that BEZ235 treatment triggers, as early as 4 hours, decreased amplitude in myc, ribosome, and proteasome functional signatures, as well as in signatures for high-risk MM, proangiogenic state and for undifferentiated embryonic stem cells (Fig. 4). In addition, we observed changes in specific transcript levels in various pathways, including cell cycle (Supplementary Fig. S6A), cytokines (Supplementary Fig. S6B), apoptosis (Supplementary Fig. S6C), heat shock proteins (Supplementary Fig. S6D), and proteasome-related genes (Supplementary Fig. S6E).

In vivo activity of BEZ235. *In vivo* testing of BEZ235 in a s.c. xenograft model was performed in nude mice injected with MM.1S-GFP/luc cells (10⁶ per mouse). BEZ235 treatment significantly decreased tumor burden compared with controls ($P = 0.033$, unpaired one-tailed *t* test; Fig. 5A). In addition, median overall survival was not reached after a median follow-up of 48 days in BEZ235-treated mice, whereas control mice had median overall survival of 23 days (95% confidence interval, 21–26 days; $P = 0.028$, log-rank test; Fig. 5B). No significant changes in weight or other signs of potential toxicity were observed during treatment with BEZ235 (Fig. 5C).

Combinations of BEZ235 with other anti-MM agents. We evaluated combinations of BEZ235 with agents currently available for the clinical management of MM (dexamethasone, bortezomib, and doxorubicin). Combinations of BEZ235 with bortezomib (Fig. 6A), doxorubicin (Fig. 6B), or dexamethasone (Fig. 6C) exhibited additive effects against MM.1S cells across a broad range of doses of either agent, suggesting that BEZ235 does not interfere with anti-MM activity of these agents. Comparable results were obtained with these combinations against KMS-18, KMS-12PE, and ARP-1 MM cell lines (Supplementary Fig. S7).

Discussion

In this study, we evaluated the activity of the PI3K/mTOR inhibitor BEZ235 in preclinical models of MM. Inhibition of mTOR triggers a compensatory activation of PI3K/Akt signaling (28). This has been attributed to activation of upstream growth factor receptors (e.g., IGF-1R) as a result of increased secretion of their corresponding growth factors and has been considered a stress

response that tends to attenuate the activity of mTOR inhibitors. This has provided a rationale for dual targeting of PI3K and mTOR. Although this can be achieved with the combination of selective inhibitors for each target, another strategy is to use agents, such as BEZ235, which have activity against both PI3K and mTOR. In this study, we show that this dual inhibitor is active against a large panel of MM cell lines at low nanomolar levels. BEZ235 also induces rapid commitment to cell death, requiring less than 16 hours of exposure in the most sensitive MM cell line. This is particularly important for activity *in vivo*, where drug levels drop as a result of metabolism and excretion and thus important for translation to clinical applications. Importantly, MM cell lines and patient samples are sensitive to BEZ235 at concentrations that have been previously reported to be achievable *in vivo* (6).

Because the bone microenvironment plays such an important role in the resistance to conventional anti-MM therapies, the ability of BEZ235 to overcome these factors is encouraging. In our studies, BEZ235 overcomes the protection conferred to MM cells by IL-6 and IGF-1 cytokines, stromal cells, and osteoclasts. The activity of BEZ235 in MM cocultures with human stromal cells and osteoclasts suggests that the drug will be active in the bone where MM lesions interact with these cells, resulting in increased RANKL levels and the maturation of osteoclasts at these sites (29). We show that BEZ235 overcomes these factors *in vitro* and causes apoptotic MM cell death, shown by Annexin V/PI and immunoblotting analysis, in response to BEZ235 exposure.

The pattern of *in vitro* anti-MM activity of BEZ235 is distinct from other currently available agents, thus suggesting that BEZ235 can be used to treat MM patients resistant to other anti-MM agents. Specifically, we show that BEZ235 is active against Dox40, a cell line that is resistant to chemotherapy, and MM.1R, a cell line resistant to dexamethasone. In addition, molecular signatures associated with high-risk MM, angiogenesis, myc activity, and proteasome function are all down-regulated in response to BEZ235 exposure. These effects on key pathways involved in MM pathophysiology may explain, in part, why BEZ235 may be able to overcome resistance to other anti-MM agents. In addition, our data showing no antagonism with various anti-MM agents *in vitro* indicate that it is possible to combine BEZ235 with other clinically used anti-MM agents.

An important consideration for the development of any anticancer agent is its therapeutic index. Because the PI3K pathway also plays a central role in normal cell proliferation and survival, targeting of this pathway might result in serious toxicities (30). In this study, however, the majority of MM cell lines are more sensitive than all of the nonneoplastic tissues. Treatment of various stromal and hepatocyte cell lines, as well as PBMCs, at doses of BEZ235 that induced killing of both MM cell lines and patient samples *in vitro*, had minimal effects on these normal cells.

PI3K signaling is critical for many cell and tumor types, but these data indicate that PI3K pathway inhibition may be particularly active in MM. PI3K mutations are present, but infrequent, in MM

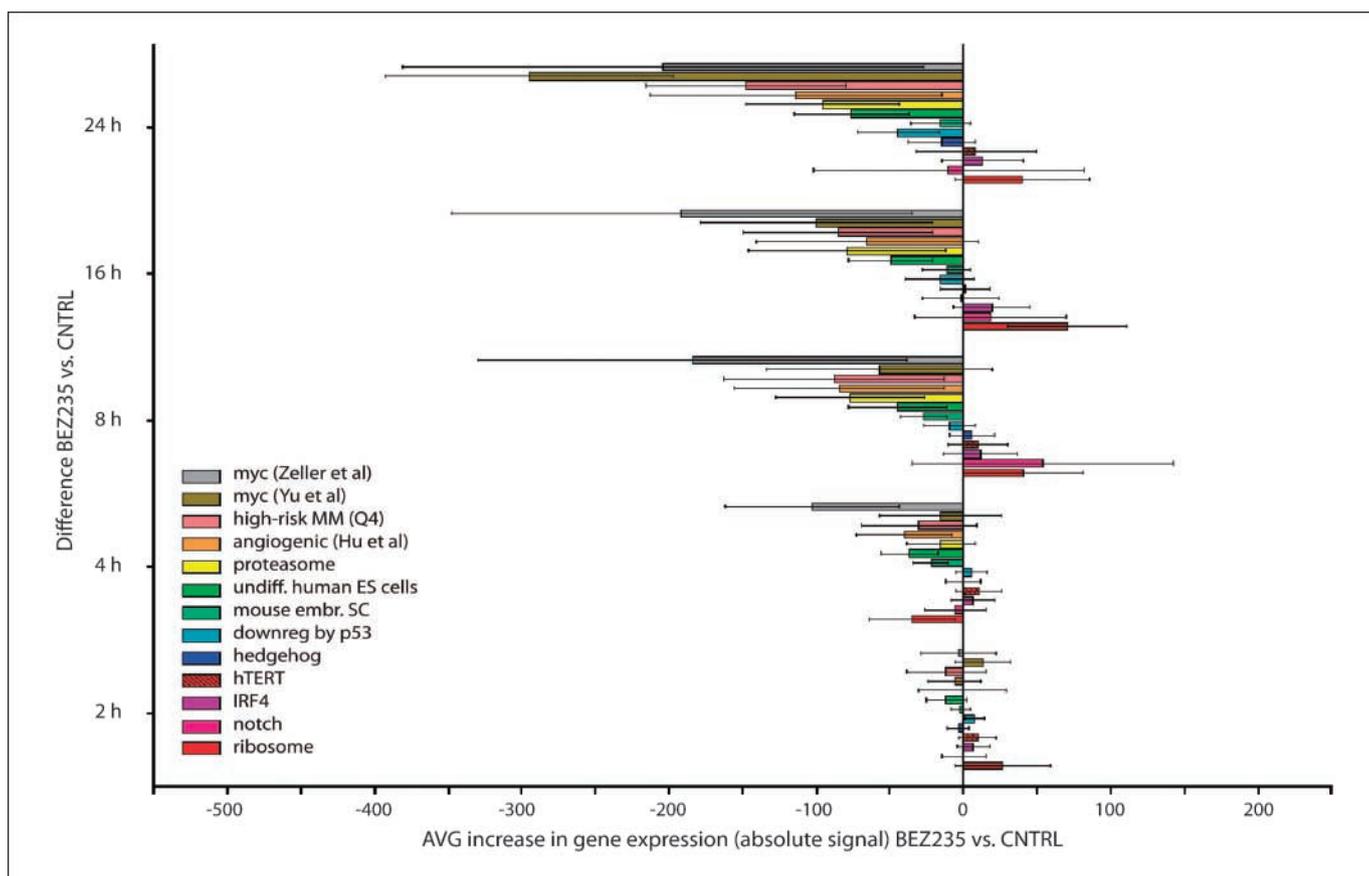


Figure 4. Pathway signature changes triggered by BEZ235 treatment. MM.1S cells were cultured for 2 to 24 h in the presence or absence of 100 nmol/L BEZ235. Total RNA was extracted and purified, cDNA synthesized, and cRNA labeled before hybridization to the HT-U133A and HT-U133B arrays. The average (\pm 95% confidence intervals) increase in gene expression (absolute values) in BEZ235 treated versus control is shown for various pathway signatures.

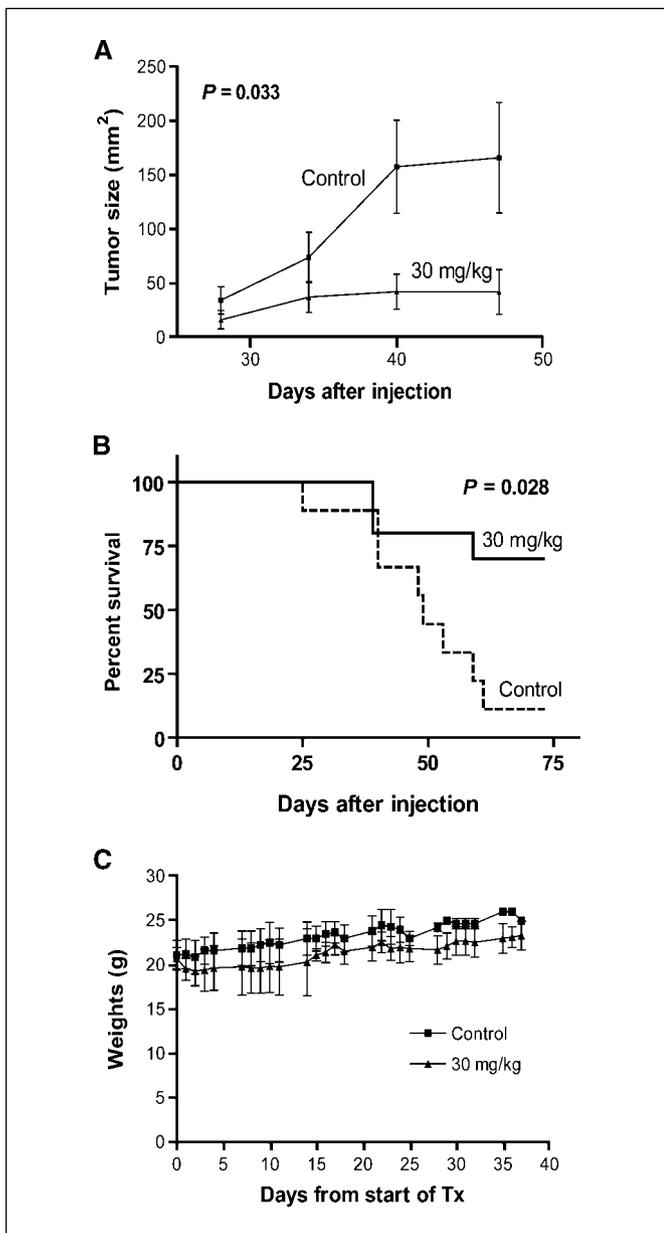


Figure 5. Activity of BEZ235 *in vivo*. MM.1S-GFP/luc cells (10^6) were injected s.c. into nude mice. Following 2 wk of tumor engraftment, mice were randomly assigned to receive 30 mg/kg BEZ235 or vehicle orally. Tumor size, survival, and weights were assessed. Mouse tumor burden was significantly reduced (A; $P = 0.033$, unpaired one-tailed t test) and overall survival was significantly increased (B; $P = 0.028$, log-rank test) in the treated mice compared with controls. Points, average tumor size; bars, SE. C, in addition, the weights of treated mice did not significantly change over the course of treatment with BEZ235. Points, average mouse weight; bars, SD.

cells (31). However, it is plausible that MM cells can have constitutive activation of the PI3K pathway through other mechanisms. This can be explained, at least in part, by the critical role of IL-6 and IGF-1 signaling in mediating the survival and growth of MM cells via the PI3K/Akt pathway (4). Because BEZ235 inhibits the PI3K/Akt pathway at multiple levels, it may overcome the compensatory drug resistance mechanisms that have developed with other selective inhibitors against individual targets of this pathway. In our study, BEZ235 overcomes the protective effect

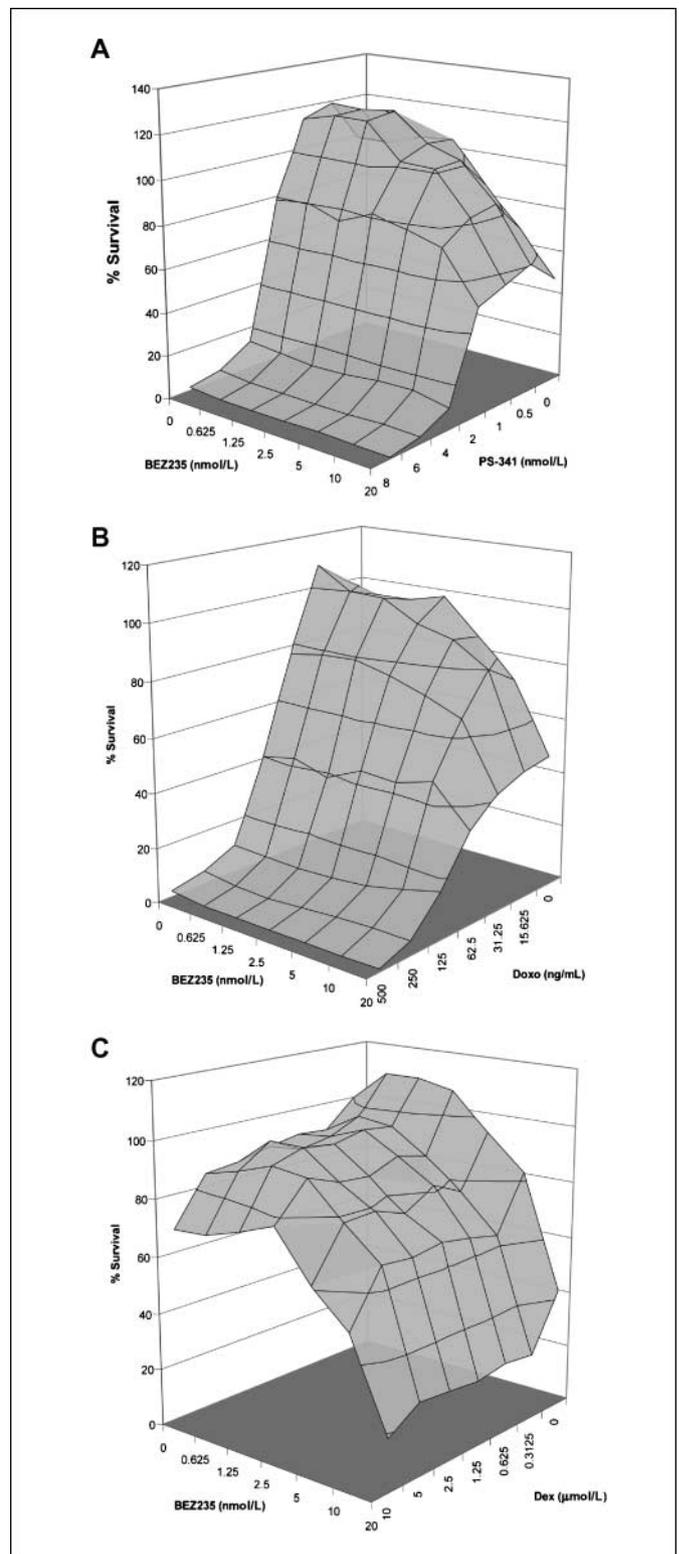


Figure 6. BEZ235 in combination with Velcade, doxorubicin, and dexamethasone *in vitro*. MM.1S cells were plated in 384-well format and treated with increasing doses of BEZ235 in combination with increasing doses of bortezomib (PS-341; A), doxorubicin (Doxo; B), or dexamethasone (Dex; C). Following 48 h of treatment, cell viability was assessed using the CellTiter-Glo viability assay. Cell viability after combination and single-agent treatment was compared and normalized to nontreated controls. BEZ235 induced additive effects with these agents.

conferred to MM.1S cells by overexpression of constitutively active Akt, likely due to its downstream inhibition of mTOR. The ability of BEZ235 to block the PI3K pathway at multiple levels may also explain its potent (submicromolar) single-agent activity. We believe that the multilevel activity of BEZ235, in addition to the profound sensitivity of the MM cell lines to this compound, may explain why we did not observe significant synergy with other anti-MM agents. Our data do not exclude the use of BEZ235 with other agents clinically. In fact, the lack of antagonism in combination with bortezomib (PS-341), doxorubicin, or dexamethasone suggests the feasibility of future clinical trial combinations of BEZ235 in MM.

Because PI3K is a central signaling node mediating tumor cell proliferation, survival, and drug resistance in many cancers, BEZ235 may be active in a broad spectrum of other tumor models (6–8). Although we have documented more pronounced submicromolar activity in MM compared with a panel of select solid tumor cell lines, it is possible that other tumor models that depend on PI3K and/or mTOR (i.e., prostate cancer) may also respond to BEZ235. Nonetheless, the ability of BEZ235 to overcome cytokine and stromal protection, Akt overexpression, as well as its *in vivo* activity in MM suggest that targeting the PI3K/Akt/mTOR pathway

at multiple levels provides a promising framework for clinical trials of BEZ235, in combination with established anti-MM agents, to improve patient outcome.

Disclosure of Potential Conflicts of Interest

N.C. Munshi: Honoraria from speakers bureau, Celgene, Novartis, and Millennium. P.G. Richardson: Honoraria from speakers bureau and consultant/advisory board, Celgene and Millennium. K.C. Anderson: Commercial research grant, Celgene and Millennium; honoraria from speakers bureau and consultant/advisory board, Celgene, Novartis, and Millennium. C.S. Mitsiades: Consultant/advisory board, Novartis, Millennium, and Pharmion. The other authors disclosed no potential conflicts of interest.

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