Evaluation of Apoptosis Induction by Concomitant Inhibition of MEK, mTOR, and Bcl-2 in Human Acute Myelogenous Leukemia Cells

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Abstract

Aberrant activation of multiple signaling pathways is common in acute myelogenous leukemia (AML) cells, which can be linked to a poor prognosis for patients with this disease. Previous research with mTOR or MEK inhibitors revealed cytostatic, rather than cytotoxic, effects in \textit{in vitro} and \textit{in vivo} AML models. We evaluated the combination effect of the mTOR inhibitor AZD8055 and the MEK inhibitor selumetinib on human AML cell lines and primary AML samples. This combination demonstrated synergistic proapoptotic effects in AML cells with high basal activation of MEK and mTOR. We next incorporated the BH3 mimetic ABT-737 into this combination regimen to block Bcl-2, which further enhanced the apoptogenic effect of MEK/mTOR inhibition. The combination treatment also had a striking proapoptotic effect in CD33⁺/CD34⁺ AML progenitor cells from primary AML samples with NRAS mutations. Mechanistically, upregulation of the proapoptotic protein Bim, accompanied by the downregulation of the antiapoptotic protein Mcl-1 (mainly via protein degradation), seemed to play critical roles in enhancing the combination drug effect. Furthermore, the modulation of survivin, Bax, Puma, and X-chromosome-linked inhibitor of apoptosis protein (XIAP) expression suggested a role for mitochondria-mediated apoptosis in the cytotoxicity of the drug combination. Consequently, the concomitant blockade of prosurvival MEK/mTOR signaling and the deactivation of Bcl-2 could provide a mechanism-based integrated therapeutic strategy for the eradication of AML cells. \textit{Mol Cancer Ther; 13(7);} 1–12. ©2014 AACR.

Introduction

Aberrant gene expression/signaling pathways like the RAS/Raf/MAPKMEK/ERK and the PI3K/Akt/mTOR pathways can promote tumorigenesis in many tissues, including the hematopoietic system. Indeed, the deregulation of RAS/Raf/MEK/ERK or PI3K/Akt/mTOR signaling has been shown to contribute to leukemogenesis (1–3), and the NRAS-activating mutations, which normally stimulate RAF/MEK/ERK and PI3K/AKT/mTOR, are found in about a third of hematopoietic malignances, including acute myelogenous leukemia (AML; ref. 1). Furthermore, we have previously reported that the simultaneous activation of the aforementioned signaling pathways is consistently associated with poor disease prognosis in patients with AML (4).

In several studies by our group and others, the MEK inhibitors CI1040 and selumetinib (also known as AZD6244) exerted mainly cytostatic effects in \textit{in vitro} and \textit{in vivo} AML models. This was also the case for the mTOR inhibitors CCI779 and RAD001 (5–7). Thus, interrupting one of these signaling pathways independently seems to be insufficient to trigger cell death in AML cells (5, 8, 9). The B-cell lymphoma 2 (Bcl-2) family of proteins are key regulators of cancer cell apoptosis. The antiapoptotic Bcl-2 proteins Bcl-2, Bcl-xL, and myeloid cell leukemia sequence 1 (Mcl-1) prevent cellular apoptosis via their expression and dimerization with the proapoptotic proteins Bim and Bax. The overexpression of the antiapoptotic Bcl-2 proteins correlates with an overall lower survival rates for patients with AML (10, 11). Several small-molecule Bcl-2 inhibitors have been developed, and they have shown encouraging single-agent activity in preclinical and clinical trials (12, 13). We hypothesize that the concomitant blockade of the MAPK and mTOR signaling, in conjunction with interference with antiapoptotic Bcl-2 family members, could promote marked cytotoxic activity in AML cells, including AML stem cells. The purpose of this study was to determine...
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summarized in Supplementary Table S1.

manufacturer’s instructions (Applied Biosystems). Genetic
using the AmpFlSTR Identifier Kit according to the man-
temember 2010 by short tandem repeat DNA fingerprinting
MOLM13, and OCI/AML3 cells were validated in Sep-
von Mikroorganismen und Zellkulturen. U937, KG-1,
cell line was obtained from the Deutsche Sammlung
was kindly provided by Dr. M. Minden (Princess Mar-
were obtained from the ATCC. The OCI/AML3 cell line
Puma were purchased from CalBiochem.
Bim, cyclin D1, Cdc2, and Cdk4 were purchased from Santa Cruz Biotechnol-
purchased from Upstate; antibodies against ERK2, Cdk2,
apurchased from BD Biosciences; antibody against Bcl-2
inhibitor of apoptosis protein (XIAP), and p27Kip-1 were
Antibodies against Bax, Mcl-1, X-chromosome-linked
antibodies against AKT, S6K, 4E-BP1, Bad, Bid, Bcl-xL,
purchased from Cell Signaling Technology, as were the
4E-BP1 (Thr37/46), p-Bad (Ser136), p-MEK1/2 were
MAPK (ERK1/2)(Thr202/Tyr204), p-AKT(Ser473),
mention reagents are shown in Supplementary Fig. S1.
142886) and AZD8055 were provided by AstraZeneca.
Selumetinib (also known as AZD6244 and ARRY-
Knockdown of Bim or Mcl-1 protein
Peripheral blood and bone marrow samples were
obtained from patients with newly diagnosed, relapsed,
or refractory AML after written informed consent had
been obtained from each patient according to institutional
guidelines of the University of Texas MD Anderson Can-
center. All cells, including those obtained from
peripheral blood and bone marrow samples, were cul-
tured in RPMI 1640 culture medium supplemented with
10% fetal calf serum.

Cell viability and apoptosis assays
Cell viability was assessed using an automated cell
counter employing the Trypan blue dye exclusion meth-
od, and cell apoptosis was examined by flow cytometry
as described previously (16). To evaluate apoptosis
induction by treatment with AZD8055 plus selumetinib
in 2 sets of peripheral blood and bone marrow samples
(with and without NRAS mutations), mononuclear cells
were separated by Ficoll–Hypaque density-gradient
centrifugation (Sigma Chemicals). Apoptosis of bulk
leukemic and leukemic progenitor cells (i.e., gating the
AML cells with CD34+ or CD33+ ) was determined as
described above. Induction of specific apoptosis was
calculated using the following formula: specific apo-
ptosis (%) = 100 (drug-induced apoptosis – spontane-
ous apoptosis)/(100 – spontaneous apoptosis) (see
ref. 17).

Cell proliferation assay
AML cells were treated with the indicated agent for 24
hours, and bromodeoxyuridine (BrdUrd; BD Pharmin-
gen) was added to the medium at the last 45 minutes
of treatment to allow BrdUrd incorporation into newly
synthesized cellular DNA. The cells were harvested and
fixed in 70% cold ethanol, and BrdUrd was labeled with an anti–
BrdUrd-fluorescein isothiocyanate antibody and mea-
ured using flow cytometry (18).

Immunoblot and immunoprecipitation analyses
AML cells were treated with AZD8055, selumetinib,
or ABT-737 alone or in combination as indicated
and then collected for analysis. Semiquantitative immu-
noblotting data were generated using the Scion imaging
software program (Beta version 4.03; Scion; ref. 18).
For the immunoprecipitation studies, AML cells were
lysed and the cell lysates (containing ~0.5 mg of total
protein in each sample) were incubated overnight with
a primary anti–Bcl-2 antibody. Protein A/G PLUS-aga-
rose (Santa Cruz Biotechnology) was added to the lysis
buffer for an additional 4 hours of incubation at 4°C.
Bcl-2 family proteins were resolved by Ficoll–Hypaque density-gradient
and cell apoptosis was examined by flow cytometry

Knockdown of Bim or Mcl-1 protein
Bim protein was knocked down by transfecting siRNAs
(sequence: 5'-GACCCAGAGGUAGACAACUUUGdTdT-
3') and mock control siRNAs (Dharmacon RNAi Technologies) into OCI/AML3 and U937 leukemia cells using an electroporation method (T-solution, X-001 and C-solution, W-001, respectively; Amaxa Biosystems) following the manufacturer's instructions. The final concentration of siRNA in the electroporation buffer was 200 nmol/L.

Mcl-1 protein was knocked down by lentiviral transfection with a gene-specific shRNA transfer vector targeting residues 2421–2440 on RefSeq NM_021960.4. Lentivirus was prepared by cotransfection of HEK293T cells (ATCC) with an equal molar mix of transfer vector and packaging plasmids (psPAX2 and pMD2.G; plasmids 12260 and 12259, respectively, from Addgene) using JetPrime transfection reagent as directed in the manufacturer's instructions (Polyplus). The OCI/AML3 cells underwent lentivirus transfection with Mcl-1 shRNA vector or nonspecific control vector (pLKO.1 TRC control, plasmid 10879; Addgene) and selected with puromycin (Invitrogen) starting at 0.5 µg/mL. Knockdown was verified by immunoblot analysis and real-time PCR.

**Real-time PCR assays**

The OCI/AML3 and MOLM13 cells were treated with the indicated drugs for 6 hours, and total RNA was extracted with Trizol (Life Technologies) following the manufacturer's protocol. cDNA was synthesized as described previously (19), and duplicated TaqMan gene expression assays were carried out in a 25-µL reaction containing the cDNA equivalent of 1.0 ng of total RNA with TaqMan gene expression assays (Life Technologies) for Mcl-1 (Hs03043899_m1), Bim (Hs00708019_s1), or housekeeping gene 18S (Hs03928985_g1) using an ABI model 7900HT fast real-time PCR system. Data were analyzed by the ΔΔCt method using RQ Manager 1.2.1 software (Life Technologies), and the relative abundance of target in treated cells, normalized to that of vehicle control, was calculated by dividing the ΔCt of the sample by the ΔCt of the control and multiplying by 2. Knockdown was confirmed by measuring the phosphorylation of target in treated cells, normalized to that of vehicle control.

**Establishment of NRAS-mutated leukemia cells**

A plasmid harboring the NRAS cDNA (IMAGE:3826638; Open Biosystems) was used as a template to generate the NRAS open reading frame (ORF) flanked by Nhel (5') and XhoI (3') sites by PCR with HiFi Hot Start polymerase (Kapa Biosystems) and the primers TGGTAAAGGTACCCGCATGACTGATCAACTGG (forward) and CCTCGAGTTACATCACCACACATGGCAATCC (reverse). An expression vector of interest was constructed by replacing the ORF for enhanced green fluorescent protein in EGFPc2 with the ORF for NRAS as a Nhel-to-XhoI construction. This expression vector was used to generate the G12D mutant with a QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) and the primers GTG GTG GTG GTT GGA GCA GAT GGT GTG GGG AAA AGC GCA CTG AC (forward) and GTC AGT GCG CTT TTC CCA ACA CCA TCT GCT CCA ACC ACC AC (reverse). Lentiviral transfer vectors were then constructed by inserting the wild-type or mutant G12D between the Nhel and NolI sites of pCDH-CMV-MSC-EF1-Puro (System Biosciences). The vectors were introduced into MV4-11 cells by lentiviral transduction as described (20). Positive cells were selected and maintained with puromycin (up to 1 µg/mL). NRAS activation was confirmed by measuring the phosphorylation level of MEK by immunoblotting.

**Mitochondrial inner transmembrane potential (ΔΨm) analysis**

MV4-11-NRAS-wild-type and MV4-11-NRAS-mutated cells were treated with AZD8055, selumetinib, or ABT-737 alone or in combination as indicated for 24 hours and then collected for staining with CMXRos (300 nmol/L; both from Molecular Probes) for 1 hour at 37°C. The ΔΨm was determined using flow cytometry by measuring CMXRos retention (red fluorescence) while simultaneously adjusting for the mitochondrial mass (green fluorescence as described previously; ref. 21).

**Statistical analyses**

The Student t test was used to analyze immunoblot, cell growth, and apoptosis data. P values ≤ 0.05 were considered statistically significant. The Chou–Talalay method (22) was used to determine combination indices for the agents. A combination index value of 1 indicated an additive effect, <1 synergism, and >1 antagonism. The mean combination index values were calculated at different effect levels (50%, 75%, and 90% effective concentrations). All statistical tests were two-sided, and the results are expressed as the mean ± SD (error bars) of triplicate samples.

**Results**

**Cotargeting of signaling pathways with AZD8055 and selumetinib enhanced the growth-inhibitory effect of either agent alone in AML cells**

Targeted treatment with MEK inhibitor selumetinib or the allosteric mTOR inhibitors CCI779 or RAD001 had only limited effects in most leukemia cell lines in our previous studies (6, 7). Therefore, for the current study, we assessed the effect of combining selumetinib with the novel mTOR kinase inhibitor AZD8055 in AML cell lines. Synergistic effects on cell growth inhibition were observed with OCI/AML3, U937, or MOLM13 (Fig. 1A, Supplementary Fig. S2). AML cells that displayed synergistic effects in response to the combination treatment expressed relatively high levels of p-ERK and p-AKT (Fig. 1B). However, KG-1 cells lack detectable p-ERK and p-AKT and showed an antagonistic effect. Furthermore, the combination treatment notably suppressed the levels of ERK1/2 (Thr202/Tyr204) and...
AKT (Ser473) phosphorylation in the OCI/AML3, MOLM13, and U937 cells, but had no consistent effect on the phosphorylation of AKT Thr308 (Fig. 1C).

We next measured BrdUrd incorporation by flow cytometry to determine whether the combination treatment affected cell-cycle progression. In all lines, cell-cycle progression was modestly impaired by the single and combination agent treatments as there were fewer cells in G1-S phase of the cell cycle compared with the controls (Fig. 1D). Although KG-1 cells lack detectable p-AKT and express only trace levels of p-ERK, approximately 50% of these cells were apparently blocked from entering S-phase by mTOR or combined inhibition compared with controls (Fig. 1D).

The KG-1 cells expressed p-S6K (Ser240/244), which was markedly suppressed by the combination treatment (Fig. 1C), suggesting that S6 protein may be critical in the G1-S-phase transition of the cell cycle. Interestingly, the expression of p-S6K, which is best known as a downstream target of PI3K/mTOR/AKT signaling rather than as a target of one of the MEK/ERK signaling cascades, decreased significantly when MEK signaling was suppressed by selumetinib only in OCI/AML3 and U937 cells, but not in MOLM13 or KG-1 cells.
In contrast, the phosphorylation of eukaryotic initiation factor 4E-binding protein (4E-BP1), a downstream protein activated by mTORC1/S6K signaling (23), was inhibited by AZD8055 but not selumetinib in all four cell lines (Fig. 1C). Upregulation of the cell-cycle inhibitor p27Kip-1 and downregulation of the G1 phase–related checkpoint protein cyclin D1/cdk4 complexes were observed in OCI/AML3, MOLM13, and U937 cells. However, downregulation of cdk2, cdc2, and p-Rb was generally observed in OCI/AML3, MOLM13, and U937 cells, but not in KG-1 cells, suggesting that these proteins may be not specific to the G1–S-phase transition of the cell cycle.

Blockade of mTOR/MEK signaling has synergistic cytotoxic effects that are associated with modulation of proapoptotic Bim and antiapoptotic Mcl-1 proteins

The AZD8055 and selumetinib agent combination produced synergistic proapoptotic effects in OCI/AML3, MOLM13, and U937 cells (Fig. 2A). In addition, in OCI/AML3, MOLM13, and U937 cells, the proapoptotic protein Bim was upregulated, and the antiapoptotic proteins Mcl-1, p-Bad(Ser136), and survivin were downregulated by AZD8055 and even more so by the combination with selumetinib (Fig. 2B). We also observed modulated expression of the p53-regulated proteins.
Puma and Bad in certain leukemia cell lines. However, no notable changes in the expression of Bcl-2, Bcl-xl, or Bax were observed. Of note, the upregulation of Bim expression and downregulation of Mcl-1 expression correlated with cleavage of caspase-3, implying that Bim and Mcl-1 play important roles in apoptosis induction. This combination was seemingly antagonistic in the KG-1 cells (Fig. 2A), which have a low basal level of ERK and AKT activation, and did not affect Mcl-1 expression in these cells (Fig. 2B).

We therefore suspected that the modulation of Mcl-1 and Bim levels might play a key role in the combination treatment–induced apoptosis. Further investigation of the mRNA transcription level showed that the combination upregulated Bim only in MOLM13, but not in OCI/AML3 cells. No statistically significant modulation was observed in Mcl-1 levels (Fig. 2C). We next treated OCI/AML3 cells in the presence of the protein translation inhibitor cycloheximide, which further induced apoptosis in the treatment groups (Fig. 2D). Cycloheximide treatment moderately decreased protein levels of Bim, but not of Mcl-1 after 6-hour exposure (Fig. 2D, inset). On the other hand, suppression of protein degradation with proteasome inhibitors bortezomib or MG132 did not markedly influence the combination-induced cell apoptosis (Supplementary Fig. S3). Taken together, the combination-induced changes of Mcl-1 and Bim protein levels might result from translational, rather than transcriptional or posttranslational degradation, effects.

To further evaluate the roles of Bim and Mcl-1 in apoptosis induction, we silenced Bim expression with siRNA or Mcl-1 expression with shRNA and assessed apoptosis induction after treatment with AZD8055 and selumetinib for 48 hours. The knockdown of Bim markedly reversed the combination effect of apoptosis induction by the treatment with AZD8055 and selumetinib in OCI/AML3 (Fig. 3A) and U937 (Supplementary Fig. S4) cells, whereas the knockdown of Mcl-1 enhanced this effect in OCI/AML3 cells (Fig. 3B). These results suggested that Bim and Mcl-1 play opposite roles in apoptosis induction by this combination treatment in the AML cells.

**Interference with Bcl-2 protein dimerization enhanced apoptotic effects induced by combined AZD8055 and selumetinib in AML cells**

Because the induction of apoptosis in leukemia cells is largely controlled by dimerization of proapoptotic and antiapoptotic proteins (11), we hypothesized that, in addition to the inhibition of mTOR/MEK signaling, the inhibition of binding of Bax or Bim with their respective partners, Bcl-2 and Bcl-xl, would further enhance apoptosis induction. We, and others, have reported previously that the Bcl-2 antagonist ABT-737 can bind to Bcl-2 family members resulting in leukemic cell death (12, 24). In addition, the expression level of the antiapoptotic Mcl-1 protein is inversely correlated with cell sensitivity to ABT-737 due to the inability of the latter to bind and inhibit Mcl-1 (12, 25). We tested the three-drug combination of ABT-737, AZD8055, and selumetinib at low concentrations (0.06, 0.1, and 0.3 μmol/L, respectively), and observed significantly enhanced proapoptotic responses in KG-1, OCI/AML3, and MOLM13 cells compared with ABT-737 only or with the AZD8055/selumetinib combination only (Fig. 4A). In the U937 cells which had the lowest basal level of Bcl-2 protein of the four AML cell lines tested (Fig. 1B), this drug combination did not induce marked apoptosis (Fig. 4A). However, apoptosis induction in the U937 cells could be boosted by increasing the concentrations of AZD8055 to 0.3 μmol/L and selumetinib to 0.9 μmol/L (Fig. 4B), suggesting that at higher doses of the mTOR inhibitors were still capable of inducing apoptosis in leukemic cells possessing a high basal level of p-AKT driven by PTEN deletion. Impressively, the KG-1 cells that were resistant to selumetinib and AZD8055 (Supplementary Figs. S5 and S6) demonstrated enhanced sensitivity to the three-drug combination after a 48-hour treatment (Fig. 4A).
We treated the ABT-737–sensitive KG-1 cells and ABT-737–resistant OCI/AML3 cells with ABT-737 alone or with AZD8055/selumetinib combination to gain an insight into the mechanism of apoptosis induction by the triple-drug combination treatment. An immunoprecipitation assay pull-down of the Bcl-2 complex with anti-Bcl-2 antibody indicated that Bcl-2/Bax heterodimerization was reduced in both cell lines after a 6-hour
ABT-737 treatment, and even more markedly so in cells treated with all the three drugs (Fig. 4C). After a 24-hour combination treatment, we observed the cleavage of caspase-9, caspase-8, caspase-3, and Bid, cytochrome c release, and the localization of Bax to the mitochondria (Fig. 4D). Furthermore, Bax was upregulated resulting in an increased Bax:Bcl-2 ratio, and the expression of Mcl-1 and the XIAP was decreased possibly because of caspase-3–mediated cleavage. These findings suggested that ABT-737 enhances the proapoptotic effect of the AZD8055/selumetinib combination by interfering with Bcl-2 function, thus triggering mitochondria-mediated cell death.

Three-drug combination treatment exerted synergistic proapoptotic effects in AML blast cells with NRAS mutations

We next tested the three-drug combination on peripheral blood or bone marrow cells obtained from patients with AML (the patient cytogeneric characteristics are summarized in Supplementary Table S2). Because RAS mutation is known to activate both the MEK and mTOR pathways, we assessed apoptosis induction in these samples based on their NRAS mutational status. Selumetinib or AZD8055 alone induced modest apoptosis in five of seven samples harboring NRAS mutations (i.e., cases 3, 4, 5, 6, and 7; Fig. 5A), and in one sample with wild-type RAS that had an FLT3-ITD mutation (i.e., case 11; Fig. 5B). By comparison, the combination treatment with both agents enhanced apoptosis in most NRAS-mutated samples (except case 2; Fig. 5A), and in two of the seven NRAS wild-type samples (i.e., cases 11 and 13; Fig. 5B). Both of the latter cases harbored mutations of FLT3. Except for cases 1 and 3, ABT-737 was highly effective in inducing apoptosis in primary AML cells, and the combination of all three agents further induced apoptosis in bulk leukemic, as well as in CD34+/CD33+, cells in 12 of 14 patient samples. Overall, the specific apoptosis induction by the three-agent combination was higher in the CD33+/CD34+ AML progenitor cells of NRAS-mutated AML samples compared with those with NRAS wild type (P < 0.01; Fig. 5C). These results indicate that NRAS-mutated AML progenitor cells might be more dependent on MEK/mTOR signaling than NRAS wild-type cells.

To confirm that these agents effectively blocked the corresponding signaling pathway in primary leukemia cells, we measured the p-ERK and p-AKT levels in one NRAS-mutated AML sample (case 7) using immunoblotting. The results indicated that targeting MEK or mTOR with the corresponding drugs or their combination markedly suppressed phosphorylation of ERK and AKT (Fig. 5D). The pattern of phosphorylation suppression of ERK/AKT correlated with apoptosis induction caused by these inhibitors (Fig. 5A, case 7).

Finally, we investigated the molecular mechanisms governing apoptotic cell death in the NRAS-mutant AML cells. We compared the modulation of the expression of the key apoptotic and signaling proteins in isogenic-paired NRAS wild-type and NRAS-mutated AML cells MV4–11. Compared with wild-type cells, cells with mutant NRAS were considerably more sensitive to treatment with selumetinib and ABT-737, or to the three-drug combination (Fig. 6A). Immunoblotting demonstrated enhanced MAPK signaling in NRAS-mutated cells manifesting as high basal levels of p-ERK, in concert with a high expression of Mcl-1 (Fig. 6B). However, the suppression of p-ERK with selumetinib failed to markedly suppress Mcl-1, and it triggered only a modest induction of apoptosis as shown by the level of cleaved caspase-3. In turn, the combined selumetinib and AZD8055 further diminished the level of Mcl-1 in NRAS-mutant cells, and this combination paired with ABT-737 caused a marked decrease of Mcl-1 protein and triggered a discernible cleavage of caspase-3 (Fig. 6B). Furthermore, at low concentrations of three inhibitors, neither ABT-737 alone nor selumetinib/AZD8055 combination triggered the loss of ΔΨm. However, ABT-737 plus selumetinib and AZD8055 showed a major ΔΨm depolarization, which was even more pronounced in the NRAS-mutant cells (Fig. 6C), suggesting a critical role for mitochondrial-mediated apoptosis in the triple-drug combination–induced leukemia cell death, especially in the cells with NRAS mutations.

Discussion

In this study, we tested the combination effects of the MEK inhibitor selumetinib and the mTOR inhibitor AZD8055 in human AML cells. Simultaneous inhibition of ERK and mTOR signaling by these agents led to an enhanced cytostatic effect in the OCI/AML3, MOLM13, and U937 cells. However, this treatment showed no synergistic effect in the KG-1 cells, which exhibit only trace basal levels of phospho-ERK and -AKT. Interestingly, we noticed that targeting MEK signaling with selumetinib drastically suppressed the p-S6K level in the OCI/AML3 and U937 cells, which was not accompanied by a decrease in the level of p-4E-BP1. The S6K and 4E-BP1 proteins are well-known mTOR substrates that contribute to cell proliferation and survival (26). S6K modulation by MAPK signaling is not well understood, although the PI3K/Akt and Ras/MAPK pathways are known to signal in a parallel manner to regulate Tsc1 and 2, and mediate the phosphorylation of S6K (27). Our data indicate that MEK/ERK signaling in conjunction with mTOR signaling plays a prominent role in regulating S6K activity in AML cells. However, the blockade of S6K activity alone is not the major predictor of cell sensitivity to MEK inhibition as exemplified by the vastly different mean half maximal inhibitory concentrations required for inhibiting the growth of OCI/AML3 cells (0.03 μmol/L; 95% confidence interval, CI, 0.01–0.08 μmol/L) and U937 cells (392 μmol/L; 95% CI, 0.5–2960 μmol/L; Supplementary Fig. S5) despite a similar suppression level of p-S6K.
Conversely, mTOR inhibition not only activated MAPK signaling, which is reportedly dependent on a feedback loop of the S6K/PI3K/Ras pathway (14), but it also relieves the negative feedback loop, resulting in increased phosphorylation of AKT at Thr308 (28). Our findings support the notion that multiple circuitry feedback mechanisms are operational in these cells, and may also explain, at least in part, why the blockade of the AKT/mTOR/S6K axis had limited activity in phase I/II clinical trials in patients with hematologic malignancies (29).

The interaction and balance between pro- and anti-apoptotic Bcl2 family proteins dictate cell fate (30). Bim is a positive effector of apoptosis induction and is involved in mitochondria-mediated apoptosis induction (31). Our previous studies demonstrated that upregulation of Bim expression, which triggers mitochondria-mediated apoptosis, plays a critical role in the antileukemia efficacy of Raf/MEK/ERK inhibitors (32). In the present study, we showed that selumetinib, or both selumetinib and AZD8055, markedly upregulated Bim expression, and that the further addition of ABT-
ABT-737 enhanced this upregulation as well as apoptosis induction. In contrast, silencing the expression of Bim markedly diminished apoptosis induction by the agents.

The level of Mcl-1 is a predictive biomarker for sensitivity to therapy-induced apoptosis induction in a variety of hematopoietic malignancies, and its upregulation was mediated via ERK/Elk-1 or AKT/mTOR signaling (33, 34). Consistent with these reported findings, targeting of MEK or mTOR decreased Mcl-1 expression. Mcl-1 expression was further diminished in the presence of ABT-737, and resulted in enhanced apoptosis induction in AML cells. Moreover, lowering the Mcl-1 level by shRNA enhanced drug-induced apoptosis (Supplementary Fig. S7). Specifically, compared with NRAS wild-type MV4-11 cells, the NRAS-mutant MV4-11 cells expressed a higher basal level of Mcl-1 protein that was subsequently diminished by the combined targeting of MEK, mTOR, and Bcl-2 pathways to trigger apoptosis induction. Thus, the three-drug combination was much more effective against AML cells with NRAS mutations compared with those with wild-type NRAS.

The interaction of Bim with Mcl-1 interferes with the cytochrome c-releasing capability of Bim and protects cells against Bim-mediated apoptosis (35). The downregulation of Mcl-1 expression (or cleavage of Mcl-1) may lead to release of cytochrome c, which then triggers apoptosis. ERK-mediated phosphorylation of Bim EL (i.e., the extra-long spliced form of the Bim protein) promotes its proteasomal degradation (36), and phosphorylation of Mcl-1 slows this turnover (37).

![Figure 6](image-url)
Furthermore, Akt-dependent glucose metabolism and ERK/ELK-1 activation promote Mcl-1 synthesis to maintain cell survival and differentiation (38, 39). Our findings suggest that the simultaneous suppression of PI3K/mTOR signaling and interference with Bcl-2 family protein dimerization are essential for drug-induced death of AML cells with a high AKT expression. A recent study using U937 cells indicated that the combination of PI3K/mTOR inhibitors with ABT-737 abrogated Bim binding to Bcl-2/Bcl-xL, which allowed Bim to activate Bak and Bak to exert proapoptotic activity (40). Recent studies indicate that leukemia stem cells (LSC) are characterized by relatively low levels of reactive oxygen species with an aberrant overexpression of BCL-2. Interestingly, the inhibition of BCL-2 reduced oxidative phosphorylation and selectively eradicated these quiescent LSCs (41). In our study, AML cells with high p-AKT and p-ERK levels (e.g., the MV4-11-NRAS–mutated cells) were efficiently killed by the triple-drug combination. This was accompanied by loss of ΔΨm indicating the high antileukemia potency of this treatment strategy against AML cells with gain-of-function NRAS mutations. This would also suggest a selective potency of this combination for targeting quiescent LSCs.

In summary, cotargeting the MEK and mTOR pathways may be an effective treatment strategy for patients who have AML cells that express high levels of MEK and mTOR, or in patients whose AML cells harbor NRAS mutations. This treatment may be further potentiated using the BH3 mimetic ABT-737. This three-drug combination could pave the way for the development of integrated therapeutic strategies that take advantage of the synergistic antileukemia effects, thereby leading to the design of novel, clinically viable combinations for AML therapy.

Disclosure of Potential Conflicts of Interest
P. Smith has ownership interest in AstraZeneca PLC. No potential conflicts of interest were disclosed by the other authors.

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Evaluation of Apoptosis Induction by Concomitant Inhibition of MEK, mTOR, and Bcl-2 in Human Acute Myelogenous Leukemia Cells

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