

Flavopiridol and Histone Deacetylase Inhibitors Promote Mitochondrial Injury and Cell Death in Human Leukemia Cells That Overexpress Bcl-2

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ABSTRACT

Interactions between the cyclin-dependent kinase (CDK) inhibitor flavopiridol and histone deacetylase (HDAC) inhibitors (suberoylanilide hydroxamide and sodium butyrate) were examined in human leukemia cells (U937 and HL-60) ectopically expressing Bcl-2/Bcl-x_L and in primary AML cells. Coadministration of flavopiridol with HDAC inhibitors synergistically potentiated mitochondrial damage (cytochrome c, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI, and apoptosis-inducing factor release), caspase activation, poly(ADP-ribose) polymerase degradation, and cell death in both wild type and Bcl-2- or Bcl-x_L-overexpressing cells and induced a pronounced loss of clonogenicity. In contrast, Bcl-2 and Bcl-x_L largely blocked these events in cells exposed to the cytotoxic agent 1- β -D-arabinofuranosylcytosine (ara-C). Enforced expression of dominant-negative Fas-associated death domain failed to protect cells from the flavopiridol/histone deacetylase inhibitor (HDACI) regimen, arguing against the in-

volvement of the receptor pathway in lethality. Ectopic expression of a phosphorylation loop-deleted Bcl-2 or Bcl-2 lacking the serine₇₀ phosphorylation site, which dramatically protected cells from ara-C lethality, delayed but did not prevent flavopiridol/HDAC inhibitor-induced mitochondrial injury, cell death, or loss of clonogenicity. Ectopic expression of Bcl-2 or Bcl-x_L was also unable to prevent the flavopiridol/HDACI regimen from inducing a conformational change in and mitochondrial translocation of Bax, and it did not attenuate Bax dimerization. As a whole, these findings indicate that in contrast to certain conventional cytotoxic agents such as ara-C, overexpression of Bcl-2 or Bcl-x_L are largely ineffective in preventing perturbations in Bax, mitochondrial injury, and cell death in human leukemia cells subjected to simultaneous CDK and HDAC inhibition. They also raise the possibility that a strategy combining CDK and HDAC inhibitors may be effective against drug-resistant leukemia cells overexpressing Bcl-2 or Bcl-x_L.

Histone acetylation represents an important epigenetic mechanism by which gene expression is regulated (Cress and Seto, 2000). The acetylation status of chromatin is reciprocally regulated by two classes of enzymes: histone acetylases and histone deacetylases (HDACs) (Kurdistani and Grunstein, 2003). The latter consist of three broad families: class I HDACs, analogous to the yeast RPD3 HDAC; class II HDACs, analogous to yeast HDAC1; and the sirtuins (e.g., SIRT1), which have recently been implicated in responses to

DNA damage and caloric restriction (Bjerling et al., 2002). In leukemic cells, HDACs represent a component of corepressor complexes thought to be involved in disruption of the differentiation program (Redner and Liu, 2005). This has led to the clinical development of diverse HDAC inhibitors (HDACIs), and preliminary evidence of activity in patients with leukemia has been observed (Drummond et al., 2005). The mechanism by which HDACIs induce cell death in neoplastic cells is not known with certainty, but it has been attributed to multiple actions, including induction of mitochondrial injury (Yu et al., 2003), generation of oxidative damage (Ruefli et al., 2001), up-regulation of death receptors (Insinga et al., 2005), disruption of heat shock protein 90 function (George et al., 2005), and down-regulation of antiapoptotic proteins (Vrana et al., 1999), among others.

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ABBREVIATIONS: HDAC, histone deacetylase inhibitor; HDACI, histone deacetylase inhibitor; CDK, cyclin-dependent kinase; NF- κ B, nuclear factor- κ B; AML, acute myelogenous leukemia; SAHA, suberoylanilide hydroxamide; PI, propidium iodide; DSP, dithiobis(succinimidyl propionate); PBS, phosphate-buffered saline; DTT, dithiothreitol; PARP, poly(ADP-ribose) polymerase; Smac, second mitochondria-derived activator of caspases; AIF, apoptosis-inducing factor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; ara-C, 1- β -D-arabinofuranosylcytosine; DIABLO, direct IAP binding protein with low pI; FADD, Fas-associated death domain.

The semisynthetic flavone flavopiridol (NSC 649890) was the first cyclin-dependent kinase (CDK) inhibitor to undergo trials in humans (Senderowicz, 1999). It acts broadly to inhibit most CDKs and induces cell death in human leukemia cells when administered in the nanomolar concentration range (Carlson et al., 1996). The mechanism by which flavopiridol induces cell death is also not known with certainty, but it may stem from various actions other than or in addition to CDK inhibition, including disruption of the CDK9/cyclin T transcription complex and inhibition of phosphorylation of the carboxyl-terminal domain of RNA PolII (Chao and Price, 2001), induction of mitochondrial injury (Almenara et al., 2002), down-regulation of antiapoptotic proteins such as Mcl-1 (Gojo et al., 2002), and inhibition of NF- κ B (Takada and Aggarwal, 2004). In a series of recent studies, we and other groups have observed that flavopiridol lowers the threshold for HDACI-mediated cell death through multiple mechanisms, including disruption of p21^{CIP1} induction (Almenara et al., 2002), down-regulation of antiapoptotic proteins such as XIAP (Rosato et al., 2004), and attenuation of NF- κ B activation. Such findings raise the possibility that a therapeutic strategy combining CDK and HDAC inhibition may have relevance for the treatment of hematologic and potentially nonhematologic malignancies.

Bcl-2 and Bcl-x_L are multidomain members of the Bcl-2 family, which block activation of the intrinsic, mitochondrial apoptotic pathway by preventing release of proapoptotic mitochondrial proteins such as cytochrome *c* into the cytosol (Yang et al., 1997). Cytosolic cytochrome *c* activates a multiprotein complex referred to as the apoptosome, which leads in turn to cleavage/activation of procaspase-9 and downstream effector caspases (e.g., caspase-3), culminating in cell death. Increased expression of Bcl-2 or Bcl-x_L has been associated with poor response to chemotherapy in various malignancies, including leukemia (Campos et al., 1993). In some preclinical systems, Bcl-2 overexpression has been shown to delay but not to prevent cell death, or to restore the clonogenic potential of malignant progenitor cells (Yin and Schimke, 1995). Furthermore, both HDACIs and flavopiridol have been reported to induce cell death in certain cell types through Bcl-2-independent mechanisms (Achenbach et al., 2000; Duan et al., 2005). It therefore seemed logical to postulate that a regimen containing both flavopiridol and HDACIs might be effective in killing cells overexpressing these antiapoptotic proteins. A systematic analysis of the effects of flavopiridol and HDACIs on leukemic cells overexpressing Bcl-2 or Bcl-x_L has not yet been carried out. To address this question, we have examined the response of human leukemia cells ectopically expressing these proteins to the flavopiridol/HDACI regimen, emphasizing effects on mitochondrial injury, caspase activation, cell death, and loss of clonogenic survival. Parallel studies have also been performed using leukemic cells ectopically expression Bcl-2 lacking either the phosphorylation loop region or a critical phosphorylation site (Ser₇₀), both of which enhance resistance to certain cytotoxic agents (Wang et al., 1999a). Our results indicate that in contrast to conventional cytotoxic agents, the flavopiridol/HDACI regimen is highly effective in inducing mitochondrial injury as well as cell death and in reducing clonogenic survival in leukemia cells ectopically expressing Bcl-x_L and wild-type or mutant Bcl-2. These findings raise the possibility that combined CDK/HDAC inhibition overcomes resis-

tance to mitochondrial injury conferred by Bcl-2 and Bcl-x_L and may therefore represent an effective strategy against leukemia cells overexpressing certain proteins conferring resistance to standard agents.

Materials and Methods

Cell Lines. U937 cells were obtained from American Type Culture Collection (Manassas, VA), cultured in RPMI 1640 medium, and transfected with either full-length Bcl-2 or Bcl-2 lacking residues 32 to 80 as described previously in detail (Wang et al., 1999a). For all studies, two clones (U937-Bcl-2 G3 and D9; U937-pCEP4 B9 and B11) were used. Bcl-x_L overexpressing U937 cells and their empty-vector counterparts (pcDNA3.1) were generated as described previously (Wang et al., 1999b). U937 cells expressing Bcl-2 lacking the phosphorylation loop region (Δ_{32-80}) or Bcl-2 containing a serine₇₀ site mutation (e.g., serine to alanine; S70A) with a empty-vector pSFFV control were constructed as described previously (Wang et al., 1999a). The point mutant and loop deletion were verified by direct DNA sequencing using a D-rhodamine terminator (PerkinElmer Life and Analytical Sciences, Boston, MA). The human promyelocytic leukemia cell line HL-60 was derived from a patient with acute promyelocytic leukemia as described previously (Wang et al., 1997). Bcl-2-overexpressing HL-60 cell variant was generated using a commercially available vector (pCEP4; Invitrogen, Carlsbad, CA) and human Bcl-2 cDNA (provided by M. Cleary, Stanford University, Stanford, CA) was generated as described previously (Wang et al., 1997). All transfectants were maintained under appropriate selection pressure [400 μ g/ml G418 (Geneticin)] while in culture.

Collection and Processing of Primary Cells. Patient-derived leukemic blasts were obtained with informed consent from the peripheral blood of patients with AML. The percentage of blasts was >70% for all samples. CD34⁺ cells were isolated from the bone marrow of patients undergoing routine bone marrow aspirations for nonmyeloid hematologic disorders and isolated using an immunomagnetic bead separation technique as described previously (Yu et al., 2003). These studies have been approved by the investigational review board of Medical College of Virginia/Virginia Commonwealth University (Richmond, VA). Bone marrow samples were collected in sterile syringe containing heparin and processed and treated as described previously (Yu et al., 2003).

Drugs and Chemicals. Flavopiridol was kindly provided by Dr. Dimitrios Colevas (Cancer Therapy and Evaluation Program, National Cancer Institute, Bethesda, MD). SAHA was purchased from BioVision (Mountain View, CA). Sodium butyrate and human recombinant tumor necrosis factor and cycloheximide were supplied by Calbiochem (San Diego, CA). Annexin V/PI was supplied by BD Biosciences PharMingen (San Diego, CA) and was formulated as per the manufacturer's instructions. DSP cross-linker was obtained from Pierce Chemical (Rockford, IL). All drugs were dissolved in dimethyl sulfoxide and diluted in PBS before use. Dynabeads (M-450) goat anti-mouse IgG was obtained from Dynal Biotech (Lake Success, NY).

Experimental Format. Logarithmically growing cells were placed in sterile plastic T-flasks (Corning Glassworks, Corning, NY) to which the designated drugs were added, and the flasks were replaced in the incubator for various intervals. At the end of the incubation period, cells were transferred to sterile centrifuge tubes, pelleted by centrifugation at 600g for 10 min at room temperature, and prepared for analysis as described below.

Assessment of Cell Death. After drug exposures, cells were stained with Annexin V/PI as described previously (Wang et al., 1999a; Yu et al., 2003). In short, cells were washed with PBS and stained with Annexin V/PI for 30 min at room temperature. Cells were then acquired and analyzed using a FACScan cytofluorometer (BD Biosciences, Mansfield, MA) with the help of CellQuest software (BD Biosciences). In some cases, cytocentrifuge slides were prepared

with Wright-Giemsa stain, and 10 random fields were viewed by light microscopy to evaluate the extent of cell death (e.g., cell shrinkage, nuclear condensation, and formation of apoptotic bodies) as described previously (Yu et al., 2003). For these studies, the percentage of dead cells was determined by evaluating ≥ 500 cells/condition in triplicate. Results with each of these methods were found to be highly concordant.

Assessment of Clonogenic Potential. Cells were washed three times in drug-free medium, counted, and plated in triplicate at 500 cells/well in 1 ml of RPMI 1640 medium supplemented with 20% fetal bovine serum, 0.3% Bacto agar (Difco, Detroit, MI) as described in detail previously (Almenara et al., 2002). Plates were incubated at 37°C, and colonies consisting of ≥ 50 cells were scored at the 10th day after plating.

Preparation of S-100 (Cytosolic) Fractions. Cells were harvested after drug treatment by centrifugation at 600g for 10 min at 4°C and washed in PBS. Cells (4×10^6) were lysed by incubating in 100 μ l of lysis buffer for 1 min containing 75 mM NaCl, 8 mM Na_2HPO_4 , 1 mM NaH_2PO_4 , 1 mM EDTA, 250 mM sucrose, and 350 μ g/ml digitonin. The lysates were centrifuged at 12,000g for 5 min at 4°C, and the supernatant was collected and quantified using Coomassie protein assay reagent (Pierce). The protein samples were denatured with 4 \times loading buffer (Invitrogen) supplemented with 50 mM dithiothreitol (DTT). Denatured protein samples were separated by 4 to 12% gradient Bis-Tris precast gel from NuPAGE (Invitrogen).

Isolation of Mitochondrial Fraction. Mitochondrial fraction of the cells was isolated using a mitochondria isolation kit obtained from Pierce as per manufacturer's instruction. Cells (2×10^7) were pelleted by centrifugation at 850g for 2 min and were resuspended in 800 μ l of reagent A in a microcentrifuge tube. Then, cells were incubated in ice for 2 min and subsequently homogenized in a pre-cooled Dounce tissue grinder applying 40 to 50 strokes. Reagent C (800 μ l) was added to the homogenized solution and thoroughly mixed by repeated inversion. The entire mixed solution was centrifuged at 700g for 10 min, and the pellet was discarded. The supernatant was further centrifuged at 12,000g for 15 min, and the pellet was considered as intact mitochondria. This fraction was further lysed in lysis buffer (Wang et al. 1999a,b) and subjected to Western blot analysis.

Western Blot Analysis. Immunoblotting was performed as described previously (Wang et al., 1999a). In brief, after drug treatment, cells were pelleted by centrifugation and lysed immediately in lysis buffer. Protein content of the homogenates was quantified using a Coomassie protein assay reagent (Pierce) and denatured with 4 \times loading buffer (Invitrogen) supplemented with 50 mM DTT. Equal amounts of protein were separated by 4 to 12% gradient Bis-Tris gel and probed with appropriate antibodies. Sources of antibodies were as follows: Bcl-2 was from DakoCytomation California Inc. (Carpinteria, CA); Bid was from R&D Systems (Minneapolis, MN); cytochrome c, caspases-3 and -9 were from BD Biosciences Pharmingen; PARP was from BIOMOL Research Laboratories (Plymouth Meeting, PA); cleaved caspase-3 was from Cell Signaling Technology Inc. (Beverly, MA); Bax, Smac, AIF, XIAP, Bad, phospho-Bad, cytochrome c, tubulin, and cytochrome c oxidase IV were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); caspase-8 was from Alexis Corp. (San Diego, CA); and Bax antibody directed against conformationally changed protein (6A7) was obtained from Sigma-Aldrich (St. Louis, MO). Blots were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.), washed, and developed by enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences).

Detection of Bax Conformational Change. Mitochondrial-rich fraction of cells with or without adequate treatment was prepared as described above. Then, mitochondrial-rich fraction was washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and lysed with the CHAPS lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% CHAPS, and 1 mM DTT) supplemented with protease and phosphatase inhibitors. Two milligrams of anti-Bax (6A7) monoclonal antibody was preincubated with

30 μ l of Dynabeads for 2 h at 4°C on a rotor. In total, 500 to 1000 μ g of protein was then added to the anti-Bax antibody loaded with Dynabeads and maintained on a rotor for overnight at 4°C. Dynabeads were collected using a Dynal Biotech magnetic particle concentrator and washed four times with CHAPS lysis buffer. Conformationally changed Bax protein was eluted from the Dynabeads by heating with 4 \times protein gel loading buffer (Invitrogen) and subjected to Western blotting (Yamaguchi and Wang, 2002; Liu et al., 2003).

Detection of Bax Oligomerization. Cells were treated with various agents for appropriate interval and then pelleted by centrifugation at 600g. Mitochondria-rich fragment was prepared as described above, and it was incubated for 0.5 to 1 h at room temperature with 2 mM DSP in dimethyl sulfoxide for the cross-linking reaction to take place. Cross-linking reaction was terminated by adding Tris-HCl to a final concentration of 20 mM and by incubating for 15 min at room temperature. The mitochondrial pellet was separated by centrifugation at 10,000g for 30 min and redissolved in CHAPS lysis buffer. Last, protein content of the samples were quantified and subjected to Western blot analysis (Yamaguchi and Wang, 2002; Liu et al., 2003).

Statistical Analysis. The significance of differences between experimental conditions was determined using the two-tailed Student's *t* test.

Results

Wild-type U937 cells or U937 cells ectopically expressing either an empty-vector control (pCEP4) or full-length Bcl-2 were exposed to marginally toxic concentrations of flavopiridol (100 nM), sodium butyrate (1 mM), or SAHA (1 μ M) alone or in combination for 24 h (Fig. 1A). As described previously (Almenara et al., 2002), coadministration of flavopiridol with either sodium butyrate or SAHA resulted in a substantial increase in cell death in untransfected cells. However, essentially identical results were observed in pCEP4 and Bcl-2 cells. The Western blot in Fig. 1A (inset) shows Bcl-2 expression in empty-vector control (pCEP4) versus full-length Bcl-2 transfectants and demonstrates that the latter express approximately 3-fold more Bcl-2 than their empty-vector counterparts. Results were equivalent in cells expressing Bcl- x_L versus control pcDNA3.1 (Fig. 1B). In contrast, ectopic expression of Bcl-2 (Fig. 1C) or Bcl- x_L (data not shown) significantly protected cells from cell death induced by the cytotoxic agent ara-C. Consistent with these findings, ectopic expression of Bcl-2 failed to prevent flavopiridol/sodium butyrate or flavopiridol/SAHA-induced caspase-3 activation/cleavage, the formation of a Bcl-2 cleavage product, or PARP degradation (Fig. 1, D and E). In contrast, cleavage of caspase-3 and Bcl-2 as well as PARP degradation was essentially abrogated in U937/Bcl-2 cells exposed to ara-C (Fig. 1F). Similar results were obtained with U937/Bcl- x_L cells (data not shown). These findings indicate that ectopic expression of Bcl-2 or Bcl- x_L is relatively ineffective in protecting leukemia cells from combined exposure to flavopiridol and HDAC inhibitors.

To establish whether this phenomenon was restricted to U937 cells, parallel studies were carried out in another human leukemia cell line (HL-60). HL-60 cells stably transfected with an empty pCEP4 vector (HL-60-pCEP4) or full-length Bcl-2 (e.g., HL-60-Bcl-2) were treated with flavopiridol (125 nM)/SAHA (1.5 μ M) either alone or on combination for 24 h. It is noteworthy that combined treatment with flavopiridol and SAHA induced cell death to a similar extent (e.g., 60–65%) in both HL-60-pCEP4 and HL-60-Bcl-2 cells,

whereas individual treatment exerted either very modest (flavopiridol) or no effects (SAHA) (Fig. 2A). Consistent with findings in U937 cells, ectopic expression of Bcl-2 failed to prevent flavopiridol/SAHA-induced caspase-3 cleavage, the formation of a Bcl-2 cleavage product, or PARP degradation in HL-60 cells (Fig. 2, B and C).

To determine whether similar events might occur in primary human leukemia cells, blasts robustly expressing Bcl-2 were obtained from the peripheral blood of a patient with AML and exposed for 24 h to flavopiridol (100 nM) and SAHA (1 μ M) alone and in combination as in the case of U937 cells. As shown in Fig. 3A, exposure of blasts to flavopiridol or SAHA individually resulted in relatively little toxicity, whereas combined exposure was associated with a marked increase in cell death (e.g., 55–65%). Western blot analysis revealed that exposure of blasts to combined (but not individual) treatment with flavopiridol and SAHA for 24 h resulted in a marked increase in PARP cleavage accompanied by caspase activation, cleavage of Bcl-2 (Fig. 3B), analogous

to results observed in U937 and HL-60 cells ectopically expressing Bcl-2.

Studies were then undertaken to determine whether ectopic expression of Bcl-2 could protect cells from flavopiridol/HDACI-mediated mitochondrial injury. Exposure to 100 nM flavopiridol for 12 h modestly induced cytochrome *c*, Smac/DIABLO, and AIF release into the cytosol in U937/pCEP4 cells, and results were essentially identical in U937/Bcl-2 cells (Fig. 4A). Such findings are consistent with previous reports demonstrating that ectopic expression of Bcl-2 was relatively ineffective in preventing flavopiridol-mediated mitochondrial injury (Decker et al., 2002). SAHA by itself (1 μ M) minimally induced cytochrome *c* and AIF release in empty-vector controls, but only the former was opposed by ectopic Bcl-2 expression. However, combined exposure of cells to these agents resulted in a marked increase in mitochondrial injury that was not diminished by ectopic expression of Bcl-2 (Fig. 4A). Similar results were obtained with combinations of flavopiridol and sodium butyrate and in

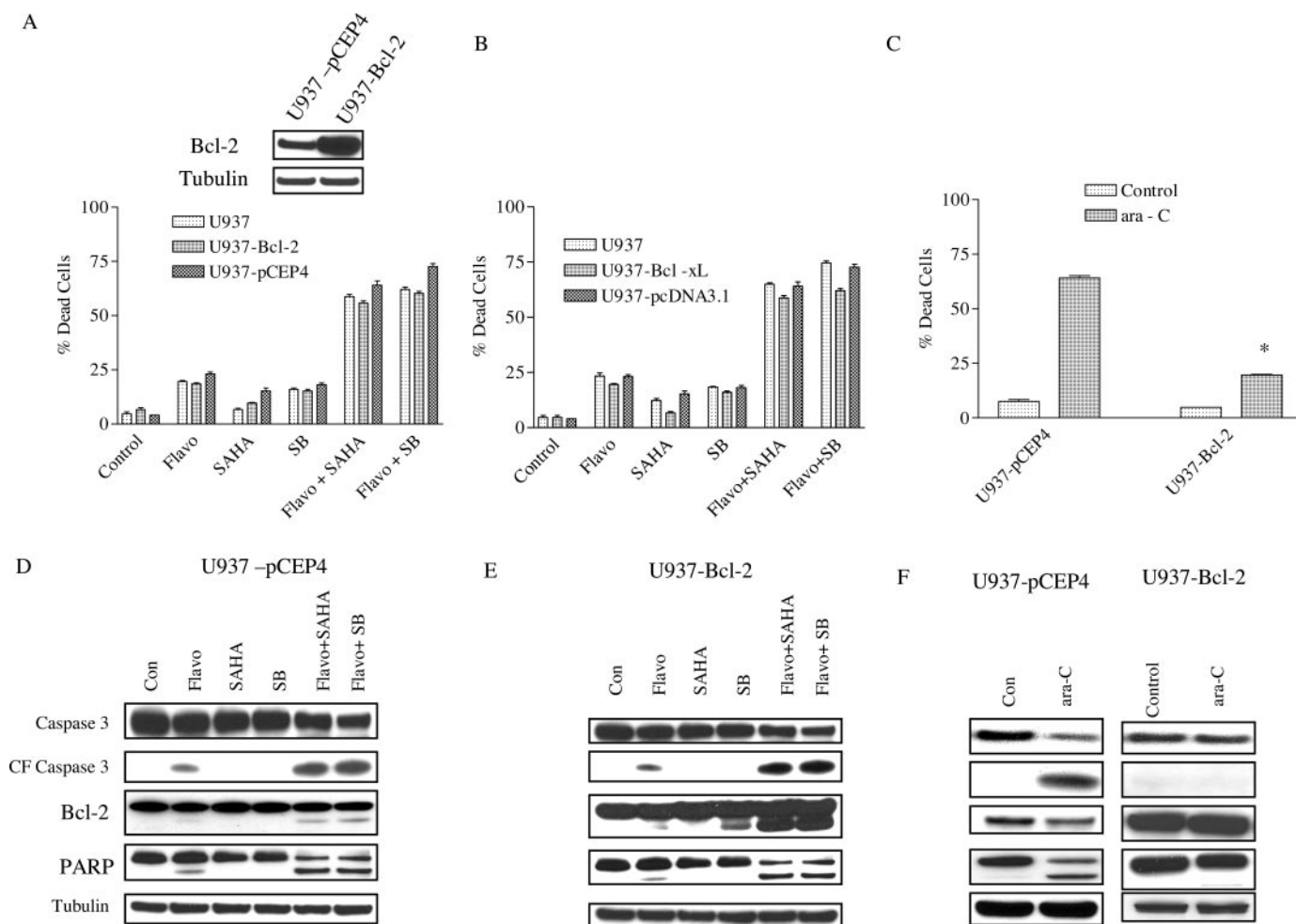


Fig. 1. Ectopic expression of full-length Bcl-2 and Bcl-x_L fails to protect U937 cells from flavopiridol/HDACI-induced lethality. **A**, U937, U937-Bcl-2, and U937-pCEP4 cells; the inset (Western blot) displays expression of Bcl-2 in U937-pCEP4 and U937-Bcl-2 cells with tubulin serving as a loading control. **B**, U937, U937-Bcl-x_L and U937-pcDNA3.1 cells were treated with flavopiridol (100 nM) or SAHA (1 μ M)/sodium butyrate (1 mM) individually or in combination. **C**, U937-Bcl-2 and U937-pCEP4 cells were treated with 1 μ M ara-C. The percentage of dead cells was monitored by Annexin V-fluorescein isothiocyanate staining and flow cytometry and compared with untreated controls after 24 h of drug exposure. Values represent the means \pm S.D. for three separate experiments performed in triplicate. *, significantly less than values for empty-vector controls; $P < 0.001$. U937-pCEP4 cells (**D**) or U937-Bcl-2 cells (**E**) were treated with flavopiridol (100 nM) \pm SAHA (1 μ M)/sodium butyrate (1 mM), and U937-Bcl-2 and U937-pCEP4 cells (**F**) were treated with 1 μ M ara-C for 12 h. At the end of this interval, cells were lysed, denatured, and subjected to Western blot analysis using the indicated primary antibodies as described under *Materials and Methods*. Equal protein (30 μ g) was loaded in each lane. Blots were stripped and reprobed with anti-tubulin antibodies to ensure equal loading and transfer of protein. Two additional studies yielded equivalent results.

Bcl-x_L cells (data not shown). Bcl-2 overexpression also did not block flavopiridol/SAHA-mediated caspase-8 cleavage/activation, and this regimen did not modify total expression of various Bcl-2 family members, including Bad, phospho-Bad, Mcl-1, or XIAP in either empty vector or U937/Bcl-2 cells (Fig. 4B). Last, consistent with the previous results, ectopic expression of Bcl-2 (Fig. 4C) or Bcl-x_L (data not shown) markedly diminished ara-C-induced cytochrome *c*, Smac/DIABLO,

and AIF cytosolic release, and caspase-8 cleavage. Together, these findings indicate that ectopic expression of the anti-apoptotic proteins Bcl-2 or Bcl-x_L are relatively ineffective in preventing the pronounced induction of mitochondrial injury in leukemia cells exposed to flavopiridol and HDAC inhibitors.

It has previously been shown that loss of the Bcl-2 phosphorylation loop increases resistance to various cytotoxic

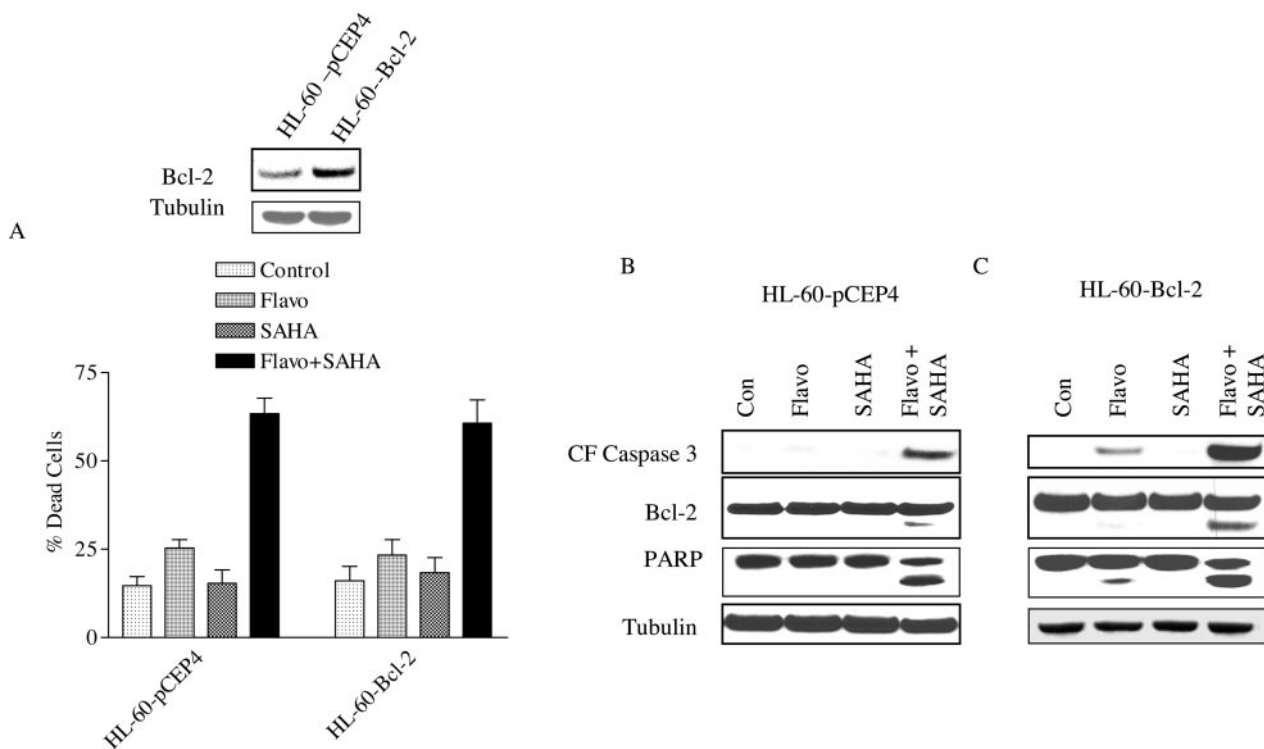


Fig. 2. Combined treatment with flavopiridol and SAHA synergistically induces cell death in HL-60 cells ectopically expressing Bcl-2. A, HL-60-pCEP4 cells and HL-60-Bcl-2 cells were treated with flavopiridol (125 nM) or SAHA (1.5 μ M) individually or in combination. At the end of drug treatment, cell death was measured as described under *Materials and Methods*; the inset (Western blot) displays expression of Bcl-2 in HL-60-pCEP4 and HL-60-Bcl-2 cells with tubulin serving as a loading control. HL-60-pCEP4 cells (B) or HL-60-Bcl-2 cells (C) were treated with flavopiridol (125 nM) \pm SAHA (1.5 μ M) for 12 h. At the end of drug treatment, cells were lysed, denatured, and subjected to Western blot analysis using the indicated primary antibodies as described under *Materials and Methods*. Equal protein (30 μ g) was loaded in each lane. Blots were stripped and reprobed with anti-tubulin antibodies to ensure equal loading and transfer of protein. Two additional studies yielded equivalent results.

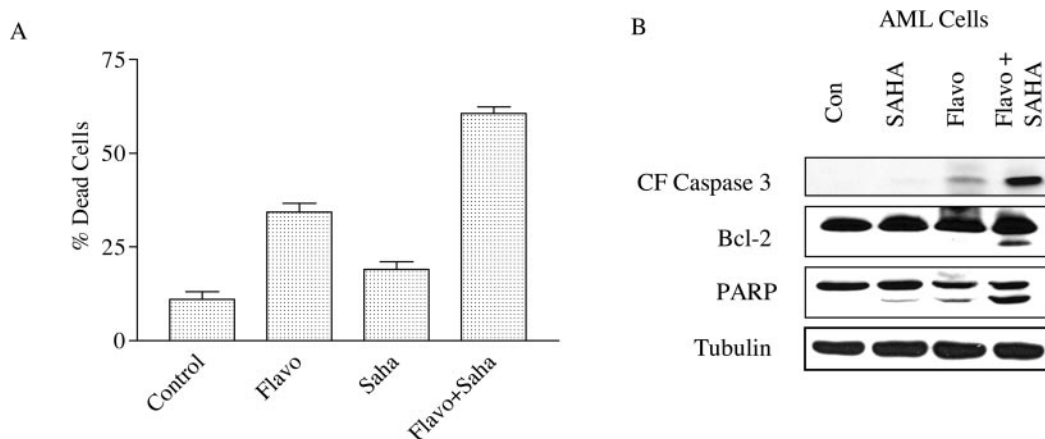


Fig. 3. Combined treatment with flavopiridol and SAHA induce cell death in primary AML blast expressing high levels of Bcl-2. A, primary AML blasts obtained from the bone marrow of a patient, and which robustly expressed Bcl-2, were isolated and exposed to flavopiridol (100 nM) and SAHA (1 μ M) alone and in combination for 24 h. At the end of this period, the percentage of dead cells was determined by Annexin V/PI staining and flow cytometry. Values represent the means \pm S.D. for triplicate determinations. B, primary AML cells were treated with flavopiridol (100 nM) \pm SAHA (1 μ M) for 24 h. At the end of drug treatment, cells were lysed, denatured, and subjected to Western blot analysis using the indicated primary antibodies as described under *Materials and Methods*. Equal protein (30 μ g) was loaded in each lane. Blots were stripped and reprobed with anti-tubulin antibodies to ensure equal loading and transfer of protein. Two additional studies yielded equivalent results.

agents, including flavopiridol (Wang et al., 1999a; Decker et al., 2002), suggesting, albeit indirectly, that Bcl-2 phosphorylation may play a role in proapoptotic actions. To test this possibility directly, the effects of flavopiridol/HDACI administration were examined in U937 cells ectopically expressing a phosphorylation loop-deleted Bcl-2 mutant (Bcl-2- Δ N). As shown in Fig. 5A, cells expressing this mutant protein were slightly more resistant to a low concentration of flavopiridol (100 nM) administered alone. Similar effects were noted with both SAHA and sodium butyrate. It is interesting that the combination of flavopiridol and either HDACI resulted in a marked increase in cell death in untransfected and empty-vector cells (pSFFV), but this response was significantly blunted in the loop-deleted mutants (e.g., 35 versus 78%; $P < 0.01$ compared with empty-vector controls). However, after 48 h of treatment with flavopiridol (75 nM)/HDACI (0.75 μ M SAHA or 0.75 mM sodium butyrate) regimens, cell death in the loop-deleted mutants was comparable with that of controls (Fig. 5B). Very similar results were obtained in U937 cells stably transfected with a construct encoding a mutant Bcl-2 protein lacking a key phosphorylation site (Ser70 \rightarrow Ala) (Wang et al., 1999a). Such mutant cells were also resistant to flavopiridol/HDACI-mediated cell death at 24 h, but this effect was also markedly reduced at 48 h (Fig. 5, C and D). Together, these findings indicate that N-termi-

nal loop deletion and loss of a critical Bcl-2 phosphorylation site delay but do not ultimately prevent cell death induced by the flavopiridol/HDACI regimen.

Parallel studies were performed to assess the effects of interruption of Bcl-2 phosphorylation on flavopiridol/HDACI-mediated mitochondrial injury and activation of the caspase cascade in the two mutant cell lines (Bcl-2- Δ N and Bcl-2-S70A). In empty-vector cells, flavopiridol (100 nM) modestly increased cytochrome *c* and Smac/DIABLO release after 12 h of treatment, whereas SAHA alone was ineffective (Fig. 6A). However, combined treatment resulted in a marked increase in cytosolic release of these proapoptotic effectors. However, in Bcl-2- Δ N cells, neither flavopiridol nor flavopiridol + SAHA, induced mitochondrial injury after 12 h of exposure, although after 24 h, there was a marked increase in both cytochrome *c* and Smac/DIABLO release in flavopiridol/SAHA-treated cells. As anticipated, ara-C-mediated cytochrome *c* and Smac/DIABLO release was not observed in Bcl-2- Δ N transfectants at 24 h. Similar results were obtained in U937-Bcl-2-S70A cells and in cells treated with flavopiridol + sodium butyrate (data not shown).

Consistent with these results, ectopic expression of Bcl-2- Δ N delayed but did not prevent flavopiridol/SAHA-mediated PARP degradation as well as caspase-3 and Bcl-2 cleavage, but it was highly effective in blocking these events after

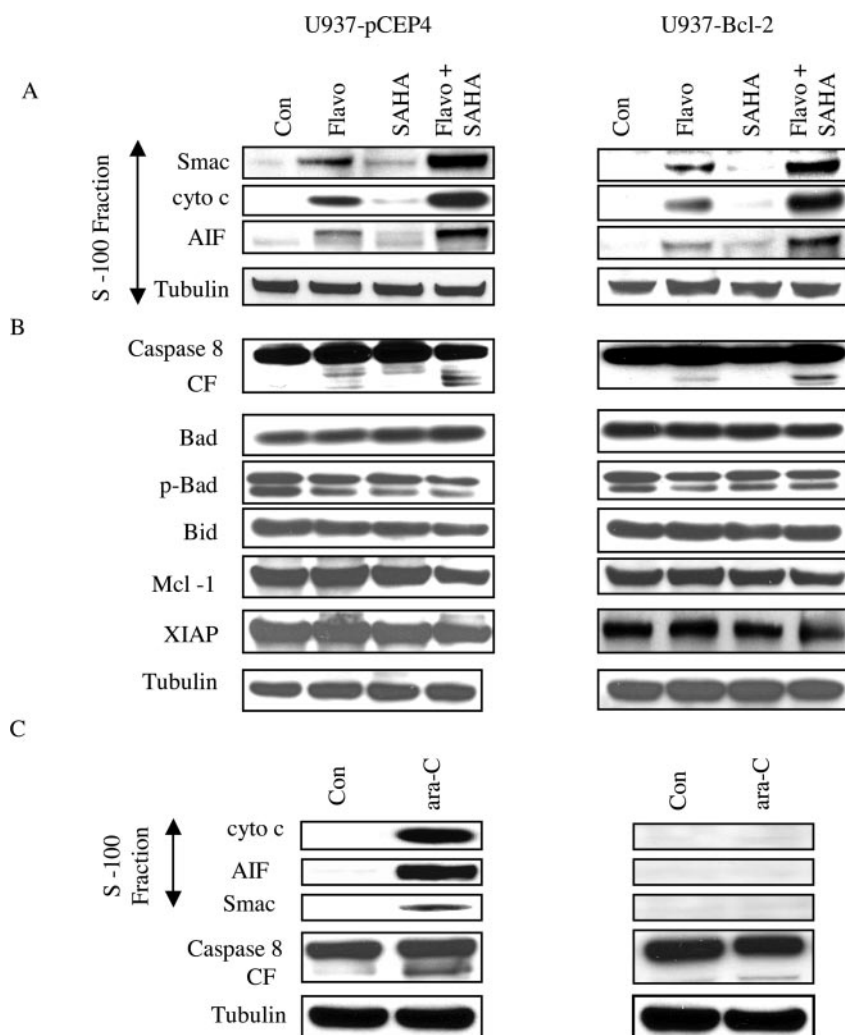


Fig. 4. Ectopic expression of Bcl-2 protects U937 cells from release of proapoptotic mitochondrial proteins, caspase activation, and PARP cleavage after exposure to ara-C, but not to combined treatment with flavopiridol and SAHA. A and B, U937-Bcl-2 and empty-vector U937-pCEP4 cells were treated with flavopiridol (100 nM) or SAHA (1 μ M) individually as well as in combination for 12 h. C, U937-Bcl-2 and empty-vector U937-pCEP4 cells were treated with 1 μ M ara-C for 12 h. After drug exposure, cytosolic (S-100) fractions and whole cell lysates were obtained as described under *Materials and Methods*. Protein samples were subjected to Western blot analysis using the indicated primary antibodies. Each lane was loaded with 30 μ g of protein; blots were stripped and reprobed with anti-tubulin antibodies to ensure equal loading and transfer of protein. Representative results are shown; two additional experiments yielded equivalent findings.

exposure of cells to ara-C (Fig. 6B). Together, these findings indicate that loss of the Bcl-2 phosphorylation loop or the Ser70 phosphorylation site can delay but cannot ultimately prevent mitochondrial injury and activation of the caspase cascade in cells exposed to the combination of flavopiridol and HDACIs.

Previous studies have shown that ectopic expression of Bcl-2 may attenuate cell death without restoring clonogenic potential in cells exposed to cytotoxic agents (Yin and Schimke, 1995). Therefore, colony-forming studies were performed to determine whether similar events occurred in cells exposed to flavopiridol and SAHA. As shown in Fig. 7, exposure of empty-vector control cells or cells ectopically expressing full-length Bcl-2, Bcl-2-S70A, or Bcl-2-ΔN to flavopiridol or SAHA individually had relatively little effect on clonogenic potential. However, in all cases, combined exposure to flavopiridol and SAHA resulted in a pronounced reduction in colony formation in each of the cell lines (e.g., by 75–80%). Furthermore, despite delaying flavopiridol/SAHA-induced cell death, transfection with Bcl-2-S70A or Bcl-2-ΔN was no more effective than full-length Bcl-2 in protecting clonogenic cells from this regimen. Together, these findings suggest that wild-type and mutant Bcl-2 proteins are equally ineffective in preventing reproductive cell death after flavopiridol/SAHA exposure.

Because activation of the extrinsic, receptor-mediated pathway can circumvent Bcl-2-mediated resistance, an attempt was made to determine whether this mechanism might account for the activity of the flavopiridol/SAHA regimen in Bcl-2-overexpressing cells. To this end, U937 cells ectopically expressing dominant-negative FADD (Cartee et al., 2002) were used. As anticipated, U937-FADD-DN cells were significantly more resistant to cell death induced by tumor necrosis factor/cycloheximide than their empty-vector counterparts (Fig. 8; $P < 0.02$). In contrast, no resistance was observed in cells exposed to flavopiridol + SAHA ($P > 0.05$), arguing against a role for activation of the extrinsic pathway in mediating flavopiridol/SAHA-associated antileukemic effects.

The effects of flavopiridol and HDACIs were then examined in relation to perturbations in the BH3 domain-only Bcl-2 family member Bax, which is known to undergo mitochondrial translocation, conformational transformation, and dimerization after exposure of cells to various noxious stimuli (Jia et al., 2001; Liu et al., 2003). Furthermore, the antiapoptotic actions of proteins such as Bcl-2 have been related to antagonism of perturbations in Bax involved in initiation of mitochondrial injury (Yamaguchi and Wang, 2002). Whereas flavopiridol alone modestly induced translocation of Bax to the mitochondrial fraction, SAHA had no effect (Fig.

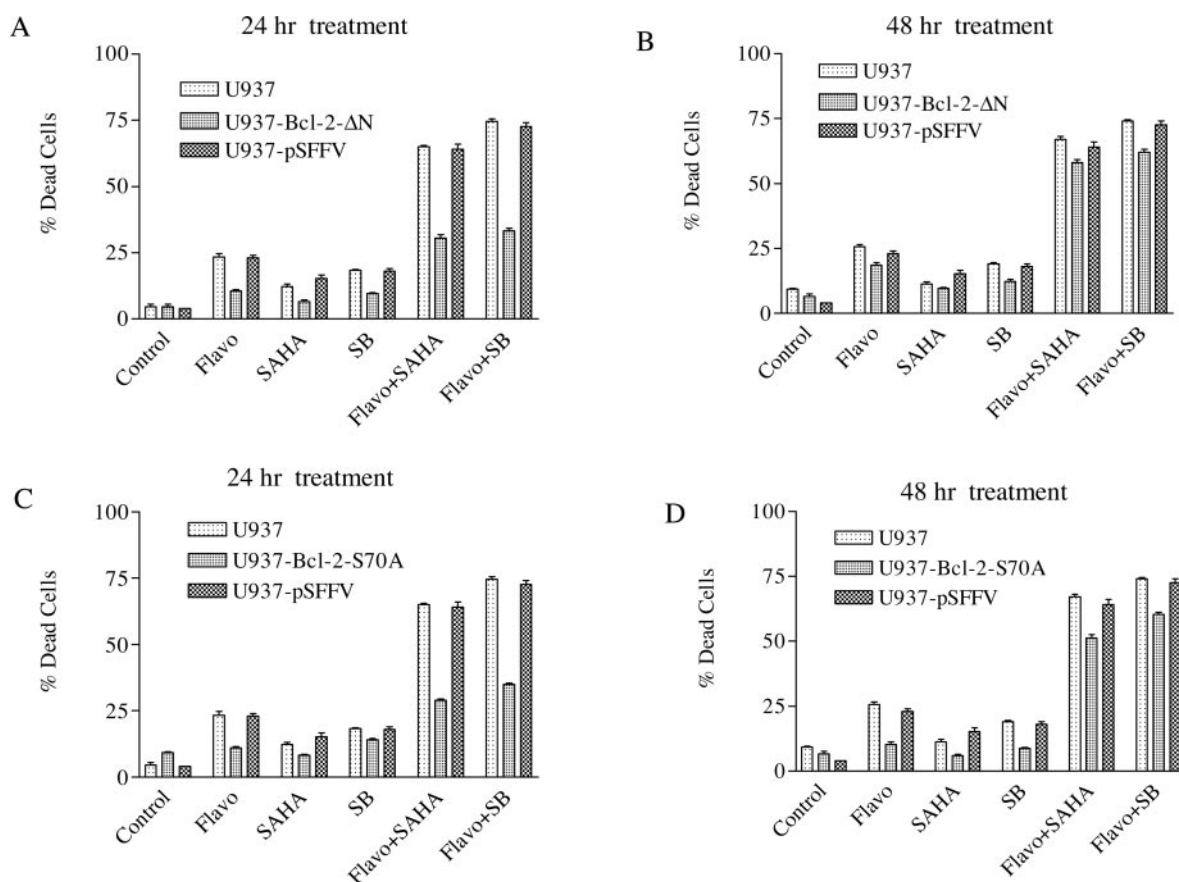


Fig. 5. Ectopic expression of loop-deleted Bcl-2 (Bcl-2 ΔN) or a Ser70 site mutation (Bcl-2-S70A) fails to protect against flavopiridol and SAHA or sodium butyrate-induced cell death. U937, U937-Bcl-2-ΔN, and U937-pSFFV cells were treated with flavopiridol (100 nM) ± SAHA (1 μM)/sodium butyrate (1 mM) for 24 h (A) or flavopiridol (75 nM) ± SAHA (0.75 μM)/sodium butyrate (0.75 mM) for 48 h (B). U937, U937-Bcl-2-S70A, and U937-pSFFV cells were treated with flavopiridol (100 nM) ± SAHA (1 μM)/sodium butyrate (1 mM) for 24 h (C) or flavopiridol (75 nM) ± SAHA (0.75 μM)/sodium butyrate (0.75 mM) for 48 h (D). At the end of this interval, the percentage of dead cells was monitored by Annexin V-fluorescein isothiocyanate staining and flow cytometry as described under *Materials and Methods*. Values represent the means ± S.D. for three separate experiments performed in triplicate.

9A). However, combined exposure of cells to flavopiridol and SAHA resulted in a large increase in Bax translocation. It is noteworthy that these effects, particularly those induced by flavopiridol/SAHA, were not attenuated by ectopic expression of Bcl-2. Furthermore, exposure to flavopiridol alone modestly increased Bax conformational change, an event that has been shown to be associated with induction of cell death (Fig. 9A) (Rosato et al., 2004), whereas SAHA alone was ineffective. However, combined treatment resulted in a significant increase in Bax conformational change. As in the

case of Bax translocation, flavopiridol/SAHA-mediated Bax conformational change was not attenuated in cells ectopically expressing Bcl-2.

Last, the ability of flavopiridol and SAHA to induce Bax dimerization, an event that has been linked to release of cytochrome *c* (Zha et al., 1996), was investigated. Expression of Bax dimers, reflected by a 52-kDa species, in pCEP4 cells was increased by flavopiridol alone and to a lesser extent by SAHA (Fig. 9B). However, coadministration of flavopiridol and SAHA resulted in a substantially greater increase in Bax

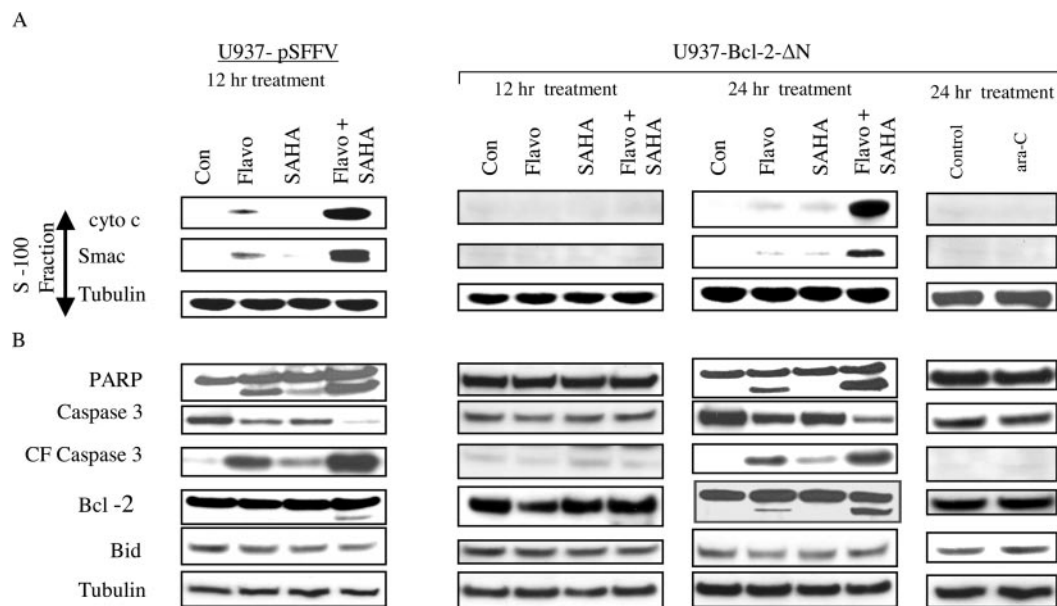


Fig. 6. Prolonged exposure to flavopiridol/SAHA but not ara-C induces release of proapoptotic mitochondrial proteins, activation/cleavage of caspase-3, and PARP degradation in U937 cells ectopically expressing loop-deleted Bcl-2 (Bcl-2 Δ N). U937-Bcl-2- Δ N and U937-pSFFV cells were treated with flavopiridol (100 nM) or SAHA (1 μ M) alone and in combination for 12 and 24 h. U937-Bcl-2- Δ N cells were also treated with 1 μ M ara-C for 24 h. After drug exposure, cytosolic (S-100) fractions (A) and whole cell lysates (B) were obtained as described under *Materials and Methods*. Protein samples were subjected to Western blot analysis using the indicated primary antibodies. Each lane was loaded with 30 μ g of protein; blots were stripped and reprobed with anti-tubulin antibodies to ensure equal loading and transfer of protein. Representative results are shown; two additional experiments yielded equivalent findings.

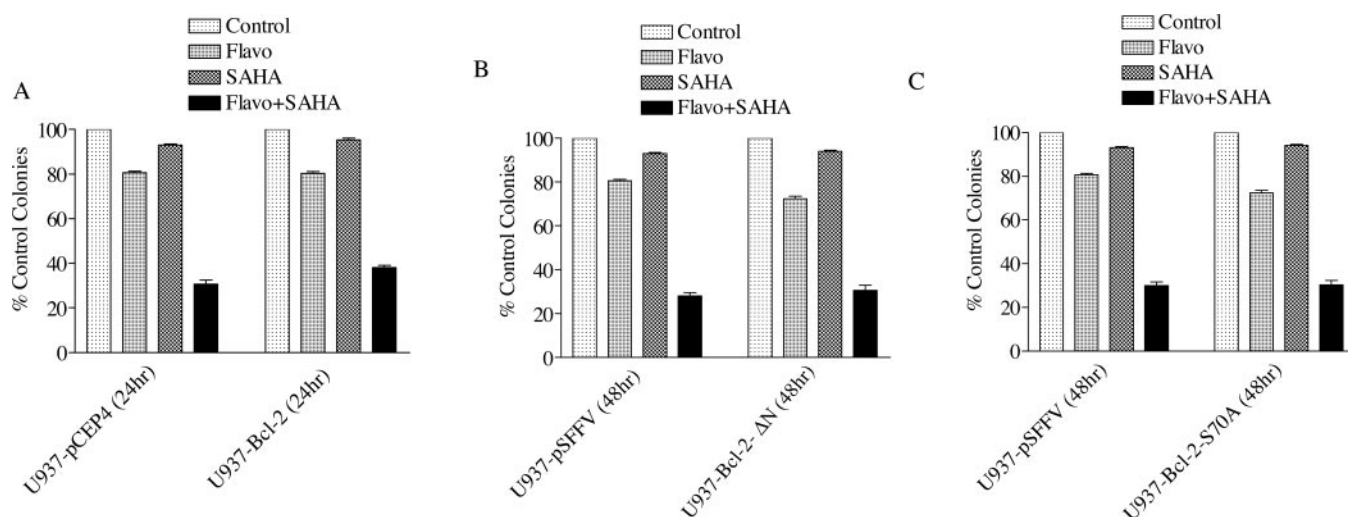


Fig. 7. Ectopic expression of full-length Bcl-2, loop-deleted Bcl-2 (Bcl-2 Δ N), or Ser70 site mutation (Bcl-2-S70A) fails to protect U937 cells from flavopiridol/SAHA-mediated loss of clonogenic potential. A, U937-Bcl-2 and empty-vector U937-pCEP4 were treated with flavopiridol (100 nM) and SAHA (1 μ M) for 24 h. B, U937-Bcl-2- Δ N and empty-vector U937-pSFFV were treated with flavopiridol (75 nM) and SAHA (0.75 μ M) for 48 h. C, U937-Bcl-2-S70A and empty-vector U937-pSFFV were treated with flavopiridol (75 nM) and SAHA (0.75 μ M) for 48 h. At the end of drug treatment, cells were washed and plated in soft agar as described under *Materials and Methods*. Colonies, consisting of groups ≥ 50 cells, were scored at day 10. Values for each condition were expressed as a percentage of control colony formation. For A to C, values represent the means \pm S.D. for three separate experiments performed in triplicate.

dimerization. It is significant that very similar results were observed in U937/Bcl-2 cells. It should be noted that a separate band occurring between the Bax monomer and dimer was detected, which might reflect a nonspecific protein or a cross-linked monomer, as reported previously (Kim et al., 2004). Together, these findings indicate that ectopic expression of Bcl-2 is relatively ineffective in preventing mitochondrial translocation, conformational change, and dimerization of Bax in cells exposed to the flavopiridol/SAHA regimen.

Discussion

Induction of cell death represents an important mechanism by which diverse chemotherapeutic agents trigger the cell death process in neoplastic cells (Danial and Korsmeyer, 2004). In the classic, intrinsic mitochondrial pathway, various noxious stimuli induce changes in mitochondrial membrane pores that permit proapoptotic proteins, particularly cytochrome *c*, to exit the intermitochondrial membrane space and enter the cytoplasm (Harris and Thompson, 2000). Although controversy exists concerning the mechanism by which this process occurs, it is generally agreed that cell death involves conformational and other changes in BH3-only domain Bcl-2 members such as Bax and Bak (Yamaguchi and Wang, 2002; Liu et al., 2003). In particular, it has been suggested that dimerization of Bax facilitates the formation of membrane pores implicated in cytochrome *c* release (Yamaguchi and Wang, 2002). Cytoplasmic cytochrome *c* activates the apoptosome, a multiprotein complex consisting of apoptotic protease activating factor-1, pro-caspase 9, and deoxy-ATP, which in turn leads, through caspase-9 activation, to engagement of effector caspases such as caspase-3 (Hu et al., 1998). Antiapoptotic multidomain Bcl-2 family members such as Bcl-2 and Bcl-x_L may act directly to block cytochrome *c* release, presumably by interfering with the function of proteins such as Bax, or indirectly, by binding to and disrupting the function of other proapoptotic proteins such as Bad, which promote mitochondrial injury (Cheng et al., 2001). Whatever the underlying mechanism of cell death, increased expression of proteins such as Bcl-2 has been associated with chemoresistance, particularly in the case of hematologic malignancies (Campos et al., 1993). Therefore, the development of strategies

capable of inducing cell death in tumor cells overexpressing Bcl-2 or Bcl-x_L represents a logical goal.

The present results suggest that a regimen involving simultaneous CDK and HDAC inhibition effectively induces cell death in leukemia cells overexpressing Bcl-2 or Bcl-x_L and is resistant to more conventional cytotoxic agents (e.g., ara-C). Findings from several recent studies indicate that despite inducing cell cycle arrest, flavopiridol disrupts several signaling and survival pathways, including those related to p21^{CIP1}, NF-κB, and Mcl-1, which prevent HDAC-mediated maturation, and instead promotes mitochondrial injury and cell death (Almenara et al., 2002; Gojo et al., 2002; Takada and Aggarwal, 2004). Although resistance of such regimens to Bcl-2- or Bcl-x_L-mediated cytoprotection could not have been predicted a priori, preexisting evidence might have suggested that this would be the case. For example, flavopiridol is known to induce mitochondrial injury in various neoplastic cells, including those overexpressing Bcl-2 (Achenbach et al., 2000; Decker et al., 2002). Furthermore, HDACs have been shown to trigger cell death in some cells through a caspase-8-dependent and Bcl-2-independent mechanism (Duan et al., 2005). In addition, HDAC-mediated lethality in human leukemia cells has recently been attributed to induction of death receptor pathways (Insinga et al., 2005). Because activation of the latter pathways are generally resistant to inhibition by Bcl-2 (Keogh et al., 2000), it was tempting to speculate activation of the extrinsic apoptotic pathway might be responsible for flavopiridol/HDAC lethality in Bcl-2 or Bcl-x_L-overexpressing cells. However, several considerations argue against this possibility. First, we have found that death receptor induction by HDACs is cell type-specific and does not occur in U937 cells (R. Rosato and S. Grant, unpublished observations). More importantly, leukemia cells ectopically expressing dominant-negative FADD, and resistant to tumor necrosis factor-induced lethality, did not display resistance to the flavopiridol/HDAC regimen. Instead, such results argue that the combination of these agents are able to overcome blockade of cytochrome *c* release and mitochondrial injury conferred by antiapoptotic multidomain Bcl-2 family members, rather than by engaging the Bcl-2-independent extrinsic cascade.

It is noteworthy that the flavopiridol/HDAC regimen effectively induced cell death in cells ectopically expressing Bcl-2 constructs lacking phosphorylation sites, either through loss of the phosphorylation loop (residues 32–80) (Decker et al., 2002) or mutation of the Ser70 site (Wang et al., 1999a). Such proteins typically confer greater resistance than wild-type Bcl-2 to cell death induced by various cytotoxic agents, including paclitaxel (Wang et al., 1999a), ara-C (Tang et al., 2000), and flavopiridol (Decker et al., 2002). In fact, the presence of the phosphorylation loop has previously been shown to be necessary for complete circumvention of Bcl-2-mediated resistance by flavopiridol in human leukemia cells (Decker et al., 2002). The mechanism by which this phenomenon occurs is unknown, but in the case of paclitaxel, it has been postulated that phosphorylation of Bcl-2 by the stress-related mitogen-activated protein kinase c-Jun NH₂-terminal kinase is required for lethality (Wang et al., 1999a). It should be recognized that the consequences of Bcl-2 phosphorylation might differ with respect to cell type and stimulus. For example, phosphorylation of Bcl-2 (e.g., at Ser70) by the macrocyclic lactone bryostatins is required to

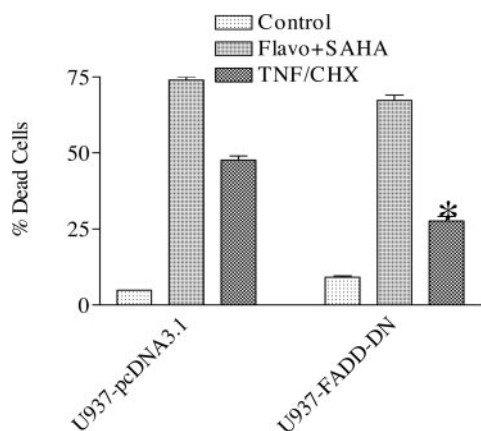


Fig. 8. U937 cells stably transfected with an empty vector (pcDNA3.1) or dominant-negative FADD were exposed to combination of flavopiridol (100 nM) and SAHA (1 μ M) or tumor necrosis factor (10 ng/ml)/cycloheximide (1 μ M). The percentage of cell death was monitored by Annexin V-fluorescein isothiocyanate staining and flow cytometry against an untreated control after 24 h of drug exposure. Values represent the means \pm S.D. for three separate experiments performed in triplicate.

prevent growth factor deprivation-induced cell death in murine hematopoietic cells (Deng et al., 1998). It is noteworthy that although both the loop-deleted Bcl-2 protein and the Ser70 mutant blocked flavopiridol/HDACI-induced cell death at early intervals, a progressive loss of viability was observed over time. Such results are consistent with the relative inability of these proteins to protect clonogenic cells from flavopiridol/HDACI lethality. They are also in accord with other studies indicating that Bcl-2 may delay but does not ultimately protect neoplastic cells from a reproductive form of cell death (Yin and Schimke, 1995). It is therefore tempting to speculate that the ability of mutant Bcl-2 protein to delay but not prevent SAHA/flavopiridol mitochondrial injury (e.g., cytochrome *c* release) is responsible, at least in part, for the failure of these proteins to restore clonogenic growth. In this context, it is important to note that previous studies from our laboratory demonstrated that coadministration of the Chk1 and CDK inhibitor UCN-01 effectively circumvented ara-C resistance in cells ectopically expressing Bcl-2 containing the N-terminal loop deletion (Tang et al., 2000). These findings,

which are in accord with the present results, raise the possibility that certain kinase inhibitors may share the capacity to circumvent resistance to mitochondrial injury and cell death conferred by both wild-type and mutant forms of Bcl-2.

Dimerization of Bax has been linked to the induction of cell death (Zha et al., 1996), and it has been proposed, although not yet proven, that this process promotes the formation of mitochondrial membrane channels that facilitate the cytosolic translocation of cytochrome *c* (Yamaguchi and Wang, 2002). In addition, these events as well as cell death in general are associated with a conformational change in Bax (Yamaguchi and Wang, 2002; Liu et al., 2003). Therefore, the possibility that interference with these phenomena by Bcl-2 and related proteins contribute to antiapoptotic actions seems plausible. In this context, it is significant that combined treatment with flavopiridol and SAHA resulted in a clear increase in Bax conformational change and Bax dimerization in both wild-type and Bcl-2-overexpressing cells. Furthermore, the extent of these processes was similar in the two cell types. Because Bcl-2 and related proteins can act at

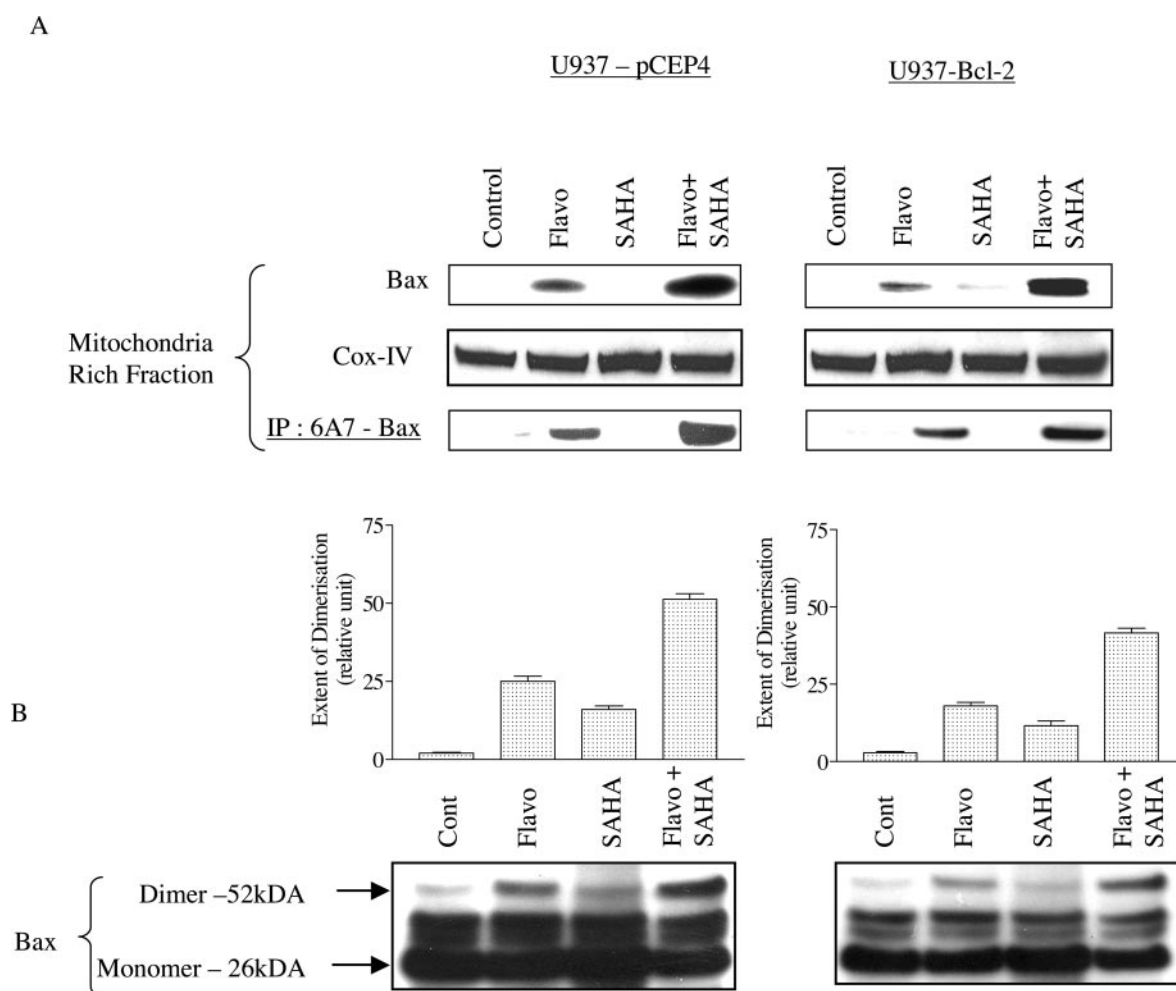


Fig. 9. Simultaneous exposure of U937 cells ectopically expressing full-length Bcl-2 or empty vector to flavopiridol/SAHA induces translocation of cytosolic Bax to mitochondria, Bax conformational change, and dimerization. A, U937-Bcl-2 and U937-pCEP4 cells were treated with 100 nM flavopiridol and 1 μ M SAHA alone or in combination for 24 h. Cells were harvested by centrifugation at 600g for 10 min at 4°C, and the mitochondrial-rich fraction was prepared as described under *Materials and Methods*. Protein content of the mitochondrial-rich fraction was quantified and subjected to Western blot to monitor Bax translocation. Conformational change of the Bax was monitored by immunoprecipitating mitochondrial protein with a specific antibody (6A7-Bax) directed against conformationally changed Bax as described under *Materials and Methods*. B, mitochondrial-rich fraction was prepared from U937-Bcl-2 and U937-pCEP4 cells treated as described above and incubated with DSP cross-linker to allow oligomerization to take place as described under *Materials and Methods*. At the end of the reaction, protein samples were subjected to Western blot analysis using Bax primary antibody. Representative results are shown; two additional experiments yielded equivalent findings.

multiple levels to inhibit apoptosis (Yang et al., 1997), it is noteworthy that cells that sustain mitochondrial injury but in which cell death is blocked (e.g., due to caspase inhibition) can undergo an alternative form of cellular demise (e.g., necrosis and autophagy) (Edinger and Thompson, 2004). Thus, whether or not the flavopiridol/SAHA regimen can overcome resistance to classic apoptosis conferred by Bcl-2 or Bcl-x_L overexpression, it may nevertheless induce an alternative form of cell death through induction of mitochondrial injury. Results of the clonogenic assays in which wild-type or mutant Bcl-2 failed to protect self-renewing cells from the flavopiridol/SAHA regimen were consistent with this concept. Whatever the underlying mechanism(s), the present findings indicate coadministration of flavopiridol and HDACIs does not induce cell death in leukemic cells by bypassing the capacity of multidomain antiapoptotic proteins to prevent mitochondrial injury (e.g., Bax translocation and conformational change, and cytochrome *c* release) and activating downstream or parallel components of the cell death pathway. Instead, this strategy seems to act directly to antagonize Bcl-2- and Bcl-x_L-mediated protection of cells from mitochondrial damage. Such findings support further efforts to explore the therapeutic potential of a strategy combining CDK with HDAC inhibitors in leukemias resistant to cell death induction by standard forms of therapy secondary to increased expression of Bcl-2 or related proteins.

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