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ORIGINAL ARTICLE

MEK inhibition enhances ABT-737-induced leukemia cell apoptosis via prevention of ERK-activated MCL-1 induction and modulation of MCL-1/BIM complex

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Recently, strategies for acute myeloid leukemia (AML) therapy have been developed that target anti-apoptotic BCL2 family members using BH3-mimetic drugs such as ABT-737. Though effective against BCL2 and BCL-X_L, ABT-737 poorly inhibits MCL-1. Here we report that, unexpectedly, ABT-737 induces activation of the extracellular receptor activated kinase and induction of MCL-1 in AML cells. MEK inhibitors such as PD0325901 and CI-1040 have been used successfully to suppress MCL-1. We report that PD0325901 blocked ABT-737-induced MCL-1 expression, and when combined with ABT-737 resulted in potent synergistic killing of AML-derived cell lines, primary AML blast and CD34+38-123+ progenitor/stem cells. Finally, we tested the combination of ABT-737 and CI-1040 in a murine xenograft model using MOLM-13 human leukemia cells.Whereas control mice and CI-1040-treated mice exhibited progressive leukemia growth, ABT-737, and to a significantly greater extent, ABT-737+CI-1040 exerted major anti-leukemia activity. Collectively, results demonstrated unexpected anti-apoptotic interaction between the BCL2 family-targeted BH3-mimetic ABT-737 and mitogen-activated protein kinase signaling in AML cells: the BH3 mimetic is not only restrained in its activity by MCL-1, but also induces its expression. However, concomitant inhibition by BH3 mimetics and MEK inhibitors could abrogate this effect and may be developed into a novel and effective therapeutic strategy for patients with AML.
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Introduction

The BCL2 family of proteins contains members such as BCL2, MCL-1 and BCL-X_L that possess potent anti-apoptotic function as well as pro-death members such as BAX, BAK, BAD and BIM.[1–5] BCL2 family members contain at least one of four common structural domains termed BCL2-homology domains 1–4 (BH1, BH2, BH3 and BH4). The anti-apoptotic members contain all four BH3 domains. Pro-apoptotic BCL2 family members are separated into two classes based on function and structure. BAX and BAK contain BH1, BH2 and BH3. These proteins disrupt the outer mitochondrial membrane when activated, and genetic studies have determined that at least one of these two proteins is required for the induction of apoptosis.[2] The other class of pro-apoptotic BCL2 family members contains only the BH3 domain (for example, BAD and BIM). The BH3-only proteins can support apoptosis by activating BAX or BAK, or by sequestering anti-apoptotic members such as BCL2.[1–5]

Aberrant expression of anti-apoptotic members such as BCL2 or suppression of pro-apoptotic members such as BAX or BIM can lead to tumor formation and promote resistance to therapy in many types of cancer, including acute myeloid leukemia (AML). A recent model of tumorigenesis/chemoresistance suggests that cancer cells become dependent on pro-survival molecules including BCL2.[6,7] This model suggests that the 'addicted' malignant cells can be eliminated by targeting the survival molecule that supports the tumor cell.[2] Expression of BCL2 is an unfavorable prognostic factor in some groups of AML patients such as those with favorable and intermediate prognosis cytogenetics.[8] A high BCL2 to BAX ratio (which would favor prevention of apoptosis) has been found to be an unfavorable prognostic factor in AML patients.[8–10] A recent study suggests that the nucleophosmin mutation promotes a high ratio of BAX to BCL2 (which would favor the promotion of apoptosis) in AML patients and might explain in part why patients with this generally have a favorable outcome.[11] These and other findings support a role in targeting anti-apoptotic BCL2 family members for therapy. One approach involves suppressing expression of anti-apoptotic BCL2 family members. Oblimersen is an anti-sense oligonucleotide against human BCL2 mRNA in clinical trials.[12] Its efficacy is, at best, uncertain. A constraint of antisense approaches is that it is limited by targeting individual BCL2 family members. Recent efforts to target a broad group of BCL2 family members has involved using small molecule inhibitors to target the BH3 domain as association between the two groups of BCL2 family members involves this domain.[13] At present, at least a dozen compounds including ABT-737 have been developed as BH3-mimetic small molecule inhibitors.[2,13–15] ABT-263, the orally active analog of ABT-737 is currently in the clinic for small-cell lung and lymphoid malignancies, and has shown promising results.[15,16] However, ABT-737 and ABT-263 bind poorly to MCL-1 and related BCL2 family members such as BCL2A1, and thus cells that rely on these anti-apoptotic BCL2 family members display resistance to the compound.[17–19] A recent study has shown that lymphoma cells can develop resistance to ABT-737 by upregulating MCL-1 by a transcriptional mechanism.[20] ABT-737's ability to kill leukemia cells is

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greatly improved when MCL-1 is suppressed.[17–19] Expression of MCL-1 is regulated by mitogen activated protein kinase (MAPK) at multiple levels. The extracellular receptor activated kinase (ERK) 1/2 has been shown to promote MCL-1 gene expression by ELK1.[21] ERK 1/2 also stabilizes MCL-1 protein expression by phosphorylating the protein within a PEST site.[22] Thus, a strategy to suppress MCL-1 expression in leukemia cells by targeting MAPK should sensitize the cells to the drug. Benzhydroxamate esters derived from their precursor anthranilic acids have been found to be potent MEK inhibitors.[23] These compounds include CI-1040 (2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropylmethoxy-3,4-difluoro-benzamide) and PD0325901. In the current study, the selective MEK inhibitor PD0325901, in combination with ABT-737, was used with human AML-derived cell lines. The combination of drugs was synergistic in killing AML cell lines and primary AML blast cells. In a murine xenograft model, tumor burden of human AML MOLM13 cells was most effectively reduced in mice treated with both ABT-737 and PD0325901.

Patients and methods

Reagents and antibodies

ABT-737, a cell-permeable small molecule inhibitor of BH3 function was synthesized at the University of Texas MD Anderson Cancer Center on the basis of previously published structure[13] and dissolved in dimethyl sulfoxide (DMSO). PD0325901 and CI-1040, cell-permeable MEK inhibitors, were kindly provided by Pfizer Global Research & Development (Ann Arbor, MI, USA). DMSO and trypan blue were purchased from Sigma Chemical Co. (St Louis, MO, USA). Annexin V FITC was purchased from Roche Diagnostic Co. (Indianapolis, IN, USA). CD34-APC, CD38-PE-Cy7 and CD123-PE were purchased from BD Biosciences (San Jose, CA, USA).

Phospho ERK 1/2 antibodies were purchased from Cell Signaling Technologies Inc. (Beverly, MA, USA), BAX and MCL-1 antibodies from BD Biosciences, the BCL2 antibody from Dako (Carpinteria, CA, USA), and Bak from Upstate (Lake Placid, NY, USA). ERK 2 and the MCL-1 antibody used for immunoprecipitation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), activated-BAK (Ab-1) from EMD Biosciences (Gibbstown, NJ, USA), NOXA from Abcam (Cambridge, MA, USA), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody from Chemicon International (Temecula, CA, USA), goat anti-mouse and goat anti-rabbit-horse radish peroxidase conjugate secondary antibodies from Bio-Rad (Hercules, CA, USA).

Cell lines and primary AML samples

HL60 were purchased from The American Type Culture Collection (ATCC, Rockville, MD, USA). MOLM13 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). OCI-AML3 cells were kindly provided by MD Minden (Ontario Cancer Institute, Toronto, ON, Canada). Mouse-embryo fibroblasts (MEF), wild-type and with Bak and/or Bax knocked down, were kindly provided by Anthony Letai (Dana-Farber Cancer Institute, Boston, MA, USA). MEFs with BIM knocked out were provided by Philippe Bouillet (Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia).

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 under informed consent in accordance with regulations and protocols approved by the Human Subjects Committee of the University of Texas MD Anderson Cancer Center. Mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemical Co.) density-gradient centrifugation. The clinical features of the patients are listed in Table 1. Cells were cultured in RPMI-1640 medium (Mediatech Inc., Herndon, VA, USA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA, USA), 1 mM L-glutamine and 50 μg/ml penicillin or streptomycin.

Cell culture

HL60, OCI-AML3 and MOLM13 cells were cultured in RPMI-1640. WT MEF, Bim-, Bax-, Bak- and double-knockout (Bak and Bax) MEFs were cultured in Dulbecco's modified Eagle medium (Mediatech Inc.). All media were supplemented with 10% fetal bovine serum, 1 mM L-glutamine and 50 μg/ml penicillin or streptomycin (Gibco Laboratories, Grand Island, NY, USA).

Leukemic cell lines were cultured at a density of 3.0×10^5 cells/ml in medium supplemented with 10% fetal bovine serum and treated with ABT-737, PD0325901 or vehicle (DMSO final concentration, 0.1%). MEFs were plated at a density of 1.0×10^5 cells/ml in medium supplemented with 10% fetal bovine serum, allowed to attach for 24 h, and then treated with ABT-737, PD0325901 or DMSO. Both ABT-737 and PD0325901 were dissolved in DMSO. In all experiments, cells were treated in log-phase growth.

Viability assay

Cell viability was assessed using a Vi-CELL XR cell viability analyzer (Beckman Coulter, Fullerton, CA, USA). The instrument assesses cell viability by trypan blue exclusion.

Flow cytometric analysis of apoptosis

Apoptosis was determined by the flow cytometric measurement of phosphatidylserine exposure using annexin V FITC (Roche Diagnostics, Indianapolis, IN, USA). Briefly, cells were washed twice with binding buffer (10 mmol/l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 140 mmol/l NaCl and 5 mmol/l $CaCl_2$ (pH 7.4), all from Sigma Chemical Co.), and stained with FITC-conjugated annexin V for 15 min at room temperature. Annexin V fluorescence was determined with a BD Biosciences Calibur flow cytometer, and the membrane integrity of the cells was simultaneously assessed by the propidium iodide exclusion method. In the case of cells collected from patient samples, cells were simultaneously stained with CD34 APC, CD38 PE-Cy7, CD123 PE and annexinV FITC, and then analyzed with a BD Biosciences LSRII flow cytometer.

Western blot analysis

Cells were lysed at a density of 1×10^6/50 μl in protein lysis buffer (0.25 M Tris-HCl, 2% SDS, 4% β-mercaptoethanol, 10% glycerol and 0.02% bromophenol blue). For determination of phospho-specific proteins, cells were lysed in buffer containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM NaF, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1% Triton-X-100, 10 mM iodacetamide, 1 mM Na_3VO_4, 0.1% NaN_3 and 3 mM phenylmethyl sulfonyl fluoride. All lysis buffers were supplemented with a protease inhibitor cocktail (Roche Diagnostic Co.). Cell lysates were then loaded onto a 12% SDS-PAGE gel (Bio-Rad). After electrophoresis, proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, England, UK), followed by

Table 1 Clinical data for patients: (a) samples used for apoptosis assay; (b) samples used for enumeration of leukemic progenitor cells (LPC)

Patient no.	*Source*	*Blast, %*	*FAB*	*Cytogenetics*	*Status*
(a)					
1	PB	96	ND	der(11)t(11;19)(q23;p13.3),der(18)t(18;19)(q21;p13.3)t(11;19),der(19)t(18;19)	Primary refractory
2	PB	69	M1	3,Y,del(X)(p22.1),−3,add(4)(q35),−5,del(7)(q22q34),add(12)(p13),−17, der(18)del(18)(p11.2)del(18)(q21.1),add(21)(p11.1)[6];44,s1,+mar[3];42−45, sdl1,+0−2mar[cp6]	Relapse
3	PB	82		Hyperdiploid clone 47,+15	Refractory
4	BM	98	M0	DEL20(Q13.1)[15];DEL20(Q13.1)X2[5]	Newly diagnosed
5	PB	60	ND	5,+i(8)(q10)x2,add(11)(p15),−13,−16,−17,−18,+3mar[6];47,XX,−5,+i(8)(q10)x2, add(11)(p15),−13,−16,−17,−18,+4mar[5];44−45,XX,−5,+i(8)(q10)x2, add(11)(p15),−13,−16,−17,−18,+2−3mar[cp5];88,XXXX,−3, add(3)(q27),−5,−5,+add(8)(p23)x3,add(11)(p15),add(11)(p15),−13,−13,−14,−16, −16,−17,−17,−18,−18,+5mar[1];Diploid[3]	Refractory
6	BM	93	M1	45−46,ADD(17)(P13)[CP5]	Newly diagnosed
7	PB	33	ND	Diploid	Refractory
8	BM	90	M1	Diploid	Relapse
(b)					
1	PB	76	M2	Diploid	Refractory
2	PB	68	ND	45−46,del(2)(q21q31),del(3)(q12q29),−5,del(6)(q21q25),−7, del(12)(p11.2p13),add(16)(p12),idic(19)(p11),+mar[cp13];Diploid46[7]	Newly diagnosed (secondary, post MDS)
3	PB	68	ND	47,del(5)(q13q33),del(7)(q22q34),i(8)(q10),t(11;16)(p11.2;q11.1), add(14)(p11.1),i(21)(q10),+mar[9];45−47,del(5)(q13q33), del(7)(q22q34),i(8)(q10),t(11;16)(p11.2;q11.1), add(14)(p11.1),+0−1mar[cp11]	Newly diagnosed (therapy-related, post CLL)
4	BM	74	ND	46,der(16)t(1;16)(q21;q12.1)[9];46,t(5;15)(q31;q15),der(16)t(1;16) (q21;q12.1) [2];Diploid[9]	Refractory
5	BM	30	M2	+8	Refractory
6	PB	83	M4	Diploid	Newly diagnosed

Abbreviations: BM, bone marrow; CLL, chronic lymphocytic leukemia; FAB, French–American–British; MDS, myelodysplastic syndrome; ND, not determined; PB, peripheral blood.

immunoblotting. Signals were detected using a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics, Sunnyvale, CA, USA). Immunoblot band quantitation was calculated using Image J software (version 1.44p; National Institutes of Health, Bethesda, MD, USA).

Immunoprecipitation and immunoblotting

Cells were washed with 1 × PBS and resuspended in ice-cold 1% CHAPS lysis buffer (150 mM NaCl, 10 mM HEPES (pH 7.4), 1% CHAPS and protease inhibitors (Roche Diagnostic Co.)) on ice for 30 min. Insoluble debris was removed by centrifugation at 4 °C for 10 min at 13 000 r.p.m. Protein A-coated 96-well strips (Pierce, Rockford, IL, USA) were washed four times with CHAPS lysis buffer. For each 5×10^6 cells, 2.5 μg of antibody (activated BAK IP: mouse anti-activated BAK (Ab1, EMD Biosciences); BIM/BAK/MCL-1 co-IP: rabbit anti-BIM (202000, EMD Biosciences); MCL-1/Bim/Bak co-IP: rat anti-MCL-1 (Santa Cruz sc-819)) was incubated in each well in 100 μl CHAPS lysis buffer with shaking for 1 h at room temperature. The strips were then washed four times with CHAPS lysis buffer. The cell extracts (5×10^6 cell equivalent) were added to the antibody-bound wells and shaken overnight at 4 °C. The wells were washed four times with CHAPS lysis buffer.

Immunoprecipitated proteins were solubilized from the protein A- antibody wells with 2 × SDS-PAGE sample buffer (0.25 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 4% β-mercaptoethanol and 0.02% bromophenol blue). The samples were heated for 5 min by placing the well strip directly on a 95 °C heating block. Proteins were separated by 12% SDS-PAGE gels, which were then transferred to Hybond-P membranes (Amersham Pharmacia Biotech) and detected by immunoblotting using rabbit anti-Bim (Calbiochem, La Jolla, CA, USA), rabbit-anti-bak (Upstate Biotechnology, Santa Cruz, CA, USA), or mouse-anti-MCL-1 (BD) antibodies. Signals were detected using a PhosphorImager (Storm 860, Molecular Dynamics).

Small interfering RNA (siRNA) transfection

Silencing of Bim gene expression in leukemic cells was achieved by the siRNA technique. siRNAs were obtained as duplexes in purified and desalted form (Option C) from Dharmacon (Lafayette, CO, USA). The sense strand of the siRNA-silencing *bim* gene (Bim-siRNA) was 5′-GACCGAGAAGGUAGACAAUU GdTdT-3′. *Bak* gene expression was silenced using human ON-TARGET and SMARTpool bak1 siRNA from Dharmacon (L-003305-00). A non-specific control pool containing four pooled non-specific siRNA duplexes was also used as a negative control (referred to as NS-siRNA, Dharmacon-Upstate). Transfection of leukemic cells was carried out by electroporation using the Nucleofection system (Amaxa, Köln, Germany), by following the manufacturer's instructions. Briefly, 3×10^6 cells were resuspended in 100 μl of V-cell nucleofector solution containing 4 μM of double-stranded siRNAs. After electroporation, 500 μl of cultured medium were added to the cuvette, and the cells were transferred into culture plates containing 1.5 ml pre-warmed

culture medium. At the optimal time of gene silencing, various concentrations of ABT-737 and PD0325901 were added to the cells, and protein expression was monitored by western analysis.

Flow cytometric analysis of AML stem cells

The frequency of AML stem cells was determined as described.[24] Leukemic (AML) stem cells have the unique phenotype $CD34^+38^-123^+$. A BD Biosciences LSR II flow cytometer was used and the frequency of AML stem 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.

Murine leukemia model

Five-week-old 01B74 athymic nude (nu/nu) mice (NCI, Frederick, MD, USA) were injected intravenously with 2.5×10^6 M M13 cells stably expressing a dual renilla luciferase-GFP (green fluorescent protein) reporter 6 h after irradiation (dose 2.5 Gy). Mice were ear-tagged at the time of leukemia transplantation and monitored twice per week for engraftment and tumor growth. At 2 weeks after cell injection, mice were randomized into four treatment groups of eight mice per group and treated as follows: liposomal ABT-737 (i.v. 20 mg/kg, q.o.d. for 3 weeks), CI1040 (i.p. 50 mg/kg q.o.d. for 3 weeks) or ABT-737 in combination with CI1040 (ABT-737 + CI1040). Control mice were injected with empty liposomes. Briefly, ABT-737 was solubilized in 30% DMSO/70% t-butanol solution at 37 °C at a concentration of 8.54 mg/ml. Phospholipid distearoyl phosphatidyl choline (DSPC) was solubilized in t-butanol at 65 °C, at a concentration of 82.16 mg/ml. DSPC and ABT-737 (20:1) were then mixed together and frozen. The mixture containing ABT-737 was lyophilized overnight, then reconstituted in normal saline at 75 °C, and sonicated. Liposomes were resuspended at room temperature in normal saline at a concentration of 2 mg/ml (100 μM) for the *in vivo* studies. Empty liposomes were made using the same lipids and following the same protocol, but without adding ABT-737. Leukemia burden was monitored by weekly noninvasive imaging of isoflurane-anesthetized mice injected intraperitoneally with luciferin in the *in vivo* imaging system (Xenogen/Caliper Life Sciences, Hopkinton, MA, USA) with total imaging time of 1 min. Before imaging, mice were placed in an acrylic chamber, anesthetized with 1.5% isofluorane–air mixture, and injected i.p. with 15 mg/ml of luciferin–potassium salt in PBS at a dose of 150 mg/kg body weight. A digital gray-scale image of each mouse was acquired, followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the mouse.

Mice were killed when they became moribund or were unable to obtain food or water. In addition, three randomly assigned mice in each group were sacrificed on day 35 after transplantation for assessment of engraftment by GFP immunohistochemical staining. Survival was estimated with the product-limit estimator of Kaplan and Meier and the log-rank statistics was used to test for differences in survival distributions between groups. Mice killed for engraftment analysis were censored on day 35.

Statistics

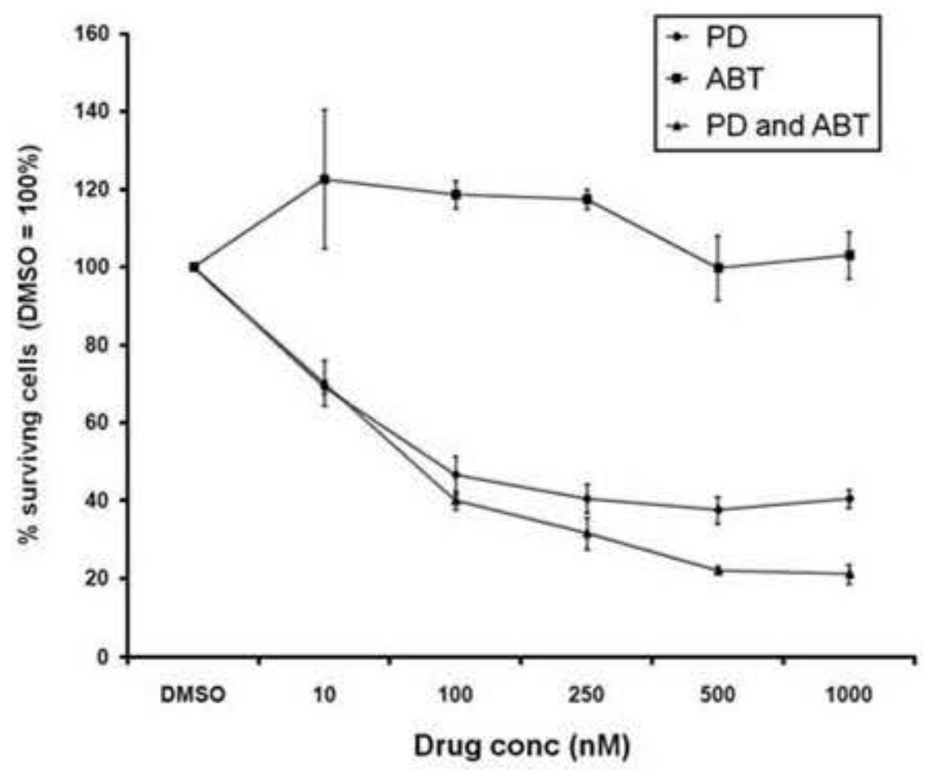
Results are expressed as means ± s.e.m. of three separate replicate experiments unless otherwise indicated. Synergism, additive effects and antagonism were assessed with the Chou–Talay method and Calcusyn software (Biosoft, Ferguson, MO, USA); the combination index (CI) for each experimental combination was calculated. When CI = 1, the equation represents the conservation isobologram and indicates additive effects. CI values <1.0 indicate synergism.

Results

ABT-737 and PD0325901 are synergistic in leukemic cells lines

To examine whether inhibition of MAPK would promote ABT-737-induced cell death in AML cells, OCI-AML3, HL60 and MOLM13 cells were treated with increasing concentrations of ABT-737 and the MEK inhibitor PD0325901, alone and in combination, for 72 h. As shown in Figure 1a, each drug alone was ineffective at promoting apoptosis in OCI-AML3 cells at the maximum dose used (that is, 1 μM; <40 and <20% annexin V-stained cells with ABT-737 or PD0325901, respectively). However, when both drugs were used in combination, >50% of OCI-AML3 cells underwent apoptosis even at 0.1 μM concentration and >90% at 1 μM concentration (Figure 1a). Using Calcusyn software to analyze the dose curves generated in Figure 1a, the combination of ABT-737 and PD0325901 gave a highly synergistic annexinV(+) CI value of 0.021 (values <1

a



b

Cell Line	ED50	ED75	ED90	AVG	SEM
OCI-AML3	0.048	0.013	0.0034	0.021	0.014
HL60	0.40	0.15	0.054	0.20	0.13
MOLM13	0.26	0.16	0.098	0.17	0.046

Figure 1 ABT-737 and PD0325901 are synergistic in leukemic cell lines. (**a**) OCI-AML3 cells were cultured in the presence of escalating doses of ABT-737 (10, 100, 250, 500 and 1000 nM), PD0325901 (10, 100, 250, 500 and 1000 nM), or the combination of the two agents at a fixed ratio added simultaneously. After 48 h, apoptosis was measured by annexin V flow cytometry. Results are expressed as means ± s.e.m. of the results of three replicates. (**b**) CI values were calculated using the Calcusyn software. CI values of <1.0 indicate synergism. Annexin V CI values are shown for OCI-AML3, HL60 and MOLM13 cell lines. ABT-737 and PD0325901 doses used for HL60: 10, 100, 250 and 500 nM for both drugs. Doses used for MOLM13: ABT-737 (10, 100, 250, 500 and 1000 nM) and PD0325901 (1, 10, 25, 50 and 100 nM).

representing a synergistic relationship; Figure 1b). HL60 and MOLM 13 showed similar sensitivity to the combination of drugs (data not shown). The synergism of ABT-737 and PD0325901 observed in OCI-AML cells was likewise seen in the HL60 and MOLM13 cells (CI values of 0.2 and 0.17 respectively; Figure 1b).

PD0325901 reduces MCL-1 protein levels

It has previously been shown that cell lines with high levels of MCL-1 protein are resistant to killing by ABT-737; however, if MCL-1 levels are reduced, the cells become sensitive to ABT-737-induced apoptosis.[17] It has also been shown before that MEK inhibitors can reduce MCL-1 gene expression and reduce MCL-1 protein levels by destabilizing the protein.[21,22] We show that, similar to previously published results, in human AML-derived OCI-AML3 cells, PD0325901 is a potent MEK inhibitor and can completely block ERK phosphorylation at 100 nM (Figure 2a). Surprisingly, as shown in Figure 2a, ABT-737 at doses as low as 10 nM was found to induce ERK phosphorylation by an as yet undetermined mechanism. The MEK inhibitor was able to suppress induction of ERK phosphorylation by the ABT-737 (Figure 2a). To examine this phenomenon in another AML-derived cell line, the effect of ABT-737 on ERK phosphorylation was tested in MOLM13 cells. As shown in Figure 2b, after 24 h, 100 nM ABT-737 alone promoted phosphorylation of the kinase. As seen in the OCI-AML3 cells, the MEK inhibitor blocked ABT-737-induced ERK phosphorylation (Figure 2b).

Consistent with activation of ERK by the BH3 mimetic, 100 nM ABT-737 alone promoted expression of MCL-1 after only 6 h of treatment (Figure 2c). At this same concentration, PD0325901 significantly reduced MCL-1 protein levels (Figure 2c). These changes are evident 6 h after treatment with the MEK inhibitor (Figure 2c) suggesting that the mechanism likely involves post-translational regulation of MCL-1. Whereas the MEK inhibitor suppressed MCL-1 protein levels as expected, ABT-737 alone appeared to induce both ERK phosphorylation and MCL-1 protein expression (Figures 2a and c). The effect is seen within 6 h so it is likely that a post-translational mechanism is involved. As shown in Figure 2c, MCL-1 levels are reduced in cells that are treated with both MEK inhibitor and ABT-737. These results suggest that ABT-737 may promote MCL-1 expression by a mechanism involving activation of ERK, and that this effect can be blocked when ERK is inhibited.

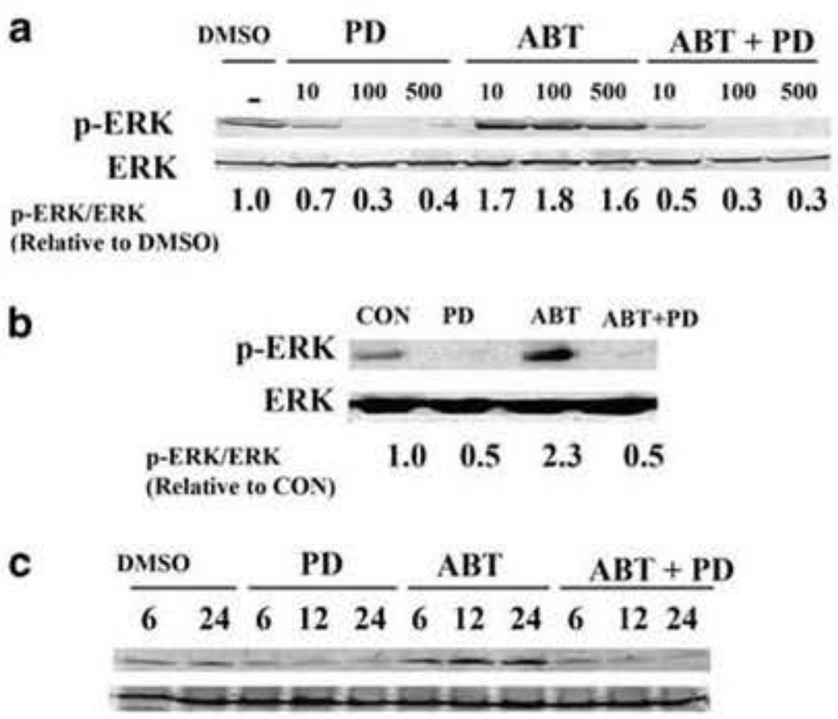


Figure 2 PD0325901 reduces MCL-1 protein levels and alters Bim phosporylation. (**a**) OCI-AML3 cells were treated with 10, 100 or 500 nM of ABT-737 and/or PD0325901 for 24 h. Levels of pERK and ERK2 were analyzed by immunoblot. Ratio of p-ERK/ERK was determined by densitometry of bands using Image J software. (**b**) MOLM13 cells were treated with 40 nM of ABT-737 and/or 80 nM PD0325901 for 24 h. Levels of pERK and ERK were analyzed by immunoblot. Ratio of p-ERK/ERK was determined by densitometry of bands using Image J software. (**c**) OCI-AML3 cells were treated with 100 nM of ABT-737 and/or PD0325901 for 6, 12 or 24 h. Levels of MCL-1 and GAPDH were analyzed by immunoblot. Ratio of MCL-1/GAPDH was determined by densitometry of bands using Image J software.

PD0325901 and ABT-737 activates BAK and disrupts MCL-1:BIM association

Activation of BAK promotes the induction of apoptosis. MAPK has been implicated in the regulation of BAK and BIM.[25–27] Recent study has suggested that ERK phosphorylation of non-receptor tyrosine phosphatase 5 prevents BAK activation by preventing dephosphorylation of the molecule at tyrosine 108 (a necessary but not sufficient event for BAK activation).[27] A previous study has shown that the fatty-acid synthase inhibitor orlistat sensitized leukemia cells to ABT-737 by a mechanism that involves BAK-dependent mitochondrial permeability transition.[28] Cells treated with PD0325901 showed an increase in the level of activated BAK after 6 h. ABT-737 treatment alone did not significantly increase the level of activated BAK, but the combination of ABT-737 and PD0325901 further increased the level of activated BAK over PD0325901 alone (Figure 3a).

We have previously shown that ABT-737 disrupts BCL2/BIM heterodimers, hence increasing the free, unbound BIM.[17,29] However, ABT-737 has a low-affinity binding to MCL-1. To determine the changes in the ability of BIM to bind MCL-1, we performed immunoprecipitation of MCL-1 protein from cells treated with ABT-737, PD0325901 or their combination. PD0325901 diminished the amount of total and immunoprecipitated MCL-1 as shown before (Figure 3b). As shown in Figure 3b, ABT-737 treatment resulted in increased binding of BIM, but not of BAK, to MCL-1. With combined treatment, more BIM was found to be bound to MCL-1, which would lead to

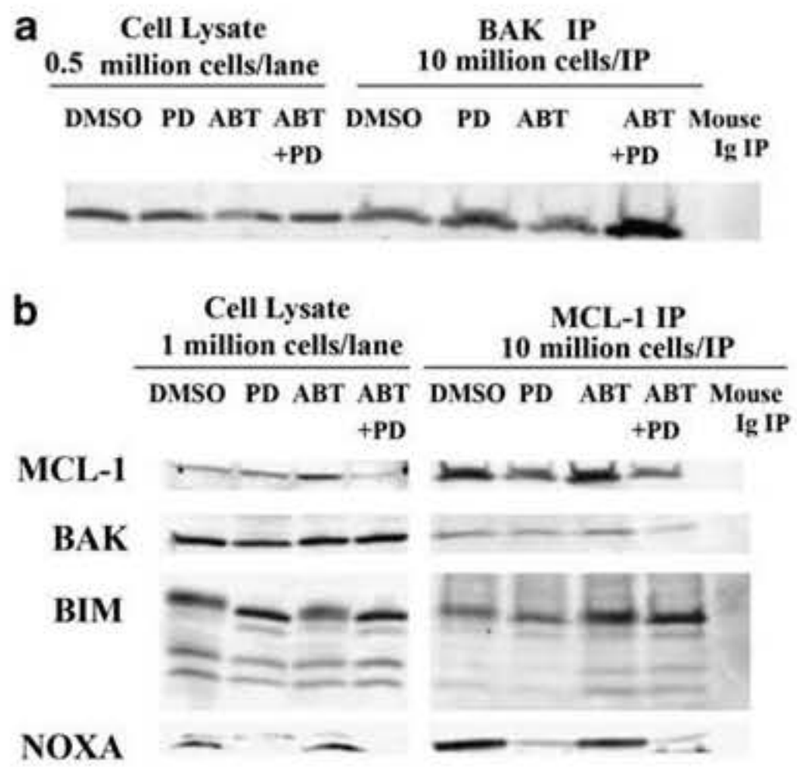


Figure 3 ABT-737 and PD0325901 treatment activates Bak and increases the association of MCL-1 with Bim. (**a**) OCI-AML3 cells were treated with ABT-737 and/or PD0325901 for 6 h. Cell lysates were immunoprecipitated with an antibody to activate Bak (Calbiochem), and then immunoblotted for total Bak. (**b**) OCI-AML3 cells were treated with ABT-737 and/or PD0325901 for 1 h. Cell lysates were immunoprecipitated with an antibody to MCL-1 (Santa Cruz), and then immunoblotted for Bim and Bak.

inactivation of MCL-1 anti-apoptotic function. Association of MCL-1 with pro-apoptotic partner Noxa was decreased in cells treated with PD0325901, concomitant with decrease in MCL-1 levels.

BIM, BAK and BAX contribute to ABT-737- and PD0325901-induced apoptosis

To assess the importance of Bim, Bak and Bax in ABT-737- and PD0325901-induced apoptosis, MEFs were treated with ABT-737 and/or PD0325901 for 48 h. Wild-type MEF cells are resistant to ABT-737, but are killed by the combination of ABT-737 and PD0325901 (Figure 4a). Loss of Bim, Bak or Bax, or both Bak and Bax expression partially protects the cells from ABT-737- and PD0325901-induced apoptosis (Figures 4a and b).

To further investigate the contribution of BIM and BAK to ABT-737- and PD0325901-induced apoptosis in AML cells, BIM and BAK expression were silenced by siRNA in OCI-AML3 cells using Amaxa transfection. BIM and BAK levels were significantly reduced (Figure 4c). As shown in Figure 4d, reduction in BIM expression effectively protected cells from the combination of ABT-737 and PD0325901, as there was an ~50% reduction in apoptosis in cells with BIM shRNA compared with control cells that were transfected with nonsense shRNA. Surprisingly, silencing of BAK expression did not suppress and in fact may have slightly stimulated apoptosis, and reduction of both BIM and BAK expression by shRNA had no increased protection from apoptosis over silencing of BIM alone (Figure 4d).

ABT-737 and PD0325901 in primary AML cells

The ability of ABT-737 to synergize with CI-1040 in primary AML blast cells was tested in eight samples (Table 1a) using annexin V staining to detect apoptosis. With the exception of patient 3, 10 nM CI-1040 was not toxic to the primary AML blast cells (Figure 5a). ABT-737 promoted apoptosis in all the samples but with varying effectiveness. When CI-1040 was used in combination with ABT-737, levels of apoptosis was increased in all patients with the exception of patients 5 and 8. Induction of apoptosis by ABT-737 or ABT-737, in combination with CI-1040 was significantly higher in AML cells compared with normal CD34+ progenitor cells (Figure 5b). Effective leukemia therapy has to target leukemia stem cells to generate sustained responses. AML stem cells have been phenotypically defined as $CD34^+38^-123^+$.[30] AML samples (sample nos. 1–6, Table 1b) were treated with vehicle, ABT-737 (50 nM), PD0325901 (50 nM) or their combination for 48 h. PD0325901 decreased the proportion of the surviving stem cells in three of six samples (nos. 2, 4 and 5, Figure 5c). Conversely, ABT-737 decreased leukemia stem cells in all samples consistent with our published data, and this effect was enhanced by PD0325901 in three of the six samples (no. 1, 4 and 5). Of note, these three patients were refractory to standard chemotherapy. This demonstrates the potential of this combination to target AML stem cells in a proportion of AML cases, including those who failed conventional chemotherapy.

CI1040 enhances the therapeutic efficacy of ABT-737 in a murine model of human AML

To determine whether CI1040 could potentiate the antileukemic effects of ABT-737 *in vivo,* we conducted an experiment in nude mice that were injected with GFP-/luciferase-bearing MOLM13 human leukemia cells. Two weeks after leukemia transplantation, mice were randomized and treated with liposomal ABT-737 (i.v. 20 mg/kg, q.o.d. for 3 weeks), CI1040 (i.p. 50 mg/kg q.o.d. for 3 weeks), ABT-737 in combination with

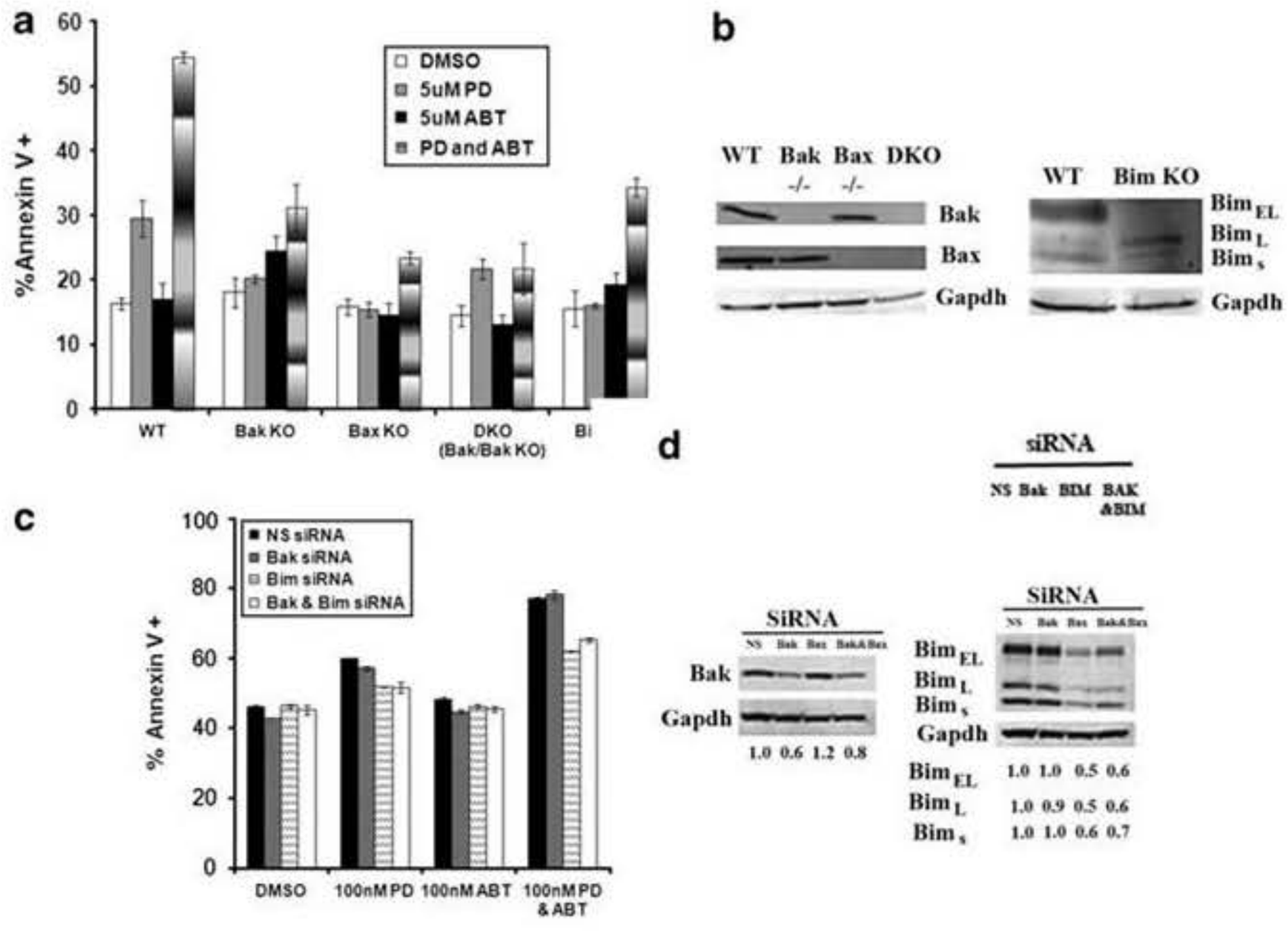


Figure 4 Loss of Bim and/or Bak expression partially protects cells from ABT-737- and PD0325901-induced apoptosis. (**a**) MEF cells with Bak, Bax, Bak and Bax, or Bim expression knocked out were treated for 48 h with 5 μM ABT-737 and/or PD0325901. Apoptosis was assessed by measuring annexinV by flow cytometry. Results are expressed as means ± s.e.m. of the results of three replicates. (**b**) Bak, Bax and Bim immunoblots of the MEF cells lines. (**c**) OCI-AML3 cells were Amaxa transfected with Bim siRNA and/or Bak ON-TARGETplus SMARTpool siRNA. Cells were treated with 100 nM ABT-737 and/or PD0325901 24 h after transfection, and after 24 h of drug treatment, apoptosis was assessed by annexinV flow cytometry. Results are expressed as means ± s.e.m. of the results of three replicates. (**d**) Bak and Bim immunoblots showing the extent of protein level reduction by siRNA. Bands were quantitated using Scion Image software and gel-loading differences were corrected for using the matching GAPDH immunoblot. The DMSO condition was set to one.

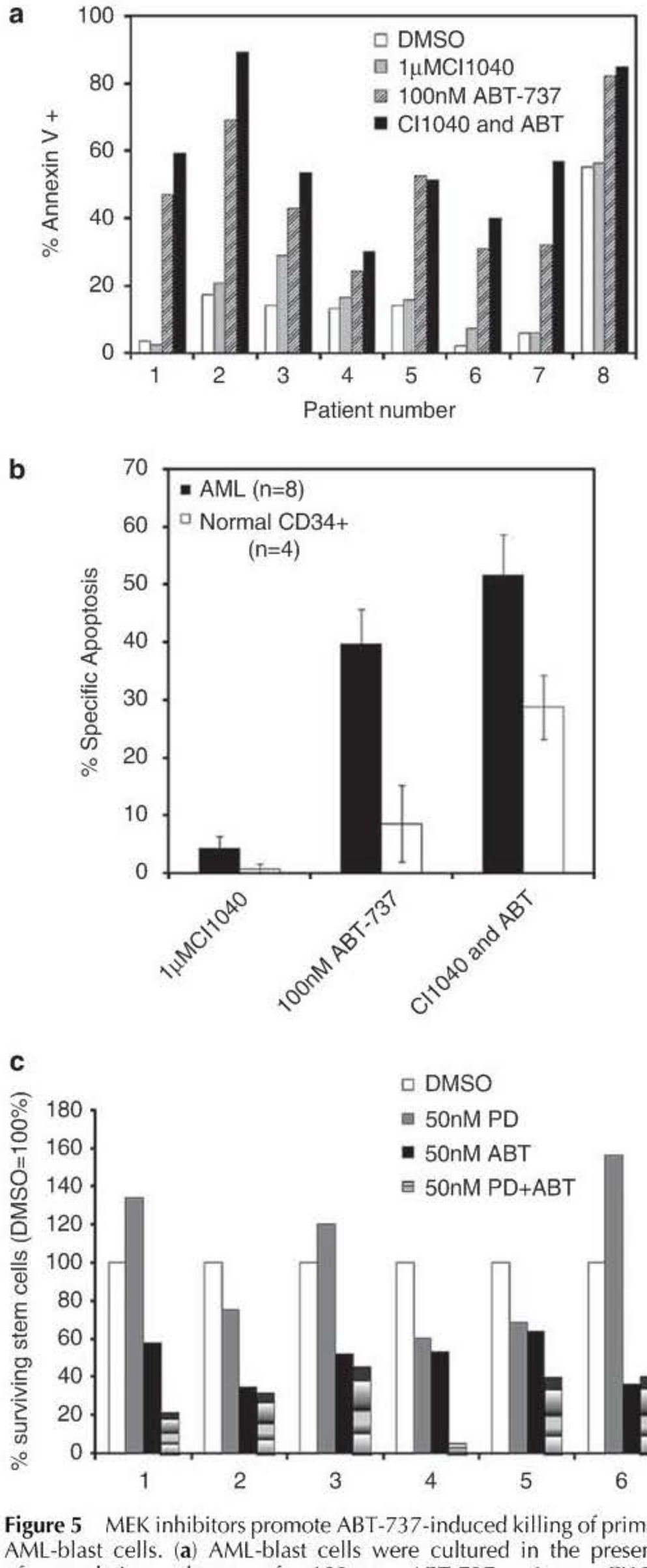


Figure 5 MEK inhibitors promote ABT-737-induced killing of primary AML-blast cells. (**a**) AML-blast cells were cultured in the presence of escalating doses of 100 nM ABT-737, 1 μM CI1040 or the combination of the two agents. After 48 h, apoptosis was measured by annexin V flow cytometry. (**b**) Induction of apoptosis by ABT-737 and CI1040 (doses as in A) in eight primary AML samples (same as in A) and in three samples of CD34+ cells isolated from normal apheresis samples. Results are expressed as the mean ± s.e.m. (**c**) Effects of ABT-737 and PD0325901 on AML-stem cells. The frequency of $CD34^+CD38^-CD123^+$ annexin V+ cells was determined by multicolor flow cytometry. The percentage of non-apoptotic (annexin V(-)) stem cells was calculated after ABT-737 and PD0325901 treatment (number of stem cells in DMSO-treated cultures = 100%). Results are expressed as the mean ± s.e.m.

CI1040 (ABT-737 + CI1040) or with empty liposomes (i.v.; control). Engraftment of MOLM13 cells was shown by immunohistochemical detection of GFP-positive cells in the spleen of control mice 5 weeks after transplantation (data not shown). Notably, whereas the control mice and CI1040-treated mice demonstrated progressive increase in leukemia-derived bioluminescence, ABT-737-treated mice, and to a significantly greater extent ABT-737 + CI1040-treated mice, exhibited much reduced tumor progression (Figure 6a). In addition, as shown in Figure 6b, quantitation of leukemia-derived bioluminescence demonstrated that ABT-737 + CI1040-treated mice had significantly ($P<0.00001$) lower leukemia burden than control mice or ABT-737-treated mice at all time points (7, 14 and 21 days of treatment).

Discussion

It has been established that simultaneous inhibition of MAPK signaling and BH3-mimetic targeting of BCL2/BCL-X_L is optimally effective at promoting apoptosis in tumor cells.[17,19] Fenretinide has been shown to promote proteolytic cleavage of MCL-1, and synergizes with ABT-737 to kill acute lymphoblastic leukemia cells.[30,31] Although it is clear that disabling MCL-1 is important for the synergistic killing of leukemia cells, the mechanism how MEK inhibition promotes ABT-737-mediated killing is not clear. A recent study in lymphoma suggests that resistance to mimetic drugs can develop as cells increase gene expression of MCL-1.[20] In the present study, we find that ABT-737 induces MCL-1 expression (Figure 2c). Though the mechanism for acquired resistance to ABT-737 in lymphoma appears to involve transcription,[20] this mechanism is not likely involved in the cells treated with ABT-737, as the increased MCL-1 expression observed in the OCI-AML3 cells occurs within 6 h of introduction of the BH3-mimetic drug, and thus the drug likely promotes MCL-1 expression by a post-transcriptional mechanism in the AML cells. However, the mechanism how this might occur is not clear. ABT-737 alone promotes phosphorylation (that is, activation) of ERK (Figures 2a and b), so it is possible that activation of ERK by the drug promotes MCL-1 protein expression. Phosphorylation of MCL-1 regulates its protein stability and the kinases (including ERK) that phosphorylate the many MCL-1 sites are well characterized.[32] Although ERK appears to be the likely candidate for ABT-737-induced expression of MCL-1, it is possible that ABT-737 activates a non-ERK kinase that may promote MCL-1 expression or suppresses a protein phosphatase that could be responsible for dephosphorylation of MCL-1. The possibility of an activated kinase intriguing as JNK has been implicated in MCL-1 phosphorylation.[32] JNK would be expected to be activated during induction of apoptosis (a likely consequence of ABT-737 treatment). As for the suppression of a MCL-1 phosphatase, PP2A has been implicated as the MCL-1 phosphatase.[22] However, the specific isoform that dephosphorylates the protein is currently unknown. Hopefully, with the availability of better reagents to investigate MCL-1 phosphorylation, this question can be answered. Still, the inclusion of an MEK inhibitor with ABT-737 overcomes the problem of ABT-737 induction of MCL-1 (Figure 2c), in addition to the sensitization to ABT-737 in cells already expressing ABT-737-insensitive MCL-1, as described by us and others. Consistent with previous reports,[21,22] MEK inhibition suppressed MCL-1 expression in the AML cells, likely by a proteolytic mechanism as the effect is seen within 6 h (Figure 2c).

There are precedents for activation of MAPKs such as ERK and p38 by various chemotherapeutic drugs in both solid tumors and

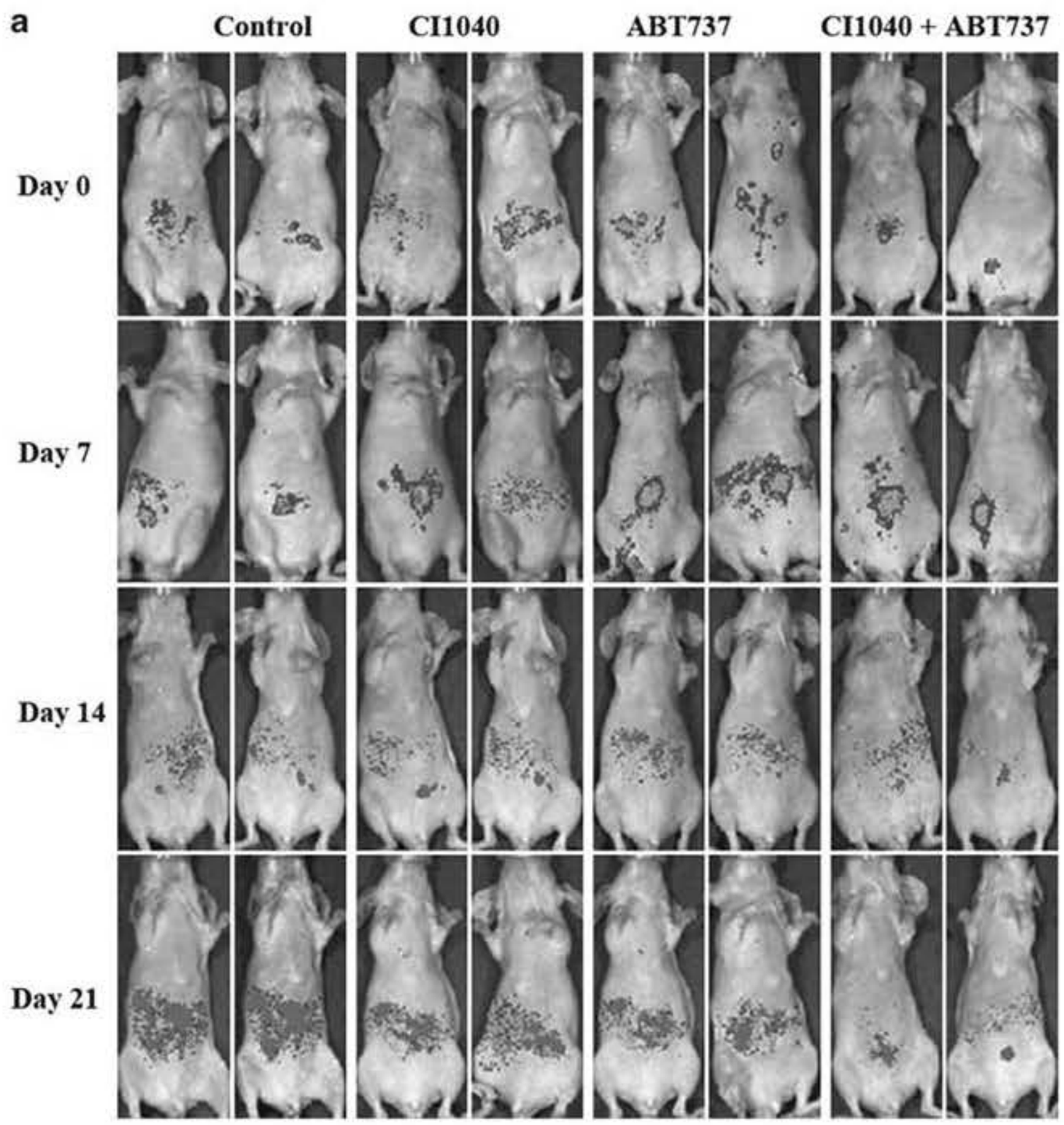


Figure 6 *In vivo* effects of combined BCL2/BCL-X_L (ABT-737) and MEK (CI-1040) inhibition in Molm-13-transplanted nude mice. (**a**) Human leukemia luciferase-expressing Molm-13 cells were injected i.v. into *nu/nu* mice, and leukemia dissemination was monitored by bioluminescence imaging. Two weeks after cell injection, mice were treated with ABT-737, CI-1040 or combination ($n = 8$ per group). Serial images of mice are shown. (**b**) Results were averaged from the peak light-emitting exposure from each group and displayed as photons/s.

hematologic malignancies.[33–39] Thus, use of appropriate inhibitors to suppress survival signaling cascades may be necessary in many chemotherapeutic strategies. An understanding of the kinases that are activated by a particular chemotherapy agent will be necessary to determine the type of inhibitors that would be useful for inclusion of the regimen in most effective treatments. Imatinib treatment of chronic myelogenous leukemia cells results in increased ERK activity.[33] ERK can also be activated in response to rapamycin[34] and arsenic trioxide.[35–38] Like ERK, p38 is activated in response to arsenic trioxide.[39]

Apoptosis induction by ABT-737 involves the activation of pro-apoptotic BCL2 family members including BAX, BAK and BIM.[13,17,18,28,29,40,41] BIM has been identified as an important regulator of ABT-737-mediated apoptosis.[17,29,40,41] A previous study has implicated ERK in promoting proteasome-mediated degradation of BIM.[29] In the present study, we did not find that inhibition of MAPK promoted BIM expression (data not shown).

A recent study has shown that histone deacetylase inhibition can promote BIM expression and promote ABT-737-induced cell death.[42,43] In that study, it appears that BCL2 and BCL-X_L, rather than MCL-1, is critical for sequestering histone deacetylase-inhibitor-induced BIM. Consistent with these data, Morales *et al.*[43] have found in multiple-myeloma cell lines that acquired resistance to ABT-737 is due to MCL-1 in only half the cell lines tested, and that a cell's dependence on BCL2/BCL-X_L versus MCL-1 was due to interactions with BIM rather than expression of any particular anti-apoptotic BCL2 family member. Suppression of MAPK signaling with PD0325901 did not promote BIM binding to MCL-1 alone, but the MEK inhibitor did augment ABT-737-induced binding of BIM to MCL-1 (Figure 3). The enhanced binding of BIM to MCL-1 in cells treated with both drugs was likely due to PD0325901-mediated reductions of total MCL-1 (Figure 3). These findings suggest that MCL-1 in AML cells likely has some role in sequestering BIM.

Cragg *et al.*[44] demonstrated that there was pro-apoptotic synergism between MEK inhibition and ABT-737 in various solid tumor cell lines. In a xenograft model using either melanoma (SkMel-28)- or colorectal cancer (Colo205)-derived cell lines, a combination of PD0325901 and ABT-737 was effective at promoting survival of mice with observed tumor regression, whereas each drug alone was at best only mildly effective.[44] ABT-737 has been shown to be effective at killing leukemic cells in a murine xenograft model.[17] In the present study, we similarly find that ABT-737 has an effective anti-tumor effect on our human-AML cell line murine-xenograft model (Figure 6). Whereas CI-1040 alone was ineffective at preventing tumor growth in the animals, MEK inhibition with the drug did augment suppression of MOLM13 tumor growth by ABT-737 in a murine xenograft model (Figure 6). Importantly, the combination of MEK inhibitor and ABT-737 was in most (6/8) primary AML samples more effective compared with ABT-737 alone, causing significantly less toxicity to normal progenitor cells (Figures 5a and b). Furthermore, combined blockade of MEK and BCL2 pathways targeted AML stem cells (CD34+38−123+) effectively in half of the samples from refractory AML cases (Figure 5c). These finding suggest that inhibition of MAPK signaling in conjunction with BH3-mimetic therapy will be a most effective means of selectively eradicating leukemia cells and progenitor/stem cells. Future clinical studies will be necessary to validate this hypothesis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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