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FLAVOPIRIDOL AND HISTONE DEACETYLASE INHIBITORS PROMOTE MITOCHONDRIAL INJURY AND CELL DEATH IN HUMAN LEUKEMIA CELLS OVEREXPRESSING BCL-2

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Running Title: CDK and HDAC inhibition in Bcl-2 overexpressing cells

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Non-standard abbreviation: SAHA – suberoylanilide hydroxamide, HDACI - histone deacetylase inhibitor

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ABSTRACT

Interactions between the cyclin-dependent kinase (CDK) inhibitor flavopiridol and histone deacetylase (HDAC) inhibitors (SAHA and sodium butyrate; SB) were examined in human leukemia cells (U937, HL-60) ectopically expressing Bcl-2/Bcl-x_L and in primary AML cells. Co-administration of flavopiridol with HDAC inhibitors synergistically potentiated mitochondrial damage (cytochrome c, Smac/DIABLO, and AIF release), caspase activation, PARP 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 ara-C. Enforced expression of dominant-negative FADD failed to protect cells from the flavopiridol/HDACI regimen, arguing against the involvement of the receptor pathway in lethality. Ectopic expression of a phosphorylation loop-deleted Bcl-2 or Bcl-2 lacking the $serine_{70}$ 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, nor did it attenuate Bax dimerization. Collectively, 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.

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INTRODUCTION

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 (HATs) 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 co-repressor 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 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 Hsp90 function (George et al., 2005), and down-regulation of anti-apoptotic proteins (Vrana et al., 1999), among others.

The semi-synthetic 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 may stem from various actions other than

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or in addition to CDK inhibition, including disruption of the CDK9/cyclin T transcription complex and inhibition of phosphorylation of the carboxy-terminal domain of RNA polII (Chao and Price, 2001), induction of mitochondrial injury (Almenara et al., 2002), down-regulation of anti-apoptotic proteins such as Mcl-1 (Gojo et al., 2002), and inhibition of NF-kB (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 anti-apoptotic proteins such as XIAP (Rosato et al., 2004), and attenuation of NF-κB 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 non-hematologic malignancies.

Bcl-2 and Bcl-x_L are multi-domain members of the Bcl-2 family which block activation of the intrinsic, mitochondrial apoptotic pathway by preventing release of pro-apoptotic mitochondrial proteins such as cytochrome c into the cytosol (Yang et al., 1997). Cytosolic cytochrome c activates a multi-protein 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 appeared logical to postulate that a regimen containing both flavopiridol and

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HDACIs might be effective in killing cells overexpressing these anti-apoptotic proteins. Currently, a systematic analysis of the effects of flavopiridol and HDACIs on leukemic cells overexpressing Bcl-2 or Bcl-x_L has not 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 utilizing leukemic cells ectopically expression Bcl-2 lacking either the phosphorylation loop region or a critical phosphorylation site (Ser_{70}), 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 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 resistance to mitochondrial injury conferred by Bcl-2 and Bcl-x_L, and may therefore represent an effective strategy against leukemia cells over-expressing certain proteins conferring resistance to standard agents.

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MATERIALS AND METHODS

Cell Lines.

U937 cells were obtained from ATCC (Rockville, MD), cultured in RPMI 1640 medium, and were transfected with either full-length Bcl-2 or Bcl-2 lacking residues 32-80 as previously described in detail (Wang et al.1999a)· For all studies, two clones (U937-Bcl-2 G3 and D9; U937- pCEP4 B9 and B11) were employed. Bcl-x_L over-expressing 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 ($\Delta_{32\text{-}80}$) or Bcl-2 containing a $serine_{70}$ site mutation (e.g., serine to alanine; S70A) with a empty-vector pSFFV control were constructed as previously described (Wang et al.1999a). The point mutant and loop deletion were verified by direct DNA sequencing using a D-rhodomine terminator (Perkin-Elmer, Wellesley, 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) and a 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) as described earlier (Wang et al. 1997). All transfectants were maintained under appropriate selection pressure (400ug/mL of G418) 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 non-

myeloid hematologic disorders and isolated using an immunomagnetic bead separation technique as previously described (Yu et al., 2003). These studies have been approved by the investigational review board of MCV/VCU. Bone marrow samples were collected in sterile syringe containing heparin and processed/ treated as described earlier (Yu et al., 2003)

Drugs and Chemicals.

Flavopiridol was kindly provided by Dr. Dimitrios Colevas of the Cancer Treatment and Evaluation Program of the 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 PharMingen, San Diego, CA, and was formulated as per the manufacturer's instructions. DSP crosslinker was obtained from PIERCE Biotechnology, Rockford, IL. All drugs were dissolved in DMSO and diluted in PBS before use. Dynabeads (M-450) Goat anti-Mouse IgG was obtained from Dynal Biotech, Luke Success, NY.

Experimental Format

Logarithmically growing cells were placed in sterile plastic T-flasks (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 600 x g for 10 min at room temperature, and prepared for analysis as described below.

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Assessment of cell death

After drug exposures, cells were stained with Annexin V/PI as described previously (Yu et al. 2003; Wang et al.1999a). In short, cells were washed with PBS and stained with Annexin V/PI for 30min. at room temperature. Cells were then acquired and analyzed using a Becton-Dickinson FACScan cytofluorometer (Mansfield, MA) with the help of Cell Quest software (Becton-Dickinson). In some cases, cytocentrifuge slides were prepared with Wright-Giemsa and 10 random fields were viewed by light microscopy to evaluate the extent of cell death (i.e., cell shrinkage, nuclear condensation, formation of apoptotic bodies, etc.) 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 RPMI supplemented with 20% FBS, 0.3% Bacto agar (Difco, Detroit, MI) as previously described in detail (Almenara et al.,2002)· Plates were incubated at 37˚ C and colonies consisting of ≥ 50 cells were scored at the 10^{th} 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 µl of lysis buffer for 1 min containing 75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, 250mM Sucrose and 350 µg/ml digitonin. The lysates were centrifuged at 12,000 x g for 5 min at 4^0C, and the supernatant was

collected and quantified using Coomassie protein assay reagent (Pierce, Rockford, IL). The protein samples were denatured with 4X loading buffer (Invitrogen) supplemented with 50 mM DTT (dithiothreitol). Denatured protein samples were separated by 4-12% gradient Bris-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, Rockford, IL as per manufacturer instruction. 2x 10^7 cells were pelleted by centrifugation at 850g for 2 minutes and were resuspended in 800µl of reagent A in micro centrifuge tube. Then cells were incubated in ice for 2.0 minutes and subsequently homogenized in a pre-cooled Dounce tissue grinder applying 40-50 strokes. 800µl of reagent C was added to the homogenized solution and thoroughly mixed by repeated inversion. The entire mixed solution was centrifuged at 700g for 10 minutes and pellet was discarded. The supernatant was further centrifuged at 12000g for 15 minutes and the pellet was considered as intact mitochondria. This fraction was further lysed in lysis buffer (Wang et al.1999a; Wang et al., 1999b) 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, lysed immediately in lysis buffer. Protein content of the homogenates was quantified using a Coomassie protein assay reagent from Pierce, Rockford, IL and denatured with 4X loading buffer (Invitrogen) supplemented with 50 mM

DTT. Equal amounts of protein (25μg) was separated by 4-12% gradient Bris-Tris gel and probed with appropriate antibodies. Sources of antibodies were as follows; Bcl-2 from Dako; Carpenteria, CA; Bid from R&D Systems, Minneapolis, MN; cytochrome c, caspases-3, and -9 from Pharmingen, San Diego, CA.; PARP from Biomol, Plymouth, MA.; cleaved caspase 3 from Cell Signaling, Beverly, MA; Bax, Smac, AIF, XIAP, Bad, p-Bad, cytochrome C, Tubulin, CoxIV from Santa Cruz Biotechnology Inc., Santa Cruz, CA ; Caspase-8 from Alexis, San Diego,CA; Bax antibody directed against conformationally changed protein (6A7) was obtained from Sigma, St. Louis, MO. Blots were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz,CA), washed, and developed by enhanced chemiluminescence (Perkin Elmer Bioscience, Boston, MA).

Detection of Bax conformational change

Mitochondria-rich fraction of cells with or without adequate treatment was prepared as described above. Then mitochondria rich fraction was washed with Ca^{2+}/Mg^{2+} free PBS and lysed with the Chaps lysis buffer (10mM Hepes, pH 7.4, 150 mM NaCl, 1% chaps, 1mM DTT) supplemented with protease and phosphatase inhibitors. Two milligrams of anti–Bax (6A7) monoclonal antibody was pre-incubated with 30μl of Dynabeads for 2 hrs. at 4^0C on a rotor. A total of 500-1000ug of protein was then added to the anti-Bax antibody loaded with dynabeads and maintained on a rotor for overnight at 4^0C. Dynabeads were collected utilizing a Dynal Magnetic Particle concentrator and washed four times with Chaps lysis buffer. Conformationally changed Bax protein was eluded from the dynabeads by heating with 4x 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. Mitochondrial-rich fragment was prepared as described above and it was incubated for 0.5-1hr. at room temperature with 2mM of DSP in DMSO in order cross-linking reaction to take place. Cross linking reaction was terminated by adding Tris –HCl to a final concentration of 20mM and by incubating for 15 min. at room temperature. The mitochondrial pellet was separated by centrifugation at 10000g for 30 min. and re-dissolved in chap's lysis buffer. Lastly, 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 *t* test.

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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 (SB: 1.0 mM), or SAHA (1.0μM) alone or in combination for 24 hr (Figure 1A). As previously described (Almenara et al., 2002), co-administration 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 shown in Fig.1A (insert) 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 that their empty vector counterpart Results were equivalent in cells expressing Bcl-x_L versus control pcDNA3.1 (Figure 1B). In contrast, ectopic expression of Bcl-2 (Figure 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 (Figures 1D and E). In contrast, cleavage of caspase-3 and Bcl-2 as well as PARP degradation were essentially abrogated in U937/Bcl-2 cells exposed to ara-C (Figure 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 are 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 flavopridol (125nM)/SAHA (1.5μM) either alone or on combination for 24 hrs. Notably, combined treatment with flavopiridol and SAHA induced cell death to a similar extent (e.g., 60-65%) in both HL-60-pCEP4 or HL-60-Bcl-2 cells, whereas individual treatment exerted either very modest (flavopiridol) or no effects (SAHA) (Figure 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 (Figures 2B 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 hr to flavopiridol (100 nM) and SAHA (1.0μM) alone and in combination as in the case of U937 cells. As shown in Figure 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 hr resulted in a marked increase in PARP cleavage accompanied by caspase activation, cleavage of Bcl-2 (Figure 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

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flavopiridol for 12 hr 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 (Figure 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 (1.0μM) 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 which was not diminished by ectopic expression of Bcl-2 (Figure 4A). Similar results were obtained with combinations of flavopiridol and sodium butyrate, and in Bcl-x_L cells (data not shown). Bcl-2 overexpression also did not block flavopiridol/SAHA-mediated caspase-8 cleavage/activation, nor did this regimen 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 (Figure 4B). Lastly, consistent with the previous results, ectopic expression of Bcl-2 (Figure 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 agents, including flavopiridol (Wang et al.,1999a ; Decker et al., 2002), suggesting, albeit indirectly, that Bcl-2 phosphorylation may play a role in pro-apoptotic actions. To test this possibility directly, the effects of flavopiridol/HDACI administration were

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examined in U937 cells ectopically expressing a phosphorylation loop-deleted Bcl-2 mutant (Bcl-2-ΔN). As shown in Figure 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. Interestingly, 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% vs 78%; $P < 0.01$ compared to empty-vector controls). However, after 48 hr of treatment with flavopiridol (75nM) / HDACI (SAHA-0.75μM or sodium butyrate -0.75mM) regimens, cell death in the loop-deletant mutants was comparable to that of controls (Figure 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 ($_{ser}70\rightarrow_{ala}$) (Wang et al., 1999a). Such mutant cells were also resistant to flavopiridol/HDACI-mediated cell death at 24 hr, but this effect was also markedly reduced at 48 hr (Figures 5C and 5D). Together, these findings indicate that N-terminal loop deletion and loss of a critical Bcl-2 phosphorylation site delays but does 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-ΔN and Bcl-2-S70A). In empty-vector cells, flavopiridol (100 nM) modestly increased cytochrome c and smac/DIABLO release after 12 hr of treatment whereas SAHA alone was ineffective (Figure 6A). However, combined treatment resulted in a marked increase in cytosolic release of these pro-apoptotic effectors. However, in Bcl-2-ΔN cells, neither flavopiridol nor flavopiridol + SAHA induced mitochondrial injury after 12 hr of exposure, although after 24 hr, there was a marked increase in both cytochrome c and

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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-ΔN transfectants at 24 hr. 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-ΔN delayed but did not prevent flavopiridol/SAHA-mediated PARP degradation, as well as caspase-3 and Bcl-2 cleavage, but was highly effective in blocking these events following exposure of cells to ara-C (Figure 6B). Together, these findings indicate that loss of the Bcl-2 phosphorylation loop or the serine$_{70}$ 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). Consequently, colony forming studies were performed to determine whether similar events occurred in cells exposed to flavopiridol and SAHA. As shown in Figure 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 following flavopiridol/SAHA exposure.

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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 employed. As anticipated, U937-FADD-DN cells were significantly more resistant to cell death induced by TNF/CHX than their empty-vector counterparts (Figure 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 following exposure of cells to various noxious stimuli (Liu et al., 2003; Jia et al., 2001). Furthermore, the anti-apoptotic 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 (Figure 9A). However, combined exposure of cells to flavopiridol and SAHA resulted in a large increase in Bax translocation. Interestingly, 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 (Figure 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.

Lastly, 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 (Figure 9B). However, co-adminstration of flavopiridol and SAHA resulted in a substantially greater increase in Bax dimerization. Significantly, very similar results were observed in U937/Bcl-2 cells. It should be noted that a separate band appearing between the Bax-monomer and dimer was detected, which might reflect a non-specific protein or a cross-linked monomer, as previously reported (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 pro-apoptotic 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 multi-protein complex consisting of apaf-1, pro-caspase 9, and dATP, which in turn leads, through caspase 9 activation, to engagement of effector caspases such as caspase-3 (Hu et al., 1998). Anti-apoptotic multi-domain 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 pro-apoptotic 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). Consequently, the development of strategies capable of inducing cell death in tumor cells overexpressing Bcl-2 or Bcl-x_L represents a logical goal.

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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 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 HDACI-mediated maturation, and instead promote mitochondrial injury and cell death (Almenara et al., 2002; Takada and Aggarwal, 2004; Gojo et al., 2002). 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, HDACIs 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, HDACI-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/HDACI 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 HDACIs is cell type specific, and does not occur in U937 cells (Rosato and Grant, unpublished observations). More importantly, leukemia cells ectopically expressing dominant-negative FADD, and resistant to TNF-induced lethality, did not display resistance to the flavopiridol/HDACI 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 anti-

apoptotic multidomain Bcl-2 family members, rather than by engaging the Bcl-2-independent extrinsic cascade.

It is noteworthy that the flavopiridol/HDACI 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 serine$_{70}$ site (Wang et al.1999a). Such proteins classically confer greater resistant 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 MAP kinase JNK (c-Jun N-terminal kinase) is required for lethality (Wang et al., 1999a). It should be recognized that the consequences of Bcl-2 phosphorylation may differ with respect to cell type and stimulus. For example, phosphorylation of Bcl-2 (e.g., at serine$_{70}$) by the macrocyclic lactone bryostatin is required to prevent growth factor deprivation-induced cell death in murine hematopoietic cells (Deng et al., 1998). Interestingly, while both the loop-deleted Bcl-2 protein and the serine$_{70}$ 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 co-administration 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). Consequently, the possibility that interference with these phenomena by Bcl-2 and related proteins contribute to anti-apoptotic 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 multiple levels to inhibit apoptosis (Yang et.al.1997), it is noteworthy that cells that sustain mitochondrial injury but in which cell death in blocked (e.g., due to caspase inhibition) can undergo an alternative form of cellular demise (e.g., necrosis, autophagy etc.) (Edinger and Thompson, 2004). Thus, whether or not the flavopiridol/SAHA regimen can overcome resistance to classical 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 entirely consistent with this concept. Whatever the underlying mechanism(s), the present findings indicate co-administration of flavopiridol and HDACIs does not induce cell death in leukemic cells by bypassing the capacity of multi-domain anti-apoptotic proteins to prevent mitochondrial injury (e.g., Bax translocation and conformational change; cytochrome c release) and activating downstream or parallel components of the cell death pathway. Instead, this strategy appears 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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LEGENDS

Figure 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 or (B) U937,U937-Bcl-x_L and U937-pcDNA3.1 cells were treated with flavopiridol (100nM) or SAHA (1.0μM)/sodium butyrate (1.0mM) individually or in combination (C) U937-Bcl-2 and U937-pCEP4 cells were treated with 1.0μM of ara-C. The percentage of dead cells was monitored by Annexin V-FITC staining and flow cytometry and compared to untreated controls after 24hr of drug exposure. Values represent the means ± S.D. for three separate experiments performed in triplicate. * = significantly less than values for empty-vector controls; $P < 0.001$. (D) U937-pCEP4 cells or (E) U937-Bcl-2 cells were treated with flavopiridol (100 nM) $\pm$ SAHA (1.0 μM)/sodium butyrate(1.0 mM) (F) U937-Bcl-2 and U937-pCEP4 cells were treated with 1.0 μM ara-C for 12 hr. At the end of this interval, cells were lysed, denatured, and subjected to Western blot analysis using the indicated primary antibodies as described in Methods. 30 μg of protein were 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.

Figure 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 μM) individually or in combination. At the end of drug treatment, cell death was measured as described in 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 (B) HL-60-pCEP4 cells or (C) HL-60-Bcl-2 cells were treated with flavopiridol (125 nM) ± SAHA (1.5 μM) for 12 hrs. At the end of drug treatment, cells were lysed, denatured, and subjected to Western blot analysis using the indicated primary antibodies as described in Methods. 30 μg of protein were 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.

Figure 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 (1.0 μM) alone and in combination for 24hrs. At the end of this period, the percentage of dead cells were determined by Annexin V/PI staining and flow cytometry. Values represent the means ± S.D. for triplicate determinations. (B) Primary AML cells were treated with flavopiridol (100 nM) ± SAHA (1.0 μM) for 24 hr. At the end of drug treatment, cells were lysed, denatured and subjected to Western blot analysis using the indicated primary antibodies as described in methods. 30 μg of protein were 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.

Figure 4. Ectopic expression of Bcl-2 protects U937 cells from release of pro-apoptotic mitochondrial proteins, caspase activation, and PARP cleavage following 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 (1.0 μM) individually as well as in combination for 12hrs. (C) U937-Bcl-2 and empty vector U937-pCEP4 cells were treated with 1.0μM of ara-C for 12 hrs. After drug exposure, cytosolic (S-100) fractions and whole cell lysates were obtained as described in methods. Protein samples were subjected to Western blot analysis using the indicated primary antibodies. Each lane was loaded with 30 μg 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

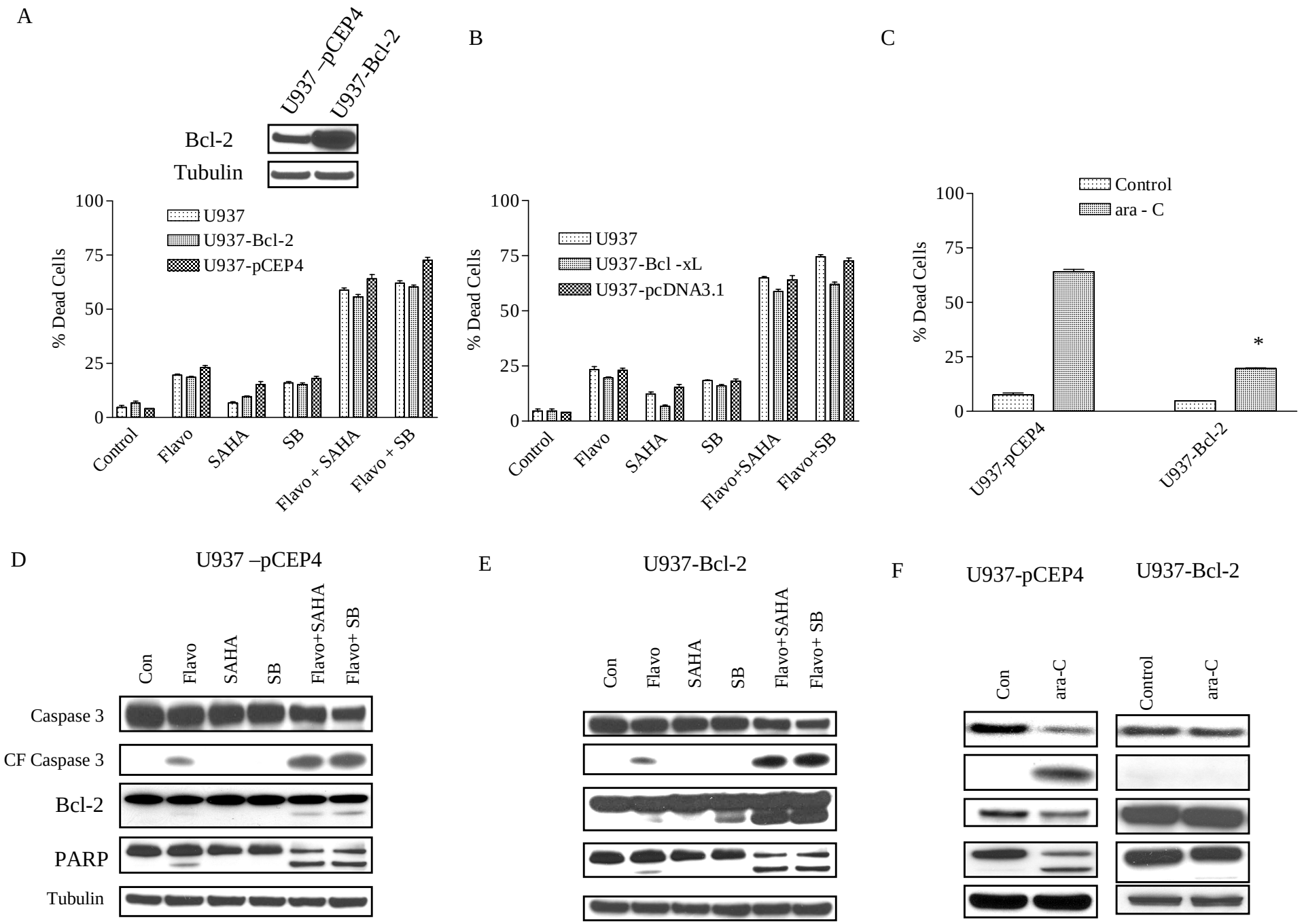
Figure 5. Ectopic expression of loop-deleted Bcl-2 (Bcl-2 ΔN) or a serine$_{70}$ site mutation (Bcl-2-S70A), fail to protect against flavopiridol and SAHA or sodium butyrate-induced cell death. U937, U937-Bcl-2-ΔN, U937- pSFFV cells were treated with (A) flavopiridol (100 nM) $\pm$ SAHA (1.0 μM)/sodium butyrate (1.0 mM) for 24 hrs or (B) flavopiridol (75 nM) $\pm$ SAHA (0.75 μM)/sodium butyrate (0.75 mM) for 48hrs. U937, U937-Bcl-2-S70A, U937-pSFFV cells were treated with (C) flavopiridol (100 nM) $\pm$ SAHA (1.0 μM)/sodium butyrate (1.0 mM) for 24 hrs.(D) flavopiridol (75 nM) $\pm$ SAHA (0.75 μM)/sodium butyrate (0.75 mM) for 48hrs. At the end of this interval, the percentage of dead cells were monitored by Annexin V-FITC staining and flow cytometry as described in Methods. Values represent the means ± S.D. for three separate experiments performed in triplicate.

Figure 6. Prolonged exposure to flavopiridol/SAHA but not ara-C induces release of pro-apoptotic mitochondrial proteins, activation/cleavage of caspase-3, and PARP degradation in U937 cells ectopically expressing loop-deleted Bcl-2 (Bcl-2 ΔN). U937-Bcl-2-ΔN and U937-pSFFV cells were treated with flavopiridol (100 nM) or SAHA (1.0 μM) alone and in combination for 12 hr and 24hr. U937-Bcl-2-ΔN cells were also treated with 1.0 μM ara-C for 24 hrs. After drug exposure, (A) cytosolic (S-100) fractions and (B) whole cell lysates were obtained as described in methods. Protein samples were subjected to western blot analysis using the indicated primary antibodies. Each lane was loaded with 30 μg 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.

Figure 7. Ectopically expression of full length Bcl-2, loop deleted Bcl-2 (Bcl-2-ΔN) or serine$_{70}$ 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 (100nM) and SAHA (1.0μM) for 24 hr. (B) U937-Bcl-2-ΔN and empty-vector U937-pSFFV were treated with flavopiridol (75nM) and SAHA (0.75μM)for 48 hrs. (C) U937-Bcl-2- S70A and empty-vector U937-pSFFV were treated with flavopiridol (75nM) and SAHA (0.75μM) for 48 hrs. At the end of drug treatment, cells were washed and plated in soft agar as described in methods. Colonies, consisting of groups $\geq$ 50 cells, were scored at day 10. Values for each condition were expressed as a percentage of control colony formation. For (A), (B) (C), values represent the means $\pm$ S.D. for three separate experiments performed in triplicate.

Figure 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 (1.0 μM) or tumor necrosis factor (10ng/ml)/cycloheximide (1.0 μM). The percentage of cell death was monitored by Annexin V-FITC staining and flow cytometry against an untreated control after 24hrs of drug exposure. Values represent the means $\pm$ S.D. for three separate experiments performed in triplicate.

Figure 9 Simultaneous exposure of U937 cells ectopically expressing full length Bcl-2 or empty vector to flavopiridol/SAHA induce translocation of cytosolic Bax to mitochondria, and Bax conformational change and dimerization (A) U937-Bcl-2 and U937-pCEP4 cells were treated with 100 nM of flavopiridol and 1.0 μM SAHA alone or in combination for 24 hr . Cells were harvested by centrifugation at 600g for 10min. at 4^0C and the mitochondrial-rich fraction was prepared as described in 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 in methods (B) Mitochondria reach fraction were prepared from U937-Bcl-2 and U937-pCEP4 cells treated as above and was incubated with DSP cross linker to allow oligomerization to take place as described in 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



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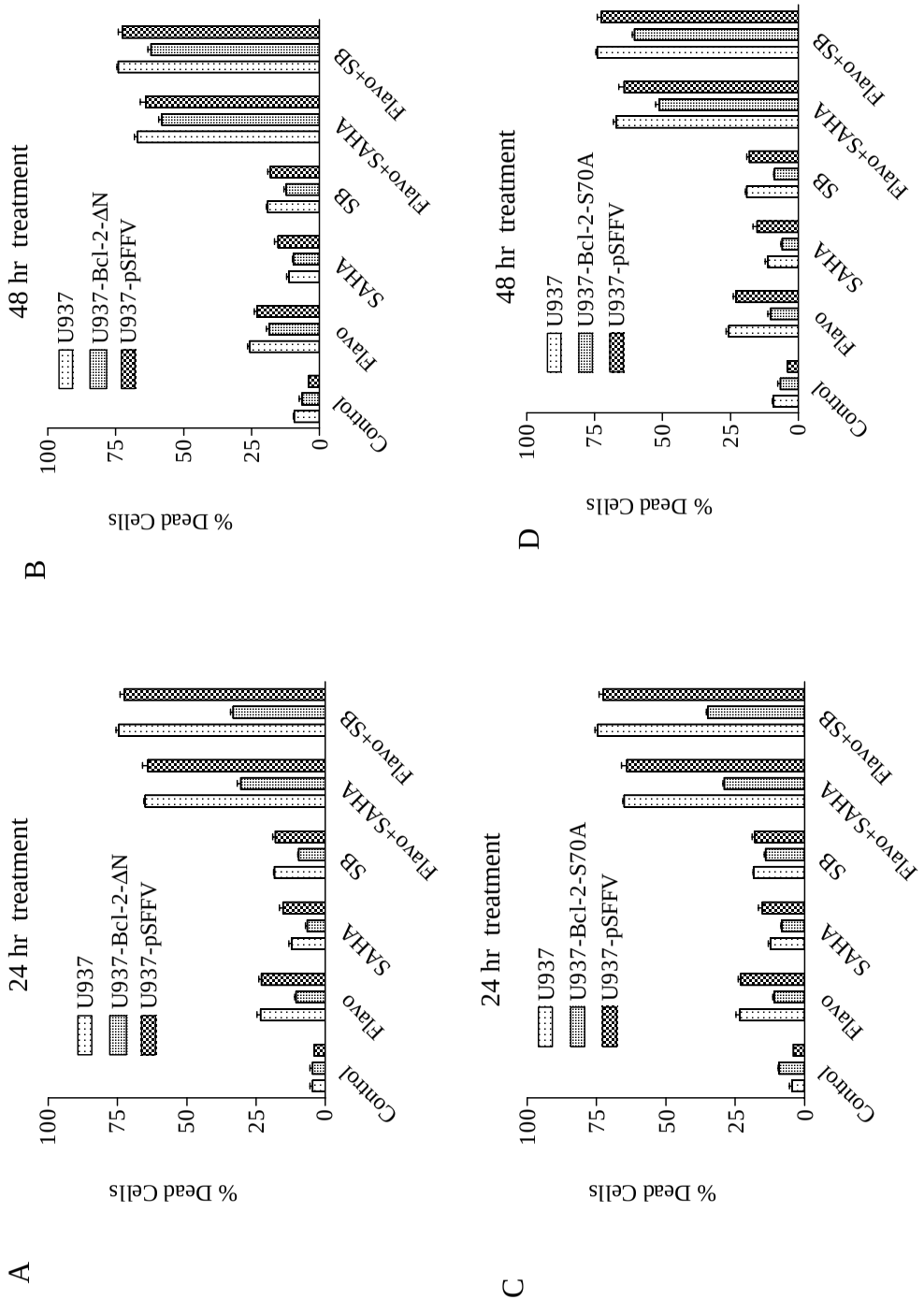

Fig.5

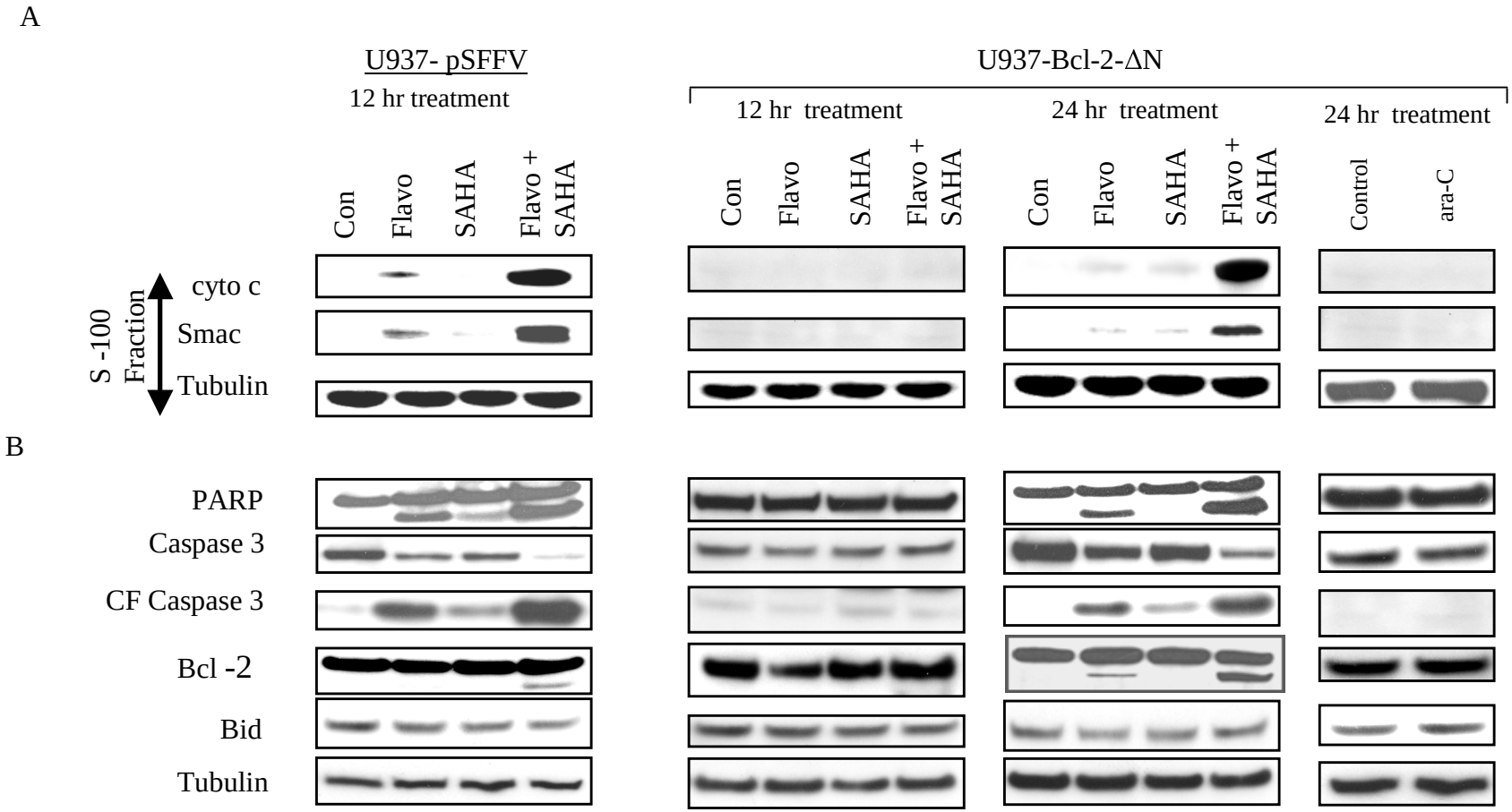

Fig.6

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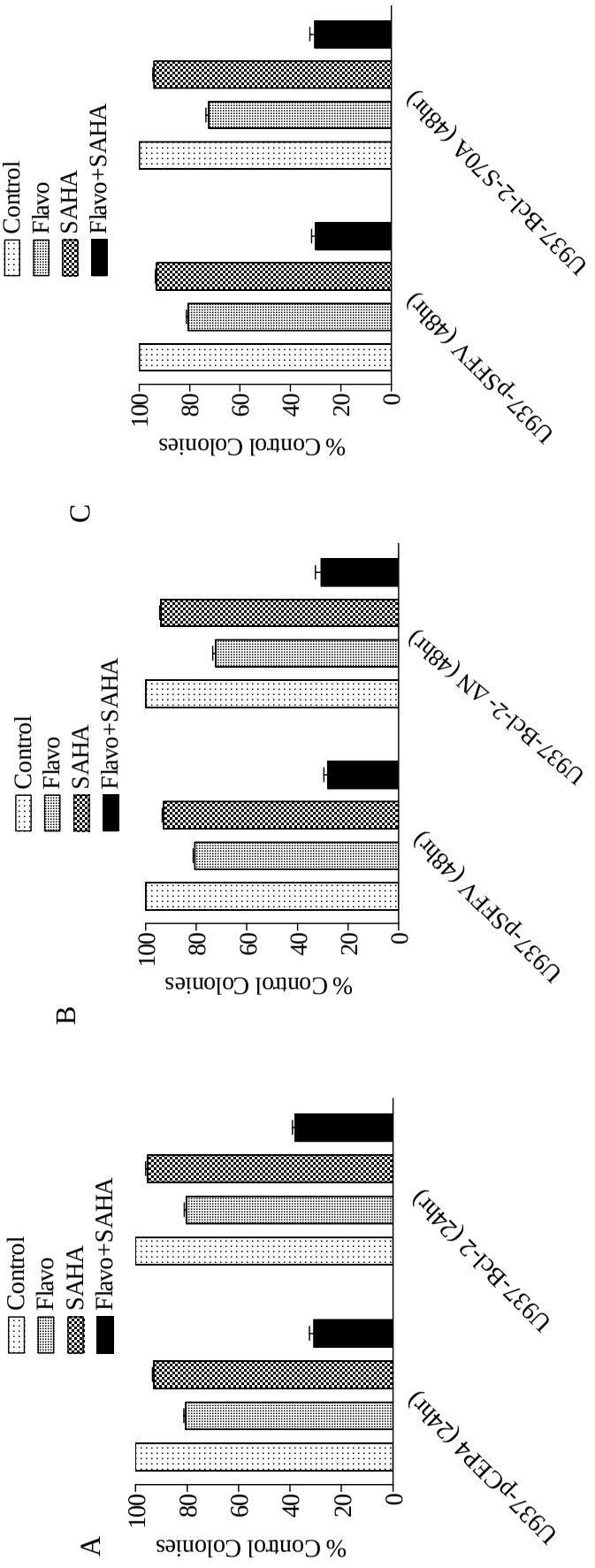

Fig.7

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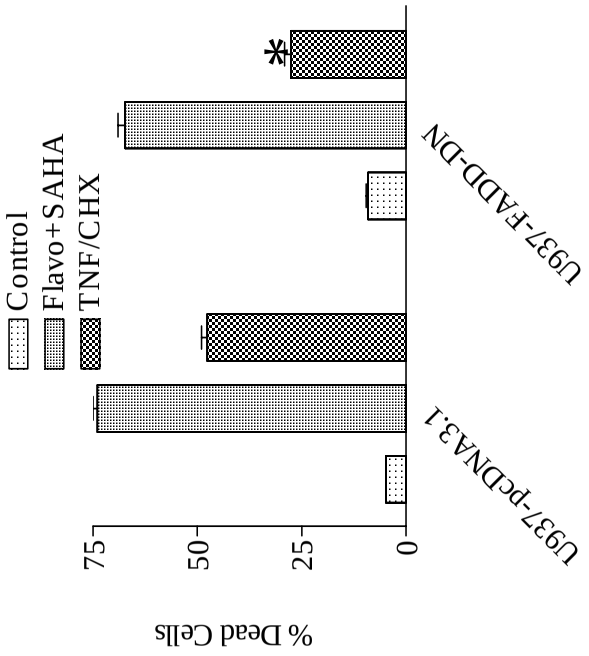

Fig.8

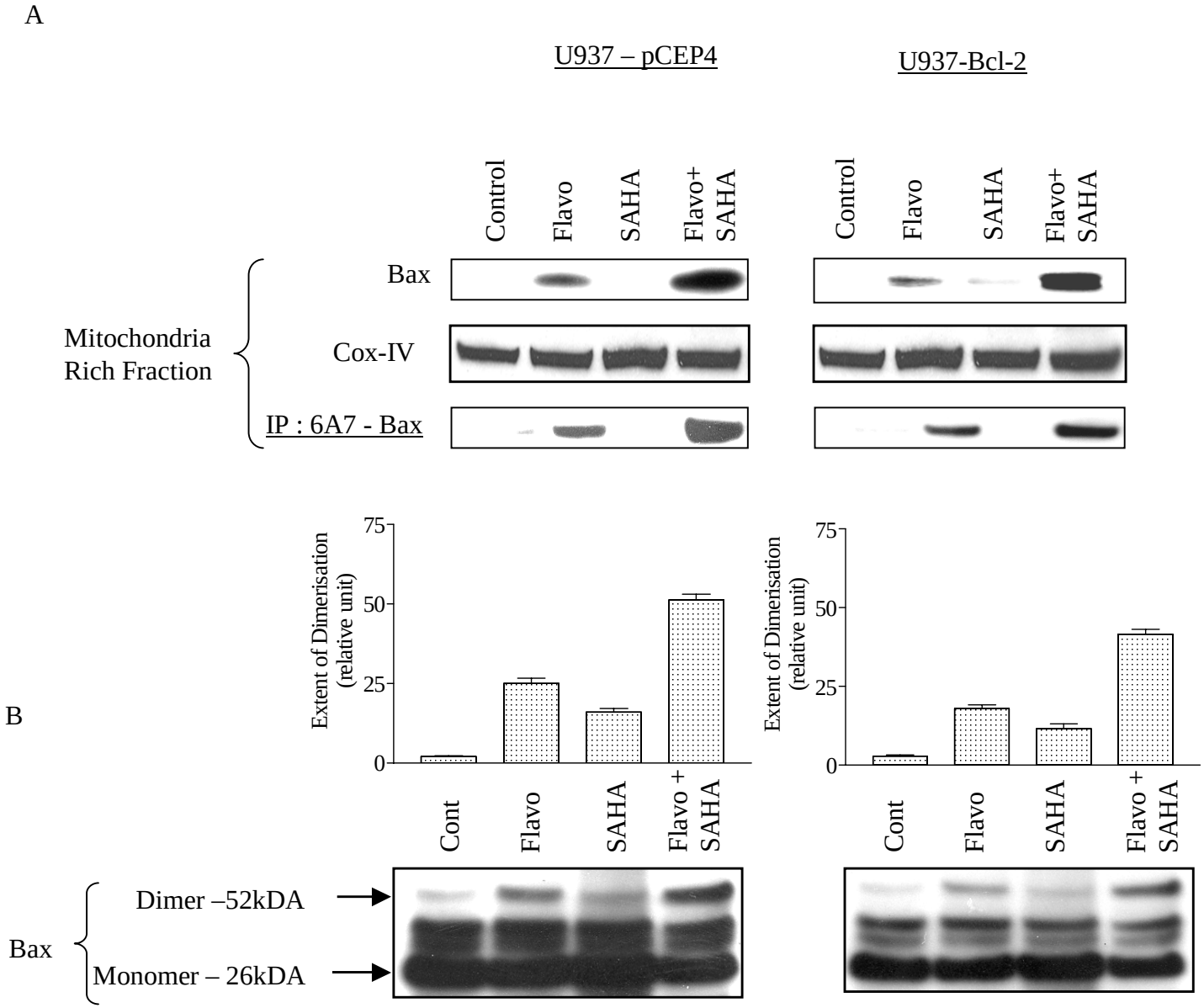

Fig.9