Potentiation of the lethality of the histone deacetylase inhibitor LAQ824 by the cyclin-dependent kinase inhibitor roscovitine in human leukemia cells

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Abstract

Interactions between the novel histone deacetylase inhibitor LAQ824 and the cyclin-dependent kinase inhibitor roscovitine were examined in human leukemia cells. Pretreatment (24 hours) with a subtoxic concentration of LAQ824 (30 nmol/L) followed by a minimally toxic concentration of roscovitine (10 μmol/L; 24 hours) resulted in greater than additive effects on apoptosis in U937, Jurkat, and HL-60 human leukemia cells and blasts from three patients with acute myelogenous leukemia. These events were associated with enhanced conformational changes in Bax; mitochondrial release of cytochrome c, Smac/DIABLO, and apoptosis-inducing factor; and a marked increase in caspase activation. LAQ824/roscovitine–treated cells displayed caspase-dependent down-regulation of p21CIP1 and Mcl-1 and a pronounced caspase-independent reduction in X-linked inhibitor of apoptosis (XIAP) expression. The lethality of this regimen was significantly attenuated by ectopic expression of XIAP, a nuclear localization signal–defective p21CIP1 mutant, Mcl-1, and Bcl-2. Combined exposure to LAQ824 and roscovitine resulted in a significant reduction in XIAP mRNA levels and diminished phosphorylation of the carboxyl-terminal domain of RNA polymerase II. Notably, roscovitine blocked LAQ824-mediated differentiation. Finally, LAQ824 and roscovitine individually and in combination triggered an increase in generation of reactive oxygen species; moreover, coadministration of the free radical scavenger N-acetylcysteine prevented LAQ824/roscovitine–mediated mitochondrial injury and apoptosis. Collectively, these findings suggest that combined treatment of human leukemia cells with LAQ824 and roscovitine disrupts maturation and synergistically induces apoptosis, lending further support for an anti-leukemic strategy combining novel histone deacetylase and cyclin-dependent kinase inhibitors.

Introduction

Histone deacetylase (HDAC) inhibitors (HDACI) represent a diverse group of agents that regulate histone acetylation status (1). Histone acetylation is reciprocally regulated by two enzymes: HDACs and histone acetylases (1, 2). HDACIs, by enhancing histone acetylation, modulate the expression of cell cycle regulation, survival, and maturation-related genes (3). HDACIs also promote gene expression by disrupting interactions between HDAC/corepressor complexes (4). A variety of second-generation HDACIs have been developed, including the hydroxamic acid SAHA (suberoylanilide hydroxamic acid) and, most recently, the cinnamic acid NVP-LAQ824, a novel HDACI active at nanomolar concentrations against human leukemia and multiple myeloma cells (5, 6). Although HDACIs are potent differentiation inducers, they trigger apoptosis rather than maturation when administered above threshold concentrations (3, 4). Events regulating HDACI-mediated cell death involve diverse factors, including generation of reactive oxygen species (ROS; refs. 7, 8) or ceramide (9), induction of the cyclin-dependent kinase (CDK) inhibitor (CDKI) p21CIP1 (10), disruption of cell cycle checkpoints (11), perturbations in signaling pathways (12), up-regulation of death receptors (5, 13, 14), induction of mitochondrial damage (15), etc.

Whereas HDACIs inhibit cell cycle progression indirectly (e.g., through the induction of p21CIP1; ref. 3), CDKIs act more directly by blocking activation of CDKs. Among these, the purine analogue roscovitine primarily targets CDK1 and CDK2 (16). The R-enantioromer of roscovitine, CYC202, exhibits antitumor activity in preclinical models (17) and has recently entered the clinic (18). In addition to triggering cell cycle arrest, CDKIs, such as flavopiridol and roscovitine, also induce apoptosis (19), although it is unclear whether the lethality stems primarily from CDK inhibition, cell cycle–independent phenomena, or both.

Because the maturation process requires G1 arrest (20), cell cycle inhibitors have been viewed as potential differentiation inducers (21). Previously, we showed that flavopiridol, instead of promoting HDACI-mediated...
differentiation in human leukemia cells, blocked this process and causes cells to engage an alternative cell death program (22–24). This may stem from the capacity of flavopiridol, as a transcriptional repressor, to down-regulate the expression of various antiapoptotic proteins, including p21CIP1, Mcl-1, and X-linked inhibitor of apoptosis (XIAP; refs. 22, 25, 26). It is currently unknown whether this phenomenon is restricted to flavopiridol or whether it can be extended to other CDKIs as well as alternative second-generation HDACIs with marked antileukemic activity. The purpose of the present study was to investigate the effects of the CDKI roscovitine on differentiation and apoptotic responses of human leukemia cells to the potent HDACI LAQ824. Our results indicate that roscovitine opposes LAQ824-mediated maturation while synergistically enhancing apoptosis, events associated with XIAP and Mcl-1 down-regulation, ROS generation, and mitochondrial injury. These findings provide further support for the concept of combining CDK with HDAC inhibitors as a novel antileukemic strategy.

Materials and Methods

Cells and Cell Culture
U937, HL60, and Jurkat human leukemia cells were obtained from American Type Culture Collection (Rockville, MD) and cultured and maintained as described previously (22). U937 cells stably expressing Bcl-2, FADD dominant-negative (DN), a nuclear localization signal (NLS), or XIAP and their empty vector counterparts were obtained as reported previously (27–29). U937/Mcl-1 cells were generated by cloning a cDNA containing the entire Mcl-1 coding region in pCEP4 vector (Invitrogen, Carlsbad, CA). All experiments used cells in logarithmic phase at 2.5 × 105 cells/mL.

Peripheral blood samples were obtained with informed consent from three patients (French-American-British classifications M2, M4, and M5B), with acute myelogenous leukemia (AML) undergoing routine diagnostic aspirations with approval from the institutional review board of Virginia Commonwealth University. Informed consent was provided according to the Declaration of Helsinki. AML blasts were isolated and cultured as described (7). Normal mononuclear cells were also obtained with informed consent from the bone marrow of patients with nonmalignant hematopoietic disorders in which the WBC series was uninvolved (e.g., iron deficiency anemia and immune thrombocytopenia) as well as from peripheral blood donated by healthy volunteers. Mononuclear cell preparations were obtained as described for the isolation of AML blasts.

Drugs and Chemicals
LAQ824 was kindly provided by Novartis Pharmaceuticals, Inc. (East Hanover, NJ). The pancaspase inhibitor Boc-d-fmk was purchased from Enzyme System Products (Livermore, CA). Roscovitine was purchased from Calbiochem (San Diego, CA).

Assessment of Apoptosis
Apoptotic cells were evaluated by Annexin V/propidium iodide (PI; BD PharMingen, San Diego, CA) staining according to the manufacturer’s instructions as described previously (23) and by morphologic assessment of Wright-Giemsa–stained cytospin preparations.

Cell Cycle Analysis
Cell cycle analysis by flow cytometry was done as described previously (10) using a Becton Dickinson (Mansfield, MA) FACScan flow cytometer and Verity Winlist software (Verity Software, Topsham, ME).

Assessment of Mitochondrial Membrane Potential
At the indicated intervals, cells (2 × 106) were harvested and incubated with 40 nmol/L DiOC6 (15 minutes, 37°C). Loss of mitochondrial membrane potential (Δψmit) was determined by flow cytometry as described previously (7).

Measurement of ROS Production
Cells were treated with 20 μmol/L, 2,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) for 30 minutes at 37°C, fluorescence monitored by flow cytometry on a FACScan, and analyzed with CellQuest software.

Analysis of Cytosolic Cytochrome c and Apoptosis-Inducing Factor
A previously described technique was employed to isolate the S-100 (cytosolic) cell fraction of treated cells (7). For each condition, proteins (30 μg) isolated from the S-100 cell fraction were separated and detected by Western blot as described.

Determination of Clonogenicity
Pelleted cells were washed extensively and prepared for soft-agar cloning as described previously (9). Cultures were maintained for 10 to 12 days in a 37°C, 5% CO2 incubator, after which colonies, defined as groups of ≥50 cells, were scored.

Western Blot Analysis
Whole-cell pellets were washed, resuspended in PBS, and lysed with loading buffer (Invitrogen) as described previously (23). Total proteins (30 μg) for each condition were separated by 4% to 12% Bis-Tris NuPAGE vector-containing entire Mcl-1 coding region in pCEP4 vector (Invitrogen, Carlsbad, CA). All experiments used cells in logarithmic phase at 2.5 × 105 cells/mL.

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Bax Conformational Change
To analyze conformational changes in Bax, cells were lysed in CHAPS lysis buffer and immunoprecipitated in lysis buffer by using 500 μg total cell lysate and 2.5 μg anti-Bax 6A7 monoclonal antibody, which recognizes conformationally changed Bax (Sigma-Aldrich, St. Louis, MO). The resulting immune complexes were analyzed by Western blot and probed with an anti-Bax polyclonal rabbit antiserum (Santa Cruz Biotechnology, Santa Cruz, CA).

Antibodies for Western Blot Analysis
Primary antibodies for the following proteins were used at the designated dilutions: poly(ADP-ribose) polymerase (PARP; 1:1,000; Biomol, Plymouth Meeting,
PA); p21cip1, p27kip1, procaspase-3, procaspase-9, cytochrome c, Mcl-1, pRb, underphosphorylated Rb, and XIAP (1:1,000; BD Transduction Laboratories, San Diego, CA); Bcl-2 (1:2,000; DAKO, Glostrup, Denmark); caspase-8 (1:2,000; Alexis Corp., San Diego, CA); Bid (1:1,000; Cell Signaling, Beverly, MA); actin (1:2,000; Sigma Chemical, St. Louis, MO); and Bcl-xL, RNA polymerase II carboxyl-terminal domain (CTD), Bad, Bax, and apoptosis-inducing factor (1:1,000; Santa Cruz Biotechnology). Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

**Real-time Reverse Transcription-PCR**

Real-time reverse transcription-PCR (RT-PCR) experiments were done in triplicate as described previously (30). Results for the experimental gene were normalized to 18S.

**Figure 1.** A, U937 cells were sequentially exposed to 30 nmol/L LAQ824 (L) for 24 h, after which they were either left untreated or exposed to roscovitine (R) without washing for an additional 24 h. The extent of apoptosis was then determined by Annexin V/PI analysis as described in Materials and Methods. Inset, median-dose-effect analysis of apoptosis induction in U937 cells sequentially exposed to LAQ824 followed by roscovitine as in A administered over a range of concentrations at three different concentration ratios (i.e., 1:400, 1:333, and 1:266). Combination indices for each fraction affected < 1.0 correspond to synergistic interactions. B, time-course analysis of LAQ824 (30 nmol/L; LAQ)/roscovitine (10 μmol/L; ROS) – induced cell death in U937 cells sequentially treated as in A, after which the percentage of apoptotic cells was monitored by Annexin V/PI staining at the indicated intervals as described above. Points, mean of three separate experiments done in triplicate; bars, SD. C, T lymphoblastic leukemia Jurkat cells and promyelocytic HL-60 leukemia cells (left), three primary patient-derived AML samples (French-American-British classifications M2, M4, and M5B; right), and normal bone marrow mononuclear cells, peripheral blood mononuclear cells (PBMC), and CD34⁺ bone marrow progenitor cells were treated sequentially as above and apoptosis was determined by Annexin V/PI analysis. The concentrations of the corresponding agents used were as follows: HL-60 cells: LAQ824, 14 nmol/L; roscovitine, 15 μmol/L; Jurkat cells: LAQ824, 20 nmol/L; rosvitine, 10 μmol/L; primary patient-derived AML samples: LAQ824, 20 nmol/L; roscovitine, 18 μmol/L for patients 1 and 2 and LAQ824, 10 nmol/L; rosvitine, 12 μmol/L for patient 3; LAQ824, 30 nmol/L; roscovitine, 10 μmol/L for normal bone marrow mononuclear cells, peripheral blood mononuclear cells, and CD34⁺ cells. D, U937 cells were sequentially exposed to 30 nmol/L LAQ824 (48 h) and 10 μmol/L roscovitine (Ros 10; 24 h), after which cells were washed free of drugs and plated in soft agar as described in Materials and Methods. Colonies consisting of groups of ≥50 cells were scored after 12 days. E, Western blot analysis of lysates of U937 cells collected at the same time intervals as in B. After treatment, cells were pelleted and lysed and proteins (30 μg) were separated by SDS-PAGE as described in Materials and Methods. Blots were then probed with the indicated antibodies directed against caspase cleavage products, Bid and PARP, c-casp 9, c-casp 3, and c-casp 7, cleavage products of caspase-9, caspase-3, and caspase-7, respectively. Blots were subsequently stripped and probed with antibodies directed against actin to ensure equivalent loading and transfer. Representative study; two additional experiments yielded similar results. *, P < 0.01, significantly greater than values for cells treated with either drug alone. F, U937 cells expressing either caspase-8-DN (C8DN) or the corresponding empty vector (EV) controls were sequentially treated with 30 nmol/L LAQ824 (24 h) and 10 μmol/L roscovitine for additional 24 h, after which apoptosis was monitored by Annexin V/PI staining as described in Materials and Methods. T/CHX, tumor necrosis factor-α (7 ng/mL)/cyclohexamide (CHX; 1 μmol/L). Inset, Western blot analysis of U937/caspase-8-DN and U937/empty vector cells treated as above, after which cells were collected and analyzed for caspase-8 activation. Blots were subsequently stripped and probed with antibodies directed against actin to ensure equivalent loading and transfer. Representative study; two additional experiments yielded similar results.
rRNA levels as specified by the manufacturer. The following primers and probe sequences for human Mcl-1 were used (real-time RT-PCR): forward primer 5'-GGGCAGGATTGTGACTCTCATT-3', reverse primer 5'-GA-TGCAGCTTTCTTGGTATGG-3', and TaqMan probe: 5'-TCAAGTGTATGAAGGCACCAAAAG-3'.

Statistical Analysis

The significance of differences between experimental conditions was determined using the Student’s t test for unpaired observations. To assess the interaction between agents, median-dose analysis (31) was used (CalcuSyn, Biosoft, Ferguson, MO). The combination index was calculated for a two-drug combination involving a fixed concentration ratio. Combination indices of <1.0 indicate a synergistic interaction.

Results

Pretreatment of Human Leukemia Cells with LAQ824 Potentiates Roscovitine-Induced Cell Death

To investigate antileukemic interactions between roscovitine and LAQ824, several sequences and schedules of drug administration were investigated. Treatment of cells with 30 nmol/L LAQ824 for 24 hours was minimally toxic to U937 cells, whereas 10 µmol/L roscovitine induced cell death in <20% of cells (Fig. 1A). Higher concentrations of roscovitine (i.e., 20 and 30 µmol/L) induced a pronounced apoptotic response, which was accompanied by a dose-dependent activation of caspase-9 and caspase-8 (Supplementary Data).5 Notably, simultaneous exposure to these agents, while significantly increasing apoptosis compared with the controls, was not more toxic than roscovitine (10 µmol/L) alone (P > 0.05; data not shown). However, when cells were first treated with LAQ824 for 24 hours followed by roscovitine for an additional 24 hours, marked lethality (e.g., 65%) was observed (Fig. 1A). Median-dose-effect analysis of apoptosis induction for cells exposed sequentially to these agents at multiple concentrations at fixed ratios (i.e., LAQ824/roscovitine, 1:400, 1:333, and 1:266) yielded combination indices of <1.0, most notably at the 1:333 LAQ824/roscovitine ratio (Fig. 1A, inset), indicating synergistic interactions. The latter concentration ratio was therefore used in all subsequent studies. In contrast, the sequence roscovitine followed by LAQ824 resulted in only additive effects (data not shown). Based on these observations, studies in U937 cells were done following the optimal sequential administration schedule (i.e., 30 nmol/L LAQ824 for 24 hours followed by the addition of 10 µmol/L roscovitine for additional 24 hours). A time-course study revealed a significant potentiation of apoptosis in LAQ824-pretreated cells as early as 4 hours following roscovitine exposure, which increased further over the ensuing 20 hours (Fig. 1B). The sequence LAQ824 followed by roscovitine also resulted in supra-additive effects in T lymphoblastic leukemia Jurkat cells and promyelocytic HL-60 leukemia cells (Fig. 1C, left) as well as in three primary patient-derived AML samples (Fig. 1C, right). Significantly, the combination of these agents exerted relatively modest toxicity toward normal bone marrow mononuclear cells and peripheral blood mononuclear cells as well as CD34+ bone marrow progenitor cells (Fig. 1C, right). Clonogenic assays involving U937 cells exposed sequentially to LAQ824 followed by roscovitine showed a pronounced reduction in clonogenicity compared with individual drug exposure (Fig. 1D). Western blot analysis revealed exposure to the two agents in sequence, but not individually, exhibited marked cleavage of procaspase-9, procaspase-3, procaspase-7, procaspase-8, Bid, and PARP, indicating activation of the caspase cascade (Fig. 1E). Activation of caspase-8 has been reported to play a significant role in a mitochondrial-dependent amplification loop involving caspase-3 and caspase-8 (32). As caspase-8 seemed to be activated concomitantly with caspase-9, caspase-3, and caspase-7, we investigated the possible contribution of this apoptotic amplification loop using U937 cells ectopically expressing a caspase-8-DN cDNA. As shown in Fig. 1F, a modest but significant (P > 0.05) decrease in the extent of apoptosis was observed in U937/caspase-8-DN cells. However, this decrease was significantly less pronounced than that observed when the same cells were exposed to tumor necrosis factor-α/cyclohexamide, which primarily induces apoptosis through activation of the extrinsic, receptor-mediated apoptotic cascade. Thus, these observations indicate that caspase-8 activation may play a modest but significant role in an apoptotic amplification loop, which maximizes the cell death response to the LAQ824/roscovitine combination.

Sequential LAQ824/Roscovitine Administration Activates the Intrinsic/Mitochondrial Apoptotic Pathway while Down-Regulating Antiapoptotic Mcl-1 and XIAP Proteins

Effects of these agents were then examined in relation to release of mitochondrial proteins and expression of Bcl-2 family members. In U937 cells, LAQ824 and roscovitine administered individually resulted in minimal losses in ΔΨm, whereas sequential administration induced significantly more mitochondrial injury (Fig. 2A). LAQ824 alone (30 nmol/L; 24 and 48 hours) resulted in essentially no release of apoptosis-inducing factor, cytochrome c, or Smac/DIABLO into the cytosolic S-100 fraction, whereas roscovitine alone (10 µmol/L; 24 hours) had only a marginal effect (Fig. 2B). However, sequential treatment resulted in a clear increase in apoptosis-inducing factor, Smac/DIABLO, and cytochrome c release after 3 to 4 hours of roscovitine exposure and a very substantial increase by 8 to 16 hours. Effects of LAQ824/roscovitine were then examined in relation to expression of Bcl-2 family members. Individual or combined treatment with LAQ824 or roscovitine exerted little effect on the expression of Bcl-2, total Bax, survivin, or Bad in these cells, whereas very slight

5 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org).
reductions in Bcl-xL levels were observed in cells exposed to LAQ824 alone for 48 hours slightly diminished XIAP expression, combined treatment of cells with LAQ824 and roscovitine triggered a pronounced reduction in XIAP levels (Fig. 2C).

Figure 2. Effects of LAQ824/roscovitine on mitochondrial function and antiapoptotic proteins. A, U937 cells were incubated with 30 nmol/L LAQ824 for 24 h, after which they were either left untreated or exposed to roscovitine for an additional 24 h. The percentage of cells exhibiting a loss of Δψm was determined by flow cytometry as described in Materials and Methods. Columns, mean of three separate experiments; bars, SE. B and C, U937 cells were sequentially exposed to 30 nmol/L LAQ824 for 24 h, after which 10 μmol/L roscovitine was added for the indicated intervals (e.g., LAQ824/roscovitine, 2–24 h). R, cells exposed to roscovitine alone for 24 h (i.e., without LAQ824 pretreatment). At the end of the treatment interval, cells were pelleted and lysed, and protein was extracted from either the cytosolic S-100 fraction (apoptosis-inducing factor (AIF), cytochrome c (Cyto c), and Smac; B) or whole-cell lysates (Bcl-xL, Bcl-2, Bax, Bad, Mcl-1, surviving, and XIAP; C). In each case, proteins (30 μg) were separated by SDS-PAGE, after which blots were then probed with the corresponding antibodies. Blots were subsequently stripped and probed with antibodies directed against actin to ensure equivalent loading and transfer. Representative study; two additional experiments yielded similar results. D, top, translocation of Bax was analyzed by monitoring expression of the protein in the cytosolic and pelleted fractions as described in Materials and Methods; bottom, LAQ824/roscovitine–induced Bax conformational change. Cells were treated as described above, after which levels of conformationally changed Bax were determined by first immunoprecipitating lysates with an anti-Bax 6A7 antibody, which recognizes only the conformationally changed protein. The proteins were then immunoblotted with an anti–Bax rabbit polyclonal antibody as described in Materials and Methods. Representative study; two additional experiments yielded similar results. E, top, U937 cells expressing either FADD-DN or the corresponding empty vector were sequentially treated with 30 nmol/L LAQ824 (24 h) ± 10 μmol/L roscovitine and apoptosis was determined by Annexin V/PI staining as described previously. Parallel studies were done in which cells were exposed to the nucleoside analogue ara-C (10 μmol/L) for 24 h. Note that empty vector controls varied in their sensitivity to the LAQ824/roscovitine regimen. *, P < 0.01, significantly lower than values for U937/empty vector cells. F, both U937/empty vector and U937/Bcl-2 cells were treated as in E, after which cells were washed free of drugs and plated in soft agar as described in Materials and Methods. Colonies consisting of groups of >50 cells were scored after 12 d. **, P < 0.01, significantly lower than values for the corresponding untreated control cells. Inset, Western blot analysis of the cytosolic S-100 fraction extracted from both U937/empty vector and U937/Bcl-2 cells sequentially exposed to 30 nmol/L LAQ824/10 μmol/L roscovitine and probed for cytochrome c. Proteins (30 μg) were separated by SDS-PAGE and blotted with anti–cytochrome c antibody. Representative study; two additional experiments yielded similar results.
Individual treatment of cells with LAQ824 or roscovitine (24 hours each) minimally affected cytosolic Bax, whereas sequential treatment induced a marked redistribution from the cytosolic to the particulate (mitochondrial) compartment within 4 hours of roscovitine treatment (Fig. 2D). Moreover, whereas roscovitine alone was ineffective and LAQ824 minimally affected Bax conformational change, an event associated with apoptosis (33), sequential treatment dramatically increased expression of conformationally changed Bax 4 hours after roscovitine administration (Fig. 2D). Thus, sequential LAQ824/roscovitine administration was associated with potentiation of mitochondrial damage, a marked conformational change in Bax, and diminished expression of Mcl-1 and XIAP.

To gain insights into the relative contribution of the intrinsic and extrinsic apoptotic pathways to LAQ824/roscovitine–mediated lethality, particularly in view of reports that LAQ824 may up-regulate death receptors (DR4 and DR5) in some leukemia cells (5), U937 leukemia cells ectopically expressing either FADD-DN (27) or Bcl-2 (28) were employed. U937 FADD-DN cells displayed no protection from LAQ824/roscovitine lethality, whereas pronounced protection from tumor necrosis factor-α/cyclohexamide was observed (Fig. 2E, top). Exposure of cells to LAQ824 and roscovitine also did not increase expression of DR4 or DR5 in these cells (data not shown). In contrast, ectopic expression of Bcl-2, which conferred marked protective effects in cells exposed to the nucleoside analogue 1-β-D-arabinofuranosylcytosine (ara-C; 10 μmol/L; ref. 34), also protected cells, although partially, from LAQ824/roscovitine–mediated lethality at both 48 and 72 hours (P < 0.05 and 0.02, respectively; Fig. 2E, bottom). Because Bcl-2-mediated protection from apoptosis may not correlate with restoration of clonogenicity (35), clonogenic assays were done. Ectopic expression of Bcl-2 reduced, albeit partially, LAQ824/roscovitine–mediated cytochrome c release into the S-100 cytosolic fraction (Fig. 2F, inset). However, enforced expression of Bcl-2 was minimally effective in protecting clonogenic cells from LAQ824-mediated lethality (Fig. 2F). Together, these findings indicate that in U937 cells the LAQ824/roscovitine regimen primarily induces apoptosis via the intrinsic, mitochondrial pathway but that increased Bcl-2 expression is relatively ineffective in protecting clonogenic cells from this drug combination.

**Effects of Sequential Administration of LAQ824 and Roscovitine on Cell Cycle Progression and Differentiation in U937 Cells**

Effects of combined exposure of cells to LAQ824 and roscovitine were then examined in relation to cell cycle perturbations and leukemic cell maturation. LAQ824 (30 nmol/L) had little effect on cell cycle traverse at 24 hours, although a 48 hours a striking increase in GI arrest was noted (e.g., 40–64%; Fig. 3A). A 24-hour exposure of cells to 10 μmol/L roscovitine by itself minimally affected cell cycle distribution. However, when cells were sequentially exposed to LAQ824 followed by roscovitine, a large increase in the subdiploid, apoptotic fraction was noted, but the distribution of the non-apoptotic population was similar to that observed in cells exposed to LAQ824 alone (Fig. 3A).

Previous studies revealed that the CDK1 flavopiridol promotes HDACI-mediated lethality, at least in part, through p21CIP1 down-regulation (22). Western analysis revealed that treatment with LAQ824 (48 hours) robustly induced p21CIP1 in U937 cells but that this effect was attenuated by roscovitine exposure (Fig. 3B). Such results are similar to, although less dramatic than, those observed in leukemic cells exposed to flavopiridol and HDACIs or phorbol 12-myristate 13-acetate (22, 36). However, in contrast to previous results with flavopiridol, the pancaspase inhibitor Boc-D-fmk substantially restored p21CIP1 expression in LAQ824/roscovitine–treated cells, suggesting that down-regulation represented at least in part a secondary, caspase-dependent event. In addition, a p27KIP1 cleavage fragment could be faintly discerned in LAQ824/roscovitine–treated cells. Lastly, LAQ824 treatment induced pRb dephosphorylation, whereas subsequent exposure of cells to roscovitine induced extensive cleavage of the underphosphorylated pRb species.

To assess the functional significance of reduced expression in LAQ824/roscovitine–mediated lethality, two separate U937 cell clones (ΔNLS-1 and ΔNLS-23) ectopically expressing a p21-ΔNLS mutant were employed. These cells express high levels of p21CIP1 in the cytoplasm (37). Both clones were significantly less sensitive than empty vector controls to LAQ824/roscovitine–mediated lethality (P < 0.01 in each case; Fig. 3C), suggesting that diminished expression of p21CIP1 may contribute to the enhanced lethality of this combination. A specific role for p21CIP1 down-regulation in LAQ824/roscovitine–induced lethality was supported by the observation that p21-ΔNLS cells were as sensitive as the empty vector controls to the nucleoside analogue ara-C, which, in contrast to LAQ824, does not induce p21CIP1 expression in human leukemia cells at this concentration (10 μmol/L; ref. 38). Together, these findings indicate that combined treatment of leukemia cells with LAQ824 and roscovitine results in p21CIP1 down-regulation, but in contrast to interactions with flavopiridol this phenomenon, along with cleavage/degradation of p27KIP1 and pRb, largely represents a secondary, caspase-dependent event.

The effects of roscovitine on LAQ824-mediated differentiation were then examined. LAQ824 alone resulted in a robust increase in expression of the myeloid differentiation markers CD11a, CD11b, and CD11c in U937 cells, whereas roscovitine alone had essentially no effect (Fig. 3D). However, sequential exposure of cells to LAQ824 followed by roscovitine resulted in a significant attenuation of leukemic cell maturation (P < 0.01 versus LAQ824 alone). Finally, sequential exposure to LAQ824 and roscovitine failed to modify expression of Raf-1 or phospho–extracellular
signal-regulated kinase, Akt, or c-Jun NH2-terminal kinase (data not shown), arguing against a contribution of these signaling proteins to synergistic interactions between these agents.

LAQ824/Roscovitine–Induced Lethality Involves Generation of ROS

Induction of apoptosis by HDACIs has been related to oxidative injury (7, 8). Therefore, the effects of LAQ824 and roscovitine were examined in relation to ROS generation. Thirty minutes after treatment with LAQ824 or roscovitine individually, a substantial percentage of cells exhibited an increase in ROS generation (i.e., 1.36- and 1.39-fold increases, respectively; Fig. 4A). In cells exposed to LAQ824 alone, a peak was reached after 2 hours (i.e., 1.66-fold increase over basal levels) followed by a decline to basal levels after 6 hours of exposure. No evidence of increased ROS generation was apparent 24 hours after LAQ824 administration (i.e., immediately before the addition of roscovitine). A second increase in ROS generation was noted early as 30 minutes after administration of roscovitine to LAQ824-pretreated and control cells. Although the initial increase was slightly but significantly ($P < 0.05$) greater for LAQ824-treated cells, values were equivalent at intervals over the ensuing 4 hours. Dose-response

Figure 3. A, cell cycle analysis following exposure of cells to agents alone (LAQ824, 30 nmol/L; roscovitine, 10 μmol/L) or in combination for the indicated interval. After treatment, U937 cells were 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. The percentages exclude the apoptotic, sub-G0 cell population. B, Western blot analysis of p21CIP1 expression in U937 cells exposed to 30 nmol/L LAQ824 (48 h; La4), 10 μmol/L roscovitine (24 h; R24), or, sequentially, LAQ824 (24 h) followed by 10 μmol/L roscovitine for additional 24 h (LR) in the presence or absence of the pancaspase inhibitor Boc-d-fmk (20 mmol/L; LR+I). At the end of this period, proteins (30 μg) were separated by SDS-PAGE and blotted with antibodies directed against p21CIP1. Alternatively, cells were treated as above, and lysates were probed with antibodies directed against p27KIP1, pRb, or underphosphorylated pRb. Representative study; two additional experiments yielded similar results. C, U937 cells ectopically expressing cytoplasmic p21CIP1 (ΔNLS-1 and ΔNLS-23) were exposed sequentially to LAQ824/roscovitine as described above, after which apoptosis was determined by Annexin V/PI staining as described in Materials and Methods. *, $P < 0.01$, significantly lower than values for U937/empty vector cells. D, effects of roscovitine on LAQ824-induced differentiation. The expression of the myeloid differentiation markers Cd11a, Cd11b, and Cd11c was determined by flow cytometry in U937 following 72 h exposure to either the agents alone or in sequence as described above. Columns, mean of three separate experiments done in triplicate; bars, SD. *, $P < 0.01$, significantly lower than values for cells exposed to 30 nmol/L LAQ824 alone.
analysis revealed that increasing the concentration of LAQ824 from 20 to 50 nmol/L only slightly increased roscovitine (10 μmol/L)–induced ROS generation (e.g., a 1.6- to 1.8-fold increase over control values; Fig. 4B, left). Similarly, increasing the roscovitine concentration from 6 to 12 μmol/L modestly increased LAQ824 (30 nmol/L)–mediated ROS generation (i.e., a 1.3- to 1.8-fold increase over controls).

To assess the functional significance of these events, the free radical scavenger N-acetylcysteine (NAC) was employed. When NAC was added 21 hours after the initial administration of LAQ824 (i.e., 3 hours before the addition of roscovitine), modest but statistically significant protection from LAQ824/roscovitine–mediated lethality was observed (P < 0.05 versus cells not exposed to NAC; Fig. 4C). However, when NAC was added 3 hours before the initial LAQ824 exposure, a significantly greater degree of protection was achieved (P < 0.02 versus NAC administered after LAQ824). A time-course study examining mitochondrial injury (ΔΨm) revealed several findings. First, cells exposed to LAQ824 for 24 hours exhibited a modest but statistically significant (P < 0.05) reduction in ΔΨm compared with controls (Fig. 4D).

Second, LAQ824 markedly increased the loss of ΔΨm in cells exposed to roscovitine over a 4-hour interval. Finally, the latter effect was blocked by coadministration of NAC. Such findings suggest that oxidative damage following sequential exposure to LAQ824 and roscovitine contributes to mitochondrial injury and apoptosis in these cells.

**LAQ824/Roscovitine–Induced XIAP and Mcl-1 Down-Regulation Plays a Functional Role in Lethality**

XIAP blocks apoptosis by inhibiting the action of several effector caspases, including caspase-3, caspase-7, and caspase-9 (39), and is down-regulated by the CDKI flavopiridol (26). Consequently, XIAP expression in LAQ824/roscovitine–treated cells was investigated in detail. Down-regulation of XIAP following LAQ824/roscovitine treatment was not reversed by the caspase inhibitor Boc-D-fmk (Fig. 5A, top), showing the caspase independence of this event. Similar results were noted in Jurkat cells (Fig. 5A, bottom). Time-course analysis revealed a marked reduction in XIAP expression in LAQ824-treated cells 4 hours after exposure to roscovitine, which approached 90% after 24 hours of treatment (Fig. 5B).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effects of LAQ824/roscovitine on ROS generation. A, U937 cells were sequentially exposed to 30 nmol/L LAQ824, 10 μmol/L roscovitine, or the two agents in combination for the indicated intervals, after which cells were labeled with the oxidation-sensitive dye (2',7'-dichlorodihydrofluorescein diacetate) and analyzed by flow cytometry to determine the percentage of cells displaying an increase in ROS production relative to untreated controls. Points, mean of three separate experiments; bars, SE. B, dose-response analysis of U937 either (right) sequentially exposed to 30 nmol/L LAQ824 for 24 h followed by increasing concentrations of roscovitine for 2 additional h or (left) preincubated with variable concentrations of LAQ824 followed by the addition of 10 μmol/L roscovitine for an additional 2 h, after which ROS generation was monitored as described. Points, mean of three separate experiments; bars, SE. C, U937 cells were sequentially exposed to LAQ824/roscovitine; the free radical scavenger NAC (15 mmol/L) either 3 h before the administration of LAQ824 (LR/NAC-3h) or after 21 h of treatment with LAQ824 (i.e., 3 h before the addition of roscovitine to LAQ824 pretreated cells; LR/NAC-21h). The cells were then labeled as above and analyzed by flow cytometry to determine the percentage of Annexin V/PI–positive cells. *, P < 0.01, significantly lower than values for cells exposed sequentially to 30 nmol/L LAQ824/roscovitine in the absence of NAC; **, P < 0.05. D, time-course analysis of drug-induced mitochondrial injury. After exposure to either LAQ824 or roscovitine alone or together in sequence as described above, the percentage of cells exhibiting a loss of ΔΨm was determined by flow cytometry as described in Materials and Methods. Parallel studies were done in the presence of 15 mmol/L NAC. R4h, roscovitine alone for 4 h; L24h, LAQ824 alone for 24 h; LR, LAQ824 for 24 h followed by roscovitine for 30 min or 1, 2, 3, or 4 h. Columns, mean of three separate experiments; bars, SE. *, significantly greater than untreated control U937 cells; #, significantly lower than values for cells coincubated in the presence of the free radical scavenger NAC (NAC-); **, significantly greater than values for LAQ824-treated U937 cells (24 h); P < 0.05 for LAQ824/roscovitine 30 min and 1, 2, and 3 h; P < 0.01 for LAQ824/roscovitine 4 h.
Furthermore, ectopic XIAP expression (29) significantly protected cells from LAQ824/roscovitine–mediated lethal-ity \( (P < 0.02; \text{Fig. 5C}) \). Similarly, XIAP cells showed marked protection against the cytotoxic agents VP-16 (25 \( \mu \text{mol/L}; 5 \) hours) and ara-C (10 \( \mu \text{mol/L}; 24 \) hours). Whereas clonogenic assays involving U937/XIAP cells exposed sequentially to LAQ824 followed by roscovitine showed significant protection \( (i.e., \sim 1 \text{ log}) \) of clonogenic cells compared with the corresponding empty vector control cells \( (\text{Fig. 5D}) \), a marked reduction in clonogenic potential was nevertheless observed in XIAP-overexpressing cells. Ectopic expression of XIAP also diminished but did not prevent LAQ824/roscovitine–mediated loss of \( \Delta \psi_m \) \( (\text{Fig. 5E}) \) and cytosolic release of cytochrome c \( (\text{Fig. 5F}) \). However, reduced activation/cleavage of procaspase-9, -3, -7, and -8 and degradation of PARP \( (\text{Fig. 5F}) \). Finally, quantitative RT-PCR analysis revealed that, whereas LAQ824 and roscovitine...
administered individually modestly reduced XIAP transcription, combined treatment significantly reduced XIAP mRNA levels as early as 4 hours after roscovitine administration (P < 0.02; Fig. 6A).

In addition to XIAP, the antiapoptotic Bcl-2 family member Mcl-1, which is repressed at the transcriptional level by flavopiridol (26), was also down-regulated after exposure of U937 cells to LAQ824/roscovitine (Figs. 2C and 6B, left). However, clear evidence of protein degradation was noted, manifested by the appearance of a cleavage fragment of ~27 kDa as described previously (40). Coincubation in the presence of pancaspase inhibitor Boc-D-fmk restored Mcl-1 expression levels and prevented formation of the Mcl-1 cleavage fragment. Analysis of Mcl-1 mRNA by Taqman real-time RT-PCR (Fig. 6D, right) showed no changes, indicating that LAQ824/roscovitine–mediated Mcl-1 down-regulation proceeds through a post-translational, caspase-dependent mechanism. Studies done with two separate U937/Mcl-1 clones (M14 and M16) ectopically expressing Mcl-1 displayed significant protection from LAQ824/roscovitine–mediated lethality, loss of ΔΨm (data not shown), and a significant reduction in cytosolic release of cytochrome c and apoptosis-inducing factor as well as procaspase-3 activation and PARP degradation (Fig. 6C). Together, these findings suggest a functional role for XIAP and Mcl-1 down-regulation in the lethality of this regimen and indicate that both transcriptional (XIAP) and post-translational (Mcl-1) mechanisms may be involved in these events.

Finally, effects of sequential administration of LAQ824/roscovitine on the expression and phosphorylation of RNA polymerase II (Fig. 6D) were examined. Recent studies indicate that both flavopiridol (41) and the R-enantiomer of roscovitine, CYC202, inhibit phosphorylation of the RNA polymerase II CTD (42). As shown in Fig. 6D, LAQ824 or roscovitine (10 μmol/L) individually had no or minimal effect on the phosphorylation status of RNA polymerase II. By contrast, cells exposed to LAQ824/roscovitine exhibited a clear reduction in CTD phosphorylation after 4 hours of drug administration, which became

Figure 6. A, U937 cells were exposed to 30 nmol/L LAQ824 alone for 24 h (L24), 10 μmol/L roscovitine alone for the indicated interval (R), or the sequential combination of these agents, after which expression of XIAP mRNA was monitored by real-time RT-PCR as described in Materials and Methods. Data for each condition are expressed as the percentage of specific XIAP mRNA normalized to levels corresponding to the untreated control U937 cells (100%). Columns, mean of three separate experiments done in triplicate; bars, SD. * P < 0.05, significantly lower than values for both drugs alone. ** P < 0.01, significantly lower than values for U937/empty vector cells. Inset, Western blot analysis of whole-cell lysates extracted from U937 cells treated as indicated above. Proteins (30 μg) were separated by SDS-PAGE and blotted with antibodies directed against Mcl-1. LR, cells exposed to the combination LAQ824/roscovitine in the presence of the pancaspase inhibitor Boc-D-fmk; CF, cleavage fragment. Right, analysis of Mcl-1 mRNA levels by real-time RT-PCR. U937 cells were exposed to the drugs alone or in combination as indicated (LAQ824, 30 nmol/L; roscovitine, 10 μmol/L). L24, L48, and R24, cells exposed to either LAQ824 or roscovitine alone for 24 to 48 h; LR24, U937 cells sequentially exposed to LAQ824 (24 h) followed by 24 h exposure to roscovitine. Data for each condition are expressed as the percentage of specific XIAP mRNA normalized to levels corresponding to untreated control U937 cells (100%). Columns, mean of three separate experiments done in triplicate; bars, SD. C, U937 cells ectopically expressing Mcl-1 (U/Mcl1-14 and U/Mcl1-16) were exposed sequentially to LAQ824/roscovitine as described, after which apoptosis was determined by Annexin V/PI staining as described in Materials and Methods. *, P < 0.01, significantly lower than values for U937/empty vector cells. Insert, Western blot analysis of whole-cell lysates extracted from U937/Mcl-1 cells treated as indicated above. Proteins (30 μg) were separated by SDS-PAGE and blotted with antibodies directed against Mcl-1. Numbers, percentage of protein expression determined by densitometric analysis relative to control values (100%) and normalized to actin. Right, Western blot analysis of cytosolic (S-100) and whole-cell lysates extracted from both U937/empty vector (U-EV) and U937/Mcl-1 clone 14 (U-Mcl-1) cells sequentially exposed to 30 nmol/L LAQ824/10 μmol/L roscovitine. LR, sequential exposure LAQ824 24 h/roscovitine 24 h. At the end of the incubation period, proteins (30 μg) were separated by SDS-PAGE and blotted with the corresponding antibodies. Representative study; two additional experiments yielded similar results. D, Western blot analysis of whole-cell lysates extracted from U937 cells as above and monitored at the indicated time points. For each condition, proteins (30 μg) were separated by SDS-PAGE and blotted with antibodies directed against phospho–RNA polymerase II (pol II) or total RNA polymerase II (CTD). Blots were subsequently stripped and reprobed with antibodies directed against actin to ensure equivalent loading and transfer. Representative study; two additional experiments yielded similar results. Numbers, percentage of protein expression determined by densitometric analysis relative to control values (100%) and normalized to actin.
more pronounced over the ensuing 12 hours, and was not restored by coadministering the pancaspase inhibitor Boc-D-fmk. No significant changes in total levels of the RNA polymerase II CTD were detected. Together, these findings raise the possibility that enhanced inhibition of RNA polymerase II in LAQ824/roscovitine–treated cells might be involved in transcriptional repression of short-lived proteins like XIAP, thereby contributing to the lethality of this combination.

Discussion

The present results indicate that nontoxic concentrations of LAQ824 interact synergistically with concentrations of the purine CDKI rosvitine below those necessary to induce apoptosis in human leukemia cells (43). Despite similarities to antileukemic interactions involving flavopiridol and HDACIs (22, 23), the present results suggest that that mechanisms underlying LAQ824/roscovitine interactions are not identical. For example, whereas concurrent administration of flavopiridol and HDACIs resulted in maximal antileukemic effects (22, 23), sequential treatment with LAQ824 followed by rosvitine resulted in optimal lethality. Differences in the mechanism of p21<sup>CIP1</sup> down-regulation were also noted. For example, flavopiridol, in addition to its CDKI actions, also blocks induction of the endogenous CDKI p21<sup>CIP1</sup>, presumably through its inhibitory effects on the PTEF-b CDK9/cyclin T transcription apparatus (44). Multiple studies have shown that disruption of p21<sup>CIP1</sup> expression promotes HDACI-related lethality (10, 45); moreover, interference with p21<sup>CIP1</sup> induction by flavopiridol contributes functionally to synergistic interactions with HDACIs in leukemia cells (22, 23, 25). However, in contrast to results obtained with flavopiridol, addition of the pancaspase inhibitor Boc-D-fmk largely restored p21<sup>CIP1</sup> levels, indicating that down-regulation represented a secondary, caspase-dependent effect in the LAQ824/roscovitine regimen. Thus, down-regulation of p21<sup>CIP1</sup> in LAQ824/roscovitine–treated cells may nevertheless amplify the apoptotic response, consistent with the significant protection afforded by ectopic expression of cytoplasmic p21<sup>CIP1</sup>.

A key question to be resolved is why rosvitine promotes LAQ824-mediated apoptosis rather than maturation. It is well known that differentiation and apoptosis represent alternative and often mutually exclusive cell fates (46), and differentiating leukemic cells exhibit resistance to apoptosis induced by cytotoxic drugs (47). In addition, because cell cycle arrest in G<sub>1</sub> is required for leukemic cell maturation, it seemed reasonable to hypothesize that CDKIs, which by themselves can induce differentiation (19, 21, 48), might enhance HDACI-mediated maturation. However, flavopiridol blocks leukemic cell differentiation by HDACIs (22, 23) as well as the phorbol ester phorbol 12-myristate 13-acetate (36). Analogously, a subtoxic concentration of LAQ824 (e.g., 30 nmol/L) potentely induced expression of the myeloid maturation markers CD11a, CD11b, and CD11c, whereas coadministration of rosvitine antagonized this process. One plausible possibility is that the observed potentiation of mitochondrial injury may disrupt the differentiation process. Alternatively, rosvitine may interfere with certain cell cycle and/or differentiation-related events in LAQ824-treated cells, triggering a default apoptotic program. In this regard, Matushansky et al. (49) observed that, whereas sequential inactivation of CDK4 by induction of the CDKI p16<sup>INK4a</sup> followed by CDK2 inhibition by rosvitine led to maturation of MEL cells, the reverse sequence resulted in apoptosis. Such findings suggest that differentiation-related changes in the cell cycle traverse are tightly regulated and that pharmacologic inhibition of CDKs, particularly at inappropriate times, culminate in cell death rather than in maturation.

The present results indicate that oxidative damage and mitochondrial injury play important roles in LAQ824/roscovitine lethality. The observation that NAC protected cells from LAQ824/roscovitine lethality and blocked the loss of Δψ<sub>m</sub> argues that in LAQ824/roscovitine–treated cells ROS generation represents a primary rather than a secondary event and supports the concept that oxidative damage is involved in the antileukemic actions of this regimen. Although the capacity of HDACIs to trigger ROS generation is known (7, 8), the mechanism underlying this phenomenon is uncertain but may reflect HDACI-mediated perturbations in genes involved in oxidative homeostasis (e.g., thioredoxin; refs. 50, 51). Our findings suggest that the initial increase in LAQ824-mediated ROS generation, although not lethal by itself, may lower the threshold for subsequent oxidative injury, a phenomenon that could account for the observation that sequential administration of LAQ824/roscovitine induced maximal lethality. For example, attention has focused recently on the ability of ROS to function as phosphatase inhibitors and, in so doing, to activate certain stress-related signaling pathways (e.g., c-Jun NH<sub>2</sub>-terminal kinase) that promote cell death (52). It is therefore possible that certain proapoptotic signaling perturbations triggered by the initial increase in ROS generation will potentiate the lethal consequences of subsequent CDKI administration. Additional studies will be required to confirm or refute this hypothesis.

Although LAQ824 has been shown to up-regulate death receptors in some leukemia cells (5), and similar events have been observed in some primary AML blasts exposed to other HDACIs (53), this was not observed in U937 cells, suggesting a cell type–specific phenomenon. Consistent with this finding, FADD-DN failed to attenuate LAQ824/roscovitine–mediated lethality. It is nevertheless noteworthy that combined treatment was associated with cleavage/down-regulation of Bid. Bid activation by HDACIs has been described (8) and may, through induction of mitochondrial injury, amplify apoptosis, particularly in type II cells in which the lethal consequences of caspase-8 activation are limited (54). In addition, recent studies have shown that acetylation by HDACIs can interfere with the function...
of Hsp90, leading to degradation of prosurvival client proteins, such as Raf-1, Akt, extracellular signal-regulated kinase, and Bcr/Abl (12, 55). However, prior exposure to LAQ824 failed to alter expression/activation of Raf-1, extracellular signal-regulated kinase, or Akt in roscovitine-treated cells. Nevertheless, the possibility that LAQ824-mediated Hsp90 acetylation might lead to alterations in other signaling or cell cycle proteins and, as a consequence, promote roscovitine lethality cannot be excluded.

Recent studies suggest that roscovitine, like flavopiridol, can transcriptionally down-regulate the expression of short-lived antiapoptotic proteins (e.g., Mcl-1 and XIAP) in B-cell malignancies (56). The present data indicate that combined exposure to roscovitine and LAQ824 at subeffective concentrations leads to a marked potentiation of these events through both transcription-dependent and transcription-independent mechanisms. XIAP inhibits the function of multiple caspases, most notably caspase-3, caspase-9, and caspase-7 (39), and, in so doing, attenuates apoptosis. In this context, XIAP has been implicated in apoptosis resistance in leukemia cells undergoing differentiation (57). Recently, roscovitine has been shown to regulate the expression of XIAP through inhibition of STAT5 activity in human T-lymphotrophic virus-1–transformed T-cell line MT2 (43). However, in U937 cells, basal STAT5 activity was very low and exposure to LAQ824/roscovitine failed to induce detectable changes, suggesting that the role of STAT5 in XIAP regulation may vary with cell type. On the other hand, it is unlikely to be coincidental that flavopiridol, which blocks phosphorylation of the RNA polymerase II CTD (41), down-regulates XIAP expression in association with induction of leukemic cell apoptosis (26). Interestingly, CYC202, the R-enantiomer of roscovitine, has been shown recently to inhibit phosphorylation of the RNA polymerase II CTD at least when administered at toxic concentrations (42). In addition, LAQ824 by itself, like the HDACI MS-275 (7), modestly diminished XIAP expression. Based on the present results, it is tempting to speculate that LAQ824 enhances transcriptional repression by roscovitine, which, like flavopiridol, acts through inhibition of the CDK9/cyclin T complex (42), resulting in pronounced XIAP down-regulation. However, it is important to note that ectopic expression of XIAP did not fully restore clonogenic potential of LAQ824/roscovitine–treated cells, suggesting that XIAP down-regulation may primarily function to accelerate the apoptotic process. Similarly, down-regulation of Mcl-1, an antiapoptotic protein that plays a key role in hematopoietic cell survival (26), seemed to contribute to the striking induction of apoptosis by this regimen as well. Roscovitine-mediated Mcl-1 down-regulation, in contrast to flavopiridol, which also blocks RNA polymerase II CTD phosphorylation (44), represented a caspase-dependent rather than a transcriptional event in our system. Although transcriptional repression customarily has the greatest effect on the expression of short-lived proteins, other factors determining the specificity of gene repression by agents that inhibit CDK9/cyclin T apparatus remain to be fully elucidated.

In summary, the present findings indicate that antileukemic synergism between HDAC and CDKIs can be extended to the novel and potent HDACI LAQ824 as well as to roscovitine, a CDKI that is less pleiotropic in its CDKI actions than flavopiridol. Thus, the combination of two classes of cell cycle inhibitors (i.e., HDACIs and CDKIs), which act via separate mechanisms, may have more generalized applicability as an antileukemic strategy. Despite certain similarities, particularly the induction of mitochondrial injury and disruption of the differentiation process, it is clear that the mechanisms underlying synergism between LAQ824 and roscovitine differ from those involved in flavopiridol/HDACI interactions, including the lack of transcriptional repression of Mcl-1 and p21/CIP1 and the involvement of oxidative damage. Nevertheless, the present findings provide further support for the general strategy of combining CDK and HDACIs as an antileukemic strategy and for extending this approach to include additional clinically relevant compounds. Accordingly, such studies are currently under way.

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Potentiation of the lethality of the histone deacetylase inhibitor LAQ824 by the cyclin-dependent kinase inhibitor roscovitine in human leukemia cells

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