HHS Public Access

Author manuscript

Leukemia. Author manuscript; available in PMC 2010 April 01.

Published in final edited form as:

Leukemia. 2009 October ; 23(10): 1744–1754. doi:10.1038/leu.2009.80.

MEK Inhibitors Potentiate Dexamethasone Lethality in Acute Lymphoblastic Leukemia Cells through the Pro-apoptotic Molecule BIM

Anila A. Rambal[1,2], **Zarah Liz G. Panaguiton**[1,2], **Lora Kramer**[1], **Steven Grant**[1], and **Hisashi Harada**[1]

[1]Department of Internal Medicine, Massey Cancer Center, Virginia Commonwealth University

Abstract

Glucocorticoids (GC) are common components of many chemotherapeutic regimens for lymphoid malignancies. GC-induced apoptosis involves an intrinsic mitochondria-dependent pathway. We and others have shown that BIM (BCL-2 interacting mediator of cell death), a BH3-only pro-apoptotic protein, is up-regulated by dexamethasone (Dex) treatment in acute lymphoblastic leukemia (ALL) cells and plays an essential role in Dex-induced apoptosis. Furthermore, BIM is inactivated by extracellular signal-regulated kinase (ERK)-mediated phosphorylation. We therefore hypothesized co-treatment with Dex and MEK/ERK inhibitors would promote apoptosis in ALL cells through BIM up-regulation and activation. We show here that MEK inhibitors (PD184352 and PD98059) synergistically enhance Dex lethality in a variety of ALL cells and in two primary ALL specimens. Co-treatment with Dex and PD184352 results in BIM accumulation, pro-apoptotic BAX/BAK activation, and cytochrome c release from mitochondria. Down-regulation of BIM by short-hairpin RNA in ALL cells suppressed BAX/BAK activation, cytochrome c release, and cell death by Dex/PD184352 co-treatment. BIM accumulated by this treatment sequesters anti-apoptotic BCL-X_L/MCL-1, resulting in the release of BAK from these anti-apoptotic molecules. This study provides a rational foundation for future attempts to improve the activity of glucocorticoids with clinically relevant pharmacologic MEK inhibitors in the treatment of ALL and possibly other hematologic malignancies.

Keywords

acute lymphoblastic leukemia; glucocorticoid; MEK inhibitor; apoptosis; BIM

Introduction

Glucocorticoids (GC) are common components in many chemotherapeutic protocols for lymphoid/myeloid malignancies, including ALL, multiple myeloma, chronic lymphocytic leukemia, and non-Hodgkin's lymphoma.1–6 GC-induced apoptosis is essentially divided

Correspondence: Hisashi Harada, Ph.D., Department of Internal Medicine, Massey Cancer Center, Virginia Commonwealth University, 401 College St., Richmond, VA 23298, Phone: 804-628-0064, Fax: 804-827-1276, Email: hharada@vcu.edu.

[2]These authors contributed equally to this work.

into three stages: (1) an initiation stage, which involves glucocorticoid receptor (GR) activation and GR-mediated gene regulation; (2) a decision stage, which engages pro- and anti-apoptotic BCL-2 family proteins at the mitochondrial level; and (3) an execution stage, which involves caspase and endonuclease activation. Although the effects of GC on lymphocytes have been scrutinized for many years, the molecular mechanisms of sensitivity and resistance are still elusive. Since GC-resistant leukemia and myeloma are often associated with GR defects, bypassing the receptor by targeting downstream molecules may lead to the development of new therapeutic strategies. Numerous novel drug combinations are currently being tested to prevent resistance and improve GC efficacy in the therapy of lymphoid malignancies. However, it is still not entirely clear how the signaling pathways and their downstream target molecules, including the BCL-2 family members, participate in GC-induced cell death.

The BCL-2 family is subdivided into three main groups based on regions of BCL-2 homology (BH) and function: multi-domain anti-apoptotic (e.g., BCL-2, MCL-1, BCL-X_L), multi-domain pro-apoptotic (e.g., BAX, BAK), and BH3-only pro-apoptotic (e.g., BAD, BID, BIM, PUMA).7,8 It is now clear that activation of BH3-only proteins by apoptotic stimuli initiates mitochondria-dependent cell death pathway. BH3-only proteins cause cytochrome c release by activating BAX and/or BAK, and the anti-apoptotic BCL-2 family of proteins prevents this process. BIM (BCL-2-interacting mediator of cell death) was identified as a BH3-only protein that induces apoptosis and is antagonized by anti-apoptotic BCL-2 family members.9 BIM activity can be modulated at the transcriptional and post-translational level.10,11 Transcriptional control of BIM involves contributions from Jun N-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) pathways. It has been reported that phosphorylation and ubiquitination of BIM can regulate its protein level.10 We have previously shown that the phosphorylation status of BIM controls its pro-apoptotic activity.12 Interleukin-3 (IL-3), a hematopoietic survival factor, induces ERK-mediated phosphorylation of BIM on three serine sites (Ser55, Ser65, Ser100). IL-3 withdrawal results in the dephosphorylation of BIM and only non-phosphorylated BIM interacts with the multi-domain pro-apoptotic effector BAX at the mitochondrial membrane. Phosphorylation of BIM upon exposure of cells to IL-3 dramatically reduces the BIM/BAX interaction. A non-phosphorylatable BIM molecule (S55A/S65A/S100A) demonstrates enhanced interaction with BAX and enhanced pro-apoptotic activity. Thus, ERK-dependent phosphorylation of BIM in response to survival factor regulates BIM/BAX interaction and the pro-apoptotic activity of BIM. Several reports suggest that BIM has a unique character among the BH3-only members; BIM has the capacity to interact with all of the multi-domain anti-apoptotic BCL-2 family members (BCL-2, MCL-1, and BCL-X_L) and also directly activate multi-domain pro-apoptotic BCL-2 family members (BAX, BAK).13–15 This mechanism is thought to be critical for the activation of the downstream apoptotic machinery through BIM.

Studies of BIM- or PUMA (p53 up-regulated modulator of apoptosis)-deficient mice/cells demonstrate that BH3-only proteins such as BIM and PUMA play important roles in dexamethasone (Dex)-induced cell death.16–18 Moreover, thymocytes from double knockout mice lacking both BAX and BAK, which have a complete block in the intrinsic apoptotic pathway, are GC resistant.19 We have demonstrated that up-regulation of BIM in

response to Dex in a T-ALL cell line, CCRF-CEM (CEM), is critical to the induction of apoptosis.20 It has also been demonstrated that induction of BIM is necessary for GC-mediated apoptotic response in B-acute lymphoblastic leukemia (B-ALL) cells and the *Bim* gene is induced in childhood ALL patients sensitive to Dex treatment.21,22 Thus, BIM could be a target for the development of new therapeutic strategies against GC resistance.

Growth factors, cytokines, and proto-oncogenes transduce their growth and differentiation promoting signals through MEK/ERK cascade.23–27 Overexpression or constitutive activation of this pathway has been shown to play an important role in the pathogenesis and progression of many tumors. Thus, the components of this signaling cascade are potentially important as therapeutic targets. While MEK activity appears restricted to only one class of substrates, ERK activates more than 70 substrates including nuclear transcription factors. For this reason, several pharmacologic MEK inhibitors have recently entered the clinic, and have been shown to inhibit phosphorylation of their targets including ERK when administered at well-tolerated doses.28–30

Collectively, these considerations suggest a novel and potentially effective way to potentiate GC activity against ALL cells based on the concept that, a) GCs up-regulate BIM; and b) pharmacologic MEK inhibitors may further potentiate BIM activation by blocking BIM phosphorylation and degradation. We show here that MEK inhibitors synergistically promote Dex lethality in a variety of ALL cell lines, and that BIM plays a central role in apoptosis induced by this regimen.

Materials and methods

Cell lines and culture

CCRF-CEM (T-ALL), SUP-B15, (B-ALL), RS4;11 (B-ALL), and Molt-4 (T-ALL) were purchased from the American Tissue Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, streptomycin, and penicillin G at 37°C in a humidified, 5% CO_2 incubator.

Chemicals and antibodies

Dexamethasone and PD98059 were purchased from Sigma (St. Louis, MO). PD184352 was kindly provided by Dr. Steven Grant (Virginia Commonwealth University), which was chemically synthesized in house based on the published structure of the drug. Reagents were dissolved in sterile DMSO and stored at −20°C under light protection. Antibodies for Western blot were purchased as follows: BIM (202000) from Calbiochem (San Diego, CA); BAX (N-20), β-tubulin, phospho-ERK, and ERK from Santa Cruz Biotechnology (Santa Cruz, CA); BAK from Upstate/Millipore (Billerica, MA); BCL-2 from Sigma; MCL-1 from Assay Designs (Ann Arbor, MI); BAD, PUMA, and BCL-X_L from Cell Signaling Technology (Beverly, MA); MCL-1 and cytochrome c from BD-Pharmingen (San Diego, CA); GAPDH from Abcam (Cambridge, MA). A phospho-S65 BIM antibody was developed in our lab as described previously.12

Plasmid construction and transfection

For down-regulation of BIM by short-hairpin RNA (shRNA), pSR-BIM and pSR-con (control) were constructed as described previously.20 For down-regulation of BAD by shRNA, a microRNA-adapted shRNA construct designed against human *Bad* (5'-acgtgctcactaccaaatgtta-3') was purchased from Open Biosystems (Huntsville, AL). HA-tagged constitutive-active MEK1 (ca-MEK1) was obtained from Upstate/Millipore. Transfection was performed by electroporation using a Bio-Rad electroporator (Hercules, CA). The cells were suspended in RPMI 1640 (4×10^6/400 μl) with 10 μg of DNA and electroporated in 0.4 cm cuvettes at 300 V, 500 μF for CCRF-CEM cells and at 900 V, 200 μF for RS4;11 cells. Puromycin (2 μg/ml for CCRF-CEM and 0.5 μg/ml for RS4;11 cells) or G418 (800 μg/ml for ca-MEK1) selection to establish stable clones began twenty four hours after electroporation.

Immunoprecipitation and Western blot analyses

Whole cell lysates were prepared with CHAPS lysis buffer [20 mM Tris (pH 7.4), 137 mM NaCl, 1 mM dithiothreitol (DTT), 1% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), 20 mM NaF, 10 mM β-glycerophosphate, and a protease inhibitor cocktail (Sigma)]. For immunoprecipitation, equal amounts of protein were precleared with protein A/G beads (Pierce, Rockford, IL), and incubated with the appropriate antibodies on ice for 2 hrs. Then the antibody complexes were captured with protein A/G beads at 4 °C for 1 hr. After washing three times with the same lysis buffer, the beads were re-suspended in the sample buffer and separated by SDS-PAGE. For Western blot analyses, equal amounts of proteins were loaded on SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by immunoblotting.

Cell Viability Assay

Cell death was quantified by Annexin-V-FITC (BD Pharmingen)-propidium iodide (PI, Sigma) staining according to the manufacturer's protocol, followed by flow cytometric analysis using FACScan (BD Biosciences).

Analysis of BAX and BAK conformational change

Cells were lysed in CHAPS buffer and 300 μg of protein were immunoprecipited with anti-BAX (6A7, Sigma) or anti-BAK (Ab-1, Calbiochem), which only recognizes BAX or BAK that have undergone conformation change. Immunoprecipitated protein was then subjected to Western blot analysis using rabbit anti-BAX or anti-BAK as primary antibodies.

Subcellular fractionation

Two million cells were washed in PBS and lysed by incubating for 30 seconds at room temperature in digitonin lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, and 350 μg/ml digitonin). Lysates were centrifuged (×12,000g) for 1 min, and the supernatant (cytosolic fraction) was collected. The pellets (membrane fractions) were washed once in cold PBS and lysed in CHAPS lysis buffer. The cytosolic and membrane samples were quantified, separated by SDS-PAGE, and subjected to Western blot analysis.

Isolation of patient-derived acuye lymphoblastic leukemia blasts

Peripheral blood samples were obtained from 2 patients diagnosed with ALL. Informed consent was obtained following institutional guidelines and approval was obtained from the institutional review board of Virginia Commonwealth University. Consent was provided according to the Declaration of Helsinki. Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. The samples, which consisted of >70% blasts, were cryopreserved and stored in liquid nitrogen before use. Viability after thawing was determined by trypan blue dye exclusion and was greater than 90%.

Statistical Analysis

For flow cytometric analyses of Annexin-V/PI, values represent the means ± SD for three separate experiments. The significance of differences between experimental variables was determined using the Student's t test. Values were considered statistically significant at $P<0.05$.

Results

Dexamethasone interacts synergistically with MEK inhibitors in CCRF-CEM cells and other ALL cell lines

We first tested our hypothesis that up-regulation of BIM (by Dex) together with diminished phosphorylation of BIM (by MEK inhibitors) enhances cell death in ALL cells. Treatment with Dex (100 nM) alone induced about 15% of apoptosis at 48 hr, and treatment with a MEK inhibitor, PD184352 (5 μM) was minimally toxic to CEM cells. In contrast, combined treatment resulted in pronounced increase in apoptosis (~60%) at the same time period (Figure 1a). Treatment with Dex or PD184352 alone reduced growth rates and co-treatment further enhanced the reduction. Dex alone or in combination with PD184352 induced G1 cell cycle arrest, but only co-treatment induced significant amount of sub-G1 population, indicative of apoptosis (Supplemental Figure 1). Treatment of as low as 30 nM Dex significantly increased cell death in combination with 5 μM of PD184352 (Figure 1b). The PD184352 dose-response curve revealed that concentrations as low as 1 μM PD184352 significantly increased the toxicity of 100 nM Dex (Figure 1c). Time course analysis indicated that simultaneous exposure of 100 nM Dex and 5 μM PD184352 resulted in little apoptosis after 24 hrs, but extensive cell death at later time points (Figure 1d). We also observed Dex and PD184352 interactions in RS4;11 (B-ALL), SUP-B15 (B-ALL), and Molt-4 (T-ALL)(Figure 1e–g). Of note, Molt-4 was highly resistant to Dex treatment alone (1 μM Dex treatment did not induce cell death); however, co-treatment with PD184352 induced marked cell death (Figure 1g). These results indicate that minimally toxic concentrations of MEK inhibitors markedly potentiate the lethality of low concentrations of dexamethasone in ALL cells.

PD184352 enhances dexamethasone-induced BIM accumulation, BAX/BAK conformational changes, and cytochrome c release from mitochondria in ALL cells

We then examined the expression of the BCL-2 family proteins before the onset of apoptosis, which begins at 24 hrs in CEM cells (Figure 1d), at 16 hrs in SUP-B15 cells, and

at 24 hrs in Molt-4 cells (data not shown) after treatment. BIM was slightly accumulated with PD184352 or Dex treatment alone (about 2~3-fold increase compared with untreated cells), but significantly increased with co-treatment in all cell lines (about 5~10-fold increase). The phosphorylation of BIM was increased by Dex treatment alone, but was decreased by Dex+PD184352 co-treatment, as judged by a phosphorylation-specific antibody (Figure 2a, pBIM). Although non-malignant thymocytes from PUMA (a BH3-only member)-deficient mice are resistant to Dex-induced apoptosis,17,18 the expression of PUMA was little altered following Dex and/or PD184352 treatment (Figure 2a). Another BH3-only protein, BAD, is a downstream target of the ERK pathway which regulates survival/apoptosis,31 but its expression was constant (Figure 2a), suggesting that perturbations in PUMA or BAD may not be essential for apoptosis induced by Dex/PD184352 co-treatment, at least in these ALL cell lines (see also below). The overall expression of the multi-domain pro-apoptotic effectors, BAX and BAK, was unchanged. We also examined the expression of anti-apoptotic members, BCL-2, BCL-X_L, and MCL-1 (Figure 2a). The expression of BCL-2 and BCL-X_L was not significantly altered. The expression of MCL-1 was slightly (CEM and SUP-B15) or significantly (Molt-4) induced by Dex treatment alone, but then restored to basal levels when cells were treated with Dex and PD184352. The phosphorylation of ERK (i.e. ERK activity) was inhibited by PD184352 regardless of Dex treatment (Figure 2a).

Following apoptotic stimuli, the multi-domain pro-apoptotic BCL-2 family, BAX and BAK undergo conformational changes (active forms), resulting in the mitochondrial outer membrane permeability transition and release of cytochrome c. We examined the effects of PD184352 and Dex on BAX and BAK conformation and release of cytochrome c from mitochondria (Figure 2b and 2c). Treatment of CEM cells for 24 hrs with Dex or PD184352 alone induced modest conformational changes in BAX and BAK. Dex treatment alone also slightly induced cytochrome c release but not with PD184352 treatment alone. However, combined treatment with both agents resulted in marked conformational changes in both BAX and BAK and significant amount of cytochrome c release from mitochondria. These findings suggest that non-phosphorylated, accumulated BIM induces BAX/BAK conformational changes (activation), cytochrome c release, and ultimately apoptosis in this treatment.

BIM is essential for cell death induced by Dex/PD184352 co-treatment in ALL cells

To confirm the significance of BIM in this regimen, we established CEM cell clones that express shBIM to reduce the expression of endogenous BIM. As a control, a scrambled, non-specific shRNA construct was transfected. The accumulation of BIM in treatment with Dex and/or PD184352 was strongly and partially inhibited by shBIM in clone 2 and clone 15, respectively (Figure 3a). However, down-regulation of BIM by shRNA did not affect ERK phosphorylation status following exposure of PD184352 with or without Dex, indicating that ERK inactivation acts upstream of BIM. We then asked whether BIM is required for BAX and BAK conformational changes, cytochrome c release from mitochondria, and ultimately cell death induced by co-treatment with Dex and PD184352. BAX and BAK conformational changes were completely inhibited by down-regulation of BIM in clone 2 (Figure 3b). Cytochrome c release was also completely abrogated (Figure

3c). Finally, cell death in response to combination treatment was sharply reduced by the introduction of shBIM. The inhibition of cell death correlated well with the reduction of BIM expression by shRNA in clone 2 and clone 15 (Figure 3a and 3d). In contrast, similar amounts of cell death were observed in all of these clones when they were treated with etoposide or staurosporine (data not shown), suggesting that the BIM-independent intrinsic pathway(s) is still functional. We also introduced a shBIM or a control construct into RS4;11 cells and established several stable clones. The accumulation of BIM following treatment with Dex and/or PD184352 was strongly inhibited by shBIM, but not by control shRNA (Figure 3e). Cell death in response to combination treatment was also strongly reduced by the introduction of shBIM (P <0.001; Figure 3f). Taken together, these findings indicate that minimally toxic concentrations of MEK inhibitors markedly enhance the lethality of low concentrations of Dex mostly through BIM in ALL cells, and BIM is essential for the activation of BAX and BAK, cytochrome c release, and cell death in this treatment.

BAD is dispensable for cell death induced by Dex and PD184352 co-treatment

Although total protein expression of BAD was constant with Dex and/or PD184352 treatment (Figure 2), it has been well demonstrated that the phosphorylation status of this protein regulated by the ERK pathway contributes to survival/apoptosis.31 Thus, we investigated whether BAD also plays a role in Dex/PD184352-induced apoptosis. We introduced a shBAD (short hairpin BAD) construct into CEM cells and established several clones. As shown in Figure 4, down-regulation of BAD affected neither BIM expression nor cell death induced by co-treatment of Dex/PD184352 (P>0.05). These data strongly suggest that BAD is dispensable for this apoptotic stimulus.

Modulation of MEK1 expression affects Dex-induced BIM accumulation and apoptosis

To evaluate the significance of ERK activity in cell death induced by Dex/MEK inhibitors co-treatment, we first established the CEM clones that express constitutively active MEK1 (ca-MEK1). These cells exhibited increased basal ERK activation compared with the empty vector control cells (Figure 5a, lane 1 versus lane 7). It has been shown that the MEK inhibitor PD184352, but not PD98059, inhibits ca-MEK1-induced ERK activation, presumably due to different targets in RAF/MEK/ERK signaling cascade.32–34 Therefore, we compared the abilities of PD184352 and PD98059 to promote Dex-induced cell death in CEM/ca-MEK1 clones. In CEM/control cells co-treatment with Dex/PD184352 or Dex/PD98059 blocked ERK phosphorylation and induced BIM accumulation and caspase-3 cleavage, indicative of apoptosis (Figure 5a, lanes 4 and 6). Co-treatment with Dex/PD184352 blocked ERK phosphorylation and induced BIM accumulation and caspase-3 cleavage, even in the expression of ca-MEK1 (Figure 5a, lane 10). In contrast, PD98059 partially reduced ERK phosphorylation in CEM/ca-MEK1 cells, resulted in less BIM accumulation and caspase-3 cleavage compared with those in CEM/control cells (Figure 5a, lane 6 versus lane 12). As a consequence, CEM/ca-MEK1 cells were substantially resistant to Dex/PD98059-mediated apoptosis (P <0.005; Figure 5b, Lane 6 versus lane 12), while displaying equivalent sensitivity to the Dex/PD184352 regimen compared with the CEM/control cells (P >0.05; Figure 5b, lane 4 versus lane 10).

Reciprocally, we established the CEM cell clones that express MEK1 shRNA to reduce the expression of endogenous MEK1. Stable transfection into CEM cells with a construct encoding MEK1 shRNA resulted in reduction of MEK1 expression, accompanied with a pronounced reduction of phospho-ERK1/2. Increased concentration of Dex resulted in more accumulation of BIM and cleaved caspase-3 compared with control cells (Figure 5c). Furthermore, down-regulation of MEK1 significantly sensitized CEM cells to Dex-induced lethality compared with control (P <0.001; Figure 5d). Together, these results support the notion that the ERK activity controls the amount of BIM and Dex-induced lethality.

Blasts from ALL Patients Are Sensitive to Dex/PD184352-mediated Apoptosis

To determine whether primary ALL patient samples also display interactions between Dex and MEK inhibitors, leukemic blasts obtained from the peripheral blood of two ALL patients were isolated and exposed for 48 hr to Dex and PD184352 alone or in combination. When cells were co-treated with 10 nM Dex and 2 μM PD184352, the viability of both specimens significantly decreased (P<0.005 or P<0.01; Figure 6a), and in each case, was significantly less than for cells exposed to the agents individually (P<0.01). Western blot analysis of blasts obtained from patient #1 displayed changes similar to those observed in CEM cells. Specifically, co-administration of Dex and PD184352 resulted in a marked increase in BIM and decreased expression of MCL-1 (Figure 6b). Thus, these findings indicate that combined treatment with Dex and PD184352 may result in an increase in apoptosis in at least some primary ALL blast specimens, analogous to findings in continuously cultured ALL cell lines.

BIM binds BCL-X_L and MCL-1, causing the release of BAK

It has been proposed that BAK is regulated through activation by BH3-only proteins and sequestered by anti-apoptotic BCL-2 family proteins.35 Therefore, we examined whether accumulated BIM regulates BAK activation mediated by co-treatment with Dex and PD184352. We first performed immunoprecipitation with an anti-BIM antibody using CEM cells treated with Dex and/or PD184352 for 24 hrs, the time before the onset of massive apoptosis. When cells were co-treated with Dex and PD184352, an anti-BIM antibody co-immunoprecipitated BCL-2, BCL-X_L, and MCL-1 most abundantly, indicating that accumulated BIM interacts with anti-apoptotic BCL-2 family proteins in response to apoptosis induced by Dex/PD184352 co-treatment (Figure 7a, upper panel). Reciprocal co-immunoprecipitation experiments with an anti-BCL-X_L or an anti-MCL-1 antibody confirmed this observation (Figure 7a, middle and bottom panel). Furthermore, immunoprecipitation with an anti-BCL-X_L or an anti-MCL-1 antibody revealed that both BCL-X_L and MCL-1 still interacted with BAK in cells treated with a single agent, whereas BAK was released from the complexes with both BCL-X_L and MCL-1 upon co-treatment (Figure 7a, middle and bottom panel). These results strongly suggest that the interaction of BIM with both BCL-X_L and MCL-1 leads to displacement and release of BAK from these complexes, resulting in the activation of BAK when cells are treated with Dex and PD184352 (Figure 7b).

Discussion

Glucocorticoids have been used in chemotherapy for leukemia, lymphoma, and myeloma for decades. Although they are effective in the initial stages, resistance often emerges, and the molecular mechanisms of sensitivity/resistance to this agent are still not fully understood. We and others have recently demonstrated BIM, a BH3-only pro-apoptotic BCL-2 family, is transcriptionally induced by Dex treatment in various cell types and plays a critical role in Dex-induced cell death.20–22 The activity of BIM is regulated not only by transcription, but also by post-translational mechanisms. Among these, ERK-mediated phosphorylation, ubiquitylation, and subsequent protein degradation has been demonstrated in a variety of cells and MEK inhibitors abrogate such regulation.10 Thus, we hypothesized combined treatment with Dex and MEK inhibitors might act synergistically in their cell killing activity. We demonstrate here that Dex interacts in a highly synergistic manner with a clinically relevant MEK inhibitor to induce apoptosis in both B- and T-ALL cells. We also observed similar interactions in a multiple myeloma cell line, MM.1S (Harada et. al., unpublished results). Furthermore, marked cell death was observed by co-treatment with Dex and PD184352 in Molt-4, an ALL cell line which was highly resistant to Dex treatment alone (Figure 1g). Consequently, this strategy may represent an attractive strategy to overcome or circumvent at least some forms of intrinsic Dex resistance. Finally, it is important to note that evidence of enhanced apoptosis, including BIM accumulation, was also observed in two primary ALL blast samples after *ex vivo* exposure to the combination of Dex and PD184352. The appearance of apoptotic cells in the peripheral blood of leukemic patients undergoing chemotherapy has been well documented, and it is conceivable that these agents might exert similar effects when administered *in vivo*. However, whether the combination of the above regimens promotes apoptosis in a larger series of primary ALL specimens and whether the observed *in vitro* interactions occur in the *in vivo* setting remain to be determined.

The observation that down-regulation of BIM by shRNA almost completely suppressed BAX/BAK activation, cytochrome c release, and cell death induced by Dex/MEK inhibitors co-treatment (Figure 3) strongly suggests that BIM is a central regulator in this regimen at least in CCRF-CEM T-ALL and RS4;11 B-ALL cells. Recent results involving epithelial breast cancer cells suggest that BAD phosphorylation status represents the primary integrator of cell death following interruption of the AKT and ERK pathways.36 However, down-regulation of BAD with shRNA, in marked contrast to BIM, failed to protect CEM cells from Dex/PD184352-mediated lethality (Figure 4), suggesting that BAD is not a critical molecule in lethality in this setting. Differences between current and previous reports may therefore reflect cell type-specific roles of BAD in integrating death signals following concomitant interruption of the MEK/ERK and AKT pathways. It has been shown that non-malignant thymocytes from PUMA-deficient mice are resistant to Dex-induced apoptosis.17,18 Thus, it is possible that this BH3-only protein may contribute to apoptosis induced by Dex/MEK inhibitors regimen, although its role may be relatively minor.

The evidence presented here suggests that the amount of BIM protein regulated by ERK plays a significant role in apoptosis induced by Dex/MEK inhibitors regimen. Constitutively active MEK1 (ca-MEK1) reduced BIM expression level and apoptosis induced by Dex in

combination with PD98059, a MEK inhibitor that primarily inhibits the RAF/MEK1 interaction.32 In contrast, ca-MEK1 was ineffective in overcoming the effects of PD184352, which directly inhibits both RAF/MEK1 interaction and MEK1 activity (Figure 5a, b).33,34 Reciprocally, down-regulation of MEK1 by shRNA significantly increased BIM expression and sensitized CEM cells to Dex-induced lethality (Figure 5c, d). These findings indicate that stabilization of BIM protein proceeds through ERK inactivation. In addition to BIM, inactivation of ERK has been shown to induce apoptosis by multiple mechanisms, including activation of procaspase-9.37 Thus, it is possible that other mechanism(s) may also contribute to synergistic interactions between Dex and MEK inhibitors.

The function of BH3-only proteins is linked to specific stimuli and/or specific cell types, and likely represents one of the control points that provide the specificity for apoptotic signaling pathways that ultimately converge on BAX and BAK. In recent years, two models have been proposed to account for the various experimental results in activation of BAX and BAK.38,39 The first model is referred as the direct binding model. According to this model, BH3-only proteins directly bind and activate the multi-domain pro-apoptotic protein BAX (and presumably BAK). Another model is referred as displacement (or indirect activation): BAX and BAK are constitutively active and must be continuously bound and inhibited by multi-domain anti-apoptotic proteins for cells to survive. In apoptotic cells, BH3-only proteins displace BAX and BAK from anti-apoptotic proteins such as MCL-1 and BCL-X_L. BIM has the capacity to interact with all of the multi-domain anti-apoptotic BCL-2 family members (BCL-2, MCL-1, and BCLX_L) and also to activate BAX and BAK directly.13–15 Our results in Figure 7a are consistent with the displacement model to activate BAK: the accumulation of BIM induced by Dex plus dephosphorylated/stabilized by MEK inhibitors leads to sequester MCL-1 and BCL-X_L, resulting in the release of BAK to be activated (Figure 7b). BIM is also required for BAX activation in Dex/PD184352 co-treatment (Figure 3b), but we could not detect BIM/BAX direct interaction by co-immunoprecipitation (data not shown). Thus, further studies are needed to clarify the mechanisms for BAX activation.

It is widely recognized that the RAS/RAF/MEK/ERK signaling pathway mediates survival signaling in diverse transformed cell types. The implication of the present findings is that in ALL cells, phosphorylation/degradation of BIM mediated by ERK may represent a pro-survival mechanism by which such cells escape the lethal consequences of glucocorticoids (GC) treatment. A corollary of this hypothesis is that MEK inhibition may potentiate the lethal effects of Dex and potentially other novel agents by preventing BIM phosphorylation/degradation. Thus, BIM phosphorylation/expression status may represent a determinant of the activity of such strategies. If validated, this concept could have implications for the development of novel anti-leukemia regimens involving the combined administration of clinically relevant agents targeting at the RAS/RAF/MEK/ERK pathway (e.g. MEK inhibitors, farnesyltransferase inhibitors, HMG CoA-reductase inhibitors) and GC. A recent study has demonstrated that the receptor tyrosine kinase inhibitor, SU11657 (potentially inactivating the RAS pathway) interacts synergistically with Dex to modulate signaling through BIM and to induce apoptosis in a highly GC-resistant ALL xenograft model.40 BIM also plays an important role in cell death induced by other chemotherapeutic drugs such as STI571 (imatinib mesylate)41–43 and histone deacetylase inhibitors (HDACI).44,45 In

these cases, BIM is transcriptionally induced through FOXO and E2F, respectively. If our hypothesis that combination of BIM up-regulation and stabilization synergistically promotes cell death is validated, it will be interesting to test whether combinations of STI571 or HDACI with MEK inhibitors interacts synergistically in ALL cells. In fact, it has been shown that this is the case in BCR/ABL$^+$ leukemia cells and other adherent malignant cells.46,47 To date, several pharmacological MEK inhibitors including PD184352 (or CI-1040), PD0325901, and AZD6244 (ARRY142886) have been developed clinically.28–30 Results of early clinical trials indicate that it is feasible to achieve the desired pharmacodynamic effect (e.g. ERK inactivation) at well-tolerated doses of MEK inhibitors. Collectively, our findings could have implications for understanding the mechanisms underlying synergistic interactions between MEK inhibitors and other targeted agents in ALL and potentially other hematologic malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

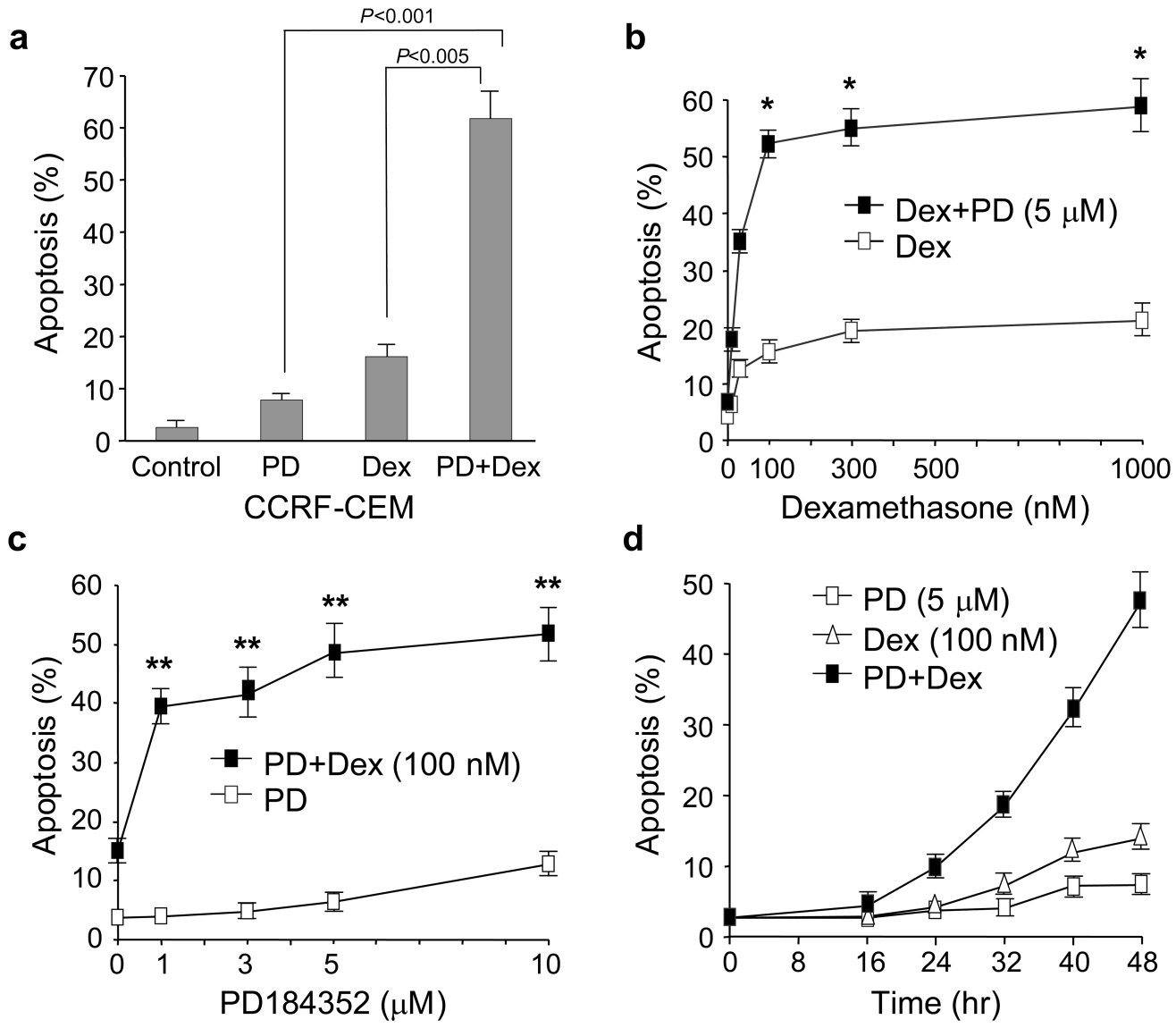
We thank members of Dr. Grant's lab for valuable advice and discussion. This work was supported by the Leukemia Research Foundation (to HH) and NIH RO1CA100866 (to SG).

References

1. Planey SL, Litwack G. Glucocorticoid-induced apoptosis in lymphocytes. Biochem Biophys Res Commun. 2000; 279:307–312. [PubMed: 11118283]
2. Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Differ. 2002; 9:6–19. [PubMed: 11803370]
3. Tissing WJ, Meijerink JP, den Boer ML, Pieters R. Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia. 2003; 17:17–25. [PubMed: 12529655]
4. Frankfurt O, Rosen ST. Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates. Curr Opin Oncol. 2004; 16:553–563. [PubMed: 15627017]
5. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med. 2004; 350:1535–1548. [PubMed: 15071128]
6. Schmidt S, Rainer J, Ploner C, Presul E, Riml S, Kofler R. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ. 2004; 11(Suppl 1):S45–S55. [PubMed: 15243581]
7. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004; 116:205–219. [PubMed: 14744432]
8. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene. 2007; 26:1324–1337. [PubMed: 17322918]
9. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, et al. Bim: a novel member of the Bcl-2 family that promotes apoptosis. EMBO J. 1998; 17:384–395. [PubMed: 9430630]
10. Ley R, Ewings KE, Hadfield K, Cook SJ. Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. Cell Death Differ. 2005; 12:1008–1014. [PubMed: 15947788]
11. Strasser A. The role of BH3-only proteins in the immune system. Nat Rev Immunol. 2005; 5:189–200. [PubMed: 15719025]
12. Harada H, Quearry B, Ruiz-Vela A, Korsmeyer SJ. Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. Proc Natl Acad Sci USA. 2004; 101:15313–15317. [PubMed: 15486085]

13. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell. 2002; 2:183–192. [PubMed: 12242151]
14. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, et al. Differential Targeting of Prosurvival Bcl-2 Proteins by Their BH3-Only Ligands Allows Complementary Apoptotic Function. Mol Cell. 2005; 17:393–403. [PubMed: 15694340]
15. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, et al. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol Cell. 2005; 17:525–535. [PubMed: 15721256]
16. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science. 1999; 286:1735–1738. [PubMed: 10576740]
17. Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell. 2003; 4:321–328. [PubMed: 14585359]
18. Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, et al. p53-and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science. 2003; 302:1036–1038. [PubMed: 14500851]
19. Rathmell JC, Lindsten T, Zong WX, Cinalli RM, Thompson CB. Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. Nat Immunol. 2002; 3:932–939. [PubMed: 12244308]
20. Lu J, Quearry B, Harada H. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. FEBS Lett. 2006; 580:3539–3544. [PubMed: 16730715]
21. Bachmann PS, Gorman R, Mackenzie KL, Lutze-Mann L, Lock RB. Dexamethasone resistance in B-cell precursor childhood acute lymphoblastic leukemia occurs downstream of ligand-induced nuclear translocation of the glucocorticoid receptor. Blood. 2005; 105:2519–2526. [PubMed: 15572593]
22. Schmidt S, Rainer J, Riml S, Ploner C, Jesacher S, Achmuller C, et al. Identification of glucocorticoid-response genes in children with acute lymphoblastic leukemia. Blood. 2006; 107:2061–2069. [PubMed: 16293608]
23. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature. 2001; 410:37–40. [PubMed: 11242034]
24. English JM, Cobb MH. Pharmacological inhibitors of MAPK pathways. Trends Pharmacol Sci. 2002; 23:40–45. [PubMed: 11804650]
25. Lee JT Jr, McCubrey JA. The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. Leukemia. 2002; 16:486–507. [PubMed: 11960326]
26. Platanias LC. Map kinase signaling pathways and hematologic malignancies. Blood. 2003; 101:4667–4679. [PubMed: 12623839]
27. O'Neill E, Kolch W. Conferring specificity on the ubiquitous Raf/MEK signalling pathway. Br J Cancer. 2004; 90:283–288. [PubMed: 14735164]
28. Allen LF, Sebolt-Leopold J, Meyer MB. CI-1040 (PD184352), a targeted signal transduction inhibitor of MEK (MAPKK). Semin Oncol. 2003; 30:105–116. [PubMed: 14613031]
29. Lorusso PM, Adjei AA, Varterasian M, Gadgeel S, Reid J, Mitchell DY, et al. Phase I and pharmacodynamic study of the oral MEK inhibitor CI-1040 in patients with advanced malignancies. J Clin Oncol. 2005; 23:5281–5293. [PubMed: 16009947]
30. Wang JY, Wilcoxen KM, Nomoto K, Wu S. Recent advances of MEK inhibitors and their clinical progress. Curr Top Med Chem. 2007; 7:1364–1378. [PubMed: 17692026]
31. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science. 1999; 286:1358–1362. [PubMed: 10558990]
32. Racke FK, Lewandowska K, Goueli S, Goldfarb AN. Sustained activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway is required for megakaryocytic differentiation of K562 cells. J Biol Chem. 1997; 272:23366–23370. [PubMed: 9287350]

33. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J. 2000; 351:95–105. [PubMed: 10998351]
34. Ohren JF, Chen H, Pavlovsky A, Whitehead C, Zhang E, Kuffa P, et al. Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition. Nat Struct Mol Biol. 2004; 11:1192–1197. [PubMed: 15543157]
35. Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev. 2005; 19:1294–1305. [PubMed: 15901672]
36. She QB, Solit DB, Ye Q, O'Reilly KE, Lobo J, Rosen N. The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer Cell. 2005; 8:287–297. [PubMed: 16226704]
37. Allan LA, Morrice N, Brady S, Magee G, Pathak S, Clarke PR. Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. Nat Cell Biol. 2003; 5:647–654. [PubMed: 12792650]
38. Letai AG. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. Nat Rev Cancer. 2008; 8:121–132. [PubMed: 18202696]
39. Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol. 2008; 18:157–164. [PubMed: 18314333]
40. Bachmann PS, Gorman R, Papa RA, Bardell JE, Ford J, Kees UR, et al. Divergent mechanisms of glucocorticoid resistance in experimental models of pediatric acute lymphoblastic leukemia. Cancer Res. 2007; 67:4482–4490. [PubMed: 17483364]
41. Kuribara R, Honda H, Matsui H, Shinjyo T, Inukai T, Sugita K, et al. Roles of Bim in apoptosis of normal and Bcr-Abl-expressing hematopoietic progenitors. Mol Cell Biol. 2004; 24:6172–6183. [PubMed: 15226421]
42. Essafi A, Fernandez de Mattos S, Hassen YA, Soeiro I, Mufti GJ, Thomas NS, et al. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. Oncogene. 2005; 24:2317–2329. [PubMed: 15688014]
43. Kuroda J, Puthalakath H, Cragg MS, Kelly PN, Bouillet P, Huang DC, et al. Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. Proc Natl Acad Sci U S A. 2006; 103:14907–14912. [PubMed: 16997913]
44. Zhao Y, Tan J, Zhuang L, Jiang X, Liu ET, Yu Q. Inhibitors of histone deacetylases target the Rb-E2F1 pathway for apoptosis induction through activation of proapoptotic protein Bim. Proc Natl Acad Sci U S A. 2005; 102:16090–16095. [PubMed: 16243973]
45. Gillespie S, Borrow J, Zhang XD, Hersey P. Bim plays a crucial role in synergistic induction of apoptosis by the histone deacetylase inhibitor SBHA and TRAIL in melanoma cells. Apoptosis. 2006; 11:2251–2265. [PubMed: 17051334]
46. Yu C, Dasmahapatra G, Dent P, Grant S. Synergistic interactions between MEK1/2 and histone deacetylase inhibitors in BCR/ABL+ human leukemia cells. Leukemia. 2005; 19:1579–1589. [PubMed: 16015388]
47. Ozaki K, Minoda A, Kishikawa F, Kohno M. Blockade of the ERK pathway markedly sensitizes tumor cells to HDAC inhibitor-induced cell death. Biochem Biophys Res Commun. 2006; 339:1171–1177. [PubMed: 16338224]



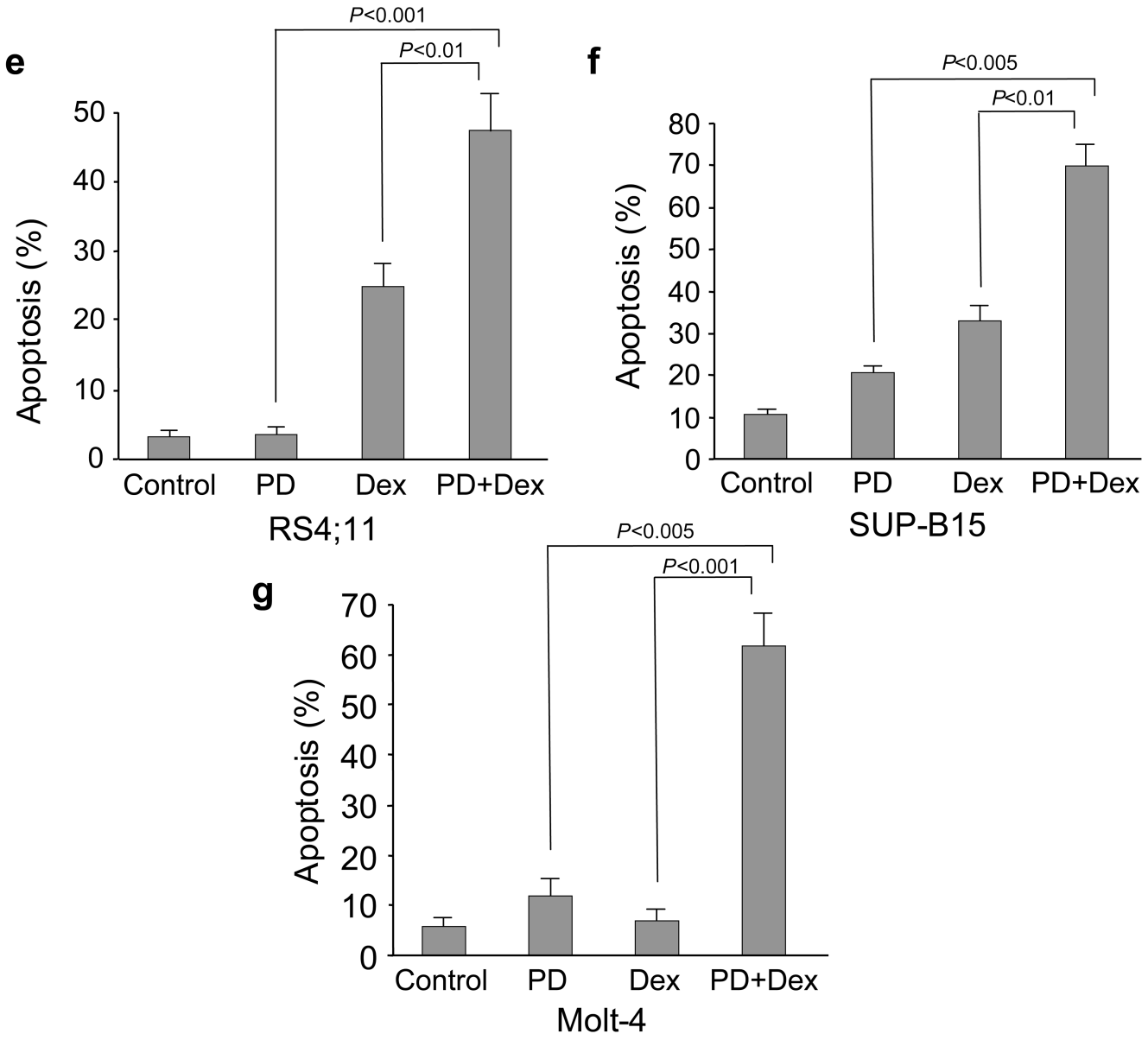


Figure 1. Co-treatment with dexamethasone and a MEK inhibitor, PD184352, markedly increases apoptosis in ALL cells

(a) CEM cells were exposed to 5 μM PD184352 (PD) and 100 nM dexamethasone (Dex) alone or in combination for 48 hrs.

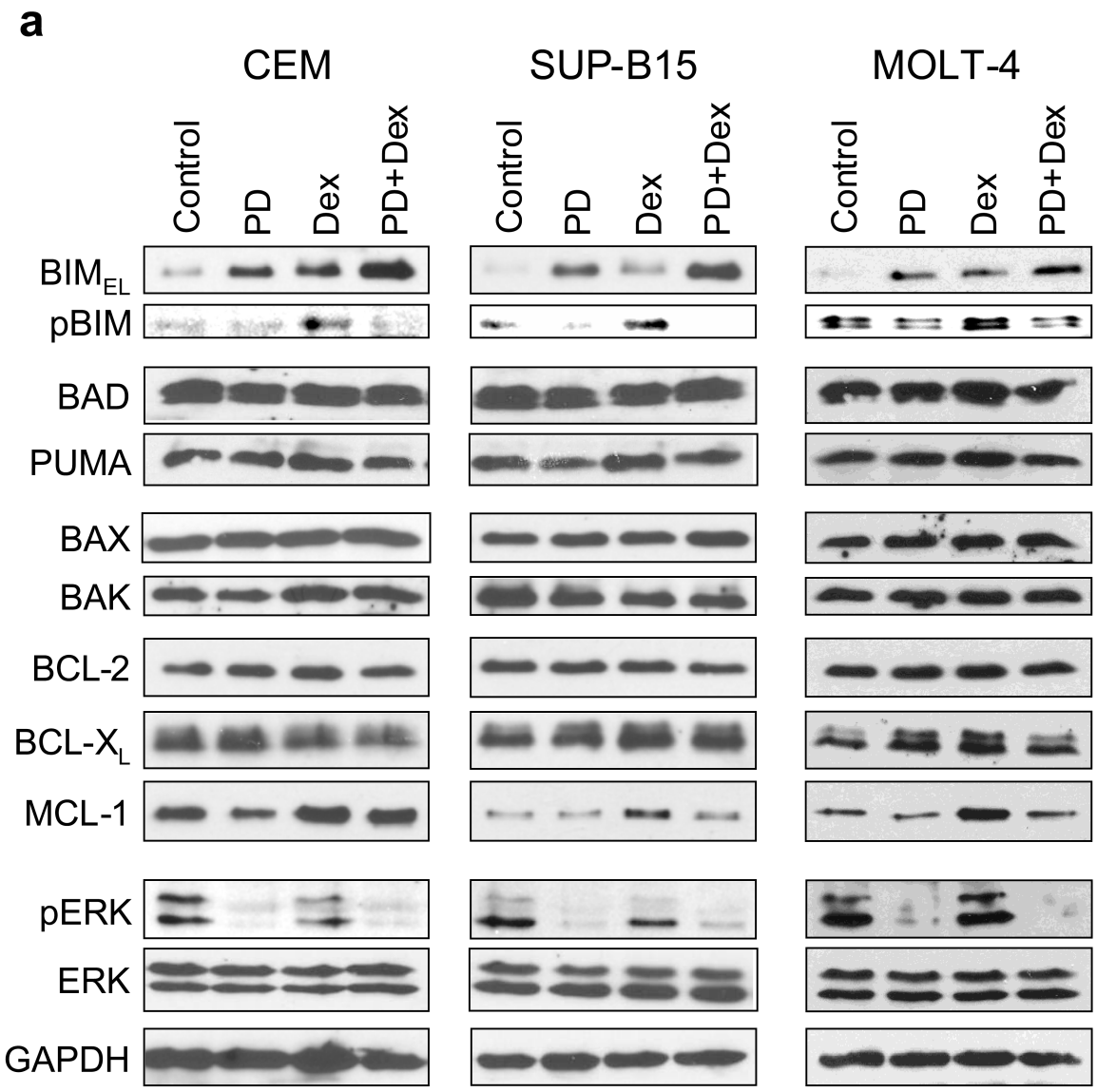
(b) CEM cells were exposed for 48 hrs to the designated concentration of dexamethasone (Dex) alone or in conjunction with 5 μM PD184352 (PD). * $P < 0.001$.

(c) CEM cells were exposed for 48 hrs to the designated concentration of PD184352 (PD) alone or in conjunction with 100 nM dexamethasone (Dex). ** $P < 0.001$

(d) CEM cells were exposed to 5 μM PD184352 (PD) and 100 nM dexamethasone (Dex) alone or in combination for the indicated time.

(e) RS4;11 (B-ALL) cells were exposed to 5 μM PD184352 (PD) and 30 nM dexamethasone (Dex) alone or in combination for 48 hrs.

(f) SUP-B15 (B-ALL) cells were exposed to 3 μM PD184352 (PD) and 30 nM dexamethasone (Dex) alone or in combination for 48 hrs.
(g) Molt-4 (T-ALL) cells were exposed to 10 μM PD184352 (PD) and 1 μM dexamethasone (Dex) alone or in combination for 72 hrs.
The percentage of apoptotic cells was determined by Annexin V-propidium iodide (PI) staining followed by FACS analysis. In all the results, values represent the mean ± SD of three independent experiments.



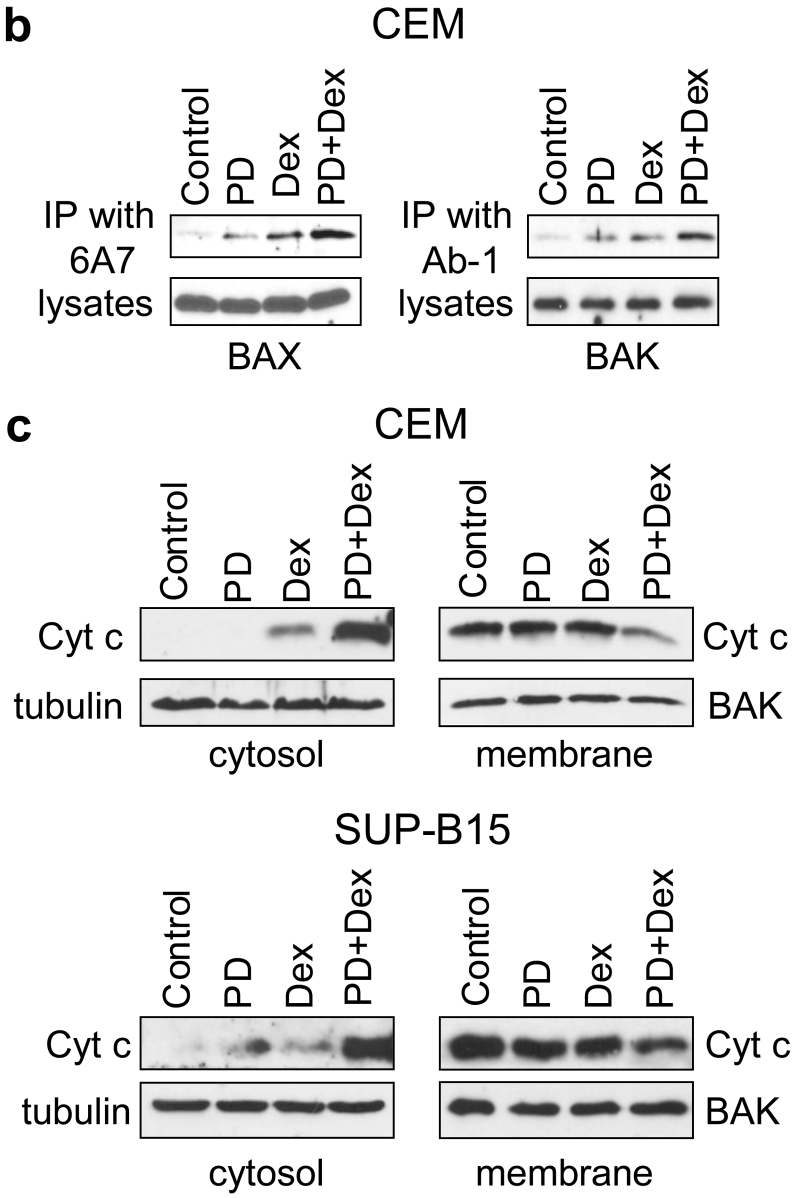
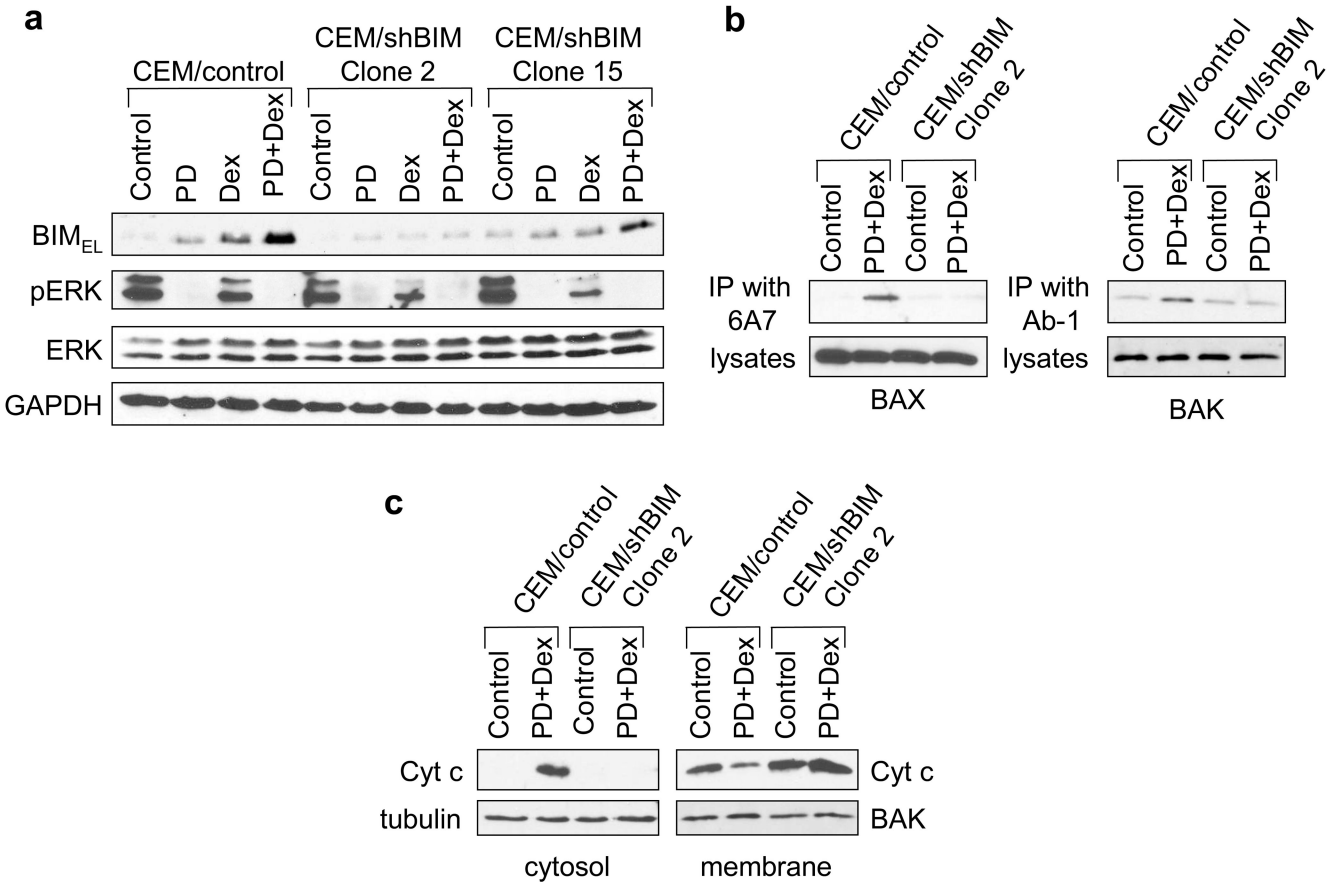


Figure 2. Co-treatment with dexamethasone and PD184352 results in BIM accumulation, pro-apoptotic BAX/BAK conformational change, and cytochrome c release from mitochondria

(a) Cells were treated with dexamethasone (Dex) and/or PD184352 (PD) for 24 hrs (CEM and Molt-4) or 16 hrs (SUP-B15), respectively, with the same doses as Figure 1. Equal amounts of total cell extracts were subjected to Western blotting with the indicated antibodies.

(b) The CEM cell extracts in (a) were subjected to immunoprecipitations with a BAX (6A7) or a BAK (Ab-1) conformational change-specific antibody, respectively. Western blotting was performed on precipitated samples and on lysates collected before immunoprecipitation with an anti-BAX or an anti-BAK antibody.

(c) Cells were treated as (a) and mitochondria-free cytosol and membrane fractions were prepared. Cytochrome c release from mitochondria was monitored by Western blotting with an anti-cytochrome c antibody.



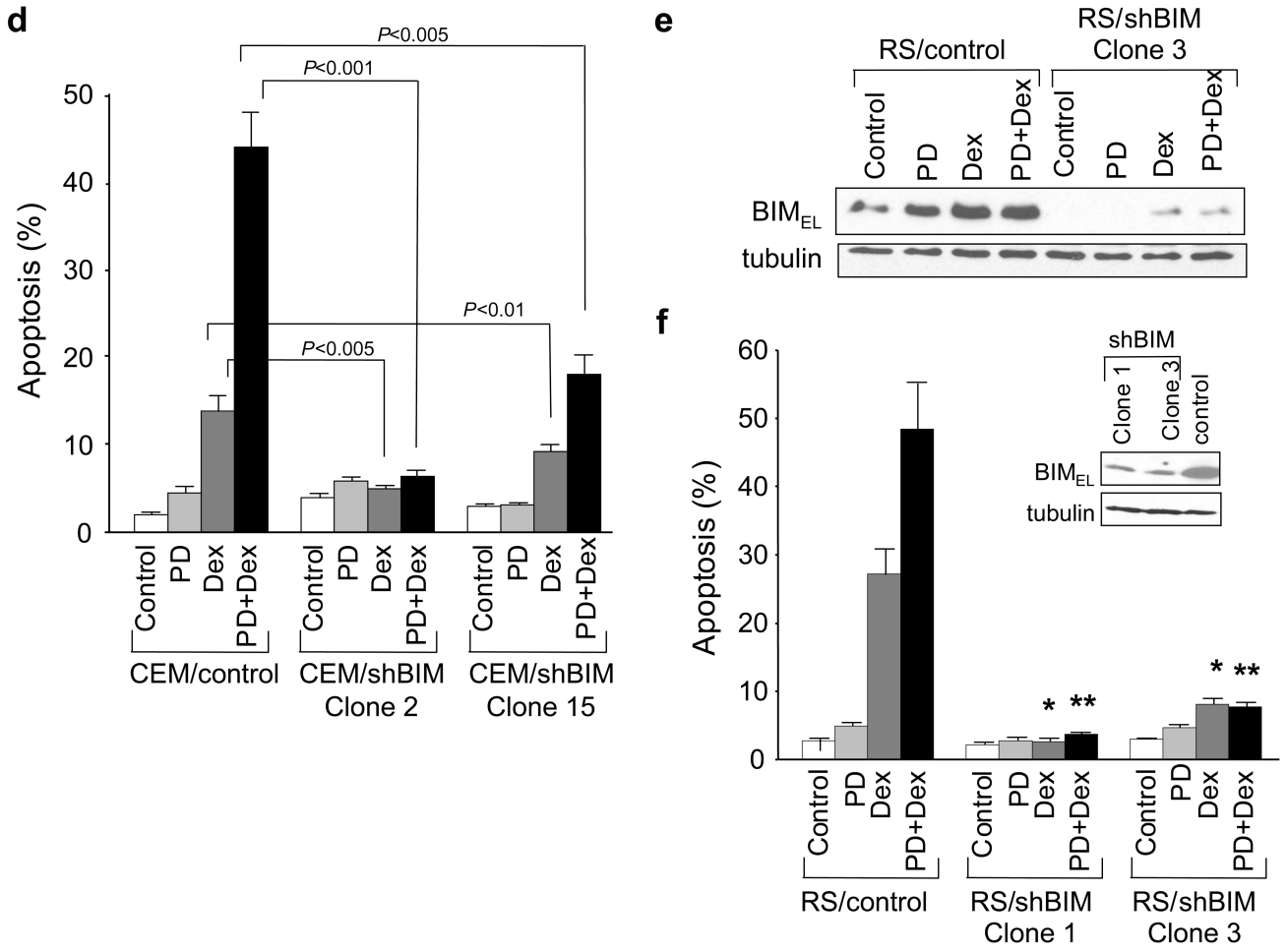


Figure 3. BIM is required for apoptosis in CEM and RS4;11 cells treated with dexamethasone and PD184352

(a) CEM cells were transfected with either pSR-BIM (shBIM) or pSR-con (control). Puromycin-resistant clones were established from each transfection. Cells were treated with dexamethasone (Dex, 100 nM) and/or PD184352 (PD, 5 μM) for 24 hrs. Equal amounts of total cell extracts were subjected to Western blotting with the indicated antibodies.

(b) The cells were treated as (a) and BAX or BAK conformational changes were determined as Figure 2b.

(c) The cells were treated as (a) and cytochrome c release from mitochondria was monitored as Figure 2c.

(d) The cells were treated as (a) for 48 hrs and cell viabilities were determined by Annexin V-PI staining followed by FACS analysis. Values represent the mean ± SD of three separates experiments.

(e) RS4;11 cells were transfected with either pSR-BIM (shBIM) or pSR-con (control) and puromycin-resistant clones were established from each transfection. Cells were treated with dexamethasone (Dex, 30 nM) and/or PD184352 (PD, 5 μM) for 24 hrs and equal amounts of total cell extracts were subjected to Western blotting with the indicated antibodies.

(f) Down-regulation of BIM expression in the shBIM clones were monitored by Western blotting (inset). The cells were treated as (e) for 48 hrs and cell viabilities were determined by Annexin V-PI staining followed by FACS analysis. Values represent the mean ± SD of three separate experiments. * or **, $P<0.001$ when comparing Dex-treated or Dex +PD184352-treated shBIM cells versus control cells, respectively.

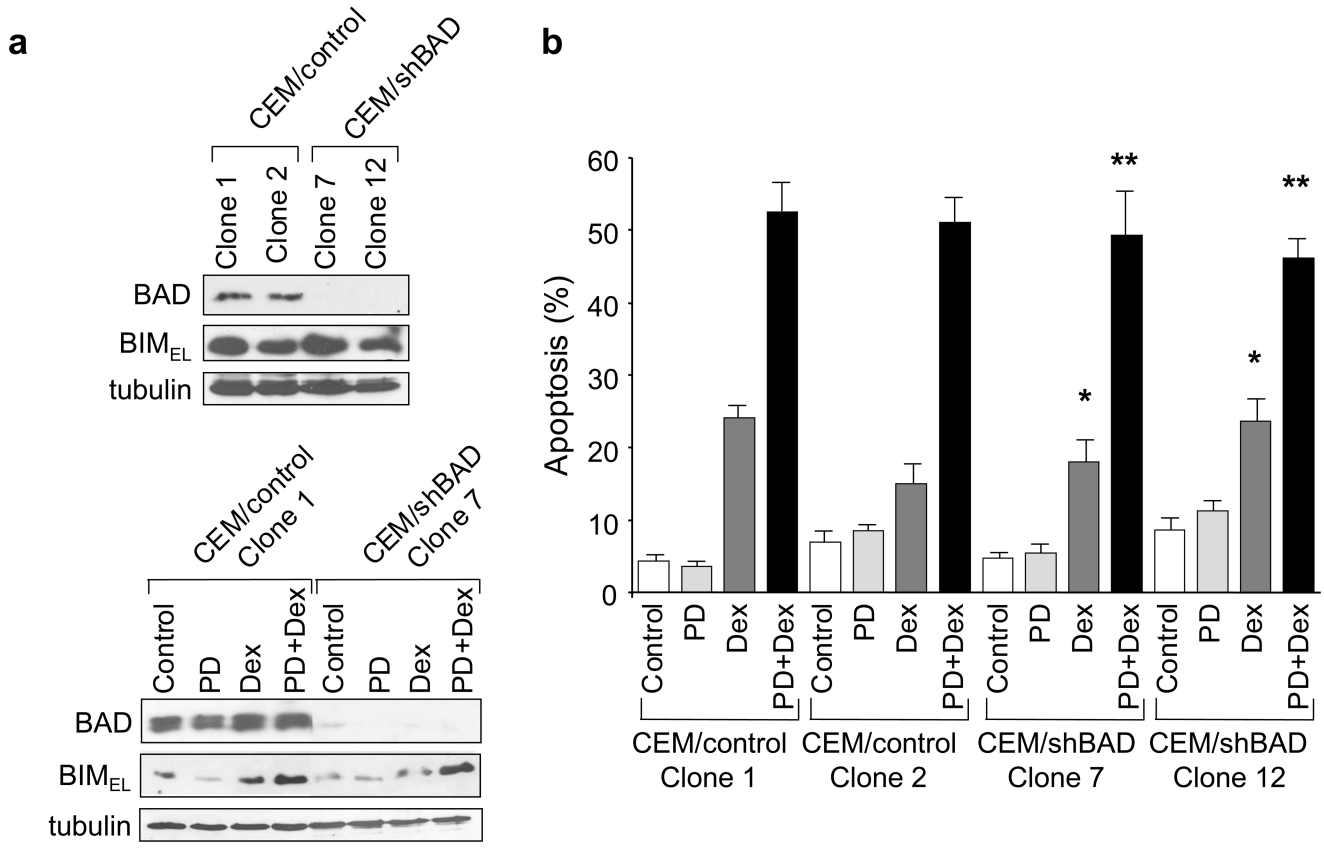


Figure 4. BAD is dispensable for cell death induced by dexamethasone and PD184352 co-treatment

(a) CEM cells were transfected with either shBAD or a scrambled, non-specific shRNA control. Puromycin-resistant clones were established from each transfection. Cells were untreated (Upper panel) or treated with dexamethasone (Dex, 100 nM) and/or PD184352 (PD, 5 μM) for 24 hrs (Lower panel). Equal amounts of total cell extracts were subjected to Western blotting with the indicated antibodies.

(g) The cells were treated as (a) for 48 hrs and cell viabilities were determined by Annexin V-PI staining followed by FACS analysis. Values represent the mean ± SD of three separate experiments. * or **, $P>0.05$ when comparing Dex-treated or Dex+PD184352-treated shBAD cells versus control cells, respectively.

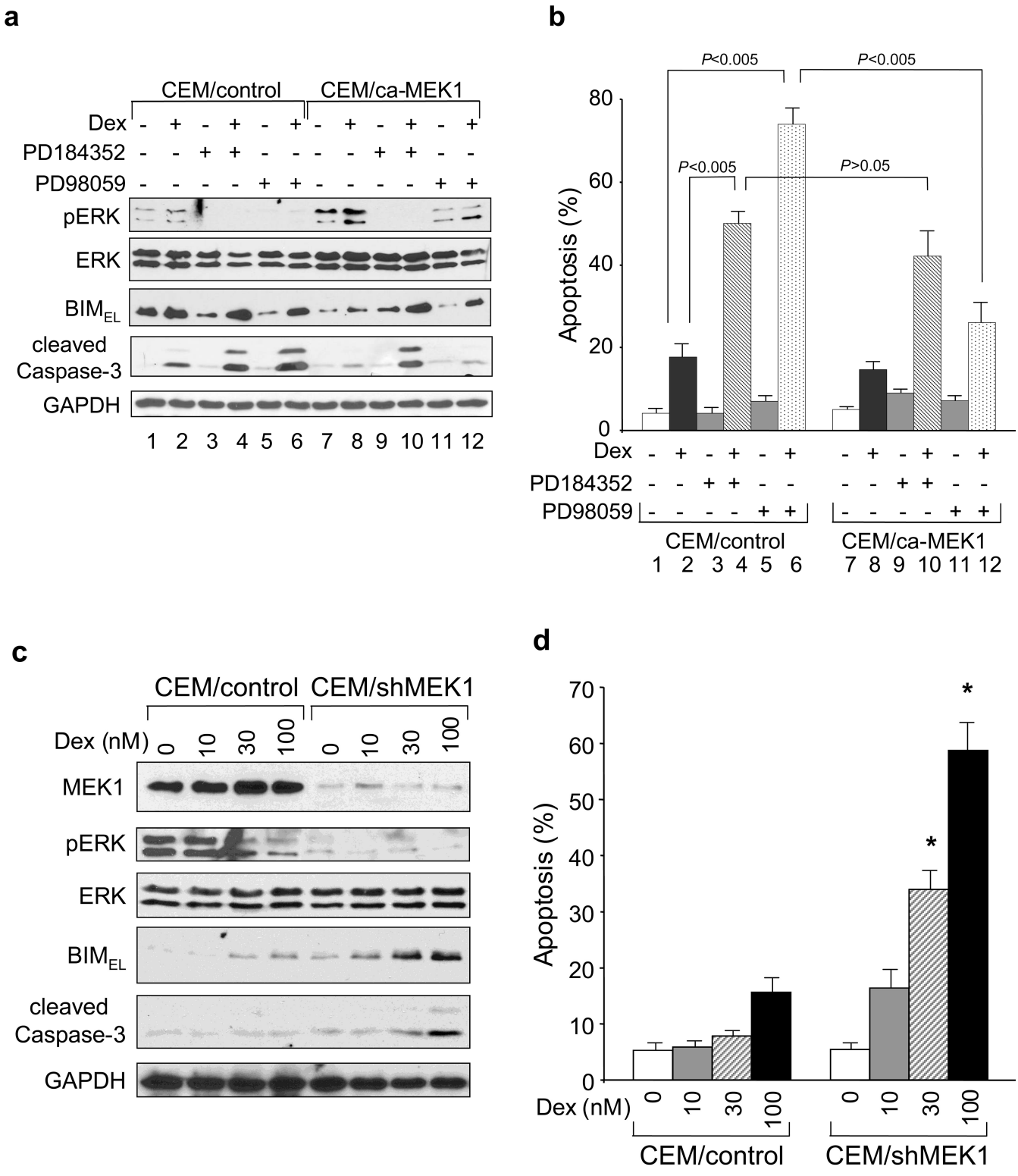


Figure 5. Modulation of MEK expression affects dexamethasone-induced BIM expression and apoptosis

(a) CEM cells were stably transfected with a HA-tagged constitutively-active MEK1 (ca-MEK1) construct or its empty vector (control). CEM/control and CEM/ca-MEK1 cells were exposed to dexamethasone (Dex, 100 nM) plus or minus 50 μM PD98059 or 5 μM PD184352 for 24 hours, respectively, after which cells were lysed and subjected to Western blot analysis with the indicated antibodies.

(b) The cells were treated with Dex plus minus PD184352 or PD98059 for 48 hrs and cell viabilities were determined by Annexin V-PI staining followed by FACS analysis. Values represent the mean ± SD of three separate experiments. Another set of each clone yielded equivalent results.

(c) CEM cells were stably transfected with constructs encoding MEK1 shRNA or a scrambled sequence as a control. The cells were treated with the indicated doses of Dex for 24 hrs, lysed, and subjected to Western blot analysis with the indicated antibodies.

(d) The cells were treated with the indicated doses of Dex for 48 hrs and cell viabilities were determined by Annexin V-PI staining followed by FACS analysis. Values represent the mean ± SD of three separate experiments.*, $P<0.001$ when comparing Dex-treated shMEK1 cells versus control cells. Another set of each clone yielded equivalent results.

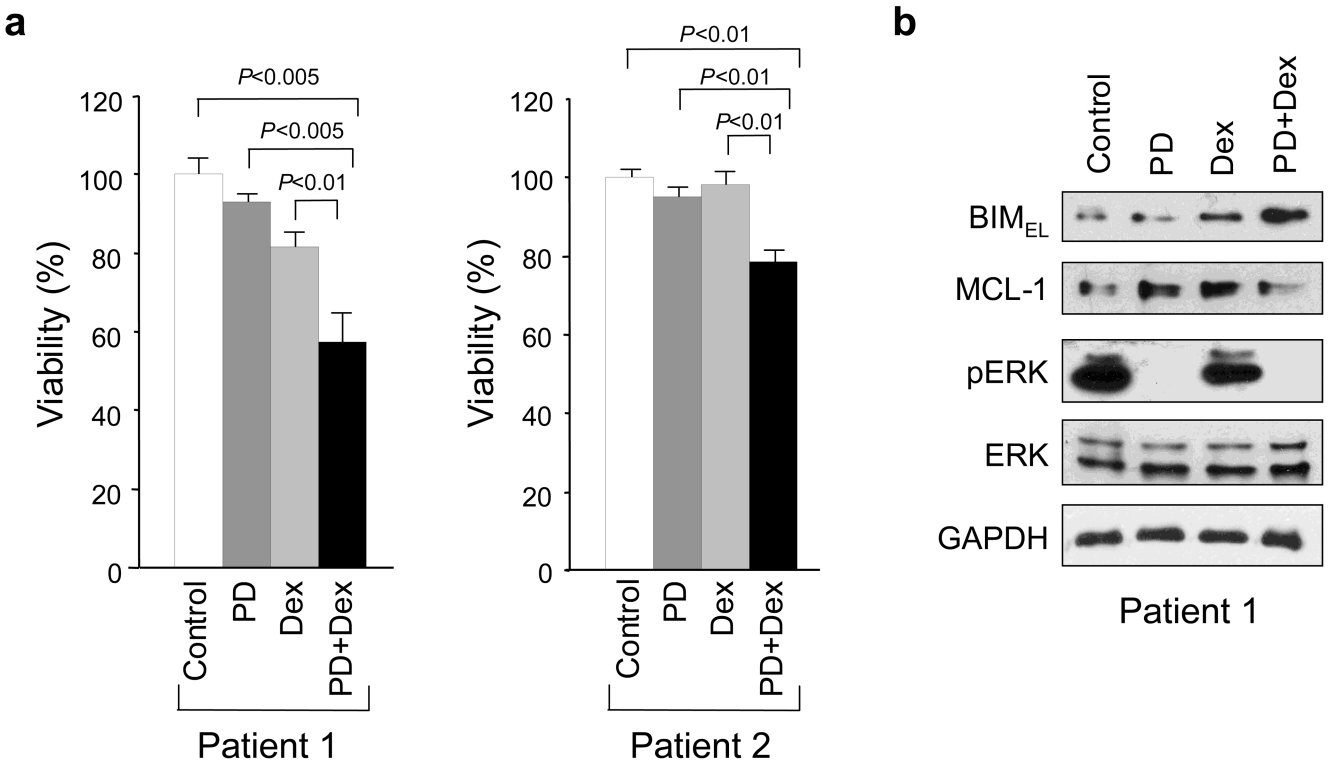


Figure 6. Blasts from ALL Patients Are Sensitive to Dex/PD184352-mediated Apoptosis

(a) Blasts were isolated from the peripheral blood of 2 patients with ALL, after which they were exposed *ex vivo* to dexamethasone (Dex, 10 nM) and/or PD184352 (PD, 2 μM) for 48 hrs. Apoptotic cells were identified by Annexin V-PI staining followed by FACS analysis. Cell viability was calculated by the percentage of annexin V-negative and PI-negative population, and the values with mock-treatment were considered as 100%. Values represent the means ±SD of triplicates.

(b) The cells from patient 1 were treated as (a) for 24 hrs and equal amounts of total cell extracts were subjected to Western blotting with the indicated antibodies.

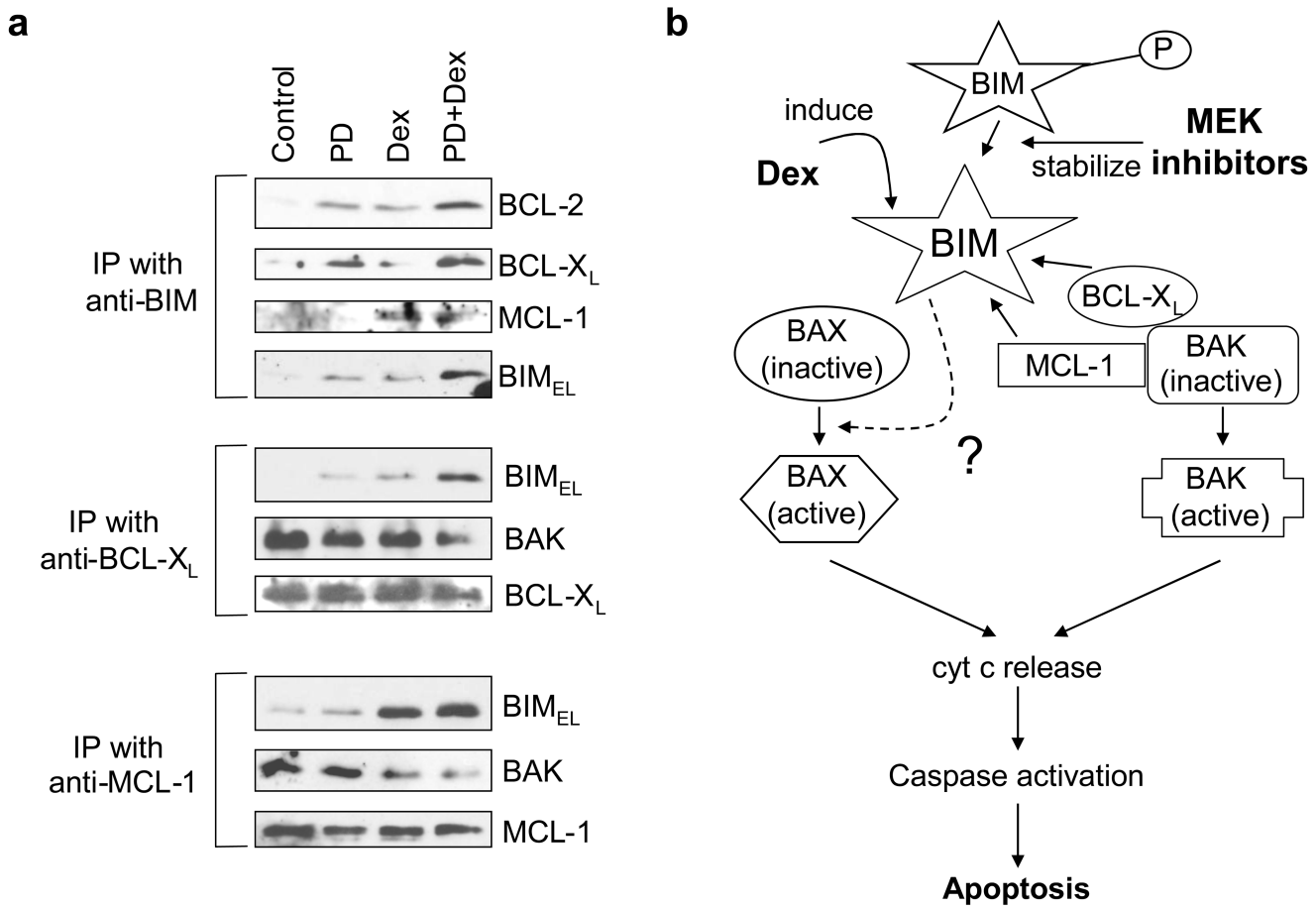


Figure 7. Accumulated BIM interacts with BCL-X_L and MCL-1 to activate BAK in apoptosis induced by co-treatment with dexamethasone and MEK inhibitors

(a) Immunoprecipitations with the extracts from Figure 2a were carried out with an anti-BIM, an anti-BCL-X_L, or an anti-MCL-1 antibody. Western blotting was performed on precipitated samples with the indicated antibodies.

(b) Apoptosis pathway induced by co-treatment with dexamethasone and MEK inhibitors. See text for details.