

Mechanism and functional role of XIAP and Mcl-1 down-regulation in flavopiridol/vorinostat antileukemic interactions

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Abstract

The mechanism and functional significance of XIAP and Mcl-1 down-regulation in human leukemia cells exposed to the histone deacetylase inhibitor vorinostat and the cyclin-dependent kinase inhibitor flavopiridol was investigated. Combined exposure of U937 leukemia cells to marginally toxic concentrations of vorinostat and flavopiridol resulted in a marked increase in mitochondrial damage and apoptosis accompanied by pronounced reductions in XIAP and Mcl-1 mRNA and protein. Down-regulation of Mcl-1 and XIAP expression by vorinostat/flavopiridol was associated with enhanced inhibition of phosphorylation of RNA polymerase II and was amplified by caspase-mediated protein degradation. Chromatin immunoprecipitation analysis revealed that XIAP and Mcl-1 down-regulation were also accompanied by both decreased association of nuclear factor- κ B (XIAP) and increased E2F1 association (Mcl-1) with their promoter regions, respectively. Ectopic expression of Mcl-1 but not XIAP partially protected cells from flavopiridol/vorinostat-mediated mitochondrial injury at 48 h, but both did not significantly restore clonogenic potential. Flavopiridol/vorinostat-mediated transcriptional repression of XIAP, Mcl-1-enhanced apoptosis, and loss of clonogenic potential also occurred in primary acute myelogenous leukemia (AML) blasts. Together, these findings indicate that transcriptional repression of XIAP and Mcl-1 by flavopiridol/vorinostat

contributes functionally to apoptosis induction at early exposure intervals and raise the possibility that expression levels may be a useful surrogate marker for activity in current trials. [Mol Cancer Ther 2007;6(2):692–702]

Introduction

Histone deacetylase inhibitors (HDACI) represent a group of compounds that increase chromatin acetylation, thereby inducing relaxation of its structure and a more open configuration. These events permit various factors (e.g., coactivators) to gain access to the transcriptional machinery and facilitate gene expression (1, 2). Histone acetylation is reciprocally regulated by histone acetylases and histone deacetylases (1, 2). Both histone acetylases and HDACs are subdivided into several groups based on structural features, functional characteristics, and subcellular localization (3, 4). In addition to their capacity to regulate gene expression, HDACIs induce apoptosis in transformed cells, particularly those of hematopoietic origin (5–7). Multiple actions have been implicated in HDACI lethality in addition to modulation of histone acetylation and regulation of gene expression (8, 9). These include, among others, generation of reactive oxygen species, activation of the extrinsic apoptotic pathways, modulation of the nuclear factor- κ B (NF- κ B) survival pathway, disruption of heat shock protein function, and perturbations in the expression of Bcl-2 family members (reviewed in refs. 8, 9). Although HDACIs have shown initial evidence of single drug activity in certain hematopoietic malignancies (e.g., cutaneous T-cell lymphoma and acute leukemia; ref. 10), preclinical studies suggest that their activity may be significantly enhanced when they are combined with other targeted agents, particularly cyclin-dependent kinase (CDK) inhibitors. For example, such regimens have proven active against human leukemia cells (11–13), malignant pleural mesothelioma cells (14), and lung and esophageal cancer cells (15).

Previously, we reported the antileukemic effects of the HDACI vorinostat (also known as suberoylanilide hydroxamic acid) were dramatically enhanced by the CDK inhibitor flavopiridol, and that these events were associated with inhibition of p21^{WAF1/CIP1} expression, disrupted cellular maturation (11, 12), and inactivation of the NF- κ B pathway (16). Flavopiridol is a semisynthetic flavonoid that inhibits CDKs by competing with ATP for the active site of these kinases (17). Consistent with this action, flavopiridol blocks cell cycle progression at the G₁-S and G₂-M boundaries in actively proliferating cells (17). In addition, flavopiridol acts as a potent transcriptional modulator by inhibiting CDK-7 and CDK-9, which are responsible for phosphorylation of the carboxyl-terminal domain of RNA

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polymerase II, a component of the positive transcription elongation factor-b complex (17, 18). In this context, flavopiridol-mediated lethality in human leukemia cells has been associated with diminished expression of certain short-lived antiapoptotic proteins, such as Mcl-1 and XIAP (19, 20). Currently, however, the precise mechanisms by which Mcl-1 and XIAP are down-regulated in flavopiridol/vorinostat-treated leukemia cells have not been fully elucidated, and the functional significance of these events in apoptosis and loss of leukemic self-renewal capacity have not been defined. The purpose of the present study is to define the factors responsible for transcriptional repression of Mcl-1 and XIAP by flavopiridol/vorinostat and to assess the effect of down-regulation of these antiapoptotic proteins on the leukemic cell death process.

Materials and Methods

Cells and Cell Culture

U937 human leukemia cells were obtained from American Type Culture Collection (Rockville, MD), and cultured and maintained as described previously (12). U937 cells stably expressing XIAP or Mcl-1 and their empty vector counterparts were obtained as reported previously (7, 13). All experiments used cells in logarithmic phase at 2.5×10^5 /mL. Peripheral blood blasts were obtained with informed consent from two patients (French-American-British classification M2) with AML undergoing routine diagnostic aspirations with approval from the institutional review board of Virginia Commonwealth University. Informed consent was provided according to the Declaration of Helsinki. AML blasts (which comprised >70% of the mononuclear cell population in each case) were isolated and cultured as previously described (6).

Drugs and Chemicals

Sodium butyrate was supplied as a powder (Calbiochem, La Jolla, CA) and dissolved in PBS before use, and suberoylanilide hydroxamic acid was purchased from Alexis (San Diego, CA). Flavopiridol FP (L86 8275; NSC 649890) was kindly provided by Dr. Edward Sausville (Cancer Treatment and Evaluation Program, National Cancer Institute, Bethesda, MD). FP was formulated in DMSO (Sigma-Aldrich, St. Louis, MO) and 10^{-2} mol/L stock solution was stored at -20°C . The pan-caspase inhibitor BOC-D-fmk was purchased from Enzyme System Products (Livermore, CA) and dissolved in DMSO.

Assessment of Apoptosis

Apoptotic cells were evaluated by Annexin V/propidium iodide (BD PharMingen, Franklin Lakes, NJ) staining, according to the manufacturer's instructions as previously described (11), and by morphologic assessment of Wright-Giemsa-stained cytospin preparations.

Assessment of Mitochondrial Membrane Potential ($\Delta\psi_m$)

At the indicated intervals, cells were harvested and 2×10^5 were incubated with 40 nmol/L DiOC₆ (15 min, 37°C). Loss of mitochondrial membrane potential was determined by flow cytometry as previously described (6).

Analysis of Cytosolic Cytochrome c and Apoptosis-Inducing Factor

A previously described technique was used to isolate the S-100 (cytosolic) cell fraction of treated cells (6). For each condition, 30 μg of protein isolated from the S-100 cell fraction were separated and detected by Western blot as described.

Determination of Clonogenicity in Cell Lines and Primary Blast Cells

Following drug exposure, pelleted cells were washed extensively and prepared for soft-agar cloning as previously described in detail (21). Cultures were maintained for 10 to 12 days in a 37°C , 5% CO₂ incubator after which colonies, defined as groups of ≥ 50 cells, were scored using an inverted microscope. The colony assay of hematopoietic cells was done using methylcellulose medium (StemCell Technologies Vancouver, BC, Canada). The assay was carried out according to the manufacturer's instructions. Briefly after treatment, cells were washed and suspended in fresh medium at final concentration of 5×10^5 /mL of methylcellulose. Cells were seeded in 12-well plates and the colonies (expressed in leukemic colony-forming units; ≥ 20 cells) were scored after 14 to 16 days of incubation. The calculations were done as before.

Western Blot Analysis

Whole-cell pellets were washed and resuspended in PBS, and lysed with loading buffer (Invitrogen, Carlsbad, CA) as previously described (11). Thirty micrograms of total protein for each condition were separated by 4% to 12% Bis-Tris NuPAGE precast gel system (Invitrogen) and electroblotted to nitrocellulose. After incubation with the corresponding primary and secondary antibodies, blots were developed by enhanced chemiluminescence (New England Nuclear, Boston, MA).

Antibodies for Western Blot Analysis

Primary antibodies for the following proteins were used at the designated dilutions: poly(ADP)ribose polymerase (1:1,000; BioMol, Plymouth Meeting, PA); Mcl-1, pro-caspase-3, cytochrome c, pro-caspase-9, caspase-7, and XIAP (1:1,000, BD PharMingen, San Diego, CA); caspase-8 (1:2,000; Alexia Corporations, San Diego, CA); Bid (1:1,000; Cell Signaling, Beverly, MA); tubulin (1:4,000; Calbiochem, San Diego, CA); apoptosis-inducing factor (AIF), RNA polymerase II carboxyl-terminal domain (1:1,000, Santa Cruz); and phosphorylated RNA polymerase II carboxyl-terminal domain (Upstate, Charlottesville, VA). Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

Real-time Reverse Transcription PCR

Real-time reverse transcription-PCR (RT-PCR) experiments were done in triplicate as described previously (20). Results for the experimental gene were normalized to 18S rRNA levels as specified by the manufacturer. The following primers and probe sequences for were used. Human Mcl-1: primers, forward 5'-GGGCAGGATTGTGACTCT-CATT-3'; reverse 5'-GATGCAGCTTTCTTGGTTTATGG-3';

TaqMan probe 5'-TCAAGTGTTCAGCCACAAAGGCAC-CAAAAAG-3'. Human XIAP: primers, forward 5'-TGGGACATGGATATACTCAGTTAACAA-3'; reverse 5'-GTTAGCCCTCCTCCACAGTGAA-3'; TaqMan probe 5'-ACCTT-CACCTAAAGCATAAAAATCCAGCTCTTGC-3'.

Determination of Caspase-3 and Caspase-9 Activity

Treated cells were lysed, and equivalent quantities were assayed according to the manufacturer's instructions (caspase-3 CP322 and caspase-9 Assay kits; BioVision, Palo Alto, CA). The fold increase in activity was calculated as the ratio between values obtained for treated samples versus those obtained in untreated controls.

Chromatin Immunoprecipitation Assay

After treatment, U937 cells were harvested and processed with a two-step fixation method. The cross-linkers used were 2 mmol/L disuccinimidyl glutarate (Sigma-Aldrich) followed by the 0.5% formaldehyde to increase the sensitivity of the technique. Cross-linking, chromatin sonication, immunoprecipitation, and DNA recovery were done following the protocol described in the detail by Nowak et al. (22). PCR amplification was done using 1 to 2 μ L of the bound fractions and 1/20th of the inputs. The specific PCR primer sequences for XIAP (accession no. AY886519) were as previously reported (23), whereas for *Mcl-1*, they were designed based on the sequence of the promoter region of *Mcl-1* gene (accession no. DQ088966) by using the Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA). *Mcl-1*, sense 5'-CGCCCCTTCCCCTTTTATGG-3'; antisense 5'-GAAGACCCCACTCCTTACTGG-3'.

Statistical Analysis

The significance of differences between experimental conditions was determined using the Student's *t* test for unpaired observations. To assess the interaction between agents, median dose analysis (24) was used (CalcuSyn; Biosoft International, Ferguson, MO). The combination index was calculated for a two-drug combination involving a fixed concentration ratio. Combination index values <1.0 indicate a synergistic interaction.

Results

Flavopiridol Potentiates XIAP Down-Regulation in U937 Cells Coexposed to Vorinostat or Sodium Butyrate

Consistent with our previous findings (11, 12), simultaneous exposure to flavopiridol (150 nmol/L) and either vorinostat (1.5 μ mol/L) or sodium butyrate (1 mmol/L) potently induced apoptosis in U937 human leukemia cells within 24 h of treatment (i.e., $\geq 70\%$; Fig. 1A). In contrast, individual drug exposure was minimally or modestly toxic. A marked reduction in lethality occurred in the presence of the pan-caspase inhibitor fmk-BOC, indicating a caspase-dependent process (Fig. 1A). As shown in Fig. 1B, detailed time course analysis of XIAP levels by Western blot showed that although moderate XIAP down-regulation was observed in U937 cells exposed to HDACIs alone, flavopiridol induced a relatively rapid and progressive decrease in

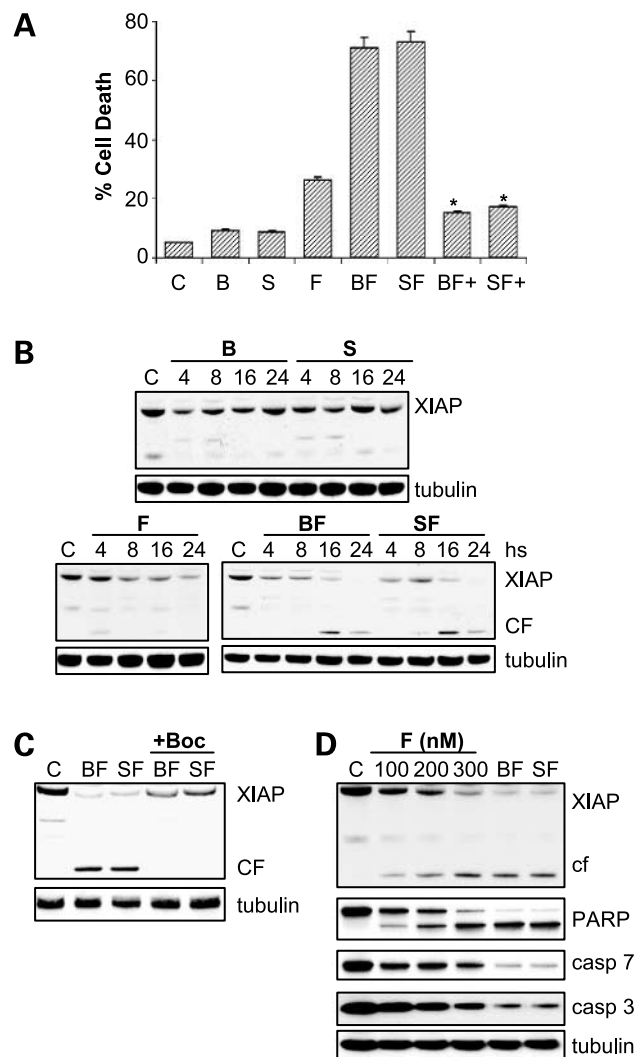


Figure 1. Flavopiridol mediates XIAP down-regulation in U937 cells coexposed to HDACIs. U937 cells were exposed for 24 h to flavopiridol (F, 150 nmol/L) \pm vorinostat (S, 1.5 μ mol/L) or sodium butyrate (B, 1 mmol/L), after which cells were analyzed. C, control. **A**, the extent of cell death was monitored by determining Annexin V/propidium iodide staining by flow cytometry as described in Materials and Methods. BF+ and SF+, cells were coincubated in the presence of the pan-caspase inhibitor fmk-BOC (20 μ mol/L). **B** to **D**, after treatment as above, 30 μ g of protein were separated by SDS-PAGE and analyzed by Western blot as described in Materials and Methods. Blots were probed with antibodies directed against XIAP, poly(ADP)ribose polymerase (PARP), caspase-7 (casp 7) and caspase-3 (casp 3), and tubulin to ensure equivalent loading and transfer. Results of a representative study; two additional experiments yielded similar results. CF, cleavage fragment.

XIAP levels (Fig. 1B). Moreover, FP-mediated XIAP down-regulation was enhanced by coaddition of HDACIs and accompanied by the appearance of an XIAP cleavage fragment at 16 and 24 h. Coincubation with the caspase inhibitor fmk-BOC abolished the appearance of the XIAP cleavage fragment but only partially restored XIAP levels (Fig. 1C). These results are consistent with previous reports describing a role for caspase activation in mediating XIAP

cleavage or degradation (7, 25). Finally, a low concentration of flavopiridol (150 nmol/L) in combination with vorinostat or butyrate was more effective than higher, considerably more lethal concentrations of flavopiridol alone (300 nmol/L) in inducing XIAP down-regulation, poly(ADP)ribose polymerase cleavage, and caspase-3 and caspase-7 activation (Fig. 1D).

Enforced Expression of XIAP Delays but Does Not Ultimately Protect Cells from Vorinostat/Flavopiridol-Induced Lethality

Although flavopiridol, alone or in combination, has previously been shown to induce down-regulation of XIAP, the functional consequences of this phenomenon have not been investigated. To address this issue, genetically modified U937 cells expressing either a cDNA coding for full-length XIAP or corresponding empty vector-transfected cells were exposed simultaneously to either vorinostat/flavopiridol or butyrate/flavopiridol, and cell death was determined by Annexin V/propidium iodide analysis. As shown in Fig. 2A, although significant inhibition of drug-induced lethality was observed during

the first 24 h in XIAP-expressing cells after vorinostat or butyrate/flavopiridol exposure ($P < 0.05$ in each case), no protection was observed at 48 h. Consistent with these findings, analysis of the long-term survival by clonogenic analysis showed that protection in U937/XIAP cells after 24 h treatment with butyrate/flavopiridol or vorinostat/flavopiridol was minimal compared with U937/empty vector cells ($1.2 \pm 0.1\%$ versus $0.3 \pm 0.02\%$, respectively; Fig. 2B).

Subsequent efforts were then undertaken to characterize further the relationship between XIAP down-regulation and vorinostat/flavopiridol-induced lethality. Time course analysis of mitochondrial integrity showed that enforced expression of XIAP partially but significantly protected cells from loss of mitochondrial membrane potential ($\Delta\psi_m$) after 16 or 24 h exposure to vorinostat or butyrate/flavopiridol, but not at 48 h (Fig. 2C). Moreover, Western blot analysis of the cytosolic fraction (S100) revealed similar levels of mitochondrial apoptosis-inducing factor and cytochrome *c* release into the cytosol at early intervals (e.g., 8–24 h) in both U937/XIAP and U937/

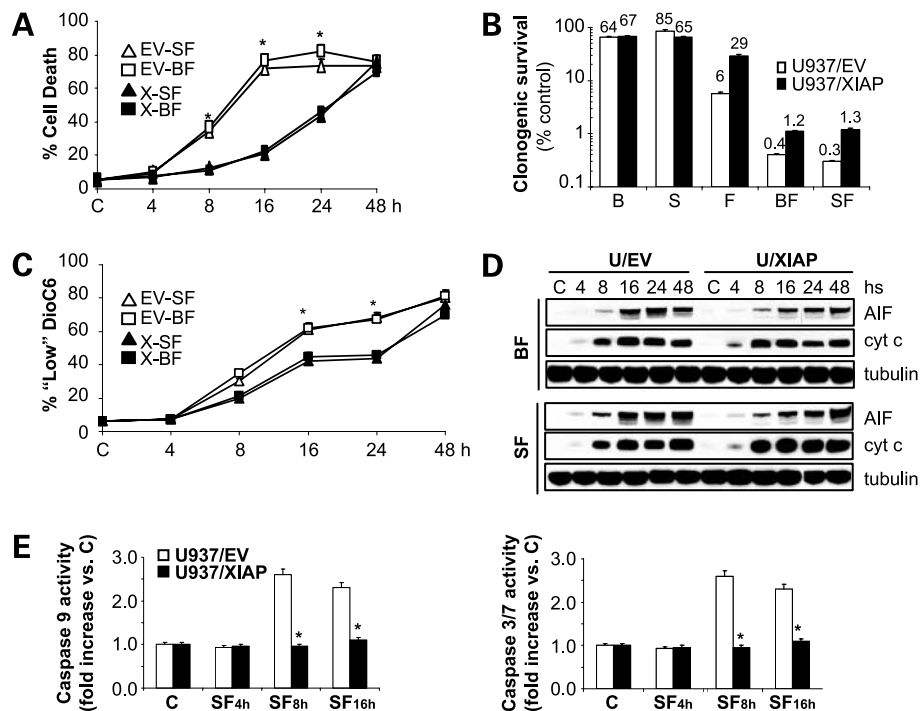


Figure 2. Effects of flavopiridol-mediated XIAP down-regulation on HDAC1/flavopiridol-induced lethality and mitochondrial function. **A**, U937 cells ectopically expressing XIAP (X) or their corresponding empty vector (EV) counterparts were cocultured with vorinostat (1.5 $\mu\text{mol/L}$) or sodium butyrate (1 mmol/L), and flavopiridol (150 nmol/L) as indicated, and cell death was monitored by Annexin V/propidium iodide staining by flow cytometry. **B**, both U937/XIAP and U937/empty vector cells were coexposed to vorinostat (1.5 $\mu\text{mol/L}$), sodium butyrate (1 mmol/L), and flavopiridol (150 nmol/L) either alone or in combination for 24 h, washed free of drugs, and plated in soft agar as described in Materials and Methods. Colonies, consisting of groups of ≥ 50 cells, were scored after 12 d. **C**, U937/XIAP and U937/empty vector cells were treated as in **A**, and loss of $\Delta\psi_m$ was determined by flow cytometry as described in Materials and Methods. **D**, U937/XIAP and U937/empty vector cells were coexposed to agents as above, pelleted, and lysed, and protein was extracted from the cytosolic S-100 fraction. In each case, 30 μg of protein were separated by SDS-PAGE and probed with the corresponding antibodies against apoptosis-inducing factor (AIF), cytochrome *c* (cyt *c*), or tubulin. A representative study. Two additional experiments yielded similar results. **E**, caspase-3 and caspase-9 activities were monitored in U937/empty vector and U937/XIAP cells treated for 4, 8, or 16 h with the combination of vorinostat and flavopiridol (1.5 $\mu\text{mol/L}$ and 150 nmol/L, respectively) as described in Materials and Methods. Values are expressed as the ratios of activity for treated samples relative to untreated controls. Columns, means for three separate experiments done in triplicate; bars, SD. *, $P < 0.01$, significantly lower than values obtained with U937/empty vector cells.

empty vector cells (Fig. 2D). On the other hand, ectopic expression of XIAP significantly reduced activation of pro-caspase-9 and pro-caspase-3/7, well-described XIAP targets (26), after 8 and 16 h of exposure to vorinostat/flavopiridol (Fig. 2E) or butyrate/flavopiridol (data not shown). Together, these findings suggest that down-regulation of XIAP by HDAC1/flavopiridol-containing regimens contributes to the early induction of apoptosis in leukemia cells, but that XIAP is relatively ineffective in protecting cells from the late consequences of mitochondrial injury.

Flavopiridol Regulates XIAP Expression at a Transcriptional Level

As caspase-mediated degradation could not fully account for the decline in XIAP expression in vorinostat/flavopiridol-treated cells, transcriptional mechanisms were investigated by real-time RT-PCR. As shown in Fig. 3A, whereas vorinostat or butyrate modestly induced a decline in XIAP mRNA levels after 4-h exposure, flavopiridol administered alone resulted in a more pronounced decrease. However, coexposure of cells to vorinostat or butyrate and flavopiridol resulted in a profound reduction in XIAP mRNA levels, for example, from ~30% expression at 8 h to 7% expression at 16 to 24 h (Fig. 3A). Values were not altered by coincubation with the caspase inhibitor fmk-BOC, arguing against the possibility that these events represented secondary, caspase-dependent effects.

Studies were then done to elucidate mechanisms involved in the regulation of XIAP transcription. Flavopiridol is a global transcriptional regulator through inhibition of the positive transcription elongation factor-b (CDK-9/cyclin T) complex (27, 28). As shown in Fig. 3B, lysates from vorinostat/butyrate-treated cells did not exhibit altered expression of either phosphorylated or total carboxyl-terminal domain RNA polymerase II. In contrast, expression levels were significantly reduced in cells exposed to flavopiridol alone, an effect that was somewhat more pronounced in cells coexposed to vorinostat or butyrate. Because the antiapoptotic functions of NF- κ B are mediated in part through regulation of XIAP expression (29), and in view of evidence that flavopiridol acts as an I κ B kinase/NF- κ B inhibitor (16), the possibility that NF- κ B might also be involved in the transcriptional repression of XIAP was investigated. To this end, chromatin immunoprecipitation (ChIP) was done using extracts from U937 cells exposed to vorinostat, flavopiridol, or vorinostat/flavopiridol for 8 h. Cross-linked chromatin was immunoprecipitated with protein A beads alone (ChIP, IgG) or with antibody to the p65 subunit of NF- κ B (ChIP, p65) and analyzed by PCR using primers directed to the XIAP promoter. As shown in Fig. 3B, whereas vorinostat alone did not significantly modify the association of p65 with the XIAP promoter, flavopiridol, administered either alone or in combination with vorinostat, dramatically reduced that association. Together, these results raise the possibility that XIAP transcriptional down-regulation in vorinostat/flavopiridol-treated cells may involve both flavopiridol-mediated inhibition of RNA polymerase II as well as diminished NF- κ B activation of the specific XIAP promoter.

Flavopiridol Induces Mcl-1 Down-Regulation and Modulates Vorinostat/Flavopiridol-Induced Lethality

Mcl-1 is a short-lived antiapoptotic protein that plays a critical role in the survival of human leukemia cells (18, 19) and is down-regulated by flavopiridol (12, 16, 18). As shown in Fig. 4A, flavopiridol induced a marked decline in Mcl-1 levels in U937 cells detectable as early as 4 h of incubation. A similar pattern was observed in lysates obtained from cells coexposed to vorinostat or butyrate, although in the latter case the presence of an Mcl-1 cleavage

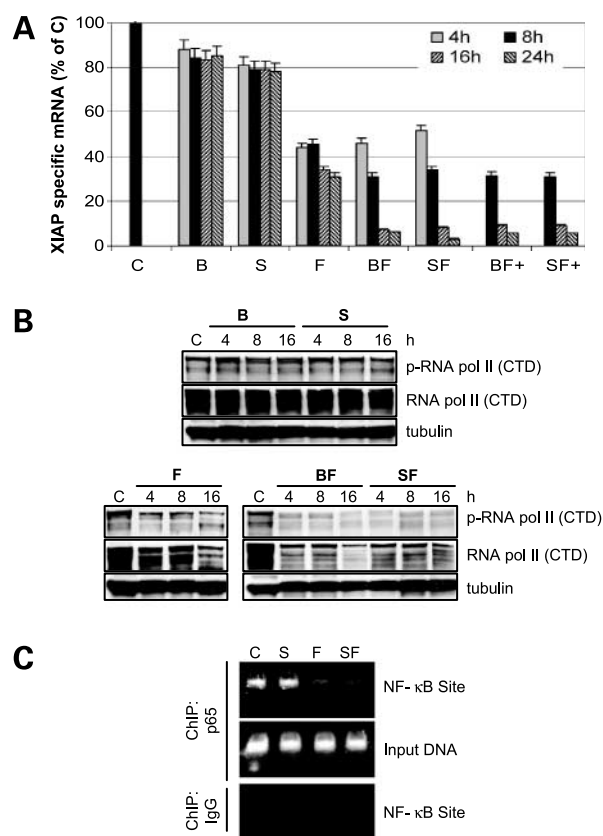


Figure 3. XIAP is transcriptionally regulated in cells exposed to HDAC1/flavopiridol. **A**, time course analysis of XIAP mRNA expression by real-time RT-PCR in U937 cells incubated with sodium butyrate (1 mmol/L), vorinostat (1.5 μ mol/L), and flavopiridol (150 nmol/L) either alone or in combination for the indicated intervals. Values for each condition are expressed as the percentage of specific Mcl-1/18S mRNAs normalized to levels corresponding to untreated control U937 cells (100%). Columns, mean for three separate experiments done in triplicate; bars, SD. **B**, Western blot analysis of whole-cell lysates extracted from U937 cells treated as in **A** and monitored at the indicated time intervals. For each condition, 30 μ g of protein were separated by SDS-PAGE and blotted with antibodies directed against phosphorylated RNA polymerase II (*p-RNA pol II*), total RNA polymerase II (*CTD*, carboxyl-terminal domain), and tubulin (to ensure equivalent loading and transfer). Results of a representative study; two additional experiments yielded similar results. **C**, association of p65/RelA NF- κ B to XIAP promoter region was evaluated by ChIP assay in U937 cells treated with vorinostat, flavopiridol, or these agents in combination for 8 h as described in Materials and Methods. Chromatin was immunoprecipitated by either p65/RelA antibody or IgG. The immunoprecipitated DNA was amplified by PCR using primers targeting the region around the NF- κ B site present in the promoter region of the XIAP gene as described in Materials and Methods.

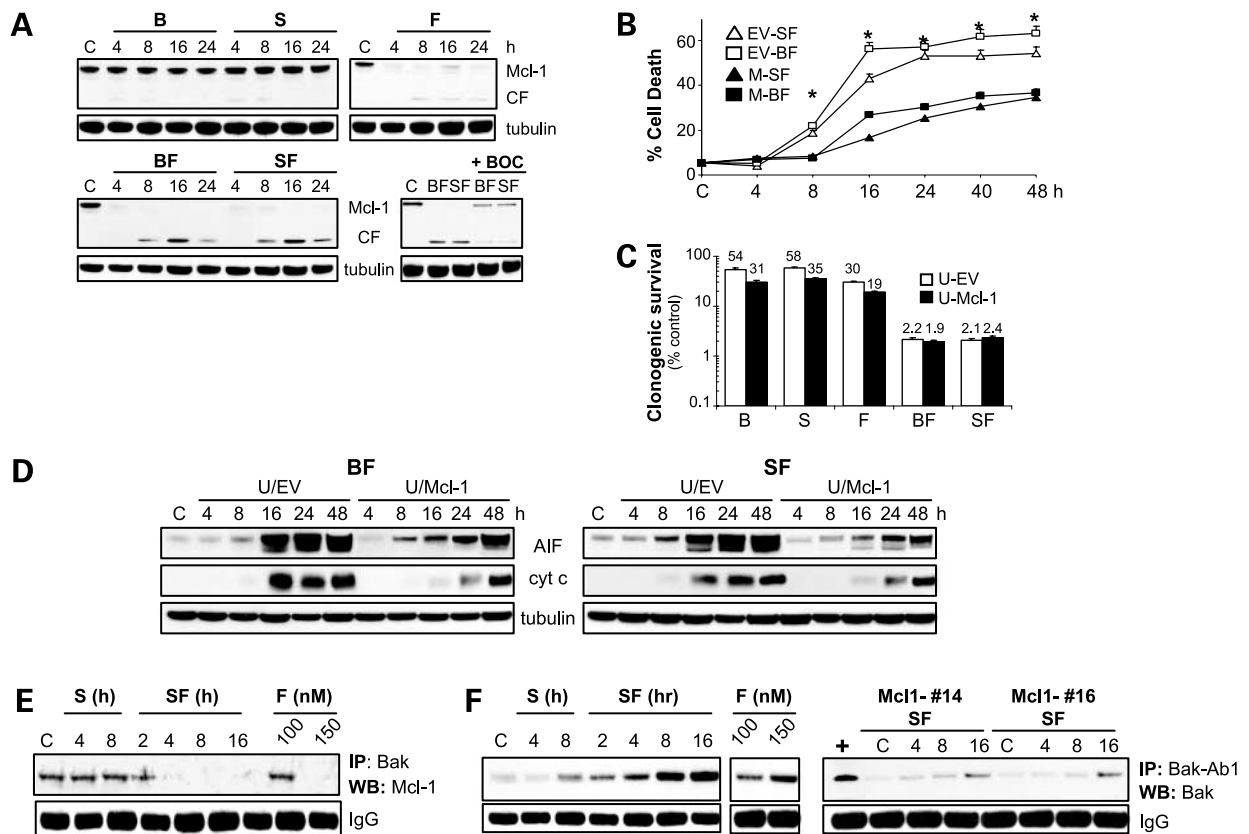


Figure 4. Flavopiridol mediates Mcl-1 down-regulation in cells exposed to the vorinostat/flavopiridol regimen. **A**, U937 cells were treated with sodium butyrate (1 mmol/L), vorinostat (1.5 μ mol/L), flavopiridol (150 nmol/L), or the corresponding combinations for the indicated intervals and analyzed by Western blot. Blots were probed with antibodies directed against Mcl-1 and tubulin. Results of a representative study; two additional experiments yielded similar results. **B**, U937 cells ectopically expressing Mcl-1 (*M*) or their corresponding empty vector counterpart were exposed to butyrate/flavopiridol and vorinostat/flavopiridol as above, after which apoptotic cells were monitored by Annexin V/propidium iodide staining by flow cytometry. **C**, U937/Mcl-1 and U937/EV cells were coexposed to sodium butyrate (1 mmol/L), vorinostat (1.5 μ mol/L), and flavopiridol (150 nm) either alone or in combination for 24 h, after which cells were washed free of drugs and plated in soft agar as described in Materials and Methods. Colonies, consisting of groups of ≥ 50 cells, were scored after 12 d. **D**, U937/Mcl-1 and U937/EV cells were exposed to vorinostat or butyrate and flavopiridol as indicated, and 30 μ g of proteins from the cytosolic S-100 fraction were analyzed by Western blot and probed with the corresponding antibodies against AIF, cytochrome *c*, or tubulin. Results of a representative study; two additional experiments yielded similar results. **E**, U937 cells were exposed to vorinostat (1.5 μ mol/L), vorinostat/flavopiridol (150 nmol/L), or to flavopiridol alone (100 or 150 nmol/L) for the indicated intervals and monitored for coimmunoprecipitation of Bak and Mcl-1. Lysates were immunoprecipitated with anti-Bak antibody, after which they were subjected to Western blot analysis using Mcl-1 antibody. **F**, analysis of Bak conformational change; U937 and U937/Mcl-1 cells were treated as described in **E**, after which levels of conformationally changed Bak were determined by first immunoprecipitating (IP) lysates with an anti-Bak-Ab1 antibody, which recognizes only the conformationally changed protein, followed by immunoblotting (WB) with an anti-Bak rabbit polyclonal antibody as described in Materials and Methods. Each lane was loaded with 30 μ g of protein; IgG controls confirm equivalent loading and transfer. Results of a representative study; two additional experiments yielded similar results. In all cases, values are means \pm SD for three separate experiments done in triplicate. +, whole lysate.

fragment was clearly discernible (Fig. 4A). As Mcl-1 is a known caspase substrate (30, 31), parallel studies were done in the presence or absence of the caspase inhibitor fmk-BOC (Fig. 4A), which revealed that caspase inhibition abrogated the appearance of the cleavage fragment and restored, although only partially, Mcl-1 levels (Fig. 4A).

To investigate the functional role of Mcl-1 down-regulation, genetically modified U937 cells ectopically expressing full-length Mcl-1 were used (13). Ectopic expression of Mcl-1 clearly protected against vorinostat or butyrate/flavopiridol-induced lethality ($P < 0.05$ in each case; Fig. 4B). In contrast to findings in cells ectopically expressing XIAP, in which protection from apoptosis was lost after 48 h of drug exposure, ectopic expression of Mcl-1

afforded partial protection against HDAC1/flavopiridol lethality over the entire 48-h exposure interval. However, as indicated by clonogenic survival assays (Fig. 4C), despite protecting cells from apoptosis for as long as 48 h, enforced expression of Mcl-1 was unable to provide long-term protection against the vorinostat or butyrate/flavopiridol regimen. Experiments were then conducted to identify the level at which Mcl-1 modifies vorinostat/flavopiridol lethality. Analysis of mitochondrial membrane potential ($\Delta\psi_m$) showed marked protection in U937/Mcl-1 cells compared with U937/empty vector cells. For example, whereas loss of $\Delta\psi_m$ in U937/empty vector cells exposed to vorinostat/flavopiridol or butyrate/flavopiridol was 75.4 ± 3.2 and 71.2 ± 3.5 respectively, these percentages

were reduced to 34.1 ± 2.3 and 31.7 ± 1.8 , respectively, in U937/empty vector cells ($P < 0.01$ in each case). Reduced mitochondrial injury was also reflected by a diminution in the amount of proapoptotic mitochondrial proteins (e.g., AIF and cytochrome *c*) released into the cytosol after 24- and 48-h exposure to these combination regimens (Fig. 4D). Together, these data indicate that, in contrast to XIAP, Mcl-1-mediated antiapoptotic functions operate upstream of the mitochondria. However, they also suggest that increased expression of Mcl-1, like XIAP, may delay but not completely protect cells from mitochondrial injury after vorinostat/flavopiridol exposure.

Recent reports indicate that Mcl-1 antiapoptotic activity operates in part through binding and regulation of the proapoptotic molecule Bak (32). Immunoprecipitation analysis of Mcl-1/Bak interactions revealed the disappearance of Mcl-1 from its complex with Bak between 2 and 4 h of exposure to vorinostat and flavopiridol (Fig. 4E), most likely reflecting pronounced Mcl-1 down-regulation by this regimen (Fig. 4A). Notably, although vorinostat (1.5 $\mu\text{mol/L}$) or flavopiridol alone had negligible effects, the reduction in Mcl-1 associating with Bak in cells exposed to 150 nmol/L flavopiridol and 1.5 $\mu\text{mol/L}$ vorinostat was similar to that observed in cells exposed to 150 nmol/L flavopiridol alone (Fig. 4E). This suggests that dissociation of Bak from Mcl-1 in vorinostat/flavopiridol-treated cells primarily reflects flavopiridol-mediated Mcl-1 down-regulation. Finally, analysis of Bak activation, reflected by conformational change (33) in lysates from U937/empty vector and U937/Mcl-1 cells exposed to vorinostat/flavopiridol for varying intervals, revealed a marked increase in active Bak in parental cells (Fig. 4F). In contrast, vorinostat/flavopiridol-mediated Bak conformational change was markedly attenuated in two U937 clones ectopically expressing Mcl-1 (clones 14 and 16, Fig. 4F). Last, a concentration-dependent increase in activated Bak was evident in lysates from U937 cells exposed to increasing concentrations of flavopiridol, reflecting the negative regulation of Mcl-1 by this agent (Fig. 4F).

Mcl-1 Expression Is Transcriptionally Regulated

Expression of Mcl-1 can be regulated at both the transcriptional (34, 35) and/or posttranslational levels (30, 36). Analysis of mRNAs from U937 cells exposed for 4, 8, 16, and 24 h to either the drugs alone (sodium butyrate, vorinostat, and flavopiridol) or their corresponding combinations (vorinostat/flavopiridol and butyrate/flavopiridol) was done by real-time RT-PCR. Extracts from U937 cells exposed to vorinostat or butyrate alone displayed a transient increase in levels of Mcl-1 mRNA over the initial 8-h interval, declining to basal levels by 24 h (Fig. 5A). On the other hand, cells exposed to flavopiridol alone, or flavopiridol in combination with vorinostat or butyrate, exhibited a dramatic decrease in Mcl-1 mRNA level after 4-h exposure, which persisted throughout the treatment interval (Fig. 5A). These findings suggest transcriptional repression of Mcl-1 mRNA by flavopiridol.

Recent reports have shown that the Mcl-1 promoter is repressed directly by E2F1 (34, 37) and that flavopiridol-

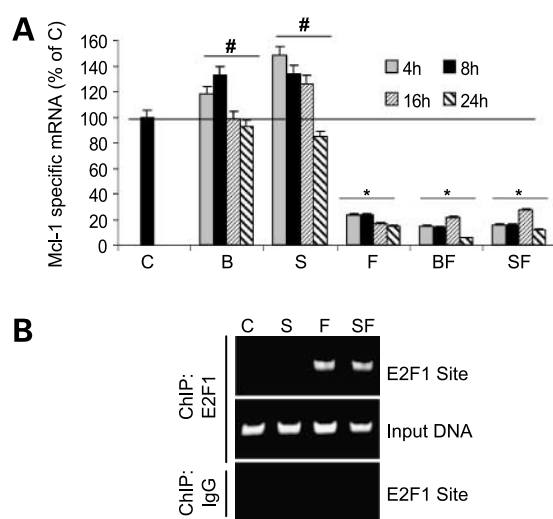


Figure 5. Regulation of Mcl-1 expression by flavopiridol. **A**, U937 cells were exposed to sodium butyrate (1 mmol/L), vorinostat (1.5 $\mu\text{mol/L}$) \pm flavopiridol (150 nmol/L) for 4, 8, 16, and 24 h after which total RNAs were extracted and expression of Mcl-1 mRNA was monitored by real-time RT-PCR as described in Materials and Methods. Values for each condition are expressed as the percentage of specific Mcl-1/18S mRNAs normalized to levels corresponding to the untreated control U937 cells (100%). *Columns*, means for three separate experiments done in triplicate; *bars*, SD. *, $P < 0.01$, significantly less than values for untreated cells. #, $P < 0.05$, significantly greater than values for untreated cells. **B**, association of E2F1 with the Mcl-1 promoter was evaluated by ChIP assay in U937 cells treated with vorinostat, flavopiridol, or vorinostat/flavopiridol for 4 h as described in Materials and Methods. Chromatin was immunoprecipitated using E2F1 antibody or IgG. The immunoprecipitated DNA was amplified by PCR using primers targeting regions around the E2F1 site present in the promoter region of the *Mcl-1* gene.

induced apoptosis in H1299 lung cancer cells involves a concentration-dependent increase in E2F1 protein levels accompanied by Mcl-1 down-regulation (38). In addition, we previously reported that HDACI/flavopiridol-induced apoptosis in human leukemia cells was associated with disruption of pRb/E2F1 complexes, resulting in increased availability of E2F1 (12). Western blot analysis of whole-cell lysates from U937 cells exposed to vorinostat \pm flavopiridol revealed no significant changes in the levels of E2F1 protein (data not shown). To determine whether E2F1 may be involved in the negative regulation of Mcl-1, ChIP assays were done using extracts from U937 cells exposed to vorinostat \pm flavopiridol for 4 h (Fig. 5B). As shown, a marked increase in the association of E2F1 with the Mcl-1 promoter was observed in lysates from cells exposed to flavopiridol alone or vorinostat/flavopiridol. These results raise the possibility that enhanced association of E2F1 with the Mcl-1 promoter in vorinostat/flavopiridol-treated cells may contribute to the observed Mcl-1 down-regulation.

Analysis of XIAP and Mcl-1 Regulation in Primary AML blasts

To determine whether the events described here in continuously cultured cell lines also occurred in primary AML specimens, parallel studies were done in leukemic blasts obtained from the bone marrow of two patients with

AML (French-American-British classification M2). Both samples exhibited a clear increase in apoptosis when exposed for 24 h to the combination of vorinostat and suberoylanilide hydroxamic acid (Fig. 6A). Interestingly, long-term survival as determined by clonogenic assay (expressed in leukemic colony-forming units) was able to be determined for one of the specimens (patient 1) and revealed a significant decline in clonogenic survival after combined drug treatment (Fig. 6B). Analysis of protein lysates by Western blot from the two AML primary cell samples after exposure to vorinostat \pm flavopiridol for 8 or 24 h showed a clear increase in poly(ADP)ribose polymerase degradation for the drug combination. Analogous to findings in U937 cells, combined exposure to vorinostat and flavopiridol resulted in a dramatic decline in XIAP levels in both AML samples, associated with the faint appearance of a cleavage fragment (Fig. 6C). Flavopiridol also induced Mcl-1 down-regulation in both samples, whereas coexposure to vorinostat induced essentially the complete loss of full-length Mcl-1, an event accompanied by extensive degradation.

Finally, RNA extracts from sample 1 collected after 8 and 24 h of treatment as above were analyzed by real-time RT-PCR (Fig. 6D). Quantitative analysis of specific XIAP mRNAs showed that whereas exposure to vorinostat or

flavopiridol alone modestly reduced XIAP mRNA levels, combined exposure reduced levels by over 90% relative to baseline. In the case of Mcl-1, vorinostat alone had little effect, whereas flavopiridol slightly reduced mRNA levels. However, combined treatment resulted in a substantial reduction (i.e., 90%). Together, these findings indicate that exposure of at least some AML blast specimens to vorinostat/flavopiridol induces similar changes in XIAP and Mcl-1 protein and mRNA levels as those observed in established leukemia cell lines.

Discussion

The goal of this study is to investigate the functional role played by down-regulation of the short-lived antiapoptotic proteins XIAP and Mcl-1 in leukemic cell apoptosis induced by the HDACi vorinostat and the CDK inhibitor flavopiridol, and to clarify the mechanisms underlying these events. Previously, flavopiridol was shown to block vorinostat-mediated induction of p21^{WAF1/CIP1}, thereby disrupting cell cycle arrest and differentiation and redirecting human leukemia cells to an alternative cell death program (11, 12, 39). Similar findings involving other HDACi have been described in lung, esophageal, and malignant pleural mesothelioma cells (14, 15). However, the inability

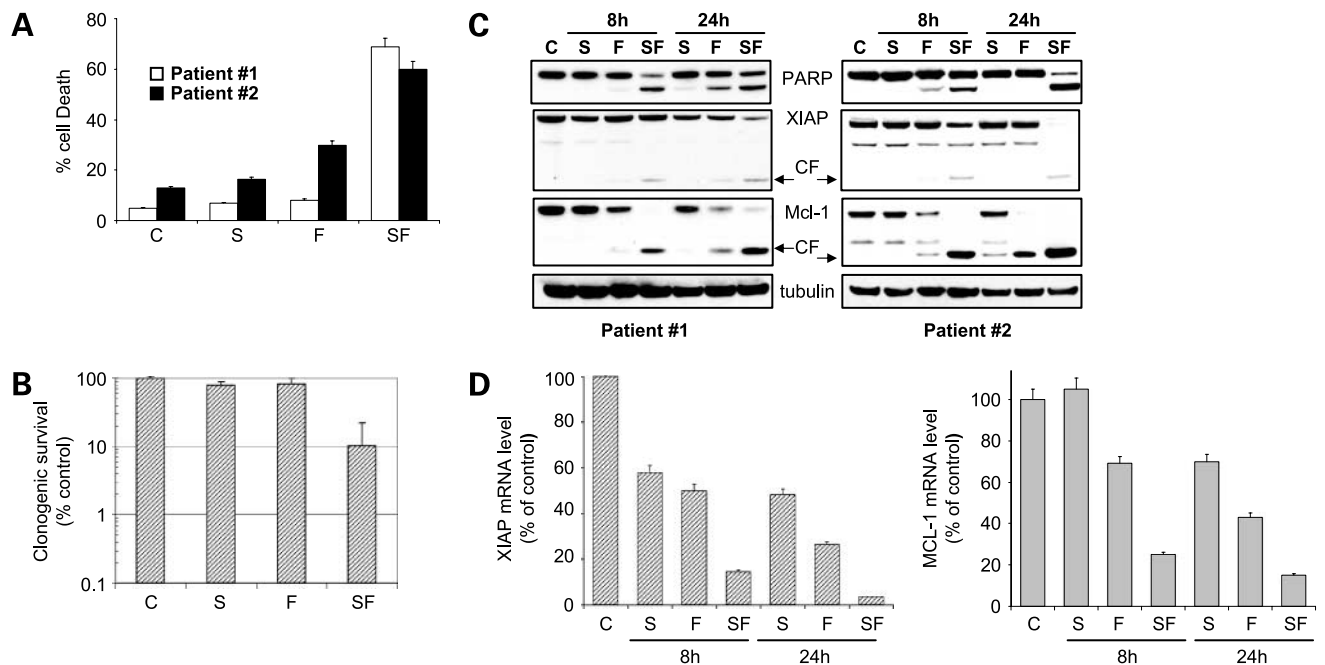


Figure 6. Flavopiridol and vorinostat modulate XIAP and Mcl-1 expression in primary AML blasts. **A**, primary blasts from the peripheral blood of two patients with AML (French-American-British classification F2; > 70% blasts) were obtained as described in Materials and Methods and exposed for 24 h to vorinostat (1.5 μ mol/L), flavopiridol (150 nmol/L), or their combination, and evaluated for cell death by Annexin V/propidium iodide staining. Columns, means for three separate experiments; bars, SE. **B**, blasts from patient 1 treated as described in **A** were washed free of drugs and plated in methylcellulose. Colonies (expressed in leukemic colony-forming units), consisting of groups of ≥ 20 cells, were scored after 12 d. Columns, means for triplicate determinations; bars, SD. **C**, after treatment with vorinostat \pm flavopiridol as above for 8 or 24 h, cell lysates (30 μ g) were analyzed by Western blot and probed with antibodies directed against poly(ADP)ribose polymerase, XIAP, Mcl-1, and tubulin. Bone marrow blasts from two AML patients were isolated and treated as indicated above for 8 and 24 h, after which total RNAs were extracted and expression of XIAP (left) and Mcl-1 (right) were monitored by real-time RT-PCR. **D**, values for each condition are expressed as the percentage of specific mRNA/18S mRNA normalized to levels corresponding to the untreated control cells (100%). Columns, mean for experiments done in triplicate; bars, SD.

of enforced expression of p21^{WAF1/CIP1} to block HDACi/flavopiridol-induced lethality completely (40) argues that additional mechanisms are likely be involved. The present studies were prompted by recent evidence, suggesting a critical role for the antiapoptotic proteins XIAP and Mcl-1 in regulating drug-induced cell death in leukemia cells (18, 20, 41), as well as the established capacity of flavopiridol to down-regulate expression of these short-lived proteins in malignant human hematopoietic cells (18, 42). Currently, direct evidence of a role for perturbations in XIAP and Mcl-1 expression in flavopiridol/vorinostat-mediated apoptosis is currently lacking, as is a clear understanding of the mechanisms by which changes in expression of these proteins occur.

The present results show that flavopiridol-mediated inhibition of XIAP transcription is markedly enhanced by coexposure to vorinostat. Both HDACi and flavopiridol, administered individually, have previously been shown to reduce XIAP expression, most likely by different mechanisms (7, 19, 20, 43). For example, down-modulation of both XIAP mRNA and protein by the HDACi MS-275 (6) and LAQ-824 (7) has been described. Consistent with these findings, vorinostat induced a modest reduction in XIAP mRNA levels. On the other hand, flavopiridol-mediated XIAP down-regulation has been observed in a variety of cell types, including human breast cancer cells (43) and leukemia cells (18–20). This action presumably reflects inhibition of CDK-7 and CDK-9, and, by extension, phosphorylation of the carboxyl-terminal domain of the large subunit of RNA polymerase II, resulting in inhibition of transcriptional initiation and elongation (28, 44). The present results revealed clear flavopiridol-mediated inhibition of carboxyl-terminal domain phosphorylation and that this effect was enhanced in cells coexposed to HDACi. In view of evidence of interplay between alterations in chromatin conformation as well RNA polymerase II in transcription regulation (45), it is possible that HDACi such as vorinostat may cooperate with flavopiridol to inhibit the transcription of XIAP at multiple levels. Finally, the partial caspase dependence of XIAP down-regulation suggests that this event may amplify the apoptotic response.

The present results also raise the possibility of NF- κ B involvement in regulation of XIAP expression. In this context, the antiapoptotic effects of NF- κ B are mediated in part through regulation of XIAP expression (29). In addition, flavopiridol has previously been reported to oppose NF- κ B activation by inhibiting I κ B kinase (46), and recent evidence suggests that this action contributes functionally to HDACi/flavopiridol lethality (16). Notably, ChIP analysis showed that flavopiridol induced a dramatic decline in the association of p65/RelA with the XIAP promoter, suggesting that flavopiridol-mediated XIAP down-regulation may proceed, at least in part, through an NF- κ B-dependent process. Thus, flavopiridol may act directly to block XIAP transcription in vorinostat-treated cells, by inhibiting RNA polymerase II, and also indirectly, by interfering with NF- κ B-dependent gene induction.

XIAP exerts its antiapoptotic actions primarily downstream of mitochondria through inhibition of caspase-3, caspase-7, and caspase-9 (26). Consistent with this notion, ectopic expression of XIAP blocked caspase activation in flavopiridol/vorinostat-treated cells, at least at early exposure intervals, but was ineffective in preventing mitochondrial injury. Furthermore, increased XIAP expression delayed, but did not ultimately prevent, lethality, consistent with previous reports that cells experiencing mitochondrial injury in the presence of caspase inhibition undergo caspase-independent cell death (47). Moreover, ectopic expression of XIAP was minimally effective in protecting clonogenic cells from flavopiridol/vorinostat-mediated lethality. Such findings are similar to those of an earlier report in which increased expression of Bcl-2 failed to restore clonogenic survival in transformed cells exposed to cytotoxic agents (48). Collectively, these findings suggest that down-regulation of XIAP may accelerate apoptosis in flavopiridol/vorinostat-treated leukemia cells, but that increased expression of this antiapoptotic protein may not protect self-renewing leukemia cells from the lethal effects of this regimen.

In striking contrast to the effects of flavopiridol, vorinostat induced an early, albeit transient, increase in Mcl-1 mRNA. Induction of Mcl-1 in response to various cytotoxic stimuli (49) or differentiation-inducing agents (50) is a well-described phenomenon. Notably, coexposure to flavopiridol antagonized this process and resulted in a dramatic decrease in mRNA and protein levels. Although these events may reflect the inhibitory effects of flavopiridol on RNA polymerase II, recent studies have shown that the proapoptotic transcription factor E2F1 exerts a negative regulatory effect on *Mcl-1* gene expression (34, 37) and that this interaction is modulated by flavopiridol (38). The present finding that flavopiridol increased the association of E2F1 with the Mcl-1 promoter is consistent with this notion. Collectively, these findings raise the possibility that flavopiridol may inhibit vorinostat-mediated Mcl-1 induction by both disrupting RNA polymerase II function and by enhancing the negative regulatory effects of E2F1 on Mcl-1 transcription.

Although XIAP primarily exerted its antiapoptotic effects downstream of mitochondria, enforced expression of Mcl-1 was more effective in blocking flavopiridol/vorinostat-mediated mitochondrial injury, at least at early intervals. There is abundant evidence of a role for Mcl-1 in suppressing mitochondrial damage and cytochrome *c* release by forming heterodimers with and neutralizing the actions of the proapoptotic molecules such as Bak (51). The finding that flavopiridol-mediated Mcl-1 down-regulation was associated with its disappearance from the Bak/Mcl-1 complex is consistent with this mode of action. In addition, recent reports have also shown that the carboxyl-terminal domain of Mcl-1 exerts a proapoptotic effect after caspase-3 cleavage through the generation of a fragment that interacts with tBid and Bak to induce mitochondrial injury (30). However, although the finding that ectopic expression of Mcl-1 attenuated flavopiridol/vorinostat-mediated

mitochondrial injury and lethality are consistent with previous studies showing protection from DNA-damaging agents (41, 52), it is important to note that enforced expression of Mcl-1 only partially protected cells from release of proapoptotic mitochondrial proteins and apoptosis. Significantly, ectopic expression of Mcl-1 failed to protect clonogenic cells from the flavopiridol/vorinostat regimen. Together, these observations suggest that although Mcl-1 down-regulation is likely to play a significant functional role in early apoptosis induced by this drug combination, it primarily delays rather than prevents mitochondrial injury, indicating that additional events (e.g., perturbations in p21^{WAF1/CIP1}, XIAP, and NF- κ B; refs. 16, 40) very likely to contribute to lethality. Furthermore, although down-regulation of both antiapoptotic proteins (XIAP and Mcl-1) may not constitute the primary cause of HDACI/flavopiridol-induced cell death, it is clear from these studies that they represent important factors that significantly contribute to the dramatic apoptotic response observed in leukemia cells exposed to both of these agents.

The present findings share some common features with results of a recent study examining interactions between the purine nucleoside analogue and CDK inhibitor roscovitine and the novel hydroxamic acid HDACI NVP-LAQ-824 in human leukemia cells. However, they also exhibit important differences. For example, although both regimens were associated with diminished expression of Mcl-1 and XIAP, pretreatment for 24 h with NVP-LAQ-824 before the addition of roscovitine was required to achieve optimal effects (22), whereas simultaneous exposure to vorinostat and flavopiridol was the most effective regimen. In addition, when administered alone, roscovitine failed to exert an inhibitory effect on XIAP or Mcl-1 mRNA levels, whereas flavopiridol was very effective in this regard. Moreover, coexposure to roscovitine and NVP-LAQ-824 had no inhibitory effect on Mcl-1 transcription; instead, down-regulation of this protein was principally caspase dependent (13). In contrast, in flavopiridol/vorinostat-treated cells, Mcl-1 down-regulation chiefly occurred at the transcriptional level but was enhanced by caspase-mediated degradation. These findings suggest that the mechanism(s) underlying down-regulation of Mcl-1 and XIAP in human leukemia cells may vary significantly after exposure to different CDK and HDACIs. Whether such disparate findings reflect differential effects on other involved pathways (e.g., p21^{WAF1/CIP1} and NF- κ B) remains to be determined.

These findings could have implications for the use of Mcl-1 and XIAP expression as surrogate end points in future clinical trials combining HDACIs and CDK inhibitors in leukemia. In this context, it is worth noting that vorinostat/flavopiridol-mediated transcriptional repression of Mcl-1 and XIAP occurred in primary AML samples and, in at least one sample, was associated with a marked reduction in leukemic cell clonogenic growth. It is therefore possible that down-regulation of XIAP and Mcl-1 may, in conjunction with other relevant molecular determinants (e.g., down-regulation or inactivation of p21^{WAF1/CIP1} and/or NF- κ B), provide surrogate markers for disease respon-

siveness. In view of the very recent initiation of phase I trials of vorinostat and flavopiridol in leukemia and other malignant diseases, this hypothesis should be subject to validation.

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