Dual Inhibition of Bcl-2 and Bcl-xL Strikingly Enhances PI3K Inhibition-Induced Apoptosis in Human Myeloid Leukemia Cells through a GSK3- and Bim-Dependent Mechanism

Mohamed Rahmani1, Mandy Mayo Aust1, Elisa Attkisson1, David C. Williams Jr5, Andrea Ferreira-Gonzalez5, and Steven Grant1,2,3,4

Abstract

Effects of concomitant inhibition of the PI3K/AKT/mTOR pathway and Bcl-2/Bcl-xL (BCL2L1) were examined in human myeloid leukemia cells. Tetracycline-inducible Bcl-2 and Bcl-xL dual knockdown sharply increased PI3K/AKT/mTOR inhibitor lethality. Conversely, inducible knockdown or dominant-negative AKT increased, whereas constitutively active AKT reduced lethality of the Bcl-2/Bcl-xL inhibitor ABT-737. Furthermore, PI3K/mTOR inhibitors (e.g., BEZ235 and PI-103) synergistically increased ABT-737-mediated cell death in multiple leukemia cell lines and reduced colony formation in leukemic, but not normal, CD34+ cells. Notably, increased lethality was observed in four of six primary acute myelogenous leukemia (AML) specimens. Responding, but not nonresponding, samples exhibited basal AKT phosphorylation. PI3K/mTOR inhibitors markedly downregulated Mcl-1 but increased Bim binding to Bcl-2/Bcl-xL; the latter effect was abrogated by ABT-737. Combined treatment also markedly diminished Bax/Bak binding to Mcl-1, Bcl-2, or Bcl-xL. Bax, Bak, or Bim (BCL2L11) knockdown or Mcl-1 overexpression significantly diminished regimen-induced apoptosis. Interestingly, pharmacologic inhibition or short hairpin RNA knockdown of GSK3α/β significantly attenuated Mcl-1 downregulation and decreased apoptosis. In a systemic AML xenograft model, dual tetracycline-inducible knockdown of Bcl-2/Bcl-xL sharply increased BEZ235 antileukemic effects. In a subcutaneous xenograft model, BEZ235 and ABT-737 coadministration significantly diminished tumor growth, downregulated Mcl-1, activated caspases, and prolonged survival. Together, these findings suggest that antileukemic synergism between PI3K/AKT/mTOR inhibitors and BH3 mimetics involves multiple mechanisms, including Mcl-1 downregulation, release of Bim from Bcl-2/Bcl-xL as well as Bak and Bax from Mcl-1/Bcl-2/Bcl-xL, and GSK3α/β, culminating in Bax/Bak activation and apoptosis. They also argue that combining PI3K/AKT/mTOR inhibitors with BH3 mimetics warrants attention in AML, particularly in the setting of basal AKT activation and/or addiction. Cancer Res; 73(4); 1340–51. ©2012 AACR.
and Bcl-xL but not Mcl-1 (8). Preclinical studies have shown that ABT-737 induces apoptosis and potentiates the antitumor activity of multiple agents in various cancers, including leukemia (8). ABT-263, a clinical derivative of ABT-737, is currently undergoing phase I and II clinical evaluation in various tumor types including leukemia (9).

Recent studies indicate that PI3K inhibitors efficiently downregulate Mcl-1; an event that plays an important role in transformed cell lethality (10–12). Furthermore, data from several laboratories, including our own, indicate that Mcl-1, as well as Bim, which is also tightly regulated by the PI3K pathway (13, 14), play important roles in determining ABT-737 sensitivity (15–18). These considerations, together with the evidence of a contribution of Bcl-2 and Bcl-xL dysregulation in leukemogenesis (7), raise the possibility that interference with Bcl-2 and Bcl-xL function might potentiate PI3K inhibitor activity in this disease. The purpose of the present studies was to determine whether, and by what mechanisms, dual inhibition of Bcl-2/Bcl-xL might cooperate with PI3K/mTOR inhibition to induce cell death in AML cells.

Materials and Methods
Cells
Human leukemia U937, KG1, and MV4-11 cells were purchased from American Type Culture Collection (ATCC) and cultured as described earlier (11). These cells were authenticated by ATCC (basic short tandem repeat profiling) at the end of the studies. Various stable or inducible cell lines used in these studies are described in Supplementary Materials.

Isolation of patient-derived leukemic blasts and normal CD34+ cells
Bone marrow or peripheral blood were collected from patients with acute myeloblastic leukemia (AML), FAB subtype M2, with a preponderance of blasts, and further enrichment of mononuclear cell populations achieved by Ficoll–Hypaque gradient separation as previously described (19). Normal bone marrow CD34+ cells were obtained with informed consent from patients undergoing routine diagnostic procedures for nonmyeloid hematopoietic disorders as before (20). These studies have been sanctioned by Virginia Commonwealth University Institutional Review Board.

Mutation analysis
Mutation analysis was conducted on genomic DNA extracted from primary blasts as previously described (11).

Reagents
ABT-737 was provided by Abbott laboratories (Abbott Park). BEZ235 was purchased from Chemie Tek. PI-103, LY294002, GSK3 inhibitor IX (2’E,3’E)-6-bromoindirubin-3’-oxime (BIO), and its inactive analogue MeBIO were purchased from Calbiochem. CHIR-98014 was purchased from Sellek chemicals. MK-2206 was provided by Merck.

Assessment of apoptosis
Apoptosis was routinely assessed by Annexin V/propi- dium iodide (PI) analysis as previously described (21). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis was also used in some experiments as described earlier (22), and visualized by confocal microscopy.

Cell growth and viability
Cell growth and viability were assessed by CellTiter-Glo Luminescent Assay (Promega).

Clonogenicity
Colony-formation assays were conducted in methylcellulose as previously described (11).

Immunoprecipitation and immunoblotting
Immunoprecipitation and immunoblotting were conducted as previously described (19, 21). Antibodies are listed in Supplementary Materials.

Bax and Bak conformational change
Bax and Bak conformational change was assessed as previously described (11).

Subcellular fractionation
Cytosolic and membrane fractions were separated as previously described (19).

In vivo studies
Animal studies were conducted under an approved protocol by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Two murine models were used:

1) Systemic xenograft model: female NOD/SCID-γ (Jackson laboratories) were injected intravenously via tail vein with 5 × 10⁶ luciferase-expressing U937 cells in which double knockdown of Bcl-2 and Bcl-xL is achieved by doxycycline. Mice were monitored for tumor growth using the IVIS 200 imaging system (Xenogen Corporation), separated into 2 groups, one of which was fed with doxycycline-supplemented pellet (200 mg/kg, Bio-Serv). Both groups were treated every 24-hour 6 days a week with BEZ235 administered by gavage.

2) Subcutaneous model: female athymic nude mice (Charles River laboratories) were inoculated subcutaneously in the flank with 2.5 × 10⁶ U937 cells. Once tumors became apparent, mice were treated twice daily 5 days per week with BEZ235 ± ABT-737 administered intraperitoneally. Tumor volumes were calculated using the formula (length × width²)/2, and when tumor length reached 2 cm, mice were euthanized.

Statistical analysis
The significance of differences between experimental conditions was determined using the Students t test for unpaired observations. Survival rates were analyzed by Kaplan–Meier and comparisons of survival curves and median survival were analyzed by log-rank test.

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Results

**Ectopic expression of Bcl-2 or Bcl-xL increases leukemia cell resistance to apoptosis induced by inhibitors of the PI3K/AKT pathway**

To test the hypothesis that Bcl-2 and Bcl-xL confer resistance to PI3K/AKT pathway inhibition in leukemia cells, U937 cells ectopically overexpressing Bcl-2 or Bcl-xL were used. These cells displayed significant resistance to the dual PI3K/mTOR inhibitors BEZ235 and PI-103, and the AKT inhibitor perifosine (Supplementary Fig. S1A), as well as the PI3K inhibitor LY294002 (data not shown), raising the possibility that Bcl-2 and Bcl-xL inhibition may potentiate leukemia cell apoptosis induced by PI3K/AKT/mTOR pathway inhibitors.

**Dual knockdown of Bcl-2 and Bcl-xL strikingly potentiates PI3K inhibitor-mediated apoptosis in U937 cells**

To determine whether Bcl-2 and Bcl-xL disruption in U937 cells enhances PI3K-mediated apoptosis, inducible knockdown of Bcl-2 or Bcl-xL or both was carried out using a tetracycline-inducible short hairpin RNA (shRNA) lentiviral system. Time course analysis (Fig. 1A, top) revealed that doxycycline induced a sharp decline in levels of Bcl-2, Bcl-xL, or both in the corresponding cells, which was observed after 48-hour exposure and persisted during the ensuing 48 hours. Interestingly, while individual knockdown of Bcl-2 or Bcl-xL modestly but significantly increased BEZ235, PI-103, or MK-2206 lethality following 6- to 24-hour exposure (Fig. 1A; bottom), rapid and striking (e.g., 3 hours) apoptosis was observed in cells in which both Bcl-2 and Bcl-xL were knocked down (Fig. 1A; bottom). Similar results were obtained with LY294002 (data not shown) and when cell growth and viability were monitored (Supplementary Fig. S1B). These events were associated with pronounced Bac and Bak conformational changes (e.g., by PI-103; Fig. 1B, top), reflecting activation, rapid, and profound cytosolic release of cytochrome c and AIF (Fig. 1B, bottom), loss of mitochondrial membrane potential (by PI-103 and BEZ235; Fig. 1C), and apoptosis, reflected by caspase-3, -9, and PARP cleavage (Fig. 1D). In contrast, agents exhibited minimal effects in the absence of doxycycline (Fig. 1A–D). Notably, AKT was rapidly dephosphorylated/inactivated by PI3K/mTOR inhibitors (e.g., BEZ235 or PI-103) with or without doxycycline (Fig. 1D). Conversely, inducible expression of DN-AKT or shAKT knockdown significantly increased, whereas constitutively active AKT-DD significantly decreased ABT-737 lethality, reflected by caspase-3/PARP cleavage and Annexin V/PI positivity (Supplementary Fig. S2A–C). Collectively, these findings indicate that the PI3K/AKT/mTOR pathway and Bcl-2/Bcl-xL interact coordinately to promote cell survival, suggesting that simultaneous targeting of these pathways may represent an effective antileukemic strategy.

**Cotreatment with PI3K/mTOR inhibitors and ABT-737 strikingly induces apoptosis in human leukemia cells**

Dose–response analysis of U937 cells revealed that while 0.5 μm/L ABT-737 was minimally toxic by itself, it
substantially increased cell death induced by BEZ235 (e.g., 50–1,000 nmol/L; Fig. 2A) or PI-103 (e.g., 1–4 μmol/L; Fig. 2B). Conversely, marginally toxic concentrations of BEZ235 (0.5 μmol/L) or PI-103 (3 μmol/L) sharply increased ABT-737 lethality (e.g., 0.25–2 μmol/L; Fig. 2C). Time course analysis of cells exposed simultaneously to BEZ235 (0.5 μmol/L) and ABT-737 (0.5 μmol/L) revealed approximately 20% cell death at 8 hours, and substantially more pronounced lethality after 18 hours (70%, Supplementary Fig. S3). Similar results were obtained in multiple other leukemia cell lines, including FLT3-ITD-dependent MV4-11 and KG1 cells (Fig. 2D). Treatment of U937 cells with BEZ235 and ABT-737 induced pronounced cytosolic release of cytochrome c and AIF, membrane translocation of Bax (Fig. 2E), and cleavage/activation of caspases-3, and -9, and PARP (Fig. 2F). In contrast, BEZ235 or ABT-737 alone had only minimal effects (Fig. 2E and F).

Cotreatment with BEZ235 and ABT-737 sharply increases lethality and reduces colony formation of primary AML blasts while largely sparing normal CD34+ cells

TUNEL assays of primary AML blasts revealed that simultaneous, but not individual, administration of BEZ235 and ABT-737 markedly increased apoptosis (Fig. 3A), associated with marked AKT dephosphorylation and pronounced caspase-3 and PARP cleavage (Fig. 3B), similar to results obtained in U937 cells. Moreover, BEZ235/ABT-737 coexposure enhanced cell death in 4 of 6 AML specimens assayed (Fig. 3C). In contrast, comparable BEZ235 and ABT-737 exposures, alone or in combination, exerted only minimal toxicity toward normal CD34+ cells (Fig. 3D). Interestingly, all responding AML samples exhibited discernible basal AKT phosphorylation/activation (Fig. 3E). In contrast, nonresponding specimens (#1 and #6), like normal CD34+ cells, displayed no detectable phospho-AKT (Fig. 3E). Protein levels of Bcl-2, Bcl-xL, Mcl-1, and Bim exhibited considerable variability among the samples analyzed. Importantly, 2 responding samples exhibited high Mcl-1 protein levels. Mutation analysis revealed that 2 of the responding specimens harbored FLT3 mutations, including a point mutation (FLT3/D835) in patient #2 and internal tandem duplication (FLT3/ITD) in patient #3. However, no mutations in PI3Kα, AKT1, Kras, Nras, or MEK1 were detected (Supplementary Table S1).

Parallel colony-forming assays revealed that combined, but not individual, treatment with BEZ235 or LY294002 and ABT-737 strikingly reduced leukemic cell colony formation (Fig. 3F). In contrast, the colony-forming capacity of normal CD34+ cells was not significantly reduced by agents alone or in combination (Fig. 3G).

Mcl-1 downregulation contributes to BEZ235/ABT-737 lethality

BEZ235 significantly reduced Mcl-1 protein levels in U937 cells (Fig. 4A) as well as primary AML blasts (Fig. 4B, top) and KG1 cells (data not shown). Moreover, Mcl-1 down-regulation by BEZ235 persisted in cells cotreated with ABT-737, which by itself increased Mcl-1 protein levels (Fig. 4A), consistent with reports involving other cells (23). Notably, ABT-737 significantly increased Noxa protein levels, but, expression was sharply diminished by treatment with BEZ235 administered alone or in combination with ABT-737 (Fig. 4A). A modest increase in Bax expression was observed following exposure to agents alone or in combination (Fig. 4A). In contrast, no major changes occurred in the expression of other antiapoptotic family members, that is, Bcl-2 or Bcl-xL or proapoptotic members Bim, Bad, Bak, or NBK (Fig. 4A). Notably, increased Mcl-1 protein levels induced by the Bcl-2/Bcl-xL antagonist ABT-737 were recapitulated genetically by dual Bcl-2/Bcl-xL knockdown (Fig. 4B, bottom).

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Finally, U937 cells ectopically expressing Mcl-1 were significantly more resistant than control cells to combined treatment with BEZ235/ABT-737 or PI-103/ABT-737 (Fig. 4C), arguing for a significant functional role for Mcl-1 downregulation in PI3K/mTOR inhibitor/ABT-737 lethality.

**Bax and Bak play important functional roles in BEZ235/ABT-737 antileukemic activity**

Immunoprecipitation studies revealed that combined, but not individual, treatment with BEZ235 and ABT-737 induced pronounced Bax and Bak conformational change (Fig. 4D). Furthermore, the amount of Bax or Bak bound to Mcl-1 sharply declined following 4- to 8-hour treatment with BEZ235 (Fig. 4E and F, respectively). Decreased Bak/Bcl-xL or Bax/Bcl-2 binding was also observed at 8-hour of treatment. In addition, ABT-737 markedly diminished binding of Bak to Bcl-xL and of Bak to both Bcl-2/Bcl-xL, but not to Mcl-1. However, combined treatment with BEZ235/ABT-737 sharply reduced Bak and Bax binding to all antiapoptotic members Bcl-2, Bcl-xL, and Mcl-1 (Fig. 4E and F).

Finally, Bax or Bak knockdown significantly protected cells from BEZ235/ABT-737 lethality, reflected by diminished caspase-3 and PARP cleavage (Supplementary Fig. S4A) and Annexin V/PI positivity (Fig. 4G). Together, these findings have shown that BEZ235 and ABT-737 exposure releases Bak and Bax from the major neutralizing molecules Bcl-2, Bcl-xL, and Mcl-1, and that Bax and Bak play significant functional roles in regimen antileukemic activity.

**BEZ235/ABT-737–mediated apoptosis involves Bim but not Bad**

Immunoprecipitation studies revealed that while BEZ235 reduced the amount of Mcl-1 bound to Bim presumably reflecting Mcl-1 downregulation, it markedly increased Bim binding to Bcl-2 and Bcl-xL (Fig. 5A). Significantly, these effects were essentially abrogated by ABT-737 cotreatment.
Similar events occurred with LY29402 and PI-103 (Supplementary Fig. S4B). Notably, U937 Bim knockdown cells displayed marked resistance to combined treatment with ABT-737 and BEZ235 or PI-103 compared with controls (Fig. 5B and C). Similar results were obtained in Bim knockdown MV4-11 cells (Supplementary Fig. 4C). In contrast, Bad knockdown cells were fully sensitive to these treatments (Fig. 5C). Comparable results were observed when ABT-737 was combined with LY294002 (data not shown). In accordance with evidence of a significant role for Bim and Bax/Bak in BEZ235/ABT-737 lethality, ectopic expression of Bcl-2 or Bcl-xL, which, like Mcl-1 sequester Bim, and Bax/Bak (24, 25), significantly protected cells against ABT-737 and BEZ235 or PI-103 lethality (Supplementary Fig. S5).

**GSK3α/β plays a functional role in BEZ/ABT lethality**

To determine whether GSK3α/β, a major downstream target of AKT, contributed to the lethality of concomitant
PI3K pathway and Bcl-2/Bcl-xL inhibition, multiple approaches were used. Exposure to BEZ235 alone or with ABT-737 sharply decreased β-catenin and c-Myc, 2 well-established GSK3α/β substrates, indicating GSK3α/β activation (refs. 26, 27; Fig. 6A). A modest decrease in GSK3 phosphorylation also occurred after BEZ235/ABT-737 treatment. Notably, the GSK3 inhibitors CHIR-98014 or BIO, but not the inactive analogue MeBIO, significantly attenuated BEZ235 antileukemic activity in Bcl-2/Bcl-xL doxycycline knockdown cells (Fig. 6B). Furthermore, BEZ235/ABT-737 lethality was also significantly reduced by CHIR-98014 or BIO, but not MeBIO (Fig. 6C). Of note, these GSK3 inhibitors alone exhibited no lethal effects (Supplemental Fig. 6A). Similar results were obtained when cell growth and viability were assessed (Supplemental Fig. 6B). Interestingly, effects of GSK3 inhibitors on BEZ235/ABT-737 lethality correlated with significant attenuation of Mcl-1 downregulation, complete reversal of β-catenin and c-Myc downregulation, and substantially decreased caspase-3 activation by CHIR-98014 or BIO, but not by MeBIO (Fig. 6D).

Parallel studies revealed that GSK3α or GSK3β shRNA knockdown also significantly reduced BEZ235/ABT-737-mediated caspase-3 and PARP cleavage (Fig. 6E), and cell death (Fig. 6F). Notably, these effects were associated with attenuation of Mcl-1 downregulation (Fig. 6E). Together, these findings argue for a functional role for GSK3α/β activation in lethality induced by concurrent Bcl-2/Bcl-xL and PI3K pathway inhibition, and implicate Mcl-1 downregulation/degradation in this phenomenon.

**Concomitant PI3K and Bcl-2/Bcl-xL inhibition increases apoptosis, inhibits tumor growth, and enhances survival in in vivo leukemia xenograft models**

To determine whether antagonizing Bcl-2 and Bcl-xL functions enhances PI3K inhibition lethality in vivo, a systemic xenograft mouse model using luciferase-labeled U937 cells expressing doxycycline-inducible shRNAs against both Bcl-2 and Bcl-xL was used. Interestingly, BEZ235 significantly reduced in vivo tumor growth following dual Bcl-2/Bcl-xL knockdown compared with control tumors with intact Bcl-2/Bcl-xL (Fig. 7A), associated with prolonged median survival, from 13 to 23 days (Supplementary Fig. S7A). Survival curves also differed significantly; P < 0.03 by log-rank test. No effects of doxycycline alone on tumor growth or survival were observed (not shown).

Parallel studies using a subcutaneous U937 xenograft mouse model revealed that ABT-737/BEZ235 cotreatment significantly reduced tumor growth (Fig. 7B and C and Supplementary Fig. S7B). In contrast, effects were less pronounced with BEZ235 and no effect was observed with
ABT-737. Western blot analysis conducted on excised tumor tissue from treated animals revealed that BEZ235 alone or with ABT-737 markedly decreased Mcl-1 protein levels and AKT phosphorylation, analogous to in vitro observations (Fig. 7D). Interestingly, combined, but not individual, treatment sharply potentiated apoptosis manifested by a pronounced increase in caspase-3 processing and PARP cleavage (Fig. 7D). Finally, Kaplan–Meier analysis (Fig. 7E) revealed that combined treatment significantly prolonged survival (*P < 0.01 versus single-agent treatment; log-rank test), whereas no effect was observed with ABT-737, and only a modest prolongation with BEZ235. Notably, mouse weights did not exhibit major changes (e.g., > 10%) in mice treated with agents alone or in combination (Supplementary Fig. S7C). Together, these findings indicate that combined treatment with BEZ235 and ABT-737 significantly inhibits tumor growth in vivo in association with diminished AKT phosphorylation, Mcl-1 downregulation, apoptosis induction, and prolonged survival in leukemia-bearing mice.

Discussion

Preclinical studies have shown that AML cells are susceptible to PI3K inhibitors (28, 29). However, as in the case of other targeted agents, interruption of a single signaling pathway may be insufficient to induce cell death (30). Strategies to enhance the antitumor activity of PI3K inhibitors include simultaneously interrupting the MEK/ERK pathway (11), inhibiting histone deacetylases (31, 32), or cyclin-dependant kinases (33) among others. Here, dual inhibition of Bcl-2 and Bcl-XL by the BH3-mimetic ABT-737 was used to potentiate PI3K inhibitor antileukemic activity based on several considerations. First, Bcl-2 family members are frequently dysregulated in transformed cells, for example, overexpression of Bcl-2, Bcl-XL, or Mcl-1 (7), and/or diminished expression/loss of Bim, Bax, Noxa, and Bik (34–36). Second, PI3K inhibitors disrupt the balance between the pro- and antiapoptotic Bcl-2 members via downregulation of Mcl-1 and upregulation/activation of Bak, Bax, Bim, and Bad (10). Third, these events, particularly...
Mcl-1 downregulation, play important roles in determining sensitivity to Bcl-2/Bcl-xL inhibitors (e.g. ABT-737: refs. 15–17).

The observation that tetracycline-inducible dual knockdown of Bcl-2 and Bcl-xL sharply increases, whereas ectopic expression significantly diminishes PI3K inhibitor lethality indicate that these antiapoptotic proteins play critical functional roles in protecting leukemic cells from lethality triggered by PI3K pathway interruption. Conversely, evidence that expression of tetracycline-responsive dominant-negative or shRNA constructs against AKT substantially increase, whereas constitutively active AKT decrease ABT-737 lethality argues that the PI3K/AKT pathway plays an important functional role in protecting leukemia cells from BH3 mimetic-induced cell death. Notably, individual Bcl-2 or Bcl-xL knockdown also potentiated PI3K/AKT inhibitor lethality, but enhancement was significantly less than for dual Bcl-2 and Bcl-xL knockdown. Such findings suggest that Bcl-2 and Bcl-xL cooperate to prevent apoptosis induced by PI3K/AKT pathway activation. Consistent with this notion, simultaneous pharmacologic inhibition of the PI3K/AKT pathway (e.g., by BEZ235) and Bcl-2/Bcl-xL (e.g., by ABT-737) recapitulated the lethality of genetic interventions, and markedly reduced colony formation of multiple AML cell lines and primary blast specimens. Importantly, this regimen exerted only modest toxicity toward normal hematopoietic progenitors (CD34+). This may reflect the preferential toxicity of BH3 mimetics such as ABT-737 toward transformed cells (8, 15), as well as the dependence of leukemic cells on PI3K/AKT activation for survival (2).

Between 50% and 80% of patient-derived AML cells display high basal levels of Mcl-1, an antiapoptotic protein implicated in leukemogenesis (37, 38), raising the possibility that this strategy may be effective in AML characterized by high Mcl-1 expression.

The ability of dual PI3K/mTOR inhibitors such as BEZ235 or PI-103 to downregulate Mcl-1 in leukemia cells is consistent with results involving other cell types (39). Classically, this effect has been attributed to multiple mechanisms: (i) transcriptional, via inhibition of the CREB transcription factor (40), (ii) translational, via inhibition of the mTOR/p70S6K/4EBP axis (41), and (iii) posttranslational, through enhanced Mcl-1 proapoptotic degradation (42, 43). The finding that ectopic Mcl-1 expression significantly attenuated PI3K/mTOR inhibitor/ABT-737 lethality argues that Mcl-1 downregulation contributes functionally to cell death. This is supported by the observation that ABT-737 administration or Bcl-2/Bcl-xL knockdown, increased Mcl-1 protein levels, a phenomenon shown to confer a survival advantage to leukemia cells (17, 23).

Interestingly, while BEZ235 alone downregulated Mcl-1, it did not significantly increase lethality. It is likely that Mcl-1 downregulation cooperates with disruption of Bcl-2 and Bcl-xL function, and potentially Mcl-1-independent effects of BEZ235, to trigger an increase in apoptosis (15–17).

PI3K/AKT pathway inhibition leads to GSK3 activation, which may exert antitumor effects through multiple downstream targets, including Mcl-1, Bim, FOXO-3, β-catenin, and c-Myc, among others (44, 45). The finding that direct inhibition of GSK3 by BIO or CHIR-98014 significantly diminished BEZ235 lethality in cells in which Bcl-2 and Bcl-xL were knocked down, along with diminished BEZ235/ABT-737 lethality in cells in which GSK3 was disabled (e.g., by BIO or CHIR-98014 or by shRNA knockdown) argue that GSK3 acts as a significant functional role in lethality. Because GSK3 promotes Mcl-1 proapoptotic degradation (42, 43), GSK3 knockdown or inhibition may protect cells from BEZ235/ABT-737 lethality by preventing this process. In support of this concept, Mcl-1 downregulation in BEZ235/ABT-737-treated cells was attenuated when GSK3 was inhibited pharmacologically or by shRNA. However, Mcl-1 downregulation was not completely prevented, suggesting alternative mechanism(s) of Mcl-1 regulation. It is recognized that Mcl-1 downregulation may not be the sole mechanism by which GSK3 promotes BEZ235/ABT-737 lethality, and that alternative or additional GSK3 downstream events may contribute to this process. Nevertheless, these data are compatible with the notion that GSK3 contributes functionally to BEZ235/ABT-737 lethality through a mechanism involving Mcl-1 downregulation.

PI3K/mTOR inhibitors and ABT-737 coadministration induced multiple perturbations in the expression of and interactions between Bcl-2 family members, which collectively may have contributed to cell death. Recent studies have highlighted the importance of interactions involving proteins such as Bim, rather than simply expression patterns (e.g., of Mcl-1) in determining cell fate (46). In the present study, PI3K/mTOR inhibitors profoundly modulated Bim distribution manifested by a marked increase of Bim binding to Bcl-2 and Bcl-xL. This phenomenon, which to the best of our knowledge, has not previously been described, could have contributed to cell death attenuation. Significantly, ABT-737 completely abrogated Bim binding to Bcl-2/Bcl-xL, which may have allowed Bim to
activate Bax and Bak, thereby exerting proapoptotic actions. This notion is supported by the observed release of Bak and Bax from major antiapoptotic Bcl-2 members. Specifically, ABT-737 exposure abrogated binding of Bak to Bcl-xL, and Bax to Bcl-2 and Bcl-xL. Moreover, binding of both Bak and Bax to Mcl-1 was also markedly reduced by PI3K inhibitors, presumably a consequence of Mcl-1 downregulation. In this context, Bak and Bax are normally maintained in an inactive state in part by tethering to Bcl-2, Bcl-xL, and Mcl-1, and simultaneous inhibition of these processes dramatically increases cell death (47). It should be noted that while many studies have described a physical binding between Mcl-1 and Bak in diverse cell types (46, 48), other studies reported weak or no binding between these proteins (47, 49). A possible explanation for these discrepancies may reflect the stoichiometric abundance of other Mcl-1 partners (e.g., Noxa, Bak) as well as Bax partners (e.g., Bcl-2 and Bcl-xL) in various cells. Collectively, these findings support a model wherein combined PI3K inhibitor and ABT-737 exposure leads to multiple events promoting cell death including (i) Bim release from Bcl-2/Bcl-xL (by ABT-737) or from Mcl-1 (by PI3K/mTOR inhibitor); (ii) Bak and Bax release from Bcl-2/Bcl-xL/Mcl-1; and (iii) Bax/Bak activation and apoptosis. This interpretation is supported by evidence that Bim, Bak, or Bax shRNA knockdown, or ectopic expression of Mcl-1, Bcl-2, or Bcl-xL significantly diminished PI3K/mTOR inhibitor/ABT-737-mediated lethality. Finally, previous studies have implicated BAD in PI3K inhibitor lethality (50). However, Bad shRNA knockdown, in sharp contrast to Bim, failed to protect leukemia cells from BEZ235/ABT-737 lethality, suggesting that Bad does not play a major role in this setting. Finally, in vivo studies have shown that simultaneous inhibition of Bcl-2/Bcl-xL and the PI3K/AKT pathway markedly inhibited leukemia growth and increased survival in an AML xenograft model in association with several events observed in vitro, that is, AKT dephosphorylation/inactivation, Mcl-1 downregulation, and caspase activation, suggesting that sufficiently high BEZ235 and ABT-737 concentrations can be achieved in vivo to recapitulate in vitro actions. Of note, little toxicity was observed in animals with the doses and schedules used in these studies. The present findings also suggest that multiple factors contribute to these interactions, including Mcl-1 downregulation, perturbations in associations between pro- (e.g., Bim, Bak, and Bax) and anti- (e.g., Bcl-2/Bcl-xL/Mcl-1) proteins, and raise the possibility that basal AKT activation status may predict susceptibility to this approach. Collectively, these studies argue that strategies using PI3K/mTOR inhibitors to enhance the antileukemic activity of BHS-mimetics such as ABT-737 warrants attention in AML, particularly in the setting of disease in which cells may be addicted to AKT. Accordingly, further efforts to test this concept are currently underway.

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

Authors’ Contributions
Conception and design: M. Rahmani, S. Grant
Development of methodology: M. Rahmani, A. Ferreira-Gonzalez, S. Grant
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.M. Aust, E. Atkinson, J.C. Williams, S. Grant
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Rahmani, A. Ferreira-Gonzalez, S. Grant
Writing, review, and/or revision of the manuscript: M. Rahmani, A. Ferreira-Gonzalez, S. Grant
Study supervision: M. Rahmani, S. Grant

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Mohamed Rahmani, Mandy Mayo Aust, Elisa Attkisson, et al.


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