Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia

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Summary

BCL-2 proteins are critical for cell survival and are overexpressed in many tumors. ABT-737 is a small-molecule BH3 mimetic that exhibits single-agent activity against lymphoma and small-cell lung cancer in preclinical studies. We here report that ABT-737 effectively kills acute myeloid leukemia blast, progenitor, and stem cells without affecting normal hematopoietic cells. ABT-737 induced the disruption of the BCL-2/BAX complex and BAK-dependent but BIM-independent activation of the intrinsic apoptotic pathway. In cells with phosphorylated BCL-2 or increased MCL-1, ABT-737 was inactive. Inhibition of BCL-2 phosphorylation and reduction of MCL-1 expression restored sensitivity to ABT-737. These data suggest that ABT-737 could be a highly effective antileukemia agent when the mechanisms of resistance identified here are considered.

Introduction

Aberrant overexpression of BCL-2 is associated with tumorigenesis and increased resistance to chemotherapy in multiple malignancies, including acute myeloid leukemia (AML) (Campos et al., 1993; Vaux et al., 1988). Similar findings have been made for other antiapoptotic members of the BCL-2 family, such as BCL-X_L and MCL-1 (Fennell et al., 2001; Kaufmann et al., 1998). BCL-2 is a potent antiapoptotic protein that protects cells from diverse stress challenges including chemotherapeutic drugs. A key function of BCL-2 is to act as guardian of mitochondrial integrity by opposing proapoptotic BCL-2 family members (Oltvai et al., 1993; Korsmeyer et al., 1993; Sedlak et al., 1995; Sharpe et al., 2004). BCL-2 function depends on posttranslational modification, whereby phosphorylation of BCL-2 can either promote (May et al., 1994; Deng et al., 2004) or inhibit (Haldar et al., 1998; Srivastava et al., 1998) its antiapoptotic function.

Antiapoptotic BCL-2 family proteins are normally kept in check by endogenous antagonists that contain a conserved dimerization motif termed BH3 (Oltvai et al., 1993; Zha et al., 1996). The BH3 domain consists of an amphipathic α helix that binds a hydrophobic groove on antiapoptotic BCL-2 family members, negating their cytoprotective activity (Fesik, 2000). Proof of concept experiments using BH3 peptides have suggested that docking at the BH3 domain of BCL-2 and its related antiapoptotic proteins could deactivate BCL-2 to increase the sensitivity of tumor cells to apoptosis. Indeed, several small-molecule chemicals have been identified that bind to BCL-2 family proteins by interacting with the BH3-binding groove and promote apoptosis (Pellecchia and Reed, 2004). ABT-737 is a synthetic small-molecule inhibitor produced by NMR-guided,

SIGNIFICANCE

BCL-2 family proteins are key regulators of apoptosis and are known to promote tumorigenesis and chemoresistance. Strategies to target BCL-2 could translate into effective anticancer therapies. We investigated mechanisms of action of the BCL-2 (BH3) inhibitor ABT-737 and found that ABT-737 disrupts BCL-2/BAX heterodimers and induces apoptosis in AML stem cells through activation of the intrinsic apoptotic pathway. ABT-737 synergized with chemotherapy, but its activity was largely diminished in cells overexpressing MCL-1 or displaying phosphorylated BCL-2. Reducing MCL-1 levels and suppressing BCL-2 phosphorylation potentiated ABT-737-induced apoptosis. Furthermore, the agent was effective in vivo. These findings suggest that ABT-737 may be an effective antileukemia agent with a unique mode of action.

structure-based drug design that binds BCL-2, BCL-X_L, and BCL-W with high affinity (K_i < 1 nM) but binds weakly (K_i > 460 nM) to other antiapoptotic BCL-2 family members, including MCL-1 and BFL-1 (Oltersdorf et al., 2005). In a cell-free system of purified mitochondria, the inhibitor antagonized the antiapoptotic function of BCL-2 and BCL-X_L but did not directly activate BAX or BAK (Oltersdorf et al., 2005).

In the current study, we report that ABT-737 can promote apoptosis as a single agent in cells from AML-derived cell lines, and in primary blasts from AML patients. The drug inhibited growth of clonogenic AML progenitor and stem cells and was effective in vivo in a xenograft model of AML. We demonstrated that ABT-737 disrupts BCL-2/BAX association in leukemia cells and promotes activation of BAX and mitochondrial dysfunction in these cells, in part through BAK. High MCL-1 expression and BCL-2 phosphorylation markedly reduced the ability of ABT-737 to induce apoptosis. The inhibition of MEK1/ERK is highly synergistic with ABT-737 in killing leukemia cells via suppression of BCL-2 phosphorylation and downregulation of MCL-1 levels in AML. The criticality of MCL-1 as a resistance factor to ABT-737 was established by MCL-1 small interfering RNA (shRNA) knockdown, which completely restored sensitivity in leukemic cells that were resistant to ABT-737 alone. These findings demonstrate the potential of ABT-737 as a mechanism-based therapeutic agent, whose activity can be further enhanced by targeting the resistance factors described in this report.

Results

ABT-737 induces apoptosis and synergizes with chemotherapy in AML cells

ABT-737 was created as a small-molecule chemical that would bind the BH3-binding groove of BCL-X_L and BCL-2 (Oltersdorf et al., 2005). Using competitive fluorescence polarization assays (FPAs), we compared the affinity of ABT-737 to various antiapoptotic BCL-2 family members. ABT-737 was shown to compete with the fluorochrome-conjugated BIM BH3 peptide for binding to BCL-X_L, BCL-2, BCL-W, and to a lesser degree, BCL-B, but not to MCL-1 and BFL-1 (Figure 1A). We next examined effects of ABT-737 and its less active enantiomer (Oltersdorf et al., 2005) on the viability of AML cell lines with different basal expression of BCL-2 family proteins (Figures 1B–1D). The IC_{50} for the inactive enantiomer in all cell lines was at least 10-fold higher as compared to the respective IC_{50} for ABT-737 (data not shown and Figures 1C and 1D). HL-60 cells displayed the greatest sensitivity to the compound (IC_{50} = 50 nM), while OCI-AML3 cells were the most resistant (IC_{50} = 5000 nM). MCL-1 expression was highest in the two cell lines (U937 and OCI-AML3) that exhibited the greatest resistance to the compound (Figure 1B). Since ABT-737 binds poorly to MCL-1 (Figure 1A and Oltersdorf et al., 2005), the possibility arises that MCL-1 may substitute for BCL-2 or BCL-X_L and thus confer resistance.

HL-60 cells treated with ABT-737 (100 nM) exhibited classic signs of apoptosis: early (within 1 hr) loss of mitochondrial membrane potential ($\Delta\psi_m$), followed by phosphatidyl serine externalization as indicated by Annexin V staining (at 12–24 hr; Figure 1E), DNA fragmentation as indicated by DNA laddering (data not shown), and finally loss of viability (at 24–48 hr; Figure 1C). ABT-737 had no effect on cell cycle distribution (data not shown). These data indicate that in AML cells ABT-737 can induce apoptosis as a single agent.

To determine if the inhibition of BCL-2 and/or family members might potentiate the effects of chemotherapeutic regimens used in the treatment of AML, OCI-AML3 cells were treated with ABT-737 for 24 hr before the addition of Ara-C (IC_{50} = 3.64 μM) or Dox (IC_{50} = 0.17 μM). The combination of ABT-737 and Ara-C in these cells, which are relatively resistant to ABT-737, Ara-C, and Dox, individually revealed synergistic effects on apoptosis induction, with an average combination index (CI) of 0.57 ± 0.05 (Figure 1F). Similarly, the combination of ABT-737 and Dox synergistically induced apoptosis with an average CI of 0.37 ± 0.01 (Figure 1G). Simultaneous treatment with ABT-737 and chemotherapeutic agents yielded essentially identical results (CI of ABT-737 and Ara-C cotreatment in OCI-AML3 cells, 0.41 ± 0.006). These results demonstrate that ABT-737 synergizes potently with both Ara-C and Dox in AML cells.

ABT-737 disrupts BCL-2/BAX heterodimerization and induces BAX conformational change in AML cells

A classic model of BCL-2 function suggests that the noncovalent heterodimerization of BCL-2 and BAX serves to quench BAX's apoptotic function by inhibiting proapoptotic BH3-only family members from activating BAX (Chen et al., 2005; Kuwana et al., 2005; Oltvai et al., 1993). The BCL-2/BAX complex was immunoprecipitated using a BAX antibody from cell lysates disrupted in 1% CHAPS lysis buffer, since a previous report indicated that the nonionic detergent NP-40 may alter BAX conformation and affect the BCL-2/BAX interaction (Hsu and Youle, 1997). As a control for dimerization studies, we used HeLa cells, in which no BAX/BCL-2 dimerization was detected using CHAPS buffer, consistent with published reports (Figure 2A) (Wolter et al., 1997; Hsu et al., 1997). In contrast, in DMSO- or enantiomer-treated HL-60 leukemic cells, abundant amounts of BCL-2 are coimmunoprecipitated with BAX, and this BCL-2/BAX association was effectively disrupted by ABT-737 (Figure 2B). Curiously, even though the total amount of BAX did not change, the amount of BAX being immunoprecipitated increased in ABT-737-treated cells, possibly reflecting more efficient recognition of BAX by polyclonal antibody after disruption of BCL-2/BAX complex. These findings suggest that ABT-737-induced cell death is associated with decreased BCL-2/BAX heterodimerization. Similar results were obtained when cells were disrupted in 0.5% NP-40 lysis buffer (data not shown). This inhibition of BCL-2/BAX association occurs 1 and 3 hr after treatment with ABT-737, concomitant with cytochrome *c* release but prior to cleavage of caspase-3, caspase-8, or BCL-2 and BAX itself (Figure 2C).

Recent studies have indicated that activated BAX assumes a specific structure that can be identified by an antibody that recognizes its "prodeath" conformation. As shown in Figure 2D, untreated cells display BAX but not in the prodeath conformation. After treatment with ABT-737, prodeath BAX is readily detectable, indicating that the compound promotes conformational changes in BAX that are associated with apoptosis. No change in either BCL-2/BAX association or BAX conformation was seen after treatment with the inactive enantiomer (Figures 2B and 2D).

While nonionic detergents may artificially promote BCL-2/BAX interaction (Hsu and Youle, 1997), a recent report demonstrated that BCL-2/BAX complexes may indeed exist at the

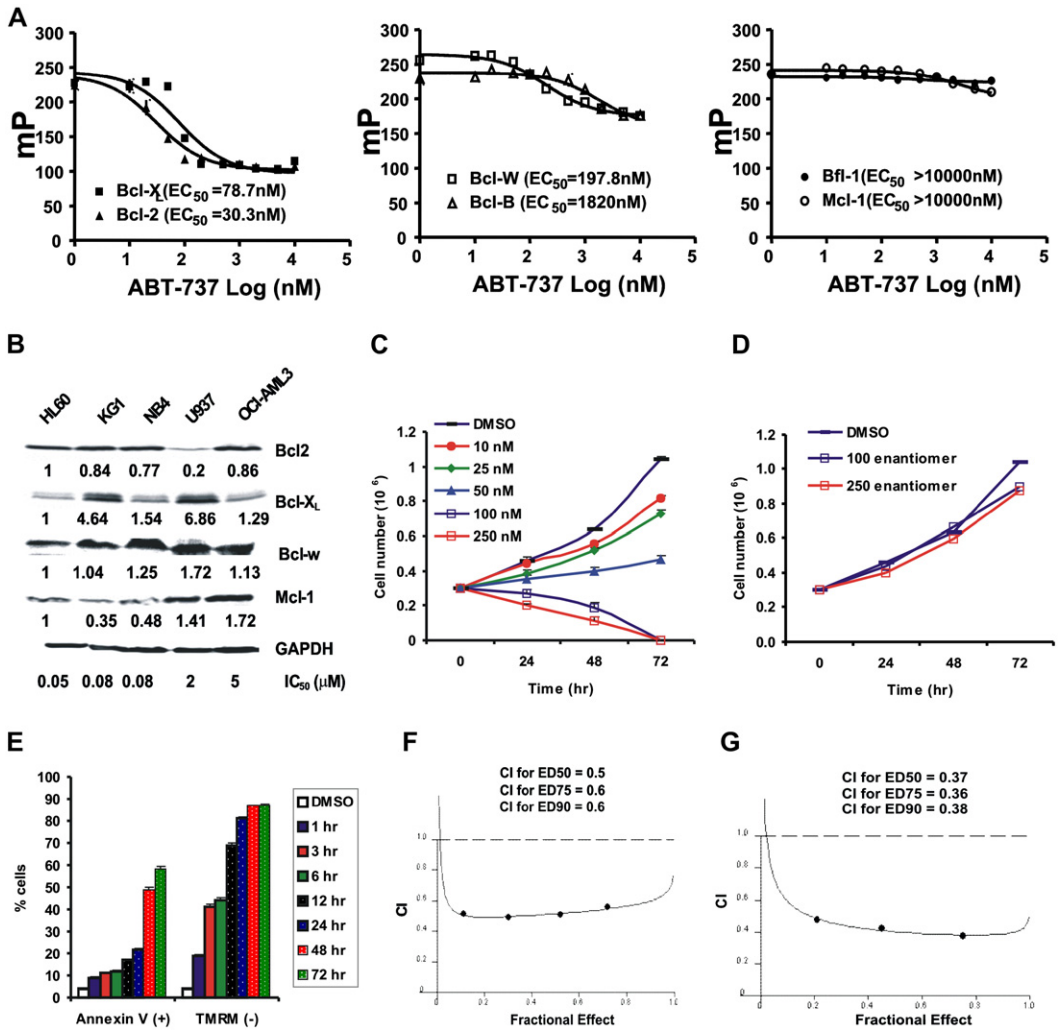

Figure 1. ABT-737 inhibits cell growth and induces apoptosis in leukemic cells

A: Competition inhibition of GST-BCL-2 family fusion protein binding to FITC-BIM BH3 peptide by ABT-737. BCL-2 proteins (100 nM) including BCL-X_L, BCL-2, BCL-W, BCL-B, BFL-1, and MCL-1were incubated with ABT-737 for 2 min in PBS buffer. FITC-conjugated-BIM BH3 peptide (20 nM) was added, and fluorescence polarization was measured after 10 min.

B: Basal expression levels of BCL-2 family members in leukemic cell lines was examined by western blotting, and the intensity of the bands relative to that in HL-60 cells was quantified by densitometry. The effect of ABT-737 on the viability of leukemic cells was determined at 72 hr, and IC_{50}s were calculated using CalcuSyn software.

C and D: Exponentially growing HL-60 cells were treated with indicated concentrations of ABT-737 (**C**) or its inactive enantiomer (**D**), and effects on cell growth were assessed by viable cell counts at 24, 48, and 72 hr.

E: Phosphatidylserine externalization in HL-60 cells was determined by Annexin V staining, and reduction in mitochondrial membrane potential ($\Delta\psi_m$) was determined by decrease in TMRM fluorescence. Results are expressed as mean ± SEM of three independent experiments.

F and G: OCI-AML3 cells were cultured in the presence of escalating doses of ABT-737 (100, 250, 500, and 1000 nM), Ara-C (**F**, 0.25, 0.625, 1.25, and 2.5 μM), doxorubicin (**G**, 0.025, 0.05, and 0.1 μM), or combinations of the two agents at a fixed (1:1) ratio. After 48 hr, apoptosis was measured by Annexin V flow cytometry. CI plots were then generated using the Chou-Talay method and Calcusyn software.

mitochondria and that mitochondrial BAX could be co-immunoprecipitated with BCL-2 using CHAPS detergent lysis (Zhou et al., 2005). To determine the composition of leukemic cell mitochondria, we assessed BAX expression in purified mitochondria from HL-60 cells. BAX was abundantly expressed in the mitochondrial fraction; however, it appeared to be loosely associated with HL-60 mitochondria, since the supernatants from isolated mitochondrial fractions contained similar or even higher amounts of BAX than the mitochondria (Figure 2E). The proapoptotic BH3-only protein BIM was detected almost exclusively in the mitochondrial fraction, consistent with recent reports in other hematopoietic cell lines (Zhu et al., 2004; Harada et al., 2004), demonstrating that leukemic cell mitochondria contain components of the proapoptotic machinery required for triggering mitochondrial permeability transition. Consistent with this scenario, ABT-737 induced cytochrome *c* release from purified mitochondria (Figure 2E).

To determine if ABT-737-induced apoptosis requires BAX, a well-characterized BAX knockout cell line (HCT116) was used. Parental cells were sensitive to 10 μM ABT-737 with approximately 80% cell death after 72 hr. The BAX knockout variant, however, was completely resistant to the drug (Figure 2F). To determine the requirement of BAK in ABT-737-induced cell death, we compared effects of ABT-737 in WT, BAX, BAK, or

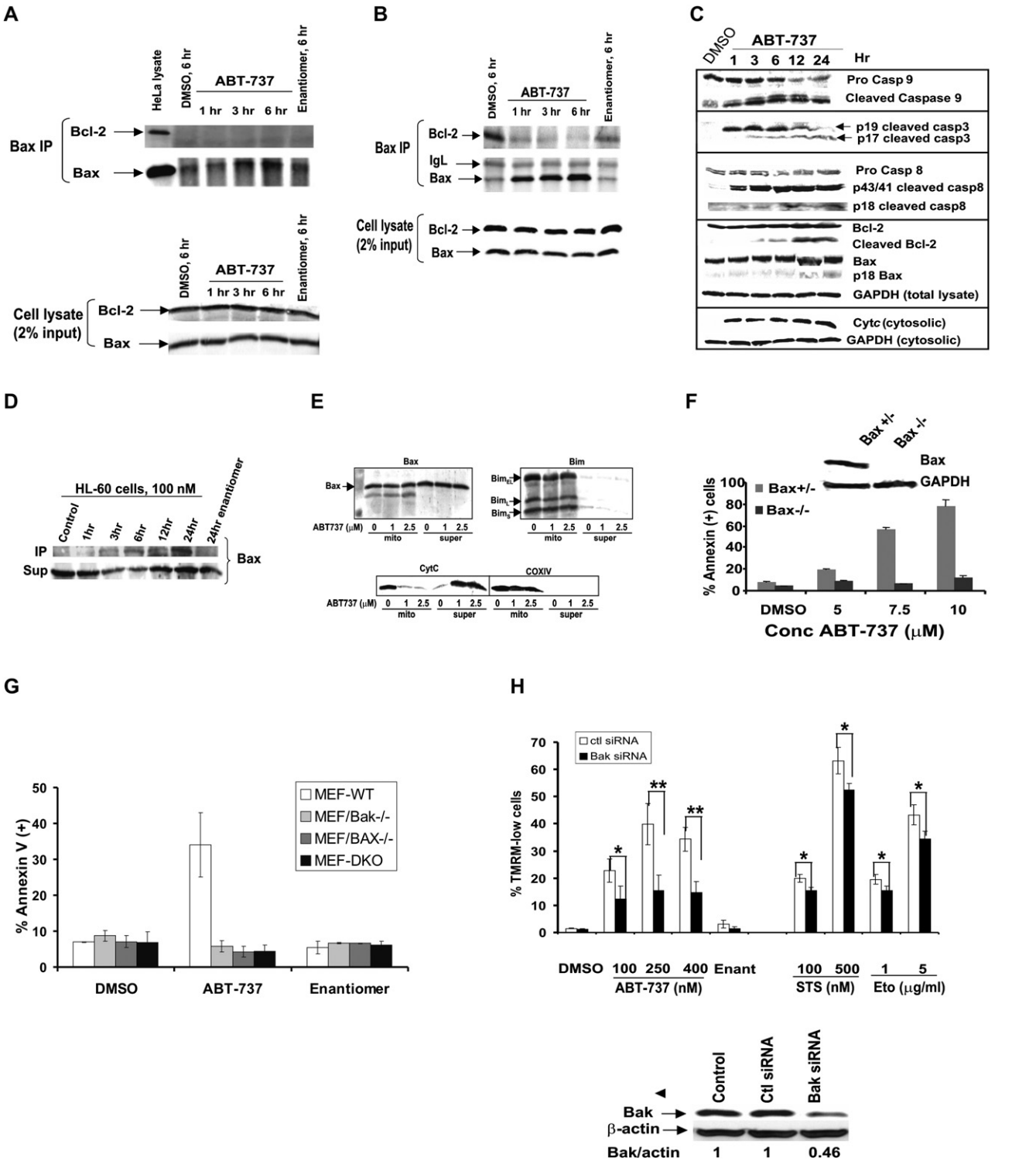

Figure 2. ABT-737 disrupts BCL-2/BAX heterodimerization and induces BAX conformational change in AML cells

A: Endogenous BCL-2 does not bind endogenous BAX in HeLa cells. Healthy HeLa cells were lysed in buffer containing 1% CHAPS. Immunoprecipitation was then performed with an anti-BAX antibody and examined for the presence of BCL-2. First lane: lysate of untreated HeLa cells. Lower panel: expression of BCL-2 and BAX in cell lysates prior to immunoprecipitation.

B: BAX was immunoprecipitated from HL-60 cells treated for the indicated time points with 100 nM ABT-737, and immunoprecipitates (CHAPS buffer) were subjected to western blot analysis using anti-BCL-2 antibody. The amount of BAX immunoprecipitated from each sample was determined using anti-BAX antibody. IgL, immunoglobulin light chain.

C: Cleavage of caspase-3, -8, and -9, cytosolic release of cytochrome *c*, and expression of BCL-2 and BAX after ABT-737 treatment were assessed by western blot.

double knockout (DKO) mouse embryonic fibroblasts (MEFs). While ABT-737 induced apoptosis in WT MEFs, induction of apoptosis was minimal in cells deficient in either BAX, BAK, or both (Figure 2G). These data indicate that ABT-737-induced killing requires both BAX and BAK. To further establish the role of BAK in ABT-737-induced apoptosis of leukemic cells, we treated HL-60 cells transfected with a pool of siRNAs targeting BAK or with control siRNAs with increasing concentrations of ABT-737. Remarkably, knockdown of BAK expression by 50% resulted in significant blockade of ABT-induced mitochondrial depolarization determined by loss of TMRM fluorescence (Figure 2H), and a less pronounced inhibition of staurosporine- or etoposide-induced MMP loss.

BIM is traditionally characterized as a direct "activator" of BAX/BAK, and in certain experimental systems these BH3-only proteins are required for activation of BAX and BAK and subsequent mitochondrial permeability (Letai et al., 2002; Kuwana et al., 2005). However, because antiapoptotic proteins can sequester these direct activator BH3-only proteins, neutralization of the antiapoptotic proteins may be necessary for apoptosis to occur. Based on the finding of the mitochondrial, rather than microtubular, localization of the BH3-only protein BIM in leukemic cells, we examined the potential role of BIM in ABT-737-induced apoptosis utilizing a siRNA approach. Knockdown of BIM isoforms of up to 70% did not significantly affect ABT-737-induced apoptosis (Figure S1 in the Supplemental Data available with this article online) but did inhibit Taxol-induced cell death, consistent with published reports (Tan et al., 2005).

Inhibition of BCL-2 phosphorylation enhances ABT-737-induced apoptosis

BCL-2 phosphorylation may also play a role in determining sensitivity to ABT-737 since the antiapoptotic function of BCL-2 is regulated by phosphorylation (May et al., 1994; Haldar et al., 1998; Srivastava et al., 1998; Deng et al., 2000). ERK was identified as a BCL-2 kinase, and we reported that MEK1 inhibitor PD98059 (Figure 3A) potently suppresses BCL-2 phosphorylation and sensitizes cells to apoptosis (Deng et al., 2000; Konopleva et al., 2002). OCI-AML3 cells treated with PD98059 show potent inhibition of BCL-2 phosphorylation (Figure 3B). The combination of PD98059 and ABT-737 was highly synergistic in killing leukemic cells (CI = 0.08 ± 0.003) (Figure 3C).

These data suggest that BCL-2 phosphorylation modulates sensitivity of AMLs to ABT-737. To further explore this mechanism, BCL-2 phosphorylation mutants were employed. In NSF/N1.H7 murine myeloid cells, conversion of the reported phosphorylated sites in BCL-2 (i.e., threonine 69, serine 70, and serine 87) to alanine residues (AAA mutant) results in increased apoptosis sensitivity. In contrast, conversion of the three amino acid residues to glutamate residues (EEE) produces a gain-of-function mutant that protects cells from Ara-C and etoposide (Deng et al., 2004). Our experiments demonstrated that BCL-2 phosphomimetic (EEE) NSF/N1.H7 cells are more resistant to ABT-737 than cells expressing WT BCL-2 (Figure 3D), suggesting that BCL-2 phosphorylation opposes the proapoptotic action of ABT-737. Consistent with a mechanism whereby increased BCL-2 phosphorylation impedes ABT-737 suppression of BCL-2 dimerization with BAX, ABT-737 potently blocked BCL-2/BAX association in cells expressing exogenous WT BCL-2 and AAA mutant, but not EEE mutant BCL-2 (Figure 3E).

Suppression of MCL-1 expression enhances ABT-737-induced apoptosis

As indicated in Figure 1B, MCL-1 expression levels correlated with sensitivity to the drug. Compared to BCL-2, MCL-1 has a short half-life that is regulated by ERK phosphorylation of a PEST sequence (Schubert and Duronio, 2001). Inactivation of ERK massively enhanced ABT-737-induced apoptosis (CI < 0.1) (Figure 3C). At least part of this synergism likely involves loss of ERK as a BCL-2 kinase. However, loss of MCL-1 expression as result of ERK suppression may also contribute to the observed synergism, and PD98059 indeed downregulated MCL-1 in OCI-AML3 cells (Figure 4A). Prolonged ABT-737 exposure induced modest increase of MCL-1 expression, likely as a result of the death of low MCL-1-expressing cells, and PD98059 potently suppressed expression of this antiapoptotic protein (Figure 4A).

To confirm the inability of ABT-737 to inhibit MCL-1 function that was demonstrated by FPA (Figure 1A), coimmunoprecipitation assays were performed. As shown in Figure 4B, when BIM or BAK was immunoprecipitated from OCI-AML3 cells, coimmunoprecipitation of MCL-1 was unimpaired in ABT-737-treated cells. In contrast, ABT-737 effectively disrupted BCL-2/BAX and BCL-2/BIM association in these cells (Figure 4B, BAX IP and BCL-2 IP). No association of MCL-1 with BAX was found in these cells either before or after ABT-737 (data not shown). On the contrary, in ABT-737-sensitive HL-60 cells, no detectable MCL-1 was found in BAK or BIM complexes (Figure S2).

To test the hypothesis that MCL-1 expression modulates ABT-737 cytotoxicity, MCL-1 levels were reduced in OCI-AML3 cells using shRNA. Infection with lentiviral bicistronic

D: Protein lysates from HL-60 cells treated for the indicated time points with 100 nM ABT-737 were subjected to immunoprecipitation using anti-BAX antibody clone 6A7, which recognizes only conformationally changed BAX protein. Supernatants (Sup) and immunoprecipitates (IP) were subjected to western blot analysis with anti-BAX antibody.

E: Mitochondria of HL-60 cells were isolated as described in the Experimental Procedures, resuspended in M buffer at 0.8 mg/ml protein, and equilibrated at room temperature for 2 min prior to the addition of ABT-737. The concentration of DMSO did not exceed 0.2%. Mitochondrial suspensions were incubated for 15 min at room temperature, and mitochondria were collected by centrifugation at 14,000 rpm for 5 min. The presence of cytochrome c, BIM, and BAX was evaluated by western blotting of the mitochondrial pellet and the supernatant. The purity of mitochondrial preparations was assessed by COXIV immunoblotting. The amount of ABT-737 per milligram of mitochondrial protein used in these experiments is comparable to the amount of ABT-737 used per milligram of protein in cell culture experiments (4–10 nmol/mg protein).

F: HCT116 $BAX^{+/-}$ and HCT116 $BAX^{-/-}$ cells were treated with the indicated concentrations of ABT-737 for 48 hr. Western blot shows levels of BAX in +/− and −/− HCT116 cells.

G: WT and DKO SV40-MEFs were cultured in DMEM medium supplemented with 10% FCS for 72 hr with or without 100 nM ABT-737 and its enantiomer control. Apoptosis was evaluated by Annexin V staining. Results are expressed as mean ± SEM of three independent experiments.

H: Depletion of BAK by siRNA preserves mitochondrial integrity in ABT-737-treated leukemic cells. HL-60 cells transfected with a pool of BAK siRNAs or control siRNA by Amaxa nucleofection were treated with the indicated concentrations of ABT-737, staurosporine (STS), etoposide (Eto), or 400 nM enantiomer for 24 hr, and loss of the mitochondrial membrane potential was determined by TMRM fluorescence. Statistically significant differences were determined by ANOVA test (*$p < 0.05$; **$p < 0.01$). BAK expression was examined by western blotting, and the intensity of the bands relative to that in mock-transfected HL-60 cells was quantified by densitometry. Results are expressed as mean ± SEM of three independent experiments.

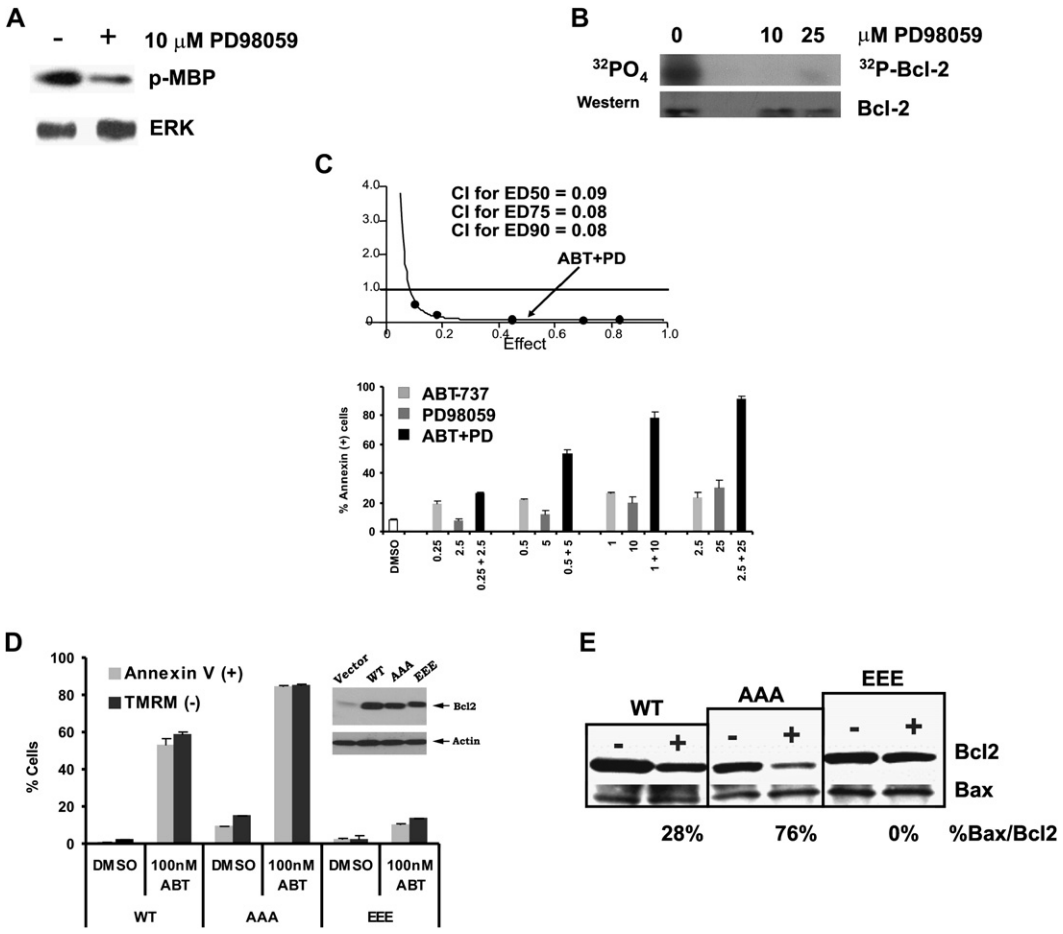

Figure 3. Inhibition of BCL-2 phosphorylation enhances ABT-737-mediated apoptosis

A: Inhibition of ERK signaling by PD98059 in OCI-AML3 cells was determined by ERK kinase assay. MAP kinase activity was measured by the ability to phosphorylate the specific substrate, MBP, in the in vitro kinase assay. Total ERK immunoblot was used as a loading control.

B: OCI-AML3 cells were radiolabeled with [^{32}P]orthophosphate, treated with 10 and 25 μM PD98059, and lysed, after which BCL-2 was immunoprecipitated. Protein was subjected to SDS/PAGE, transferred to nitrocellulose membrane, and exposed to X-ray film to identify phosphorylated BCL-2. The identity of the protein was confirmed by western blot analysis using the same filter.

C: OCI-AML3 cells were cultured in the presence of escalating doses of ABT-737 (100, 250, 500, and 1000 nM) and PD98059 (2.5, 5, 10, and 25 μM) or combinations of the two agents at a 1:25 ratio. After 72 hr, apoptosis was measured by Annexin V flow cytometry, and CI plots were generated. Results are expressed as mean ± SEM of three independent experiments.

D: NSF.N1/H7 cells expressing WT, AAA (phosphorylation deficient), or EEE (gain of function) mutants were treated with 100 nM ABT-737 for 48 hr, and apoptosis was determined by Annexin positivity and mitochondrial membrane potential loss (TMRM). Equal levels of transfected BCL-2 in these cells were assured by immunoblotting. Results are expressed as mean ± SEM of three independent experiments.

E: DMSO- or ABT-treated NSF.N1/H7 WT, AAA, or EEE cells were lysed in buffer containing 0.5% NP-40. Immunoprecipitation was performed with an anti-BAX antibody and examined for the presence of BCL-2. The amount of BAX immunoprecipitated from each sample was determined by anti-BAX antibody. Association of endogenous BCL-2 with BAX was recently confirmed in these cells by comparing 1% CHAPS lysis buffer with 0.5% NP-40 lysis buffer (Deng et al., 2006). The intensity of the bands was quantitated by densitometry and expressed as a percentage of BAX/BCL-2 relative to control.

constructs containing shRNA against transcript 1 of the MCL-1 gene encoding the long isoform 1 gene product and a GFP reporter gene resulted in a 63% reduction of MCL-1 protein expression (Figure 4D). When these MCL-1-depleted cells were treated with ABT-737, impressive apoptosis was induced, as compared to mock-transfected cells or cells transfected with irrelevant shRNA, which were resistant to ABT-737 (Figures 4E). These experiments established MCL-1 as a major resistance factor to ABT-737.

ABT-737 induces apoptosis and selectively inhibits colony formation of primary AML cells

The effect of ABT-737 on primary AML blast cells was tested by measuring clonogenic AML cell growth in the CFU-blast assay. All samples were obtained from relapsed/refractory AML patients (Table S1). Colony formation was significantly reduced to 18% ± 9% at 100 nM and to only 7% ± 4% at 250 nM ($p < 0.05$) (Figure 5A), with IC_{50}s between 1.4 nM and 103.2 nM. BCL-2 expression did not show significant variations in this sample set (Figure 5B) and did not correlate with the ability to inhibit colony formation ($R^2 = 0.01$, $p = 0.87$). In contrast, MCL-1 levels varied 10-fold among different samples and inversely correlated with IC_{50} for inhibition of clonogenicity ($R^2 = 0.96$, $p = 0.003$).

Importantly, only minimal inhibition of colony formation by normal bone marrow cells (n = 3) was observed at up to 250 nM ABT-737 ($p > 0.05$) (Figures 5C–5E). The difference in inhibition of AML and normal GM-progenitor cells was highly significant ($p < 0.001$). MCL-1 protein was highly expressed in cell lysate from

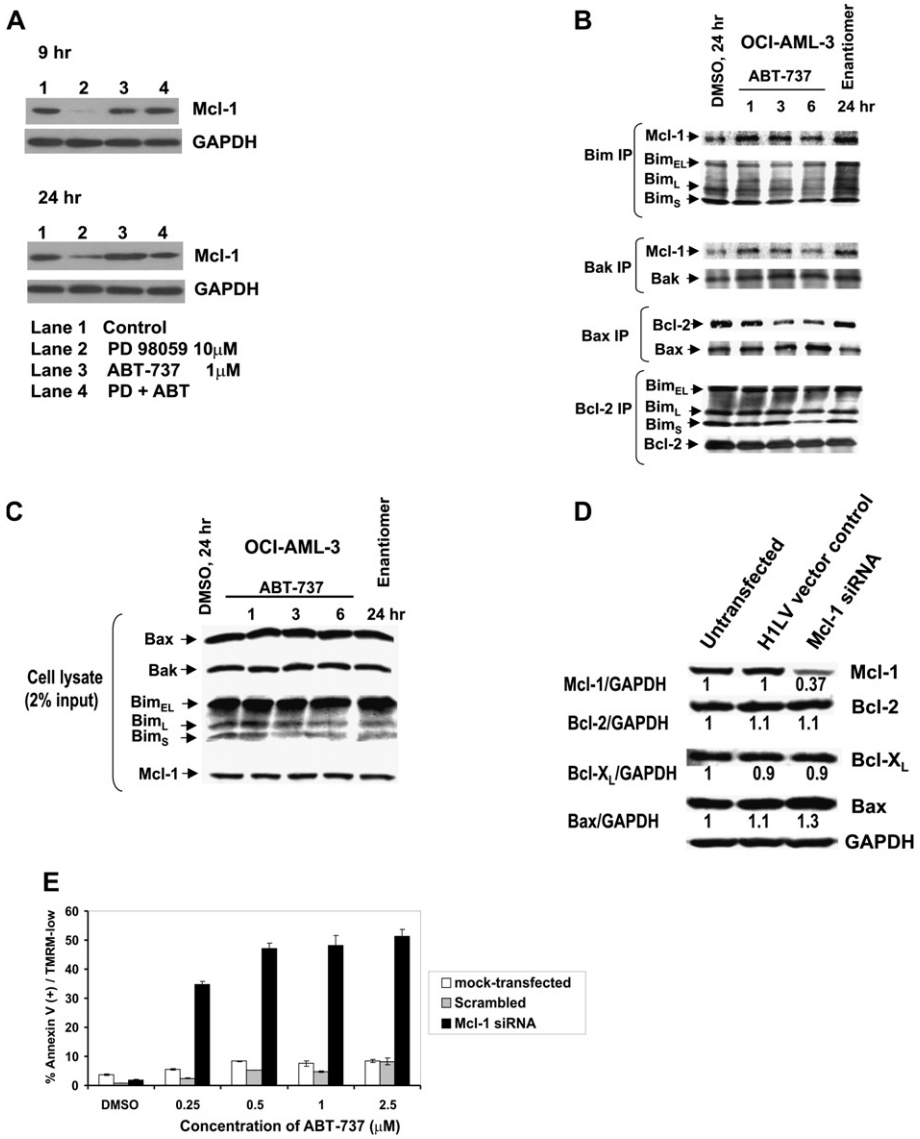

Figure 4. Suppression of MCL-1 expression enhances ABT-737-induced apoptosis

A: OCI-AML3 cells were treated with 1 μM ABT-737, 10 μM PD98059, or their combination for 9 and 24 hr, and MCL-1 expression was determined by western blot analysis.

B: BIM, BAK, BAX, or BCL-2 were immunoprecipitated from OCI-AML3 cells treated for the indicated times with 250 nM ABT-737, and immunoprecipitates were subjected to western blot using anti-MCL-1 (BIM, BAK IP), anti-BCL-2 (BAX IP), or anti-BIM (BCL-2 IP) antibodies. The amount of BIM, BAX, BAX, or BCL-2 immunoprecipitated from each sample was determined using the respective antibodies.

C: Expression of BIM, BAX, BAX, and BCL-2 in cell lysates prior to immunoprecipitation.

D and E: OCI-AML3 cells were infected with lentiviral MCL-1 siRNA, control vector (H1LV), or scrambled siRNA as described in the Experimental Procedures. GFP-positive and -negative cells were FACS sorted and treated with the indicated concentrations of ABT. MCL-1 expression was analyzed by western blotting (**D**). Induction of apoptosis was analyzed by Annexin V/TMRM flow cytometry (**E**). Results are expressed as mean ± SEM of three independent experiments.

CD34-enriched normal marrow sample (lane 1, Figure 5B), while BCL-2 expression was low, consistent with published observations (Park et al., 1995; Andreeff et al., 1999). MCL-1 mRNA expression was significantly higher in normal hematopoietic progenitor cells (n = 4) compared to AML blasts (n = 58) (Figure 5F; $p < 0.016$), consistent with the reported crucial role for MCL-1 in normal hematopoiesis (Opferman et al., 2005).

Supporting the direct effects on AML progenitors, ABT-737 induced apoptosis (Annexin V positivity) in eight of nine $CD34^+$ AML progenitor cells (Tables S2 and S3, samples 1–9). No difference in sensitivity to ABT-737-induced apoptosis was noted between newly diagnosed and relapsed/refractory patients, while ABT-737 induced moderate apoptosis only in one out of four normal $CD34^+$ samples (Tables S2 and S3, samples 10–13), confirming preferential apoptosis induction in leukemic but not in normal progenitor cells.

High sensitivity of AML stem cells to ABT-737

Effective leukemia therapy has to target leukemia stem cells to generate sustained responses. AML stem cells have been phenotypically defined as $CD34^+38^-123^+$ (Jordan et al., 2000). In samples from six relapsed AML patients, the frequency of these stem cells was as follows: 0.23%, 0.61%, 0.33%, 39.18%, 2.97%, and 1.51%, respectively (samples 1–6, Table S4). AML samples were treated with ABT-737 (100 nM), Ara-C (1 and 5 μM), or vehicle for 24 hr. Three of six samples (1, 2, and 5) had 2.8%, 0%, and 0% surviving stem cells following treatment with ABT-737 (Figure 5H). Ara-C, at the concentrations used, was without exception less cytotoxic against AML stem cells than ABT-737, and ABT-737 completely eliminated all stem cells in one of the two Ara-C-resistant samples (Figures 5G and 5H). This demonstrates what appears to be an exquisite sensitivity of AML stem cells to ABT-737 in most but not all cases.

Antileukemia activity of ABT-737 in vivo

Next, the effects of in vivo administration of ABT-737 were examined in the conditional leukemia model driven by Raf-transformed myeloid cells (Konopleva et al., 2005). FDCP-ΔRaf1 cells were infected with AdLux-F/RGD vector to monitor leukemia dissemination by bioluminescence imaging (BLI) and injected

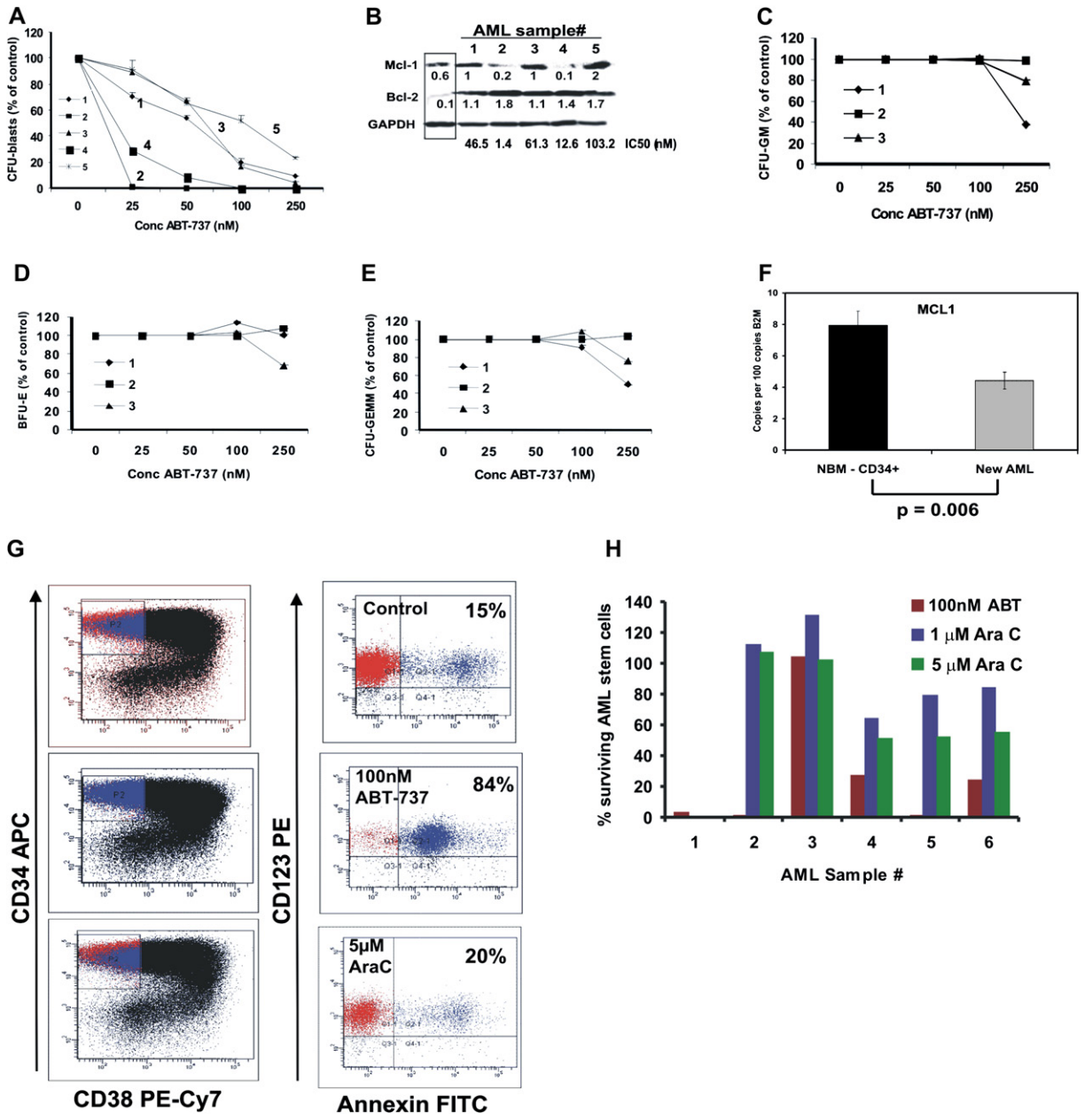

Figure 5. ABT-737 inhibits clonogenic progenitor growth and causes selective apoptosis in AML stem cells

A, C, D, and E: Results are expressed as the mean ± SEM of the percentage of colonies compared with the number in DMSO-treated control cells. Inhibition of colonies (CFU) in AML bone marrow samples (n = 5) (**A**) and CFU-GM (**C**), BFU-E (**D**), and CFU-GEMM (**E**) in normal bone marrow samples (n = 3) in the presence of increasing concentrations of ABT-737 (25, 50, 100, and 250 nM). Results are expressed as mean ± SEM of three independent experiments. **B:** Western blot analysis of BCL-2, BAX, and MCL-1 expression in five AML samples (sample numbers correspond to those in Figure 5A) and in one $CD34^+$-enriched sample from normal bone marrow used for clonogenic studies. Numbers represent densitometry quantitation of the intensity of the bands normalized to GAPDH expression in each sample and to the levels in HL-60 cells (=1). IC_{50}s for AML samples were calculated based on the inhibition of the colony-forming ability of AML blasts using CalcuSyn. **F:** Abundance of MCL-1 transcript was measured in samples from 58 AML samples and from 4 normal marrow $CD34^+$ cells by real-time PCR, and results are reported as the mean number of transcripts per hundred transcripts of β-2-microglobulin. Error bars denote the standard error of the mean. **G:** Cells from a primary AML sample were treated with DMSO (control), 100 nM ABT-737, or 1 μM Ara-C for 24 hr. Induction of apoptosis was measured by Annexin V positivity on gated $CD34^+CD38^-CD123^+$ AML stem cells. **H:** Effects of ABT-737 and Ara-C on AML stem cells. The frequency of $CD34^+CD38^-CD123^+$ PS/Annexin V+ cells was determined by multicolor flow cytometry. The percentage of nonapoptotic (Annexin V−) stem cells was calculated after ABT-737 and Ara-C treatment (number of stem cells in DMSO-treated cultures = 100%). In sample 1, cell numbers were insufficient to examine effects of Ara-C.

intravenously (i.v.) into SCID mice. BLI demonstrated that ABT-737 suppressed the leukemia burden by 48% and 53% at the 20 and 30 mg/kg dose levels, respectively ($p < 0.02$; Figures 6A and 6B) and significantly extended survival of mice in this aggressive leukemia model (median survival, 28–32.5 days compared to 19.5 days survival of controls, $p < 0.01$) (Figure 6C). Similar inhibition of leukemia burden was observed in a human xenograft leukemia model with KG-1 cells (Figures 6D and 6E). TUNEL demonstrated induction of apoptosis in spleen and liver infiltrated with leukemic cells (Figure S3). In healthy SCID mice liver,

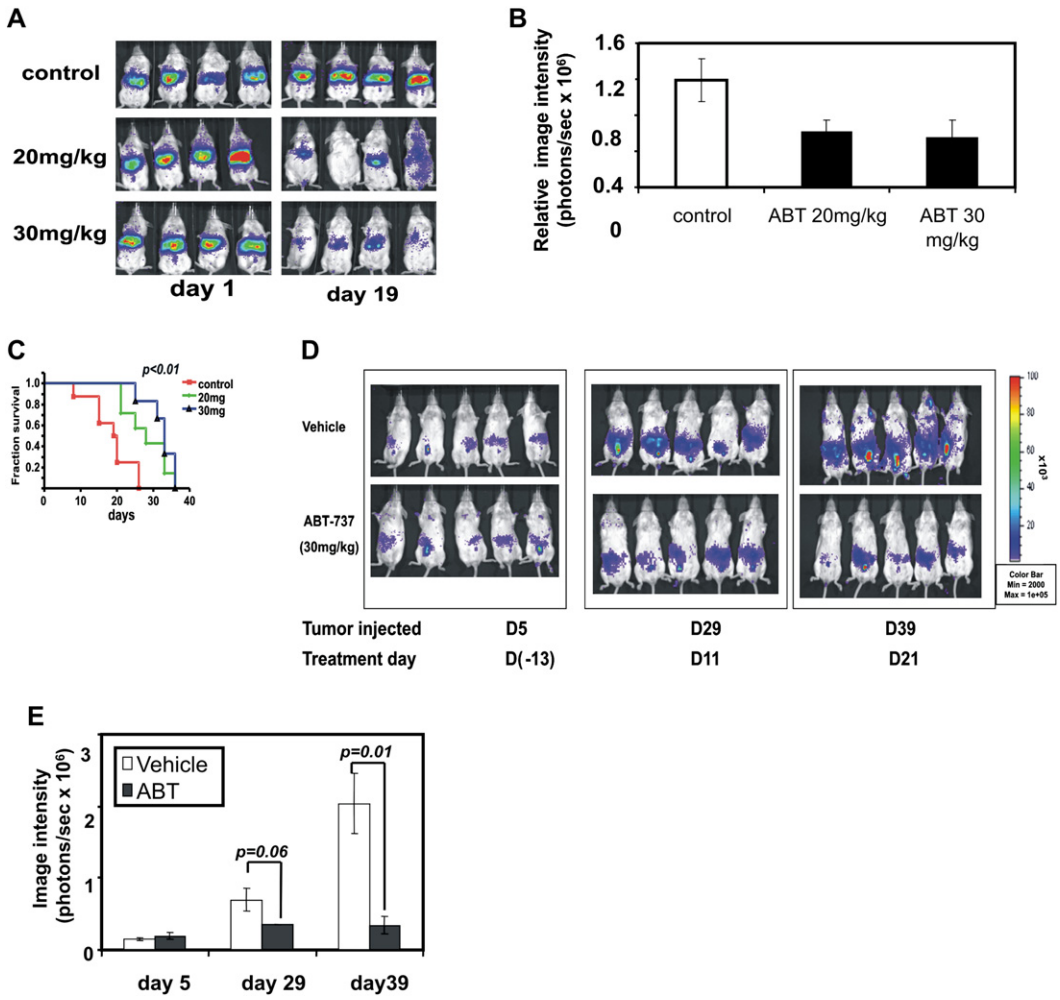

Figure 6. ABT-737 inhibits leukemia in vivo

A and B: FD/GFPΔRaf-1:ER *luc* cells were injected i.v. into SCID mice carrying β-estradiol-release pellets. Mice were treated with either ABT-737 (from days 1–21, n = 8 per group) or vehicle (n = 8). On day 19, mice were imaged after D-luciferin injection. Serial images of representative mice are shown in **A**. Results were averaged from the peak light-emitting exposure from each group and displayed as photons/s after normalizing for baseline luminescence intensity (**B**). Error bars represent SEM.

C: Kaplan-Meier curves for these three groups of mice are shown. p value was determined by log-rank test.

D: Human leukemia luciferase-expressing KG-1 cells were injected i.v. into SCID mice, and leukemia dissemination was monitored by bioluminescence imaging. Eighteen days post-cell injection, mice were treated with ABT-737 (n = 8 per group) or vehicle (n = 8). Serial images of representative mice are shown.

E: Results were averaged from the peak light-emitting exposure from each group and displayed as photons/s. Error bars represent SEM.

kidney, and spleen remained histologically normal, and ABT-737 treatment did not induce significant abnormalities in blood cell counts or serum chemistries, although platelet counts tended to be lower in ABT-737-treated animals ($p = 0.15$; Figure S4).

Discussion

The mechanism of action of many traditional anticancer drugs involves programmed cell death and raises the possibility of designing strategies aimed at enhancing apoptosis. While several regulators of apoptosis are candidates for such strategies, BCL-2 family proteins have assumed a central role. ABT-737 is representative of a class of BH3 mimetics developed for this purpose (Oltersdorf et al., 2005; Pellecchia and Reed, 2004). The results presented here support the use of ABT-737 as an antileukemia agent, as the compound potently kills AML-derived cell lines and blast cells from AML patients. Most importantly, our results demonstrated killing of leukemic progenitors (CFU) and stem cells ($CD34^+38^-123^+$), a critical property of any truly innovative treatment for leukemias and cancer. Normal progenitor cells were remarkably resistant to ABT-737, suggesting a very favorable therapeutic window. In contrast, Ara-C, the most active cytotoxic agent used in AML therapy, was less effective against AML stem cells, providing excellent rationale for combination approaches. Our data in aggressive murine and human xenograft leukemia models clearly suggest that ABT-737 is effective in vivo, in the absence of unwanted side effects on normal tissues. These findings provide rationale for the development of ABT-737 or related compounds for the treatment of AMLs.

Our mechanistic studies suggest that the cellular composition of BCL-2 family proteins critically determines sensitivity to ABT-737. It was recently postulated that MCL-1 and/or BFL-1 might account for resistance to ABT-737 (Cory and Adams, 2005).

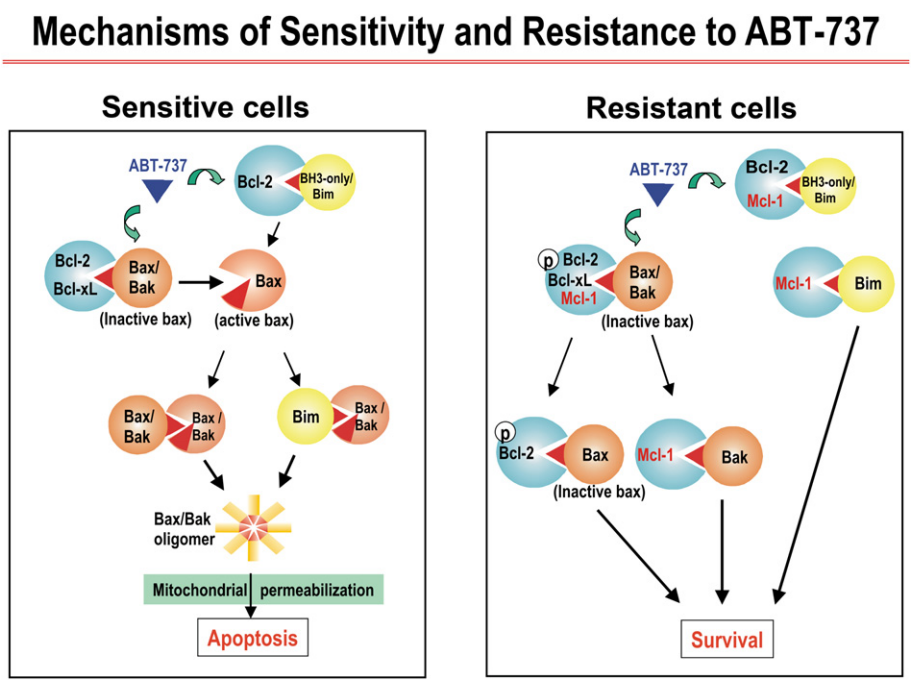

Figure 7. Mechanisms of sensitivity and resistance to ABT-737

In sensitive cells with low or absent levels of MCL-1 (left panel), BAK, BAX, and "activating" BH3-only proteins, including BIM and possibly other unidentified BH3-only proteins, are bound and neutralized by BCL-2 prosurvival proteins. ABT-737 displaces active BAK from BCL-X_L, BAX from BCL-2, or BIM/BH3-only protein from BCL-2. This is followed by activation of freed BAX by BAK or BH3-only proteins, MPT, caspase activation, and apoptosis. In ABT-resistant cells expressing high MCL-1 levels and/or phospho-BCL-2 (right panel), both BAK and the "activating" BH3-only proteins are sequestered by MCL-1 in addition to BCL-2 and BCL-X_L prosurvival proteins. ABT-737 fails to displace BAX from phosphorylated BCL-2, and BAK/BIM (or other BH3-only proteins) remains bound and inactivated by MCL-1, thus hindering BAX/BAK activation and promoting cell survival.

Our FPA results and coimmunoprecipitation experiments confirmed the inability of ABT-737 to inhibit binding of BIM or BAK to MCL-1, and resistance to ABT-737 appears to correlate with high MCL-1 expression in AML cell lines (Figure 1B), in a subset of primary AML samples (Figure 5B) and in normal $CD34^+$ progenitor cells (Figures 5B and 5F). Downregulation of MCL-1 by siRNA (Figure 4E) resulted in striking sensitization to ABT-737. Thus, future small molecule inhibitors targeting MCL-1 would be expected to act synergistically with ABT-737. However, MCL-1 inhibitors may also target normal leukemic stem cells, as MCL-1 has recently been shown to be a critical regulator of early hematopoietic development in mice (Opferman et al., 2005), consistent with our expression data in human $CD34^+$ cells (Figure 5E). In the absence of MCL-1 small molecule inhibitors, the MEK/ERK inhibitor PD98059 proved effective at both suppressing MCL-1 expression (Figure 4A) and acting synergistically in an unprecedented manner with ABT-737 (Figure 3C). We propose that, in AML cells with constitutive activation of MAPK/ERK signaling, MCL-1 expression is largely dependent on this pathway; hence inhibition of ERK activation with MEK inhibitors may overcome resistance to ABT-737. Importantly, we and others have shown complete lack of phospho-ERK expression in normal progenitor cells (Milella et al., 2001), while activation of ERK signaling confers poor survival in AML (Kornblau et al., 2006).

Synergistic use of MEK/ERK inhibitors and ABT-737 has the added advantage of blocking ERK's role as a BCL-2 kinase (Figure 3B). We demonstrated that BCL-2 phosphorylation abrogates the activity of ABT-737 cells expressing a phosphomimetic mutant of the serine 70 phosphorylation site of BCL-2 were extremely resistant (Figure 3D). Of importance, we recently reported phosphorylation of BCL-2 in primary AML samples, and its negative impact on patient prognosis (Kurinna et al., 2006). BCL-2 phosphorylation at serine 70 promotes heterodimerization with BAX (Ito et al., 1997). We therefore postulate that phosphorylation-induced conformational changes in BCL-2 may impede access of ABT-737 to the BH3 binding groove, a hypothesis currently under investigation.

Molecular analysis of apoptotic events initiated by ABT-737 suggested several mechanisms of sensitivity and resistance to ABT-737 (Figure 7). The existing functional models acknowledge the criticality of BAX and BAK for the execution of apoptosis, which was confirmed by our finding that MEF cells devoid of BAX or BAK are resistant to ABT-737. Coimmunoprecipitation experiments demonstrated that, in leukemic but not in HeLa cells, BAX and BCL-2 form complexes, and that these complexes are disrupted upon exposure to ABT-737. Our data further showed that in leukemic cells, perhaps in contrast to other cell types, a large fraction of BAX is associated with mitochondria. Identical results were reported in other studies (Letai et al., 2002; Deng et al., 2006), although this cell type-specific phenomenon is not widely acknowledged in the literature. It remains unclear, however, whether mitochondria-localized BAX is already activated. While a recent report demonstrated the existence of BCL-2-inhibitable autoactivation of mitochondrial membrane-bound BAX (Tan et al., 2006), we were unable to detect it in healthy cells (Figure 2D). In addition, no association of BAX with MCL-1 was detected in ABT-resistant cells, suggesting involvement of additional protein(s) capable of activating BAX in sensitive cells that are sequestered and inactivated by MCL-1 in resistant cells. Of all BH3-only proteins examined, only tBID and BIM are known to act as direct "activators" of BAX and BAK (Letai et al., 2002). We first focused on BIM, which has recently emerged as being critical for hematopoiesis (Bouillet et al., 1999) and is localized at the mitochondria of hematopoietic cells (Zhu et al., 2004; Harada et al., 2004; and Figure 2E), but not other cell types (Puthalakath et al., 1999). Using synthetic peptides (Figure 1A) and coimmunoprecipitation studies (Figures 4B), we indeed demonstrated the ability of ABT-737 to disrupt BCL-2/BIM interactions. However, BIM knockdown did not reduce ABT-triggered apoptosis. While we did not achieve complete BIM silencing in hematopoietic cells,

which are notoriously difficult to transfect, a recent report suggests that other types of cell death may proceed independent of BIM (Ekert et al., 2006), in sharp contrast to complete resistance of cells deficient in BAX and BAK. Furthermore, ABT-737 was shown to dramatically increase Imatinib-induced killing of bcr-abl-transformed hematopoietic cells from $BIM^{-/-}BAD^{-/-}$ knockout mice (Kuroda et al., 2006), thus arguing against the criticality of BIM in ABT-737-induced apoptosis and BAX activation. While we did not examine the role of tBID in ABT-737-induced cell death, BID requires either cleavage via activated caspase-8 into truncated tBID (Clohessy et al., 2006) or membrane targeting (Oh et al., 2006) to serve as an activating BH3 protein. Our studies in caspase-8-deficient Jurkat cells documented undiminished ABT-737-induced apoptosis (Figure S5), arguing against the role of caspase-8 and hence tBID in apoptosis induction by this agent. These findings also do not support the recently proposed model of cell death that implies requirements of ''stress'' to engage the direct activators of BAX and BAK (i.e., BIM or tBID) in cells that are ''addicted'' to the antiapoptotic BCL-2 proteins (Certo et al., 2006). Alternatively, mitochondrially localized BAK may play a principal role in sensitivity to ABT-737: BAK binds BCL-X_L and MCL-1 and can assume a BH3-exposed, active conformation in healthy cells, which is kept in check by MCL-1/BCL-X_L (Willis et al., 2005). In HL-60 cells with low endogenous MCL-1, we failed to detect MCL-1/BAK association, implying that neutralization of BCL-X_L by ABT-737 triggers apoptosis through activated BAK in cooperation with BAX (Figure S2). In contrast, in resistant OCI-AML3 cells overexpressing MCL-1, BAK was tightly associated with MCL-1, and ABT-737 was unable to disrupt this interaction (Figure 4B). Further, BAX-expressing MEF cells deficient in BAK were resistant to ABT-737 (Figure 2G), and depletion of BAK by siRNA in BAX-expressing HL-60 cells profoundly inhibited mitochondrial depolarization caused by ABT (Figure 2H). These findings indicate that BAK is indeed participating in apoptosis of sensitive cells.

While these hypotheses warrant further investigations, findings presented here strongly suggest that cellular abundance and mitochondrial composition of certain BCL-2 family proteins define patterns of sensitivity or resistance to BH3 mimetics. Our studies define hematologic malignancies as tumors exquisitely sensitive to ABT-737 as a single agent, although elevated MCL-1 expression and basal BCL-2 phosphorylation may contribute to resistance. In cases where ERK signaling is antiapoptotic, both MCL-1 expression and phosphorylation of BCL-2 can be blocked by ERK inhibitors. Alternatively, combinations with traditional chemotherapeutic agents may overcome resistance in certain cases, one possible mechanism being MCL-1 degradation. In summary, the findings presented here strongly suggest that ABT-737 will be an effective antineoplastic agent and a potent weapon in the anti-AML arsenal.

Experimental procedures

Reagents and antibodies

ABT-737 was synthesized as described previously (Oltersdorf et al., 2005). Tetramethylrhodamine methyl ester (TMRM) was purchased from Molecular Probes (Eugene, OR). Annexin V FITC was purchased from Roche Diagnostic Co. (Indianapolis, IN). Mouse IgG_1 FITC, IgG_1 PE, and CD34 PE were purchased from BD Biosciences (San Jose, CA). Caspase-3, -8, and -9 (anti-human and anti-mouse), phospho-BCL-2 (S-70), and phospho-ERK 1/2 antibodies were purchased from Cell Signaling Technologies Inc. (Beverly, MA); the cytochrome *c*, BAX, BCL-2 (6C8), and MCL-1 antibodies were from BD Biosciences; the BCL-2 antibodies were from Dako (Carpinteria, CA); BAK antibodies were from Upstate (Lake Placid, NY) and Calbiochem (Ab-1); and the BIM antibody (22-40) was from Calbiochem (La Jolla, CA). ERK 2 antibody and control anti-rat IgG, anti-rabbit, or anti-Syrian hamster isotype-matched control antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), BAX 2D2 and BAX clone 6A7 antibodies were from Sigma Chemical Co., GAPDH antibody was from Chemicon International (Temecula, CA), and goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad (Hercules, CA). Rat monoclonal anti-BIM (3C5) was kindly provided by Drs. L.A. O'Reilly and A. Strasser (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), and polyclonal anti-human anti-BAX antibody was from Dr. W.S. May (Ito et al., 1997). TUNEL labeling kit was purchased from Roche Diagnostics Corporation (Mannheim, Germany).

Cell lines and primary AML samples

U937, HL-60, KG-1, and NB4 cells were purchased from The American Type Culture Collection (ATCC, Rockville, MD). OCI-AML3 cells were kindly provided by M.D. Minden (Ontario Cancer Institute, Toronto, ON, Canada), and HCT116 $BAX^{+/-}$ and $BAX^{-/-}$ cells were provided by Dr. B. Vogelstein (John Hopkins University, Baltimore, MD). WT, BAX, BAK, and DKO SV40-MEFs were a kind gift of Dr. S.J. Korsmeyer (Howard Hughes Medical Institute, Harvard Medical School, Boston, MA). Various phospho mutants of the murine IL-3-dependent cell line NSF/N1.H7 were constructed using site-directed mutagenesis, as described (May et al., 1994).

Bone marrow or peripheral blood samples were obtained for in vitro studies from patients with newly diagnosed or recurrent AML during routine diagnostic work-up following informed consent. All studies performed with human specimens were done with approval from the M.D. Anderson Institutional Review Board. The clinical features of the patients are listed in Tables S1–S4. Specimens from bone marrow transplant donors underwent $CD34^+$ separation to greater than 95% purity by positive-selection magnetic-bead sorting using a VarioMACS device (Miltenyi Biotec, Auburn, CA). Cells were either used for colony assays or cultured in AIM-V medium (Gibco Laboratories) supplemented with 5% FBS, 1 mM L-glutamine, and 50 μg/ml penicillin/streptomycin.

Flow cytometric analysis of apoptosis

Apoptosis was determined by the flow cytometric measurement of phosphatidylserine exposure using Annexin V FITC as described (Milella et al., 2001). In the case of cells from patient samples, cells were stained with PE-labeled anti-CD34 and Annexin V FITC. The mitochondrial transmembrane potential ($\Delta\psi_m$) was determined by measuring TMRM retention (red fluorescence) (Kaufmann et al., 1998).

FPAs

To determine the binding affinity of GST-BCL-2 family proteins to the FITC-conjugated BH3 domain of BIM (FITC-Ahx-DMRPEIWIAQELRRIGDEF NAYYAR), FPAs were performed as described (Zhai et al., 2005). Briefly, 100 nM of GST-BCL-2 family fusion proteins were incubated with serial dilutions of ABT-737 in PBS for 2 min. Then, 20 nM of FITC-BIM BH3 peptide (FITC-Ahx-DMRPEIWIAQELRRIGDEFNAYYAR) was added. Fluorescence polarization was measured using an Analyst TM AD Assay Detection System (LJL Biosystem, Sunnyvale, CA) after 10 min using the 96-well black plate (Greiner bio-one). IC_{50}s were determined using GraphPad Prism software (GraphPad, Inc., San Diego, CA).

BAX conformational change

Cells (5×10^6) were lysed in buffer (125 μl) containing 150 mM NaCl, 10 mM HEPES (pH 7.4), 1% CHAPS, and protease inhibitors. Protein lysates were subjected to immunoprecipitation using anti-BAX antibody clone 6A7, which recognizes conformationally changed BAX protein. Supernatants and immunoprecipitates were then resuspended in SDS gel-loading buffer and subjected to western blot analysis with anti-BAX antibody (Craig, 2002).

Mitochondrial cytochrome *c* release

HL-60 mitochondria were isolated as described previously (Samudio et al., 2006). Mitochondrial suspensions (0.8 mg/ml protein) were energized with succinate in the presence of rotenone and treated with DMSO or various concentrations of ABT-737 for 15 min at room temperature. Mitochondria were

collected by centrifugation at 14,000 rpm for 5 min, and the presence of cytochrome *c* was evaluated by western blotting of the mitochondrial pellet and the supernatant.

Quantitative real-time PCR

Bone marrow and peripheral blood samples were lysed with RNA Stat 60 (Tel-Test, Friendswood, TX). Real-time PCR was done using an ABI Prism 7700 instrument. As primer and probe sets to detect MCL-1 and housekeeping gene β-2-microglobulin, we used TaqMan Gene Expression Assays Hs0000172036_m1 and Hs99999907_m1 (for details, see the Supplemental Experimental Procedures). The abundance of each transcript relative to that of β-2-microglobulin was calculated as follows: relative expression (RE) = 100 × 2 exp [−ΔCt], where ΔCt is the mean Ct of the transcript of interest less the mean Ct of the transcript for β-2-microglobulin.

siRNA transfection

For BIM knockdown, siRNAs were obtained as duplexes in purified and desalted form from Dharmacon. The sense strand of the siRNA silencing *BIM* gene (BIM-siRNA) was GACCGAGAAGGUAGACAAUUGdTdT (Han et al., 2005). A nonspecific control pool containing four pooled nonspecific siRNA duplexes was used as a negative control (referred to as NS-siRNA, Dharmacon-Upstate). For BAK knockdown, ON-TARGETplus SMARTpool and siCONTROL Non-Targeting siRNA 1 were obtained from Dharmacon. Transfection of leukemic cells was carried out by electroporation using the Nucleofection system (Amaxa, Köln, Germany), following the manufacturer's instructions. The final concentration of siRNA was 67 nM. At the optimal time of gene silencing monitored by western blot (24 hr posttransfection), various concentrations of ABT-737 were added to the cells.

Preparation of lentiviral construct for shRNA MCL-1 knockdown

To downregulate the expression of MCL-1 by RNA interference, sense and antisense oligonucleotides were designed to generate a short 19 bp hairpin corresponding to nucleotides 2343–2361 of transcript variant 1 of the MCL-1 gene encoding the long isoform 1 gene product (GenBank accession number NM 021960). As control, sense and antisense oligonucleotides were designed to generate a 19 bp hairpin targeting the irrelevant sequence, CGTACGCGGAATACTTCGA. The oligonucleotides were annealed and ligated into a self-inactivating lentiviral vector (Lois et al., 2002; Miyoshi et al., 1998) under control of the HI promoter. The vector was also designed to carry the GFP reporter gene under control of the human ubiquitin-C promoter to monitor infection efficiency.

Each lentiviral shRNA construction was transfected into 293T cells using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). Cells were monitored for GFP fluorescence, and at 72 hr posttransfection, virus-containing supernatants were collected and used to infect OCI-AML3 cells. At 72 hr postinfection, the cells were sorted for GFP fluorescence, plated, and treated with ABT-737.

AML blast colony assay and CFU-granulocyte-macrophage assays were conducted as described (Milella et al., 2001) (for details, please refer to the Supplemental Experimental Procedures).

Flow cytometric analysis of AML stem cells

The frequency of AML stem cells was determined as described (Jordan et al., 2000). One million total cells were measured per sample using a Becton Dickinson LSR II flow cytometer, and the frequency of $CD34^{+}38^{-}123^{+}$ cells was calculated. Induction of apoptosis in AML stem cells was determined by a four-color multiparametric flow cytometry assay using CD34 APC, CD38 PE Cy7, CD123 PE, and Annexin V FITC.

Statistics

Results are expressed as means ± SEM of three separate replicate experiments unless otherwise indicated. Levels of significance were evaluated by a two-tailed paired Student's t test, and $p < 0.05$ was considered significant. Synergism, additive effects, and antagonism were assessed with the Chou-Talay method (Chou and Talalay, 1984) and Calcusyn software (Biosoft, Ferguson, MO); the CI for each experimental combination was calculated. When CI = 1, the equation represents the conservation isobologram and indicates additive effects. CI values < 1.0 indicate an additive effect characteristic of synergism.

Xenograft studies in Scid mice

All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee. We utilized a Raf-induced model of AML established in our laboratory (Konopleva et al., 2005). To generate *luc*-expressing FD/ΔRaf-1:ER cells, they were infected with fiber-modified adenoviral vector expressing firefly luciferase. Four- to six-week-old CB.17 Scid mice (Harlan Sprague Dawley, Madison, WI) were implanted with 1.7 mg, 60 day release, 17β-estradiol pellets (Innovative Research, Sarasota, FL). The next day, 1×10^6 luciferase-expressing FD/ΔRaf-1:ER cells suspended in 200 μl PBS were injected i.v. In a model of human leukemia xenograft, 5×10^6 human leukemic luciferase-expressing KG-1 cells were injected i.v. into Scid mice.

For intraperitoneal (i.p.) administration, 1 g ml^{-1} stock solution of ABT-737 in DMSO was added to a mixture of 30% propylene glycol, 5% Tween 80, 65% D5W (5% dextrose in water) (pH 4−5; final concentration of DMSO ≤ 1%). Mice injected with FD/ΔRaf-1:ER cells were treated with either ABT-737 (20 and 30 mg/kg/mouse every day i.p. for 21 days starting on day 1 post-cell injection (n = 9–10 mice per group) or vehicle or left untreated (control); mice injected with human KG-1 cells were treated with 30 mg/kg ABT-737 starting on day 18 post-cell injection. For noninvasive imaging of FD/ΔRaf-1:ER-*luc* cells, anesthetized mice were injected with 150 mg/kg of D-luciferin (potassium salt, Xenogen Corp., Alameda, CA) and placed for imaging in the In Vivo Imaging System (IVIS, Xenogen) with total imaging time of 2 min. Total body bioluminescence was quantified as described (Konopleva et al., 2005).

Mice were sacrificed when they became moribund or unable to obtain food or water or if they lost >20% of their body weight. Effects of ABT-737 on survival were estimated with the method of Kaplan and Meier, and log-rank statistics was used to test for differences in survival distributions.

Other procedures are in the Supplemental Data.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, five supplemental figures, and four supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/5/375/DC1/.

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