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CONTRIBUTION OF DISRUPTION OF THE NF-κB PATHWAY TO INDUCTION OF APOPTOSIS IN HUMAN LEUKEMIA CELLS BY HISTONE DEACETYLASE INHIBITORS AND FLAVOPIRIDOL

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The abbreviations used are: CDKI, cyclin-dependent kinase inhibitor; HDACIs, histone deacetylase inhibitors; FP, flavopiridol; NaB, sodium butyrate; SAHA, suberoylanilide hydroxamic acid; NF-κB, Nuclear factor-kappaB; IKK, IkappaB kinase; EMSA, electrophoretic mobility shift assay.

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ABSTRACT

Interactions between the cyclin-dependent kinase inhibitor (CDKI) flavopiridol and the histone deacetylase inhibitors (HDACIs) sodium butyrate (NaB) and SAHA have been examined in human leukemia cells in relation to effects on NF-κB activation. Exposure (24 hr) of U937 human leukemia cells to NaB (1 mM) or SAHA (1.5 μM) resulted in a marked increase in NF-κB DNA binding, effects that were essentially abrogated by co-administration of flavopiridol (100 nM). These events were accompanied by a marked increase in mitochondrial injury, caspase activation, and apoptosis. Mutant cells expressing an IκBα super-repressor exhibited impairment of NF-κB DNA binding in response to HDACIs and a significant although modest increase in apoptosis. However, disruption of the NF-κB pathway also increased mitochondrial injury and caspase activation in response to flavopiridol and to an even greater extent to the combination of flavopiridol and HDACIs. Co-administration of flavopiridol with HDACIs down-regulated XIAP, Mcl-1, and $p21^{CIP1/WAF1}$ and activated JNK; moreover, these effects were considerably more pronounced in IκBα mutants. Similar responses were observed in U937 mutant cells stably expressing RelA/p65 siRNA. In all cases, flavopiridol was significantly more potent than genetic interruption of the NF-κB cascade in promoting HDACI-mediated lethality. Together, these findings are consistent with the notion that while inhibition of NF-κB activation by flavopiridol contributes to antileukemic interactions with HDACIs, other NF-κB-independent flavopiridol actions (e.g., down-regulation of Mcl-1, XIAP, and $p21^{CIP1/WAF1}$) play particularly critical roles in this phenomenon.

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INTRODUCTION

Histone acetylation status plays a major role in the control of gene transcription in diverse cell types, including those of leukemic origin (Carrozza et al., 2003). Histones acetylation is reciprocally regulated by two classes of enzymes: histone deacetylases (HDAC) and histone acetyl transferases (HATs) (Peterson, 2002). In general, acetylation of histones allows chromatin to assume a more relaxed conformation, thereby promoting gene transcription (Gray and Teh, 2001). Histone deacetylase inhibitors (HDACIs) represent a diverse group of compounds that block histone deacetylation, and thereby modulate expression of genes involved in multiple cellular processes, including differentiation (Rosato and Grant, 2004). In leukemic cells, HDACIs including the short chain fatty acid butyrate and the hydroxamic acid suberoylanilide hydroxamic acid (SAHA) potently induce differentiation *in vitro* (Rivero and Adunyah, 1998; Richon et al., 1998). However, when administered at higher concentrations, such agents trigger leukemic cell apoptosis (Vrana et al., 1999). Recently it has been shown that dysregulation of the endogenous cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ in human leukemia cells blocks HDACI-mediated maturation and reciprocally promotes cell death (Rahmani et al, 2003). Clinical trials of butyrate derivatives (e.g., phenylbutryate) have been carried out in patients with hematologic malignancies (Gilbert et al., 2001), and Phase I and II trials of SAHA are underway (Kelly et al., 2003).

Flavopiridol, a semi-synthetic rohitukine alkaloid that potently inhibits essentially all cyclin-dependent kinases (CDKs) (De Azevedo et al., 1996), was the first CDK inhibitor to enter clinical trials (Senderowicz, 1999). Flavopiridol induces G_1 arrest in tumor cells (Carlson et al., 1996), and triggers leukemic cell apoptosis at sub-micromolar concentrations (Parker et al., 1998). Recently, flavopiridol has been reported to inhibit the cyclin T/CDK9 transcriptional regulatory complex (Positive Transcription Elongation Factor-b; PTEF-b) (Chao et al., 2000)

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and to repress transcription of various genes, including $p21^{CIP1/WAF1}$ and Mcl-1 among others (Rosato et al., 2004, Gojo et al., 2002). Very recently, flavopiridol has been shown to inhibit activation of NF-κB, a transcription factor intimately involved in diverse cellular processes, including cell survival, proliferation, and differentiation (Takada and Aggarwal, 2004). NF-κB regulation involves, among other mechanisms, sequestration in the cytoplasm as an inactive form by members of the IκB family (e.g., IκBα) (Goldberg and Rock, 2002). Recently, flavopiridol has been shown to disrupt NF-κB signaling by inhibiting the IKK kinase, thus sparing IκBα from proteasomal degradation (Takada and Aggarwal, 2004). The role that flavopiridol-mediated interruption of NF-κB signaling plays in antileukemic interactions remains largely unexplored.

Studies from several groups have shown that co-administration of flavopiridol with HDACIs such as sodium butyrate (NaB), SAHA, and depsipeptide dramatically increases mitochondrial injury and apoptosis in leukemic and epithelial cancer cells (Almenara et al., 2002, Rosato et al., 2004, Nguyen et al., 2004). It has been postulated that such interactions may stem from flavopiridol-mediated transcriptional repression of anti-apoptotic genes (e.g., $p21^{CIP1/WAF1}$, Mcl-1, XIAP) (Almenara et al., 2002;Rosato et al., 2004). However, it is also known that HDACIs trigger perturbations in NF-κB activity, and that such events may protect cells from apoptosis. For example, HDACIs such as trichostatin A (TSA) and sodium butyrate (NaB) activate NF-κB in non-small cell lung cancer cells; moreover, pharmacologic or genetic NF-κB inhibition promotes HDACI-mediated apoptosis (Mayo et al., 2003). Analogously, disruption of the NF-κB pathway in human leukemia cells blocks HDACI-mediated $p21^{CIP1/WAF1}$ induction, inhibits differentiation, and enhances apoptosis (Dai et al., 2003a). Such findings raise the possibility that an intact NF-κB pathway may be required for HDACIs and other differentiation-inducing agents to trigger a maturation program in malignant hematopoietic cells.

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Because NF-κB activation is required for leukemic cell maturation (Dai et al., 2003a), and in view of recent evidence that flavopiridol acts as an IKK inhibitor (Takada and Aggarwal, 2004), the possibility that flavopiridol-mediated NF-κB inhibition might contribute to synergistic interactions with HDACIs appeared plausible. To define the role that flavopiridol-mediated NF-κB dysregulation might play in antileukemic synergism more rigorously, we have examined interactions between flavopiridol and the HDACIs NaB and SAHA in relation to effects on NF-κB activation. Specifically, we have employed genetic strategies to assess the functional significance of perturbations in NF-κB activation on mitochondrial injury, caspase activation, and cell survival following exposure of cells to these agents alone and in combination. Our results indicate that flavopiridol dramatically blocks HDACI-mediated NF-κB activation in human leukemia cells and that this event plays a significant, albeit limited, role in antileukemic synergism. However, our findings also suggest that interactions between these agents are multifactorial, and that more direct, NF-κB-independent flavopiridol actions (e.g., down-regulation of $p21^{CIP1/WAF1}$, Mcl-1, and XIAP) in all likelihood contribute substantially to this phenomenon.

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MATERIALS AND METHODS

Reagents and Cell Culture

The pan-CDK inhibitor, flavopiridol, was kindly provided by Dr Edward Sausville (Division of Cancer Treatment, NCI/NIH, Bethesda, MD). Sodium butyrate (NaB) was purchased from Biomol (Plymouth Meeting, PA), and suberoylanilide hydroxamic acid (SAHA) was purchased from Biovision (Moutain View, CA). All reagents were dissolved in DMSO as a stock solution and stored at −80°C.

U937 human leukemia cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 medium supplemented with sodium pyruvate, MEM, essential vitamins, L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (FBS). U937 cells were stably transfected with Ser32/Ser36 mutant IκBα cDNA or an empty vector (pcDNA3.1), and clones were selected with G418 as previously reported (Dai et al., 2003). All experiments were performed using logarithmically growing cells ($3\text{-}5 \times 10^5$ cells/ml).

RelA/p65 siRNA-expressing U937 cells were obtained by standard transfection techniques. The DNA oligonucleotides encoding hairpin siRNA targeting the coding region 186–204 downstream of the first nucleotide of the start codon of human relA/p65 were designed using the siRNA Target Finder tool (Ambion). Two complementary DNA oligonucleotides (**5'gatccGATCAATGGCTACACAGGAttcaagaga TCCTGTGTAGCCATTGATCttttttggaaa-3')and (3'gCTAGTTACCGATGTGTCCT aagttctctAGGACACATCGGTAACTAGaaaaaacctttcga-5')** were synthesized, annealed and cloned into bamHI/HindIII sites of The RNA polymerase III-based expression vector *psilencer 3.1-H1 hygro* (3.1-H1 hygro-p65siRNA) using standard techiniques. The construct was verified by DNA sequencing and transfected into U937 cells using electroporation as previously

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described (Rahmani et al., 2003). Stable clones from a single cell were selected in the presence of 400 μg/ml of hygromycin and tested for p65 protein expression by Western blot.

Annexin V/PI Assays for Apoptosis

For Annexin V/PI assays, cells were stained with Annexin V-FITC and propidium iodide (PI), and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD PharMingen, San Diego, CA). Briefly, 1×10^6 cells were washed twice with PBS, and stained with 5 μl of Annexin V-FITC and 10 μl of PI (5 μg/ml) in 1× binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM $CaCl_2$) for 15 min at room temperature in the dark. The apoptotic cells were determined using a Becton Dickinson FACScan cytoflorometer (Mansfield, MA).

Western Blot Analysis

Western blot analysis was performed using the NuPAGE Bis-Tris electrophoresis system (Invitrogen, Carlsbad, CA). The total cellular samples were washed twice with cold phosphate-buffered saline (PBS) and lysed in 1× NuPAGE LDS sample buffer supplemented with 50 mM dithiothreitol (DTT, Fisher Biotech, Pittsburgh, PA). The protein concentration was determined using Coomassie Protein Assay Reagent (Pierce, Rockford, IL). The total cellular protein extracts were separated by SDS-PAGE, and transferred to nitrocellulose membrane in 20 mM Tris-HCl (pH 8.0) containing 150 mM glycine and 20% (v/v) methanol. Membranes were blocked with 5% nonfat dry milk in 1× TBS containing 0.05% Tween 20 and incubated with antibodies against phosphor-JNK (Thr183/Tyr185)(Santa Cruz, Santa Cruz, CA), SAPK/JNK (Cell Signaling, Beverly, MA), XIAP (Cell Signaling), Mcl-1 (PharMingen, San Diego, CA), $p21^{WAF1/CIP1}$ (Transduction Laboratories, Lexington, KY), $p27^{KIP1}$ (PharMingen), PARP

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(Biomol, Plymouth Meeting, PA), cleaved caspase-3 (Cell Signaling), caspase-8 (Alexis, Carlsbad, CA), caspase-9 (PharMingen). β-Actin (Santa Cruz) was used to ensure equal loading and transfer of proteins. Protein bands were detected by incubating with horseradish peroxidase-conjugated antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and visualized with enhanced chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA).

Analysis of Cytosolic Cytochrome C and AIF

After treatment, cells (2×10^6) were washed twice in PBS and lysed by incubating for 5 min in lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, and 350 μg/ml digitonin). The lysate was centrifuged for 1 min, and the supernatant was collected and added to an equal volume of 2× sample buffer. The protein concentration was determined using Coomassie Protein Assay Reagent (Pierce, Rockford, IL). 30 μg of cytosolic extract was separaed by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with antibodies against cytochrome C (PharMingen) and AIF (Santa Cruz).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein extracts were prepared as previously described (Hehner et al., 1998). Briefly, cells were harvested and resuspended in hypotonic buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) for 15 min on ice and then added 5 μl of 10% Nonidet P-40. After centrifugation at 2,000 g for 10 min at 4°C, nuclei pellets were resuspended in buffer B (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) for 30 min on ice. After centrifugation at 20,000 g for 15 min, the supernatant was collected as the

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nuclear extracts, and stored at −70 °C. For EMSA, 4 μg of nuclear extracts were incubated with DNA binding buffer containing 2 μg of poly(dI-dC), 1 μg of bovine serum albumin, and 15,000 cpm of a ^{32}P-labeled oligonucleotide. Subsequently the free and the oligonucleotide-bound proteins were separated by electrophoresis on a native 6% polyacrylamide gel. The gel was dried after electrophoresis and exposed to an X-ray film. The following oligonucleotide corresponding to NF-κB binding site was used: 5'- AGTTGAGGGGACTTTCCCAGGC-3'/3'-TACACTCCCCTGAAAGGGTCCG-5'.

Densitometric Analysis

Autoradiographic signals of EMSA were quantified using Scion Image software (Scion Corporation). Mean densitometry data from independent experiments were normalized to results obtained in cells in the control. The data were presented as the mean ± SD.

Statistical Analysis

For analysis of apoptosis, values were presented as means ± SD. Statistical differences between control and treated groups were determined by the Student's t-test. Differences were considered statistically significant for values of $p<0.05$, $p<0.01$ and $p<0.001$.

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RESULTS

Flavopiridol blocks SAHA- and NaB-mediated induction of NF-κB DNA-binding activity

To assess the effects of flavopiridol on NF-κB activation by HDACIs, U937 cells were exposed to 1 mM NaB or 1.5 μM SAHA for 24 hr in the presence or absence of 100 nM flavopiridol, after which NF-κB DNA binding was monitored by EMSA analysis (Figure 1) and quantified by densitometry. Both SAHA and NaB markedly increased while flavopiridol slightly decreased basal NF-κB DNA binding (Figures 1A and 1B). Significantly, flavopiridol dramatically reduced HDACI-mediated NF-κB activation. Supershift assays employing antibodies directed against p50 and p65 confirmed the specificity of these events (Figure 1C). These findings indicate that co-administration of flavopiridol prevents NaB- and SAHA-mediated NF-κB activation in U937 cells.

Expression of an IκBα "super-repressor" abrogates NF-κB activation in cells exposed to HDACIs ± flavopiridol

To investigate the effects of interruption of the NF-κB pathway on the response of cells to HDACIs ± flavopiridol, U937 cells ectopically expressing an IκBα super-repressor mutuant were employed. This construct exhibits serine to alanine substitutions at residues 32 and 36, which prevent proteasomal degradation of IκB, allowing this protein to bind and inactivate NF-κB in the cytoplasm (Alkalay et al., 1995). As shown in Figure 2A and 2B, expression of the IκBα super-repressor by two separate clones (2C8 and 2H6) substantially reduced NF-κB DNA binding in cells exposed to HDACIs alone, and essentially abrogated DNA binding in cells exposed to flavopiridol alone or in combination.

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Genetic blockade of NF-κB activation potentiates apoptosis by HDACIs, flavopiridol, as well as the HDACI/flavopiridol combination

The impact of genetic interruption of the NF-κB cascade on the apoptotic response of U937 cells to HDACIs ± flavopiridol was then investigated. The central premise underlying these studies was that if NF-κB disruption played a critical role in synergistic interactions between flavopiridol and HDACIs, then such synergism would be attenuated in mutant cells (because NF-κB was already disrupted). Several finding emerged from these studies. First, both mutant clones displayed a modest but statistically significant increase ($p<0.01$ in each case) in apoptosis in response to low, minimally toxic concentrations of either NaB or SAHA (Figure 3A and 3B). Second, disabling of the NF-κB pathway markedly increased the lethal effects of flavopiridol, consistent with our earlier findings (Dai et al., 2003b). Interestingly, apoptosis in IκBα mutant cells exposed to flavopiridol + HDACIs was essentially complete, and in all cases modestly but significantly greater than that exhibited by empty vector controls ($p<0.01$ for all conditions). Finally, potentiation of NaB- or SAHA-mediated apoptosis by flavopiridol was significantly greater than that observed in IκBα mutant cells ($p<0.01$ in all cases). Collectively, these findings argue that while flavopiridol-related inhibition of HDACI-mediated NF-κB activation may contribute to the marked increase in apoptosis in HDACI/flavopiridol-treated cells, other flavopiridol actions, presumably unrelated to effects on NF-κB, very likely play a role in this phenomenon.

Effects of disruption of the NF-κB cascade on mitochondrial injury and caspase activation in U937 cells

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Effects of disabling the NF-κB cascade were then investigated in relation to induction of mitochondrial injury and apoptosis by HDACIs ± flavopiridol. To this end, empty vector (pcDNA 3.1) and mutant clones (2C8 and 2H6) were exposed to NaB or SAHA ± flavopiridol as above, after which release of cytochrome c or AIF into the cytosolic S-100 fraction or cleavage/activation of caspases were monitored by Western blot analysis. As shown in Figure 4A and 4B, IκBα mutant cells displayed modest increases in cytochrome c and AIF release in response to flavopiridol. On the other hand, exposure to NaB (4A) or SAHA (4B) alone had either no or a very limited effect on cytochrome C/AIF release in mutant cells. However, IκBα mutants exhibited a slight but discernible increase in cytochrome c and a more pronounced increase in AIF release in response to the combination of HDACIs + flavopiridol. Combined treatment with HDACIs + flavopiridol also resulted in enhanced activation/cleavage of caspases-3, -8, and -9 in IκBα transfectants (Figures 5A and 5B). As observed in the case of apoptosis, Flavopiridol was more effective than blockade of the NF-κB pathway in potentiating HDACI-mediated mitochondrial injury. This finding, along with evidence that genetic interruption of the NF-κB pathway potentiates HDACI/flavopiridol-induced mitochondrial injury, caspase activation, and apoptosis, argue that factors other than or in addition to flavopiridol-mediated NF-κB inhibition contribute to the pronounced induction of apoptosis by this drug combination.

Leukemia cells stably expressing RelA/p65 siRNA display enhanced apoptosis in response to the HDACI/flavopiridol regimen

To determine whether these findings were restricted to cells expressing the IκBα mutant protein, parallel studies were performed in leukemia cells stably expressing a RelA/p65 siRNA construct. As shown in Figure 6A, transfectants cells displayed a marked reduction in expression of RelA/p65 compared to their empty vector controls. Furthermore, mutant cells showed a

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significant increase in susceptibility to flavopiridol-induced apoptosis (Figures 6B and 6C). Moreover, expression of the RelA/p65 antisense construct resulted in modest but statistically significant increases ($P < 0.01$ in each case) in the apoptotic responses of cells to low, marginally toxic concentrations of NaB or SAHA. As noted in the case of IκBα mutants, these effects were clearly less pronounced than those attributable to flavopiridol alone in control cells. Also consistent with results observed in IκBα mutants, RelA/p65 siRNA-expressing cells were significantly more sensitive to flavopiridol than controls ($p<0.01$). Finally, cells expressing the siRNA construct resulted in a modest but statistically significant ($p<0.01$) increase in apoptosis following exposure to the flavopiridol/HDACI regimen. Collectively, these findings provide further support for the notion that factors in addition to disruption of the NF-κB pathway contribute to the dramatic antileukemic synergism observed between flavopiridol and HDACIs.

Disruption of the NF-κB cascade promotes JNK activation and down-regulation of XIAP, Mcl-1, and p21$^{CIP1/WAF1}$ in response to flavopiridol/HDACIs

The effects of disabling the NFκB cascade were then examined in relation to perturbations in stress and cell cycle-related pathways. Similar to results described above, IκBα mutant cells displayed minimal increases in activation of the stress kinase JNK in response to NaB or SAHA alone, and modest increases in flavopiridol-mediated JNK activation (Figure 7). However, disabling of the NF-κB axis markedly increased JNK activation in response to flavopiridol/HDACIs. In separate studies, co-administration of the JNK inhibitor SP600125 (10 μM) failed to attenuate flavopiridol/HDACI-mediated lethality (data not shown), arguing against a major functional role for JNK activation in this phenomenon.

Flavopiridol modestly diminished XIAP expression in parental cells, and this effect was also observed in IκBα mutants. In contrast, HDACIs had little effect on XIAP expression in any

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of the cell lines. However, XIAP expression was substantially reduced in parental cells and essentially abrogated in mutant cells treated with flavopridol/HDACIs. Expression of the anti-apoptotic protein Mcl-1 was modestly reduced by flavopiridol alone, and either unchanged or slightly increased in cells treated with HDACIs. However, expression was absent in mutant cells exposed to flavopiridol/HDACIs. Both NaB and SAHA induced $p21^{CIP1/WAF1}$ and this response was attenuated by either flavopiridol or by expression of the IκBα mutant protein. Furthermore, $p21^{CIP1/WAF1}$ expression was essentially abrogated in mutant cells treated with the flavopiridol/HDACI regimen. Combined exposure to flavopiridol/HDACIs also resulted in cleavage and reduced expression of $p27^{KIP1}$, events that were more pronounced in IκBα mutant cells. Finally, the effects of flavopiridol on XIAP, Mcl-1, and $p21^{CIP1/WAF1}$ down-regulation in wild-type cells were in all cases greater than those triggered by disruption of the NF-κB pathway i.e., by expression of IκBα mutants. Together, these findings suggest that while disabling the NF-κB pathway by flavopiridol may contribute to perturbations in certain stress, cell cycle, and survival pathways in HDACI-treated cells, other events, including those related to direct actions of flavopiridol, are more likely to play primary roles in the dramatic antileukemic synergism between these agents.

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DISCUSSION

The concept of combining pharmacologic CDK inhibitors such as flavopiridol with differentiation-inducing agents such as HDACIs in leukemia therapy has several theoretical rationales. For example, HDACIs have been shown to be potent inducers of leukemic cell differentiation (Rosato et al., 2003). In addition, induction of leukemic cell maturation requires cells to undergo G_1 arrest (Dai et al., 2003a); moreover, CDK inhibitors, including flavopiridol, have been reported to induce differentiation in certain malignant cell types (e.g., non-small cell lung cancer cells) (Lee et al., 1999). Thus, the possibility that flavopiridol might enhance maturation by HDACIs as well as other maturation-inducers appeared plausible. However, it has been established that flavopiridol does not promote HDACI-mediated differentiation; instead, flavopiridol has been shown to potentiate mitochondrial injury and apoptosis by multiple HDACIs including NaB, SAHA, and depsipeptide (Almenara et al., 2002; Rosato et al., 2004, Nguyen et al., 2004). This capacity has been attributed to the ability of flavopiridol to diminish, via inhibition of the PTEF-b CDK9/cyclin T complex (Chao et al., 2000), expression of various anti-apoptotic proteins, including Mcl-1, XIAP, and $p21^{CIP1/WAF1}$, which are necessary for the normal maturation program to proceed. Recently, several other potentially pro-apoptotic flavopiridol actions have been described, including inhibition of survivin phosphorylation (Wall et al., 2003) and disruption of the NF-κB pathway through inhibition of the IKK kinase (Sizemore et al., 2002). In this regard, HDACIs have been shown to activate NF-κB (Dai et al., 2003a; Adam et al., 2003), an action which under some circumstances blocks HDACI-mediated apoptosis (Mayo et al., 2003). Furthermore, in human leukemia cells, disruption of NF-κB signaling by either pharmacologic or genetic means disrupts differentiation-induction by phorbol esters (Altuwaijri et al., 2003) as well as HDACIs (Dai et al., 2003a), resulting in a reciprocal increase in apoptosis. Based upon these considerations, it is tempting to speculate that

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flavopiridol-mediated NF-κB inhibition may contribute to synergistic antileukemic interactions that occur between this agent and HDACIs.

The results presented here indicate that NaB and SAHA do in fact strikingly activate NF-κB in U937 human leukemia cells, and that this action is largely abrogated by co-administration of flavopiridol. However, while flavopiridol-mediated inhibition of HDACI-associated NF-κB activation in all likelihood contributes to potentiation of apoptosis by this drug combination, other actions undoubtedly play a major role in this phenomenon. For example, consistent with previous results by our group and others (Dai et al., 2003b, Mayo et al., 2003), disabling of the NF-κB pathway significantly increased, albeit modestly, HDACI-mediated lethality, even when the latter was administered at low, marginally toxic concentrations. However, potentiation of HDACI-mediated apoptosis by flavopiridol was significantly greater than that induced by genetic interruption of the NF-κB pathway (e.g., in cells expressing the IκBα mutant protein or RelA/p65 siRNA). In addition, genetic disruption of NF-κB signaling resulted in a significant increase in cell death in leukemic cells exposed to the flavopiridol/HDACI regimen. Because the NF-κB pathway is already disrupted in mutant cells, this finding indicates that factors other than or in addition to flavopiridol-mediated NF-κB inhibition is involved in lowering the apoptotic threshold. Collectively, these observations suggest that while the striking ability of flavopiridol to block HDACI-mediated NF-κB activation very likely contributes to the observed potentiation of apoptosis, other flavopiridol actions, particularly those unrelated to NF-κB inhibition, play a critical role in this interaction.

One possible explanation for the present observations is that flavopiridol may modulate the expression of proteins involved in regulation of HDACI-mediated apoptosis through both NF-κB-dependent as well as –independent mechanisms. For example, the endogenous cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ is a major target of HDACIs (Gui et al., 2004) and has

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been shown in multiple systems to attenuate HDACI-mediated apoptosis (Almenara et al., 2002; Rosato et al., 2003, Nguyen et al., 2004). The latter capacity may reflect, at least in part, the ability of cytoplasmic $p21^{CIP1/WAF1}$ to bind to and inactivate procaspase-3 (Asada et al., 1999; Rosato et al., 2004). However, a functional role for NF-κB activation in induction of $p21^{CIP1/WAF1}$ in human leukemia cells exposed to phorbol esters and HDACIs has recently been demonstrated (Dai et al., 2003a). Thus, abrogation of $p21^{CIP1/WAF1}$ induction and potentiation of apoptosis in HDACI-treated cells by flavopiridol may reflect both direct effects related to flavopiridol-mediated transcriptional repression, e.g., through inhibition of PTEF-b (Chao et al., 2000), as well as indirect effects i.e., mediated by inhibition of NF-κB. In support of this notion, $p21^{CIP1/WAF1}$ induction by NaB or SAHA was attenuated in IκBα mutant cells, but was essentially abrogated when flavopiridol was added. Similar considerations apply to the anti-apoptotic protein XIAP, which is both a target of NF-κB (Tang et al., 2001) as well as of flavopiridol (Dai et al., 2003b). Finally, it is conceivable that the lethal consequences of disruption of NF-κB activation by flavopiridol may be potentiated by down-regulation of certain anti-apoptotic proteins (e.g., Mcl-1) through a direct, NF-κB-independent mechanism.

Combined treatment with flavopiridol and HDACIs resulted in a clear increase in mitochondrial injury (e.g., cytochrome c and AIF release), particularly under conditions in which the NF-κB pathway was disabled. These events were accompanied by engagement of the downstream apoptotic cascade, including activation/cleavage of procaspases-9, -3, -8, and PARP. Although the mechanism by which NF-κB inhibition promotes mitochondrial dysfunction is controversial, it is noteworthy that flavopiridol/HDACI-mediated mitochondrial injury was accompanied by JNK activation, an event that has been directly implicated in cytochrome c release (Tournier et al., 2000), including that triggered by HDACIs (Yu et al., 2003). Furthermore, NF-κB inhibition has been reported to promote JNK activation through an

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XIAP-related process (Tang et al., 2001; Lewis et al., 2004). Finally, in view of evidence that $p21^{CIP1/WAF1}$ opposes JNK actions (Huang et al., 2003), the ability of flavopiridol to block HDACI-mediated $p21^{CIP1/WAF1}$ induction through both NF-κB-dependent and –independent mechanisms may be relevant.

In summary, the present results suggest that flavopiridol effectively blocks the ability of the HDAC inhibitors NaB and SAHA to activate NF-κB in human leukemia cells, and that this phenomenon contributes, at least in part, to the pronounced mitochondrial injury and apoptosis induced by this regimen. However, based upon the present results, it is probable that NF-κB-independent flavopiridol actions, most likely related to PTEF-b inhibition (e.g., down-regulation of XIAP, Mcl-1, and $p21^{CIP1/WAF1}$), play major roles in such interactions. An additional possibility is that flavopiridol-mediated down-regulation of XIAP, Mcl-1, and $p21^{CIP1/WAF1}$ following HDACI exposure may be particularly lethal under conditions in which the NF-κB pathway is disabled. One implication of these findings is that while the search for more specific CDK inhibitors is clearly justified, less specific agents such as flavopiridol, whose actions are pleiotropic, may offer certain advantages, particularly when used in combination with other targeted agents such as HDAC inhibitors. Accordingly, efforts to determine whether similar events occur when other CDK antagonists are combined with HDAC inhibitors are currently underway.

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FIGURE LEGENDS

Figure 1. Flavopiridol markedly inhibits activation of NF-κB induced by histone deacetylase inhibitors in human leukemia cells. (A) U937 cells were untreated or treated with 1 mM sodium butyrate (NaB), 100 nM flavopiridol (FP) or the combination (FP + NaB) for 24 h, after which nuclear extracts were then prepared and subjected to EMSA as described in Materials and Methods. For C + C', a 100-fold excess of unlabeled NF-κB oligonucleotides was added to the nuclear extract obtained from untreated cells prior to addition of labeled NF-κB oligonucleotides. (B) U937 cells were untreated or treated with 1.5 μM SAHA, 100 nM FP, or the combination of FP and SAHA for 24 h. NF-κB DNA binding activity was determined by EMSA as described above. (C) For the supershift assay, nuclear extracts were preincubated with specific anti-p50 and anti-p65 antibodies and subjected to EMSA. Results are representative of three separate studies. For all studies, lanes were loaded with 4 ug of protein. NF-κB DNA binding signals were quantified using Scion Image software (Scion Corporation). Lower panels: Mean densitometric values from 3 independent experiments were normalized to controls which were assigned an arbitrary value of 1.0. The data represent the means ± SD (n = 3).

Figure 2. Inhibition of NF-κB activity in U937 cells transfected with and IκBα "super-repressor" mutant. U937 cells were stably transfected with an IκBα mutant (Ser32, 36/Ala) or an empty vector (pcDNA3.1) as described in Methods. Two U937 cell clones stably expressing IκBα mutants (designated 2C8 and 2H6) were then isolated and proparated. (A) Empty vector control cells and the two IκBα mutant transfectants (2C8 and 2H6) were treated with 1 mM NaB, 100 nM FP or the combination of FP and NaB, after which the nuclear extracts were prepared and subjected to EMSA as described in Methods. (B) Empty vector controls and the

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two IκBα mutant transfectants (2C8 and 2H6) were treated with 1.5 μM SAHA, 100 nM FP or the combination of FP and SAHA, after which nuclear extracts were prepared and subjected to EMSA analysis as described above. Lower panels: Densitometric analysis of signals from EMSA analysis shown above. Results represent values compared to vector controls, and are representative of three separate studies. NF-κB DNA binding signals were quantified and analyzed as described in Fig. 1. The data are expressed as means ± SD (n = 3).

Figure 3. Stable transfection of cells with an IκBα mutant enhances FP/NaB and FP/SAHA induced apoptosis in U937 cells. U937 cells were stably transfected with IκBα mutant (Ser32, 36/Ala) or an empty vector (pcDNA3.1) as described in Methods. U937/IκBα mutant (clones 2C8 and 2H6) and /pcDNA3.1 cells were treated with 100 nM FP, the histone deacetylase inhibitors, NaB (1 mM) or SAHA (1.5 μM), or the combination of FP/NaB (A) and FP/SAHA (B) for 24 h. Cells were stained with annexin V/PI, and apoptosis determined using flow cytometry as described in Methods. The values obtained from the annexin V assay represent the means ± SD for three separate experiments. ** = values for U937/IκBα mutant cells (clones 2C8 and 2H6) significantly greater than those for empty vector controls (pcDNA3.1 cells) by the Student's t-test; $p<0.01$. *** = $p<0.001$.

Figure 4. Stable transfection of cells with an IκBα enhances FP/NaB and FP/SAHA induced mitochondrial injury in U937 cells. U937/IκBα mutant (clones 2C8 and 2H6) and /pcDNA3.1 cells were treated with 100 nM FP, NaB (1 mM) or SAHA (1.5 μM), or the combination of FP/NaB (A) and FP/SAHA (B) for 24 h. The cytosolic S-100 fractions were prepared and subjected to Western blot assay using antibodies against cytochrome C and AIF.

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Each lane was loaded with 20 μg of protein; blots were subsequently stripped and reprobed with antibodies directed against β-actin to ensure equivalent loading and transfer. Two additional studies yielded equivalent results.

Figure 5. Stable transfection of cells with an IκBα enhances FP/NaB- and FP/SAHA-induced caspase activation in U937 cells. U937/IκBα mutant (clones 2C8 and 2H6) and /pcDNA3.1 cells were treated with 100 nM FP, NaB (1 mM) or SAHA (1.5 μM), or the combination of FP/NaB (A) and FP/SAHA (B) for 24 h. Total cellular extracts were prepared and subjected to Western blot assay using antibodies against PARP, caspase 3, caspase 8, caspase 9, and β-actin as indicated. Each lane was loaded with 20 μg of protein; blots were subsequently stripped and reprobed with antibodies directed against β-actin to ensure equivalent loading and transfer. Two additional studies yielded equivalent results.

Figure 6. Stable transfection of RelA/p65 siRNA enhances FP/NaB- and FP/SAHA- induced apoptosis in U937 cells. U937 cells were stably transfected with RelA/p65 siRNA or an empty vector (3.1-H1 hygro) as described in Methods. Comparison of the expression of RelA/p65 in U937/p65 siRNA and U937/3.1-H1 hygro cells is shown by Western blot (A). U937/p65 siRNA (p65) and /3.1-H1 hygro (3.1) cells were treated with FP (100 nM), NaB (1 mM) or SAHA (1.5 μM), or the combination of FP/NaB (B) and FP/SAHA (C) for 24 h. Cells were stained with annexin V/PI, and apoptosis was determined using flow cytometry. The values obtained from annexin V assays represent the means ± SD for three separate experiments. ** = values for RelA/p65 siRNA cells significantly greater than those for empty vector controls (3.1) by the Student t-test; $p<0.01$.

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Figure 7. Stable transfection with an IκBα mutant enhances FP/NaB and FP/SAHA-induced activation of phospho-JNK and down-regulation of XIAP, $p21^{CIP1/WAF1}$, and Mcl-1. U937/IκBα mutant (2C8 and 2H6) and /pcDNA3.1 cells were treated with 1 mM NaB, 100 nM FP or the combination of FP/NaB (A). Alternatively, transfected cells were also treated with 1.5 μM SAHA, 100 nM FP or the combination of FP/SAHA (B). Total cellular extracts were prepared and subjected to Western blot assay using antibodies against phospho-JNK, JNK, XIAP, Mcl-1, $p21^{CIP1/WAF1}$, $p27^{KIP1}$, and β-actin. Each lane was loaded with 20 μg of protein; blots were subsequently stripped and re-probed with antibodies to actin to ensure equivalent loading and transfer. Two additional studies yielded equivalent results.

Figure 1

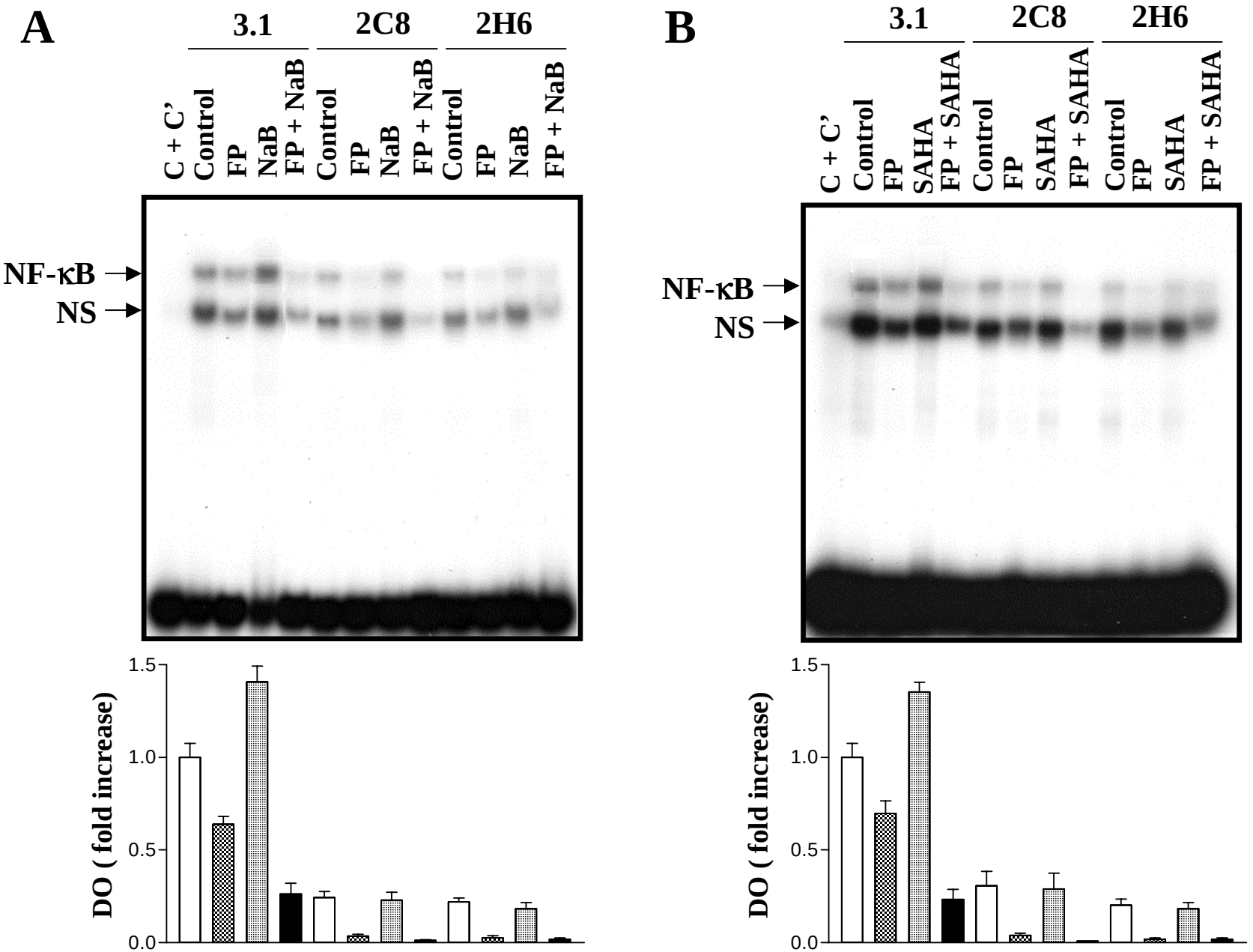

Figure 2

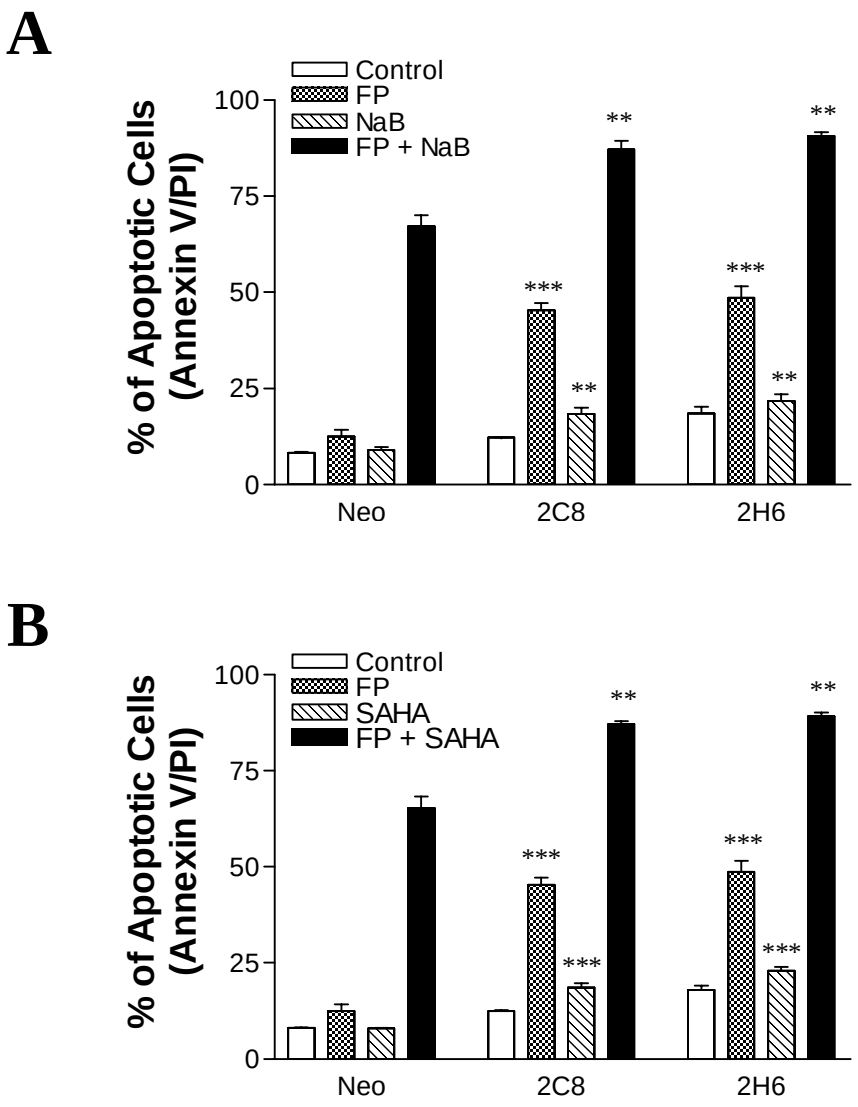

Figure 3

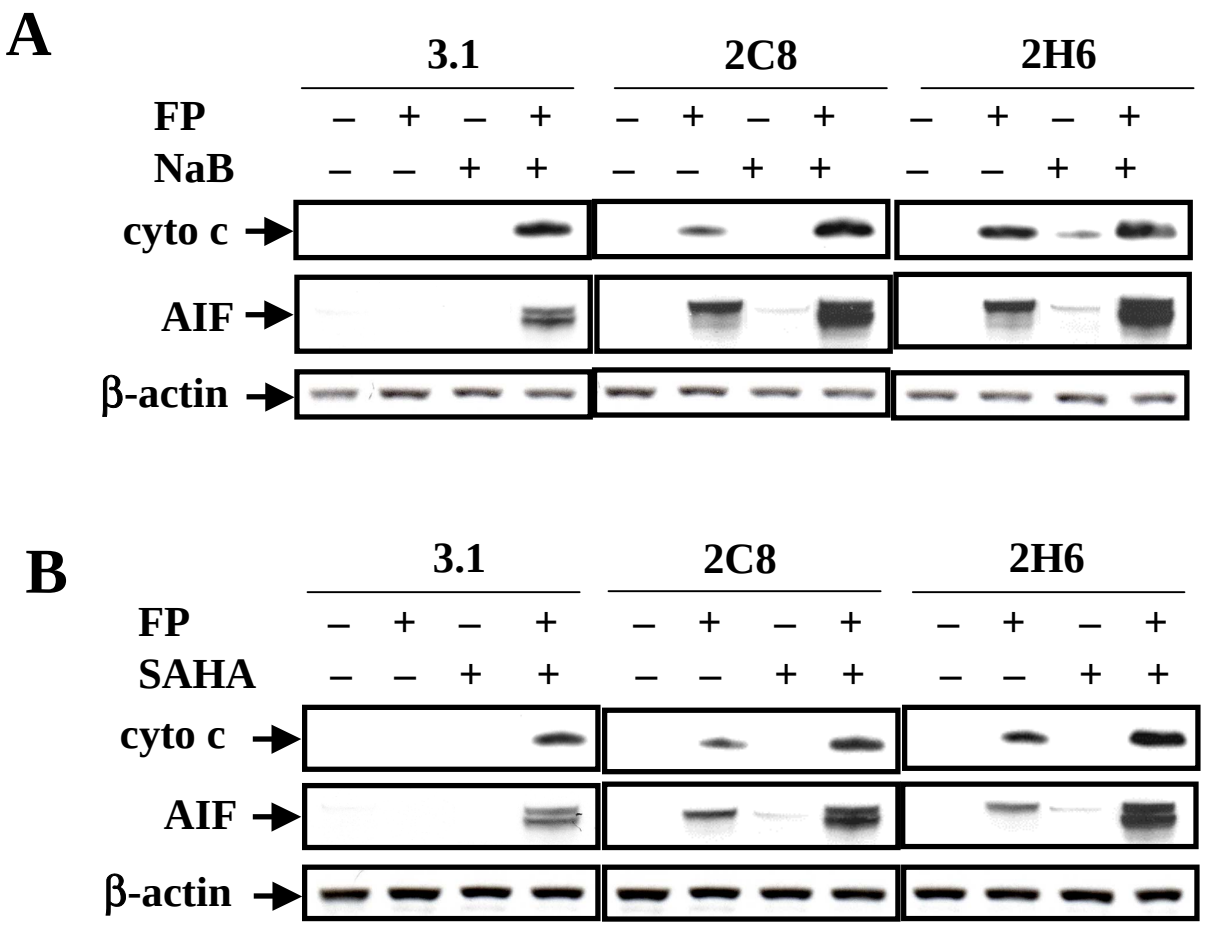

Figure 4

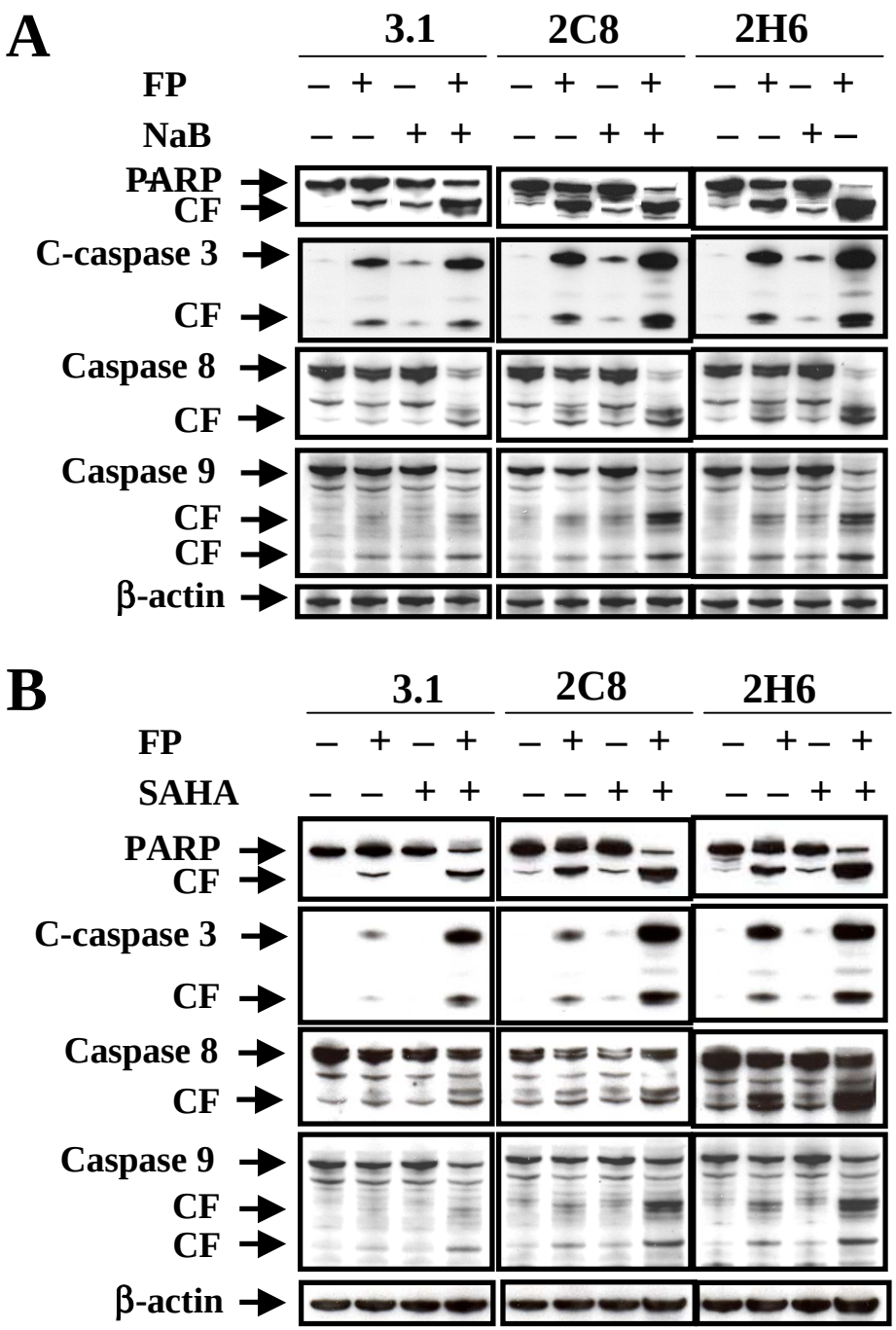

Figure 5

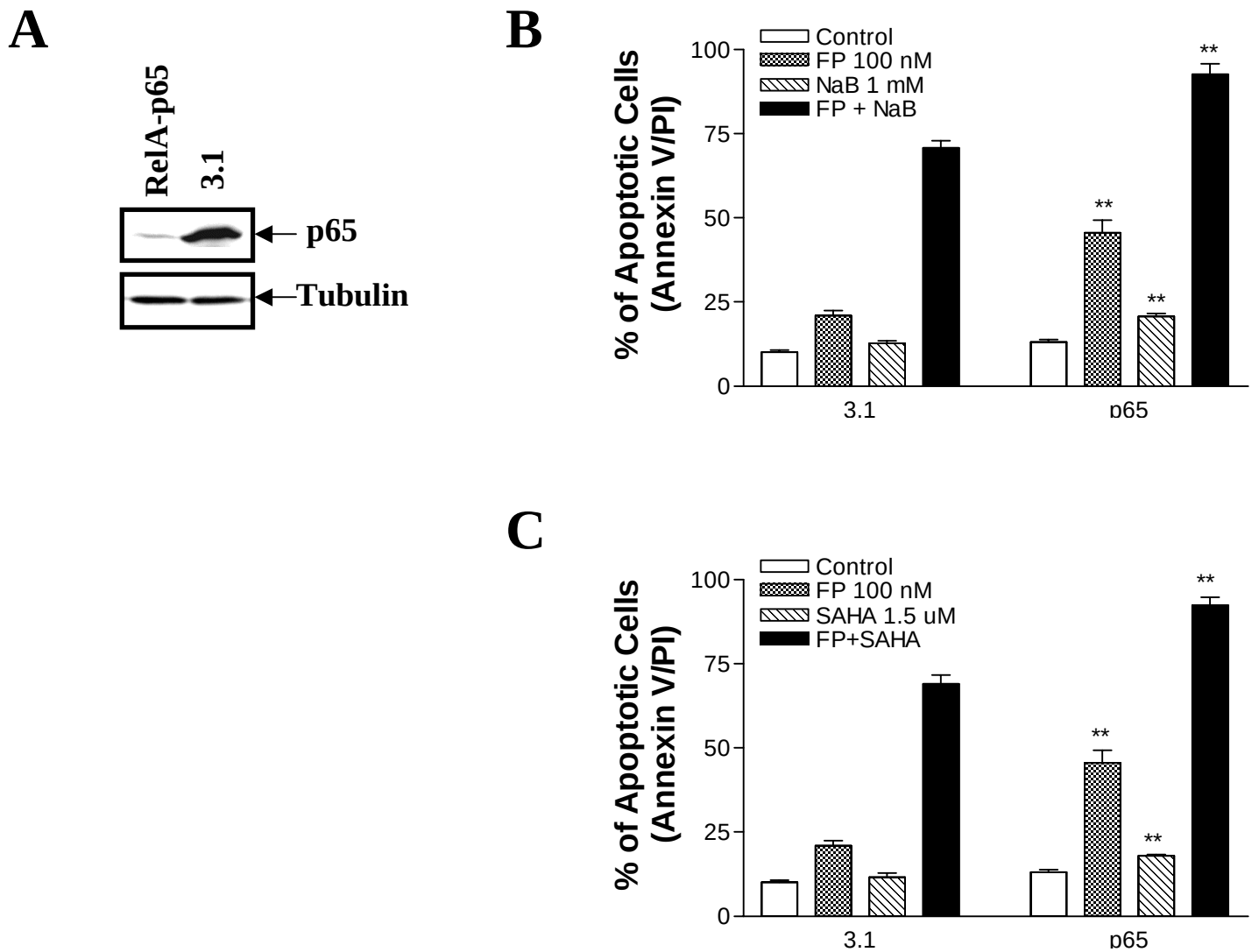

Figure 6

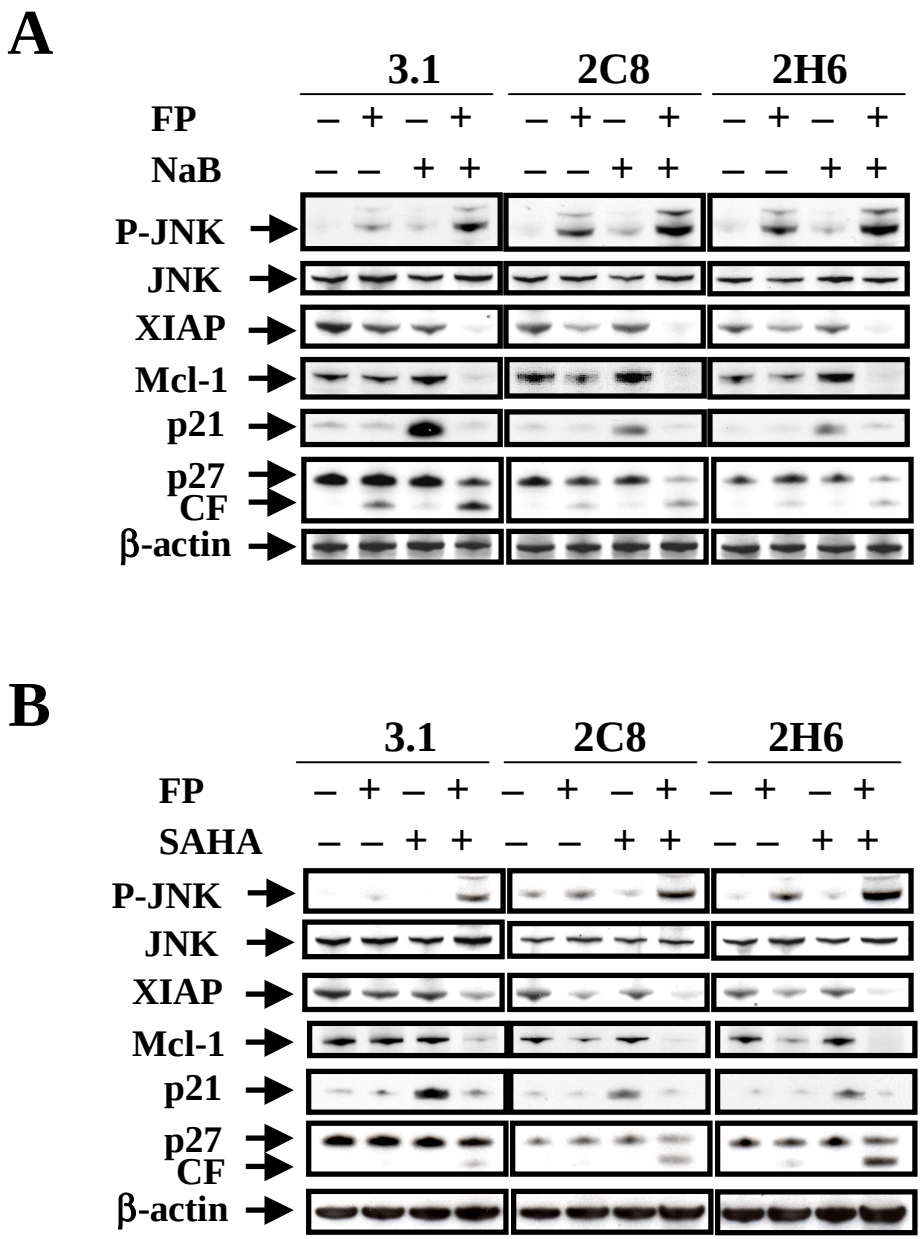

Figure 7