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RESENZITIZACE LEUKEMICKÝCH A LYMFOMOVÝCH BUNĚK K TRAILEM INDUKOVANÉ APOPTÓZE

RESENSITIZATION OF LEUKEMIA AND LYMPHOMA CELLS TO TRAIL-INDUCED APOPTOSIS

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ABSTRACT (Czech)

Apoptóza představuje přirozenou bariéru proti rozvoji nádorových onemocnění a rezistence nádorových buněk k apoptóze je jednou ze základních vlastností získaných během tumorigeneze. Protože většina v současné době používaných cytostatik vyvolává zánik nádorových buněk aktivací vnitřní apoptotické dráhy, je porucha aktivace vnitřní apoptotické dráhy spojena se selháním terapie. Cílená aktivace zevní apoptotické dráhy umožňuje indukci apoptózy i u nádorových buněk rezistentních k cytostatikům. TRAIL je cytokin patřící do rodiny TNFα, který specificky indukuje zevní apoptotickou dráhu v nádorových buňkách a je netoxický vůči normálním buňkám. Vzhledem k těmto vlastnostem představuje TRAIL slibnou protinádorovou látku. Velká část primárních nádorů je však vůči TRAILu rezistentní. Ve snaze o překonání rezistence nádorových buněk na TRAIL byla identifikována celá řada látek, které zvyšují citlivost nádorových buněk na TRAIL. Předchozí studie ukazují, že roskovitin, inhibitor cyklin dependentních kináz, zvyšuje citlivost solidních nádorů na TRAIL.

V této práci jsme studovali citlivost různých hematologických malignit k TRAILem indukované apoptóze. Následně jsme zjišťovali schopnost roskovitinu zvýšit citlivost leukemických a lymfomových buněk na TRAIL *in vitro* a *in vivo* na myším modelu lidského lymfomu. Nakonec jsme analyzovali molekulární mechanismy cytotoxického synergismu mezi roskovitinem a TRAILem

Výsledky ukazují, že roskovitin a TRAIL působí synergisticky v indukci apoptózy jak u hematologických buněčných linií, tak u primárních buněk. Léčba leukemických a lymfomových buněk roskovitinem vyvolá po jejich následném vystavení TRAILu zvýšené štěpení apikálních kaspáz. Dále jsme po léčbě roskovitinem pozorovali akumulaci pro- i anti- apoptotických BCL-2 proteinů v mitochondriích. Tyto výsledky naznačují, že roskovitin zvýšením akumulace BCL-2 proteinů v mitochondriích snižuje práh pro různé proapoptotické podněty. Předpokládáme, že zvýšené štěpení apikálních kaspáz a mitochondriální akumulace BCL-2 proteinů představují hlavní molekulární mechanizmy roskovitinem navozené senzitizace k TRAILem indukované apoptóze v leukemických / lymfomových buňkách.

ABSTRACT

Apoptosis serves as a natural barrier to cancer development, and resistance to apoptosis represents one of the key capabilities acquired during tumor development or progression. As most of the currently used cytotoxic drugs initiate tumor cell death by direct or indirect triggering of the intrinsic apoptotic pathway, impairment of the intrinsic pathway is associated with therapy failure. Targeting of the death receptors, however, enables induction of apoptosis even in chemotherapy resistant cancer cells. TRAIL is a death ligand belonging to the TNFα superfamily that specifically kills tumor cells while sparing healthy tissues. Unfortunately, many primary tumors have been shown to be TRAIL resistant. In attempt to overcome TRAIL resistance a wide array of agents have been shown to sensitize tumor cells to TRAIL. Previous studies reported that roscovitine, a cyclin-dependent kinase inhibitor, sensitized various solid cancer cells to TRAIL.

In this study we analyzed the sensitivity of diverse hematologic malignancies to TRAIL-induced apoptosis and measured the ability of roscovitine to potentiate TRAIL-induced apoptosis or to sensitize TRAIL-resistant tumor cells to TRAIL both *in vitro* and *in vivo* using a mouse xenograft model of human lymphoma. In addition, we analyzed molecular mechanisms responsible for the cytotoxic synergism between roscovitine and TRAIL.

We showed that roscovitine and TRAIL demonstrated synergistic cytotoxicity in hematologic malignant cell lines and primary cells. Pretreatment of TRAIL resistant leukemia and lymphoma 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 anti-apoptotic 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.

INTRODUCTION

Apoptosis

Apoptosis is a form of programmed cell death that has evolved in multicellular organisms in order to eliminate unwanted, damaged or potentially hazardous cells.

Extrinsic apoptotic pathway is induced by death ligands bound to their cognate death receptors. The most studied and best characterised death ligands are tumor necrosis factor α (TNFα), FAS ligand (FASL), and TNF-related apoptosis inducing ligand (TRAIL). Ligation of death receptors by death ligands leads to the formation of the death-inducing signaling complex (DISC), composed of the core adapter protein FADD, and the initiator caspases -8 and -10 [1-2]. DISC assembly enables proximity-induced homodimerization and activation of the initiator caspases [3].

Intrinsic (mitochondrial) apoptotic pathway is initiated inside the cell in response to various stimuli, such as loss of growth signals during development or sever cell stress (DNA damage, growth factor deprivation, oncogene activation, microtubule disruption) [4]. This pathway is controlled by interactions among members of the BCL-2 protein family that regulate mitochondrial outer membrane permeabilization (MOMP). MOMP results in the release of cytochrome c and other proapoptotic molecules from mitochondria. Subsequently, cytochrome c enables formation of the multiprotein complex apoptosome followed by activation of initiator caspase-9 [5-7].

The connection between extrinsic and intrinsic apoptotic pathways is mediated by proapoptotic BCL-2 family member BID that is activated by caspase-8 mediated cleavage.

Antiapoptotic members of the BCL-2 family (BCL-2, MCL-1, BCL-XL, BCL-W, BFL1, BCL-B) promote cell survival by binding of proapoptotic BCL-2 proteins. *Proapoptotic BCL-2 family members* can be divided into multi-domain proapoptotic proteins sharing BH1-3 domains and so called BH3-only proteins containing only BH3 domain. Upon activation, the multi-domain proapoptotic proteins (Bak, Bax, Bok) are thought to mediate MOMP by oligomerization and formation of pores within MOM. BH3-only proteins (BID, BAD, BIK, BIM, PUMA, NOXA, BMF, HRK) function as molecular signals corresponding to a plethora of stress impulses from the environment or from within the cell.

TRAIL

TRAIL (Apo2L, dulanermin) is a member of the TNF ligand family. It is a transmembrane glycoprotein that can be cleaved to form soluble ligand [8-9].

To date, five receptors for TRAIL have been identified - death receptor 4 (DR4), death receptor 5 (DR5), decoy receptor 1 (DcR1), decoy receptor 2 (DcR2), and osteoprotegerin (OPG) [10-13]. DR4 and DR5 are characterized by the intracellular death domain (DD) that is essential for transmission of death signal upon binding of TRAIL. Decoy receptors, and OPG are unable to transmit death signal and act as competitive inhibitors of death receptors for TRAIL binding.

The physiological function of endogenous TRAIL is not fully defined. The cytotoxic activity of TRAIL against tumor cells and its expression on the cells of immune system suggest the role of TRAIL in immune tumor surveillance. Besides its involvement in antitumor surveillance, TRAIL was implicated in innate immunity against virus infection and in modulating an autoimmune response.

TRAIL as therapeutic agent

Since its discovery, TRAIL has been investigated as a potential anti-cancer agent. Preclinical studies have shown that TRAIL induces apoptosis in a wide range of tumor cell lines and primary cells *in vitro* as well as *in vivo* in xenograft animal tumor models [8-9, 14-17]. Unlike TNFα and FASL TRAIL proved to be non-toxic for normal untransformed cells. Despite promising results of preclinical and phase I studies combination of rhTRAIL with paclitaxel, carboplatin, and bevacizumab in patients with advanced non-small-cell lung cancer did not improved outcome [18]. Similarly, addition of rhTRAIL to anti-CD20 antibody rituximab in patients with low grade non-Hodgkin's lymphomas seems to have limited efficiency (www.clinicaltrials.gov). Considering efficacy of rhTRAIL in clinical trials it is worth noting that its dose-limiting toxicity has not been reached and thus the dose of rhTRAIL used in clinical trials could be underestimated. The other possible issue that could reduce the effectiveness of rhTRAIL is primary or acquired resistance to TRAIL-induced apoptosis.

Mechanisms of resistance to TRAIL-induced apoptosis

Many tumor cells are constitutively TRAIL resistant and originally TRAIL sensitive cells can acquire resistance under selective pressure of TRAIL. A variety of mechanisms that mediate TRAIL resistance have been described so far, including impairments of death receptor signaling and mitochondrial apoptotic pathway.

Death receptors and TRAIL resistance

The expression of death and decoy receptors, their posttranslational modifications (O-glycosylation, palmitoylation), and localization (lipid rafts, endocytosis) can influence TRAIL sensitivity [19-24]. Dysfunction of other DISC components (FADD deficiency, caspase-8 deficiency) can also lead to TRAIL resistance [25-26].

Mitochondrial pathway and TRAIL resistance

Although TRAIL-induced apoptosis is initiated through the extrinsic apoptotic pathway, several studies provided evidence that at least in some cell types TRAIL-induced apoptosis is dependent on the mitochondrial pathway. Human colon cancer cells and leukemic cells deficient in Bax are TRAIL resistant despite the expression of Bak [27-31]. Bax dependency of TRAIL induced apoptosis in cancer cells and inability of Bak to induce apoptosis can be explained by selective interactions among BCL-2 family members. Deregulation of BCL-2 family is a common finding in tumor cells [30, 32-34].

Inhibitors of apoptosis (IAP) are proteins that can inhibit caspases by binding to their active sites. The activity of IAPs can be blocked by Smac/Diablo, a mitochondrial protein that is released into the cytosol during apoptosis [35-36]. High expression of IAPs has been shown to confer resistance of prostate cancer cells to TRAIL [37-38]. Resistance of melanoma cell lines to TRAIL was associated with reduced release of Smac/Diablo from mitochondria to cytosol [39].

HYPOTHESIS AND THE AIMS OF THE STUDY

Although TRAIL activates the extrinsic apoptotic pathway through death receptors, the amplification of proapoptotic signal through the mitochondrial pathway may be required to induce apoptotic cell death [40].

Recently, it has been reported that TRAIL resistant glioma, breast cancer, and thyroid carcinoma cells can be sensitized to TRAIL-induced apoptosis by roscovitine, an inhibitor of CDK [41-43]. Previous studies ascribed TRAIL-sensitizing effect of roscovitine to the downregulation of several antiapoptotic proteins, namely MCL-1, XIAP and survivin [41-42]. Roscovitine-mediated inhibition of transcription by decreased phosphorylation of CTD of RNA polymerase II through inhibition of CDK7 [44] was considered as a mechanism responsible for the downregulation of these short-lived proteins [45-47]. Roscovitine was also reported as a potent *in vitro* inducer of apoptosis in many primary hematologic malignant cells, including chronic lymphocytic leukemia (CLL) [48], diffuse large B-cell lymphoma (DLBCL) [49], mantle cell lymphoma (MCL) [50] and multiple myeloma (MM) cells [45]. However, the effect of roscovitine on the sensitization of hematologic malignancies to TRAIL-induced apoptosis has not been studied.

Based on the previous reports we hypothesize that the alteration of intrinsic apoptotic pathway in leukemia and lymphoma cells results in the resistance to TRAIL-induced apoptosis. Therefore, roscovitine will sensitize these cells to TRAIL-induced apoptosis by interfering with the expression of BCL-2 family proteins.

We set these specific aims, the fulfillment of which is the subject of this thesis:

- to determine the sensitivity of leukemia and lymphoma cell lines and primary cells to TRAIL-induced apoptosis

- to measure the cytotoxic effect of combined *in vitro* treatment of TRAIL and an inhibitor of cyclin dependent kinases roscovitne in leukemia and lymphoma cell lines and primary cells

- to identify molecular mechanisms responsible for the roscovitine-mediated sensitization to TRAIL-induced apoptosis

- to determine the cytotoxic effect of combined treatment with TRAIL and roscovitine *in vivo* using a mouse model of human lymphoma

MATERIAL AND METHODS

Material:

Cell lines

DB, DOHH-2, EB-1, GRANTA-519, HBL-2, HEK293T, HL-60, JEKO-1, JURKAT, K-562, MC-116, ML-2, NU-DHL-1, NU-DUL-1, OPM-2, RAJI, RAMOS, REC-1, RPMI-8226, SC-1, SU-DHL-1, THP-1

Primary leukemia and lymphoma cells

Peripheral blood, bone marrow aspirates or pleural effusions were acquired from patients with diverse hematologic malignancies. Mononuclear cells were purified by Ficoll-Paque density-gradient centrifugation. We collected 26 primary samples - 9 acute myeloid leukemias (AML), 2 chronic myeloid leukemias (CML), 5 mantle cell lymphomas (MCL), 6 chronic lymphocytic leukemias (CLL), 1 diffuse large B cell lymphoma (DLBCL), 1 follicular lymphoma (FL), 1 marginal zone lymphoma (MZL), 1 acute lymphocytic leukemia (ALL).

Animals

For xenotransplantation of human leukemia and lymphoma cells NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ immunodeficient mice (Jackson Laboratory, USA) were used.

Methods:

Cell culture

Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% or 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin).

For apoptosis assays cells were incubated with various concentrations of either roscovitine (R), TRAIL (T) or with their combinations (R+T) for 24 hours and 48 hours. Similar single or combined treatment regimen was used also for K-562 and RAMOS cells treated with TNFα, FASL, cycloheximide, actinomycin D, ABT-737, and TRAIL. In caspase inhibition experiments, K-562 and RAMOS cells were pre-treated with 100 μM Z-VAD-FMK, Z-IETD-FMK or Z-LEHD-FMK for 1 hour before exposure to the particular drugs. In experiments with roscovitine pre-

treatment, K-562 cells were incubated with roscovitine for 3, 6, 12 and 24 hours. Subsequently, roscovitine was washed out, TRAIL was added and apoptosis was measured 24 hours after addition of TRAIL.

Proliferation assays

Flow cytometry

- *Analysis of apoptosis*
- *Cell-mediated cytotoxicity assay*
- *Cell cycle analysis*
- *Cell surface expression of TRAIL receptors*

Gene expression analysis

- *Real-time RT-qPCR analysis*
- *Whole-genome gene expression profiling and data analysis*

Western blotting

- *Total and mitochondrial proteins extraction*
- *DISC immunoprecipitation by streptavidin-agarose beads*
- *Western blotting*

Lentivirus production and infection

Xenotransplantation and treatment

RESULTS

Sensitivity of leukemia and lymphoma cells to TRAIL-induced apoptosis.

We have analyzed sensitivity to TRAIL-induced apoptosis in 21 established leukemia and lymphoma cell lines. Only four cell lines (i.e. 19%), K-562, THP-1, SC-1, and SU-DHL-1 were inherently TRAIL resistant with less than 10% apoptosis after 24 hour exposure to 1 μg/ml TRAIL. On the other hand, four cell lines, JEKO-1, JURKAT, NU-DHL-1, and NU-DUL-1 were extremely TRAIL sensitive with high percentage of apoptosis after exposure to doses as low as 1-10 ng/ml. The majority of cell lines tested exerted a certain degree of sensitivity when high doses of TRAIL were used.

Next, we have measured TRAIL sensitivity in 26 primary cell samples obtained from patients with diverse hematologic malignancies. Compared to leukemia and lymphoma cell lines the vast majority of primary leukemia and lymphoma cells were TRAIL resistant with less than 10% apoptosis after 24-hour exposure to 1μg/ml TRAIL in 19 (i.e. 73%) from 26 samples. The most resistant samples were those obtained from patients with acute myeloid leukemia (AML) (8/9, i.e. 88%), followed by CLL samples (4/6, i.e. 67%) and by MCL samples (3/5, i.e. 60%).

Resistance of leukemia and lymphoma cells to TRAIL-induced apoptosis can be overcome by treatment with cyclin-dependent kinase inhibitor roscovitine.

Roscovitine (seliciclib) is an inhibitor of cyclin-dependent kinases (CDK) [51] with selectivity towards CDK1, CDK2, CDK5, CDK7 and CDK9 [52]. Roscovitine was reported as a potent *in vitro* inducer of apoptosis in many primary hematologic malignant cells [45, 48-50].

Treatment of 21 leukemia / lymphoma cell lines and 26 primary cell samples obtained from patients with diverse hematologic malignancies with roscovitine resulted in induction of apoptosis in a dose-dependent manner. 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. Importantly, roscovitine was able to sensitize even TRAIL resistant cell lines K-562, THP-1, SU-DHL-1 and

primary cells to TRAIL-induced apoptosis. Subsequently, we tested if roscovitine is able to sensitize cells not only to TRAIL but also to other death ligands. K-562 cells were more sensitive to TNFα-induced apoptosis while RAMOS cells were more sensitive to FASL-induced apoptosis when pretreated with low-toxic dose of roscovitine. As death ligands, particularly FASL are involved in cell-mediated cytotoxicity we asked whether roscovitine could augment this type of cell death. Pretreatment of RAMOS cells with roscovitine indeed enhanced apoptosis induced by peripheral blood mononuclear cells (PBMC) from a healthy donor.

Roscovitine induces long-term proapoptotic changes of DISC without affecting surface expression of death receptors.

To analyze molecular mechanisms of the cytotoxic synergism between roscovitine and TRAIL we used TRAIL resistant K-562 cells of the myeloid origin and TRAIL sensitive RAMOS cells of the lymphoid origin. Both K-562 and RAMOS cells demonstrated significant cytotoxic synergism between roscovitine and TRAIL.

Preincubation of K-562 and RAMOS cells with caspase-8 (Z-IETD-FMK), caspase-9 (Z-LEHD-FMK) or pan-caspase (Z-VAD-FMK) inhibitors suppressed the apoptosis induced by combination of roscovitine and TRAIL. This finding suggests that alterations of both extrinsic and intrinsic apoptotic pathways might be involved in the observed cytotoxic synergism between roscovitine and TRAIL. To unveil potential changes in the extrinsic apoptotic pathway, we measured 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 hours. However, immunoprecipitation of DISC proteins from the lysates of K-562 cells treated with biotinylated TRAIL and pre-exposed to roscovitine for 12 and 24 hours revealed enhanced processing of both caspase-8 and caspase-10 at the DISC. We also noticed lower levels of FLIP-L and its pre-processed p43 form at the DISC of roscovitine treated K-562 cells.

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 anti- apoptotic genes by real-time RT-PCR 1.5, 3, 6, 12 and 24 hours after exposure of K-562 cells to roscovitine. Transcription of all tested genes showed gradual downregulation with the maximum decrease at 12 hours after exposure to roscovitine. At 24 hours the level of expression of most tested genes returned to that of untreated controls or was even higher.

Next we analyzed gene expression of K-562 cells exposed to roscovitine using genome-wide Illumina HumanRef-8 arrays. We identified 213, 859, and 430 genes downregulated by more than 2-fold, and 6, 133, and 362 genes upregulated by more than 2-fold following exposure to roscovitine for 1.5, 6, and 24 hours, respectively. The microarray data clearly confirmed significant inhibitory effect of roscovitine on gene transcription. Indeed, treatment of K-562 cells with roscovitine was associated with decreased phosphorylation of serine 2 and serine 5 of the carboxyl-terminal domain (CTD) of RNA polymerase II implying inhibition of transcription. Moreover, treatment with roscovitine induced phosphorylation of the eukaryotic initiation factor - 2 alpha (eIF-2α) after 12 and 24 hours suggesting an inhibition of translation. To test whether inhibition of transcription or translation can sensitize cells to TRAIL-induced apoptosis we treated K-562 and RAMOS cells with actinomycin D (inhibitor of transcription) and cycloheximide (inhibitor of translation). Both drugs sensitized K-562 and RAMOS cells to TRAIL-induced apoptosis.

Roscovitine induces gradual downregulation of cytoplasmic MCL-1 and BCL-XL followed by upregulation of both proteins at 24 hours after the exposure to roscovitine.

As treatment with roscovitine resulted in downregulation of many transcripts, we decided to analyze protein expression of selected pro- and anti- apoptotic molecules by western blotting 1.5, 3, 6, 12 and 24 hours after exposure to roscovitine. We observed gradual downregulation of some of the tested proteins (e.g. BCL-XL, MCL-1, or PUMA) during the first 12 hours after exposure to roscovitine, while at 24 hours these proteins

were all upregulated compared to untreated cells. In contrast, expression of other proteins (e.g. BAK, BAD, or BCL-2) remained virtually unchanged in response to roscovitine despite the fact they had demonstrated similar changes of mRNA expression (i.e. downregulation during 12 hours, and upregulation after 24 hours). Using short hairpin (sh) RNA-mediated gene expression silencing, we confirmed that downregulation of both MCL-1 and BCL-XL in K-562 cells indeed sensitized the cells to TRAIL-induced apoptosis. Interestingly, downregulation of BCL-XL sensitized K-562 cells also to roscovitine-induced cell death. From the analyzed proapoptotic BCL-2 proteins we observed moderate upregulation of BAD, BAK, and PUMAα at 1.5 hours, and upregulation of PUMAα at 24 hours.

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

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. At least 12 hour pretreatment with roscovitine was required to induce sensitization to TRAIL, but this sensitization persisted only for 6 hours. Cells treated with roscovitine for 24 hours remained sensitive to TRAIL even 24 hours after roscovitine removal.

Roscovitine induces recruitment of both pro- and anti-apoptotic BCL-2 proteins to the mitochondria.

At least 12 hour pretreatment with roscovitine appeared essential to induce sensitization of K-562 cells to TRAIL-induced apoptosis and was associated with maximal downregulation of MCL-1 and BCL-XL. Maximal and long-lasting sensitization to TRAIL was reached after 24-hour pretreatment with roscovitine, 24-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, the protein expression levels obtained from the whole cell lysates might not reliably reflect potential compartment-specific changes of the BCL-2 family protein levels at the mitochondria. We analyzed protein expression of selected apoptosis-regulating molecules using mitochondria isolates of K-

562 cells treated with roscovitine for 3, 6, 12 and 24 hours. 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 anti-apoptotic proteins with the only exception of PUMAβ. These results imply that antiapoptotic BCL-2 proteins might sequester proapoptotic BCL-2 proteins. Indeed, K-562 cells treated with roscovitine were more sensitive to ABT-737, an inhibitor of BCL-2, BCL-XL and BCL-W.

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, single-agents or in combination. Combined treatment of roscovitine and TRAIL significantly suppressed growth of both RAMOS and HBL-2 tumors compared to single-agent approaches.

DISCUSSION

In the present study we determined the sensitivity of diverse leukemia and lymphoma cell lines and primary cells to TRAIL-induced apoptosis and measured the ability of CDK inhibitor roscovitine to sensitize these cells to TRAIL-induced apoptosis both *in vitro* and *in vivo* using a mouse model of human lymphoma. Further, we analyzed molecular mechanisms responsible for the cytotoxic synergism between roscovitine and TRAIL.

In our set of 21 established leukemia and lymphoma cell lines only four cell lines (i.e. 19%) were inherently TRAIL resistant (defined as less than 10% apoptosis after 24 hour exposure to 1μg/ml TRAIL). The majority of cell lines tested exerted a certain degree of sensitivity. On the other hand, the vast majority of primary leukemia and lymphoma cells were TRAIL resistant (19 from 26 samples, i.e. 73%). The cause of different TRAIL sensitivity between cell lines and primary samples is not clear. One of the possible explanations could be immunoselective pressure of TRAIL exerted on tumor cells *in vivo* [53].

Significant number of primary malignancies turned up to be TRAIL resistant. However, a number of studies keep on documenting that even these resistant cancer cells could be sensitized to TRAIL by various current and emerging anti-cancer drugs [54]. Recently, it was reported that glioma, breast cancer, and thyroid carcinoma cells can be sensitized to TRAIL-induced apoptosis by the purine analogue roscovitine [41-43]. R-roscovitine (seliciclib) competes with adenosine triphosphate for its binding site on CDK [51], and demonstrates selectivity towards CDK1, CDK2, CDK5, CDK7 and CDK9 [52]. Roscovitine was reported a potent *in vitro* inducer of apoptosis in many primary hematologic malignant cells [45, 48-50]. Therefore, we have tested if roscovitine is able to sensitize leukemia and lymphoma cells to TRAIL-induced apoptosis. 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. Importantly, roscovitine was able to sensitize even completely TRAIL resistant cell lines and primary cells. Moreover, combination of roscovitine and TRAIL significantly suppressed the growth of subcutaneous human lymphoma xenografts in immunodeficient mice compared to TRAIL or roscovitine used alone. Besides TRAIL, exposure of K-562 and RAMOS cells to roscovitine increased also apoptosis induced by TNFα and FASL, respectively. As the cytotoxic T and NK cells

were reported to use death ligands to kill target cells we asked 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 anti-tumor surveillance mediated by cytotoxic cells of the innate immune system.

Next, we analyzed molecular mechanisms responsible for the cytotoxic synergism between roscovitine and TRAIL. As exposure of K-562 and RAMOS cells to roscovitine did not alter cell surface expression of TRAIL receptors we suggest that roscovitine-induced proapoptotic changes should be localized downstream. We have demonstrated that activation of the initiator caspases -8 and -10 was significantly more effective in cells exposed to roscovitine compared to control cells. This increased cleavage of caspases -8 and -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 the 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 ubiquitinylation by cullin-3-based E3 ligase [55]. Alternatively, decreased expression of competitive inhibitor that impedes binding of initiator caspases to FADD could explain this observation. Recently, DJ-1 oncogene was shown to compete with caspase-8 binding to FADD thereby inhibits TRAIL-induced apoptosis [56]. As expected, inhibition of caspase-8 suppressed TRAIL-induced apoptosis in roscovitine-treated cells. Interestingly, inhibition of caspase-9 had similar effect implying requirement for augmentation of the apoptotic signal through the mitochondria. Previous studies ascribed TRAIL-sensitizing effects of roscovitine to the observed downregulation of several antiapoptotic proteins, namely MCL-1, XIAP and survivin [41-42]. Roscovitine-mediated inhibition of transcription by decreased phosphorylation of CTD of RNA polymerase II through inhibition of CDK7 [44] was considered as a mechanism responsible for the downregulation of these short-lived proteins [45-47]. In agreement with previous data, we found decreased phosphorylation of 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 cycloheximid) 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 the death ligands-induced apoptosis.

Surprisingly, the gradual mRNA and protein downregulation of MCL-1 and BCL-XL detected during the first 12 hours after exposure to roscovitine was followed by marked upregulation of these molecules 12 hours later. As we have shown, at least 12-hour pretreatment with roscovitine appeared essential to induce sensitization of K-562 cells to TRAIL-induced apoptosis. The roscovitine-induced TRAIL-sensitizing effect persisted only for 6 hours. For long-lasting sensitization to TRAIL, 24-hour pretreatment with roscovitine was indispensable. 24-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 analysed mitochondrial lysates of K-562 cells treated with roscovitine and found gradual upregulation of all tested pro- and anti-apoptotic BCL-2 family members with the only exception of PUMAβ. These results are in contrast with previous reports where downregulation of antiapoptotic proteins was proposed as sensitizing mechanism. None of these studies, however, determined the expression of these proteins on mitochondrial level. Based on the concomitant upregulation of both pro- and anti- apoptotic proteins on mitochondria following exposure to roscovitine, we suggest that the main mechanism by which roscovitine sensitizes leukemia and lymphoma cells to TRAIL-induced apoptosis could be mitochondrial priming for death. In several previous reports BH3 profiling technique evaluating the response of mitochondria to the peptides derived from the BH3 domains of proapoptotic BH3-only proteins was used to identify apoptotic defects in cancer cells [57-58]. This technique identified three classes of apoptotic block that cancer cells use to survive. The first block (or class A block) is characterized by low expression of the activator BH3-only proteins. In the second block (or class B block) the proapoptotic proteins Bax and/or Bak are either absent or functionally defective. In the third block (or class C block) cells express high amount of antiapoptotic BCL-2 proteins that are primed with BH3-only protein activators, or activated Bax or Bak. These cells are referred to as primed for death. Inhibition of the antiapoptotic proteins in primed cells (compared

to unprimed 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. We hypothesize that this occurs by displacement of proapototic BH3-only proteins from antiapoptotic BCL-2 family proteins, and subsequent activation of Bak/Bax. It was shown that treatment of K-562 cells with TRAIL induces redistribution of BCL-2 family members without induction of apoptosis (as K-562 cell line is TRAIL resistant). Specifically, PUMA and BIM increasingly bind to MCL-1 and tBID to BCL-XL [59]. Such rearrangement of BCL-2 family members in roscovitine-treated (primed) cells could trigger apoptosis. Proposed mechanism of roscovitine-mediated sensitization of K-562 cells to TRAIL-induced apoptosis is shown in Figure 1.

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 molecular mechanisms responsible for the observed roscovitine-induced sensitization to TRAIL and potentially also to cell-mediated cytotoxicity.

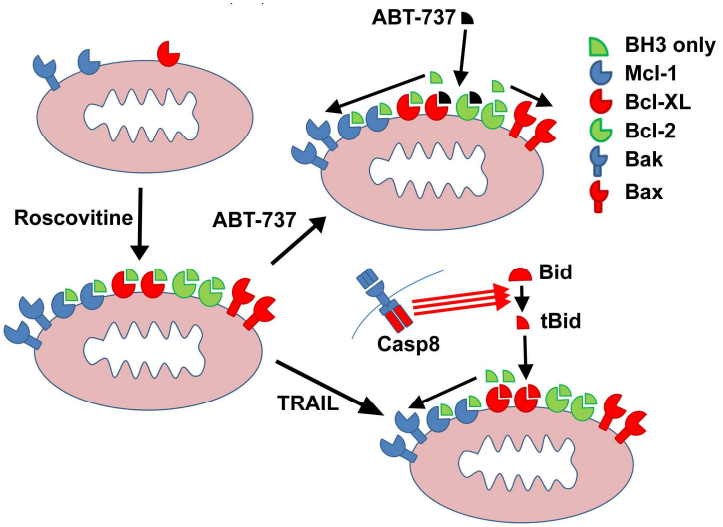

Figure 1. Proposed mechanism of roscovitine-mediated sensitization to TRAIL-induced apoptosis. Roscovitine induces upregulation of pro- and anti-apoptotic BCL-2 family members on mitochondria. Antiapoptotic BCL-2 family proteins sequester proapoptotic BH3-only proteins. Exposure of roscovitine-treated K-562 cells to ABT-737, inhibitor of BCL-2, BCL-XL and BCL-W induces apoptosis by displacement of proapototic BH3-only proteins from antiapoptotic BCL-2 family proteins that in turn activate Bak/Bax. Similarly, treatment of primed (roscovitine-treated) cells with TRAIL activates apoptotic cascade due to TRAIL-induced redistribution of BCL-2 family members in roscovitine-primed cells.

CONCLUSIONS

The present study provides experimental evidence that

- the majority of leukemia and lymphoma primary cells are resistant to TRAIL-induced apoptosis compared to leukemia and lymphoma cell lines

- an inhibitor of cyclin-dependent kinases roscovitine is able to sensitize most of leukemia and lymphoma cell lines and primary cells to TRAIL-induced apoptosis

- roscovitine sensitizes leukemia and lymphoma cells also to TNFα and FASL and enhances cell-mediated cytotoxicity

- combined treatment with roscovitine and TRAIL significantly suppressed the growth of subcutaneous human lymphoma xenografts in immunodeficient mice compared to roscovitine or TRAIL used alone

- treatment with roscovitine does not change surface expression of DR4 or DR5, however, enhances processing of initiator caspases at the DISC

- treatment of K-562 cells with roscovitine increases mitochondrial levels of both pro- and anti- apoptotic BCL-2 family proteins which implies that roscovitine induces mitochondrial priming for death

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