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The Histone Deacetylase Inhibitor MS-275 Interacts Synergistically with Fludarabine to Induce Apoptosis in Human Leukemia Cells

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ABSTRACT

Interactions between the novel benzamide histone deacetylase (HDAC) inhibitor MS-275 and fludarabine were examined in lymphoid and myeloid human leukemia cells in relation to mitochondrial injury, signal transduction events, and apoptosis. Prior exposure of Jurkat lymphoblastic leukemia cells to a marginally toxic concentration of MS-275 (*e.g.*, 500 nM) for 24 h sharply increased mitochondrial injury, caspase activation, and apoptosis in response to a minimally toxic concentration of fludarabine (500 nM), resulting in highly synergistic antileukemic interactions and loss of clonogenic survival. Simultaneous exposure to MS-275 and fludarabine also led to synergistic effects, but these were not as pronounced as observed with sequential treatment. Similar interactions were noted in the case of (*a*) other human leukemia cell lines (*e.g.*, U937, CCRF-CEM); (*b*) other HDAC inhibitors (*e.g.*, sodium butyrate); and (*c*) other nucleoside analogues (*e.g.*, 1-β-D-arabinofuranosylcytosine, gemcitabine). Potentiation of fludarabine lethality by MS-275 was associated with acetylation of histones H3 and H4, down-regulation of the antiapoptotic proteins XIAP and Mcl-1, enhanced cytosolic release of proapoptotic mitochondrial proteins (*e.g.*, cytochrome *c*, Smac/DIABLO, and apoptosis-inducing factor), and caspase activation. It was also accompanied by the caspase-dependent down-regulation of $p27^{KIP1}$, cyclins A, E, and D_1, and cleavage and diminished phosphorylation of retinoblastoma protein. However, increased lethality of the combination was not associated with enhanced fludarabine triphosphate formation or DNA incorporation and occurred despite a slight reduction in the S-phase fraction. Prior exposure to MS-275 attenuated fludarabine-mediated activation of MEK1/2, extracellular signal-regulated kinase, and Akt, and enhanced c-Jun NH_2-terminal kinase phosphorylation; furthermore, inducible expression of constitutively active MEK1/2 or Akt significantly diminished MS-275/fludarabine-induced lethality. Combined exposure of cells to MS-275 and fludarabine was associated with a significant increase in generation of reactive oxygen species; moreover, both the increase in reactive oxygen species and apoptosis were largely attenuated by coadministration of the free radical scavenger L-*N*-acetylcysteine. Finally, prior administration of MS-275 markedly potentiated fludarabine-mediated generation of the proapoptotic lipid second messenger ceramide. Taken together, these findings indicate that the HDAC inhibitor MS-275 induces multiple perturbations in signal transduction, survival, and cell cycle regulatory pathways that lower the threshold for fludarabine-mediated mitochondrial injury and apoptosis in human leukemia cells. They also provide insights into possible mechanisms by which novel, clinically relevant HDAC inhibitors might be used to enhance the antileukemic activity of established nucleoside analogues such as fludarabine.

INTRODUCTION

2-Fluoroadenine 9-β-D-arabinofuranoside-monophosphate or fludarabine (F-ara-AMP) is a purine analogue that has demonstrated significant activity in B-cell malignancies, including chronic lymphocytic leukemia and indolent non-Hodgkin's lymphoma (1). It has also shown activity, when combined with other agents, in patients with acute myelogenous leukemia (2). F-ara-AMP is rapidly dephosphorylated in the plasma to its nucleotide derivative, F-ara-A, which is subsequently transported across cell membranes by a facilitated nucleoside diffusion system (3). It is then rephosphorylated by the salvage pathway enzyme deoxycytidine kinase and ultimately converted to its lethal form, 2-fluoroadenine 9-β-D-arabinofuranoside-triphosphate (F-ara-ATP), by a mono- and dinucleoside kinase (4). F-ara-ATP inhibits multiple enzymes involved in DNA synthesis, including DNA polymerase, DNA primase, and ribonucleotide reductase (5), and kills leukemic cells by inducing apoptosis (6). Although the relative contributions of the diverse biochemical actions of F-ara-AMP to cell death are not known, incorporation of F-ara-AMP into leukemic cell DNA appears to be required for lethality (7). The mechanism by which leukemic cells develop resistance to F-ara-AMP is uncertain, although recent studies suggest that loss of deoxycytidine kinase may contribute to this phenomenon, at least in continuously cultured cell lines (8). Recently, several groups have reported that the lethal actions of F-ara-AMP are potentiated in human leukemia cells by UCN-01, an inhibitor of protein kinase C as well as Chk1 (9, 10).

Histone deacetylase (HDAC) inhibitors, including trichostatin A, sodium butyrate, suberoylanilide hydroxamic acid (SAHA), depsipeptide, MS-275, and aphicidin, among others, represent a novel and diverse class of agents that act by promoting histone acetylation (11). Histone acetylation status is reciprocally regulated by the activities of HDACs and histone acetylases (12). By blocking the actions of the former, HDAC inhibitors induce acetylation of several histones (*e.g.*, histones H3 and H4), events that favor uncoiling and relaxation of the chromatin structure (13). Chromatin relaxation, in turn, permits the expression of diverse genes, including those involved in the differentiation process (*e.g.*, $p21^{CIP1}$; Ref. 14). In fact, several HDAC inhibitors (*e.g.*, SAHA, butyrate) have been shown to induce maturation in various human leukemia cell lines (15, 16). However, under other circumstances, HDAC inhibitors induce apoptosis rather than maturation, particularly in human leukemia cells (17, 18). Although the factors that determine which response predominates remain to be fully elucidated, evidence has appeared suggesting that the generation of reactive oxygen species (ROS; Ref. 19) and/or the extent of induction of $p21^{CIP1}$ (20) may play a role in this process.

In view of the documented activity of F-ara-AMP in hematological malignancies, particularly those of lymphoid origin, as well as ongoing efforts to develop HDAC inhibitors as antineoplastic agents (21), the possibility arose that combined treatment with these agents might result in enhanced antileukemic activity. In this context, the ability of short chain fatty acids such as phenylbutyrate and phenylacetate to potentiate the lethal effects of several conventional cytotoxic agents in multiple myeloma cells has been reported previously (22). Currently, however, interactions between established antileukemic agents and novel HDAC inhibitors now entering the clinical arena have not been extensively evaluated. One such agent is MS-275, a member of the benzamide class of HDAC inhibitors, which has shown broad preclinical activity against hematopoietic and nonhematopoietic malignant cells (23, 24) and which is now in Phase I evaluation (25). To

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date, a systematic examination of possible mechanism(s) underlying interactions between HDAC inhibitors such as MS-275 and nucleoside analogs such as F-ara-AMP in human leukemia cells has not yet been attempted. To address these issues, we have examined the effects of combined administration of HDAC inhibitors, particularly MS-275 and F-ara-AMP, in several lymphoid and myeloid cell leukemia cell types. Our results indicate that MS-275 markedly increases F-ara-AMP-induced mitochondrial injury and apoptosis in a sequence-dependent manner, resulting in highly synergistic antileukemic interactions. Moreover, the enhanced lethality of this drug combination is associated with perturbations in several signal transduction pathways, *i.e.*, inactivation of extracellular signal-regulating kinase (ERK) and Akt, activation of c-Jun NH_2-terminal kinase (JNK), perturbations in the expression of cell cycle and apoptotic regulatory proteins, increases in generation of ROS, and a striking potentiation of ceramide generation. Taken together, these findings suggest that combining F-ara-AMP with clinically relevant HDAC inhibitors such as MS-275 warrants additional examination as an antileukemic strategy.

MATERIALS AND METHODS

Cells. Jurkat lymphoblastic, U937 myelomonocytic, and CCRF-CEM lymphoblastic cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured and maintained in logarithmic phase growth in RPMI 1640 supplemented with sodium pyruvate, MEM nonessential amino acids, L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) in a 37°C, 5% CO_2, fully humidified incubator. Cells were passed three times weekly. All experiments used logarithmically growing cells suspended at a concentration of 2×10^5 cells/ml.

Jurkat cells inducibly expressing constitutively active MEK1/2 or myristolated Akt under the control of a doxycycline-responsive promoter were used as previously described in detail (26).

Drugs and Chemicals. MS-275 was kindly provided by Dr. Osamu Nakanishi (Schering-Nihon, Chiba, Japan). F-ara-AMP and 1-β-D-arabinofuranosylcytosine (ara-C) were purchased from Sigma (St. Louis, MO). Sodium butyrate was purchased from Calbiochem (La Jolla, CA), and SAHA was purchased from Alexis (San Diego, CA). Gemcitabine was a gift from Eli Lilly and Company (Greenfield, IN). The pan-caspase inhibitor BOC [BOC-ASP (OME)-FMK] was purchased from ICN Biomedicals (Aurora, OH). Human recombinant tumor necrosis factor (TNF)-α (Calbiochem) and recombinant human TNF-α soluble receptor (R&D Systems, Inc., Minneapolis, MN) were dissolved in PBS +0.5% BSA. Cycloheximide was purchased from Sigma, dissolved in PBS and stored at −20°C. Purified antihuman Fas ligand was obtained from BD PharMingen (San Diego, CA). Human anti-TNF-related apoptosis-inducing ligand antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Assessment of Cell Death. Apoptotic cells were evaluated by both morphological assessment of Diff-Quik (Dade Behring, Newark, DE) stained cytospin preparations as well as by annexin V-FITC/propidium iodide (PI) staining as described previously (27). Briefly, apoptotic cells were identified by classic morphological features (*i.e.*, nuclear condensation, cell shrinkage, and formation of apoptotic bodies) in five or more randomly selected fields/slide. Annexin V/PI (BD PharMingen) analysis of cell death was carried out as per the manufacturer's instructions using a FACScan cytofluorometer in conjunction with the Cell Quest software analytic program (Becton Dickinson, Mansfield, MA). Viability was also determined by trypan blue counting or 7-aminoactinomycin D exclusion (Sigma). For the latter studies, cells were incubated in 5μg/ml 7-aminoactinomycin D for 20 min at 37°C and assayed for cell death by flow cytometry as described previously (28). In general, results obtained by morphological assessment of apoptosis, annexin V/PI staining, or 7-aminoactinomycin D exclusion were highly concordant ($r > 0.95$).

Cell Cycle Analysis. After drug treatment, cells were pelleted by centrifugation at $500 \times g \times 6$ min and resuspended in 70% ethanol. The cells were incubated on ice for at least 1 h and resuspended in 1 ml of cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml PI) at a concentration of 10×10^5 cells/ml and stored in the dark at 4°C until analysis (24 h), using a Becton-Dickinson FACScan flow cytometer and Verity Winlist software (Verity Software, Topsham, ME).

Determination of Clonogenic Potential. After treatment, pelleted cells were washed extensively in drug-free medium, resuspended in RPMI 1640 containing 20% fetal bovine serum and 1% Bacto-agar, and seeded in 12-well culture plates at fixed density (500 cells/ml/well) as described previously (29). Cultures were maintained for 8–12 days in a fully humidified incubator, after which, colonies, consisting of groups of ≥50 cells, were scored with the aid of an Olympus-inverted microscope.

F-ara-ATP Formation and [^{3}H]F-ara-A DNA Incorporation. Formation of F-ara-AMP triphosphate was monitored using a Shimadzu Class-VP high-performance liquid chromatography system as described previously (30). F-ara-A DNA incorporation was determined by monitoring the incorporation of [^{3}H]F-ara- A into DNA as we have previously reported in detail (30).

Assessment of Mitochondrial Membrane Potential. Cells were harvested and incubated with 40 nM $DiOC_6$ (Calbiochem) for 15 min at 37°C. Analysis was carried out by flow cytometry. The percentage of cells exhibiting low levels of $DiOC_6$, reflecting loss of mitochondrial membrane potential, was determined as described previously (31).

Analysis of Cytochrome *c*, Smac/DIABLO, and Apoptosis-Inducing Factor (AIF) Release into the S-100 Cytoplasmic Fraction. A previously described method for monitoring release of mitochondrial proteins into the cytosolic, S-100 cell fraction was used (31). After treatment, 4×10^6 cells/condition were harvested by centrifugation at $500 \times g \times 5$ min. Pellets were washed once in PBS and resuspended in lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, 700μg/ml digitonin, and 250 mM sucrose) at a concentration of 4×10^6 cells/50 μl (31). After incubation for 3 min at room temperature, the cells were centrifuged for 5 min at $500 \times g$, and 5× sample buffer was added to the supernatant. For each condition, 30 μg of protein were loaded onto the gel, separated by SDS-PAGE, and subsequently probed with antibodies directed against cytochrome *c*, Smac/DIABLO, or AIF (all Santa Cruz Biotechnology) at 1:2000.

Western Blot Analysis. Whole cell pellets were washed twice in PBS, resuspended in PBS, and lysed by addition of 1 volume loading buffer. Lysates were heated at 70°C for 10 min, centrifuged at $12{,}800 \times g$ for 5 min and quantified using Coomassie protein assay reagent (Pierce, Rockford, IL). Thirty μg of total protein/condition were separated on 4–12% Bis-Tris NuPAge precast gels (Invitrogen, Carlsbad, CA) and electroblotted to nitrocellulose. The blots were blocked in 5% nonfat milk in PBS-Tween and probed for 1 h with the appropriate dilution of primary antibody. Blots were then washed 3×10 min in PBS-Tween and incubated with 1:2000 peroxidase-conjugated secondary antibody (KPL, Gaithersburg, MD) for 1 h at room temperature. The blots were again washed and developed by enhanced chemiluminesence (Perkin-Elmer, Boston, MA). Where indicated, the blots were stripped and reprobed with 1:2000 actin antibody (Sigma).

Primary Antibodies. Primary antibodies for the following proteins were used at 1:1000 unless otherwise designated. Bak, Bcl-X_L, cyclins A and E, AIF (1:2000), Mcl-1 (1:500), and Bid (1:200) were from Santa Cruz Biotechnology. Caspase 9 (1:3000), retinoblastoma protein (pRb), and underphosphorylated Rb were from BD PharMingen. $p21^{WAF1/CIP1}$, p27, cyclin-dependent kinase (cdk)2, and cdk4 were from BD Transduction Labs. X-linked inhibitor of apoptosis protein (XIAP) and procaspase 3 were from Cell Signaling Technology (Beverly, MA). Phosphospecific pRb at cdk2- and cdk4-associated sites were from Biosource International (Camarillo, CA). Antiacetylated histone H3 and H4 (1:2000) were from Upstate Biotechnology (Lake Placid, NY). Poly(ADP-ribose) polymerase (1:4000) was from BioMol (Plymouth Meeting, PA), caspase 8 (1:3000) from Alexis, and Bcl-2 from Dako Cytomation (Carpinteria, CA).

ROS. Levels of ROS were determined in treated cells by monitoring the reduction of dichlorodihydrofluoroscein acetate (Calbiochem) by flow cytometry as described previously (32).

Ceramide Generation. Generation of ceramide was assayed as described previously (33). Briefly, lipids were extracted, and mass amounts of ceramide in cellular extracts were measured by the diacylglycerol kinase enzymatic method. An aliquot (10–50 nmol of total phospholipid) of the chloroform phase from cellular lipid extracts was dried under a nitrogen stream. The lipids or standard bovine brain type IV ceramides were resuspended in 40 μl of 7.5% (w/v) octyl-b-D-glucopyranoside/5 mM cardiolipin in 1 mM DETPAC/10 mM imidazole (pH 6.6) and solubilized by freeze thawing and subsequent sonica-

tion. The enzymatic reaction was started by the addition of 20 μl of DTT (20 mM), 10 μl of *Escherichia coli* diacylglycerol kinase (0.88 units/ml), 20 μl of [g-32P]ATP (10–20 μCi, 10 mM), and 100 μl of reaction buffer [100 mM imidazole (pH 6.6), 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM EGTA]. After incubation for 1 h at room temperature, lipids were extracted with 1 ml chloroform/methanol/conc.HCl (100:200:1, v/v) and 0.17 ml of 1 M KCl. Labeled phosphatidic acid and ceramide-1-phosphate were resolved by thin-layer chromatography with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v). Bands corresponding to ceramide were scraped from the plates and counted with a scintillation counter or, alternatively, quantified with a Molecular Dynamics Storm phosphoimager (Sunnyvale, CA).

In parallel, total phospholipids present in cellular lipid extracts used for ceramide analysis were quantified as previously described (34) with minor modifications. Briefly, to dried aliquots of cellular lipid extracts, 40 μl of a mixture of 10 N H_2SO_4/70% perchloric acid (3:1, v/v) were added, and samples were incubated for 30 min at 210°C. After cooling, 75 μl of water and 400 μl of 4.2% ammonium molybdate in 4 N HCl/0.045% (w/v) malachite green (1:3 v/v) were added. Samples were incubated at 37°C for 15 min and absorbances measured at 660 nm.

Statistical Analysis. The significance of differences between experimental conditions was determined using the student's *t* test for unpaired observations. Combination indices for the designation of synergism were calculated according to the method of Chou and Talalay (35) using a commercially available software program (CalcuSyn, version 1.1; Biosoft, Ferguson, MO).

RESULTS

Interactions between MS-275 and F-ara-AMP in Human Leukemia Cells. To investigate antileukemic interactions between MS-275 and F-ara-AMP in Jurkat cells, several schedules were investigated. Exposure of cells to MS-275 (500 nM) for 48 h resulted in only a modest degree of toxicity, reflected by annexin V/PI staining (*i.e.*, ~8% increase over control values, whereas treatment with 500 nM F-ara-AMP for 24 h increased killing by ~20% (Fig. 1*A*). However, when cells were pretreated with MS-275 for 24 h followed by F-ara-AMP for an additional 24 h, the extent of cell death increased to 75%. Median dose effect analysis over a range of MS-275 and F-ara-AMP concentrations yielded combination index (CI) values considerably <1.0, corresponding to a highly synergistic interaction for this schedule (Fig. 1*B*). When cells were exposed to MS-275 and F-ara-AMP simultaneously (24 h each), CI values < 1.0 were also obtained, but the degree of synergism was less pronounced than that associated with sequential drug administration (Fig. 1*C*). In separate studies, sequential administration of F-ara-AMP followed by MS-275 resulted in additive antileukemic effects (data not shown). On the basis of these findings, subsequent studies primarily focused on investigations involving MS-275 pretreatment followed by F-ara-AMP administration.

Because induction of apoptosis does not always correlate with loss of clonogenic potential (36), clonogenic assays were performed. Exposure of Jurkat cells to MS-275 (300 nM) for 48 h or to F-ara-AMP (400 nM) for 24 h substantially reduced clonogenic potential *e.g.*, by >60% (Fig. 1*D*). However, when cells were pretreated with MS-275 followed by F-ara-AMP, colony formation was reduced by ~2 logs (*i.e.*, to ~2% of control values). CI values for drugs administered at a fixed ratio (1:1) using inhibition of clonogenicity as an end point were <1.0 (*e.g.*, 0.62–0.82; data not shown). Thus, sequential administration of MS-275 followed by F-ara-AMP resulted in a very pronounced reduction in leukemic cell self-renewal capacity.

Dose response analysis revealed that when Jurkat cells were exposed to 500 nM F-ara-AMP for 24 h, cell death, reflected by 7-amino-actinomycin D uptake, was modest (*e.g.*, ~20%). However, pretreatment of cells (24 h) with 200 nM MS-275, which was nontoxic by itself, resulted in a significant increase in F-ara-AMP-mediated cell apoptosis (Fig. 2*A*). This effect was particularly marked at MS-275 concentrations ≥ 500 nM, although at the highest concentrations,

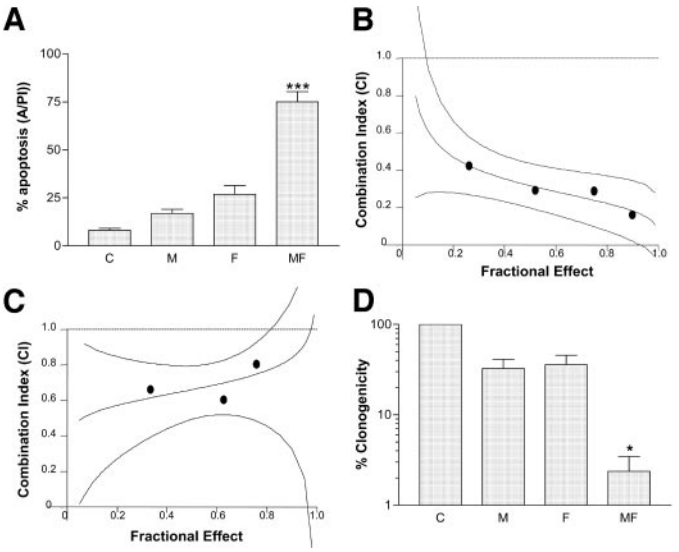

Fig. 1. *A*, Jurkat cells were exposed to 500 nM MS-275 (M) for 24 h, after which, they were either left untreated or exposed to 500 nM fludarabine (F) or an additional 24 h, after which, the extent of apoptosis was determined by annexin V/PI (A/PI) analysis as described in "Materials and Methods." Values represent the means for three separate experiments ± SE *** = significantly greater than values for fludarabine or MS-275 alone; $P < 0.001$ *B*, median dose effect analysis of apoptosis induction in Jurkat cells sequentially exposed to MS-275 followed by fludarabine as in (*A*) at a concentration ratio of 1:1. Combination index values for each fraction affected <1.0 correspond to synergistic interactions. *C*, median dose effect analysis of apoptosis induction by fludarabine and MS-275 administered simultaneously (24 h) at a concentration ratio of 1:1. *D*, Jurkat cells were sequentially exposed to 300 nM MS-275 (48 h) ± 400 nM fludarabine (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 8–12 days; *, values significantly lower than those obtained for cells exposed to MS-275 or fludarabine alone; $P < 0.02$.

MS-275-induced lethality became apparent. In addition, 24-h pretreatment with a minimally toxic concentration of MS-275 (500 nM) resulted a pronounced increase in lethality for all F-ara-AMP concentrations examined (*e.g.*, 100-1000 nM; Fig. 2*B*). A time course study revealed that prior exposure to MS-275 resulted in a small increase in F-ara-AMP-induced apoptosis at 6 h, a clear increase by 12 h, and a very pronounced increase after 24 h of exposure (Fig. 2*C*). Thus, potentiation of F-ara-AMP-mediated lethality in human leukemia cells was dose and time dependent and occurred at both low and high MS-275 and F-ara-AMP concentrations.

Effects of Combined Exposure to MS-275 and F-ara-AMP on Caspase Activation and Histone Acetylation. Treatment of Jurkat cells with MS-275 (500 nM) alone for 48 h resulted in minimal cleavage/activation of procaspases 3, 9, and 8 or Bid and poly(ADP-ribose) polymerase degradation, whereas F-ara-AMP (500 nM; 24 h) exerted modest effects (Fig. 3*A*). However, sequential exposure of cells to MS-275 followed by F-ara-AMP resulted in very marked induction of caspase activation and degradation of Bid and poly(ADP-ribose) polymerase. Each of these events was largely reversed by the pan-caspase-inhibitor Boc-D-fmk.

Effects of MS-275 ± F-ara-AMP were then examined in relation to histone acetylation. Exposure of cells to 500 nM MS-275 alone for 48 h resulted in a very clear increase in acetylation of histones H3 and H4, whereas treatment with F-ara-AMP (500 nM; 24 h) had little effect (Fig. 3*B*). Moreover, cells exposed to MS-275 followed by F-ara-AMP exhibited histone acetylation patterns similar to those observed in cells treated with MS-275 alone. These findings indicate that when administered at these concentrations, MS-275 potently induces histone acetylation but that this effect is not additionally modified by F-ara-AMP treatment.

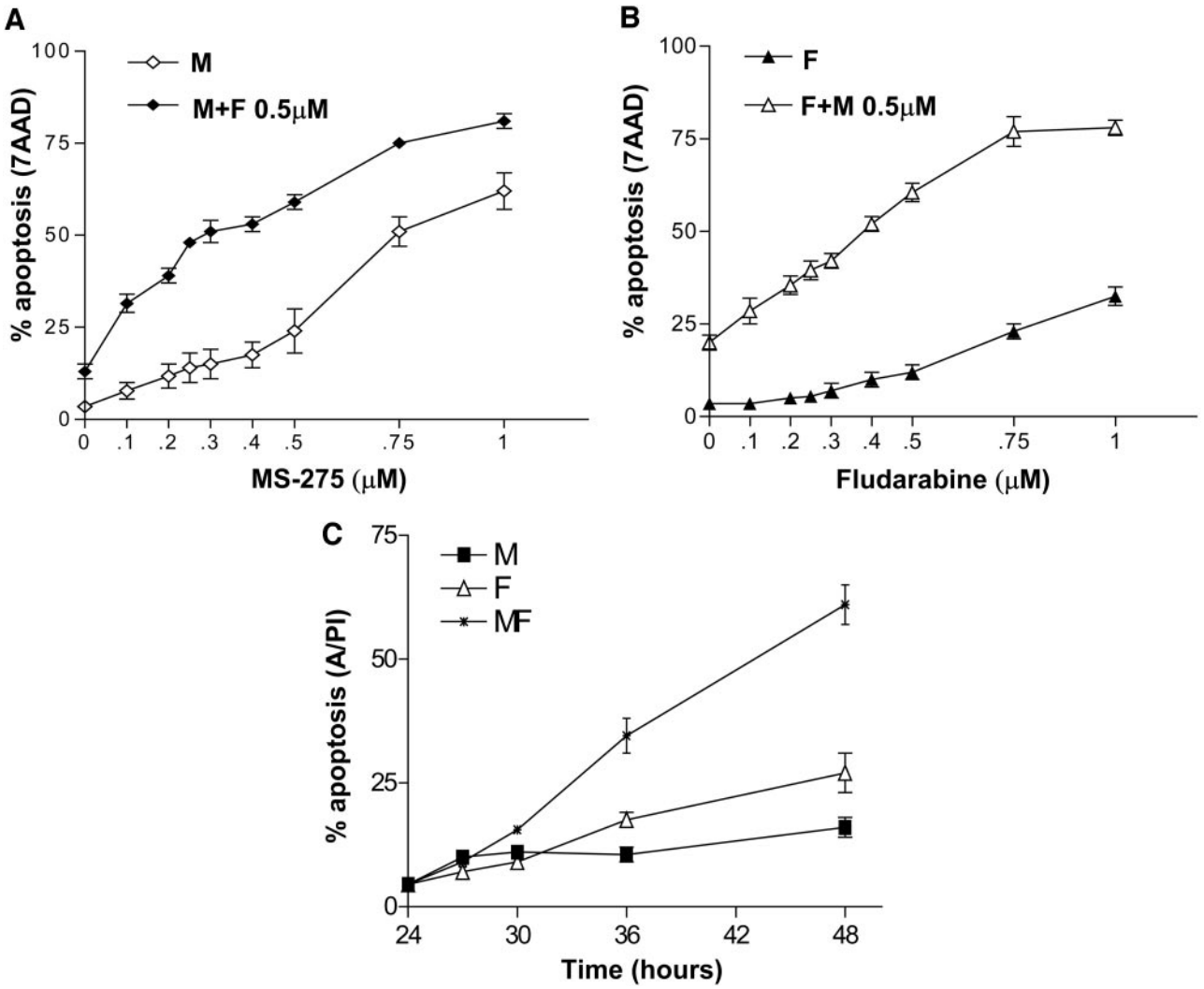

Fig. 2. *A*, Jurkat cells were exposed to 100-1000 nM MS-275 (M) for 24 h, after which, they were either left untreated or exposed to 500 nM fludarabine (F) for an additional 24 h. The extent of apoptosis was determined by 7AAD analysis as described in "Materials and Methods." *B*, cells were exposed to 500 nM MS-275 for 24 h, after which, they were either left untreated or exposed to 100-1000 nM fludarabine for an additional 24 h, after which, apoptosis was determined by 7AAD analysis. *C*, cells were incubated in drug-free medium or medium containing 500 nM MS-275 for 24 h, after which, they were exposed to 500 nM fludarabine for various intervals beyond the initial 24-h incubation period; the extent of apoptosis was then monitored by annexin V/PI staining. MF, sequential MS-275 followed by fludarabine. Values represent the means for three separate experiments ± SE

MS-275/F-ara-AMP-Mediated Lethality Does Not Involve Enhanced F-ara-AMP Metabolism, S-Phase Recruitment, or TNF-α Release. To determine whether potentiation of F-ara-AMP lethality might reflect enhanced nucleoside analogue metabolism, the effects of MS-275 pretreatment were examined in relation to formation of the lethal F-ara-AMP metabolite, F-ara-ATP (Fig. 3*C*). However, F-ara-ATP levels in MS-275-treated Jurkat cells did not differ significantly from those observed in controls ($P > 0.05$). Similarly, the extent of incorporation of [^{3}H]F-ara-AMP into DNA was not increased in MS-275-treated cells ($P > 0.05$ in each case). These findings indicate that MS-275 does not potentiate F-ara-AMP lethality by enhancing F-ara-AMP metabolism.

Recently, we have reported that potentiation of nucleoside analog lethality in human leukemia cells by the protein kinase C activator/down-regulator bryostatin 1 involved a TNF-α-related process (37). To determine whether a similar phenomenon might underlie MS-275/F-ara-AMP interactions, cells were exposed to these agents as above in the presence or absence of neutralizing TNF-α soluble receptors. As shown in Fig. 3*D*, TNF-soluble receptor had no effect on MS-275/F-ara-AMP-mediated lethality; in contrast, a significant reduction in TNF-α/cycloheximide-induced apoptosis was observed ($P < 0.02$). In related studies, coadministration of anti-FAS ligand or anti-TNF-related apoptosis-inducing ligand antibodies failed to attenuate MS-275/F-ara-AMP lethality (data not shown). Taken together, these findings argue against a major role for TNF-α release or activation of FAS- or TNF-related apoptosis-inducing ligand-related pathways as mechanisms underlying MS-275/F-ara-AMP interactions.

Effects of MS-275 and F-ara-AMP on Cell Cycle Traverse. In view of evidence that F-ara-AMP is an S-phase specific agent (7), the effects of MS-275 on the cell cycle traverse of Jurkat cells were examined (Table 1). Exposure of cells to 500 nM MS-275 for 48 h modestly but significantly reduced the S-phase fraction (*i.e.,* from 33.8 ± 0.7 to 30.4 ± 0.7%) while inducing a reciprocal increase in the G_2-M fraction (*i.e.*, from 15.5 ± 0.3 to 20.6 ± 1.0%; $P < 0.05$ and $P < 0.01$, respectively). In contrast, F-ara-AMP alone resulted in a marked increase in the G_0-G_1 fraction and a reduction in the S-phase fraction. Finally, combined treatment abrogated F-ara-AMP-mediated G_0-G_1 arrest. Thus, MS-275 increased the lethality of F-ara-AMP in Jurkat cells despite diminishing the S-phase cell fraction.

Interactions Involving Other HDAC Inhibitors, Nucleoside Analogues, and Leukemic Cell Types. As shown in Fig. 4*A*, a 48-h exposure to 500 nM MS-275 or 24-h exposure to 500 nM F-ara-AMP

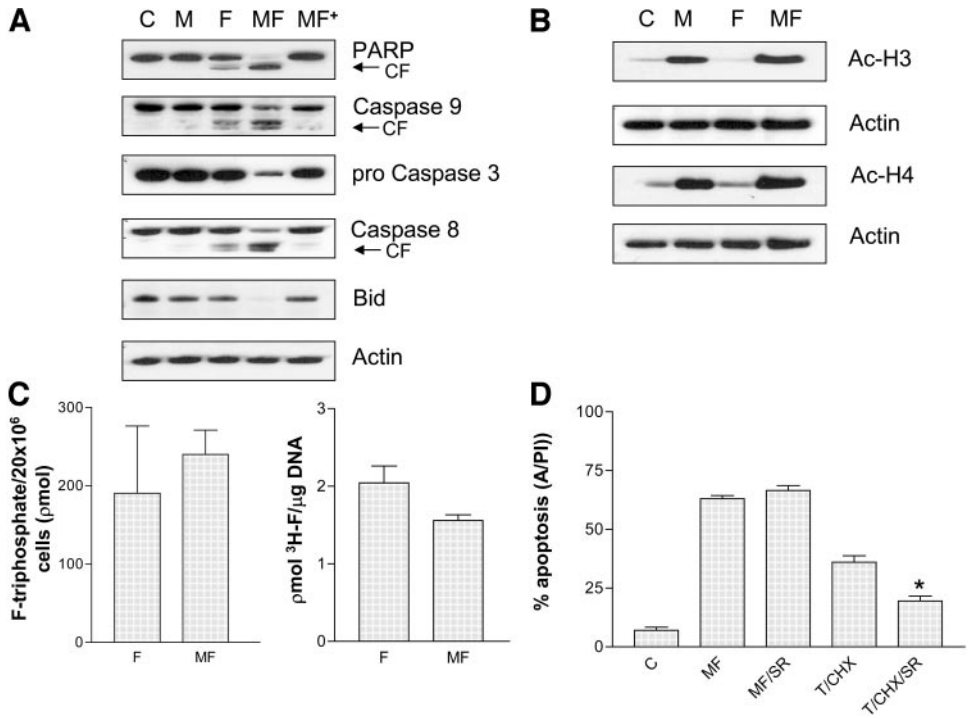

Fig. 3. *A*, Jurkat cells were sequentially exposed to 500 nM MS-275 (48 h; M), 500 nM fludarabine (24 h; F), or the combination in the presence (MF^+) or absence (MF) of 25 μM Boc-D-fmk. At the end of this period, cells were lysed, the proteins separated by SDS-PAGE, and Western blot analysis used to monitor the expression of poly(ADP-ribose) polymerase (PARP), caspase-9, caspase-3, caspase-8, and Bid. *B*, cell were exposed to MS-275 ± fludarabine as in *A*, after which, acetylated histones H3 and H4 were monitored by Western blot analysis as described in "Materials and Methods." For *A* and *B*, blots were stripped and reprobed with antibodies to actin to ensure equivalent loading and transfer. Each lane contained 30 μg of protein; two additional experiments yielded similar results. *C*, Jurkat cells were cultured in medium or medium containing 500 nM MS-275 for 44 h, after which, they were exposed to 500 nM unlabeled or [^{3}H]-labeled fludarabine for 4 h. Cells were then lysed and F-ara-ATP levels determined by high-performance liquid chromatography or [^{3}H]fludarabine (DNA) incorporation determined as described in "Materials and Methods." For both F-ara-ATP and [^{3}H]fludarabine (DNA) incorporation, values represent the means for three separate experiments ± SE. *D*, Jurkat cells were exposed to 500 nM MS-275 + 500 nM fludarabine (MF) or tumor necrosis factor (TNF)-α (T; 10 ng/ml) + cycloheximide (CHX; 1 μM) for 48 h in the absence or presence of TNF-soluble receptor (SR; 100 ng/ml), after which, apoptosis was monitored by annexin V/PI analysis. Values represent the means ± SD for three separate experiments performed in triplicate. *, significantly less than values for cells exposed to TNF/CHX in the absence of TNF-SR; $P < 0.02$.

minimally induced apoptosis in U937 myelomonocytic leukemia cells. However, sequential exposure to these agents resulted in a very marked increase in lethality (*i.e.*, to ~50% of cells). Median dose effect analysis yielded CI values very considerably <1.0, indicating a highly synergistic interaction (Fig. 4*C*). Analogous to results obtained in Jurkat cells, simultaneous exposure of U937 cells to MS-275 + F-ara-AMP also resulted in synergistic effects on cell death, although not to the extent as those observed after sequential drug administration (data not shown). In addition, synergistic interactions between MS-275 in CCRF-CEM lymphoblastic leukemia cells, although not quite as pronounced as those observed in U937 cells, were nevertheless readily apparent (Fig. 4, *B* and *D*).

Similar results were also obtained when other HDAC inhibitors were examined. For example, using the sequential drug administration paradigm, exposure of Jurkat cells to sodium butyrate or SAHA in conjunction with F-ara-AMP resulted in CI values < 1.0 in each case (Table 2). Thus, greater than additive interactions between F-ara-AMP and MS-275 could be extended to several other clinically relevant HDAC inhibitors.

To determine whether synergism involving MS-275 was restricted to F-ara-AMP, median dose effect analysis was used to characterize interactions in Jurkat cells with two other clinically relevant nucleoside analogs *i.e.*, ara-C and 2,2′-difluorodeoxycytidine (gemcitabine). As shown in Table 2, in each case, CI values were significantly <1.0, indicating synergistic interactions for each of these regimens.

Effects of MS-275/F-ara-AMP on Bcl-2 Family Members, Mitochondrial Function, and Cell Cycle-Related Proteins. Exposure of human leukemia cells to cytotoxic drugs, particularly novel cell cycle inhibitors, has been associated with down-regulation of certain apoptotic regulatory proteins (38). To determine whether this phenomenon might occur in cells exposed to MS-275 + F-ara-AMP, the expression of several antiapoptotic proteins was monitored (Fig. 5). Treatment of Jurkat cells with MS-275 and F-ara-AMP, alone or in combination, exerted little effect on expression of Bcl-2, Bcl-X_L, or Bak (Fig. 5*A*). In accord with the results of other groups (39), Bax mutations displayed by Jurkat cells precluded monitoring expression/intracellular disposition of this protein. Similarly, treatment with MS-275 failed to modify expression of the antiapoptotic proteins Mcl-1 or XIAP (Fig. 5*B*). However, F-ara-AMP alone slightly reduced Mcl-1 expression, and combined treatment was associated with virtually complete disappearance of this protein. Furthermore, cells exposed to MS-275 + F-ara-AMP exhibited a pronounced decrease in XIAP levels. Finally, down-regulation of Mcl-1 was partially reversed and XIAP largely blocked by coadministration of the pan-caspase

Table 1 *Effects of MS-275 and fludarabine on cell cycle traverse*

Logarithmically growing Jurkat cells were exposed to 500 nM MS-275 for 24 h followed by 500 nM fludarabine for an additional 24 h, after which, the percentage of cells in the G_0-G_1-, S-, and G_2 M phases of the cell cycle was determined by flow cytometric analysis of propidium iodide-stained cells as described in "Materials and Methods." Values correspond to the percentage of nonapoptotic cells and exclude the sub-G_1 population. Values represent the means for triplicate experiments ± SE.

	G_0-G_1	S	G_2-M
Control	50.6 ± 0.6	33.8 ± 0.7	15.5 ± 0.2
MS-275 (500 nM)	52.2 ± 3.1	30.4 ± 0.7	20.6 ± 0.9[a]
Fludarabine (500 nM)	64.9 ± 0.5[b]	25.2 ± 1.3[c]	11.6 ± 0.8[c]
MS-275 + fludarabine	46.3 ± 2.4[c]	35.3 ± 1.3	18.9 ± 1.15[a]

[a] Values significantly greater than controls; $P < 0.05$; [b] Values significantly greater than controls; $P < 0.01$; [c] Values significantly less than controls; $P < 0.05$.

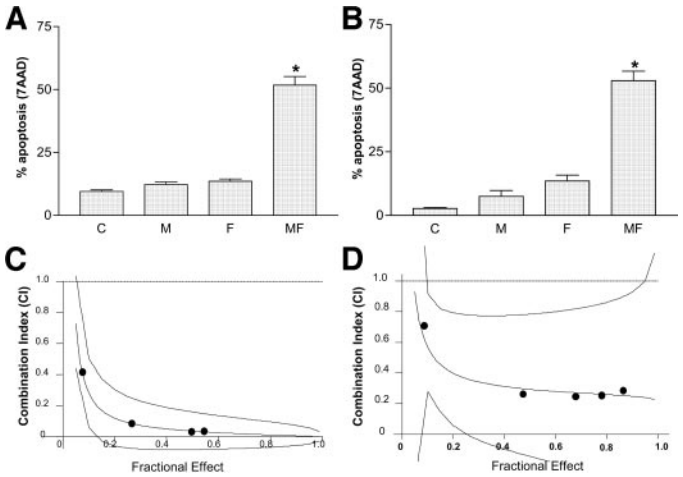

Fig. 4. *A*, U937 cells were exposed to 500 nM MS-275 (M) for 24 h, after which, they were incubated with either drug-free medium or 500 nM fludarabine (F) for an additional 24 h as above. At the end of this period, the extent of cell death was determined by monitoring 7-aminoactinomycin D (7-AAD) uptake as described in "Materials and Methods." *B*, CCRF-CEM lymphoblastic leukemia cells were exposed to MS- 275 and fludarabine as in *A*, except that the MS-275 concentration was 1 μM. For *A* and *B*, values represent the means for three separate experiments performed in triplicate ± SE. *, significantly greater than values for fludarabine or MS-275 alone; $P < 0.001$. *C* and *D*, median dose effect analysis of apoptosis induction by MS-275 and fludarabine in U937 (*C*) and CCRF (*D*) cells. A constant MS-275/fludarabine dose ratio was used in each case (1:1 for U937; 2:1 for CCRF-CEM). Values are the means of three separate determinations.

Table 2 *Interactions between HDAC inhibitors and nucleoside analogues in Jurkat cells*

Logarithmically growing Jurkat cells were exposed for 24 h to varying concentrations of the indicated HDI (*e.g.*, suberoylanilide hydroxamic acid (SAHA), MS-275, or sodium butyrate), after which, they were incubated for an additional 24 h with various concentrations of fludarabine, 1-β-D-arabinofuranosyl-cytosine (ara-C), or gemcitabine administered at a fixed ratio. At the end of this period, the extent of cell death was determined by annexin V/propidium iodide staining in conjunction with flow cytometry as described in "Materials and Methods." Combination index values (CI) for each fraction affected (Fa) were calculated using a commercially available software program (Calcusyn; Biosoft). CI values < 1.0 correspond to synergistic interactions. Values are representative for three separate experiments performed in triplicate.

		Fa ± SE	CI ± SE
I	SAHA → fludarabine	0.31 ± 0.05	0.77 ± 0.03
		0.50 ± 0.07	0.73 ± 0.04
		0.83 ± 0.01	0.69 ± 0.02
II	Butyrate → fludarabine	0.16 ± 0.03	0.63 ± 0.05
		0.41 ± 0.02	0.47 ± 0.07
		0.61 ± 0.11	0.62 ± 0.06
III	MS-275 → ara-C	0.42 ± 0.04	0.47 ± 0.06
		0.65 ± 0.06	0.45 ± 0.06
		0.82 ± 0.04	0.56 ± 0.07
IV	MS-275 → gemcitabine	0.39 ± 0.52	0.71 ± 0.25
		0.66 ± 0.20	0.43 ± 0.07
		0.78 ± 0.05	0.49 ± 0.05

inhibitor Boc-D-fmk, indicating that these events are at least partially caspase dependent.

Induction of apoptosis, particularly by cytotoxic drugs, is intimately related to mitochondrial dysfunction (40). Consequently, the effects of combined exposure to MS-275 and F-ara-AMP on leukemic cell mitochondrial function were examined. As shown in Fig. 5*C*, exposure of cells to MS-275 or F-ara-AMP alone minimally affected cytosolic release of cytochrome *c*, AIF, or Smac/DIABLO. In contrast, combined MS-275/F-ara-AMP exposure markedly increased release of cytochrome *c*, AIF, and Smac/DIABLO. Consistent with the preceding observations, combined treatment of leukemic cells with MS-275 and F-ara-AMP also resulted in a pronounced loss of $\Delta\Psi_m$ (data not shown). Taken together, these findings indicate that sequential exposure of leukemic cells to MS-275 and F-ara-AMP results in a marked potentiation of mitochondrial dysfunction.

Effects of MS-275 ± F-ara-AMP Were Then Examined in Relation to Expression of Various Cell Cycle-Related Proteins. Although MS-275 or F-ara-AMP alone exerted either no or only modest effects on expression of $p27^{KIP1}$, cyclin A, cyclin E, or cyclin D_1, combined treatment resulted in a pronounced reductions in protein levels (Fig. 6*A*). Moreover, coadministration of the pan-caspase inhibitor Boc-D-fmk substantially reversed these effects in the case of cyclin D_1 and $p27^{KIP1}$ but only partially in the case of cyclins A and E, indicating that such changes were at least in part caspase mediated. In contrast to these findings, the MS-275/F-ara-AMP regimen resulted in very modest reductions in $p21^{CIP1}$expression. Comparable changes were noted in U937 cells exposed to MS-275 and F-ara-AMP (data not shown).

Exposure to MS-275 + F-ara-AMP, but not to the drugs individually, resulted in pRb cleavage (Fig. 6*B*). Interestingly, coadministration of MS-275 + F-ara-AMP was associated with increased expression of underphosphorylated pRb, accompanied by the appearance of a corresponding pRb cleavage fragment. The latter effect, but not pRb dephosphorylation, was blocked by Boc-D-fmk. Exposure of cells to MS-275 + F-ara-AMP was also associated with diminished phosphorylation of pRb on CDK2- and CDK4-specific sites (Fig. 6*B*), effects that were partially blocked by Boc-D-fmk. Thus, diminished phospho-

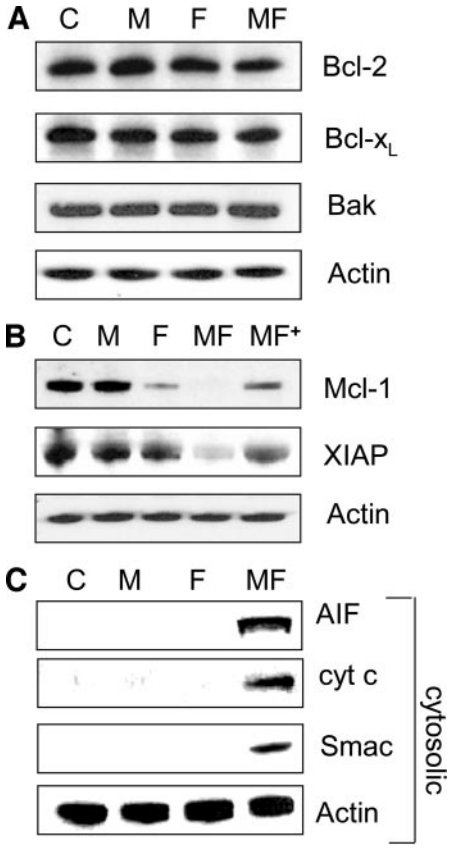

Fig. 5. *A*, Jurkat cells were exposed to either no drugs (C), MS-275 (M; 500 nM) for 48 h, fludarabine (F; 500 nM) for 24 h, or MS-275 for 48 h with fludarabine added during the last 24 h (MF). At the end of this period, cells were lysed, the proteins separated by SDS-PAGE, and Western blot analysis used to monitor expression of Bcl-2, Bcl-X_L, and Bak. *B*, Jurkat cells were exposed to MS-275 ± fludarabine as above, except that in some cases, cells were exposed to MS-275 + fludarabine in the presence of 25 μM Boc-D-fmk (MF^+). *C*, Jurkat cells were exposed to MS-275 ± fludarabine as above, after which, cytosolic S-100 fractions were obtained as described in "Materials and Methods" and the expression of cytosolic cytochrome *c*, apoptosis-inducing factor (AIF), and Smac/DIABLO was monitored by Western blot. Each lane contained 30 μg of protein; blots were stripped and reprobed with antibodies to actin to ensure equivalent loading and transfer. Results shown reflect a representative experiment; two additional studies yielded equivalent results.

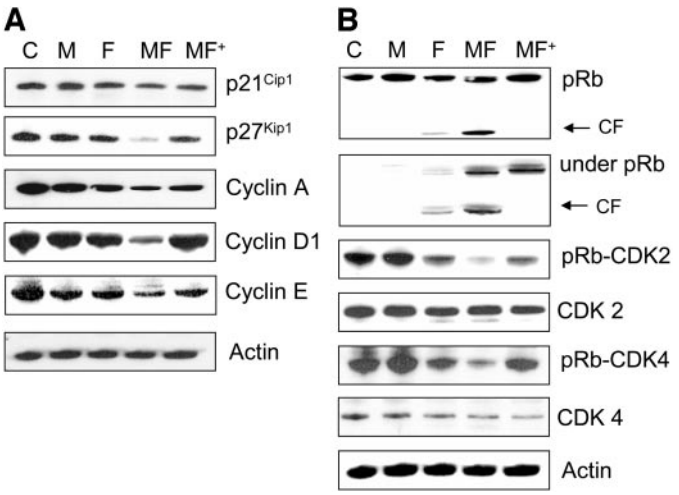

Fig. 6. *A*, Jurkat cells were exposed to 500 nM fludarabine for 24 h after incubation with either drug-free medium or 500 nM MS-275 for 24 h, after which, cell lysates were obtained and subjected to Western blot analysis as above. C, control; M, MS-275 alone (48 h); F, fludarabine alone (24 h); MF, MS-275 before fludarabine. In parallel studies, cells were exposed to MS-275 followed by fludarabine in the presence of 25 μM Boc-D-fmk. Blots were then probed with antibodies directed against $p21^{CIP1}$, $p27^{KIP1}$, cyclins A, E, and D1. *B*, cells were treated as in *A*, and blots probed with antibodies directed against total pRb, underphosphorylated pRb, CDK2, CDK4, and pRb phosphorylated on CDK2- and CDK4-specific sites. Each lane was loaded with 30 μg of protein; blots were stripped and reprobed with antibodies to actin to ensure equivalent loading and transfer. Results of a representative study are shown; two additional experiments yielded similar results. CF, cleavage fragment.

rylation of pRb in MS- 275/F-ara-AMP-treated cells may reflect both reduced expression of cyclins as well as pRb degradation.

Effects of MS-275/F-ara-AMP on Signal Transduction Pathways. Treatment of Jurkat cells with MS-275 alone for 24 h resulted in a decline in levels of phospho-MEK, phospho-ERK, and phospho-Akt, accompanied by a modest increase in levels of phospho-JNK (Fig. 7). Cells exposed to F-ara-AMP alone (3 h) exhibited no change in Akt and JNK activation but a clear increase in levels of phospho-MEK and ERK. However, prior exposure of cells to MS-275 largely abrogated F-ara-AMP-mediated mitogen-activated protein/extracellular signal-regulated kinase (MEK) and ERK activation, diminished Akt phosphorylation, and resulted in a clear increase in JNK activation. No changes in total MEK, ERK, Akt, or JNK levels were observed under any of the conditions, nor were perturbations in the expression of total or phospho-p38 mitogen-activated protein kinase observed. In separate studies, MS-275 also attenuated ERK and Akt activation and promoted JNK activation in F-ara-AMP-treated U937 cells (data not shown). Thus, prior exposure of F-ara-AMP-treated leukemia cells to MS-275 abrogated or attenuated activation of the cytoprotective MEK/ERK and Akt pathways and reciprocally promoted activation of the stress-related JNK pathway.

Effects of Enforced Activation of MEK/ERK on MS-275/F-ara-AMP Lethality. To investigate the functional significance of MEK1/2 and ERK inactivation by MS-275 in relation to F-ara-AMP-mediated lethality, Jurkat cells (designated MT-6) stably expressing a constitutively active form of MEK as well as an HA tag under the control of a doxycycline-responsive promoter were used (26). As shown in Fig. 8*A*, exposure of cells to doxycycline resulted in a clear increase in phospho-ERK (as well as expression of an HA tag) after combined exposure to MS-275 and F-ara-AMP. Moreover, annexin V/PI analysis revealed a very significant reduction in apoptosis in cells exposed to MS-275/F-ara-AMP in the presence of doxycycline ($P < 0.01$ compared with cells cultured without doxycycline in each case). Essentially identical results were obtained with a second inducible clone (MER-7; data not shown). These findings suggest that interference with the MEK/ERK cytoprotective pathway by MS-275 contributes functionally to the marked synergism exhibited by the MS-275/F-ara-AMP regimen.

Effects of Enforced Activation of Akt on MS-275/F-ara-AMP Lethality. Parallel studies were carried out using Jurkat cells (Akt 29) that express constitutively active (myristolated) Akt in the presence of doxycycline (Fig. 8*B*). Culture of cells in the presence of doxycycline resulted in a clear increase in expression of phospho-Akt, as well as a c-Myc tag, in cells exposed to either medium alone or the combination of MS-275 + F-ara-AMP. Coexposure of cells to the MS-275/F-ara-AMP regimen in the presence of doxycycline resulted in a significant reduction in cell death ($P < 0.01$ compared with cells exposed to drugs in the absence of doxycycline). This suggests that inactivation of Akt plays a functional role in promoting leukemia cells exposed to MS-275 + F-ara-AMP.

Role of ROS in MS-275/F-ara-AMP-Mediated Antileukemic Activity. Because the lethal effects of both nucleoside analogues (41) as well as HDAC inhibitors (19) have been related to the oxidative stress, interactions between these agents were examined in relation to the generation of ROS. As shown in Fig. 9, pretreatment of cells with 500 nM MS-275 was associated with a minimal increase in ROS generation, reflected by enhanced uptake of 2′,7′-dichlorofluorescein diacetate (Fig. 9*A*). However, in cells previously exposed to a subtoxic concentration of MS-275 (*e.g.*, 500 nM), F-ara-AMP treatment (500 nM) was significantly more effective in triggering ROS generation compared with effects observed in untreated control cells ($P < 0.002$). Furthermore, coadministration of the free radical scavenger L-*N*-acetylcysteine (LNAC) essentially abrogated the increase in ROS generation in MS-275/F-ara-AMP-treated cells (Fig. 9*A*, shaded bars). Significantly, attenuation of ROS generation by LNAC was accompanied by a pronounced reduction in apoptosis, reflected by diminished annexin V/PI staining (Fig. 9*B*). Taken together, these findings

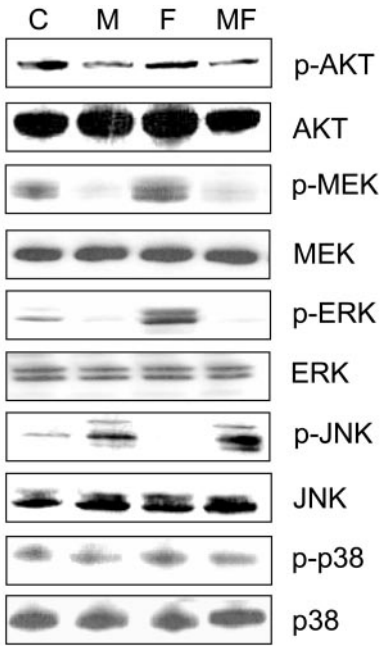

Fig. 7. Jurkat cells were incubated for 24 h in medium alone or medium containing 500 nM MS- 275, after which, they were exposed to 500 nM fludarabine for 3 h. Cells were then lysed, the protein extracts separated by SDS-PAGE, and Western blot analysis used to monitor the expression of total and phosphorylated mitogen-activated protein/extracellular signal-regulated kinase (MEK)1/2, extracellular signal-regulated kinase (ERK)1/2, c-Jun NH_2-terminal kinase (JNK), p38, and Akt as described in "Materials and Methods." Each lane was loaded with 30 μg of protein. Results of a representative experiment are shown; two additional experiments yielded equivalent results.

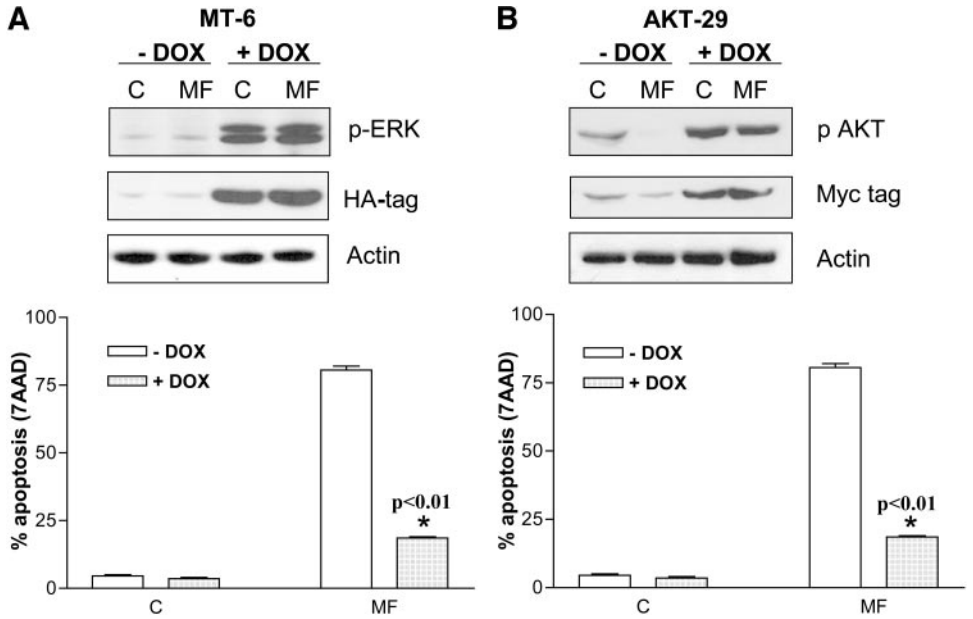

Fig. 8. *A*, Jurkat cells (MT-6) inducibly expressing constitutively active mitogen-activated protein/extracellular signal-regulated kinase 1/2 as well as a HA tag under the control of a doxycycline (DOX)-responsive promoter were exposed to 500 nM MS- 275 (24 h) followed by 1.5 μM fludarabine for an additional 24 h in the presence or absence of DOX as described in "Materials and Methods." Western blot analysis was then performed, demonstrating increased expression of phospho-ERK as well as the HA tag in cells exposed to DOX. Each lane was loaded with 30 μg of protein; blots were stripped and reprobed with antibodies to actin to ensure equivalent loading and transfer of protein. In parallel, the extent of apoptosis was determined by annexin V/PI analysis as described in "Materials and Methods." Values represent the means for three separate experiments performed in triplicate ± SE; *, significantly less than values obtained for cells cultured in the absence of DOX; $P < 0.01$. *B*, Jurkat cells inducibly expressing constitutively activated Akt (AKT29) as well as a c-Myc tag in the presence of DOX were exposed to MS-275 + fludarabine (±DOX) as described above in *A*, after which the percentage of apoptotic cells was monitored by 7-AAD analysis as previously described. Western blot analysis was also performed to demonstrate increased expression of phospho-Akt as well as the c-Myc tag in cells exposed to DOX. Each lane was loaded with 20 μg of protein; blots were stripped and reprobed with antibodies to actin to ensure equivalent loading and transfer of protein. *, significantly less than values obtained for cells cultured in the absence of DOX; $P < 0.01$. MF, sequential MS-275 followed by fludarabine; C, no drugs.

suggest that enhanced ROS generation contributes to the potentiation of F-ara-AMP lethality by MS-275 in human leukemia cells.

Effects of Combined Exposure to MS-275 and F-ara-AMP on Ceramide Generation in Leukemia Cells. Finally, previous studies have demonstrated that exposure of human leukemia cells to nucleoside analogues such as ara-C results in induction of the lipid second messenger ceramide (42), which has been implicated in apoptosis induction, particularly in human leukemia cells (43). To determine whether perturbations in ceramide metabolism might be implicated in the lethal effects of the MS-275/F-ara-AMP regimen, ceramide generation was monitored in cells exposed to F-ara-AMP, either alone, or after treatment with MS-275. As shown in Fig. 10, exposure to MS-275 for 48 h or F-ara-AMP alone for 24 h induced only modest increases in levels of ceramide (*i.e.*, ~1.3- and 3-fold, respectively, *versus* controls). The latter result is very similar to that reported by Biswal *et al.* (44) who observed approximately a 3-fold increase in ceramide levels in F-ara-AMP-treated B-lymphoblastic leukemia cells. However, sequential exposure of cells to MS-275 and F-ara-AMP resulted in a very dramatic increase in ceramide generation (*e.g.*, 11-fold). Such findings raise the possibility that MS-275 increases the ability of F-ara-AMP to trigger ceramide production, a phenomenon that may contribute to the lethality of this drug combination.

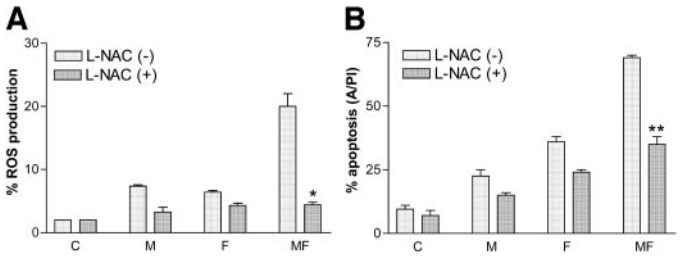

Fig. 9. Jurkat cells were incubated in either drug-free medium or medium containing MS-275 (M; 500 nM) for 24 h, after which, they were exposed to 500 nM fludarabine (F). The percentage of cells displaying an increase in reactive oxygen species (ROS) generation was determined after 1 h by monitoring uptake of dichlorodihydrofluorescein diacetate (DCF-H2) by flow cytometry as described in "Materials and Methods." Parallel studies were performed in the presence of the free radical scavenger L-*N*-acetylcysteine (LNAC; 20 mM). *B*, cells were exposed to MS-275 (500 nM; 48 h) ± fludarabine (500 nM; 24 h) in the presence or absence of LNAC, after which, the percentage of apoptotic cells was determined by annexin V/PI analysis as described in "Materials and Methods." Values represent the means for three separate experiments performed in triplicate ± SE; *, significantly less than values for sequential MS-275 followed by fludarabine (MF) in the absence of LNAC; $P < 0.02$; **, $P < 0.01$.

DISCUSSION

Recently, considerable attention has focused on HDAC inhibitors as anticancer agents, particularly in hematopoietic malignancies, in view of their ability to induce either differentiation or apoptosis in leukemic cells (15, 17). Although it is presumed that such actions result from deacetylation of histones and transcriptional activation of genes involved in these processes, the specific downstream events responsible for induction of cell death or maturation remain to be fully elucidated. Initial studies focused on the capacity of HDAC inhibitors to trigger the expression of genes such as $p21^{CIP1}$ (23) and gelsolin (45); however, more recently, other HDAC inhibitor actions, including perturbations in signal transduction pathways (44) and redox state (19), have attracted attention. In addition, HDAC inhibitors may modify acetylation of substrates other than histones, including chaperone proteins such as Hsp90 (46). Aside from their intrinsic capacity to induce apoptosis or maturation, HDAC inhibitors, as with other differentiation-inducing agents (*i.e.*, all-*trans*-retinoic acid) may also enhance the lethal effects of conventional cytotoxic agents (47). However, with the possible exception of topoisomerase inhibitors in which HDAC inhibitor-mediated increases in topoisomerase II levels appear to contribute to lethality (48), the mechanism by which HDAC

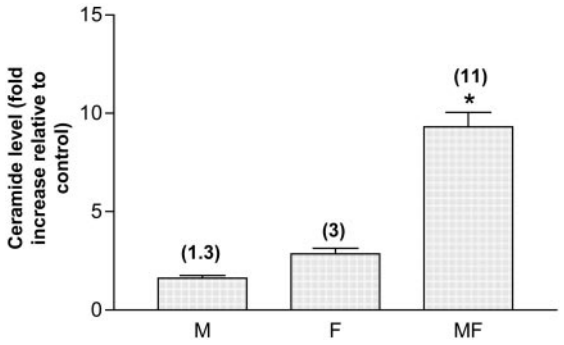

Fig. 10. Jurkat cells were exposed to 500 nM fludarabine (F; 24 h) after a 24-h exposure to either drug-free medium or medium containing 500 nM MS-275 (M). At the end of this period, ceramide levels, normalized to levels of total phospholipids, were compared with those of untreated control cells. Values are expressed as fold increases over control levels and represent the means for five separate determinations ± SE basal (unstimulated) ceramide levels were 15 ± 6 pmol ceramide/nm phospholipid. *, significantly greater than values for fludarabine alone; $P < 0.0001$.

inhibitors increase the cytotoxic activity of established cytotoxic drugs is unclear. The present results suggest that several previously reported as well as novel HDAC inhibitor actions, including promotion of free radical formation, interference with cytoprotective signaling pathways, activation of stress-related cascades, reduced expression of antiapoptotic proteins, and enhanced generation of ceramide, may all contribute to synergistic antileukemic interactions.

It is noteworthy that although prior exposure of cells to MS-275 resulted in the most pronounced increase in F-ara-AMP-mediated apoptosis, simultaneous exposure of cells to these agents also led to a clear increase in cell death. Such findings are consistent with results of an earlier study involving multiple myeloma cells in which simultaneous treatment with phenylbutyrate and various cytotoxic agents led to synergistic or additive effects on cell viability, depending upon the agent and the extent of lethality (22). However, in the present study, exposure of cells to F-ara-AMP followed by MS-275 did not lead to a major increase in toxicity. This observation stands in contrast to that of a previous report in which sequential exposure of HL-60 cells to DNA-damaging agents, including 5-azacytidine, followed by a differentiation-inducing stimulus (*i.e.*, the HDAC inhibitor sodium butyrate) resulted in a marked increase in lethality (49). In any case, the finding that prior exposure of leukemic cells to MS-275 maximized F-ara-AMP lethality is most consistent with the notion that HDAC inhibitors trigger time-dependent events that lower the threshold for F-ara-AMP-mediated apoptosis.

Combined exposure of leukemia cells to MS-275 and F-ara-AMP resulted in increase in ROS generation; moreover, the free radical scavenger LNAC blocked this effect, as well as the lethality of the regimen. These observations suggest that enhanced generation of ROS may contribute to potentiation of F-ara-AMP-induced apoptosis by MS-275. In this context, ROS generation has previously been implicated in mediating the lethal effects of ara-C in human leukemia cells, presumably a consequence of nucleoside analogue-related DNA damage (41). In addition, the contribution of ROS to HDAC inhibitor-mediated lethality has recently been described previously (19). Consistent with these findings, we have recently reported that MS-275 is a particularly potent inducer of ROS in human leukemia cells and that the lethal consequences of this action are modulated by $p21^{CIP1}$ (50). In view of these considerations, as well as the observation that LNAC substantially blocked MS-275/F-ara-AMP-related lethality, it is tempting to propose that synergistic interactions between these agents reflect, at least in part, enhanced ROS generation. However, it should be noted that LNAC has been reported to exert pleiotropic actions in addition to its role as a free radical scavenger (51), and the possibility that one or more of these may contribute to its cytoprotective capacity cannot be excluded.

The observation that exposure of leukemic cells to MS-275 in conjunction with F-ara-AMP resulted in diminished activation of ERK is consistent with previous studies suggesting a general cytoprotective role for the MEK/ERK pathway (52), as well as with earlier observations, indicating that MEK/ERK inactivation increases the antileukemic activity of nucleoside analogs such as ara-C and other cytotoxic agents (53, 54). It is also in accord with more recent data indicating that HDAC inhibitors, through as yet to be determined mechanisms, induce down-regulation of the Raf/MEK/ERK cascade (46, 55). In this regard, it is noteworthy that HDAC inhibitors can induce acetylation of nonhistone proteins such as the chaperone Hsp90 (46), raising the possibility that in so doing, they may promote the degradation of client proteins such as Raf-1. Consistent with this concept, HDAC inhibitors have been shown to interact with STI571 to promote down-regulation of the Bcr/abl protein by several groups (56, 57). In any case, the notion that relative contribution of the cytoprotective Raf/MEK/ERK and stress-related JNK modules represents a critical determinant of cell survival is well established (52). Furthermore, the observation that coadministration of MS-275 potentiated JNK activation in F-ara-AMP-treated cells while reciprocally inactivating ERK is entirely consistent with this model. Although the downstream effectors of ERK prosurvival actions remain to be fully elucidated, the Ets family of transcription factors (*i.e.*, Elk; Ref. 58) as well as cAMP-responsive element binding protein (59) have been implicated. Most recently, ERK activation has been implicated in phosphorylation and inactivation of procaspase-9 (60). On the other hand, the proapoptotic activity of JNK has been linked to multiple events, including cytochrome *c* release (61), phosphorylation of Bcl-2 (62) and Mcl-1 (63), as well as to oxidative stress (64). Thus, it is possible that HDAC inhibitors, by diminishing the protective influence of the Raf/MEK/ERK cascade and promoting stress responses, may lower the threshold for nucleoside analog-mediated mitochondrial injury. In support of this concept, we have recently reported that coadministration of histone deacetylase inhibitors such as SAHA and sodium butyrate increase the lethality of the kinase inhibitor STI571 in association with inactivation of the MEK/ERK module (57). Finally, evidence that ERK activation protects cells from oxidative stress (65) raises the possibility that HDAC inhibitor-mediated disruption of the Raf/MEK/ERK cascade may enhance F-ara-AMP-mediated oxidative injury.

It is also worth noting that prior exposure to MS-275 diminished, albeit partially, F-ara-AMP-mediated activation of Akt. Inhibition of cell death by the PI3K/Akt pathway is well described (66), and recent studies suggest that inactivation of this cascade sensitizes neoplastic cells to drug-induced apoptosis (67). Akt may protect cells from apoptosis through a variety of mechanisms, including phosphorylation of procaspase-9 and inactivation of Bad (68) among several others (69). Consistent with a cytoprotective role for the Akt cascade, enforced activation of Akt substantially diminished the lethal effects of the MS-275/F-ara-AMP regimen. Thus, the notion that HDAC inhibitor-mediated inactivation of Akt contributes to MS-275/F-ara-AMP lethality appears plausible.

Combined exposure of leukemic cells to MS-275 and F-ara-AMP induced several perturbations in cell cycle and survival regulatory proteins that might also play a role in cell death. For example, prior exposure of F-ara-AMP-treated cells with MS-275 resulted in the caspase-dependent down-regulation of the CDKI $p27^{KIP1}$, which has been shown to protect cells from apoptosis (70). It also resulted in diminished phosphorylation of pRb, an effect that might reflect both pRb degradation as well as reduced expression of cyclins A, E, and D_1. In this regard, down-regulation of cyclin D_1 has been associated

with apoptosis induced by pharmacological kinase inhibitors, including the CDK inhibitor flavopiridol (71). In addition, combined treatment with MS-275 resulted in down-regulation of Mcl-1 and XIAP, events that were also at least partially caspase dependent. Recent studies have emphasized the important role that Mcl-1 may play in malignant hematopoietic cell survival (72) and the ability of cell cycle inhibitors other than HDAC inhibitors (*e.g.*, flavopiridol) to diminish XIAP expression has previously been described (38). The relative contribution, if any, of these events to synergistic induction of apoptosis in MS-275/F-ara-AMP-treated cells remains to be elucidated.

The observation that combined treatment of leukemia cells with MS-275 and F-ara-AMP resulted in a marked increase in ceramide generation represents, to the best of our knowledge, the first evidence of a link between the actions of HDAC inhibitors and lipid signaling pathways. Ceramide, which can be generated through either *de novo* synthesis or via acid hydrolysis of sphingomyelin (73), has been established as an inducer of apoptosis in neoplastic cells, including those of hematopoietic origin (43). In this regard, induction of apoptosis in leukemic cells by ara-C has been associated with increased ceramide levels (42), and of particular relevance to the present studies, ceramide generation has been implicated in the lethal actions of F-ara-AMP in B-chronic lymphocytic leukemia cells (44, 74). Furthermore, it has been shown that perturbations in ceramide metabolism can enhance the sensitivity of tumor cells to conventional cytotoxic drugs (75). Interestingly, ceramide has been implicated in ROS-mediated lethality (76) and appears to exert its lethal effects through a JNK-dependent process (77). Finally, in some cell types, ceramide has been reported to trigger ERK (78) and Akt (79) inactivation. Thus, it is tempting to propose that enhanced ceramide generation in MS-275/F-ara-AMP-treated leukemic cells may play a central role in multiple proapoptotic signaling events, including increased ROS production, ERK, and Akt down-regulation, and activation of JNK. Whether in fact increased ceramide levels represent a cause or consequence of one or more of these events remains to be determined.

In summary, the present findings indicate that clinically relevant HDAC inhibitors, including novel agents such as MS-275, interact in a highly synergistic manner with nucleoside analogs such as F-ara-AMP in a schedule-dependent manner to trigger mitochondrial dysfunction, caspase activation, apoptosis, and loss of clonogenic survival in human leukemia cells. These interactions are unrelated to changes in cell cycle traverse or F-ara-AMP metabolism but are associated with multiple perturbations in expression/activation of cell cycle, survival, and signaling proteins, including diminished expression of Mcl-1 and XIAP, reductions in ERK and Akt activation, and stimulation of the stress-related JNK pathway. The observation that combined exposure of leukemic cells to MS-275 and F-ara-AMP resulted in a significant increase in ROS generation and that the free radical scavenger LNAC blocked this action as well as cell death suggests that oxidative stress contributes to the antileukemic synergism of this drug combination. Finally, prior exposure of leukemic cells to MS-275 strikingly potentiated F-ara-AMP-mediated ceramide generation, a phenomenon that has previously been implicated in nucleoside analog-mediated apoptosis. One issue that remains to be addressed is the extent to which potentiation the lethality of F-ara-AMP occurs in normal host tissues and whether a net gain in therapeutic index will result from this combination. Answers to such questions will clearly have a critical impact on the translational implications of the present findings. These issues notwithstanding, additional insights into the relative roles of these events in HDAC inhibitor/F-ara-AMP-related synergism may provide a rational basis for future efforts to combine newer generation HDAC inhibitors such as MS-275 with nucleoside analogues such as F-ara-AMP in the development of novel antileukemic strategies.

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