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Roscovitine sensitizes leukemia and lymphoma cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis

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Abstract
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a death ligand with selective antitumor activity. However, many primary tumors are TRAIL resistant. Previous studies reported that roscovitine, a cyclin-dependent kinase inhibitor, sensitized various solid cancer cells to TRAIL. We show that roscovitine and TRAIL demonstrate synergistic cytotoxicity in hematologic malignant cell lines and primary cells. Pretreatment of TRAIL-resistant leukemia cells with roscovitine induced enhanced cleavage of death-inducing signaling complex-bound proximal caspases after exposure to TRAIL. We observed increased levels of both pro- and antiapoptotic BCL-2 proteins at the mitochondria following exposure to roscovitine. These results suggest that roscovitine induces priming of cancer cells for death by binding antiapoptotic BCL-2 proteins to proapoptotic BH3-only proteins at the mitochondria, thereby decreasing the threshold for diverse proapoptotic stimuli. We propose that the mitochondrial priming and enhanced processing of apical caspases represent major molecular mechanisms of roscovitine-induced sensitization to TRAIL in leukemia/lymphoma cells.

Keywords: Roscovitine, TRAIL, synergism, apoptosis, leukemia, lymphoma

Introduction
Intrinsic or acquired drug resistance is one of the main causes of treatment failure in cancer therapy. One of the established mechanisms of drug resistance is evading apoptosis through impairment of apoptotic pathways [1]. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, Apo2L, dulanermin; Genetech) is a cytokine of the TNF family that activates the extrinsic apoptotic pathway through its cognate death receptors DR4 and DR5 [2]. Ligation of TRAIL death receptors leads to formation of the death-inducing signaling complex (DISC), which is, in addition to the ligand bound receptors DR4 and/or DR5, also composed of the core adapter protein FADD and the initiator caspases CASP8 and CASP10 [3,4]. An additional component of the DISC is FLIP, a competitive inhibitor of CASP8/10 binding and activation. Activated initiator caspases cleave and activate the effector caspases 3, 6 and 7, as well as the proapoptotic BCL-2 member BID. Truncated BID (tBID) in turn amplifies the proapoptotic signal through the mitochondrial pathway [5,6]. Since TRAIL was shown to selectively kill tumor cells (including those with mutated TP53) while sparing normal tissues, it has gained much attention as a potentially targeted anticancer agent [7], and has been evaluated in several clinical trials in solid and hematologic malignancies. Unfortunately, a significant number of the primary hematologic malignancies have turned out to be TRAIL resistant. However, a number of studies have continued to document that even these resistant cancer cells could be sensitized to TRAIL by various current and emerging anticancer drugs [8]. Recently, it was reported that glioma, breast cancer and thyroid carcinoma cells can be sensitized to TRAIL-induced apoptosis by the purine analog roscovitine [9–11]. R-roscovitine (seliclib) competes with adenosine triphosphate (ATP) for its binding site on cyclin-dependent kinases (CDKs) [12], and demonstrates selectivity toward CDK1, CDK2, CDK5, CDK7 and CDK9 [13]. Roscovitine-induced blockade of transcription mediated by inhibition of CDK7-dependent phosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II results in a rapid decline of intracytoplasmic levels of short-lived antiapoptotic proteins, particularly MCL-1 [14–16]. Roscovitine has been reported to be a potent in vitro inducer of apoptosis in many primary hematologic malignant cells, including chronic lymphocytic leukemia (CLL) cells.
[17], diffuse large B-cell lymphoma (DLBCL) cells [18], mantle cell lymphoma (MCL) cells [16] and multiple myeloma (MM) cells [15]. To date, roscovitine has been evaluated in several phase I and phase II clinical trials, particularly in non-small cell lung cancer and nasopharyngeal cancer.

In the present study, we assessed and analyzed molecular mechanisms of the cytotoxic synergism between roscovitine and TRAIL using a panel of hematologic malignant cell lines and primary cells, and a mouse xenograft model of human lymphoma. Our results suggest that roscovitine, as a single agent or in combination with TRAIL, might have a role in the therapy of selected hematologic malignancies.

Materials and methods

Cell culture

Cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) with the exception of HBL-2, which was a kind gift of Prof. Dreyling (University of Munich, Germany). Cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% or 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Peripherical blood, bone marrow aspirates or pleural effusions were acquired from patients with diverse hematologic malignancies after obtaining informed consent. Mononuclear cells were purified by Ficoll-Paque (GE Healthcare) density-gradient centrifugation and cultured in IMDM supplemented with 20% FBS. We collected 26 primary samples: nine acute myeloid leukemia (AML), two chronic myeloid leukemia (CML), five MCL, six CLL, one DLBCL, one follicular lymphoma (FL), one marginal zone lymphoma (MZL) and one acute lymphocytic leukemia (ALL).

Reagents

R-roscovitine was kindly provided by Prof. Strnad (Palacky University, Czech Republic) [19]. Recombinant human TRAIL and biotin-labeled recombinant TRAIL were obtained from Apronex Biotechnologies. SuperFasLigand and TNFα were purchased from Enzo Life Sciences. Caspase inhibitors Z-VAD-FMK, Z-IETD-FMK and Z-LEHD-FMK were purchased from R&D. Cycloheximide and actinomycin D were obtained from Sigma-Aldrich, and 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) from Life Technologies. ABT-737 was purchased from Selleck Chemicals. Antibodies used in this study were: anti-Bad, Bid (Santa Cruz Biotechnology), anti-Bcl-2, FADD (BD Biosciences), anti-caspase-10 (MBI International), anti-caspase-8, FLIP (Alexis Biochemicals), anti-Mcl-1, XIAP (BioVision), anti-Bax, Bak, Bel-XL, COX IV, phospho-eIF2α, PUMA (Cell Signaling Technology), anti-RNA polymerase II 8WG16, H5, H14 (Covance), horseradish peroxidase (HRP)-conjugated anti-actin (Santa Cruz Biotechnology), HRP-conjugated anti-mouse, -rabbit or -goat secondary antibodies (Jackson ImmunoResearch Laboratories), and fluorescein isothiocyanate (FITC)-conjugated anti-DR4 and -DR5 (Exbio).

Treatment of cell lines and primary cells for apoptosis assay

Cells were incubated with various concentrations of either roscovitine (R) or TRAIL (T) or their combination (R + T) for 24 h and 48 h. A similar single or combined regimen was used also for K-562 and RAMOS cells treated with TNFα, FASL, cycloheximide, actinomycin D, ABT-737 and TRAIL for 24 or 48 h. In caspase inhibition experiments, K-562 and RAMOS cells were pretreated with 100 μM Z-VAD-FMK, Z-IETD-FMK or Z-LEHD-FMK for 1 h before exposure to the particular drugs. In experiments with roscovitine pretreatment, K-562 cells were incubated with roscovitine for 3, 6, 12 and 24 h. Subsequently, roscovitine was washed out, TRAIL was added and apoptosis was measured 24 h after the addition of TRAIL.

Analysis of apoptosis

Cells were incubated with annexin V-FITC (Apronex Biotechnologies) in the dark at room temperature for 15 min and analyzed by flow cytometry (FACSCanto II cytometer; Becton Dickinson). The percentage of apoptosis (annexin-positive cells) was calculated as follows: apoptosis (%) = treatment (% apopt) – control (% apopt)/100 – control (% apopt).

Proliferation assays

To measure the effect of R, T and the combination of R + T on proliferation, we used a Quick Cell Proliferation Assay Kit (BioVision) according to the manufacturer’s instructions. Briefly, we incubated 20 000 cells with each drug alone or in combination for various time periods (0–96 h) and measured absorbance of the samples after 2 h incubation with WST-8 reagent.

Cell-mediated cytotoxicity assay

Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy volunteer by Ficoll–Paque (GE Healthcare) density-gradient centrifugation. Prior to use, PBMCs were stained by 1.5 μM CFSE. K-562 and RAMOS cells were treated for 24 h with 40 μM or 10 μM roscovitine, respectively. After washing, cell lines were incubated with CFSE-labeled PBMCs at a target to effector ratio of 1:9 for 24 h. Apoptosis was quantified in the CSF-negative population using annexin V-phycocerythrin (PE) staining.

Cell surface expression of TRAIL receptors

K-562 and RAMOS cells were treated with roscovitine for 24 h and 48 h, stained with FITC-conjugated anti-DR4 and -DR5 antibodies and analyzed by flow cytometry (FACSCanto II; Becton Dickinson). Untreated cells stained with the secondary antibodies and isotype controls served as controls.

DISC immunoprecipitation by streptavidin–agarose beads

Immunoprecipitation of the DISC was performed as described earlier [20]. Briefly, K-562 cells were left untreated or treated with 40 μM roscovitine for 12 h and 24 h. After incubation with 1 μg/mL of biotinylated TRAIL for 0, 15 and 40 min, cell lysis was performed on ice and lysates mixed with streptavidin–agarose beads (Pierce Biotechnology). Protein complexes were eluted from the beads by the addition of sodium dodecyl sulfate (SDS) sample buffer and heating at 95 °C for 5 min, and analyzed by Western blotting for the levels and status of the DISC components (CASp8, CASp10, FADD and FLIP).
Real-time RT-qPCR analysis
Total RNA was extracted from K-562 cells treated with 40 μM roscovitine for 1.5, 3, 6, 12 and 24 h by RNeasy Mini Kit (Qiagen), and reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was run for 40 cycles (95 °C for 10 s, 60 °C for 30 s) on a 7900 HT SDS PCR cycler (Applied Biosystems) using a SYBR Green-based kit (Bio-Rad Laboratories). Raw data were expressed as CT values. Data were normalized to the expression of actin and untreated cells were used as a control. Data were evaluated using Mev software (www.tm4.org/mev).

Whole-genome gene expression profiling and data analysis
Total cellular RNA was isolated from K-562 cells treated with 40 μM roscovitine for 1.5, 6 and 24 h as described above. Quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technology). Extracted RNA was amplified using an Illumina RNA Amplification Kit (Ambion). Amplified RNA was hybridized to the Illumina HumanRef-8 BeadChip (Illumina). Subsequent data analysis was performed in R-software, mainly using the limma package from Bioconductor (http://bioconductor.org). Multiple testing correction was performed using the Benjamini and Hochberg method [21]. The filtered group of genes with fold change greater/ lower than ±2 and adjusted p-value < 0.05 were annotated and arranged into biologically relevant categories using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) [22].

Total and mitochondrial protein extraction
Total cellular proteins were extracted from K-562 cells untreated or treated with 40 μM roscovitine for 1.5, 3, 6, 12 and 24 h by lysis in RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich), phenylmethylsulfonylfluoride (PMSF) and dithiothreitol (DTT). For mitochondrial extracts, K-562 cells treated with 40 μM roscovitine for 1.5, 3, 6, 12 and 24 h were incubated in a lysis buffer (250 mM sucrose, 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] pH 7.4, 10 mM KCl, 1.5 mM (250 mM sucrose, 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA [ethylene diamine tetraacetic acid], 1 mM EGTA [ethylene glycol tetraacetic acid]), passed through a 25G needle and left on ice for 20 min. The supernatant obtained after centrifugation (5 min at 3000 rpm) was centrifuged again for 30 min at 8000 rpm and the mitochondrial pellet was resuspended in RIPA buffer. Samples were used immediately or stored at −80 °C.

Western blotting
Samples were mixed with Laemmli sample buffer (Bio-Rad Laboratories) and boiled for 5 min. After separation by SDS-polyacrylamide gel electrophoresis (PAGE), proteins were transferred to an Immun-Blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories). Membranes were blocked for 1 h in 2% skim milk and incubated with appropriate primary antibodies overnight at 4 °C. Subsequently, the membranes were washed and incubated with HRP-conjugated anti-mouse, -rabbit or -goat secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Proteins were visualized using a Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). As a loading control we used HRP-conjugated anti-actin antibody.

Lentivirus production and infection
pLKO.1 plasmids expressing shRNA against BCL-XL, MCL-1 and BCL-2 were purchased from Sigma and used for preparation of the recombinant lentiviruses in HEK293T cells. Viruses were concentrated from supernatant by the PEG-it Virus Precipitation Solution (System Biosciences) and frozen in aliquots at −80 °C. K-562 cells were then infected with appropriate recombinant lentiviruses at a multiplicity of infection (MOI) of approximately 10, and the transduced cells were selected 2 days post-infection with medium containing puromycin (3 μg/mL) for 4 days. Cells were analyzed for the expression of particular proteins by Western blot.

Subcutaneous xenograft studies
The studies were approved by the institutional Animal Care and Use Committee. Female immunodeficient NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl/Sif</sup> mice (Jackson Laboratory) were maintained in individually ventilated cages. RAMOS and HBL-2 cells were harvested, suspended in phosphate buffered saline (PBS) and injected (10 x 10<sup>6</sup>/mouse) subcutaneously into the left dorsal flank of 8- to 12-week-old mice. When tumors reached 500–1000 mm<sup>3</sup>, animals were randomized into four groups and treated. Roscovitine (12.5 mg/mouse) was given by oral gavage as a slurry in 50 mM HCl in a volume of 100 μL. TRAIL (250 μg/mouse) was given intraperitoneally in a volume of 300 μL, and a control group was injected with vehicle (PBS) intraperitoneally. The treatment schedule for combined treatment was: two doses of roscovitine at 12 h intervals on day 1, followed by a single dose of TRAIL on day 2, followed by a second treatment cycle on day 4. For single treatments, roscovitine and TRAIL were dosed similarly to the combination. When tumors in the control group reached 2.5–3 cm in diameter, animals were sacrificed and tumors weighed.

Statistical analysis
Data were analyzed using GraphPad Software. Values represent the mean ± SD of at least three independent experiments. Differences between experimental groups were determined by Student’s t-test. Synergistic, additive or antagonistic effects were calculated according to the formulae: SF<sub>R+T</sub> ≤ SF<sub>R</sub> × SF<sub>T</sub>; synergism; SF<sub>R+T</sub> > SF<sub>R</sub> × SF<sub>T</sub>; antagonism; SF<sub>R+T</sub> = SF<sub>R</sub> × SF<sub>T</sub>-1; additive effect, where SF is the surviving fraction after treatment with roscovitine, TRAIL or the combination.

Results
Roscovitine sensitizes human leukemia and lymphoma cells to TRAIL-induced apoptosis
Twenty-one leukemia/lymphoma cell lines and 26 primary cell samples obtained from patients with diverse hematologic malignancies were analyzed. Treatment with roscovitine induced apoptosis of the tested cell lines and primary cells in a dose-dependent manner. Only four cell lines, K-562, THP-1, SC-1 and SU-DHL-1, were inherently TRAIL-resistant,
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Figure 1. Roscovitine sensitizes leukemia and lymphoma cells to TRAIL, TNFα and FASL-induced apoptosis and enhances cell-mediated cytotoxicity.

(A, B) Percentage of annexin V–FITC-positive, apoptotic cells induced by 24 h exposure of cell lines (A) or primary cells (B) to roscovitine (R), TRAIL (T) and combination of both (R + T). Dotted upper part represents synergism, i.e. percentage of apoptotic cells above the sum of both monotherapies. (C) K-562 and RAMOS cell lines were treated with roscovitine (R), TRAIL (T) and combination (R + T) of both (concentrations as indicated) and proliferation measured as described in “Materials and methods.” (D) Percentage of apoptotic K-562 or RAMOS cells after their 24 h treatment with TRAIL (T), TNFα, FASL and combinations with roscovitine (R). (E) After pretreatment of K-562 and RAMOS cells with roscovitine for 24 h, peripheral blood mononuclear cells (PBMCs) from healthy donor stained with CFSE were added and apoptosis measured after 24 h in CFSE unstained population. Columns represent means and error bars standard deviations of three independent experiments.

with less than 10% apoptosis after 24 h exposure to 1000 ng/mL TRAIL. As predicted, primary cells were less sensitive to TRAIL-induced apoptosis. For the study of drug synergism between roscovitine and TRAIL we decided to use a concentration of roscovitine that induced approximately 20–30% apoptotic death in the target cell population after 24–48 h. Similarly, low-toxic doses of TRAIL in the range between 0.25 and 1000 ng/mL were used to assess drug synergism in the cell lines. Primary cells were exposed to a single concentration of 1000 ng/mL TRAIL with increasing doses of roscovitine (5, 10, 15, 20, 30, 40 and 60 μM). Roscovitine used at the low-toxic dose augmented TRAIL-induced apoptosis above the additive effect in most tested cell lines and primary leukemia and lymphoma cells [Figures 1(A) and 1(B)]. Importantly, roscovitine was able to sensitize TRAIL-resistant cell lines K-562, THP-1, SC-1 and SU-DHL-1 and primary cells to TRAIL-induced apoptosis. The combination of roscovitine and TRAIL synergistically suppressed proliferation of tested cell lines with the exception of SC-1 [Figure 1(C) and Supplementary Figure 1 available online at http://informahealthcare.com/lal/doi/10.3109/10428194.2012.710331]. Molecular mechanisms of the cytotoxic synergism between roscovitine and TRAIL were subsequently analyzed using TRAIL-resistant K-562 cells of myeloid origin and TRAIL-sensitive RAMOS cells of lymphoid origin. Both K-562 and RAMOS cells demonstrated significant cytotoxic synergism between roscovitine and TRAIL despite the fact that they both harbor a homozygous p53 mutation.

Roscovitine sensitizes leukemia and lymphoma cells to TNFα- and FASL-induced apoptosis, and enhances cytotoxic cell-mediated cytotoxicity

Roscovitine sensitized K-562 and RAMOS cells not only to TRAIL but also to other death ligands. Specifically, K-562 cells were more sensitive to TNFα-induced apoptosis, while RAMOS cells were more sensitive to FASL-induced apoptosis when pretreated with a low-toxic dose of roscovitine [Figure 1(D)]. As the death ligands, particularly FASL, are involved in cell-mediated cytotoxicity, we considered whether roscovitine could augment this type of cell death. Pretreatment of RAMOS cells with roscovitine indeed significantly enhanced apoptosis induced by PBMCs from a healthy donor [Figure 1(E)].

Roscovitine induces long-term proapoptotic changes of DISC

Suppression of TRAIL-induced apoptosis in roscovitine-treated cells by caspase inhibitors suggested that alterations of both extrinsic and intrinsic apoptotic pathways might be involved in the observed cytotoxic synergism between roscovitine and TRAIL [Figure 2(A)]. To unveil potential changes in the extrinsic apoptotic pathway, we measured the cell surface expression of TRAIL receptors, and analyzed formation of the DISC in roscovitine-treated cells. Cell surface expression of TRAIL death receptors on K-562 and RAMOS cells was not affected by exposure to roscovitine for 24 h [Figure 2(B)] or 48 h. Similarly, the expression of death receptors was unchanged (data not shown). Immunoprecipitation of DISC...
Roscovitine inhibits transcription and translation

To reveal other potential mechanisms that might contribute to the observed drug synergism between roscovitine and TRAIL, we studied mRNA expression of selected pro- and antiapoptotic genes by real-time RT-PCR, 1.5, 3, 6, 12 and 24 h after exposure of K-562 cells to roscovitine. Transcription of all tested genes showed gradual down-regulation, with a nadir at 12 h after exposure to roscovitine. At 24 h the level of expression of most tested genes returned to that of untreated controls or was even higher [Figure 3(A)]. Based on the results obtained from real-time RT-PCR we decided to analyze gene expression of K-562 cells exposed to roscovitine using genome-wide Illumina HumanRef-8 arrays. We identified 213, 859 and 430 genes down-regulated by more than two-fold, and six, 133 and 362 genes up-regulated by more than two-fold following exposure to roscovitine for 1.5, 6 and 24 h, respectively [Figure 3(B)]. The microarray data clearly confirmed a significant inhibitory effect of roscovitine on gene transcription. Supplementary Figure 2 available online at http://informahealthcare.com/lal/doi/10.3109/10428194.2012.710331 shows expression patterns of selected gene functional groups. Previous studies reported that roscovitine inhibited transcription by abrogation of CDK7-mediated phosphorylation of the CTD of RNA polymerase II [16]. Hence, we analyzed protein expression of RNA polymerase II and its phosphorylation status at the indicated time-points by Western blotting. Treatment of K-562 cells with roscovitine was associated with decreased phosphorylation of serine 2 and serine 5 of the CTD of RNA polymerase II [Figure 3(C)]. Because roscovitine influences the expression of genes involved in translation and protein folding as well, we tested the phosphorylation status of eukaryotic initiation factor-2 alpha (eIF-2α) in K-562 cells after treatment with roscovitine.
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Figure 4. BCL-2 family proteins and mitochondria play an important role in proapoptotic effects of roscovitine in K-562 cells. (A) Western blotting showing protein expression of several pro- and antiapoptotic regulators 1.5, 3, 6, 12 and 24 h after treatment with roscovitine. (B) K-562 cells with down-regulated expression of MCL-1 or BCL-XL were treated with TRAIL or roscovitine for 24 h and induction of their apoptosis was determined by annexin V–FITC staining and flow cytometry. (C) K-562 cells were pretreated with 40 μM roscovitine for 3, 6, 12 or 24 h (R3h, R6h, R12h and R24h, respectively), then cells were washed and TRAIL was added to fresh medium at final concentration 1000 ng/mL. Percentage of apoptotic cells was determined by annexin V–FITC staining and flow cytometry after 24 h of TRAIL exposure. Further, K-562 cells were exposed to roscovitine for either 12 or 24 h, and then after extensive washing cells were incubated in medium without roscovitine for 0, 3, 6, 12 and 24 h (T0, T3, T6, T12 and T24, respectively). At each time point TRAIL was added and 24 h later percentage of apoptotic cells was determined as above. Columns represent means and error bars standard deviations of three independent experiments.

Figure 3(D) demonstrates that treatment with roscovitine induced phosphorylation of eIF-2α after 12 and 24 h, suggesting an inhibition of translation. As expected, treatment with actinomycin D (inhibitor of transcription) and cycloheximide (inhibitor of translation) sensitized K-562 and RAMOS cells to TRAIL-induced apoptosis (Supplementary Figure 3 available online at http://informahealthcare.com/lal doi/10.3109/10428194.2012.710331).

Roscovitine causes gradual down-regulation of cytoplasmic MCL-1 and BCL-XL followed by up-regulation of both proteins at 24 h after exposure to roscovitine

As treatment with roscovitine resulted in the down-regulation of many transcripts, we decided to analyze protein expression of selected pro- and antiapoptotic molecules by Western blotting, 1.5, 3, 6, 12 and 24 h after exposure to roscovitine [Figure 4(A)]. Similar to real-time RT-PCR and microarray data [Figure 3(A)], we observed a gradual down-regulation of some of the tested proteins (e.g. BCL-XL, MCL-1 or PUMA) during the first 12 h after exposure to roscovitine, while at 24 h these proteins were all up-regulated compared to untreated cells [Figure 4(A)]. In contrast, expression of other proteins (e.g. BAK, BAD or BCL-2) remained virtually unchanged in response to roscovitine, despite the fact that they had demonstrated similar changes of mRNA expression (i.e. down-regulation during 12 h, and up-regulation after 24 h) [Figure 4(A)]. Using short hairpin (sh) RNA-mediated gene expression silencing, we confirmed that down-regulation of both MCL-1 and BCL-XL in K-562 cells indeed sensitized the cells to TRAIL-induced apoptosis. Interestingly, down-regulation of BCL-XL sensitized K-562 cells also to roscovitine-induced cell death [Figure 4(B)]. In contrast, shRNA-mediated down-regulation of BCL-2 protein, the expression of which did not change in response to roso-
vitine, did not impact TRAIL-induced apoptosis (data not shown). From the analyzed proapoptotic BCL-2 proteins we observed moderate up-regulation of BAD, BAK, and PUMA\(\alpha\) at 1.5 h, and up-regulation of PUMA\(\alpha\) at 24 h.

**Roscovitine-induced sensitization to TRAIL is dependent on length of cell pre-incubation with roscovitine**

Co-treatment of malignant hematopoietic cells with cytotoxic doses of roscovitine and TRAIL significantly enhanced the cytotoxic activity in a wide range of hematologic malignant cell lines and primary leukemia and lymphoma cells. To elucidate which changes in protein expression are relevant to roscovitine-induced sensitization to TRAIL, we studied the dependence of TRAIL sensitization on the length of exposure of K-562 cells to roscovitine. We found that at least 12 h pretreatment with roscovitine was required to induce sensitization to TRAIL, but this sensitization persisted only for 6 h after washing off the drug. In contrast, cells treated with roscovitine for 24 h remained sensitive to TRAIL even 24 h after roscovitine removal, indicating strong establishment of long-lasting changes in cell physiology in favor of TRAIL-induced pro-death signaling [Figure 4(C)].

**Roscovitine induces recruitment of both pro- and antiapoptotic BCL-2 proteins to mitochondria**

Most of the BCL-2 family members exert their apoptosis-regulating roles at the mitochondria. As the protein expression levels obtained from whole cell lysates might not reliably reflect potential compartment-specific changes of BCL-2 family protein levels at the mitochondria, we analyzed the protein expression of selected apoptosis-regulating molecules using mitochondria isolates of K-562 cells treated with roscovitine for 3, 6, 12 and 24 h. Interestingly, unlike the data obtained from the whole cell lysates, treatment with roscovitine resulted in a gradual increase in mitochondrial levels of all tested pro- and antiapoptotic proteins, with the only exception of PUMA\(\beta\) [Figure 5(A)]. These results imply that antiapoptotic BCL-2 proteins might sequester proapoptotic BCL-2 proteins. K-562 cells treated with roscovitine were more sensitive to ABT-737, an inhibitor of BCL-2, BCL-XL and BCL-W [Figure 5(B)].

**Roscovitine and TRAIL synergistically inhibit growth of leukemia and lymphoma xenografts in immunodeficient mice**

K-562 cells engraft only inconsistently in immunodeficient mice, the reason why these leukemia cells could not be used for xenograft experiments. RAMOS and HBL-2 subcutaneously xenografted mice were treated with roscovitine and TRAIL, as single agents or in combination. Combined treatment of roscovitine and TRAIL significantly suppressed the growth of both RAMOS and HBL-2 tumors compared to single-agent approaches (Figure 6).

**Discussion**

In the present study we analyzed molecular mechanisms responsible for the cytotoxic synergism between roscovitine and TRAIL. Exposure of K-562 and RAMOS cells to roscovitine did not alter the cell surface expression of TRAIL receptors, suggesting that roscovitine-induced proapoptotic changes should be localized downstream. We have demonstrated that activation of the initiator caspases CASP8/10 was significantly more effective in roscovitine-exposed compared to control cells. This increased cleavage of CASP8/10 might be explained at least partially by decreased recruitment of the caspase inhibitor FLIP to the DISC. The precise molecular mechanisms that drive recruitment of initiator caspases to the DISC in response to roscovitine remains to be elucidated. One of the possible explanations could be enhanced stabilization/aggregation of the initiator caspases mediated by their ubiquitination by Cullin-3-based E3 ligase [23]. Besides TRAIL, exposure of K-562 and RAMOS cells to roscovitine also increased apoptosis induced by TNF\(\alpha\) and FASL, respectively. As cytotoxic T and natural killer (NK) cells have
levels following exposure of K-562 cells to roscovitine. Our previous data, we found decreased phosphorylation of the CTD of RNA polymerase II, and a decline in cellular mRNA of these short-lived proteins \[14,15,25\]. In agreement with polyoma II through inhibition of CDK7 \[24\] was considered whether roscovitine could potentiate cytotoxic cell-mediated killing of malignant cells. Indeed, pretreatment of K-562 and RAMOS cells with roscovitine enhanced the cell apoptosis induced by peripheral blood mononuclear cells isolated from healthy donors. Roscovitine thus appears to augment antitumor surveillance mediated by cytotoxic cells of the innate immune system.

As expected, inhibition of CASP8 suppressed TRAIL-induced apoptosis in roscovitine-treated cells. Interestingly, inhibition of CASP9 had a similar effect, implying requirement for augmentation of the apoptotic signal through the mitochondria. Previous studies ascribed TRAIL-sensitizing effects of roscovitine to the observed down-regulation of several antiapoptotic proteins, namely MCL-1, XIAP and survivin [9,10]. Roscovitine-mediated inhibition of transcription by decreased phosphorylation of the CTD of RNA polymerase II through inhibition of CDK7 [24] was considered the mechanism responsible for the down-regulation of these short-lived proteins [14,15,25]. In agreement with previous data, we found decreased phosphorylation of the CTD of RNA polymerase II, and a decline in cellular mRNA levels following exposure of K-562 cells to roscovitine. Our microarray data confirmed the overall inhibitory effect of roscovitine on transcription. As other established inhibitors of transcription and translation (namely actinomycin D and cycloheximide) also sensitize K-562 cells to TRAIL, we assume that the inhibition of transcription and/or translation could represent evolutionary old molecular triggers that would generally sensitize cancer cells to death ligand-induced apoptosis.

Surprisingly, the gradual mRNA and protein down-regulation of MCL-1 and BCL-XL detected during the first 12 h after exposure to roscovitine was followed by marked up-regulation of these molecules 12 h later. As we have shown, at least 12 h pretreatment with roscovitine appeared essential to induce sensitization of K-562 cells to TRAIL-induced apoptosis. The roscovitine-induced TRAIL-sensitizing effect, however, persisted only for 6 h. For long-lasting sensitization to TRAIL, 24 h pretreatment with roscovitine was indispensable. Twenty-four hour exposure to roscovitine, however, was associated with increased protein levels of MCL-1 and BCL-XL compared to untreated cells. Since most of the BCL-2 family members exert their apoptosis-regulating roles at the mitochondria, we analyzed mitochondrial lysates of K-562 cells treated with roscovitine and found a gradual up-regulation of all tested pro- and antiapoptotic BCL-2 family members, with the only exception of PUMAβ. These results are in contrast with previous reports where down-regulation of antiapoptotic proteins was proposed as a sensitizing mechanism. None of these studies, however, determined the expression of these proteins on a mitochondrial level. Based on the concomitant up-regulation of both pro- and antiapoptotic proteins on the mitochondria following exposure to roscovitine, we suggest that roscovitine induces mitochondrial priming for death. As described in several previous reports [26,27], survival of the cells primed for death depends on sufficient expression of antiapoptotic BCL-2 family proteins that sequester proapoptotic BH3-only proteins. Inhibition of the antiapoptotic proteins in primed cells results in induction of apoptosis. K-562 cells treated with roscovitine were more sensitive to ABT-737, an inhibitor of BCL-2, BCL-XL and BCL-W [Figure 5(B)]. We hypothesize that this occurs by displacement of proapoptotic BH3-only proteins from antiapoptotic BCL-2 family proteins, and subsequent activation of Bak/Bax. Meng et al. found increased binding of PUMA and BIM to MCL-1 and tBid to BCL-XL in K-562 cells following treatment with TRAIL [28]. Thus, TRAIL-induced redistribution of BCL-2 family members might trigger apoptosis in roscovitine-treated cells [Figure 5(C)].

In conclusion, we demonstrate that roscovitine sensitizes leukemia and lymphoma cells to TRAIL and other death ligands and potentiates cell-mediated cytotoxicity. Proapoptotic changes at the DISC and mitochondrial priming for death represent, in our opinion, two major complex molecular mechanisms responsible for the observed roscovitine-induced sensitization to TRAIL and potentially also to cell-mediated cytotoxicity. Roscovitine, as a single agent or in combination with TRAIL, might have a role in the experimental treatment of selected hematologic malignancies. Combinations of roscovitine with BCL-2 inhibitors require further evaluation.

![Figure 6. Roscovitine and TRAIL synergistically inhibit growth of subcutaneous lymphoma xenografts. Immune-deficient NOD.Cg-Prkdcre;129S7-Il2rgtm1Wjl/SzJ mice were subcutaneously injected with RAMOS or HBL-2 cell lines. After tumors developed, mice were randomized and treated with two cycles of roscovitine (rosco), TRAIL (TRAIL) or with their combination (R + T) as described in "Materials and methods." Control mice (ctrl) were injected with PBS. Dots represent weight of individual tumors and horizontal lines are plotted at the means.](Image 69x406 to 282x739)
Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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References


Supplementary material available online

Supplementary Figures 1–3 showing additional results to be found online at http://informahealthcare.com/doi/abs/10.3109/10420194.2012.710331.