

The Histone Deacetylase Inhibitor MS-275 Promotes Differentiation or Apoptosis in Human Leukemia Cells through a Process Regulated by Generation of Reactive Oxygen Species and Induction of p21^{CIP1/WAF1}

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ABSTRACT

Effects of the histone deacetylase (HDAC) inhibitor MS-275 have been examined in human leukemia and lymphoma cells (U937, HL-60, K562, and Jurkat) as well as in primary acute myelogenous leukemia blasts in relation to differentiation and apoptosis. MS-275 displayed dose-dependent effects in each of the cell lines. When administered at a low concentration (e.g., 1 μ M), MS-275 exhibited potent antiproliferative activity, inducing p21^{CIP1/WAF1}-mediated growth arrest and expression of differentiation markers (CD11b) in U937 cells. These events were accompanied by an increase in hypophosphorylated retinoblastoma protein and down-regulation of cell cycle-related proteins including cyclin D1. However, at higher concentrations (e.g., 5 μ M), MS-275 potently induced cell death, triggering apoptosis in ~70% of cells at 48 h. In contrast to other HDAC inhibitors such as apicidin, the extrinsic, receptor-mediated pathway played a minimal role in MS-275 lethality. However, MS-275 potently induced a very early (e.g., within 2 h) increase in reactive oxygen species (ROS), followed by the loss of mitochondrial membrane potential ($\Delta\psi_m$) and cytosolic release of cytochrome *c*. These events culminated in activation of the caspase cascade, manifested by poly(ADP-ribose) polymerase, p21^{CIP1/WAF1}, p27^{KIP}, Bcl-2, and retinoblastoma protein degradation. MS-275 exposure also resulted in diminished expression of cyclin D1 and the antiapoptotic proteins Mcl-1 and XIAP. Administration of the free radical scavenger L-N-acetylcysteine blocked MS-275-mediated mitochondrial injury and apoptosis, suggesting a primary role for ROS generation in MS-275-associated lethality. Lastly, U937 cells stably expressing a p21^{CIP1/WAF1} antisense construct were significantly more sensitive to MS-275-mediated apoptosis than controls, but they were impaired in their differentiation response. Together, these findings demonstrate that MS-275 exerts dose-dependent effects in human leukemia cells, i.e., p21^{CIP1/WAF1}-dependent growth arrest and differentiation at low drug concentrations and a marked induction of ROS, mitochondrial damage, caspase activation, and apoptosis at higher concentrations.

INTRODUCTION

Recently, HDAC⁴ inhibitors have emerged as a potentially promising new class of anticancer drugs (reviewed in Refs. 1–3). HDAC inhibitors such as SB, SAHA, apicidin, and trichostatin A represent structurally diverse compounds that share the capacity to interrupt cell cycle progression in G₁ and G₂-M phase, resulting in growth arrest, differentiation, and/or cell death (4–10). The latter represent alterna-

tive and, under some circumstances, mutually exclusive cell fates (7, 11). Currently, the factors that determine whether HDAC inhibitors induce cellular maturation *versus* apoptosis remain largely unknown. Histone hyperacetylation induces the expression or repression of a variety of genes that mediate the effects of HDAC inhibitors on cell growth/differentiation (12, 13). Of these, induction of the CDKI p21^{CIP1/WAF1} may play a particularly important role in governing the effects of HDAC inhibitors on tumor cell growth arrest and apoptosis (7, 9, 14–16).

In leukemia models, HDAC inhibitors have demonstrable activity, either alone (7, 16–18) or in combination with other agents (7, 19). During the last few years, efforts have led to the identification of new agents with HDAC-inhibitory activity. Recently, a novel synthetic benzamide derivative, MS-275, has shown activity against diverse neoplastic cell lines (20–23). In such cells, MS-275 induces p21^{CIP1/WAF1} and gelsolin, events accompanied by alterations in cell cycle distribution (20, 23). Currently, little information is available concerning the effects of MS-275 on leukemic cell maturation, and little is known about the molecular events that determine whether such cells undergo apoptosis or differentiation in response to this agent. The results described herein indicate that when administered at low concentrations, MS-275 is a strong inducer of maturation in human leukemia cells. However, at higher concentrations, MS-275 potently triggers the early release of ROS and induces mitochondrial damage, events that lead to activation of the intrinsic caspase cascade culminating in apoptosis, cleavage of p27^{KIP} and pRb, and down-regulation of cyclin D1. Our results also suggest that the CDKI p21^{CIP1/WAF1} may play an important functional role in protecting leukemic cells from MS-275-mediated cell death signals and in reciprocally promoting differentiation.

MATERIALS AND METHODS

Cells. U937, HL-60, Jurkat, and K562 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in a 37°C, 5% CO₂, fully humidified incubator and cultured in RPMI 1640 supplemented with sodium pyruvate, MEM essential vitamins, L-glutamate, penicillin, streptomycin, and 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA). U937 cells stably overexpressing Bcl-2, Bcl-X_L, CcrmA, C8DN, p21^{CIP1/WAF1} antisense, and their empty vector counterparts were obtained as reported previously (24–26) and maintained as described above in the presence of the corresponding selection antibiotics. All experiments were performed using cells in logarithmic phase growth suspended at 2.5 × 10⁵ cells/ml. Patient-derived leukemic blasts were obtained with informed consent from the peripheral blood of patients with AML. These studies have been approved by the Investigational Review Board of the Medical College of Virginia/Virginia Commonwealth University. Blood was collected in sterile syringes containing heparin, diluted 1:4 with cell culture media (RPMI 1640), and aliquoted into 50-cc sterile plastic centrifuge tubes. Only samples consisting of >70% blasts were used in these studies. To each tube was added an underlayer of sterile Ficoll-Histopaque (specific gravity, 1.077; Sigma-Aldrich, St. Louis, MO), and the tubes were centrifuged for 30 min at 400 × g at room temperature. Cells were then harvested from the interface layer, resuspended in fresh medium, and prepared for studies as described above.

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⁴ The abbreviations used are: HDAC, histone deacetylase; AML, acute myelogenous leukemia; ROS, reactive oxygen species; L-NAC, L-N-acetylcysteine; SB, sodium *n*-butyrate; SAHA, suberoylanilide hydroxamic acid; CDKI, cyclin-dependent kinase inhibitor; PI, propidium iodide; C8DN, dominant negative caspase 8; pRb, retinoblastoma protein; TNF, tumor necrosis factor; TNFSFR, TNF soluble receptor; FasL, Fas ligand; CHX, cycloheximide; CcrmA, cytokine response modifier A; PBS-T, phosphate-buffered saline-Tween 20.

Drugs and Chemicals. MS-275 was kindly provided by Dr. O. Nakanishi (Nihon Shering K. K. Pharmaceuticals, Chiba, Japan). In experiments involving examination of ROS, cells were pretreated with L-NAC (Sigma) 1 h before the addition of MS-275. The pan-caspase inhibitor BOC-D-fmk was purchased from Enzyme System Products (Livermore, CA) and dissolved in DMSO. Human recombinant TNF (Calbiochem) and TNFSR (R&D Systems, Minneapolis, MN) were dissolved in medium and kept at room temperature 20 min before use. CHX was purchased from Sigma, dissolved in PBS, and stored at -20°C . Purified antihuman FasL was obtained from BD PharMingen (San Diego, CA).

Assessment of Apoptosis. Apoptotic cells were evaluated by both morphological assessment of Wright-Giemsa-stained cytospin preparations and by annexin V/PI staining (BD PharMingen) as per the manufacturer's instructions, as described previously (19). The extent of apoptosis was determined using a Becton Dickinson FACScan flow cytometer.

Determination of Clonogenicity. Pelleted cells were washed extensively and prepared for soft agar cloning as described previously (27). Cells were resuspended in cold PBS and seeded in 35-mm culture plates at a fixed density (400 cells/ml/well) in complete RPMI 1640 containing 20% FCS, 10% 5637-conditioned media, and 0.3% Bacto agar (Difco, Detroit, MI). Cultures were maintained for 10–12 days in a 37°C , 5% CO_2 incubator, after which colonies (defined as groups of ≥ 50 cells) were scored.

Cell Cycle Analysis. After drug treatment, cells were pelleted by centrifugation at $500 \times g$ for 6 min and resuspended in 70% ethanol. The cells were incubated on ice for at least 1 h, 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).

Assessment of Mitochondrial Membrane Potential ($\Delta\psi_m$). At the indicated intervals, cells were harvested, and 2×10^5 cells were incubated with 40 nM DiOC₆ for 15 min at 37°C . Analysis was then carried out on a Becton Dickinson FACScan cytofluorometer. The percentage of cells exhibiting low levels of DiOC₆, reflecting loss of mitochondrial membrane potential, was determined as described previously (28).

Analysis of Cytosolic Cytochrome *c*. A previously described technique was used (29). The S-100, or cytosolic fraction, was subjected to Western analysis as described above. For each condition, 30 μg of the S-100 fraction were loaded on the gel and probed with the corresponding antibody.

Western Blot Analysis. Whole cell pellets were washed twice in PBS, resuspended in PBS, and lysed by the addition of 1 volume of loading buffer. Thirty μg of total proteins per point were separated by 4–12% Bis-Tris NuPage precast gel system (Invitrogen) and electroblotted to nitrocellulose. The blots were blocked in 5% nonfat milk in PBS-T and probed for 1 h with the appropriate dilution of primary antibody. Blots were washed 3×10 min in PBS-T and then incubated with a 1:2000 dilution of peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed 3×10 min in PBS-T and then developed by enhanced chemiluminescence (New England Nuclear, Boston, MA). Where indicated, blots were stripped and reprobed with antibodies directed against actin.

HDAC Assay. Acid extraction of proteins from treated cells and detection of acetylated histones H3 and H4 by Western blot analysis were performed per the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY), as described previously (30).

Antibodies for Western Blot Analysis. Primary antibodies for the following proteins were used at the designated dilutions: (a) p21^{WAF1/CIP1} (1:1000; PharMingen-Transduction Laboratories, Lexington, KY); (b) Bcl-X_L (1:1000; Trevigen, Gaithersburg, MD); (c) XIAP (1:1000; Cell Signaling Technology, Beverly, MA); (d) poly(ADP-ribose) polymerase (1:1000; BioMol Plymouth Meeting, PA); (e) pro-caspase 3 (1:1000; PharMingen-Transduction Laboratories); (f) cytochrome *c*, cyclin D1, cyclin A, cyclin E, Mcl-1, pRb, and underphosphorylated pRb (1:1000; BD PharMingen); (g) Bcl-2 (1:2000; DAKO, Glostrup, Denmark); (h) caspase 8 (1:2000; Alexia Corp., San Diego, CA); (i) Bid (1:1000; Cell Signaling Technology); (j) Ac-H3 and Ac-H4 (1:1000; Upstate Biotechnology); and (k) actin (1:2000; Sigma). Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

Measurement of ROS Production. Cells were treated with 20 μM 2',7'-dichlorodihydrofluorescein diacetate H₂DCFDA (Molecular Probes Eugene,

OR) for 30' at 37°C , and fluorescence was measured by flow cytometry on a fluorescence-activated cell-sorting scan and analyzed with CELLQuest software.

Statistical Analysis. The significance of differences between experimental conditions was determined using Student's *t* test for unpaired observations.

RESULTS

MS-275 Inhibits the Proliferation of and Induces Cell Death in Human Leukemia Cell Lines and Primary AML Blasts in a Dose-dependent Manner. Effects of treatment of human myelomonocytic leukemia U937 cells with different concentrations of MS-275 for varying intervals are depicted in Fig. 1A. MS-275 exhibited marked HDAC activity, inducing a sharp increase in acetylation of histones H3 and H4 at all of the concentrations evaluated (*i.e.*, 0.5–10.0 μM ; Fig. 1A, *inset*). Concentrations of MS-275 $< 2 \mu\text{M}$ (*i.e.*, 0.5 and 1 μM) did not affect the growth of U937 cells during the first 48 h. However, after 72 h of exposure, marked growth inhibition could be seen with 2 μM MS-275 ($\sim 50\%$), whereas 0.5 and 1 μM MS-275 induced only modest changes at 72 h. At higher MS-275 concentrations, *i.e.*, 5 and 10 μM , no increase in cell number occurred after 24 h of exposure, and declines were noted after 48 h. The differential effect of low and high MS-275 concentrations was then examined in relation to induction of cell death. As shown in Fig. 1B, MS-275 concentrations of $\leq 1 \mu\text{M}$ minimally induced cell death, whereas higher concentrations (*e.g.*, $\geq 2 \mu\text{M}$) were far more effective in this regard, with essentially 100% of cells apoptotic at later exposure intervals. It should be noted that a considerably higher concentration of the HDAC inhibitor SB (1 mM) was relatively ineffective in inducing apoptosis in these cells. Despite minimally inducing apoptosis, a 24- and 48-h exposure to 1 μM MS-275 reduced U937 cell colony formation by $\sim 60\%$ and 2 logs, respectively (Fig. 1C). However, 24-h exposure to 5 μM MS-275 reduced colony formation by ~ 3 logs, and a 48-h exposure essentially abrogated clonogenic survival. Based on these results, two MS-275 concentrations were selected for further analysis: (a) 1 μM , which resulted in growth inhibition but only modestly induced apoptosis; and (b) 5 μM , which potently triggered cell death at exposure intervals of ≥ 48 h. Consistent with these findings, treatment of cells with 5 μM MS-275 induced marked cleavage of Bid, activation of pro-caspases-3 and -8, and poly(ADP-ribose) polymerase degradation (Fig. 1D). Minimal changes were observed at 24 h with either concentration or at 48 h with 1 μM MS-275.

Parallel studies were performed in human promyelocytic leukemia cells (HL-60), human Bcr-Abl-expressing myeloid leukemia cells (K562), and Jurkat T human lymphoblastic leukemia cells. As shown in Fig. 2A, the growth-inhibitory effects of MS-275 were comparable with those observed in U937 cells. Furthermore, 5 μM MS-275 was more effective than the 1 μM concentration in inducing apoptosis in each cell line, with the exception of HL-60 cells, in which the extent of cell death was similar for both MS-275 concentrations at the 48-h interval (Fig. 2B). Finally, induction of apoptosis by MS-275 in two primary AML specimens closely paralleled results obtained in the continuously cultured cell lines (Fig. 2C). These findings indicate that MS-275 induces cell death in human leukemia cells in a dose- and time-dependent manner.

Effect of MS-275 on Cell Cycle Arrest and Differentiation in U937 Cells. The effects of 1 and 5 μM MS-275 were examined in relation to cell cycle traverse, surface markers, and expression of various cell cycle-regulatory proteins (Fig. 3). Cell cycle analysis revealed that $\sim 80\%$ of cells treated with 1 μM MS-275 underwent growth arrest in G₀-G₁ phase after 48 h of exposure (Fig. 3A). This phenomenon was accompanied by a concomitant increase in the

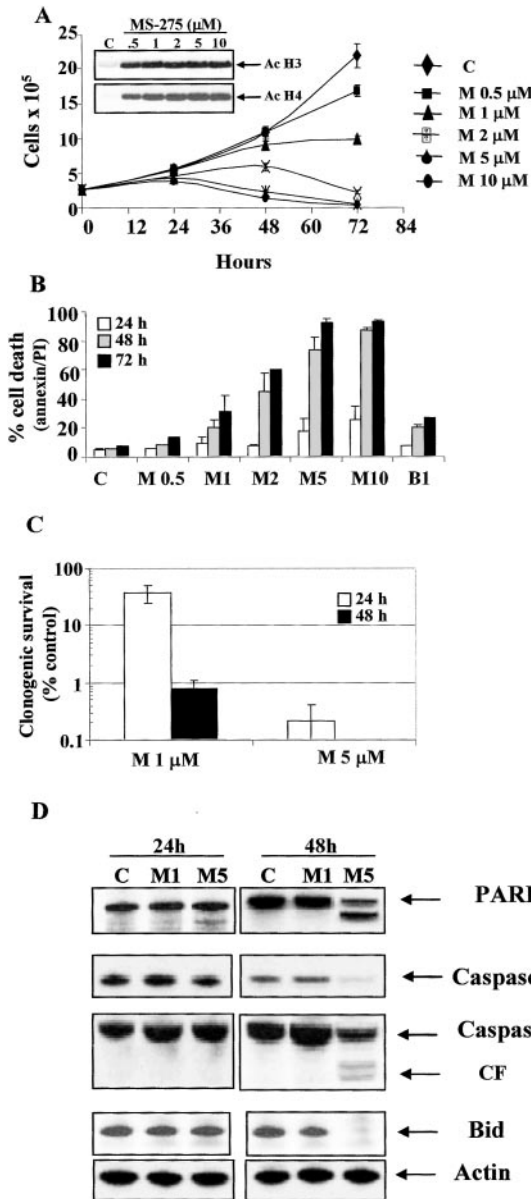


Fig. 1. Effect of MS-275 on U937 cell growth and viability. U937 cells were suspended in the presence of MS-275 at the indicated concentrations and assayed at 24, 48, and 72 h. **A**, cell growth was determined by monitoring the number of cells posttreatment using a Beckman Coulter Counter. *Inset*, after a 24-h exposure to the designated concentration of MS-275, cell lysates were prepared as described, and Western blot analysis was used to monitor the levels of acetylated histones H3 and H4. Each lane was loaded with 30 μ g of protein; an additional experiment yielded similar results. **B**, after treatment, the percentage of apoptotic cells was monitored by Annexin V/PI staining as described in "Materials and Methods." **C**, cells exposed to either 1 or 5 μ M MS-275 for 24 or 48 h were washed free of drug and plated in soft agar as described in "Materials and Methods." At the end of 10–12 days of incubation, colonies (defined as groups of ≥ 50 cells) were scored under light microscopy using an inverted microscope. For **A–C**, values represent the means \pm SD for three separate experiments performed in triplicate. **D**, Western blot analysis of U937 cell lysates incubated for 24 or 48 h with either 1 or 5 μ M MS-275. Thirty μ g of protein were separated on 4–12% Bis-Tris NuPage precast gels, blotted, and probed with the corresponding antibodies. *CF*, cleavage fragment. The results of a representative study are shown; two additional experiments yielded similar results.

expression of the differentiation marker CD11b (Fig. 3B). Notably, 0.5 and 1.0 μ M MS-275 were significantly more effective than a 1000-fold higher concentration of SB (*e.g.*, 1 mM) in inducing maturation in U937 cells. In contrast, 5 μ M MS-275 failed to induce G₀-G₁ arrest at 48 h (Fig. 3A); instead, the large majority of cells appeared in the apoptotic, subdiploid fraction (data not shown). Consistent with their primarily lethal effects, MS-275 concentrations of ≥ 5 μ M were relatively ineffective in inducing U937 cell maturation (Fig. 3B).

Previous studies have shown that HDAC inhibitors induce p21^{CIP1/WAF1} expression in hematopoietic cells and that this action is temporally associated with growth arrest (7, 9, 15, 21, 23, 31). Exposure of cells to MS-275 (1 or 5 μ M) induced up-regulation of the CDKI p21^{CIP1/WAF1}, associated with increased expression of the underphosphorylated form of pRb. However, after 48-h exposure to 5 μ M MS-275, p21^{CIP1/WAF1}, p27^{KIP1}, and pRb degradation were evident, phenomena described previously in cells undergoing apoptosis (7, 32, 33). These events were blocked by coexposure of cells to BOC-D-fmk, a pan-caspase inhibitor.

Progression through the cell cycle requires the presence of functional CDK/cyclin complexes (34), and cells traversing the G₁ to S-phase interface sequentially express cyclins D, E, and A. Western blot analysis revealed a marked, caspase-independent down-regulation of cyclin D1 in cells exposed to 5 μ M MS-275 and a less pronounced effect in cells treated with 1 μ M MS-275 (Fig. 3C). In contrast, no major changes were observed in the levels of cyclin A and E in MS-275-treated cells (data not shown). Taken together, these data demonstrate specific changes in the expression of cell cycle-related proteins that may contribute to MS-275-induced antiproliferative ef-

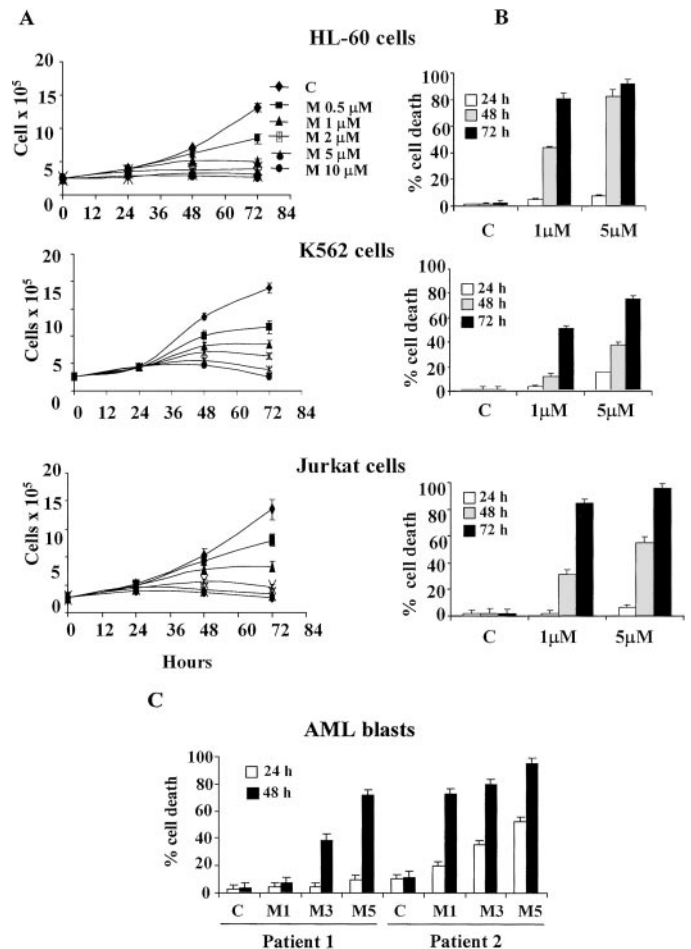


Fig. 2. Effect of MS-275 on growth and viability of leukemia cell lines and primary blasts from patients with AML. Cell lines were incubated in the absence or presence of MS-275 at the doses indicated and collected at 24, 48, and 72 h. **A**, cell growth was determined by monitoring the number of cells posttreatment using a Beckman Coulter Counter. **B**, percentage of apoptotic cells was monitored by Annexin V/PI staining. Equivalent results were obtained when determined by morphological assessment of Wright-Giemsa-stained cytospin preparations. **C**, samples were isolated from the peripheral blood of two patients with AML (in each case, French-American-British classification M2) in which the blast count was $>85\%$ as described in "Materials and Methods." The blasts were exposed *ex vivo* to 1, 3, or 5 μ M MS-275 for 24 and 48 h. The percentage of apoptotic cells was determined as described above. Values represent the means \pm SD for three separate experiments performed in triplicate.

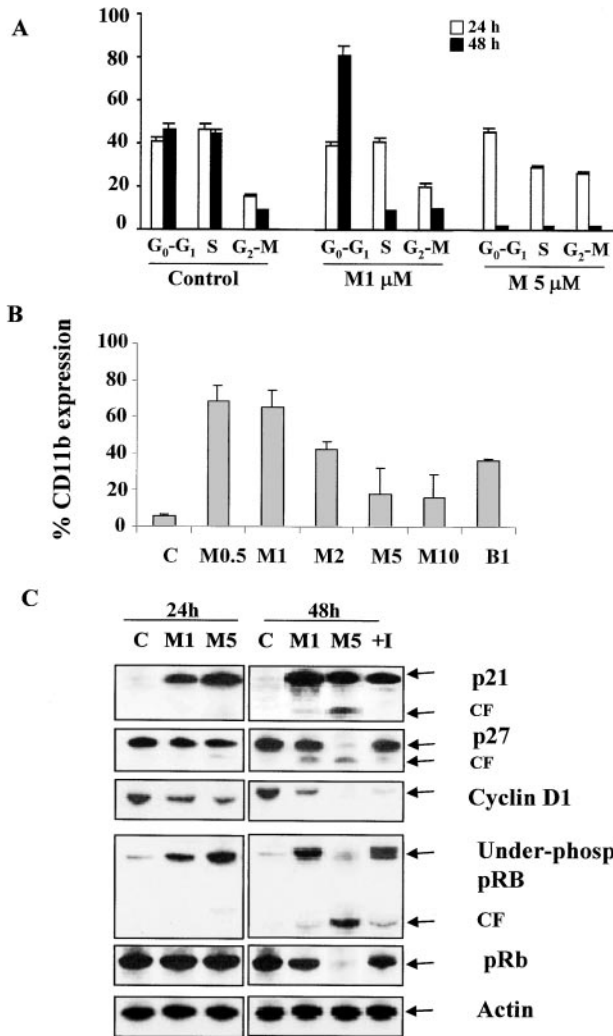


Fig. 3. Effects of MS-275 on cell cycle distribution and differentiation. **A**, U937 cells were exposed to either 1 or 5 μM MS-275 for 24 or 48 h, after which they were isolated, incubated in 70% ethanol, treated with PI, and subjected to cell cycle analysis using a Becton Dickinson FACScan flow cytometer and ModFit software as described in "Materials and Methods." Values correspond to the percentage of nonapoptotic (subdiploid) cells in G_0 - G_1 , S phase, or G_2 -M for each condition. The results of a representative study are shown; two additional experiments yielded similar results. **B**, after incubation with the indicated concentration of MS-275 for 72 h, the percentage of U937 cells expressing the CD11b surface differentiation marker was determined by flow cytometry as described in "Materials and Methods." Parallel studies were performed in cells exposed to 1 mM SB (B1). Values represent the means \pm SD for three separate experiments performed in triplicate. **C**, Western blot analysis of the cell cycle-related proteins. U937 cells were incubated for 24 or 48 h with either 1 or 5 μM MS-275, after which they were pelleted and lysed, and 30 μg of protein were separated by SDS-PAGE as described in "Materials and Methods." Blots were then probed with the corresponding antibodies. CF, cleavage fragment. The results of a representative study are shown; two additional experiments yielded similar results.

fects at low concentrations (*i.e.*, p21^{CIP1/WAF1} induction, cyclin D1 down-regulation, and underphosphorylation of pRb) or to MS-275-mediated apoptosis at higher concentrations (*i.e.*, p21^{CIP1/WAF1}, p27^{KIP1}, and pRb degradation).

MS-275-induced Apoptosis Is Largely Independent of the Extrinsic/receptor-Mediated Pathway. In view of evidence that HDAC inhibitors such as *M*-carboxycinnamic acid bishydroxamide and apicidin-induced apoptosis involve the extrinsic/receptor-mediated pathway (8, 35), attempts were made to determine whether this phenomenon might also play a role in MS-275-induced cell death. To this end, U937 cells were exposed for 48 h to 5 μM MS-275 in the presence or absence of TNFSR (100 ng/ml), which is known to oppose TNF- α -related lethality (36, 37). As shown in Fig. 4A, MS-

275-induced apoptosis was not altered by the addition of TNFSR, whereas TNFSR completely blocked TNF/CHX-induced apoptosis. Similarly, when MS-275-treated U937 cells were coincubated with an anti-FasL antibody, no changes were observed (Fig. 4B). Parallel studies were performed in U937 cells ectopically expressing either CrmA, a serpin that potently inhibits caspase-8 (38), or C8DN. As shown in Fig. 4C, ectopic expression of either CrmA or C8DN failed to protect cells from MS-275-mediated apoptosis relative to empty vector control cells (pcDNA3.1). In marked contrast, ectopic expression of these proteins essentially abrogated apoptosis induced by a stimulus known to trigger the extrinsic cascade, *i.e.*, combined exposure to TNF- α (10 ng/ml) + CHX (1 μM ; Fig. 4C). Together, these findings argue that the induction of apoptosis by MS-275, in contrast to that initiated by the HDAC inhibitors *M*-carboxycinnamic acid bishydroxamide and apicidin, proceeds in large part independently of the receptor-related, extrinsic apoptotic cascade.

Mitochondrial Dysfunction Plays a Critical Role in MS-275-induced Apoptosis. Studies were subsequently undertaken to characterize the effects of MS-275 on mitochondrial events associated with apoptosis. Western blot analysis of U937 cells revealed minimal cytochrome *c* release during the first 24 h at either 1 or 5 μM MS-275 (Fig. 5A). However, at 48 h, a modest increase in cytochrome *c* release was detected in cells treated with 1 μM MS-275, and a very pronounced increase was noted in cells at the 5 μM concentration. Cytoplasmic cytochrome *c* release was accompanied by the loss of

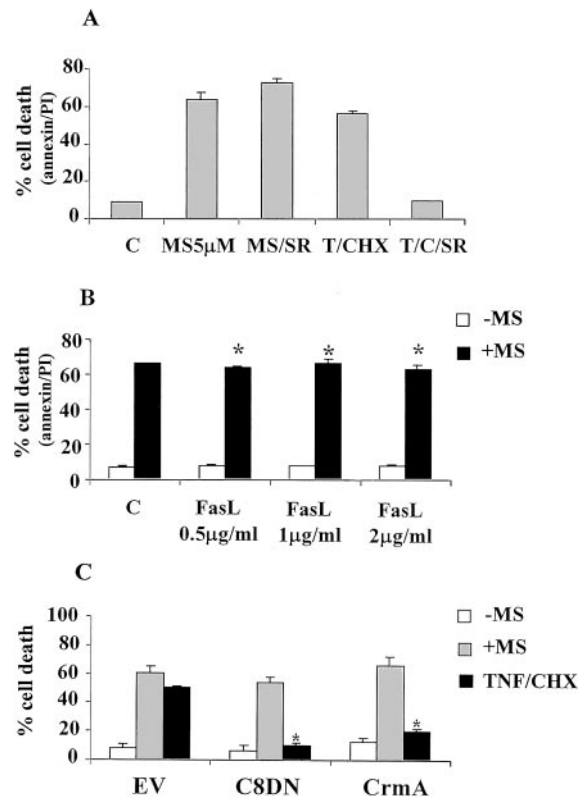


Fig. 4. Role of the extrinsic/receptor-mediated pathway in MS-275-induced apoptosis. **A**, U937 cells were exposed to either 5 μM MS-275 or TNF- α (T; 10 ng/ml) + CHX (1 μM) for 48 h in the absence or presence of TNF- α soluble receptor (SR; 100 ng/ml) and analyzed for apoptosis by AnnexinV/PI analysis. **B**, U937 cells were coincubated with MS-275 and the indicated concentration of an anti-FasL antibody, after which apoptosis was assessed as described above. *, not significantly different from values obtained in the absence of anti-FasL, $P > 0.05$. **C**, U937 cells transfected with empty vector (pcDNA3.1; EV), C8DN, or CrmA were incubated for 48 h in the absence or presence of 5 μM MS-275 or the combination of TNF- α (T; 10 ng/ml) + CHX (1 μM), after which the extent of cell death was determined as described above. Values represent the means \pm SD for three separate experiments performed in triplicate. *, values not significantly different from those obtained in empty vector controls, $P > 0.05$.

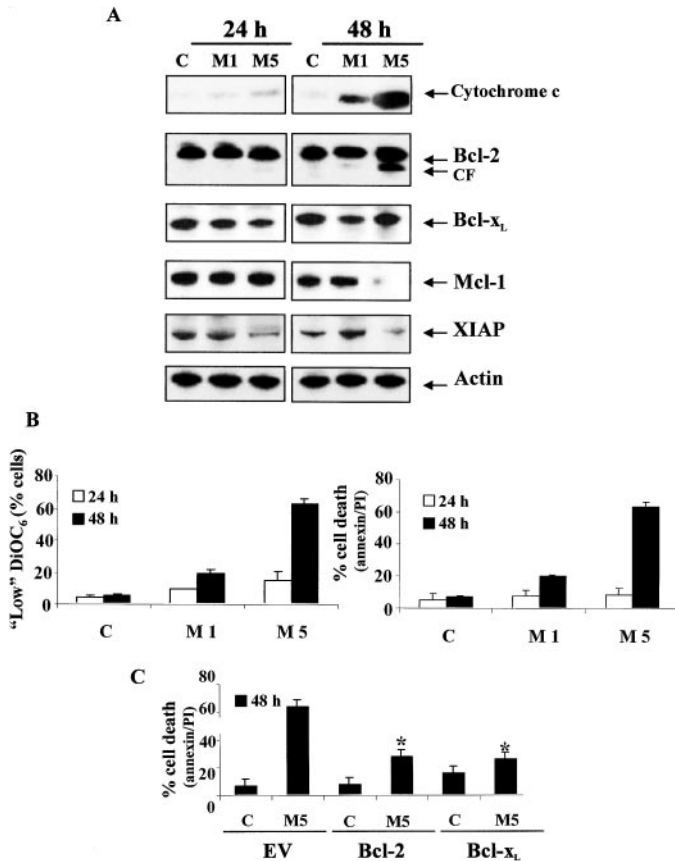


Fig. 5. Effects of MS-275 on mitochondrial function and antiapoptotic proteins. *A*, U937 cells were incubated for 24 or 48 h with either 1 or 5 μM MS-275, after which cells were pelleted and lysed, and protein was extracted from either the cytosolic S-100 fraction (cytochrome *c*) or whole cell lysates (Bcl-2, Bcl-X_L, Mcl-1, and XIAP). In each case, 30 μg of protein were separated by SDS-PAGE. Blots were then probed with the corresponding antibodies. The results of a representative study are shown; two additional experiments yielded similar results. *CF*, cleavage fragment. *B*, U937 cells were incubated with either 1 or 5 μM MS-275 for 24 or 48 h, after which the percentage of cells exhibiting a loss of $\Delta\psi_m$ (left panel) or apoptosis by annexinV/PI staining (right panel) was determined as described in "Materials and Methods." *C*, U937 cells transfected with an empty vector (EV) or plasmids encoding Bcl-2 or Bcl-X_L were incubated with 5 μM MS-275 for 48 h, after which the percentage of annexinV/PI cells was determined as described above. Values represent the means \pm SD for three separate experiments performed in triplicate. *, significantly less than values obtained for empty vector control cells, $P > 0.005$.

$\Delta\psi_m$ (Fig. 5B, left panel). A close correlation between loss of $\Delta\psi_m$ and the extent of cell death was observed (Fig. 5B, right panel).

Bcl-2-related proteins are key regulators of the mitochondrial-mediated apoptosis (39). Whereas neither 1 nor 5 μM MS-275 significantly modified expression of Bcl-2, Bcl-X_L, or Mcl-1 after 24 h of treatment, cells exposed to 5 μM MS-275 exhibited a decline in expression of XIAP (Fig. 5A). No changes in expression of these proteins were noted in cells exposed to 1 μM MS-275 for 48 h. However, cells treated with 5 μM MS-275 for 48 h displayed evidence of a Bcl-2 cleavage product, which has been associated with proapoptotic functions (40), as well as marked declines in Mcl-1 and XIAP protein levels.

Further evidence of mitochondrial involvement in MS-275-induced apoptosis emerged from studies involving U937 cells stably transfected with plasmids encoding either full-length Bcl-2 or Bcl-X_L. As shown in Fig. 5C, ectopic expression of both Bcl-2 and Bcl-X_L significantly, albeit partially, protected cells from MS-275-mediated lethality. These findings, along with those presented in Fig. 4, provide strong support for the notion that MS-275 induces apoptosis through a mechanism that involves engagement of the intrinsic, mitochondrial-related pathway.

MS-275 Potently Induces the Generation of ROS Upstream of Mitochondrial Injury. It is well established that mitochondria are sensitive to changes in cellular redox state (reviewed in Ref. 41). In fact, mitochondria represent the predominant source of ROS in most cells undergoing apoptosis (42, 43). Flow cytometric analysis of U937 cells exposed to 1 μM MS-275 revealed a modest (17.3%) production of ROS after 24 h (Fig. 6A), compatible with the primarily differentiation-related effects of this drug concentration (Fig. 3B). In marked contrast, 5 μM MS-275 induced a dramatic increase in ROS generation as early as 2 h after drug exposure that persisted over the ensuing 24 h (Fig. 6A). Moreover, ROS production was completely blocked by the free radical scavenger L-NAC (20 mM; Fig. 6A). Significantly, this effect was accompanied by a pronounced inhibition of MS-275-induced loss of $\Delta\psi_m$, release of cytochrome *c*, and cell death (Fig. 6B). Consistent with these results, U937 cells ectopically expressing Bcl-2 or Bcl-X_L were resistant to MS-275-induced ROS generation relative to their empty vector counterparts (Fig. 6C). Furthermore, MS-275 induced considerably higher levels of ROS than equitoxic concentrations of either SB (e.g., 1 mM) or SAHA (e.g., 2.5 μM), an agent whose lethal actions have recently been attributed to ROS generation (Ref. 6; Fig. 6D). Finally, MS-275 was equally effective in increasing ROS production in several other human leukemia cell types, including HL-60 promyelocytic leukemia, K562 erythroleukemia, and Jurkat lymphoblastic leukemia cells. Taken together, these data are compatible with the notion that MS-275 is a potent inducer of ROS in human leukemia cells and that this phenomenon plays an important role in MS-275-mediated mitochondrial damage and apoptosis.

Dysregulation of p21^{CIP1/WAF1} Potentiates MS-275-mediated Mitochondrial Injury and Apoptosis without Increasing ROS Generation. The CDK1 p21^{CIP1/WAF1} is known to be a key regulator of the HDAC inhibitor-induced growth arrest/differentiation and apoptosis (7, 14, 15, 44). To assess the functional significance of p21^{CIP1/WAF1} on MS-275-induced apoptosis, U937 cell lines stably transfected with a p21^{CIP1/WAF1} antisense construct (p21^{CIP1/WAF1}-AS) were used (29). As shown in Fig. 7A, pREP-empty vector-transfected cells expressed p21^{CIP1/WAF1} when exposed to either 1 or 5 μM MS-275 or 1 mM SB. In contrast, p21^{CIP1/WAF1} expression was essentially abrogated in p21^{CIP1/WAF1}-AS cells. After a 24-h exposure to 5 μM MS-275, pronounced apoptosis was observed in p21^{CIP1/WAF1}-AS cells, but not in their empty vector counterparts. Parallel effects were observed when loss of $\Delta\psi_m$ was monitored (Fig. 7C). Dysregulation of p21^{CIP1/WAF1} also disrupted the differentiation response, reflected by diminished CD11b expression, in cells treated for 72 h with 0.5 and 1.0 μM MS-275 (Fig. 7D). Lastly, despite the differential sensitivity to MS-275, no increase was observed in the level of ROS produced by p21^{CIP1/WAF1}-AS cells compared with their empty vector counterparts during a 24-h exposure to 5 μM MS-275 (Fig. 7E). Together, these findings indicate that p21^{CIP1/WAF1} promotes MS-275-mediated differentiation while reciprocally protecting cells from apoptosis through a process that operates downstream or independently of ROS generation.

DISCUSSION

MS-275 is a novel agent with HDAC-inhibitory activity that is structurally dissimilar from other HDAC inhibitors (20, 21). As in the case of other compounds of this class, MS-275-associated HDAC-inhibitory activity is accompanied by an increase in expression of the CDKI p21^{CIP1/WAF1} and accumulation of cells in G₁ phase (21). MS-275 displays antiproliferative activity toward several human cancer cell lines including breast (21), colorectal, leukemia, lung, ovarian, and pancreatic cancer cell lines (20) through a mechanism that may

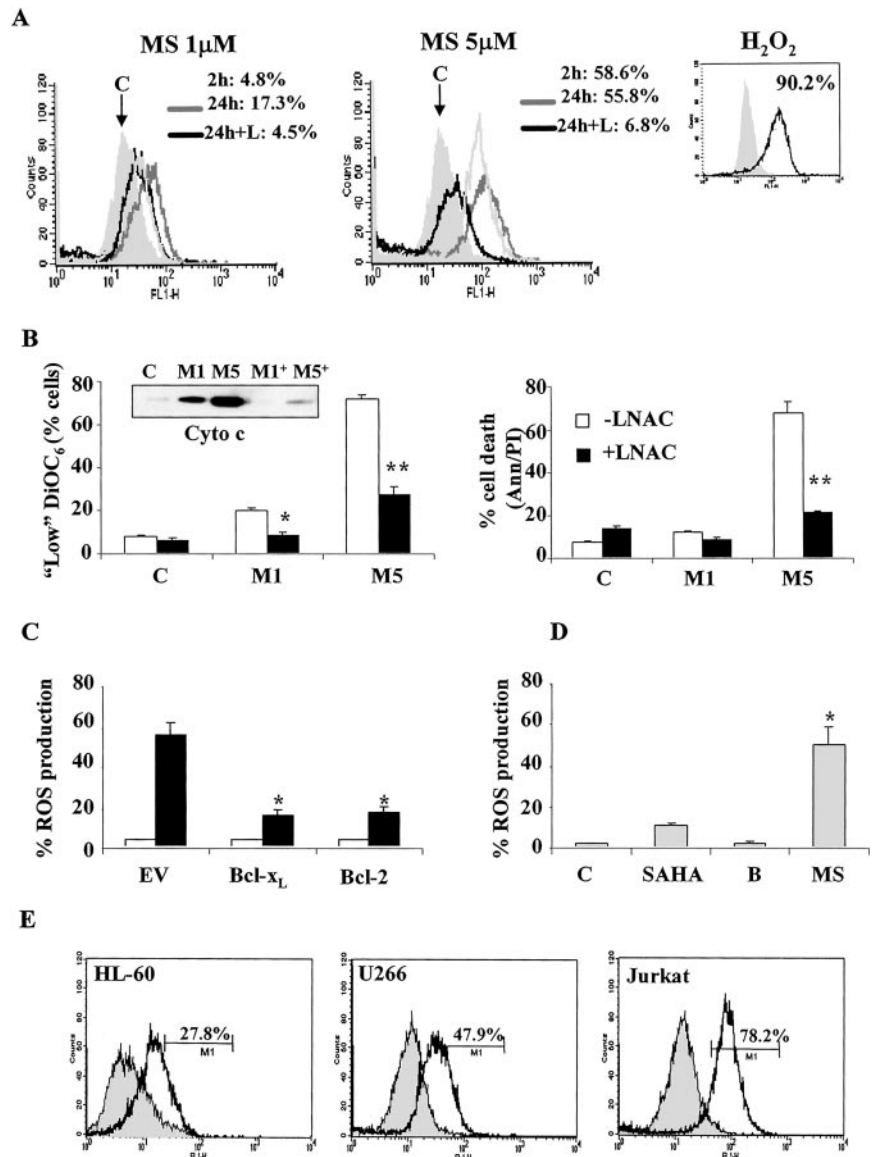


Fig. 6. Effects of MS-275 on ROS generation. **A**, U937 cells were incubated with 1 or 5 μ M MS-275 \pm the free radical scavenger L-NAC (15 mM) for 2 and 24 h, after which they were labeled with an oxidative-sensitive dye (H₂DCFDA) and analyzed by flow cytometry to determine the percentage of cells displaying an increase in ROS production (reflected by a rightward shift of the histogram). As controls, cells were treated with 10 μ M H₂O₂ for 15 min and analyzed in parallel. **B**, cells were treated for 48 h with 1 or 5 μ M MS-275 in the absence (\square) or presence (\blacksquare) of 15 mM L-NAC. At the end of the incubation period, the percentage of cells exhibiting loss of $\Delta\psi_m$ (left panel) or apoptosis (annexinV/PI⁺; right panel) was determined as described above. *Inset*, cells were treated in parallel with 1 or 5 μ M MS-275 in the absence (M1 and M5) or presence (M1⁺ and M5⁺) of 15 mM L-NAC, after which release of cytochrome *c* into the cytosolic S-100 fraction was monitored as described above. *, significantly less than values obtained for cells treated with MS-275 in the absence of L-NAC, $P < 0.05$; **, $P < 0.002$. *Cyto c*, cytochrome *c*. **C**, U937/EV, U937/Bcl-2, or U937/Bcl-X_L cells were incubated in the absence (\square) or presence (\blacksquare) of 5 μ M MS-275 for 24 h, after which the percentage of cells displaying an increase in ROS production was determined as described above. *, significantly less than values obtained in empty vector controls, $P < 0.01$. **D**, U937 cells were treated for 24 h with 2.5 μ M SAHA, 1 mM SB (**B**), or 5 μ M MS-275, and the increase in ROS production was quantified as described above. *, significantly greater than values obtained for cells exposed to SAHA or SB, $P < 0.002$. **E**, HL-60, K562, and Jurkat cells were incubated in the presence or absence of 5 μ M MS-275 for 2 h, after which the increase in ROS production was quantified as described above. For **B–D**, values represent the means \pm SD for three separate experiments performed in triplicate.

involve induction of transforming growth factor β receptor expression and induction of transforming growth factor β signaling (20, 23). Currently, however, little information exists concerning MS-275-related differentiation in human leukemia cells or the factors that determine whether this agent triggers an apoptotic *versus* a maturation response. The results presented herein suggest that these events are critically influenced by the extent of MS-275-mediated ROS generation and are further regulated by the CDKI p21^{CIP1/WAF1}.

When administered at both low (*e.g.*, 1 μ M) and high (*e.g.*, 5 μ M) concentrations, MS-275 robustly induced p21^{CIP1/WAF1}. The induction of this CDKI has been observed in cells exposed to a variety of other HDAC inhibitors, including SB (7), phenylbutyrate (31), SAHA (15), trichostatin A (10), and apicidin (9). Up-regulation of p21^{CIP1/WAF1} occurred concomitantly with the expression of underphosphorylated pRb, as well as down-regulation of cyclin D1, resulting in growth arrest in G₁. Significantly, such growth-arrested U937 cells did not display substantial evidence of apoptosis, supporting the concept that that low concentrations of MS-275 primarily exert anti-proliferative actions. Moreover, low concentrations of MS-275 effectively reduced clonogenic survival and very effectively triggered differentiation in these cells. It is noteworthy that MS-275, at least

when administered at low concentrations, was a considerably more potent inducer of U937 cell maturation than a 1000-fold higher concentration of SB. In this context, it has previously been shown that HDAC inhibitors such as SB induce the transcriptional down-regulation of cyclin D1 (45, 46). It is therefore tempting to speculate that MS-275-induced down-regulation of this cyclin and induction of p21^{CIP1/WAF1} combine to modulate the activity of the downstream pRb/E2F axis and, in so doing, trigger the G₁ arrest program required for leukemic cell differentiation (47).

In contrast to the differentiation-inducing properties of low (*i.e.*, ≥ 1.0 μ M) concentrations of MS-275, higher concentrations potentially triggered apoptosis in leukemic cells. In the case of some HDAC inhibitors, *e.g.*, apicidin, this phenomenon has been linked to engagement of the extrinsic, apoptotic cascade (8). However, the inability of TNFSRs, antibodies directed against FasL, or ectopic expression of C8DN or CrmA to attenuate lethality argues strongly against this possibility in the case of MS-275. In contrast, the bulk of evidence suggested activation of the mitochondrial pathway was critically involved in MS-275-induced cell death. Specifically, high concentrations of MS-275 triggered a very early and pronounced increase in ROS production. In this context, it is well known that disruption of

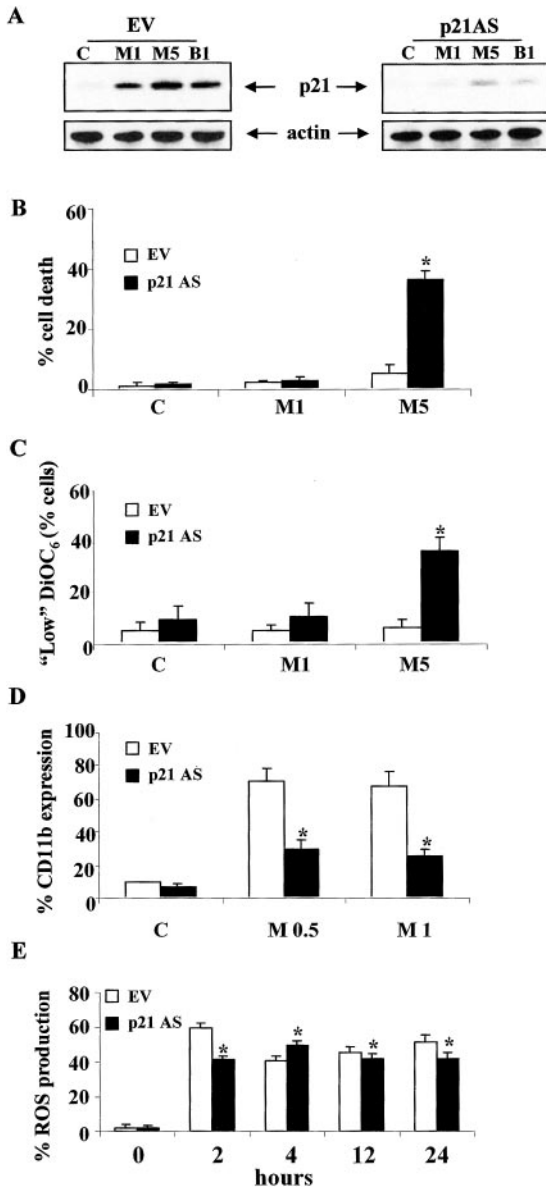


Fig. 7. Role of the CDK-inhibitor p21^{CIP1/WAF1} in MS-275-induced cell death. U937/EV or U937/antisense p21^{CIP1/WAF1} cells were exposed for 24 h to either 1 or 5 μ M MS-275. A, Western blot analysis of cell lysates; 30 μ g of protein were separated on SDS-PAGE gels, blotted, and probed with an antibody against p21^{CIP1/WAF1}. Lysates from cells exposed to 1 mM SB (BI) were examined in parallel. The results of a representative study are shown; an additional experiment yielded similar results. At the end of the 24-h exposure interval, the percentage of cells exhibiting morphological evidence of apoptosis (B) or loss of $\Delta\psi_m$ (C) was determined as described. *, significantly greater than values obtained for empty vector control cells, $P < 0.005$. D, flow cytometry analysis of cells expressing the CD11b surface differentiation marker after incubation for 72 h with 0.5 or 1.0 μ M MS-275. *, values significantly less than those obtained in empty vector controls, $P < 0.005$. E, U937/EV or U937/p21^{CIP1/WAF1}-AS cells were incubated with either 1 or 5 μ M MS-275 over the indicated treatment intervals, after which the percentage of cells displaying an increase in of ROS production was determined by flow cytometry. *, not significantly different from values obtained in empty vector controls, $P > 0.05$. For B–E, values represent the means \pm SD for three separate experiments performed in triplicate.

mitochondrial function under conditions of oxidative stress is an important contributor to the apoptotic response (41, 48, 49). Moreover, MS-275-induced ROS generation occurred at very early intervals (e.g., \sim 2 h) and clearly preceded other hallmarks of mitochondrial injury (i.e., loss of mitochondrial membrane potential and release of cytochrome *c*). Significantly, blockade of ROS production by the free radical scavenger L-NAC inhibited both mitochondrial damage (e.g., loss of $\Delta\psi_m$ and cytochrome *c* release) and apoptosis, providing

further support for the notion that MS-275-associated free radical production was causally related to lethality. It is noteworthy that cell death induced by the HDAC inhibitor SAHA has also been shown to involve ROS generation and to induce cleavage of Bid in lymphoblastic leukemia cells (6). However, when approximately equitoxic concentrations of MS-275, SAHA, and SB were compared, MS-275 induced an earlier and more pronounced increase in ROS levels. Collectively, these observations suggest that MS-275-related perturbations in redox balance and mitochondrial integrity play a critical role in the lethal actions of this agent, at least in U937 human myelomonocytic leukemia cells.

The ability of ectopic expression of Bcl-2 or Bcl-X_L to attenuate MS-275-induced ROS production, loss of $\Delta\psi_m$, and apoptosis is consistent with the known contribution of these proteins to maintenance of oxidative homeostasis. It has been proposed that the antiapoptotic effects of Bcl-2 and Bcl-X_L involve, at least to an extent, maintenance of mitochondrial-cytosolic coupling of oxidative phosphorylation (50, 51). There is also evidence that Bcl-2 family proteins may function as antioxidants and exert antiapoptotic effects by scavenging ROS (52–54). The capacity of Bcl-2 and Bcl-x_L to block MS-275-induced free radical generation and apoptosis supports the notion that ROS production represents a central mechanism underlying MS-275-associated lethality. In this regard, the formation of a putatively proapoptotic Bcl-2 cleavage fragment (55, 56) in cells exposed to toxic concentrations of MS-275 may be relevant. Similarly, down-regulation of the expression of the antiapoptotic protein Mcl-1, which has been implicated in malignant hematopoietic cell survival (57), may also contribute to MS-275-associated lethality.

The present results suggest that in addition to its antiproliferative effects, p21^{CIP1/WAF1} plays an important role in regulating apoptotic and differentiation responses to MS-275. Previous studies have shown that p21^{CIP1/WAF1} protects cells from apoptosis and attenuates the toxicity of certain cytotoxic agents in a variety of tumor cell types (58–60). The mechanism underlying this phenomenon has not been fully elucidated but may involve the capacity of p21^{CIP1/WAF1} to form a complex with caspase-3 and inhibit its activity (61). The observation that p21^{CIP1/WAF1} antisense-expressing cells were more sensitive than their wild-type counterparts to MS-275-mediated apoptosis is consistent with such a notion. Loss of p21^{CIP1/WAF1} function also diminished MS-275-mediated maturation, analogous to effects observed in the case of the HDAC inhibitor SB (7). It is tempting to speculate that loss of p21^{CIP1/WAF1}, in conjunction with dysregulation of other cell cycle-regulatory proteins (e.g., down-regulation of cyclin D1 and pRb degradation), disrupts the maturation program of leukemic cells exposed to MS-275 and causes them to engage an alternative, apoptotic program. In addition, the ability of lethal concentrations of MS-275 (e.g., 5 μ M) to induce cleavage of p21^{CIP1/WAF1} may also be relevant because this phenomenon has recently been implicated in activation of the apoptotic cascade (30, 32, 62). Lastly, failure of p21^{CIP1/WAF1} dysregulation to potentiate MS-275-mediated ROS generation indicates that this CDKI operates independently or at a point downstream of free radical production, at least in U937 cells. In this context, the recent observation that induction of p21^{CIP1/WAF1} increases ROS levels and senescence in p53-null EJ cells (63) suggests that such interactions may be cell type specific.

In summary, the results of the present study indicate that the HDAC inhibitor MS-275 exerts highly dose-dependent effects in human leukemia cells. At low concentrations, MS-275 exhibits antiproliferative activity, potently inducing growth arrest and expression of differentiation markers. These events are accompanied by induction of p21^{CIP1/WAF1}, hypophosphorylation of pRb, and down-regulation of several cell cycle-related proteins, e.g., cyclin D1. However, at higher concentrations, MS-275 provides a potent cell death-inducing signal

in both leukemia cell lines and primary, patient-derived AML blast cells. Such effects are associated with very early and pronounced increases in ROS generation, mitochondrial injury, and cleavage or down-regulation of several cell cycle and apoptotic-regulatory proteins, including p21^{CIP1/WAF1}, pRb, cyclin D₁, p27^{KIP1}, Bcl-2, Mcl-1, and XIAP. Currently, Phase I trials of MS-275 in patients with refractory hematological and nonhematological malignancies are under way. The findings described herein may provide insights into the mechanism(s) by which this agent acts *in vivo*. The present findings also raise the possibility that MS-275 may prove particularly effective in the treatment of certain hematological malignancies by virtue of its ability to induce either differentiation or apoptosis.

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