Dual PI3K/AKT/mTOR Inhibitor BEZ235 Synergistically Enhances the Activity of JAK2 Inhibitor against Cultured and Primary Human Myeloproliferative Neoplasm Cells

Warren Fiskus1, Srdan Verstovsek2, Taghi Manshouri2, Jacqueline E. Smith1, Karissa Peth1, Sunil Abhyankar1, Joseph McGuirk1, and Kapil N. Bhalla1

Abstract
Hemopoietic progenitor cells (HPC) from myeloproliferative neoplasms (MPN) such as myelofibrosis commonly express mutant JAK2-V617F or other mutations that are associated with increased activities of JAK-STAT5/3, RAS/RAF/MAPK, and PI3K/AKT/mTOR pathways. This confers proliferative and survival advantage on the MPN HPCs. Treatment with JAK tyrosine kinase inhibitor (TKI), for example, TG101209, TG101348 (SAR302503), or INCB018424 (ruxolitinib), inhibits mutant JAK2-mediated signaling. Although effective in reducing constitutional symptoms and splenomegaly, treatment with JAK-TKI does not ameliorate myelofibrosis or significantly improve survival of patients with advanced myelofibrosis. Here, we show that treatment with the dual phosphoinositide-3-kinase (PI3K)/AKT and mTOR inhibitor BEZ235 attenuated PI3K/AKT and mTOR signaling, as well as induced cell-cycle growth arrest and apoptosis of the cultured human JAK2-V617F-expressing HEL92.1.7 (HEL), UKE1 cells, and primary CD34+ myelofibrosis (MF)-MPN cells. Treatment with BEZ235 also induced significant apoptosis of the JAK2-TKI resistant HEL/TGR cells that were selected for resistance against JAK-TKI. Cotreatment with BEZ235 and JAK2-TKI (TG101209 and SAR302503) synergistically induced lethal activity against the cultured and primary CD34+ MPN cells while relatively sparing the normal CD34+ HPCs. These findings create a compelling rationale to determine the in vivo activity of dual PI3K/mTOR inhibitors in combination with JAK inhibitors against myelofibrosis HPCs. Mol Cancer Ther; 12(5): 577–88. ©2013 AACR.

Introduction
Recent studies have implicated a somatic, gain-of-function, activating, point mutation JAK2-V617F, as well as mutations in other genes (e.g., MPL, CBL, and LNK) that lead to deregulated activation of JAK-STAT signaling in myeloproliferative neoplasms (MPN; refs. 1–4). Highly sensitive assays for JAK2 have determined that the JAK2V617F mutation is the most prevalent mutation in BCR-ABL negative MPN with a frequency of more than 90% in patients with polycythemia vera, approximately 55% with ET or 65% with primary myelofibrosis (5, 6). JAK2-V617F mutation disrupts the pseudokinase (JH2) domain thus eliminating its autoinhibitory functions on the JAK2 catalytic domain (7, 8). This leads to an aberrant and deregulated activation of the kinase (JH1) domain of the mutant JAK2, which activates the downstream signaling pathways through STAT3 and STAT5 (9–11). Presence of JAK2V617F and other activating JAK2 or other mutations, (e.g., mutations in MPL, LNK, or SOCS genes), are also associated with increased activity of JAK/STAT5/3 but also of the phosphatidylinositol 3-kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK; refs. 9–14). Enhanced cytokine receptor activity in MPN cells also leads to increased activity of AKT, which can also result in downstream activation of mTOR kinase (15, 16). Collectively, activities of these pathways confer growth and survival advantage on the MPN hemopoietic progenitor cells (HPC). Recently, in addition to the activating mutations in JAK2, additional mutations have been identified in the TET2, IDH1, ASXL1, TET2, DNMT3A, and EZH2 genes, which may play a role in the self-renewal/proliferation of MPN HPCs, although their direct contribution in the pathobiology of MPN is not fully elucidated (3, 4, 14, 17–21).

Several, orally bioavailable, small-molecule, ATP-competitive, relatively JAK2-selective inhibitors have been developed (e.g., TG101209, SAR302503, INCB018424, and BMS-911543) and are currently undergoing clinical testing in patients with advanced MPNs (22–24). Results from 2 phase III clinical trials showed that the JAK-TKI ruxolitinib is relatively well-tolerated, depleted circulating inflammatory cytokines, ameliorated constitutional symptoms, and reduced splenomegaly, but did not...
normalize bone marrow histopathology or reverse myelofibrosis in patients (25–28). Recently, the long-term effects of treatment with ruxolitinib in patients with myelofibrosis were reported in 2 studies. One study reported no significant difference in the survival of ruxolitinib-treated patients compared with patients treated with standard therapy. In contrast, the other study reported that treatment with ruxolitinib significantly improved the survival of 107 patients with myelofibrosis over matched historical controls (29, 30). In addition, treatment with TG101348 (SAR302503) has been shown to reduce the allelic burden of the JAK2-V617F–mutant clone, although a favorable impact on myelofibrosis or survival has yet to be showed (28). Also, similar to first and second generation anti-BCR-ABL TKIs, JAK2-TKIs may also be less active against MPN-initiating stem cells or the acute myelogenous leukemia (AML)-transformed MPN HPCs (25, 28). These observations create a strong rationale for developing and testing novel JAK2-V617F–targeted combinations against MPN cells.

Other downstream signaling pathways that are deregulated in MPN cells and may represent additional therapeutic targets include the PI3K/AKT/mTOR pathway and its cross talk with the RAS/RAF/MEK/ERK pathway (9, 10, 31). Recently, the mTOR inhibitor, everolimus, was shown to exhibit clinical activity in a phase I/II trial in patients with myelofibrosis (32). To abrogate the feedback induction of PI3K activity seen with mTOR inhibitors, dual PI3K and mTOR inhibitors have been developed, including the dual pan-class I PI3K, mTORC1 and mTORC2 inhibitor NVP-BEZ235 (BEZ235; 33, 34). As such, it not only inhibits p-AKT but also p-4EBP1 and p-p70S6K levels, as well as the activity of the downstream targets (33). Treatment with BEZ235 was shown to exert significant in vitro and in vivo antitumor activity against a variety of hematologic malignancies, including myeloid malignancies (35–37). Preliminary results have shown that it is safe to administer BEZ235 up to 600 mg twice daily, which is associated with peak plasma levels more than 1.0 μmol/L. BEZ235 also exhibited clinical activity in patients with advanced cancers (38, 39). In the present study, we determined the in vitro activity of BEZ235 combined with JAK2-TKIs TG101209 and SAR302503 in cultured and primary human MPN HPCs. Our findings demonstrate that BEZ235 alone inhibited PI3K/AKT and mTOR signaling, as well as induced cell-cycle growth arrest and apoptosis of cultured and primary MPN cells while relatively sparing normal CD34+ bone marrow progenitor cells. Combined treatment with BEZ235 and TG101209 or SAR302503 exerted synergistic activity against MPN cells. In addition, BEZ235 showed significant activity against the cultured MPN HEL/TGR cells with acquired resistance to JAK2-TKI.

Materials and Methods

Reagents and antibodies

BEZ235 was kindly provided by Novartis Pharmaceuticals Inc. SAR302503 (SAR) and TG101209 (TG) were kindly provided by Sanofi-Aventis. Figure 1 shows the chemical structures of BEZ235, SAR302503, TG101209, and ruxolitinib.
and ruxolitinib. Anti-p-JAK2 (Tyr1007/1008) and anti-p-FOXO3A (Ser253) were obtained from Abcam. Anti-JAK2, anti-p-STAT3 (Tyr705), anti-STAT3, anti-p-AKT (Ser473), anti-AKT, anti-p-PRAS40, PRAS40, anti-FOXO3A, anti-p-ERK1/2, anti-ERK1/2, anti-p-p70S6K, anti-p70S6K, anti-p-4EBP1, anti-4EBP1, and anti-BIM antibodies were obtained from Cell Signaling. Monoclonal anti-p-STAT5 (Tyr694), and anti-p-p27Kip1 antibodies were obtained from BD Transduction Labs. Anti-STAT5 and anti-Bcl-xL antibodies were obtained from Santa Cruz Biotechnologies. Monoclonal anti-β-actin was obtained from Sigma-Aldrich.

Cell lines and cell culture

Human erythroleukemia HEL 92.1.7 (HEL) cells with homozygous expression of JAK2-V617F were obtained from American Type Culture Collection (ATCC). SET2 cells were obtained from DSMZ. All experiments with cell lines were conducted within 6 months after thawing or obtaining from ATCC or DSMZ. Cell line authentication was done by ATCC or DSMZ. The ATCC and DSMZ use short tandem repeat (STR) profiling for characterization and authentication of cell lines. Murine Ba/F3-hEpoR-hJAK2V617F, and Ba/F3-hEpoR cells (kindly provided and authenticated by Dr. Ross Levine, Memorial Sloan-Kettering Cancer Center, New York, NY), were maintained in RPMI media with 10% FBS, 1% penicillin/streptomycin, and 1% non-essential amino acids, as previously described (40). Ba/F3-hEpoR cells were supplemented with 10% WEHI pre-conditioned media. HEL cells were cultured in RPMI media with 10% heat-inactivated FBS and 1% penicillin/streptomycin. UKE1 cells (kindly provided and authenticated by Dr. Walter Fiedler, University Hospital Eppendorf, Hamburg, Germany) were cultured in Iscove’s Modified Dulbecco’s Medium containing 10% horse serum, 10% FBS, and 1 μmol/L hydrocortisone (41). Logarithmically growing cells were exposed to the designated concentrations of BEZ235 and/or TG101209 or SAR302503. Following these treatments, cells were washed free of the drug(s) before the performance of the studies. JAK2-TKI-resistant HEL92.1.7 (HEL/TGR) cells were established and maintained, as previously described (42).

Primary MF-MPN cells

Primary peripheral blood and/or bone marrow aspirate MF-MPN samples were obtained with informed consent from patients with high risk (>3) myelofibrosis (according to the International Prognostic Scoring System). The samples were collected in heparinized tubes, and mononuclear cells were separated using Ficoll Hypaque following the manufacturer’s protocol. The cells were washed once, then resuspended in complete RPMI-1640, and counted to determine the number of cells isolated before their use in the various experiments, as previously described (40, 42). Banked, delinked, and deidentified normal CD34+ bone marrow progenitor cells procured for recipients, who had since deceased, and primary myelofibrosis-MPN cells were purified by immunomagnetic beads conjugated with anti-CD34 antibody before use in the cell viability assay (StemCell Technologies), as previously described (40, 42).

Cell-cycle analysis

Following the designated treatments, cells were harvested, washed twice with 1 × PBS and fixed in 70% ethanol overnight at −20°C. Fixed cells were washed twice with 1 × PBS and stained with propidium iodide and RNase A for 15 minutes at 37°C. Cell-cycle data were collected and analyzed on a BD Accuri C6 flow cytometer (BD Biosciences).

Assessment of apoptosis by Annexin-V staining

Untreated or drug-treated cells were stained with Annexin-V (Pharmingen) and TO-PRO-3 iodide and the percentages of apoptotic cells were determined by flow cytometry, as previously described (40, 42). To analyze synergism between TG101209 or SAR302503 and BEZ235, cells were treated with TG101209 (200–1,000 nmol/L) or SAR302503 (100–1,000 nmol/L) for 48 hours and the percentages of Annexin V-positive, apoptotic cells were determined by flow cytometry. The combination index (CI) for each drug combination was calculated by median dose effect analyses (assuming mutual exclusivity) using the commercially available software Calcsyn (Biosoft; ref. 43). CI values of less than 1.0 represent a synergistic interaction of the 2 drugs in the combination.

Assessment of percentage non-viable cells

Following designated treatments, cells were stained with Trypan blue (Sigma). The numbers of non-viable cells were determined by counting the cells that exhibited trypan blue uptake in a hemocytometer, and reported as a percentage of untreated control cells. Alternatively, cells were washed with 1 × PBS, stained with propidium iodide and analyzed by flow cytometry.

Coloncy growth assay

HEL and UKE1 cells were treated with BEZ235 and/or TG101209 or SAR302503 for 48 hours. At the end of treatment, cells were washed free of the drugs and 500 cells per condition were plated in methylcellulose. Colony formation was measured after 7 to 10 days. For colony growth of primary MF-MPN cells, CD34+ MF-MPN cells were treated with BEZ235 and SAR302503 for 48 hours. Following this, cells were washed free of the drugs and plated in MethoCult H4534 Classic media containing cytokines (StemCell Technologies). Colony formation was measured after 14 days.

Cell lysis and protein quantitation

Untreated or drug-treated cells were centrifuged, and the cell pellets were incubated in lysis buffer on ice for 30 minutes, as previously described (40, 42). After centrifugation, an aliquot of each cell lysate was diluted 1:10 and protein quantitated using a BCA Protein Quantitation Kit (Pierce), according to the manufacturer’s protocol.
SDS-PAGE and immunoblot analyses

Seventy-five micrograms of total cell lysate were used for SDS-PAGE. Western blot analyses of p-JAK2, JAK2, p-STAT3 (Tyr705), STAT3, p-STAT5 (Tyr 694), STAT5, p-AKT (Ser473), AKT, p-FOXO3A (Ser253), FOXO3A, p-ERK1/2, ERK1/2, BIM, p27, p-p70S6K, p70S6K, p-PRAS40, PRAS40, p-4EBP1, and 4EBP1 were conducted on total cell lysates using specific antisera or monoclonal antibodies. Blots were washed with 1 × PBST, then incubated in IRDye 680 goat anti-mouse or IRDye 800 goat anti-rabbit secondary antibodies (LI-COR) for 1 hour, washed 3 times in 1 × PBST and scanned with an Odyssey Infrared Imaging System (LI-COR). The expression levels of β-actin were used as the loading control for the Western blots. Immunoblot analyses were conducted at least twice. Representative immunoblots were subjected to densitometry analysis. Densitometry was conducted using ImageQuant 5.2 (GE Healthcare).

Statistical analysis

Significant differences between values obtained in a population of MPN cells treated with different experimental conditions were determined using a 2-tailed, paired t test. P values of less than 0.05 were assigned significance.

Results

Treatment with dual PI3K/mTOR inhibitor induces cell-cycle arrest and apoptosis of mouse [Ba/F3] and human MPN cells expressing JAK2V617F

We first determined the effects of BEZ235 on the viability of mouse pro-B Ba/F3-hEpoR cells (which express...

Figure 2. Treatment with the dual PI3K/mTOR inhibitor BEZ235 induces cell-cycle arrest and apoptosis of mouse [Ba/F3] and human MPN cells expressing JAK2V617F. A, mouse Ba/F3 cells with ectopic expression of hEpoR (Ba/F3-hEpoR) or coexpression of hEpoR and hJAK2-V617F (Ba/F3-hEpoR-JAK2V617F) were treated with the indicated concentrations of BEZ235 for 48 hours. At the end of treatment, cells were washed with 1× PBS and stained with Annexin V and TO-PRO-3 iodide. The percentages of Annexin V-positive, apoptotic cells were determined by flow cytometry. Columns represent the mean of 3 independent experiments; bars represent the SEM. †, apoptosis values significantly less (P < 0.05) in Ba/F3-hEpoR cells compared with Ba/F3-hEpoR-hJAK2V617F cells treated with the same concentrations of BEZ235. B, HEL and UKE1 cells were treated with the indicated concentrations of BEZ235 for 24 hours. Following this, cell-cycle status was determined by flow cytometry. Columns represent the mean of 3 independent experiments; bars represent the SEM. †, apoptosis values significantly greater in cells treated with BEZ235 compared with control cells (P < 0.05). C, HEL, UKE1 and SET2 cells were treated with the indicated concentrations of BEZ235 for 48 hours. At the end of treatment, cells were washed with 1× PBS and stained with Annexin V and TO-PRO-3 iodide. The percentages of Annexin V-positive, apoptotic cells were determined by flow cytometry. Columns represent the mean of 3 experiments; bars represent the SEM. †, apoptosis values significantly greater in the BEZ235-treated cells than the control cells (P < 0.05).
murine wild-type JAK2) with or without the ectopic expression of human JAK2-V617F. As shown in Fig. 2A, treatment with 250 and 1,000 nmol/L of BEZ235 induced apoptosis of Ba/F3-hEpoR-hJAK2-V617F cells, but was significantly less \( (P < 0.05) \) active against Ba/F3-hEpoR cells without hJAK2-V617F expression (Fig. 2A). We next determined the cell-cycle effects of BEZ235 on the cultured human MPN HEL and UKE1 cells. As shown in Fig. 2B, treatment with BEZ235 for 24 hours dose dependently and significantly increased the percentage of cells in the G0–G1 phase \( (P < 0.05) \), with concomitant decline in the percentage of cells in the S and G2–M phase of the cell cycle. Treatment with BEZ235 also induced apoptosis of the 3 cultured human MPN cell types HEL, UKE1, and SET2 in a dose-dependent manner (Fig. 2C). As compared with HEL, the cultured MPN SET2 and UKE1 cells were significantly more sensitive to BEZ235-induced apoptosis \( (P < 0.05; \text{Fig. 2C}) \).

**Treatment with BEZ235 reduced PI3K/AKT and mTOR signaling in MPN cells**

We next determined the effects of treatment with BEZ235 on PI3K/AKT and mTOR pathways signaling proteins.

![Figure 3](https://www.aacrjournals.org/molcanther/issue/5/BEZ235-Inhibits-mTOR-Signaling-in-MPN-Cells.png)

**Figure 3.** Treatment with BEZ235 dose dependently inhibits PI3K/AKT and mTOR signaling in MPN cells. A and B, HEL and UKE1 cells were treated with the indicated concentrations of BEZ235 for 24 hours. At the end of treatment, cells were harvested and total cell lysates were prepared. Immunoblot analyses were conducted for the expression levels of p-FOXO3A, FOXO3A, p-PRAS40, PRAS40, p-4EBP1, p-70S6K, and \( \beta \)-actin in the cell lysates. The numbers beneath the bands represent densitometry conducted on representative immunoblots relative to the control cells. C and D, HEL and UKE1 cells were treated with the indicated concentrations of BEZ235 for 24 hours. Following this, cells were harvested and total cell lysates were prepared. Immunoblot analyses were conducted for the expression levels of BIM, p27, and \( \beta \)-actin in the cell lysates. The numbers beneath the bands represent densitometry done on representative immunoblots relative to the control cells.

Treatment with BEZ235 (50–500 nmol/L) in HEL and UKE1 cells dose dependently attenuated the levels of p-AKT and p-PRAS40, which are targets of PI3K and AKT, respectively, as well as the levels of p-p70S6K and p-4EBP1 that are known to be the targets of mTOR kinase (Fig. 3A and 3B). Densitometry of the immunoblots with SD is shown in Supplementary Fig. S1A–S1C. In addition, treatment with BEZ235 depleted the expression levels of the progrowth and prosurvival protein p-FOXO3A (Fig. 3A and 3B), whereas it simultaneously induced the levels of growth and survival-inhibitory p27 and BIM that are transactivated by FOXO3A in HEL and UKE1 cells (Fig. 3C and 3D). Densitometry of the immunoblots with SD is shown in Supplementary Fig. S1D–S1F. BEZ235 also significantly depleted the expression levels of p-p70S6K even at concentrations as low as 50 nmol/L in HEL and UKE1 cells but did not affect the total levels of p70S6K in either cell line.

**Cotreatment with BEZ235 enhances JAK2 inhibitor-mediated inhibition of PI3K/AKT/mTOR signaling and apoptosis of MPN cells**

We have previously shown the single agent anti-MPN activity of TG101209 on JAK/STAT signaling and...
induction of apoptosis in cultured MPN cells (40, 42). We next determined the effects of treatment with JAK2 inhibitor, SAR302503 on the induction of apoptosis of cultured MPN cells. As shown in Fig. 4A, SAR302503 dose dependently induced apoptosis of HEL and UKE1 cells. Dose-dependent apoptotic effects were also observed against HEL and UKE1 following treatment with TG101209 (0.2–1.0 µmol/L; Fig. 4B). We next determined the effects of cotreatment with BEZ235 on JAK2-TKI-induced apoptosis of HEL and UKE1 cells. Figure 4C shows that cotreatment with BEZ235 and TG101209 synergistically induced apoptosis of HEL and UKE1 cells following evaluation of the combination indices generated through median dose effect and isobologram analyses. Combination index (CI) values less than 1.0 indicate the synergistic anti-MPN activity of the 2 agents (Fig. 4C and Supplementary Fig. S2A and S2B). Similar synergistic apoptosis was also observed following treatment with BEZ235 and SAR302503 in the cultured MPN cells (Fig. 4D and Supplementary Fig. S2C and S2D). The combined treatment of BEZ235 and TG101209 also exerted superior inhibition of colony growth compared to treatment with either agent alone in HEL and UKE1 cells (P < 0.05; Fig. 4E and F). Similar effects on colony growth were also observed following treatment with BEZ235 and SAR302503 (data not shown). We next determined the effects of treatment.

Figure 4. Cotreatment with BEZ235 and JAK2 inhibitors synergistically induces apoptosis of MPN cells. A and B, HEL and UKE1 cells were treated with the indicated concentrations of SAR302503 (A) or TG101209 (B) for 48 hours. At the end of treatment, cells were washed with 1X PBS and stained with Annexin V and TO-PRO-3 iodide. The percentages of Annexin V-positive, apoptotic cells were determined by flow cytometry. Columns represent the mean of 3 independent experiments; bars represent the SEM. C and D, HEL and UKE1 cells were treated with BEZ235 (dose range, 50–1,000 nmol/L) and TG101209 (dose range 100–1,000 nmol/L) or SAR302503 (dose range 100–1,000 nmol/L) for 48 hours. The percentages of Annexin V-positive, apoptotic cells were analyzed by flow cytometry. The median dose effect and combination indices (CI) were calculated using CalcuSyn software (assuming mutual exclusivity). CI values less than 1.0 indicate a synergistic interaction of the 2 agents. E and F, HEL and UKE1 cells were treated with the indicated concentrations of BEZ235 and/or TG101209 for 48 hours. At the end of treatment, cells were washed free of the drug and plated in methylcellulose for 7 to 10 days. Columns represent the mean of 3 experiments; bars represent the SEM. *: colony growth values significantly less (P < 0.05) in the combination treatment compared with treatment with either agent alone.
with BEZ235 and TG101209 on the activity of JAK2-V617F and the downstream signaling proteins, as well as on the mTOR pathway proteins in HEL and UKE1 cells. Figure 5A and B show that, as compared with each agent alone, cotreatment with BEZ235 and TG101209 caused greater depletion of p-STAT3, p-AKT, p-ERK1/2 and p-4EBP1 in the MPN cells. This effect was less pronounced against p-p70S6K. The effects on the levels of the signaling proteins were accompanied with increased expression of BIM and p27 in HEL and UKE1 cells (Fig. 5C and 5D). Similar effects were also noted in the MPN cells following combined treatment with BEZ235 and SAR302503 (Supplementary Fig. S3A and S3B).

**Combined treatment with BEZ235 and JAK2 inhibitor is selective and synergistically active against primary MF-MPN cells expressing JAK2-V617F versus normal HPCs**

We next determined the effects of BEZ235 and/or SAR302503 on the viability of primary CD34\(^+\) MF-MPN HPCs expressing JAK2-V617F harvested from the peripheral blood of patients with myelofibrosis. Treatment with BEZ235 or SAR302503 alone caused greater loss of viability of primary CD34\(^+\) MF-MPN HPCs than normal HPCs (Fig. 6A). Moreover, cotreatment with BEZ235 and SAR302503 caused significantly more (\(P < 0.05\)) loss of viability of primary MF-MPN HPCs than treatment with either agent alone. In addition, the combined treatment of BEZ235 and SAR302503 induced significantly greater lethality against MF-MPN HPCs, as compared with normal HPCs (\(P < 0.05\)). Superior anti-MPN activity was also observed in primary MPN cells following treatment with BEZ235 and TG101209 (Supplementary Fig. S4). Similar to the effects observed in the cultured MPN cells, cotreatment with BEZ235 and SAR302503 exerted synergistic anti-MPN activity against primary CD34\(^+\) MF-MPN cells with CI values less than 1.0 for all combinations tested (Fig. 6B). Consistent with the effects observed in HEL and UKE1 cells treated with BEZ235 and TG101209, cotreatment with BEZ235 and SAR302503 exerted greater inhibition of colony growth than either agent alone against primary CD34\(^+\) MF-MPN HPCs (Fig. 6C). In CD34\(^+\) primary MF-MPN cells, treatment with 250 nmol/L of BEZ235 alone reduced the expression of p-AKT and Bcl-x\(_L\) concomitantly with induction of all isoforms of BIM. As compared with treatment with each agent alone, cotreatment with BEZ235 and SAR302503 caused greater depletion of p-AKT, p-ERK1/2, and Bcl-x\(_L\), as well as increased BIM levels in MF-MPN HPCs (Fig. 6D). Similar effects were also observed in primary MPN cells treated with

**Figure 5.** Cotreatment with JAK2 inhibitor enhances BEZ235-mediated inhibition of PI3K/AKT and mTOR signaling and increases expression of BIM and p27 in MPN cells. A–D, HEL and UKE1 cells were treated with the indicated concentrations of BEZ235 and/or TG101209 for 24 hours. Following this, cells were harvested and total cell lysates were prepared. Immunoblot analyses were conducted for the expression levels of p-STAT5, STAT5, p-STAT3, STAT3, p-AKT, AKT, p-ERK1/2, ERK1/2, p-4EBP1, 4EBP1, p-p70S6K, p70S6K, p27, BIM, and \(\beta\)-actin in the cell lysates. The numbers beneath the bands represent densitometry conducted on the representative immunoblots relative to the control cells.
BEZ235 and TG101209. In contrast, these alterations were not observed following treatment with BEZ235 alone or in combination with JAK2 inhibitors in the normal CD34+ bone marrow progenitor cells (Fig. 6E).

**TKI-resistant MPN cells are collaterally sensitive to treatment with BEZ235**

We had previously reported the creation of JAK2-TKI-resistant HEL92.1.7 (HEL/TGR) cells that are significantly less sensitive to TG101209 than the parental-sensitive HEL cells (42). This was confirmed in the present studies (Fig. 7A). In addition, HEL/TGR cells were reported to exhibit high expression levels of p-JAK2, p-STAT5, p-AKT, and p-FOXO3A, which were not altered by treatment with TG101209 (42). Furthermore, compared with HEL cells, HEL/TGR cells exhibited markedly lower expression of BIM, and treatment with TG101209 did not induce BIM expression in HEL/TGR cells, as compared with HEL cells (42). We next determined the effects of BEZ235 on cell-cycle status and its ability to induce apoptosis in HEL/TGR cells. Treatment with BEZ235 induced significant cell-cycle growth inhibition in the HEL/TGR cells (Supplementary Fig. S5). Treatment with BEZ235 also induced significantly more apoptosis of HEL/TGR versus normal CD34+ cells (42). This was confirmed in the present studies (Fig. 6E).

**Figure 6.** Cotreatment with BEZ235 and JAK2 inhibitor exerts selective and synergistic activity against primary MF-MPN cells compared with normal HPC’s. A, primary CD34+ MF-MPN cells (n = 6) and normal CD34+ cells (n = 5) were treated with BEZ235 and/or SAR302503, as indicated, for 48 hours. After treatment, cells were washed with 1× PBS and stained with propidium iodide. The percentages of non-viable cells were determined by flow cytometry. †, values significantly greater (p < 0.05) in the combination treatment compared with treatment with either agent alone. ††, values significantly less (p < 0.05) in normal CD34+ cells compared with the primary MF-MPN cells. B, primary CD34+ MF-MPN cells were treated as in A with a constant ratio of BEZ235 and SAR302503. The median dose effect and combination indices (CI) were calculated using Calcusyn software. CI values less than 1.0 indicate a synergistic interaction of the 2 agents. C, primary CD34+ MF-MPN cells were treated with the indicated concentrations of BEZ235 and/or SAR302503 for 48 hours. After treatment, cells were washed free of the drug and plated in methylcellulose with cytokines. Colony formation was measured after 14 days. D, primary CD34+ MF-MPN cells were treated with the indicated concentrations of BEZ235 and SAR302503 for 24 hours. Then, cells were harvested and total cell lysates were prepared. Immunoblot analyses were conducted for the expression levels of p-AKT, p-ERK1/2, Bcl-xL, BIM, and β-actin in the cell lysates. The numbers beneath the bands represent densitometry done on representative immunoblots relative to the untreated cells. E, normal CD34+ bone marrow progenitor cells (BMPC) were treated with the indicated concentrations of BEZ235 and SAR302503 for 24 hours. At the end of treatment, total cell lysates were prepared and immunoblot analyses were conducted for the expression levels of p-AKT, p-ERK1/2, Bcl-xL, BIM, and β-actin in the cell lysates.
HEL/TGR cells were treated with the indicated concentrations of TG101209 or BEZ235 for 48 hours. At the end of treatment, cells were washed with 1× PBS and stained with Annexin V and TO-PRO-3 iodide. The percentages of Annexin V-positive, apoptotic cells were determined by flow cytometry. Columns represent the mean of 3 independent experiments; bars represent the SEM. C, HEL and HEL/TGR cells were treated with the indicated concentration of BEZ235 for 24 hours. Following this, the cells were harvested and total cell lysates were prepared. Immunoblot analyses were conducted for the expression levels of p-AKT, AKT, p-FOXO3A, FOXO3A, p27, BIM, p-4EBP1, 4EBP1, and β-actin in the cell lysates. The numbers beneath the bands represent densitometry conducted on representative immunoblots relative to the control cells.

Discussion

In the present study, we determined that treatment with the dual PI3K/mTOR inhibitor BEZ235 induces apoptosis and synergistically interacts with JAK2 inhibitors TG101209 and SAR302503 against cultured and primary MF-MPN progenitor cells. Previous in vitro studies in myeloid and other transformed cells have shown that BEZ235 inhibits both mTORC1 and mTORC2 activity, as well as abrogates the feedback activation of the cytokine or growth factor-mediated PI3K/AKT, which results following treatment with rapamycin and the other "rapalogs" that allosterically inhibit the activity of the TORC1 complex of the mTOR kinase (16, 44). This has been documented to dampen the antitumor activity of rapalogs by activating the progrowth and prosurvival activity of AKT (44). The mechanism underlying this activation has been shown to be through derepression of upstream receptor tyrosine kinase signaling through TORC1-S6K1-IRS1- and TORC1-GRB10–mediated mechanisms (44–47). Our findings show for the first time in MPN HPCs that, while inhibiting the phosphorylation of mTORC1 targets p70S6K and 4EBP1, treatment with BEZ235 was also able to inhibit the upstream p-AKT and its target, p-PRAS40 levels (16). AKT-mediated phosphorylation of PRAS40 inhibits its function as an mTORC1 inhibitor (48). BEZ235-mediated inhibition of AKT and FOXO3A was associated with induction of p27 and BIM, resulting in cell-cycle growth inhibition and loss of survival of MPN HPCs (49). These findings are consistent with the previous reports showing similar effects of BEZ235 on p-AKT and p-FOXO3A levels, as well as on inducing apoptosis in AML cells (37). It is also noteworthy that the sensitivity to BEZ235-induced apoptosis was markedly higher in BaF3-hEpoR-hJAK2V617F versus BaF3-hEpoR cells, indicating that the presence of JAK2-V617F increases the susceptibility of the cytokine-dependent marrow progenitor cells to BEZ235. Treatment with BEZ235 also dose dependently depleted the phosphorylation of 4EBP1 at Thr 37 and Thr 46 in the MPN HPCs. As 4EBP1 phosphorylation is known to be the rate-limiting step for the formation of translation-initiating complexes and cap-dependent translation, BEZ235 treatment could also be causing global inhibition of protein synthesis in MPN cells (16, 37, 44).

Figure 7. JAK2-TKI-resistant HEL/TGR cells exhibit collateral sensitivity to BEZ235-mediated inhibition of PI3K/AKT and mTOR signaling and induction of apoptosis. A and B, HEL and HEL/TGR cells were treated with the indicated concentrations of TG101209 or BEZ235 for 48 hours. At the end of treatment, cells were washed with 1× PBS and stained with Annexin V and TO-PRO-3 iodide. The percentages of Annexin V-positive, apoptotic cells were determined by flow cytometry. Columns represent the mean of 3 independent experiments; bars represent the SEM. C, HEL and HEL/TGR cells were treated with the indicated concentration of BEZ235 for 24 hours. Following this, the cells were harvested and total cell lysates were prepared. Immunoblot analyses were conducted for the expression levels of p-AKT, AKT, p-FOXO3A, FOXO3A, p27, BIM, p-4EBP1, 4EBP1, and β-actin in the cell lysates. The numbers beneath the bands represent densitometry conducted on representative immunoblots relative to the control cells.
Previous studies had shown that treatment with JAK2 inhibitors not only attenuates the levels of p-STAT3 and p-STAT5 but also reduces the levels of p-AKT and p-ERK1/2 in MF-MPN cells. Our findings here show that combined treatment with BEZ235 and TG101209 caused attenuation of not only p-STAT3, p-AKT, and p-ERK1/2 but also mediated a greater decline in the levels of p-4EBP1 in MF-MPN cells. Consistent with this, cotreatment with BEZ235 and TG101209 or SAR302503 synergistically induced apoptosis in cultured and primary MPN cells. The mechanism underlying the synergistic activity of cotreatment with BEZ235 and JAK2-TKI SAR302503 or TG101209 may be the JAK2 TKI-mediated attenuation of the progrowth and prosurvival signaling through STAT5 and STAT3, as well as the BEZ235-mediated abrogation of mTOR and AKT signaling with downregulation of p-4EBP1 and greater induction of p27 and BIM, respectively. Cotreatment with BEZ235 and TG101209 or SAR302503 caused greater downregulation of AKT activity, which led to the activation of FOXO3A that is responsible for the induction of p27 and BIM (49). Recently, JAK2 was shown to directly bind and phosphorylate p27 resulting in its inactivation (51). Consequently, TG101209 or SAR302503-mediated inhibition of JAK2 may also directly activate p27, thereby contributing to growth inhibition and apoptosis of MF-MPN cells. It is noteworthy that synergistic activity of combined treatment with BEZ235 and JAK2 inhibitor was relatively specific towards CD34+ MF-MPN cells while sparing normal CD34+ bone marrow progenitor cells. This raises the possibility that the combination may be relatively safe and not exert in vivo toxicity against normal bone marrow progenitor cells.

Superior lethal activity against MPN HPCs also suggests that the combination of BEZ235, or the other new generations of mTOR inhibitors, and JAK2 inhibitors may also have an impact on the allelic burden of JAK2-V617F and potentially reduce the risk of emergence of secondary resistance to JAK2-TKI (52–54). We have previously reported that, compared with the control HEL cells, HEL/TGR cells are highly resistant to the apoptotic effects of JAK inhibitor TG101209 (42). This is explained by the much higher levels of p-JAK2, p-STAT5, p-AKT, and p-FOXO3A, but lower levels of BIM, in HEL/TGR versus HEL cells, which were not reversed by treatment with the JAK inhibitor. Our findings from present studies show that HEL/TGR cells are collaterally sensitive to BEZ235, although combined effects of BEZZ35 with JAK inhibitor were not superior to treatment of HEL/TGR cells with BEZ235 alone. On the basis of their high levels of p-JAK2 and p-AKT, HEL/TGR cells seem to be addicted to the progrowth and prosurvival signaling through PI3K/AKT/mTOR. This could be the basis of the collateral sensitivity to BEZ235 in the HEL/TGR cells, such that BEZ235 induced more apoptosis of JAK-TKI-resistant HEL/TGR versus the parental HEL cells. Treatment with BEZ235 was able to overcome the molecular mechanisms of resistance by reducing the levels of p-AKT, p-FOXO3A, and inducing BIM levels, thereby exerting lethal activity against HEL/TGR cells. As these cells were generated under the selection pressure of continuous exposure to TG101209, it is possible that the secondary resistance mechanisms documented in HEL/TGR cells may be clinically relevant in a setting where JAK2-TKI will be administered over many months, as was recently reported (54). This makes the clinical implications of the collateral sensitivity of HEL/TGR cells to BEZ235 particularly notable and relevant. Overall, these findings support a compelling rationale to test the in vivo activity of BEZ235-based combinations with JAK-TKI against MF-MPN HPCs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: S. Verstovsek, T. Manshouri, K.N. Bhalla
Development of methodology: T. Manshouri
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. McGuirk, S. Verstovsek, T. Manshouri, J. E. Smith, K. N. Bhalla
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Fiskus, J. E. Smith, S. Abhyankar
Writing, review, and/or revision of the manuscript: W. Fiskus, S. Verstovsek, K. N. Bhalla
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Verstovsek, J. McGuirk
Study supervision: K. N. Bhalla

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 5, 2012; revised January 8, 2013; accepted February 7, 2013; published OnlineFirst February 27, 2013.

References


Dual PI3K/AKT/mTOR Inhibitor BEZ235 Synergistically Enhances the Activity of JAK2 Inhibitor against Cultured and Primary Human Myeloproliferative Neoplasm Cells

Warren Fiskus, Srdan Verstovsek, Taghi Manshouri, et al.

Mol Cancer Ther 2013;12:577-588. Published OnlineFirst February 27, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-12-0862

Supplementary Material  Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2013/03/13/1535-7163.MCT-12-0862.DC1

Cited articles  This article cites 52 articles, 18 of which you can access for free at: http://mct.aacrjournals.org/content/12/5/577.full.html#ref-list-1

Citing articles  This article has been cited by 8 HighWire-hosted articles. Access the articles at: /content/12/5/577.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.