Mcl-1 Down-regulation Potentiates ABT-737 Lethality by Cooperatively Inducing Bak Activation and Bax Translocation

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Abstract

The Bcl-2 antagonist ABT-737 targets Bcl-2/Bcl-xL but not Mcl-1, which may confer resistance to this novel agent. Here, we show that Mcl-1 down-regulation by the cyclin-dependent kinase (CDK) inhibitor roscovitine or Mcl-1-shRNA dramatically increases ABT-737 lethality in human leukemia cells. ABT-737 induces Bax conformational change but fails to activate Bak or trigger Bax translocation. Coadministration of roscovitine and ABT-737 unethers Bak from Mcl-1 and Bcl-xL, respectively, triggering Bak activation and Bax translocation. Studies employing Bax and/or Bak knockout mouse embryonic fibroblasts (MEFs) confirm that Bax is required for ABT-737 + roscovitine lethality, whereas Bak is primarily involved in potentiation of ABT-737–induced apoptosis by Mcl-1 down-regulation. Ectopic Mcl-1 expression attenuates Bak activation and apoptosis by ABT-737 + roscovitine, whereas cells overexpressing Bcl-2 or Bcl-xL remain fully sensitive. Finally, Mcl-1 knockout MEFS are extremely sensitive to Bak conformational change and apoptosis induced by ABT-737, effects that are not potentiated by roscovitine. Collectively, these findings suggest down-regulation of Mcl-1 by either CDK inhibitors or genetic approaches dramatically potentiate ABT-737 lethality through cooperative interactions at two distinct levels: unleashing of Bak from both Bcl-xL and Mcl-1 and simultaneous induction of Bak activation and Bax translocation. These findings provide a mechanistic basis for simultaneously targeting Mcl-1 and Bcl-2/Bcl-xL in leukemia.

Introduction

Cell death decisions are regulated by the complex interplay between two groups of Bcl-2 family members: proapoptotic proteins (e.g., multidomain: Bax and Bak; BH3-only: Bad, Bim, Bid, and Noxa) and antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and Bfl-1/A1; refs. 1, 2). In disorders such as leukemia, increased expression of antiapoptotic proteins, such as Bcl-2, is required for disease maintenance (3), confers drug resistance (4), and is associated with poor clinical outcome (5). These observations have prompted the development of small-molecule Bcl-2 inhibitors (refs. 6, 7; e.g., HA14-1) that disable Bcl-2, resulting in induction of apoptosis in leukemia cell lines (8).

Alternative strategies include the use of antisense oligodeoxynucleotides (e.g., G3139; ref. 6) or stabilized forms of BH3 peptides (9), among others. Recently, a novel inhibitor (ABT-737) of Bcl-2, Bcl-xL, and Bcl-w, which is significantly more potent than previous compounds of this type, has been developed. This compound acts by mimicking the capacity of the BH3-only protein Bad to dock to the hydrophobic groove of antiapoptotic Bcl-2 family proteins, thereby diminishing their ability to antagonize apoptosis (10). ABT-737 lowers the apoptotic threshold for chemotherapeutic agents or ionizing radiation and has shown impressive preclinical activity against hematopoietic malignancies as well as solid tumors in vitro and in vivo (10). Recently, ABT-737 was shown to overcome drug resistance (e.g., toward imatinib) in Bcr/Abl+ leukemic cells (11). However, ABT-737 has a low affinity for other antiapoptotic Bcl-2 family proteins (e.g., Mcl-1 and A1; ref. 10) and thus may exhibit limited cytotoxic effects in cells with high endogenous levels of Mcl-1 (12). Moreover, ABT-737 efficiently kills interleukin-3 (IL-3)–dependent cells (e.g., FL5.12) only after IL-3 withdrawal (13), suggesting that additional death signals may be required for lethality. Notably, Mcl-1 is a highly expressed antiapoptotic protein (14) and a critical survival factor for various malignant hematopoietic cells (14, 15). Recent evidence suggests that more than one antiapoptotic protein (e.g., Mcl-1 and Bcl-xL) cooperate to sequester multidomain proapoptotic proteins, such as Bak, thereby preventing its activation (16). Thus, the impaired capacity of ABT-737 to induce apoptosis in tumor cells expressing high Mcl-1 levels may stem from a requirement for inhibition of multiple antiapoptotic proteins. A corollary is that down-regulation/inhibition of ABT-737–nontargeted proteins (e.g., Mcl-1 or A1) may enhance the lethality of this compound (12).

One candidate strategy to down-regulate/inhibit Mcl-1 involves the use of cyclin-dependent kinase (CDK) inhibitors. In preclinical studies, CDK inhibitors, including flavopiridol and the roscovitine derivative CYC202 (seliciclib), are potent inducers of apoptosis in malignant hematopoietic cells, including leukemia cells (17, 18). Notably, results from several laboratories have established that CDK inhibitors (e.g., flavopiridol and CYC202) act, at least in part, by inhibiting CDK9, a kinase intimately involved in transcription initiation and elongation through activation of the positive transcription elongation factor-b, resulting in down-regulation of several short-lived proteins, including Mcl-1 (17, 19). Here, we report that Mcl-1 down-regulation by either CDK inhibitors or a small hairpin RNA (shRNA) approach leads to a dramatic increase in ABT-737–mediated apoptosis in human leukemia cells. Our results also indicate that this phenomenon stems from a mechanism involving two levels of cooperation between antiapoptotic and multidomain proapoptotic proteins of the Bcl-2 family: (a) simultaneous untethering of Bak from Bcl-xL (by ABT-737) and Mcl-1 (e.g., by roscovitine) and (b) the resulting
activation of both Bak and Bax, culminating in Bax mitochondrial translocation and engagement of the apoptotic cascade. These findings may provide a theoretical framework for combinatorial approaches that target diverse antiapoptotic proteins that cooperate in the efficient induction of apoptosis in malignant cells.

Materials and Methods

Cells and reagents. Human leukemia U937, HL-60, and Jurkat cells were provided by the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 containing 10% FCS as previously reported (20). U937/Bcl-2 and U937/Bcl-xl were obtained by stable transfection of cells with full-length Bcl-2 and Bcl-xl cDNA, respectively (21). U937 cells stably overexpressing Mcl-1 were kindly provided by Dr. Roy Craig (Dartmouth Medical School, Hanover, NH; ref. 22). All experiments used logarithmically growing cells (3–5 × 10⁶ cells/ml). Peripheral blood samples were obtained with informed consent according to the Declaration of Helsinki from the peripheral blood of three patients with acute myeloblastic leukemia (AML; FAB subtype M2) undergoing routine diagnostic aspirations with approval from the Virginia Commonwealth University Institutional Review Board. Leukemic blasts were isolated as previously described (20). Wild-type (wt), Bax−/−, Bak−/−, and Bax−/−/Bak−/− (double knockout) mouse embryonic fibroblast (MEF) were kindly provided by the laboratory of Dr. Stanley Korsmeyer (Dana-Farber Cancer Institute, Boston, MA; ref. 23). Mcl-1−/−/− Mefs were kindly provided by Dr. Joseph Opferman (St. Jude Children’s Research Hospital, Memphis, TN). All experiments were initiated in cells cultured at ~60% confluence.

ABT-737 was kindly provided by Dr. Gary Gordon (Abbott Laboratories, Abbott Park, IL; ref. 10). It was dissolved in DMSO, aliquoted, and stored at ~80°C. Roscovitine and R-roscovitine were purchased from Calbiochem (San Diego, CA), dissolved in sterile DMSO, aliquoted, and stored at ~20°C. In all experiments, the final concentration of DMSO did not exceed 0.1%.

Assessment of apoptosis. The extent of apoptosis was evaluated by flow cytometric analysis using Annexin V-FITC staining as described previously (20). To analyze the extent of cell death in MEFs, cells were trypsinized and cytometric analysis using Annexin V-FITC staining as described previously (San Diego, CA), dissolved in sterile DMSO, aliquoted, and stored at ~20°C. The interaction between Bak and Mcl-1 or Bcl-xL was evaluated by coimmunoprecipitation analysis by using ExactaCruz Kit V (Amaxa GmbH, Cologne, Germany) as per manufacturer's instructions.

Results

CDK inhibitors transcriptionally down-regulate the expression of Mcl-1 and synergistically interact with ABT-737 to induce apoptosis in U937 cells. Earlier reports indicated that various CDK inhibitors, including roscovitine (27), transcriptionally down-regulate expression of short-lived proteins, such as Mcl-1. As shown in Fig. 1A, treatment with either CDK inhibitor at concentrations >10 μmol/L resulted in significant declines in Mcl-1 protein levels, but no change in expression of Bcl-2 and Bcl-xl was observed. This phenomenon was associated with inhibition of phosphorylation of pol II CTD and a pronounced reduction in Mcl-1 mRNA levels (Fig. 1B).

Attempts were then made to determine what effect CDK inhibitors would have on the response of cells to ABT-737. Whereas exposure to 12 μmol/L roscovitine alone modestly increased apoptosis, ABT-737 at a concentration range of 150 to 750 nmol/L had very little effect (Fig. 1C). However, combined treatment resulted in a dramatic increase in the loss of mitochondrial membrane potential (ΔΨm; data not shown) and apoptosis. Identical results were obtained with R-roscovitine. Moreover, lower concentrations (<10 μmol/L) of either roscovitine or R-roscovitine, which failed to down-regulate Mcl-1 (Fig. 1A), did not enhance ABT-737 lethality (data not shown). Median dose-effect analysis, employing apoptosis determined by Annexin V-FITC as an end point, yielded combination index values <1.0 (Fig. 1C, inset), denoting synergistic interactions.
Exposure of U937 cells to roscovitine resulted in marked down-regulation of Mcl-1 protein, whereas ABT-737 by itself failed to modify Mcl-1 expression (Fig. 1D). In contrast, no change in Bcl-2 or Bcl-xL expression was observed (data not shown). Lastly, effects of cotreatment with ABT-737 and roscovitine were examined in relation to mitochondrial events. Cotreatment with roscovitine and ABT-737 triggered a pronounced increase in cytochrome c and AIF release into the cytosolic fraction (Fig. 1D). Combined treatment also induced a modest but discernible increase in caspase-9 and caspase-8 cleavage, and a marked increase in cleavage of caspase-3, accompanied by cleavage of PARP (Fig. 1D). However, transfection with a dominant-negative caspase-8 construct (21) failed to protect U937 cells from mitochondrial damage and apoptosis induced by ABT-737/roscovitine (data not shown), arguing against involvement of the extrinsic apoptotic pathway.

Together, these findings indicate that CDK inhibitors dramatically increase ABT-737–mediated apoptosis in association with Mcl-1 down-regulation.

Roscovitine markedly down-regulates Mcl-1 and increases ABT-737 lethality in various human leukemia cells, including primary AML cells. Parallel studies were done in human HL-60 promyelocytic and Jurkat lymphoblastic leukemia cells. First, these leukemia cells exhibited differing susceptibilities to ABT-737–mediated lethality (IC50: 1.3 μmol/L for U937, 420 nmol/L for Jurkat, 190 nmol/L for HL-60). Immunoblot showed that Bcl-2 and Mcl-1 protein levels varied between the cell lines, whereas Bcl-xL expression was equivalent. Interestingly, HL-60 cells, which were the most sensitive of the three lines, exhibited very low Mcl-1 expression but higher levels of Bcl-2 (Fig. 2A, inset). These results suggest that levels of Mcl-1 and/or the ratio between Mcl-1 and Bcl-2 play a critical role in ABT-737–mediated apoptosis.
Bcl-2 expression may represent as determinants of ABT-737 sensitivity in leukemia cells.

Cotreatment with marginally toxic concentrations of ABT-737 and roscovitine markedly induced mitochondrial damage (data not shown) and apoptosis in Jurkat and HL-60 cells (Fig. 2A). Roughly equivalent results were obtained when R-roscovitine was coadministered (data not shown). Moreover, roscovitine, administrated alone ± ABT-737 dramatically down-regulated Mcl-1 in Jurkat cells, and combined treatment marked increased PARP cleavage (Fig. 2B).

Effects of this regimen on primary leukemia blasts isolated from three patients with AML (Fig. 2C) were similar to those obtained in leukemia cell lines. Furthermore, roscovitine ± ABT-737 almost completely abrogated Mcl-1 expression in AML blasts and induced pronounced PARP (Fig. 2D), indicating that ABT-737/roscovitine interactions occur in association with Mcl-1 down-regulation in both continuously cultured human leukemia cell lines differing in their sensitivity to ABT-737 as well as in primary AML blasts.

Roscovitine enhances ABT-737-mediated Bax conformational change, whereas Bak activation is induced only by combined ABT-737 and roscovitine administration. Bax and Bak are essential for mitochondrial outer membrane permeabilization (28, 29), a critical cell death determinant (30). Whereas Bak is monomeric and found in the cytosol of healthy cells. Following death stimuli, Bax undergoes conformational change and translocates to organellar membranes. Activation of both Bak and Bax is associated with a conformational change, which can be detected by antibodies recognizing only the active protein conformers (16). The effect of ABT-737 ± roscovitine on Bak/Bax conformational change was then examined. When U937 cells were exposed to ABT-737 (150–500 nmol/L) alone, a clear dose-dependent increase in Bak conformational change was observed (Fig. 3A). Interestingly, Bak conformational change was unaccompanied by Bak translocation (see below; Fig. 3B) nor did it induce

![Figure 2](image_url)

**Figure 2.** Roscovitine down-regulates Mcl-1 and promotes ABT-737 lethality in multiple human leukemia cell lines and primary AML cells. A, untreated U937, HL-60, and Jurkat cells were lysed and subjected to immunoblot analysis to detect protein levels of Bcl-2, Bcl-xL, and Mcl-1 (inset). Jurkat and HL-60 cells were exposed for 24 h to ABT-737 (Jurkat, 100–200 nmol/L; HL-60, 30–50 nmol/L) ± 12 μmol/L roscovitine, after which the percentage of Annexin V+ cells was determined by flow cytometry. B, Jurkat cells were treated as described in (A), after which immunoblot analysis was done to monitor Mcl-1 expression and PARP cleavage. C, blasts were isolated from the peripheral blood of three AML patients (designated as #1–3; FAB subtype M2) and then incubated (24 h) with 150 nmol/L ABT-737 ± roscovitine (#1 and #2, 10 μmol/L; #3, 12 μmol/L). At the end of this period, the percentage of Annexin V+ cells was determined by flow cytometry. Columns, means for an experiment done in triplicate; bars, SD. Representative experiment (patient #3). D, the blasts (patient #3) were incubated (24 h) with 150 and 300 nmol/L ABT-737 ± 12 μmol/L roscovitine and then lysed for immunoblot using indicated primary antibodies. C-PARP, cleaved PARP. A (inset), B, and D, representative for three separate experiments.
apoptosis (Fig. 1C). On the other hand, roscovitine (12 μmol/L) alone minimally induced Bax conformational change. Notably, cells coexposed to roscovitine and ABT-737 displayed a further increase in Bax conformational change compared with cells treated ABT-737 alone (Fig. 3A), an effect accompanied by marked increases in Bax translocation (see below; Fig. 3C) and lethality. These findings suggest that ABT-737–induced Bax conformational change by itself may not be sufficient to trigger Bax translocation and apoptosis.

Parallel studies were then done in U937 cells to assess Bak conformational change. In sharp contrast to results involving Bax, ABT-737 by itself had little or no effect on Bak activation (Fig. 3A), whereas roscovitine also failed to induce Bak activation. However, combined treatment with ABT-737 and roscovitine induced pronounced Bak activation. No change in total Bak was noted for any condition (data not shown). Together, these results indicate that (a) roscovitine enhances ABT-737–mediated Bax conformational change and cooperates to trigger Bax translocation and (b) ABT-737 alone is ineffective in triggering Bax conformational change; instead, roscovitine coadministration is required for ABT-737–mediated Bak activation.

**Coadministration of ABT-737 and roscovitine disrupts the association of Bak with both Bcl-xL and Mcl-1 and induces Bax translocation.** One of the mechanisms by which Mcl-1 opposes apoptosis is by binding/sequestering Bak and preventing its activation (31). Furthermore, there is evidence that Bcl-xL, but not Bcl-2, Bcl-w, or A1, acts analogously to block Bak activation (16), and that both Mcl-1 and Bcl-xL must be neutralized (e.g., by Noxa and Bad, respectively) to displace Bak in order to trigger cell death (16, 32). Consequently, the effects of ABT-737 and roscovitine on interactions between Bak and Bcl-xL or Mcl-1 were assessed. No change in total Bak levels was observed with any treatment (see below; Fig. 3C). U937 cells exposed to ABT-737 ± roscovitine were lysed in CHAPS buffer, and associations between Bak and Mcl-1 or Bcl-xL were assessed (Fig. 3B). Treatment with ABT-737

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**Figure 3.** Bax is necessary for induction of cell death by both ABT-737 alone and in combination with roscovitine, whereas Bak activation is only required for synergistic interactions among these agents. A, U937 cells were treated (24 h) with 150 to 500 nmol/L ABT-737 ± 12 μmol/L roscovitine, after which cells were stained with anti–conformationally changed Bax (clone 3)/FITC-conjugated goat-anti-mouse IgG and subjected to flow cytometry. In parallel, Bak activation was monitored by flow cytometry after staining with anti–conformationally changed Bak (Ab-1)/FITC-conjugated goat-anti-mouse IgG. Representative histograms (dotted, untreated controls). Values, mean ± SD for three separate experiments done in triplicate. B, U937 cells were exposed for 24 h to 150 to 300 nmol/L ABT-737 ± 12 μmol/L roscovitine, after which the cells were lysed in CHAPS buffer, and associations between Bak and anti-Bcl-xL antibody. For comparison, the right lanes (designated as C) were loaded with whole-cell lysates. C, U937 cells were treated as described in (A), after which S-100 cytosolic and pelleted organelar membrane fractions were prepared and subjected to immunoblot analysis using an anti-Bak antibody. Alternatively, total levels of Bax and Bak were monitored in whole-cell lysates. D, untreated wt, Bak−/−, Bax−/−, and Bak−/−/Bax−/− (double knockout [DKO]) MEFs were lysed and subjected to immunoblot to detect expression of both proapoptotic (Bak and Bax) and antiapoptotic (Mcl-1, Bcl-2, and Bcl-xL) proteins (inset). Various MEFs were exposed (24 h) to 300 to 500 nmol/L ABT-737 ± 12 μmol/L roscovitine, after which the cells, including those in the culture supernatant, were collected for each condition. Cells were then stained with 7AAD and subjected to flow cytometry to determine the percentage of 7AAD+ cells.* P < 0.01, significantly greater than values for treatment with each concentration of ABT-737 alone. B, C, and D (inset), representative results from one experiment, and two additional studies yielded equivalent results.
alone modestly but discernibly increased the amount of Mcl-1 associating with Bak. Notably, this effect was largely abrogated by roscovitine treatment, presumably due to Mcl-1 down-regulation (Fig. 1). Reciprocal effects were noted when the amount of Bcl-xL coimmunoprecipitating with Bak was monitored (i.e., roscovitine substantially increased the amount of Bcl-xL associating with Bak, whereas ABT-737 essentially reversed this phenomenon). Together, these findings suggest that cotreatment with roscovitine and ABT-737 antagonizes interactions of Bak with both Mcl-1 and Bcl-xL.

Lastly, the effect of coexposure to roscovitine and ABT-737 on intracellular Bax localization was examined. Treatment with either ABT-737 or roscovitine alone had little or no effect on the intracellular disposition of Bax (Fig. 3C). However, ABT-737/roscovitine coadministration induced a major translocation of Bax from the cytosolic compartment to the pellet fraction associated with organellar membranes. These findings also suggest that concomitant activation of Bax and Bak may be responsible for the dramatic induction of apoptosis in cells coexposed to roscovitine and ABT-737.

Bax knockout in MEFs substantially diminishes the lethality of ABT-737 ± roscovitine, whereas Bak knockout primarily blocks synergistic interactions between these agents. To evaluate further the functional roles of Bak and Bax in the lethality of ABT-737 and its interactions with roscovitine, Bax−/−, Bak−/−, and Bax−/−/Bak−/− (double knockout) MEFs were employed (Fig. 3D, inset). Immunoblot analysis revealed that antiapoptotic Bcl-2 family protein levels (e.g., Mcl-1, Bcl-2, and Bcl-xL) were roughly equivalent in each of the cell types. Coimmunoprecipitation of roscovitine clearly increased the lethality of ABT-737 in wt MEFs (P < 0.01, compared with cells exposed to ABT-737 alone; Fig. 3D). However, the lethality of ABT-737 ± roscovitine was substantially blunted in Bax−/− MEFs, indicating that Bax is critical for this phenomenon. In marked contrast, ABT-737 was still able to induce cell death in Bak−/− MEFs; in fact, these cells were slightly more sensitive than wt cells. Significantly, coimmunoprecipitation of roscovitine failed to increase the lethality of ABT-737 in Bak−/− MEFs. Finally, Bax/Bak double knockout MEFs displayed essentially no response to any of these treatments. Together, these findings suggest that Bax is required for induction of cell death by both ABT-737 ± roscovitine, whereas Bak activation, although dispensable for ABT-737 lethality, is nevertheless required for ABT-737/roscovitine synergism. They also support the notion that cooperation between Bak and Bax is critical for ABT-737/roscovitine lethality.

Roscovitine down-regulates Mcl-1 expression and attenuates ABT-737 resistance in leukemic cells ectopically expressing Bcl-2 or Bcl-xL. Because ABT-737 targets Bcl-2 and Bcl-xL (10), it might be predicted that the relative abundance of antiapoptotic proteins, such as Bcl-2, would be related to ABT-737 sensitivity. Consequently, the effect of Bcl-2 or Bcl-xL expression on the susceptibility of cells to ABT-737 ± roscovitine was examined using U937 cells ectopically expressing Bcl-2 or Bcl-xL. Ectopic expression of Bcl-2 or Bcl-xL provided significant protection from the lethal effects of etoposide (VP-16), a potent inhibitor of DNA topoisomerase (Fig. 4A and B). Notably, Bcl-2 or Bcl-xL overexpression attenuated ABT-737–mediated lethality but did not affect roscovitine cytotoxicity. However, Bcl-2 or Bcl-xL overexpression failed to protect cells from mitochondrial damage (i.e., loss of ΔΨm; data not shown) and apoptosis induced by roscovitine and ABT-737 coadministration (P > 0.05, for each ABT-737 concentration, compared with empty vector controls U937/pCEP or U937/3.1). Cotreatment with roscovitine/ABT-737 induced an equivalent decline in Mcl-1 expression and enhanced PARP cleavage in U937/Bcl-2, U937/Bcl-xL, and controls but did not modify Bcl-2 or Bcl-xL expression (Fig. 4C and D). Collectively, these findings indicate that although ectopic expression of either Bcl-2 or Bcl-xL reduces human leukemia cell sensitivity to ABT-737, they are unable to prevent the ABT-737/roscovitine regimen from diminishing Mcl-1 levels and inducing apoptosis.

Ectopic expression of Mcl-1 but not Bcl-2 blocks Bak activation and apoptosis triggered by combined exposure of leukemia cells to ABT-737 and roscovitine. Because Bcl-2/Bcl-xL and Mcl-1 may play disparate functional roles in blocking apoptosis (32), studies were done employing U937 cells ectopically expressing Mcl-1 (22). As in cells overexpressing Bcl-2 or Bcl-xL, ectopic expression of Mcl-1 substantially protected cells from VP-16– and ABT-737–mediated lethality (Fig. 5A). However, in striking contrast to the former cells, enforced Mcl-1 expression significantly diminished mitochondrial damage (loss of ΔΨm; data not shown) and apoptosis (Fig. 5A) induced by the ABT-737/roscovitine regimen (P < 0.001, versus U937/pCEP).

Immunoblot revealed that combined treatment with ABT-737/roscovitine clearly diminished Mcl-1 expression in U937/pCEP cells but failed to do so in their U937/Mcl-1 counterparts (Fig. 5B). Moreover, PARP cleavage induced by ABT-737/roscovitine was almost completely abrogated in U937/Mcl-1 cells. Basal Bcl-2 expression was somewhat lower in U937/Mcl-1 cells compared with controls but was not modified appreciably in either line with any treatment. Together, these findings argue strongly that down-regulation of Mcl-1 plays a functional role in potentiation of ABT-737–mediated lethality by roscovitine.

Finally, the effects of ectopic expression of Bcl-2 or Mcl-1 were compared with respect to conformational change of Bax and Bak induced by the ABT-737/roscovitine regimen. Consistent with their inability to block ABT-737/roscovitine–mediated lethality, Bcl-2 overexpression failed to attenuate conformational change of Bax or Bak in cells exposed to ABT-737 and roscovitine in combination (Fig. 5C), although it did reduce Bax conformational change induced by ABT-737 alone (data not shown). Similar results were obtained in cells ectopically expressing Bcl-xL (data not shown). However, in sharp contrast, ectopic expression of Mcl-1, which also attenuated Bax conformational change mediated by ABT-737 alone (data not shown), essentially abrogated Bak activation triggered by the ABT-737/roscovitine regimen and also partially reduced Bax conformational change after exposure to the combination (Fig. 5D).

These findings provide further evidence that disruption of Mcl-1 function plays a critical role in ABT-737/roscovitine interactions associated with Bax/Bak activation and apoptosis.

RNA interference or gene knockout of Mcl-1 dramatically sensitizes cells to ABT-737 but abrogates the capacity of roscovitine to potentiate Bak activation and lethality. To evaluate further the functional role of Mcl-1 in Bax/Bak activation as well as apoptosis mediated by ABT-737 ± roscovitine, a shRNA strategy and Mcl-1−/− MEFs were employed. First, U937 cells were transiently transfected with a construct encoding shRNA against Mcl-1 mRNA, and immunoblot analysis documented Mcl-1 down-regulation (Fig. 6A, inset). Mcl-1 down-regulation by this approach dramatically sensitized human leukemia cells to ABT-737 lethality (P < 0.002–0.001, for each ABT-737 concentration, compared with those transfected with empty vector; Fig. 6A).
Further studies were done in wt and Mcl-1−/− MEFs. Immunoblots revealed that levels of Bcl-2, Bcl-xL, Bak, and Bax in Mcl-1−/− cells were roughly equivalent to those in wt MEFs, whereas Mcl-1 was absent (Fig. 6B, inset). Mcl-1−/− cells exhibited a dramatic increase in sensitivity to ABT-737 (50–100 nmol/L) compared with wt cells (P < 0.001; Fig. 6B). Significantly, roscovitine was unable to enhance cell killing mediated by ABT-737 in Mcl-1−/− cells (P > 0.05). Moreover, Bak conformational change was observed only after coexposure of wt MEFs to ABT-737 and roscovitine, consistent with preceding findings in human leukemia cells. In striking contrast, ABT-737 alone dramatically induced Bak activation in Mcl-1−/− cells, a phenomenon that was not further enhanced by roscovitine (Fig. 6C). Analogously, Mcl-1−/− cells displayed greater Bax conformational change following ABT-737 exposure compared with their wt counterparts (Fig. 6C). However, as in the case of Bak, roscovitine failed to increase ABT-737–mediated Bax conformational change in Mcl-1−/− cells. Together, these results provide clear evidence that Mcl-1 is a critical determinant of both ABT-737 actions as well as the capacity of roscovitine to potentiate ABT-737 lethality through cooperative activation of Bak and Bax.

**Discussion**

ABT-737, a novel small-molecule Bcl-2/Bcl-xL/Bcl-w inhibitor currently in development as an anticancer agent, has a relatively low affinity for the more divergent antiapoptotic Bcl-2 family proteins (e.g., Mcl-1 and A1; ref. 10). ABT-737 is less efficient in killing tumor cells exhibiting relatively high levels of Mcl-1 (12). Because Mcl-1 has a short half-life (i.e., <2 h; ref. 33), it is particularly susceptible to down-regulation by agents that disrupt its de novo synthesis. Consequently, attention has recently focused on the capacity of several clinical relevant CDK inhibitors (e.g., flavopiridol and the roscovitine derivative CYC202) to transcriptionally down-regulate Mcl-1 through CDK9 inhibition (17, 19). Moreover, we previously reported that flavopiridol enhanced the lethality of HA14-1, although the mechanism underlying this interaction was not determined (34). Consistent with these findings, roscovitine and its R enantiomer R-roscovitine inhibited phosphorylation of RNA polymerase II CTD, diminished Mcl-1 mRNA levels, and markedly down-regulated protein levels. Significantly, such actions correlated closely with synergistic interactions with ABT-737. Although the possibility that other
activities (e.g., CDK disruption) contribute to the lethality of combination regimens involving CDK inhibitors cannot be completely excluded (35), it is noteworthy that Mcl-1 down-regulation plays an important role in apoptosis in malignant hematopoietic cells (17, 19, 36). On the other hand, in certain transformed cells, Mcl-1 down-regulation, although required, seems to be incapable by itself of initiating apoptosis (31, 37), indicating that other cooperating factors may be necessary for cell death. These findings suggest that interventions targeting more than one antiapoptotic protein may be required for optimal cell killing.

Whereas resistance to ABT-737 may reflect the compensatory actions of Mcl-1 (12), the present investigation was prompted by recent evidence that Bak activation requires simultaneous disruption of its associations with Mcl-1 (e.g., by Noxa) and Bcl-xL (e.g., by Bad; ref. 16). Moreover, increased production of Noxa can oppose Mcl-1 antiapoptotic functions, leading to simultaneous activation of Bax and Bak (38). The present results suggest that coadministration of ABT-737 and roscovitine recapitulate the actions of more physiologic proapoptotic BH3-only proteins. Specifically, ABT-737, by binding to hydrophobic groove within the Bcl-xL BH3 domain (10), untethers Bak from Bcl-xL, analogous to the actions of BH3-only proteins such as Bad (39). On the other hand, Mcl-1 down-regulation by roscovitine, mimicking the actions of Noxa in displacing Bak from Mcl-1 (16), reciprocally released Bak from Mcl-1 sequestration. Thus, coadministration of ABT-737 and roscovitine markedly diminished the association of Bak with both Bcl-xL and Mcl-1, inducing Bak activation. In support of this hypothesis, Bak activation was observed only when roscovitine and ABT-737 were administrated concomitantly, but not after ABT-737 alone. The notion of cooperativity in the regulation of ABT-737 lethality is further supported by the results obtained in Mcl-1 knockout MEFs, in which ABT-737 alone, in contrast to its actions in wt cells, markedly induced Bak activation. Significantly, roscovitine was unable to enhance ABT-737 lethality further in these cells presumably because Mcl-1 was absent and levels could not be reduced further. These findings provide strong support for the concept that disruption of more than one antiapoptotic protein of the Bcl-2 family (i.e., Mcl-1 and Bcl-2/Bc-xL) represents a highly potent apoptotic stimulus (12).

The finding that ABT-737 induced Bax conformational change but did not trigger apoptosis by itself suggests that the lethality of the ABT-737/roscovitine regimen involves not simply activation of either Bax or Bak, but cooperativity between these proteins. Untethering of Bak from both Mcl-1 and Bcl-xL allows Bak conformational change, homo-oligomerization (16), as well as possible associations with Bak (40). Nevertheless, there is evidence that Bax and Bak may interact to promote apoptosis (41). Results obtained with Bax and Bak knockout MEFs are fully compatible with a model in which Bax and Bak cooperate to trigger cell death. For example, Bax knockout cells displayed marked resistance to ABT-737 given alone or in combination with roscovitine. This suggests that the presence of Bax is essential for both ABT-737 lethality and synergistic interactions with roscovitine. In striking contrast, Bak knockout cells remained fully sensitive to ABT-737–mediated cell death.
killing, indicating that Bak is not absolutely required for the lethality of an agent targeting Bcl-2/Bcl-xL. However, it is significant that roscovitine failed to potentiate ABT-737 lethality in these cells, arguing strongly that Bak is required for full engagement of the apoptotic program following disruption of the Bcl-2/Bcl-xL axis. Moreover, Bax translocation and cell death occurred only in human leukemia cells coexposed to ABT-737 and roscovitine (Fig. 3C). Although the mechanisms responsible for potentiation of ABT-737-mediated Bax translocation by roscovitine are presently unclear, there are several plausible explanations. These include the possibilities that (a) Mcl-1 may be involved in Bax regulation, either through a process involving "activator" BH3-only proteins such as Bim and/or BH3 (13, 42–45), or directly by itself (46); or that (b) activated Bak may promote Bax translocation in an as yet to be defined way. Collectively, the present results support the notion that simultaneous interruption of Mcl-1 and Bcl-xL function frees and activates Bak, which, in the setting of Bax conformational change, results in Bax translocation, leading in turn to full engagement of the apoptotic machinery.

It is noteworthy that overexpression of Bcl-2 or Bcl-xL failed to protect leukemia cells from the ABT-737/roscovitine regimen, reflecting the important contribution of Mcl-1 down-regulation to the lethality of this regimen. The finding that ectopic expression of Mcl-1 diminished potentiation of ABT-737 lethality by roscovitine highlights the central role of Mcl-1 down-regulation in synergism between these agents. This interpretation is further supported by results showing that roscovitine was unable to enhance ABT-737-mediated apoptosis in Mcl-1 knockout MEFs. Moreover, overexpression of Mcl-1, but not Bcl-2/Bcl-xL, essentially abrogated Bak activation following exposure to ABT-737/roscovitine, strongly arguing that Mcl-1 plays a major role in regulating Bak. This concept is consistent with previous findings indicating that Mcl-1 binds with considerably greater affinity to Bak compared with Bcl-xL (IC_50_ < 10 versus < 100 nmol/L; ref. 16).

 Whereas recent studies suggest that ABT-737 and the more specific Bcl-xL inhibitor A-385358 increase the antitumor activity of conventional cytotoxic drugs (10, 47), this phenomenon may reflect a generic lowering of the apoptotic threshold. On the other hand, the present results suggest that a mechanism-based approach combining agents that target distinct antiapoptotic molecules (e.g., CDK inhibitors that down-regulate Mcl-1 expression and small-molecule Bcl-2/Bcl-xL inhibitors like ABT-737) deserve attention. These findings also highlight the importance of

![Image](https://example.com/image.png)

Figure 6. Knockout or down-regulation of Mcl-1 sensitize cells to cell death induced by ABT-737. A, U937 cells were transiently transfected with either empty vector (EV) or shMcl-1/pSUPER for 24 h, after which cells were lysed and subjected to immunoblot analysis (inset). After transfection with empty vector or shMcl-1, cells were incubated for 6 h and then exposed to the indicated concentrations of ABT-737 for an additional 24 h, after which the percentage of viable cells (7AAD−) was determined by flow cytometry after stained with 7AAD. * P < 0.02; ** P < 0.001, significantly less than values for cells transfected with empty vector. B, immunoblot analysis was done to monitor levels of antiapoptotic (Mcl-1, Bcl-2, and Bcl-xL) and multidomain proapoptotic proteins (Bax and Bak) in untreated wt and Mcl-1 knockout MEFs (inset). MEFs were exposed to 50 to 100 nmol/L ABT-737 alone or in the presence of 12 μmol/L roscovitine for 24 h, after which cells, including those in the culture supernatant, were harvested together for flow cytometry using 7AAD staining. ** P < 0.001, significantly greater than values for wt cells. C, MEFs (wt and Mcl-1−/−) were incubated (24 h) with 100 nmol/L ABT-737 + 12 μmol/L roscovitine, after which cells were stained with anti–conformationally changed Bak (Ab-2) or Bak (YTH-6A7)/FITC-conjugated goat-anti-mouse IgG and subjected to flow cytometry.
cooperative interactions between such agents at two separate but interrelated levels in cell death induction: (a) release of Bak from both Bcl-xL and Mcl-1 and (b) simultaneous activation of both Bak and Bak, which may be essential for Bax translocation and ABT-737 lethality. Whether a strategy combining CDK inhibitors, or other transcriptional repressors capable of down-regulating Mcl-1, with Bcl-2/Bcl-xL antagonists will result in enhanced therapeutic efficacy will depend upon multiple factors, including the capacity of such agents to diminish Mcl-1 expression in vivo, and whether the therapeutic index is enhanced. In this context, it is noteworthy that ABT-737 displays in vivo antitumor selectivity in preclinical studies (10). In any case, the present findings suggest that in addition to combining Bcl-2/Bcl-xL antagonists with conventional cytotoxic drugs, combination strategies involving targeted agents that down-regulate Mcl-1, a protein that can compensate for the loss of Bcl-2/Bcl-xL function, represents a potentially useful alternative approach.

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References


37. Nijhawan D, Fang M, Traer E, et al. Elimination of Bcl-xL and Mcl-1, two heat shock proteins: mechanisms, physiological roles, and thera-

38. Mcl-1 Down-regulation Enhances ABT-737 Lethality
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