

Univerzita Karlova v Praze

1. lékařská fakulta

studijní program: Biomedicína

studijní obor: Fyziologie a patofyziologie člověka



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RESENZITIZACE LEUKEMICKÝCH A LYMFOMOVÝCH BUNĚK
K TRAILEM INDUKOVANÉ APOPTÓZE

**RESENSITIZATION OF LEUKEMIA AND LYMPHOMA CELLS
TO TRAIL-INDUCED APOPTOSIS**

Dizertační práce

Ph.D. Thesis

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Praha 2013

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Podpis:

Identifikační záznam:

MOLINSKÝ, Jan. *Resenzitizace leukemických a lymfomových buněk k TRAILem indukované apoptóze* [RESENSITIZATION OF LEUKEMIA AND LYMPHOMA CELLS TO TRAIL-INDUCED APOPTOSIS]. Praha, 2013, 98 s., 3 přílohy, Dizertační práce (Ph.D.), Univerzita Karlova v Praze, 1. lékařská fakulta, Ústav patologické fyziologie. Školitel: MUDr. Pavel Klener, Ph.D.

Tato práce je věnovaná mým rodičům

Poděkování:

Na tomto místě bych rád poděkoval svému školiteli, MUDr. Pavlovi Klenerovi, Ph.D. za pomoc s formulováním cílů práce, designem experimentů a za veškerou pomoc a podporu, které se mi od něj po celou dobu doktorského studia dostávalo.

Rád bych poděkoval bývalému i současnému přednostovi Ústavu patologické fyziologie 1. LF UK, Prof. MUDr. Emanuelovi Nečasovi, DrSc. a Doc. MUDr. Martinu Vokurkovi, CSc. za cenné rady, diskuzi a podporu vedoucí ke vzniku této práce.

Rád bych poděkoval všem kolegům a kolegyním z naší laboratoře za pomoc, přátelské pracovní prostředí a stimulující vědeckou diskuzi. Jmenovitě bych rád poděkoval: Dr. Magdaleně Klánové, Dr. Lucii Latečkové, Dr. Petře Vočkové, Dr. Bokangovi Maswabimu, Dr. Daně Průkové, Dr. Janu Živnému, Dr. Ladislavu Anděrovi.

V neposlední řadě bych rád poděkoval své rodině za podporu a shovívavost v průběhu celého studia.

Disertační práce byla vytvořena za podpory následujících projektů:

IGA MZ NS/10287-3 z Ministerstva zdravotnictví ČR

LC06044 a MSM021620806 z Ministerstva školství, mládeže a tělovýchovy ČR

PRVOUK-P24/LF1/3, PRVOUK-P27/LF1/1, UNCE204021 a SVV-2013-266509 od Univerzity Karlovy v Praze.

Content

LIST OF ABBREVIATIONS	6
ABSTRACT	9
ABSTRACT (Czech)	11
1 INTRODUCTION	13
1.1 Apoptosis	13
1.1.1 Caspases	14
1.1.2 Extrinsic apoptotic pathway	16
1.1.3 Intrinsic apoptotic pathway	18
1.2 TRAIL	23
1.2.1 TRAIL and its receptors	23
1.2.2. Biological role of TRAIL	27
1.2.3. TRAIL as therapeutic agent	29
1.2.4. Mechanisms of resistance to TRAIL-induced apoptosis	31
2 HYPOTHESIS AND THE AIMS OF THE STUDY	36
3 MATERIALS AND METHODS	38
3.1 Materials	38
3.2 Methods	45
3.2.1 Cell culture	45
3.2.2 Proliferation assays	46
3.2.3 Flow cytometry	46
3.2.4 Gene expression analysis	47
3.2.5 Western blotting	48
3.2.6 Lentivirus production and infection	49
3.2.7 Xenotransplantation and treatment	50
3.2.8 Statistical analysis	50
4 RESULTS	52
4.1 Sensitivity of leukemia and lymphoma cells to TRAIL-induced apoptosis	52
4.2 Resistance of leukemia and lymphoma cells to TRAIL-induced apoptosis can be overcome by treatment with cyclin-dependent kinase inhibitor roscovitine	54
4.3 Roscovitine induces long-term proapoptotic changes of the DISC without affecting surface expression of death receptors	57
4.4 Roscovitine inhibits transcription and translation	60
4.5 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 ...	62
4.6 Roscovitine-induced sensitization to TRAIL is dependent on the length of cell pre-incubation with roscovitine	64
4.7 Roscovitine induces recruitment of both pro- and anti-apoptotic BCL-2 proteins to mitochondria	65
4.8 Roscovitine and TRAIL synergistically inhibit growth of leukemia and lymphoma xenografts in immunodeficient mice	66
5 DISCUSSION	68
6 CONCLUSIONS	73
7 REFERENCES	74
8 LIST OF PUBLICATIONS	93
9 APENDICES	94
9.1 Supplementary figure 1	94
9.2 Supplementary figure 2	97
9.3 Publications related to the dissertation topic	98

LIST OF ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ATP	adenosine triphosphate
BAD	BCL-2 antagonist of cell death
BAK	BCL-2 antagonist/killer
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2 protein
BH	BCL-2 homology domain
BID	BH3-interacting domain death agonist
BIK	BCL-2 interacting killer
BIM	BCL-2-interacting mediator of cell death
BMF	BCL-2-modifying factor
BOK	BCL-2-related ovarian killer
CARD	caspase recruitment domain
CASP	caspase
CDK	cyclin-dependent kinase
CFSE	5(6)-carboxyfluorescein diacetate <i>N</i> -succinimidyl ester
CTD	carboxyl-terminal domain
CFSE	carboxyfluorescein succinimidyl ester
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
DcR	decoy receptor
DD	death domain
DED	death effector domain

DIABLO	direct IAP binding protein with low pI
DLBCL	diffuse large B-cell lymphoma
DISC	death-inducing signaling complex
DR	death receptor
EDAR	ectodermal dysplasin receptor
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
eIF-2 α	eukaryotic initiation factor - 2 alpha
FASL	FAS ligand
FADD	FAS associated death domain
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FLICE	FADD-like interleukin-1 beta-converting enzyme
FLIP	FLICE-inhibitory protein
GPI	glycosylphosphatidylinositol
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HRK	harakiri protein
HRP	horseradish peroxidase
IAP	inhibitor of apoptosis
IMDM	Iscoe's modified Dulbecco's medium
JNK	Jun N-terminal kinase
MCL	mantle cell lymphoma
MM	multiple myeloma
MOI	multiplicity of infection
MOMP	mitochondrial outer membrane permeabilization

MTCH2/MIMP	mitochondrial carrier homologue 2/Met-induced mitochondrial protein
NF κ B	nuclear factor kappa B
NGFR	nerve growth factor receptor
OPG	osteoprotegerin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
PLAD	pre-ligand assembly domain
PMSF	phenylmethylsulfonylfluoride
PUMA	p53 upregulated modulator of apoptosis
RIP1	receptor-interacting protein kinase 1
shRNA	short hairpin RNA
SMAC	second mitochondrial-derived activator of caspases
TEMED	tetramethylethylenediamine
TNF α	tumor necrosis factor alpha
TRADD	TNF α associated death domain
(rh)TRAIL	(recombinant human) TNF-related apoptosis inducing ligand

ABSTRACT

Apoptosis serves as a natural barrier to cancer development, and the resistance to apoptosis represents one of the key capabilities acquired during tumor development or progression. Impairment of the intrinsic apoptotic pathway exemplifies one of the established mechanisms of constitutive or acquired drug resistance. 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 the chemotherapy resistant cancer cells. TRAIL is a death ligand belonging to the TNF α superfamily that specifically kills tumor cells while sparing healthy tissues. Much enthusiasm has been generated for TRAIL as a highly promising targeted anti-cancer agent. However, many primary tumors have been shown to be TRAIL resistant. In attempt to overcome such an intrinsic 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 21 hematologic malignant cell lines and 26 primary cell samples. Pretreatment of TRAIL resistant leukemia and lymphoma cells with roscovitine induced enhanced cleavage of the 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 anti-apoptotic BCL-2 proteins to pro-apoptotic 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.

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 TNF α , 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 synergizmu mezi roskovitem 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 roskovitem vyvolá po jejich následném vystavení TRAILu zvýšené štěpení apikálních kaspáz. Dále jsme po léčbě roskovitem 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 mechanismy roskovitinem navozené senzitivace k TRAILem indukované apoptóze v leukemických / lymfomových buňkách.

1 INTRODUCTION

1.1 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.

In 1972, Kerr et al. described distinctive morphological process of cell demise different from uncontrolled and accidental necrosis [1], until then the only known mechanism of cell death, and entitled it *apoptosis*. In contrast to *necrosis* that was characterized by cell and organelle swelling, plasma membrane rupture and subsequent loss of intracellular contents, apoptosis was characterized by condensation of nucleus and cytoplasm, nuclear fragmentation and breaking up of the cell into membrane-embedded apoptotic bodies that were rapidly phagocytosed without inflammatory response. Morphologic signs of apoptosis were detected during embryogenesis as well as in the adult healthy tissues and Kerr et al. therefore assumed that it is an active, programmed process regulating (together with mitosis) cell populations in animals. Moreover, apoptosis was detected in growing malignant tumors and its rate increased after therapy. Several years later a genetic program of apoptosis began to be elucidated by dint of genetic studies in *Caenorhabditis elegans* and it has been shown that this program is well conserved during evolution [2].

Apoptosis serves as a natural barrier to cancer development [3], resistance to apoptosis thus represents one of the key capabilities acquired during tumor development and exemplifies one of the established mechanisms of intrinsic or acquired drug resistance [4]. Understanding apoptotic regulatory mechanisms is essential for understanding the pathogenesis of malignant disorders as well as the development of more effective therapies.

The term apoptosis has been used as a synonym for programmed cell death for a long time. However, other mechanisms of programmed cell death have been described recently,

some of them with morphological features of necrosis [5-7]. The typical feature of apoptotic cell disassembly is a proteolytic cascade mediated by a family of enzymes called caspases.

1.1.1 Caspases

Caspases are the family of cysteine proteases that cleave substrates after aspartic acid residues. Caspases play many important roles not only in the regulation of apoptosis, but also in the cytokine processing and inflammation. Casapases are produced as inactive zymogens (procaspases) that contain three domains: N-terminal prodomain, a large subunit (p20) and a small subunit (p10). The activation of procaspases is mediated through proteolytic cleavage between the large and small subunits to form a heterodimer. Based on the structure, mechanism of activation, and role in the apoptotic cascade, caspases can be classified into two groups, initiator (caspases-2, -8,-9,-10) and effector (caspases-3,-6,-7) caspases [8-9].

The initiator caspases contain long prodomain with either the caspase recruitment domain (CARD), or the death effector domain (DED). The caspases containing DED domain (caspases-8,-10) interact with cytoplasmic side of death receptors through the adaptor proteins that also contain DED domains. The caspases with CARD domain (caspases-2,-9) interact with the CARD domain containing adaptors in specialized cytoplasmic protein complexes. Activation of the initiator caspases require homodimerization leading to the proximity-induced autoactivation and take place in the macromolecular complexes called DISC (death-inducing signaling complex), apoptosome, and PIDDosome [8, 10]. DISC is formed at the cytoplasmic side of death receptors upon binding of death ligands and mediates activation of caspases -8 and -10 [11-12]. This apoptotic pathway is referred to as *extrinsic or receptor-mediated*. Apoptosome formation is triggered in cytoplasm by *intrinsic or mitochondrial* apoptotic pathway and serves to the activation of caspase-9 [13]. PIDDosome represents cytoplasmic macromolecular activation platform for caspase-2 [14] (Figure 1).

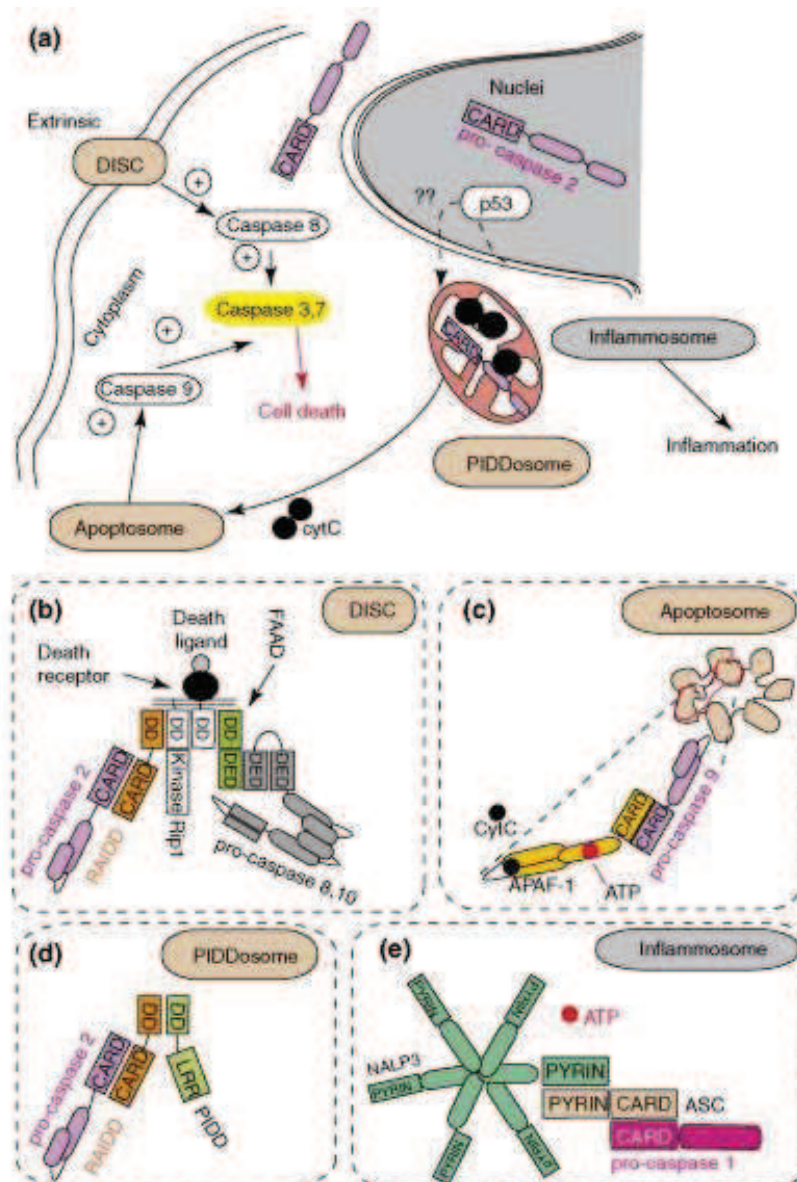


Figure 1. Overview of the protein complexes specialized for activation of caspases (a). DISC (b), apoptosome (c) and PIDDosome (d) serve as molecular platforms for activation of the apoptotic caspases, inflammasome (e) activates the inflammatory caspases. Adapted from Sanchez-Pulido et al. [15].

Compared to initiators the effector caspases (caspases-3,-6,-7) contain short prodomains and exist as dimers that are activated by initiator caspases by cleavage between the large and small domains.

Both apoptotic pathways converge to a final common pathway mediated by the effector caspases resulting in cleavage of cell regulatory and structural molecules.

1.1.2 Extrinsic apoptotic pathway

Extrinsic apoptotic pathway is induced by death ligands bound to their cognate death receptors. Death ligands are type II transmembrane proteins (extracellular C-terminus and intracellular N-terminus) belonging to the tumor necrosis factor (TNF) superfamily and specific proteases can cleave ligands into their soluble forms. The TNF family contains many ligands, most studied and the best characterized are TNF α , FAS ligand (FASL, CD95L), and TNF-related apoptosis inducing ligand (TRAIL). Death receptors are type I transmembrane proteins (extracellular N-terminus and intracellular C-terminus) belonging to the superfamily of TNF receptors (TNFR). Common feature of death receptors is the presence of cytoplasmic domain that is indispensable for ligand-induced apoptotic signaling known as the death domain (DD). The DD mediates homophilic interactions with the death domain containing adaptor proteins TRADD (TNF α associated death domain) and FADD (FAS associated death domain) [16]. So far eight death receptors have been identified, TNF receptor 1 (TNFR1), FAS (CD95), death receptors 3, 4, 5, and 6 (DR3, DR4, DR5, DR6), ectodermal dysplasin receptor (EDAR), and nerve growth factor receptor (NGFR) [17-18].

Ligation of death receptors by death ligands leads to the formation of DISC, composed of the core adapter protein FADD (and TRADD in the case of TNFR1), and the initiator caspases -8 and -10 [11-12]. Additional component of the DISC is FLIP, a competitive inhibitor of caspase -8/-10 binding and activation. On ligand binding, death receptors recruit FADD adaptor through interactions of the DD. FADD then recruits caspases -8 and/or -10 and/or FLIP through interactions of the DED. DISC assembly enables the proximity-induced homodimerization and self-processing of initiator caspases and facilitates their activation [19]. However, caspase-8 dimers are unstable and full activation of the initiator caspases requires further aggregation [20]. Jin et al. [21] proposed recently new mechanism of caspase-8 stabilization. They found that caspase-8 is polyubiquitinated at the DISC after TRAIL

treatment. This polyubiquitination is mediated by E3 ligase cullin-3. Subsequently, ubiquitin-binding protein p62/sequestosome-1 promotes translocation of polyubiquitinated caspase-8 from the DISC to the cytosolic ubiquitin-rich foci (aggresomes). Afterwards, activated initiator caspases cleave and activate the effector caspases -3, -6 and -7.

In type I cells sufficient amount of caspase-8 is created to activate the effector caspases. In type II cells proapoptotic signal must be amplified through the mitochondrial pathway [22-23]. The basis for this signaling diversity seems to be located at the receptor level. It has been shown that in type I cells mRNAs for death receptors have longer half-life and receptors are more expressed at cell surface. Type II cells can be converted to type I cells by forced expression of death receptors and, conversely, type I cells can be converted to type II cells by receptor downregulation. Interestingly, the same cells can exhibit type I signaling for one death receptor and type II signaling for other death receptor [24] (Figure 2).

The connection between extrinsic and intrinsic apoptotic pathways is mediated by the proapoptotic BCL-2 family member Bid. Under normal circumstances Bid is generally thought to be inactive. Activation of Bid is mediated by its cleavage by caspase-8. Moreover, several other proteases including granzyme B, cathepsins, and calpains have been shown to cleave and activate Bid [19]. Interestingly, Gonzalez et al. showed that in type II cells caspase-8 translocates to the mitochondrial outer membrane (MOM), where its accumulation, oligomerization and activation relies on the mitochondria-specific phospholipid cardiolipin [25]. Activated Bid (truncated Bid, tBid) is recruited to the MOM through interaction with MTCH2/MIMP protein (mitochondrial carrier homologue 2/Mit-induced mitochondrial protein) [26] and induces mitochondrial outer membrane permeabilization (MOMP) that is dependent on Bax and/or Bak pro-apoptotic BCL-2 members [27-28].

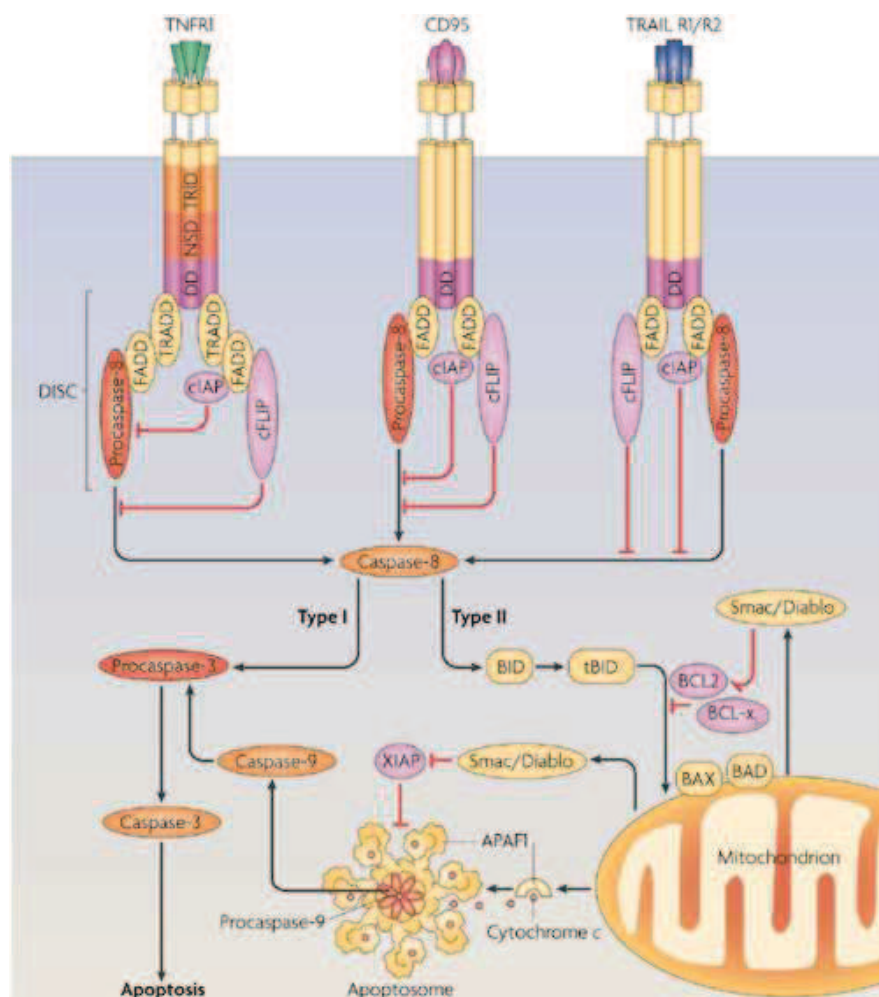


Figure 2. Extrinsic (receptor mediated) apoptotic pathway. Ligation of death receptors by death ligands (especially $\text{TNF}\alpha$, FASL and TRAIL) activates the initiator caspases. In type I cells sufficient amount of caspase-8 is created to activate the effector caspases. In type II cells proapoptotic signal must be amplified through intrinsic (mitochondrial) pathway. Adapted from Schutze et al [29].

1.1.3 Intrinsic apoptotic pathway

Intrinsic (mitochondrial) apoptotic pathway is initiated inside cell in response to various stimuli, such as loss of growth signals during development or severe cell stress (DNA damage, growth factor deprivation, oncogene activation, microtubule disruption) [30]. This pathway is controlled by interactions among members of the BCL-2 protein family that regulate MOMP. MOMP results in the release of cytochrome c, Smac/Diablo (second mitochondrial-derived activator of caspases/direct IAP binding protein with low pI),

endonuclease G and other proapoptotic proteins from mitochondria. Subsequently, cytochrome c enables formation of the multiprotein complex apoptosome followed by the activation of initiator caspase-9 [31-33].

The founder of this family, BCL-2 (B-cell lymphoma 2) protein, was the first discovered protein with antiapoptotic function. Originally it was reported that BCL-2 gene participates in the t(14;18) chromosomal translocation observed in patients with follicular lymphoma [34]. Subsequently, antiapoptotic activity of BCL-2 and its activity in regulating of conserved mitochondrial apoptotic pathway were discovered [35-37]. Since its discovery multiple members of the BCL-2 family have been discovered, including both antiapoptotic and proapoptotic proteins. These proteins are related by sequence homology in one or more BCL-2 homology (BH) regions (Figure 3).

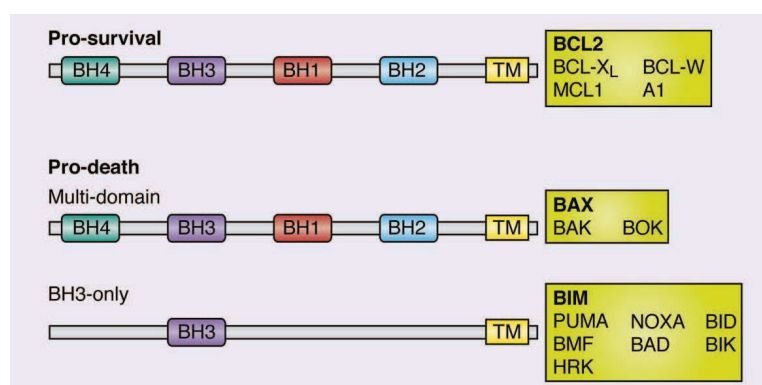


Figure 3. Structural and functional classification of BCL-2 protein family. Adapted from Happon et al. [38].

Antiapoptotic members of the BCL-2 family contain four BH domains (BH1 - BH4) and promote cell survival by binding of proapoptotic BCL-2 proteins. Apart from BCL-2, another five antiapoptotic BCL-2 family members have been identified so far, *MCL-1* (myeloid cell leukemia sequence 1) [39], *BCL-XL* (also known as BCL2-like 1; BCL2L1)

[40], *BCL-W* (BCL2L2) [41], *BFL1* (BCL2A1) [42], *BCL-B* (BCL2L10) [43]. These proteins associate with mitochondrial, nuclear and endoplasmic reticulum membranes.

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 are thought to mediate MOMP by oligomerization and formation of pores within MOM. Members identified so far are *Bak* (BCL-2 antagonist/killer) [44-45], *Bax* (BCL-2-associated X protein) [46], and *Bok* (BCL-2-related ovarian killer) [47]. *Bak* is inserted into the mitochondrial membrane, whereas *Bax* localizes predominantly as a monomer in the cytosol and upon activation translocates to the mitochondrial membrane [48-50]. BH3-only proteins comprise *Bid* (BH3-interacting domain death agonist) [51], *BAD* (BCL-2 antagonist of cell death) [52], *BIK* (BCL-2 interacting killer) [53], *BIM* (BCL-2-interacting mediator of cell death) [54], *PUMA* (p53 upregulated modulator of apoptosis) [55], *NOXA* [56], *BMF* (BCL-2-modifying factor) [57], *HRK* (harakiri) [58]. They developed during evolution as molecular signals corresponding to a plethora of stress impulses from the environment or from within the cell, and their major role lies in the conveying of stress stimuli into the apoptotic pathway. These proteins are regulated by diverse transcriptional and posttranslational mechanisms. *Bid* is activated by proteolytic cleavage by caspase-8 and several other proteases and thus conveys death signal from extrinsic apoptotic pathway to mitochondria. *BAD* phosphorylation by Akt kinase results in its binding by 14-3-3 proteins. *BIK* is phosphorylated and inhibited by casein kinase II. Transcription of *BIK* gene can be activated by E2F1 and p53. Transcription of *BIM* is activated by FOXO3a and CHOP transcription factors. It is sequestered by microtubule-associated dynein motor complex. Its phosphorylation can be activating or inactivating. ERK mediated phosphorylation targets *BIM* for the ubiquitylation and degradation in proteasome. Activity of *BIM* can be increased by the JNK mediated phosphorylation or by protein

phosphatase 2A mediated dephosphorylation. PUMA and NOXA are transcriptional targets of p53 and E2F1. HIF1 α activates transcription of NOXA. Phosphorylation of NOXA by CDK5 causes its cytoplasmic sequestration. BMF binds to cytoskeleton through myosin V motor complex and is activated by the JNK mediated phosphorylation. Similarly, JNK activates HRK [30].

The BCL-2 family members can interact each other, but individual proteins differ in their binding specificity (Figure 4). Bak was shown to interact with BCL-XL and MCL-1 but not with BCL-2, BCL-W or BFL1, whereas Bax binds to all of these proteins [59]. Considering BH3-only proteins, BIM and PUMA bind with high affinity to all antiapoptotic proteins, whereas BAD and BMF associate preferentially with BCL-2, BCL-XL and BCL-W but not with MCL-1 or BFL1. In contrast, NOXA binds selectively to MCL-1 and BFL1 [60-61]. Finally, BIK, HRK, and Bid prefer BCL-XL, BCL-W, and BFL1 over BCL-2 or MCL-1 [60].

These specific interactions among BCL-2 members allow to regulate responses to diverse apoptotic stimuli. To explain how interactions among BH3-only, multi-domain proapoptotic and antiapoptotic proteins regulate apoptosis, two models have been proposed (Figure 4). Both models agree that the activation of Bax and/or Bak is essential for induction of mitochondrial apoptosis as *bax*^{-/-}*bak*^{-/-} double knockout cells (but not single knockout cells) fail to undergo MOMP in response to diverse apoptotic stimuli [28]. Discrepancy is in that how Bax/Bak effectors are activated. According to the *indirect activation* model Bax and Bak must be sequestered by antiapoptotic proteins to prevent MOMP. Apoptosis is then triggered when BH3-only proteins bind to antiapoptotic proteins, thereby release and activate Bax/Bak [62]. According to the *direct activation* model BH3-only proteins can be divided into two groups, activators and sensitizers. Activators (namely BIM, tBid, and possibly others such as PUMA) directly interact with and activate Bax/Bak. In healthy cells activators are

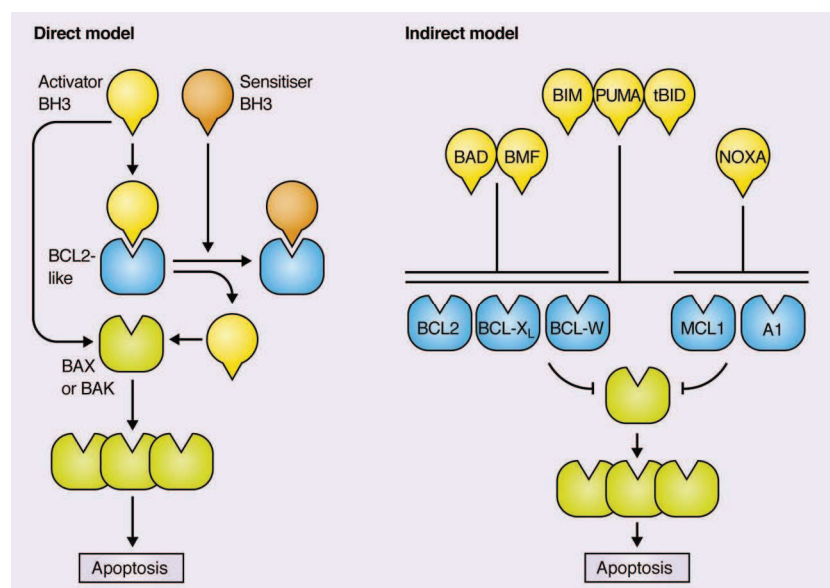


Figure 4. Direct and indirect models of Bax/Bak activation. Adapted from Hapko et al. [38].

1.2 TRAIL

1.2.1 TRAIL and its receptors

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL, Apo2L, dulanermin) is a member of the TNF ligand family discovered because of its sequence homology to TNF α and FASL. The TRAIL gene is located on the chromosome 3 at position 3q26 and contains five exons and four introns. It encodes type II transmembrane glycoprotein consisting of 281 amino acids that can be cleaved by specific metalloproteinases to form soluble ligand [65-66]. Similarly to other members of the TNF family, soluble TRAIL has a trimeric structure. A unique property of TRAIL, compared to other TNF members, is the presence of central zinc atom that binds to cysteine-230 residue of each monomer. The TRAIL structure thus represents the first example of metal binding-mediated trimerization of a cytokine. The zinc ion is important for the structure, stability and biological activity of TRAIL. Removal of the bound metal from TRAIL results in the formation of poorly active dimers, decrease in receptor-binding affinity and a 90-fold decrease in apoptotic activity [67-68].

Besides the full length TRAIL (or TRAIL α) another eight truncated isoforms of TRAIL have been described to date (TRAIL β , γ , δ , BX439, AK, E2, E3, E4) [69]. It seems that the cancer-selective apoptotic activity is mediated only by the full length TRAIL. The truncated isoforms, however, are able to activate NF κ B and thus might play a role in non-apoptotic signaling [70].

To date, five receptors for TRAIL have been identified - death receptor 4 (DR4, TRAIL-R1) [71], death receptor 5 (DR5, TRAIL-R2) [72], decoy receptor 1 (DcR1, TRAIL-R3) [72], decoy receptor 2 (DcR2, TRAIL-R4) [73], and osteoprotegerin (OPG) [74]. The TRAIL-receptor genes are all located on the chromosome 8. DR4, DR5, and DcR2 are type I

transmembrane proteins, DcR1 is tethered to the membrane via a glycosylphosphatidylinositol (GPI) anchor, and OPG lacks a membrane-interacting domain and is therefore secreted as a soluble protein. The extracellular domains of these receptors are characterized by the presence of cysteine-rich domains (CRDs). DR4 and DR5 are characterized by the intracellular DD that is essential for the transmission of death signal upon binding of TRAIL. DcR1 completely lacks the intracellular part, and DcR2 has truncated, non-functional DD. Decoy receptors, and OPG are thus unable to transmit death signal and act as competitive inhibitors of death receptors for TRAIL ligand binding (Figure 5).

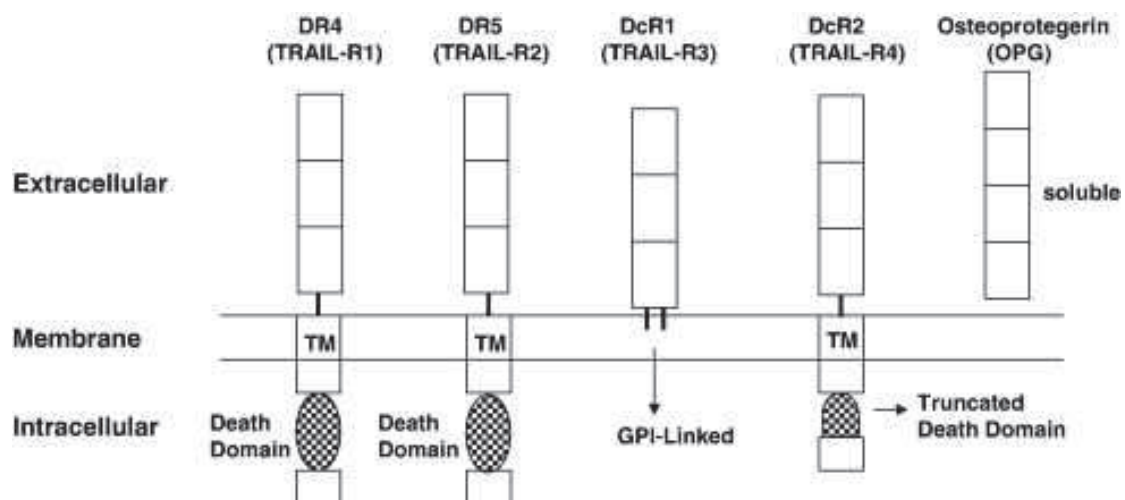


Figure 5. TRAIL receptors. Adapted from Huang et al. [75].

TRAIL receptors were thought to exist as monomers that trimerize upon binding of ligand. However, emerging evidence now indicates that TRAIL receptors can exist as pre-assembled oligomers prior to ligand stimulation [76]. Such a pre-ligand receptor binding is mediated by the pre-ligand assembly domain (PLAD) located within the membrane distal CRD of TRAIL receptors. Interestingly, the PLAD mediates homotypic as well as heterotypic receptor associations between the death and decoy TRAIL receptors [77]. The balance

between homotypic versus heterotypic complexes may represent regulatory mechanism that controls cellular sensitivity to TRAIL. Indeed, ligand-independent association between DR5 and DcR2 has been shown to be responsible for the resistance of CD8⁺ T cells and other untransformed cells to TRAIL [78]. Decoy receptors thus may protect untransformed cells against TRAIL by the sequestration of TRAIL or by formation of heterotypic complexes that are unable to transmit death signal [79].

Moreover, post-translational modifications of death receptors have emerged as an important regulator of TRAIL signaling. Ligand-induced clustering of DR4 and DR5 is promoted by O-glycosylation of death receptors on their conserved extracellular O-glycosylation sites. Interestingly, elevated expression of O-glycosylation enzymes (GALNT14, GALNT3, FUT3 and FUT6) correlated with the sensitivity of diverse cancer cell lines to TRAIL-induced apoptosis [80]. A potential N-glycosylation site has been noted on DR4 [71]. The inhibition of N-glycosylation by tunicamycin modulated DR4 protein but not DR5 [81]. Palmitoylation of DR4 is required for the localization of receptor in lipid rafts and for its homo-oligomerization and hetero-oligomerization with DR5 [82]. The localization of death receptors in lipid rafts could be important for apoptotic signaling. TRAIL resistant non-small cell lung cancer cells exhibited non lipid raft localization of death receptors compared to TRAIL sensitive cell lines. This non lipid raft localization was associated with inhibition of caspase-8 cleavage and activation of NF- κ B and ERK1/2 signaling [83]. Non lipid raft signaling was mediated by RIP and c-FLIP proteins, knockdown of either RIP or c-FLIP redistributed the DISC to lipid rafts, thereby switching the signaling from NF- κ B and ERK1/2 activation to caspase-8 initiated apoptosis [83].

Another process regulating death receptor signaling is their internalization. Receptors and ligands can internalize by clathrin-mediated endocytosis or by clathrin-independent endocytosis. These endocytotic pathways occurs at specialized membrane microdomains like

clathrin-coated pits, lipid rafts (sphingolipid and cholesterol enriched domains) or caveolae (special membrane invaginations). Internalization of cell surface receptors has long been considered as a mechanism that terminates signaling by lysosomal receptor degradation. However, experimental data indicates that internalized receptors can be active and that endocytosis is important for the diversification of intracellular signaling. Regarding death receptors, clathrin-mediated internalization of both, TNFR1 and FAS is a prerequisite for apoptosis signaling. When these receptors are not internalized, antiapoptotic signaling mediated by activation of NF- κ B and MAPKs occurs through RIP1 and TRAF2 binding to TNFR1 or low level recruitment of caspase-8 to FAS, respectively [84]. Inhibition of TNFR1 endocytosis thus results in the resistance to apoptosis induced by TNF α . Adenoviruses use the inhibition of TNFR1 internalization by adenoviral protein E3-14.7K to prevent TNF α -mediated apoptosis of infected cells [85]. The role of receptor internalization in TRAIL signaling is not so obvious. Stimulation of death receptors with TRAIL resulted in their rapid internalization together with FADD and caspase-8 by both clathrin mediated and non-clathrin mediated pathways [86-87]. The blockage of TRAIL receptor endocytosis did not inhibit TRAIL-induced apoptosis but rather amplify it. Moreover, stimulation of TRAIL receptors induced caspase-mediated cleavage of the clathrin pathway components and terminated TRAIL receptor endocytosis [86]. These reports suggest that TRAIL-stimulated internalization of its cognate receptors proceeds through both clathrin-dependent and clathrin-independent pathways, but TRAIL-induced apoptosis signaling also occurs in the absence of TRAIL receptor endocytosis. Austin et al. [86] suggest that the interaction between TRAIL death receptors and clathrin endocytic pathway represents positive feedback loop that reinforces apoptotic signaling through the extrinsic pathway. According to this model, clathrin machinery remains intact in response to subthreshold ligand levels and endocytosis of death receptors opposes apoptotic signaling to preserve cell survival. However, if death receptor

stimulation generates enough caspase activity to disrupt endocytosis, then the signal amplifies further caspase activation and promotes apoptosis. Endocytosis of TRAIL receptors can be associated with TRAIL resistance. Zhang et al. [88] found that death receptors in some TRAIL resistant breast cancer cells undergo constitutive endocytosis. Inhibition of endocytosis by pharmacologic inhibitors or disruption of clathrin endocytic pathway restores cell surface expression of death receptors and sensitizes TRAIL resistant cells to TRAIL-induced apoptosis.

1.2.2. Biological role of TRAIL

The physiological function of endogenous TRAIL is not fully defined. Mice with knockout DR5 (the only mouse DR) or TRAIL are viable and develop normally [89-90] suggesting that this death ligand and its receptors do not have an essential developmental function. The major roles of TRAIL have been found in the regulation of immune response.

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. Takeda et al. found that TRAIL is important for the protection of mice against development of tumors induced by chemical carcinogen and neutralization of TRAIL promoted development of spontaneous tumors in p53 heterozygote mice [91]. TRAIL receptor deficiency in mice enhances lymph node metastasis of chemically induced skin squamous cell carcinoma without affecting primary tumor development [92]. Moreover, TRAIL-knockout mice are more susceptible to experimental tumor metastasis [90] and develop spontaneously disseminated lymphomas during their life [93]. Schmaltz et al. found that TRAIL is involved in the graft-versus-tumor effect in the bone marrow transplantation model [94].

Besides the involvement in antitumor surveillance, TRAIL was implicated in innate immunity against virus infection. TRAIL resistant colonic epithelial cells upregulate TRAIL

receptors after infection with cytomegalovirus and become TRAIL sensitive [95]. Hepatitis C virus (HCV) infection sensitized host cells to TRAIL-induced apoptosis by upregulation of DR4 and DR5 [96]. Polymorphisms of DR4 associated with increased viral loads in patients with chronic HCV infection and increased risk of hepatocellular carcinoma in these patients [97]. Treatment of HCV infected patients with interferon alpha induced TRAIL expression on NK cells [98]. Infection of T cells with HIV upregulated the expression of TRAIL and its death receptors. Interestingly, HIV induced the expression of TRAIL splice variant that inhibited DR5 and renders cells TRAIL resistant [99]. Similarly, infection of mice with influenza virus resulted in the upregulation of TRAIL and DR5 in the lungs of infected mice [100]. Moreover, TRAIL regulates magnitude of the CD8 T cell response against influenza virus, influenza virus infected TRAIL-deficient mice exhibited increased morbidity and mortality due to increase cytotoxic activity [101]. These results suggest that TRAIL is involved both in the direct cytotoxic activity of infected cells, and in the regulation of antiviral response mediated by cytotoxic cells.

Apart from the antitumor and antiviral immunity TRAIL has also been implicated in the modulating of autoimmune response. It was supposed that TRAIL signaling is involved in intrathymic negative selection that is responsible for removal of potentially autoreactive thymocytes. Nevertheless, aged TRAIL^{-/-} mice do not show the signs of spontaneous autoimmunity compared to wild-type mice suggesting that TRAIL has not a critical role in negative selection [102]. TRAIL-deficiency in mice, however, has been shown to increase susceptibility to experimentally induced autoimmune arthritis [103], diabetes [104] and autoimmune encephalomyelitis [105].

1.2.3. TRAIL as therapeutic agent

Most of the currently used cytotoxic drugs initiate cell death by triggering the intrinsic apoptotic pathway [106]. Deregulation of the intrinsic apoptotic pathway is a common cause of drug resistance. Targeting death receptors initiates the extrinsic apoptotic pathway and thus enables induction of apoptosis even in the chemotherapy resistant cancer cells.

Since its discovery, TRAIL has been investigated as a potential anti-cancer agent. Several forms of soluble recombinant human TRAIL (rhTRAIL) have been prepared up to now. Besides untagged rhTRAIL, other forms contain exogenous polypeptide tags that enable purification and/or stabilization of a trimer (these include polyhistidine-tagged (His-TRAIL), leucine-zipper (LZ)-tagged (LZ-TRAIL), FLAG-tagged (FLAG-TRAIL), isoleucine-zipper-tagged (iz-TRAIL)) [107-108].

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 [65-66, 109-112]. Unlike TNF α and FASL TRAIL proved to be non-toxic for normal untransformed cells. Although Jo et al. reported TRAIL hepatotoxicity [113], several studies have shown that hepatocytes are sensitive to the TRAIL variants with exogenous polypeptide tags but not to the untagged TRAIL molecule [108, 114]. Safety evaluation revealed no signs of toxicity in cynomolgus monkeys and chimpanzees after administration of soluble TRAIL [115]. Pharmacokinetic studies showed rapid elimination of soluble TRAIL with a half-life approximately 3-5 min in rodents and approximately 23-31 min in nonhuman primates [115].

In a phase I study in patients with advanced cancers rhTRAIL was administered intravenously over 1 hour infusion for 5 days every 3 weeks at doses up to 30 mg/kg. At the tested dose range rhTRAIL was well tolerated and safe with mostly mild adverse events manageable with the symptomatic treatment [116]. The half-life of rhTRAIL was approximately 0.5-1 hour and the peak serum concentrations corresponded to those in

preclinical testing (147 µg/ml at 15 mg/kg dose). Despite the promising results of preclinical and phase I studies the combination of rhTRAIL with paclitaxel, carboplatin, and bevacizumab in patients with advanced non-small-cell lung cancer did not improved outcome [117]. 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).

An alternative approach to induce apoptosis by targeting the TRAIL apoptotic pathway represents utilization of the agonistic monoclonal antibodies against TRAIL death receptors that functionally act as ligands. Compared to rhTRAIL the monoclonal antibodies directed against death receptors show substantially longer half-life (approximately 10-20 days [118]). On the other hand, the disadvantage of monoclonal antibodies might be their monospecificity (they engage only one of the two death receptors). In some cancer cells, either DR4 or DR5 might be more responsive, so stimulation of both receptors (as in the case of rhTRAIL) might be beneficial [107]. Phase I and II clinical trials evaluating pharmacokinetics, safety and anti-cancer properties have been conducted with the following antibodies: anti-DR4: mapatumumab (HGS-ETR1), anti-DR5: lexatumumab (HGS-ETR2), conatumumab (AMG-655), drozitumab (Apomab), tigatuzumab (TRA-8/CS-1008), and LBY135 [118].

Considering the efficacy of rhTRAIL in clinical trials it is worth noting that its dose-limiting toxicity has not been reached and thus the doses 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.

1.2.4. Mechanisms of resistance to TRAIL-induced apoptosis

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

1.2.4.1 Death receptors and TRAIL resistance

The expression of death and decoy receptors can influence TRAIL sensitivity. The upregulation of decoy receptors has been postulated as a possible mechanism causing the resistance of tumor cells by competing for TRAIL with death receptors. Additional mechanisms by which decoy receptors could cause TRAIL resistance have been described recently. DcR1 prevents the assembly of DISC by titrating TRAIL within lipid rafts, whereas DcR2 is corecruited with DR5 and inhibits initiator caspase activation [79].

The genes coding TRAIL receptors are located on the chromosome 8p21-22, which is a frequent site of allelic deletions in tumors. Rubio-Moscardo et al. showed that hemizygous loss of the death receptor genes resulted in the downregulation of their cell surface expression, thus impaired TRAIL-induced apoptosis through a gene dosage effect [119]. In other words, loss of the death receptor genes might act in a dominant negative manner toward remaining alleles. Lee et al. found death receptor mutations in 6.8% of lymphoma samples (5 diffuse large B-cell lymphomas, 1 peripheral T-cell lymphoma, 2 mucosa-associated lymphoid tissue (MALT)-type lymphomas). Of the eight detected mutations, six were missense (two DR4 and four DR5) and one nonsense (DR5) [120]. No mutation of the death receptor genes has been reported in chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and follicular lymphoma (FL) so far, although several DR4 gene polymorphisms have been detected [121-122]. It is suggested that these polymorphisms decrease apoptosis induced by

TRAIL. Liu et al. found no mutations in 46 samples from the patients with chronic myelogenous leukemia (CML), and in 18 samples from the patients with myelodysplastic syndrome (MDS) [123].

Posttranslational modifications of death receptors have emerged as a potential mechanism regulating TRAIL sensitivity. O-glycosylation of death receptors promotes TRAIL-induced clustering of DR4 and DR5. The expression of O-glycosyl transferase GALNT14 correlated with TRAIL sensitivity in pancreatic carcinoma, non-small-cell lung cancer and melanoma cells. Similarly, the expression of GALNT3, FUT3 and FUT6 correlated with TRAIL sensitivity in colorectal cancer [80]. Palmitoylation of DR4 is required for the localization of receptor in lipid rafts [82]. TRAIL resistant non-small cell lung cancer cells exhibited non lipid raft localization of death receptors compared to TRAIL sensitive cell lines. This non lipid raft localization was associated with the inhibition of caspase-8 cleavage and the activation of NF- κ B and ERK1/2 signaling [83].

Subcellular localization of death receptors also regulates the sensitivity to TRAIL. The lack of surface TRAIL receptors with predominantly cytoplasmic expression was described in breast cancer cells and correlated with the activity of death ligands [124]. Death receptors in some TRAIL resistant breast cancer cells undergo constitutive endocytosis and the inhibition of endocytosis by pharmacologic inhibitors restores cell surface expression of death receptors and sensitize TRAIL resistant cells to TRAIL-induced apoptosis [88].

Besides TRAIL receptors, dysfunction of other DISC components can lead to TRAIL resistance. FADD^{-/-} mouse embryonic fibroblasts transfected with DR4 or DR5 are resistant to TRAIL compared to FADD^{+/-} fibroblasts or FADD^{-/-} fibroblasts with reconstituted FADD [125]. FADD-deficient Jurkat cells resist high concentrations of TRAIL, in contrast wild-type Jurkat cells undergo extensive apoptosis at low TRAIL concentrations. Similarly, caspase-8-deficient Jurkat cells are completely resistant to TRAIL, whereas the wild-type cells remain

sensitive to TRAIL [126]. Decreased or absent caspase-8 expression was also found in a variety of primary tumor samples including Ewing's tumor, neuroblastoma, malignant brain tumors, melanoma, and small-cell lung cancer. In most cases, the loss of caspase-8 expression resulted from the gene silencing by methylation of the caspase-8 regulatory sequences. Restoration of the caspase-8 expression by gene transfer or by demethylation using 5-aza-2'-deoxycytidine resensitized cells to TRAIL-induced apoptosis [127-128]. A role of caspase-10 in TRAIL mediated apoptosis is controversial. Sprick et al. showed that caspase-10 is recruited to the DISC but is not required for apoptosis induction. Moreover, resistance to apoptosis observed in caspase-8-deficient Jurkat cells could not be rescued by the caspase-10 overexpression [12]. On the other hand, the transfection of MCF-7 breast carcinoma cells with caspase-10 sensitizes them to TRAIL-induced apoptosis [129]. In addition, TRAIL resistance of the caspase-8-deficient neuroblastoma cells can be reverted by the overexpression of caspase-10A or caspase-10D isoforms, but not by caspase-10B or -10G isoforms [130]. Increased expression of the caspase-8 competitive inhibitor FLIP has been observed in several tumor types and it contributed to TRAIL resistance at the DISC level by prevention of procaspase-8 recruitment and activation [131].

1.2.4.2 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. Murine embryonic fibroblasts (MEFs) from Bax and Bak double knockout animals are TRAIL resistant compared to MEFs from Bax or Bak single knockout animals [132]. These results suggest an overlapping function of Bax and Bak in mediating of TRAIL-induced apoptosis in these cells. Nevertheless, it does not seem to be the case for other cell types. Human colon cancer cells and leukemic cells

deficient in Bax are TRAIL resistant despite the expression of Bak [133-137]. On the other hand, Bak was shown to be indispensable for apoptosis induced by cytostatics in leukemic cells [138]. Bax dependency of TRAIL-induced apoptosis in cancer cells and inability of Bak to induce apoptosis can be explained by selective interactions among the BCL-2 family members. Unlike Bax Bak binds only to MCL-1 and BCL-XL, but not to other anti-apoptotic BCL-2 members [59]. Truncated Bid generated during the induction of extrinsic apoptotic pathway binds with high affinity to BCL-XL but not MCL-1 [60]. Indeed, TRAIL treatment of leukemic K-562 cells resulted in binding of tBid to BCL-XL but not MCL-1 [139]. Moreover, TRAIL induced increased binding of MCL-1 to Bak. These data suggest that tBid is not sufficient to release Bak from its inhibitor MCL-1. Gillissen et al. confirmed that Bax dependency of TRAIL-induced apoptosis is determined by MCL-1 but not BCL-XL [140].

Deregulation of the BCL-2 family is a common finding in tumor cells. Treatment of colon cancer cell lines with TRAIL resulted in the selection of TRAIL resistant clones with mutations in Bax gene [136]. Many mantle cell lymphomas harbour homozygous deletion of BIM and the silencing of BIM by promoter methylation was described in Burkitt lymphoma, the mutation and silencing of NOXA was present in diffuse large B-cell lymphoma [141]. The expression of BCL-XL correlated with the sensitivity to TRAIL-induced apoptosis in pancreatic adenocarcinoma cell lines and the inhibition of BCL-XL sensitized these cells to TRAIL [142]. The overexpression of BCL-XL mediated by Wilms tumor 1 transcription factor contributed to TRAIL resistance in acute myeloid leukemia cells [143]. In another study, the overexpression of BCL-2 conferred protection against TRAIL in neuroblastoma, glioblastoma, and breast cancer cell lines [144].

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 [145-146]. High expression of IAPs has been

shown to confer the resistance of prostate cancer cells to TRAIL [147-148]. The resistance of melanoma cell lines to TRAIL was associated with reduced release of Smac/Diablo from mitochondria to cytosol [149].

Figure 6 provides an overview of mechanisms that mediate the resistance to TRAIL-induced apoptosis.

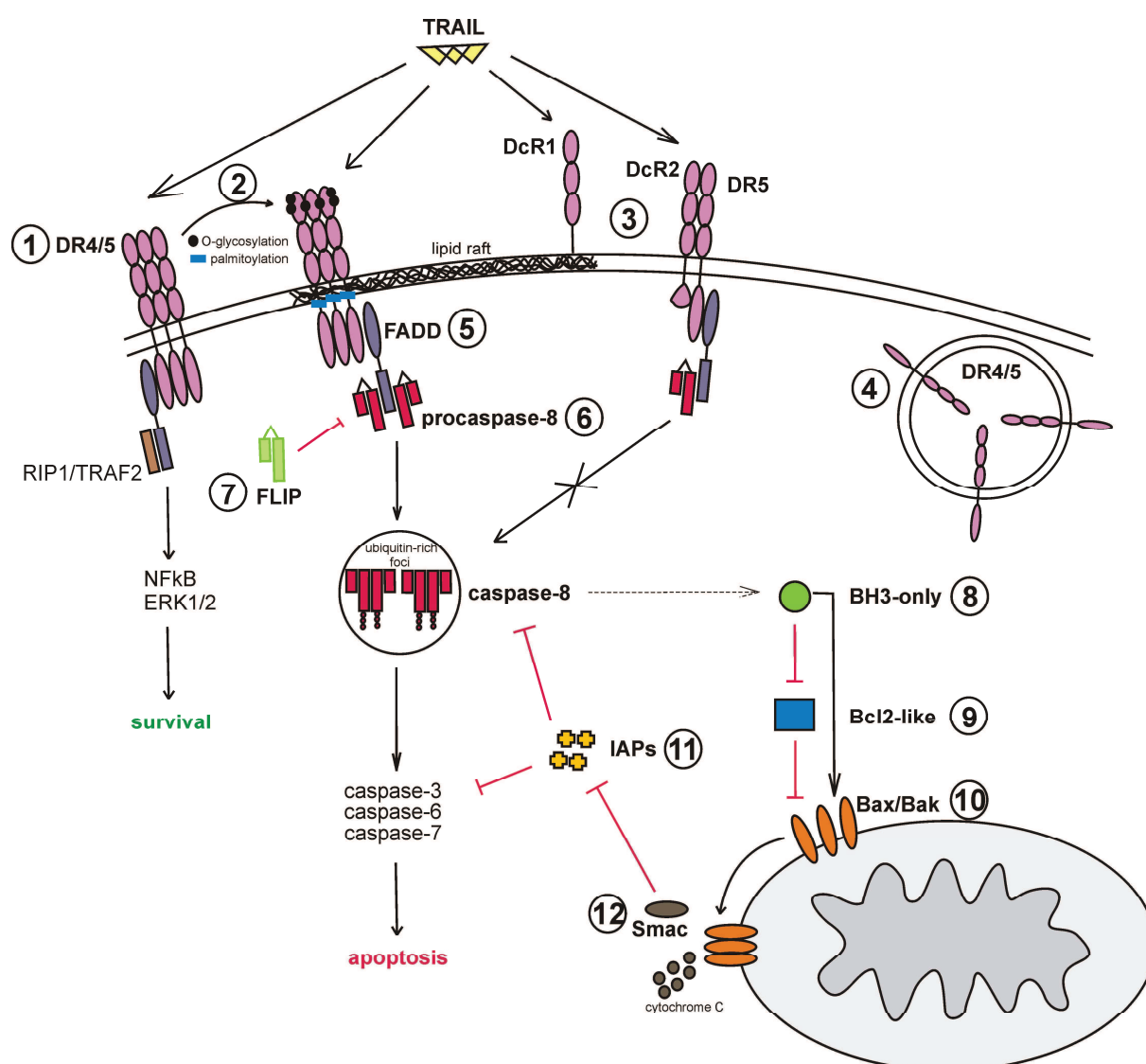


Figure 6. Overview of mechanisms that mediate the resistance to TRAIL-induced apoptosis. 1) impaired expression of DRs; 2) posttranslational modifications of DRs (mediate lipid raft localization and increase DR oligomerization); 3) expression of DcRs; 4) constitutive endocytosis of DRs; 5) FADD deficiency; 6) caspase-8 deficiency; 7) competitive inhibitors of caspase-8 binding and activation; 8) deficiency of BH3-only proteins; 9) overexpression of BCL2-like proteins; 10) Bax/Bak deficiency; 11) overexpression of IAPs; 12) Smac deficiency. For a more detailed explanation see the text.

2 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 [24].

Recently, it was reported that TRAIL resistant glioma, breast cancer, and thyroid carcinoma cells can be sensitized to TRAIL-induced apoptosis by roscovitine, an inhibitor of CDK [150-152]. Previous studies ascribed TRAIL-sensitizing effect of roscovitine to the downregulation of several antiapoptotic proteins, namely MCL-1, XIAP and survivin [150-151]. Roscovitine-mediated inhibition of transcription by decreased phosphorylation of CTD of RNA polymerase II through inhibition of CDK7 [153] was considered as a mechanism responsible for the downregulation of these short-lived proteins [154-156]. Roscovitine was also reported as a potent *in vitro* inducer of apoptosis in many primary hematologic malignant cells, including CLL [157], DLBCL [158], MCL [159] and MM cells [154]. 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 roscovitine 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

3 MATERIALS AND METHODS

3.1 Materials

Chemicals

ABT-737 (*Selleck Chemicals, USA*)

30% Acrylamide/Bis Solution 29:1 (*Bio-Rad Laboratories, USA*)

Actinomycin D (*Sigma-Aldrich, USA*)

Ammonium persulfate (*Sigma-Aldrich, USA*)

Annexin V FITC and PE conjugated (*Apronex Biotechnologies, Czech Republic*)

Bradford reagent (*Amresco, USA*)

CFSE (5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester) (*Life Technologies, USA*)

Cycloheximide (*Sigma-Aldrich, USA*)

Dithiothreitol (DTT) (*R&D, USA*)

EDTA (ethylenediaminetetraacetic acid, disodium salt: dihydrate) (*Sigma-Aldrich, USA*)

EGTA (ethyleneglycoltetraacetic acid) (*Amresco, USA*)

Fetal bovine serum (FBS) (*Lonza, Switzerland*)

Ficoll-Paque (*GE Healthcare, USA*)

Glycine (*Sigma-Aldrich, USA*)

Hydrochloric acid (*Penta, Czech Republic*)

HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) (*Sigma-Aldrich, USA*)

Iscove's Modified Dulbecco's Medium (IMDM) (*Lonza, Switzerland*)

iQ SYBR Green Supermix (*Bio-Rad Laboratories, USA*)

Laemmli Sample Buffer (*Bio-Rad Laboratories, USA*)

Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) (*Amresco, USA*)

2-Mercaptoethanol (*Sigma-Aldrich, USA*)

Methanol (methylalcohol) (*Carl Roth, Germany*)

Nonidet P40 (*Sigma-Aldrich, USA*)

PEG-it Virus Precipitation Solution (*System Biosciences, USA*)

Pen-Strep (penicillin/streptomycin) (*Lonza, Switzerland*)

Phenylmethylsulfonylfluoride (PMSF) (*Sigma-Aldrich, USA*)

Phosphatase inhibitor cocktails (*Sigma-Aldrich, USA*)

Phosphate buffered saline (PBS) (*Amresco, USA*)

Pierce ECL Western Blotting Substrate (*Thermo Fisher Scientific, USA*)

Potassium chloride (*Lach-Ner, Czech Republic*)

Precision Plus Dual Color Protein Standards (*Bio-Rad Laboratories, USA*)

Propidium iodide (*Sigma-Aldrich, USA*)

Protease inhibitor cocktail (*Sigma-Aldrich, USA*)

Puromycin dihydrochloride (*Sigma-Aldrich, USA*)

Ribonuclease A (*Life Technologies, USA*)

RIPA buffer (*Sigma-Aldrich, USA*)

RNAlater (*Qiagen, USA*)

R-roscovitine (2-(R)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine)
(*kindly provided by prof. Strnad, Palacky University, Czech Republic*)

Sodium citrate dihydrate (*Lach-Ner, Czech Republic*)

Sodium Dodecyl Sulfate (SDS) (*Promega, USA*)

Sodium fluoride (*Sigma-Aldrich, USA*)

Sodium orthovanadate (*Sigma-Aldrich, USA*)

Streptavidin-agarose beads (*Thermo Fisher Scientific, USA*)

Sucrose (*Sigma-Aldrich, USA*)

SuperFasLigand (*Enzo Life Sciences, USA*)

TEMED (tetramethylethylenediamine) (*Sigma-Aldrich, USA*)

TNF α (*Enzo Life Sciences, USA*)

TRAIL (recombinant human, and biotin-labeled) (*Apronex Biotechnologies, Czech Republic*)

Triton X-100 (polyethylene glycol *tert*-octylphenyl ether) (*Sigma-Aldrich, USA*)

Trizma base (2-amino-2-(hydroxymethyl)-1,3-propanediol) (*Sigma-Aldrich, USA*)

Z-IETD-FMK (*R&D, USA*)

Z-LEHD-FMK (*R&D, USA*)

Z-VAD-FMK (*R&D, USA*)

Antibodies

anti-Actin, HRP conjugated (*Santa Cruz Biotechnology, USA*)

anti-Bad (*Santa Cruz Biotechnology, USA*)

anti-Bak (*Cell Signaling Technology, USA*)

anti-Bax (*Cell Signaling Technology, USA*)

anti-Bcl-2 (*BD Biosciences, USA*)

anti-Bcl-xL (*Cell Signaling Technology, USA*)

anti-Bid (*Santa Cruz Biotechnology, USA*)

anti-Caspase-8 (*Enzo Life Sciences, USA*)

anti-Caspase-10 (*MBL International, USA*)

anti-COX IV (cytochrome c oxidase) (*Cell Signaling Technology, USA*)

anti-FADD (*BD Biosciences, USA*)

anti-FLIP (*Enzo Life Sciences, USA*)

anti-Mcl-1 (*BioVision, USA*)

anti-Phospho-eIF2 α (*Cell Signaling Technology, USA*)

anti-Puma (*Cell Signaling Technology, USA*)

anti-RNA Polymerase II (clones 8WG16, H5, H14) (*Covance, USA*)

anti-TRAIL-R1, FITC-conjugated (*Exbio, Czech Republic*)

anti-TRAIL-R2, FITC-conjugated (*Exbio, Czech Republic*)

anti-XIAP (*BioVision, USA*)

Peroxidase-conjugated AffiniPure F(ab')₂ Fragment donkey anti-Mouse, anti-Rabbit, and anti-Goat IgG (H+L) (*Jackson ImmunoResearch Laboratories, USA*)

Kits

High Capacity cDNA Reverse Transcription Kit (*Life Technologies, USA*)

Illumina RNA Amplification Kit (*Life Technologies, USA*)

Quick Cell Proliferation Assay Kit (*BioVision, USA*)

RNeasy Mini Kit (*Qiagen, USA*)

Cell lines

Cell lines were obtained from German Collection of Microorganisms and Cell Cultures (Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) with the exception of HBL-2, which was a kind gift of prof. Dreyling (University of Munich, Germany). We used these cell lines:

DB	established from the ascitic fluid of a patient with diffuse large B-cell lymphoma
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DOHH-2	established from the pleural effusion of a patient with refractory immunoblastic B-cell lymphoma progressed from follicular lymphoma
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EB-1	established from the maxilla tumor of a patient with Burkitt lymphoma
GRANTA-519	established from the peripheral blood of a patient with relapse of mantle cell lymphoma
HBL-2	established from the cervical lymph node of a patient with mantle cell lymphoma
HEK293T	derived from human embryonic kidney cells, expressing SV40 large T antigen
HL-60	established from the peripheral blood of a patient with acute myeloid leukemia (AML M2)
JEKO-1	established from the peripheral blood of a patient with mantle cell lymphoma
JURKAT	established from the peripheral blood of a patient with acute lymphoblastic leukemia
K-562	established from the pleural effusion of a patient with chronic myeloid leukemia in blast crisis

MC-116	established from the pleural effusion of a patient with B-cell lymphoma (undifferentiated lymphoma)
ML-2	established from the peripheral blood of a patient with acute myeloid leukemia (AML M4)
NU-DHL-1	established from the inguinal lymph node of a patient with diffuse large B-cell lymphoma
NU-DUL-1	established from the cerebrospinal fluid of a patient with diffuse large B-cell lymphoma
OPM-2	established from the peripheral blood of a patient with multiple myeloma (IgG lambda)
RAJI	established from the maxilla of a patient with Burkitt lymphoma
RAMOS	established from the ascitic fluid of a patient with Burkitt lymphoma
REC-1	established from the lymph node or peripheral blood of a patient with refractory mantle cell lymphoma
RPMI-8226	established from the peripheral blood of a patient with multiple myeloma (IgG lambda)

SC-1	established from the ascitic fluid of a patient with follicular lymphoma
SU-DHL-1	established from the pleural effusion of a patient with anaplastic large cell lymphoma (ALCL)
THP-1	established from the peripheral blood of a patient with acute monocytic leukemia

Animals

For xenotransplantation of human leukemia and lymphoma cells NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ immunodeficient mice (Jackson Laboratory, USA) were used. Mice were bred in individually ventilated cages in the specific pathogen-free facility of the Center of Experimental Biomodels, First Faculty of Medicine, Charles University in Prague, maintained in a clean conventional part of the facility during the experiments with a light-dark cycle of 12 hours, and fed ad libitum. Eight- to twelve-month-old female mice were used in the experiments. All experiments were approved by the Laboratory Animal Care and Use Committee of the First Faculty of Medicine, Charles University in Prague and were performed in accordance with national and international guidelines for laboratory animal care.

3.2 Methods

3.2.1 Cell culture

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 in 37 °C humidified atmosphere with 5% CO₂. Peripheral 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 density-gradient centrifugation and cultured in IMDM supplemented with 20% FBS. 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).

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 TNF α , FASL, cycloheximide, actinomycin D, ABT-737, and TRAIL for 24 or 48 hours. In caspase inhibition experiments, K-562 and RAMOS cells were pre-treated with 100 μ M 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.

3.2.2 Proliferation assays

To measure effect of R, T and combination of R+T on proliferation, we used Quick Cell Proliferation Assay Kit according to the manufacturer instructions. Briefly, we incubated 20 000 cells with each drug alone or in combination for various time periods (0-192 hours) and measured absorbance of the samples after 2 hour incubation with WST-8 reagent using Sunrise microplate reader (Tecan Group, Switzerland).

3.2.3 Flow cytometry

- *Analysis of apoptosis*

Cells were incubated with FITC conjugated annexin V in dark at the room temperature for 15 minutes and analyzed by flow cytometry (FACSCanto II cytometer; Becton Dickinson, USA). Percentage of apoptosis (Annexin-positive cells) was calculated as follows: Apoptosis (%) = Treatment (% apopt) – Control (% apopt) / 100 – Control (% apopt).

- *Cell-mediated cytotoxicity assay*

Peripheral blood mononuclear cells (PBMC) were isolated from a healthy volunteer by Ficoll-Paque density-gradient centrifugation. Prior to use, PBMC were stained by 1.5 μ M CFSE for 8 min at room temperature. Pre-warmed FBS was added and PBMC were incubated at 37°C for 10 min and washed with PBS. K-562 and RAMOS cells were treated for 24 hours with 40 μ M or 10 μ M roscovitine, respectively. After washing, cell lines were incubated with CFSE-labeled PBMC at a target to effector ratio 1:9 for 24 hours. Apoptosis was quantified by flow cytometry (FACSCanto II; Becton Dickinson, USA) in the CFSE-negative population using Annexin V-PE staining.

- *Cell cycle analysis*

K-562 and RAMOS cells were washed with PBS and resuspended in hypotonic buffer (50 μ g propidium iodide, 100 μ g Ribonuclease A, 1 mg Sodium citrate, 1 μ l Triton X-100,

distilled water to 1 ml). Subsequently, samples were measured by flow cytometer (FACSCanto II; Becton Dickinson, USA) and analysed using ModFit LT software (Verity Software House, USA).

- ***Cell surface expression of TRAIL receptors***

K-562 and RAMOS cells were treated with roscovitine for 24 hours and 48 hours, stained with FITC conjugated anti –TRAIL-R1 and –TRAIL-R2 antibodies and analyzed by flow cytometry (FACSCanto II; Becton Dickinson, USA). Untreated cells stained with the secondary antibodies and isotype controls served as controls.

3.2.4 Gene expression analysis

- ***Real-time RT-qPCR analysis***

Total RNA was extracted from K-562 cells treated with 40 μ M roscovitine for 1.5, 3, 6, 12 and 24 hours by RNeasy Mini Kit, and reverse-transcribed using High Capacity cDNA Reverse Transcription Kit. Real-time qPCR was run for 40 cycles (95°C for 10 seconds, 60°C for 20 seconds, 72°C for 30 seconds) on 7900 HT SDS PCR cycler (Life Technologies, USA) using SYBR Green-based kit. 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 μ M roscovitine for 1.5, 6, and 24 hours as described above. Quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Extracted RNA was amplified using the Illumina RNA Amplification Kit. Amplified RNA was hybridized to the Illumina HumanRef-8 BeadChip (Illumina, USA). Subsequent data analysis was performed in R-software, mainly in limma package from Bioconductor (<http://www.bioconductor.org>). Multiple testing correction

was performed using Benjamini & Hochberg method [160]. 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>) [161].

3.2.5 Western blotting

- **Total and mitochondrial proteins 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 hours by lysis in RIPA buffer containing protease and phosphatase inhibitors, phenylmethylsulfonylfluoride (PMSF) and dithiothreitol (DTT). For mitochondrial extracts, K-562 cells treated with 40 μM roscovitine for 3, 6, 12 and 24 hours were incubated in a lysis buffer (250 mM Sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA), passed through 25G needle and left on ice for 20 minutes. Supernatant obtained after centrifugation (5 min at 3000 rpm) was centrifuged again 30 min at 8000 rpm and mitochondrial pellet was resuspended in RIPA buffer. Samples were used immediately or stored at -80°C .

- **DISC immunoprecipitation by streptavidin-agarose beads**

K-562 cells were left untreated or treated with 40 μM roscovitine for 12 hours and 24 hours. Subsequently, cells were pre-cooled for 15 minutes in the fridge and incubated with 1 $\mu\text{g} / \text{ml}$ of biotinylated TRAIL first on ice for 15 minutes to saturate death receptors and then quickly warmed to 37°C for 15 and 40 minutes. A time 0' control was included at this point to observe receptor status before DISC complex induction with biotinylated TRAIL at 37°C . After 15 or 40 minutes cells were transferred on ice and washed with ice-cold PBS. Cell lysis was performed in 200 μl of a lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% Glycerol, 10 mM EDTA, 1% NP-40, 10 mM NaF, 10 mM PMSF, 1 mM Na_3VO_4 , with 10 μM

each leupeptine and aprotinin) on ice for 30 minutes following centrifugation at 12 000 rpm for 30 minutes at 4 °C. After centrifugation 20 µl of supernatant were collected in a separate tube for the 'cell lysate' control, and mixed with 10 µl of 2% SDS sample buffer. Remaining part of supernatant was mixed with 15 µl of equilibrated streptavidin-agarose beads according to the manufacturer and samples were rotated at 2000 rpm for 1 hour at 4 °C. Streptavidin-agarose beads were then centrifuged at 2000 rpm for 2 minutes and supernatant was discarded after 20 µl of supernatant were collected in a separate tube for the 'flow through' control, and mixed with 10 µl of 2% SDS sample buffer. Beads were washed 5 times with the cell lysis buffer and protein complexes were eluted from the beads by the addition of 30 µl SDS sample buffer and heating at 95 °C for 15 minutes. Proteins were separated in SDS-PAGE and immunoblotted for caspase-8, caspase-10, FADD, and FLIP.

- **Western blotting**

Samples were mixed with Laemmli Sample Buffer and boiled for 5 min. After separation by SDS-PAGE, proteins were transferred to Immun-Blot PVDF Membrane (Bio-Rad Laboratories, USA). Membranes were blocked for 1 hour in 2% skim milk and incubated with appropriate primary antibodies overnight at 4 °C. Subsequently, the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse, -rabbit or -goat secondary antibodies for 1 hour at room temperature. Proteins were visualized using Pierce ECL Western Blotting Substrate. As a load control we used HRP-conjugated anti-actin antibody.

3.2.6 Lentivirus production and infection

pLKO.1 plasmids expressing shRNA against BCL-XL, MCL-1 were purchased from Sigma-Aldrich and used for the preparation of the recombinant in HEK293T cells. Viruses were concentrated from supernatant by the PEG-it Virus Precipitation Solution and freezed in

aliquots at -80 °C. K-562 cells were then infected with appropriate recombinant lentiviruses at MOI approximately 10 and the transduced cells were selected 2 days post-infection with the medium containing puromycin (3 µg / ml) for 4 days. Cells were analysed for the expression of particular protein by Western blot.

3.2.7 Xenotransplantation and treatment

The studies were approved by the institutional Animal Care and Use Committee. Female immunodeficient NOD.Cg-*Prkdc^{scid}Il2rg^{tm1Wjl}*/SzJ mice were maintained in individually ventilated cages. RAMOS and HBL-2 cells were harvested, suspended in PBS, and injected (10×10^6 / mouse) subcutaneously into the left dorsal flank of 8- to 12-week-old mice. When tumors reached 500 - 1000 mm³, animals were randomized into 4 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 the control group was injected with vehicle (PBS) intraperitoneally. Treatment schedule for combined treatment was: 2 doses of roscovitine in 12 hour interval on day 1, followed by single dose of TRAIL on day 2, followed by second treatment cycle on day 4. For single treatments roscovitine and TRAIL were dosed similarly as in combination. When tumors in the control group reached 2.5 – 3 cm in diameter, animals were sacrificed and tumors weighed.

3.2.8 Statistical analysis

The data were analysed in GraphPad Prism Software (GraphPad Software, USA, www.graphpad.com). The values represent means ± standard deviation (SD) of at least three independent experiments. The differences between experimental groups were determined by

Student's *t* test. P values < 0.05 were considered statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Synergism, additive, or antagonistic effects were calculated according to the formula: $SF_{R+T} < SF_R \times SF_T$ – synergism, $SF_{R+T} > SF_R \times SF_T$ – antagonism, $SF_{R+T} = SF_R \times SF_T$ – additive effect, where SF is surviving fraction after treatment with roscovitine, TRAIL or combination.

4 RESULTS

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

Despite the ability of TRAIL to induce apoptosis selectively in tumor cells, many of these tumors are constitutively TRAIL resistant and, moreover, even TRAIL sensitive tumor cells can acquire resistance under the selective pressure of TRAIL.

We have analyzed the sensitivity to TRAIL-induced apoptosis in 21 established leukemia and lymphoma cell lines. As shown in Figure 7A, 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 1000 ng/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 (acute myeloid leukemia - AML1-9, acute lymphoblastic leukemia - ALL1, chronic myeloid leukemia - CML1-2, chronic lymphocytic leukemia - CLL1-6, mantle cell lymphoma - MCL1-5, diffuse large B-cell lymphoma - DLBCL1, follicular lymphoma - FL1, and marginal zone lymphoma - MZL1). 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 1000 ng/ml TRAIL in 19 (i.e. 73%) from 26 samples (Figure 7B). The most resistant samples were those obtained from patients with AML (8/9, i.e. 88%), followed by CLL samples (4/6, i.e. 67%) and by MCL samples (3/5, i.e. 60%).

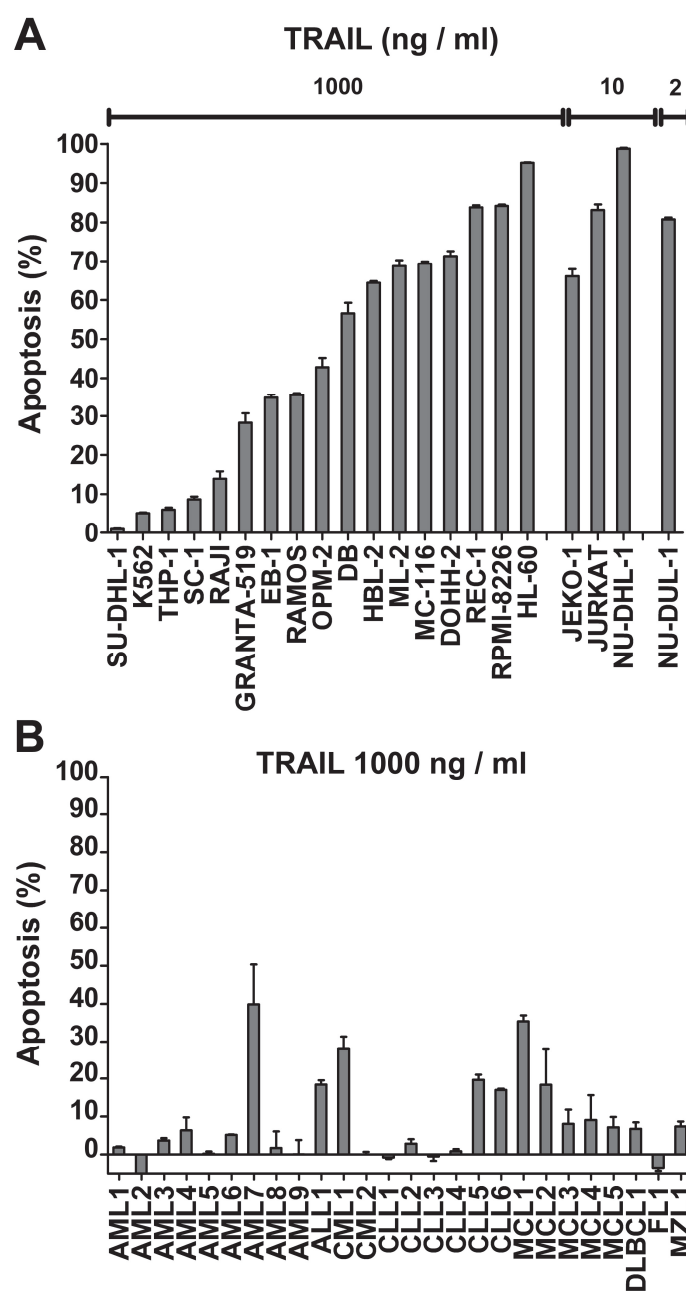


Figure 7. Sensitivity of leukemia and lymphoma cells to TRAIL induced apoptosis. (A-B) The percentage of Annexin V-FITC-positive, apoptotic cells induced by 24-hour exposure of cell lines **(A)** or primary cells **(B)** to TRAIL (concentrations as indicated). Columns represent means and error bars standard deviations of three independent experiments.

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

Roscovitine (seliciclib) is a purine analogue that competes with adenosine triphosphate (ATP) for its binding site on cyclin-dependent kinases (CDK) [162], and demonstrates selectivity towards CDK1, CDK2, CDK5, CDK7 and CDK9 [163]. Roscovitine was reported as a potent *in vitro* inducer of apoptosis in many primary hematologic malignant cells, including chronic lymphocytic leukemia cells [157], diffuse large B-cell lymphoma cells [158], mantle cell lymphoma cells [159] and multiple myeloma cells [154].

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. 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 hours. Similarly, low-toxic doses of TRAIL in the range between 0.25 to 1000 ng/ml were used to assess the drug synergism in the cell lines. Primary cells were exposed to the single concentration of 1000 ng/ml TRAIL with increasing doses of roscovitine (5, 10, 15, 20, 30, 40, and 60 μ M). 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 (Figure 8A, B). 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. Moreover, the combination of roscovitine and TRAIL synergistically suppressed proliferation of tested cell lines with the exception of SC-1 (Supplementary figure 1).

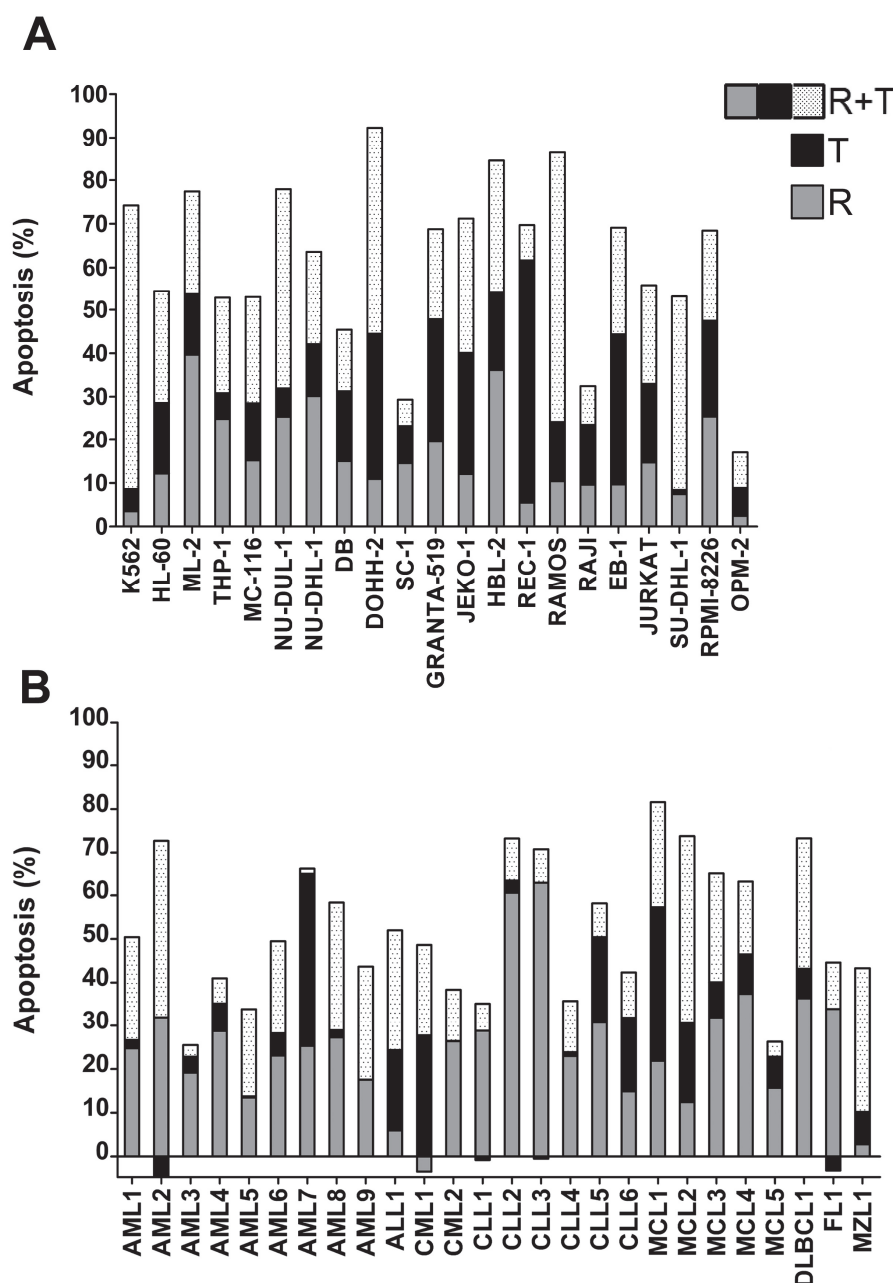


Figure 8. Roscovitine sensitizes leukemia and lymphoma cells to TRAIL induced apoptosis. The percentage of Annexin V-FITC-positive, apoptotic cells induced by 24-hour exposure of cell lines **(A)** or primary cells **(B)** to roscovitine (R), TRAIL (T) and combination of both (R+T), dotted part of the column represents synergism, i.e. percentage of apoptotic cells above the sum of both monotherapies. Columns represent means of three independent experiments, error bars are not shown for the clarity of the graphs.

Subsequently, we tested if roscovitine is able to sensitize the cells not only to TRAIL but also to other death ligands. Figure 9A shows that K-562 cells were more sensitive to TNF α -induced apoptosis while RAMOS cells were more sensitive to FASL-induced apoptosis when pretreated with low-toxic doses 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 (Figure 9B).

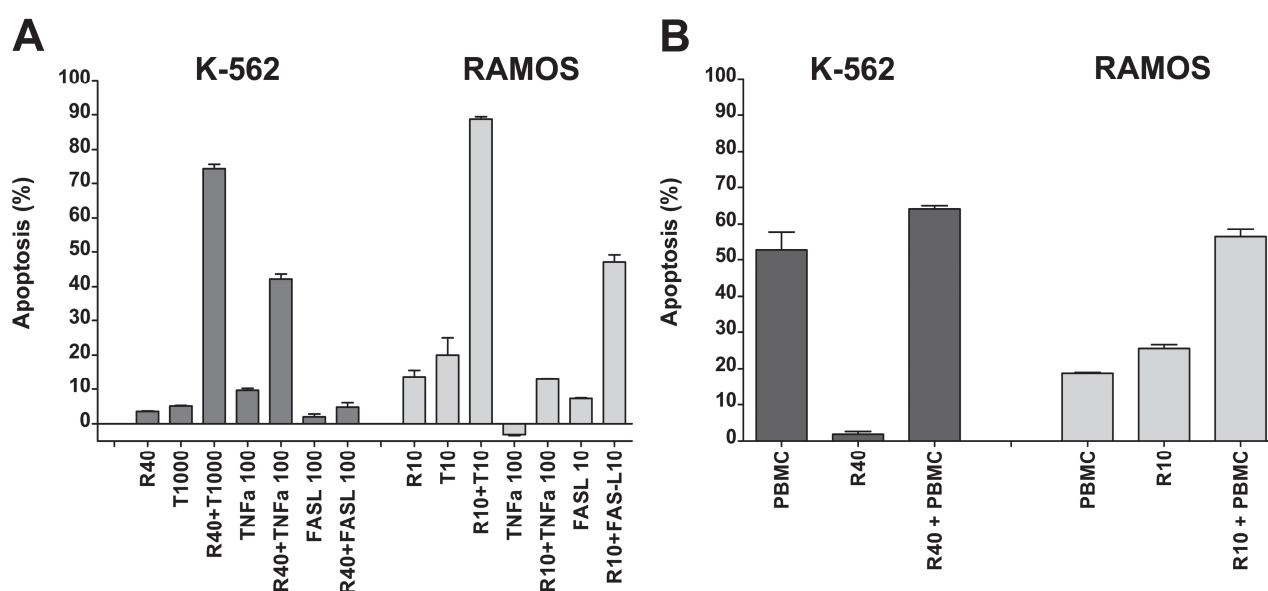


Figure 9. Roscovitine sensitizes leukemia and lymphoma cells to TNF α and FASL induced apoptosis, and enhances cell-mediated cytotoxicity. (A) The percentage of apoptotic K-562 or RAMOS cells after 24-hour treatment with TRAIL (T), TNF α , FASL and the combinations with roscovitine (R). **(B)** After pretreatment of K-562 and RAMOS cells with roscovitine for 24 hours, peripheral blood mononuclear cells from a healthy donor stained with CFSE were added and apoptosis measured after 24 hours in CFSE unstained population. Columns represent means and error bars standard deviations of three independent experiments.

4.3 Roscovitine induces long-term proapoptotic changes of the 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. As roscovitine is a selective inhibitor of CDKs we determined the effect of low-toxic doses of roscovitine on the distribution of cell cycle phases. Treatment with roscovitine decreased the number of cells in the S phase and caused partial cell cycle arrest in the G2/M phase (Figure 10A, B).

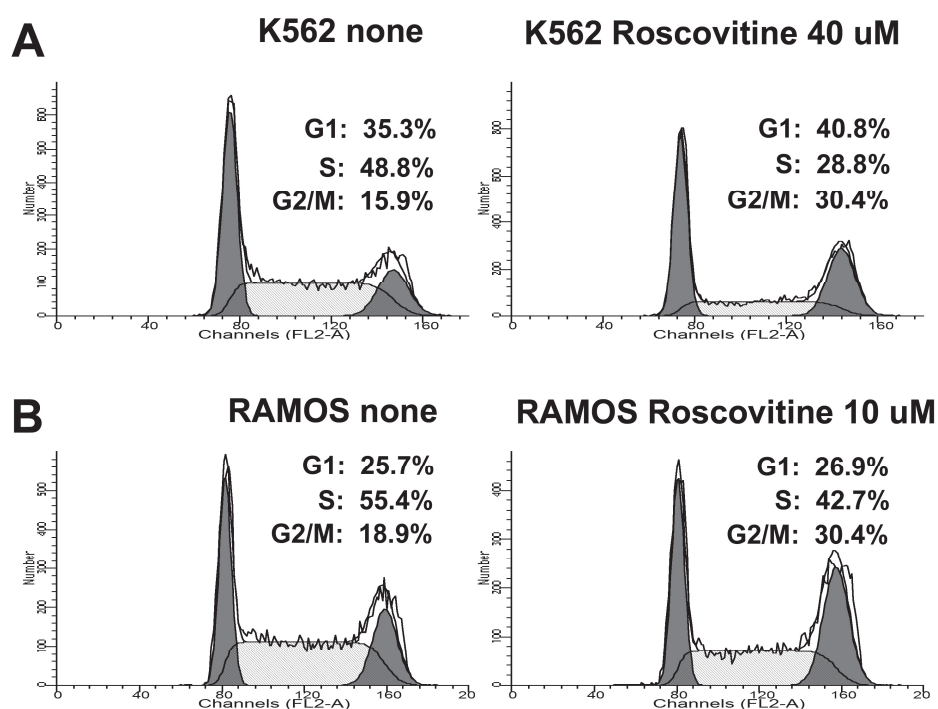


Figure 10. Roscovitine partially arrested cells in the G2/M phase of cell cycle. K-562 (A) and RAMOS (B) cells were treated with roscovitine for 24 hours (concentrations as indicated) and cell cycle distribution was measured after staining with propidium iodide by flow cytometer.

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 apoptosis induced by the combination of roscovitine and TRAIL (Figure 11A). 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 the formation of DISC in the roscovitine-treated cells. Cell surface expression of TRAIL death receptors on K-562 and RAMOS cells was not affected by the exposure to roscovitine for 24 hours (Figure 11B). 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 (increased p25/23) and caspase-10 (increased p30) proteins at the DISC (Figure 11C). We also noticed lower levels of FLIP-L and its pre-processed p43 form at the DISC of roscovitine treated K-562 cells.

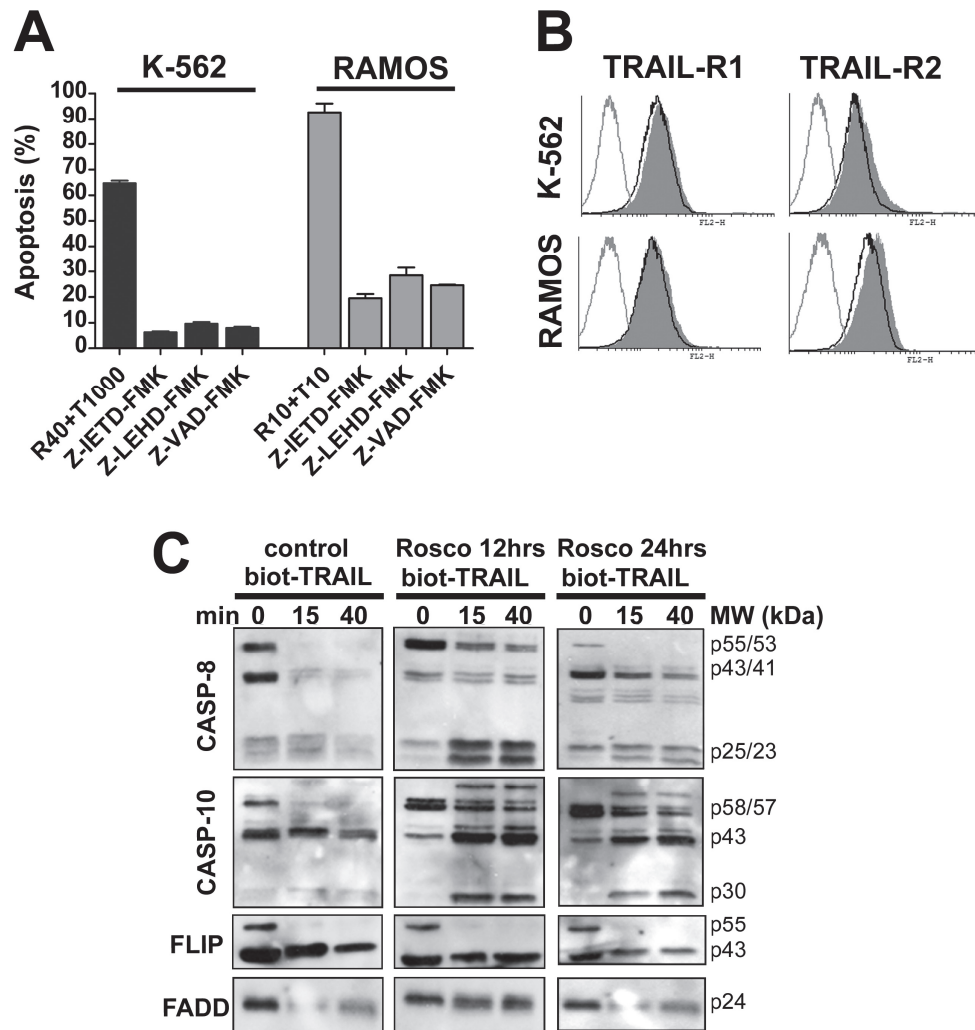


Figure 11. Roscovitine enhances DISC-mediated activation of the initiator caspases-8/10 without affecting cell surface expression of TRAIL receptors. (A) K-562 and RAMOS cells were exposed to the combination of roscovitine and TRAIL (concentrations as indicated) 1 hour after pretreatment with 100 μ M Z-VAD-FMK or Z-IETD-FMK or Z-LEHD-FMK caspase inhibitors. The level of apoptosis was determined after 24 h. Columns represent means and error bars standard deviations of three independent experiments. (B) Flow cytometry analysis of TRAIL-R1 and TRAIL-R2 expression on K-562 and RAMOS cells before (black line empty histograms) and after 24-hour treatment with roscovitine (filled histograms). Grey line empty histograms represent cells stained with the control isotype antibody. Histograms shown are representative examples from three independent experiments. (C) Western blots of the DISC proteins from biot-TRAIL-treated K-562 cells (0, 15 and 40 minutes after TRAIL addition). K-562 cell line was either untreated (control) or pre-treated with 40 μ M roscovitine for 12 and 24 hours prior to addition of biot-TRAIL.

4.4 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 (Figure 12A). Based on the results obtained from the 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 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 (Figure 12B). The microarray data clearly confirmed significant inhibitory effect of roscovitine on gene transcription. Supplementary figure 2 shows the expression patterns of selected gene functional groups (transcription factors, cell cycle genes, subunits of RNA polymerases). Previous studies reported that roscovitine inhibited transcription by abrogation of CDK7-mediated phosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II [159]. 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 12C). Because roscovitine influenced the expression of genes involved in translation and protein folding as well, we tested phosphorylation status of eukaryotic initiation factor - 2 alpha (eIF-2 α) in K-562 cells after treatment with roscovitine. Figure 12D demonstrates that the treatment with roscovitine induced phosphorylation of eIF-2 α after 12 and 24 hours suggesting an inhibition of translation. To test whether the inhibition of transcription or

translation can sensitize cells to TRAIL induced apoptosis we treated K-562 and RAMOS cells with actinomycin D (an inhibitor of transcription) and cycloheximide (an inhibitor of translation). Both drugs sensitized K-562 and RAMOS cells to TRAIL-induced apoptosis (Figure 12E).

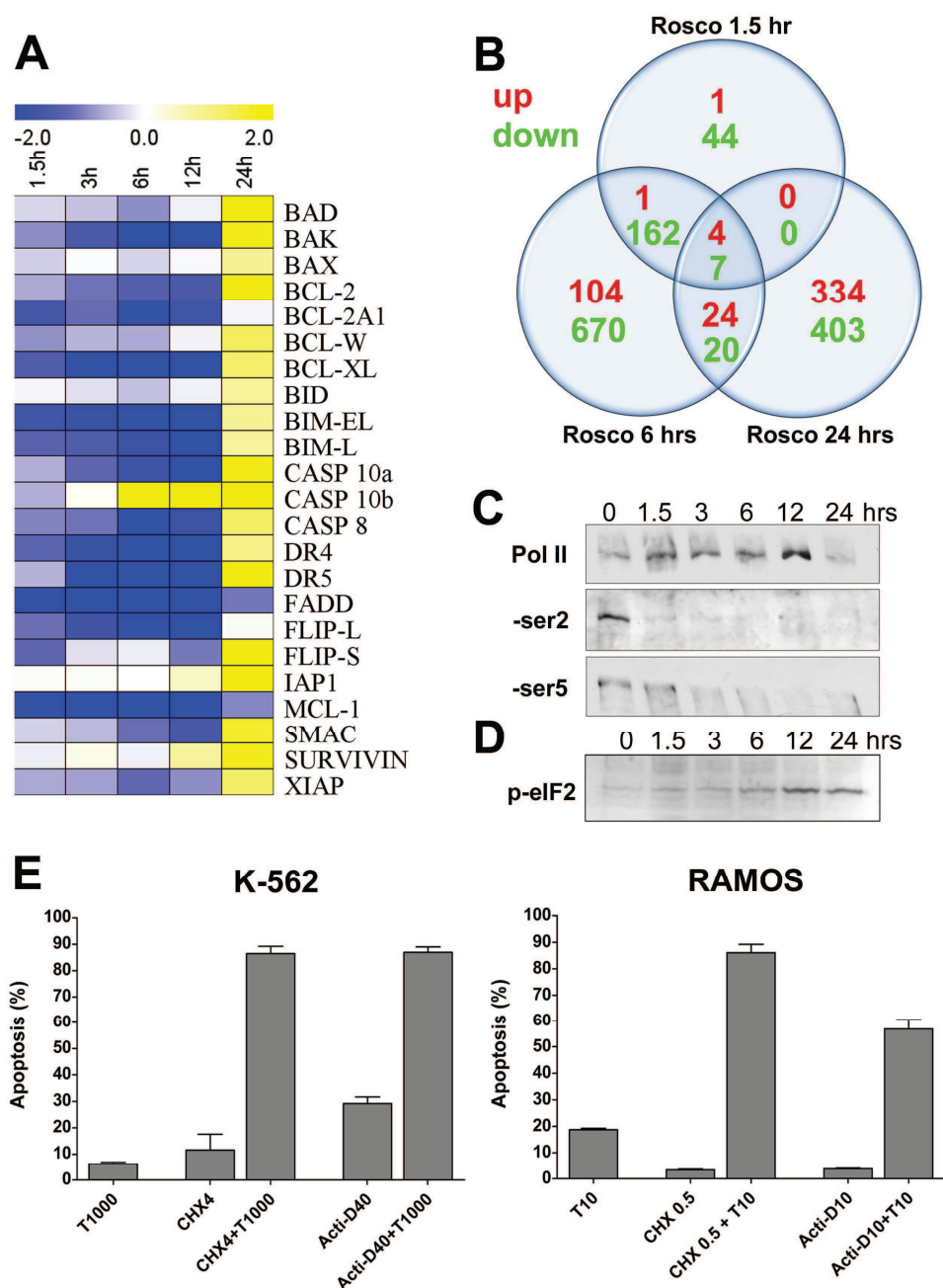


Figure 12 (previous page). Treatment with roscovitine induces the inhibition of transcription and translation. (A) The expression of several pro- and anti-apoptotic genes in K-562 cells was assessed by the real-time RT-PCR 1.5, 3, 6, 12 and 24 hours after treatment with roscovitine. The majority of genes were downregulated during 12 hours followed by the upregulation after 24 hours. Columns represent timepoints and rows correspond to genes. Color changes within a row indicate expression levels relative to the untreated controls (data analyzed using MeV4 software, color scale limits: $\log_{2}FC = \pm 2$, blue downregulation, yellow upregulation). (B) Global gene expression of K-562 cells was assessed by Illumina chips 1.5, 6 and 24 hours after treatment with roscovitine compared to untreated controls. Figure shows the Venn's diagram with number of genes upregulated or downregulated by more than 2-fold. (C) Western blot analysis of protein expression of RNA polymerase II and its phosphorylation on serine 2 and serine 5 of the CTD 1.5, 3, 6, 12 and 24 hours after treatment with roscovitine. (D) Phosphorylation status of translation initiation factor eIF2 α was assessed by western blotting of cell lysates prepared 1.5, 3, 6, 12 and 24 hours after treatment with roscovitine. (E) K-562 and RAMOS cell lines were treated with actinomycin D and cycloheximid alone or in combination with TRAIL (concentrations as indicated for actinomycin D and cycloheximid in μ M and for TRAIL in ng/ml) and apoptosis measured after 24 hours. Inhibition of transcription and translation by actinomycin D and cycloheximid, respectively, sensitized cells to TRAIL-induced apoptosis. Columns represent means and error bars standard deviations of three independent experiments.

4.5 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 the 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 (Figure 13A). Similarly to real-time RT-PCR and microarray data (Figure 12A) 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, the 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 the 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 (Figure 13B). From the analyzed proapoptotic BCL-2 proteins we observed moderate upregulation of BAD, BAK, and PUMA α at 1.5 hours, and upregulation of PUMA α at 24 hours.

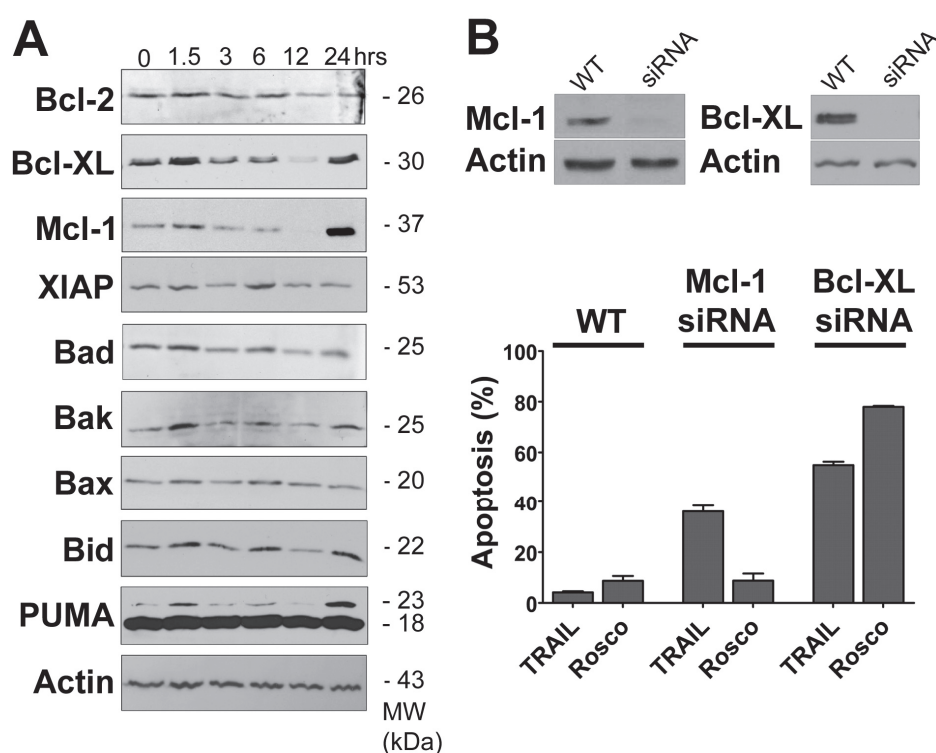


Figure 13. BCL-2 family proteins and mitochondria play an important role in proapoptotic effects of roscovitine in K-562 cells. (A) Western blots showing the protein expression of several pro- and anti-apoptotic regulators 1.5, 3, 6, 12, and 24 hours after treatment with roscovitine. **(B)** K-562 cells with down-regulated expression of MCL-1 or BCL-XL were treated with TRAIL or roscovitine for 24 hours and the induction of apoptosis was determined by Annexin V-FITC staining and flow cytometry. Columns represent means and error bars standard deviations of three independent experiments.

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

Co-treatment of malignant hematopoietic cells with low-toxic dose of roscovitine and TRAIL significantly enhanced 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-hour pretreatment with roscovitine was required to induce sensitization to TRAIL, but this sensitization persisted only for 6 hours after washing off the drug. In contrast cells treated with roscovitine for 24 hours remained sensitive to TRAIL even 24 hours after roscovitine removal, indicating strong establishment of long-lasting changes in cell physiology in favor of TRAIL-induced pro-death signaling (Figure 14A, B).

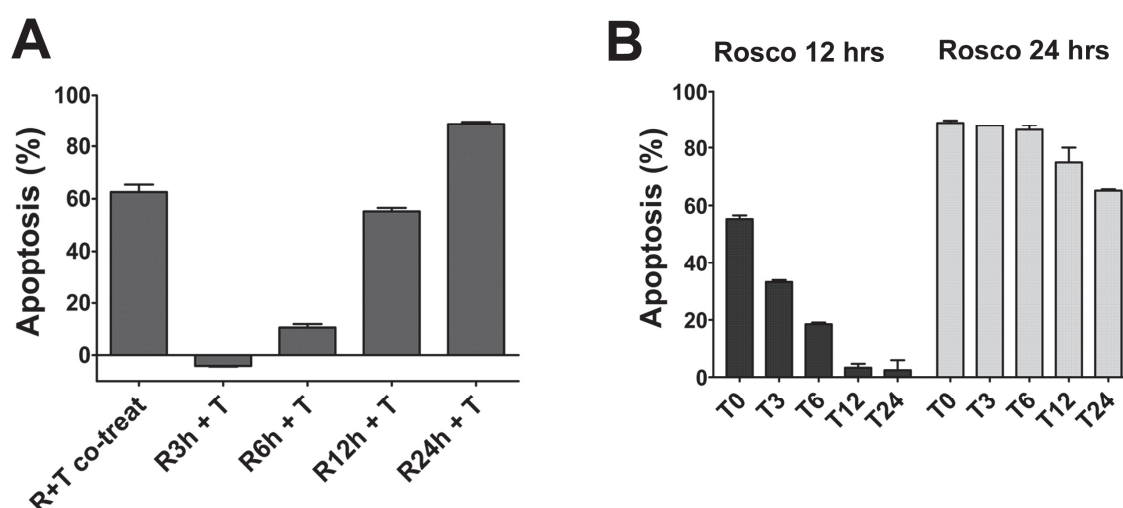


Figure 14. Roscovitine-induced sensitization to TRAIL is dependent on the length of pre-incubation. (A) K-562 cells were pre-treated with 40 μ M roscovitine for 3, 6, 12 or 24 hours (R3h, R6h, R12h, and R24h, respectively), then the cells were washed and TRAIL was added to the fresh medium at the final concentration 1 μ g/ml. Percentage of apoptotic cells was determined by Annexin V-FITC staining and flow cytometry after 24 hours of TRAIL exposure. **(B)** Further, K-562 cells were exposed to roscovitine either for 12 or 24 hours, and

then after extensive washing cells were incubated in the medium without roscovitine for 0, 3, 6, 12, and 24 hours (T0, T3, T6, T12, and T24, respectively). At each time point TRAIL was added and 24 hours later percentage of apoptotic cells was determined as above. Columns represent means and error bars standard deviations of three independent experiments.

4.7 Roscovitine induces recruitment of both pro- and anti-apoptotic BCL-2 proteins to 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 mitochondria, the protein expression levels obtained from whole cell lysates might not reliably reflect potential compartment-specific changes of the BCL-2 family protein levels at 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 PUMA β (Figure 15A). These results imply that the antiapoptotic BCL-2 proteins might sequester the 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 (Figure 15B).

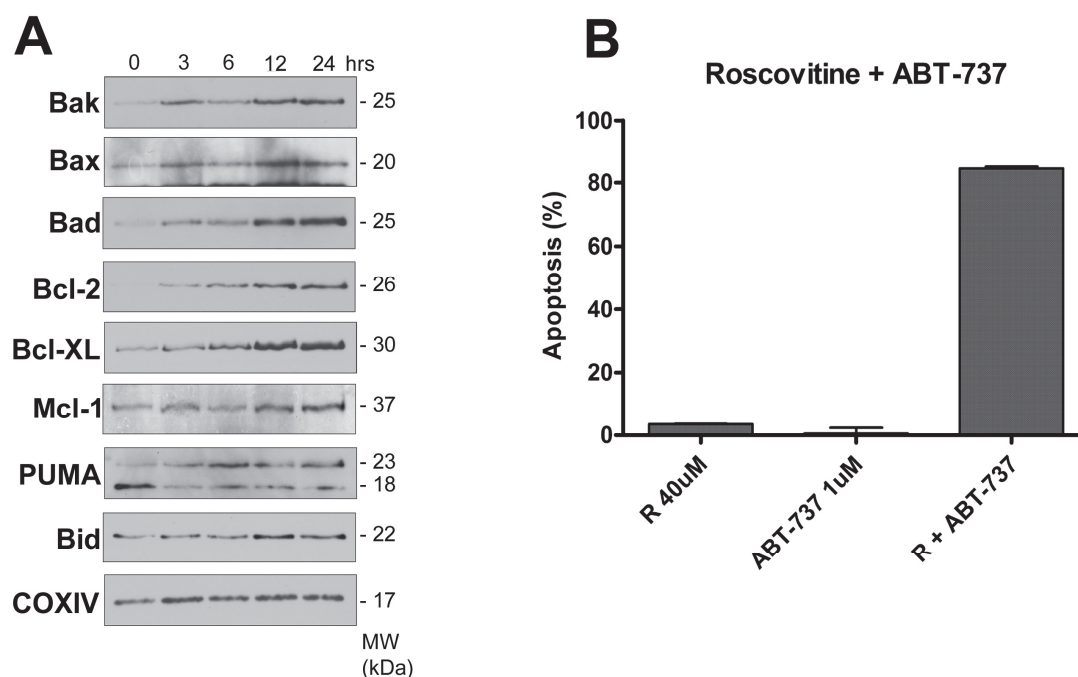


Figure 15. Roscovitine induces upregulation of BCL-2 family proteins at mitochondria. (A) Western blots showing mitochondrial expression of several pro- and anti-apoptotic regulators from the BCL-2 family 3, 6, 12, and 24 hours after treatment with roscovitine. (B) K-562 cells were treated with roscovitine (R), ABT-737 and combination of both (concentrations as indicated) and apoptosis was determined by Annexin V-FITC staining and flow cytometry. Columns represent means and error bars standard deviations of three independent experiments.

4.8 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 (Figure 16).

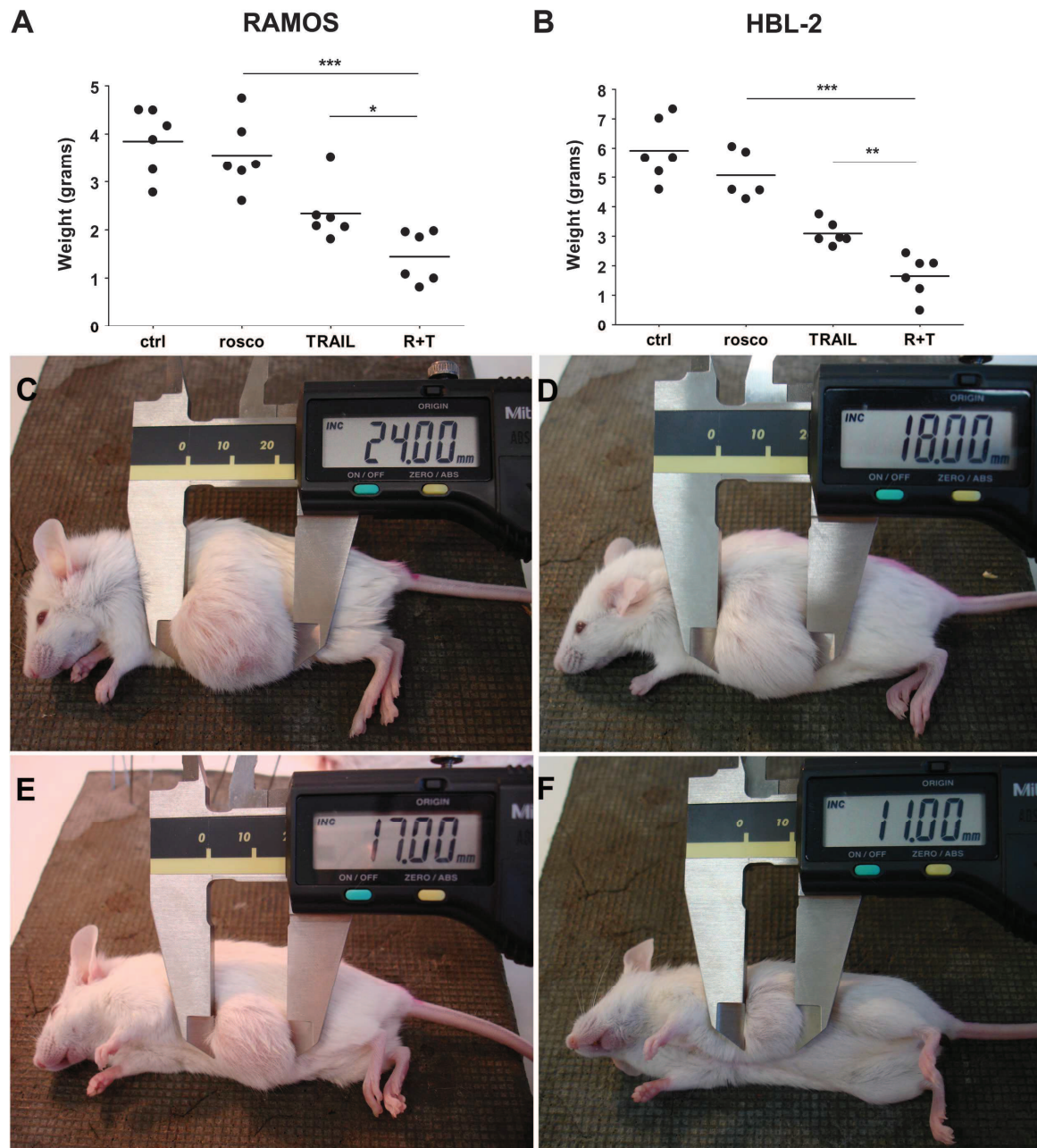


Figure 16. Roscovitine and TRAIL synergistically inhibit growth of subcutaneous lymphoma xenografts. Immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice were subcutaneously injected with RAMOS (A) or HBL-2 (B) cell lines. After tumors developed, mice were randomized and treated with 2 cycles of roscovitine (rosco), TRAIL (TRAIL) or with their combination (R+T) as described in Material and Methods. Control mice (ctrl) were injected with PBS. Dots represent weight of individual tumors and lines are plotted at the means. (C-F) Representative photographs of RAMOS cell line xenografted mice untreated (C), treated with roscovitine (D), TRAIL (E), and both (F). Significance of difference from the single treatments (R or T): *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

5 DISCUSSION

In this study we determined the sensitivity of diverse leukemia and lymphoma cell lines and primary cells to TRAIL-induced apoptosis. Next, we 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. In addition, 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 1000 ng/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 explanation could be immunoselective pressure of TRAIL exerted on tumor cells *in vivo* [164]. The most resistant samples were those obtained from the patients with AML followed by CLL and MCL samples. Our data are in agreement with previous reports [165-167].

Significant number of the 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 [168]. Recently, it was reported that glioma, breast cancer, and thyroid carcinoma cells can be sensitized to TRAIL-induced apoptosis by the purine analogue roscovitine [150-152]. Roscovitine (seliciclib) competes with adenosine triphosphate (ATP) for its binding site on CDK [162], and demonstrates selectivity towards CDK1, CDK2, CDK5, CDK7 and CDK9 [163]. Roscovitine was reported a potent *in vitro* inducer of apoptosis in many primary hematologic malignant cells, including CLL [157], DLBCL [158], MCL [159] and MM cells [154]. Therefore, we have tested if roscovitine is able to sensitize leukemia and lymphoma

cells to TRAIL-induced apoptosis. Roscovitine used at the low-toxic doses 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 TNF α 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 the 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 ubiquitination by cullin-3-based E3 ligase [21]. Alternatively, decreased expression of a competitive inhibitor that impedes binding of the 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 [169].

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 mitochondria. Previous studies ascribed TRAIL-sensitizing effects of roscovitine to the downregulation of several antiapoptotic proteins, namely MCL-1, XIAP and survivin [150-151]. Roscovitine-mediated inhibition of transcription by decreased phosphorylation of the CTD of RNA polymerase II through inhibition of CDK7 [153] was considered as a mechanism responsible for the downregulation of these short-lived proteins [154-156]. 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 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 the 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. 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 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 PUMA β . These results are in contrast with previous reports where the downregulation of antiapoptotic proteins was proposed as sensitizing mechanism. None of these studies, however, determined the expression of these proteins on the 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 the 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 [170-171]. This technique identified three classes of apoptotic blocks that cancer cells use to survive. The first block (or class A block) is characterized by low expression of 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 the 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 the proapoptotic 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 the 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 [139]. Such rearrangement of the 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 17.

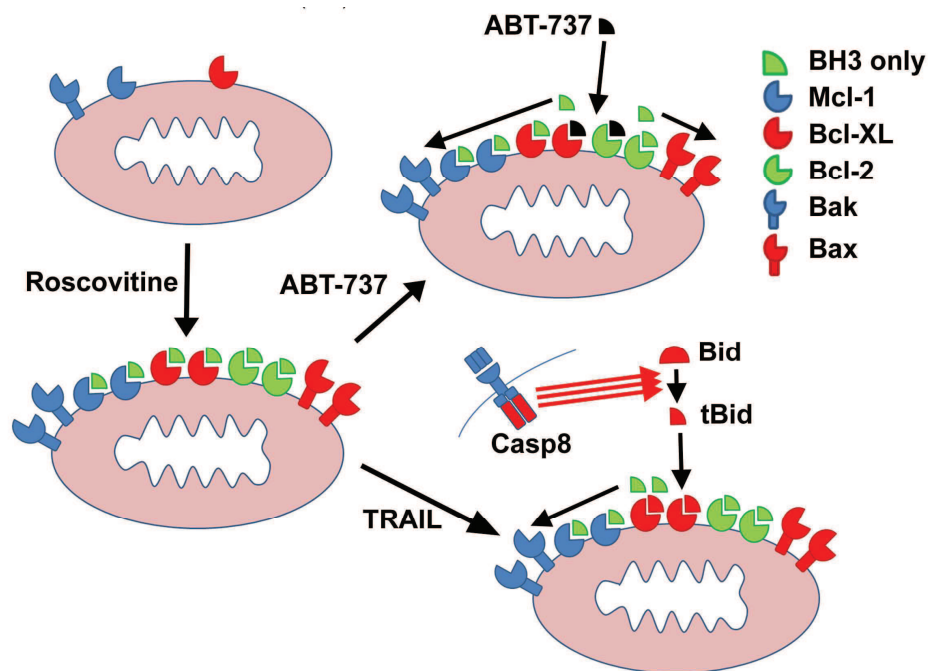


Figure 17. Proposed mechanism of roscovitine-mediated sensitization to TRAIL-induced apoptosis. Roscovitine induces upregulation of pro- and anti- apoptotic BCL-2 family members on mitochondria. Antiapoptotic BCL-2 family proteins sequester proapoptotic BH3-only proteins. Exposure of roscovitine-treated K-562 cells to ABT-737, an inhibitor of BCL-2, BCL-XL and BCL-W induces apoptosis by displacement of proapoptotic 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.

We demonstrated 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.

6 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 TNF α 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 the 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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8 LIST OF PUBLICATIONS

Related to the dissertation topic:

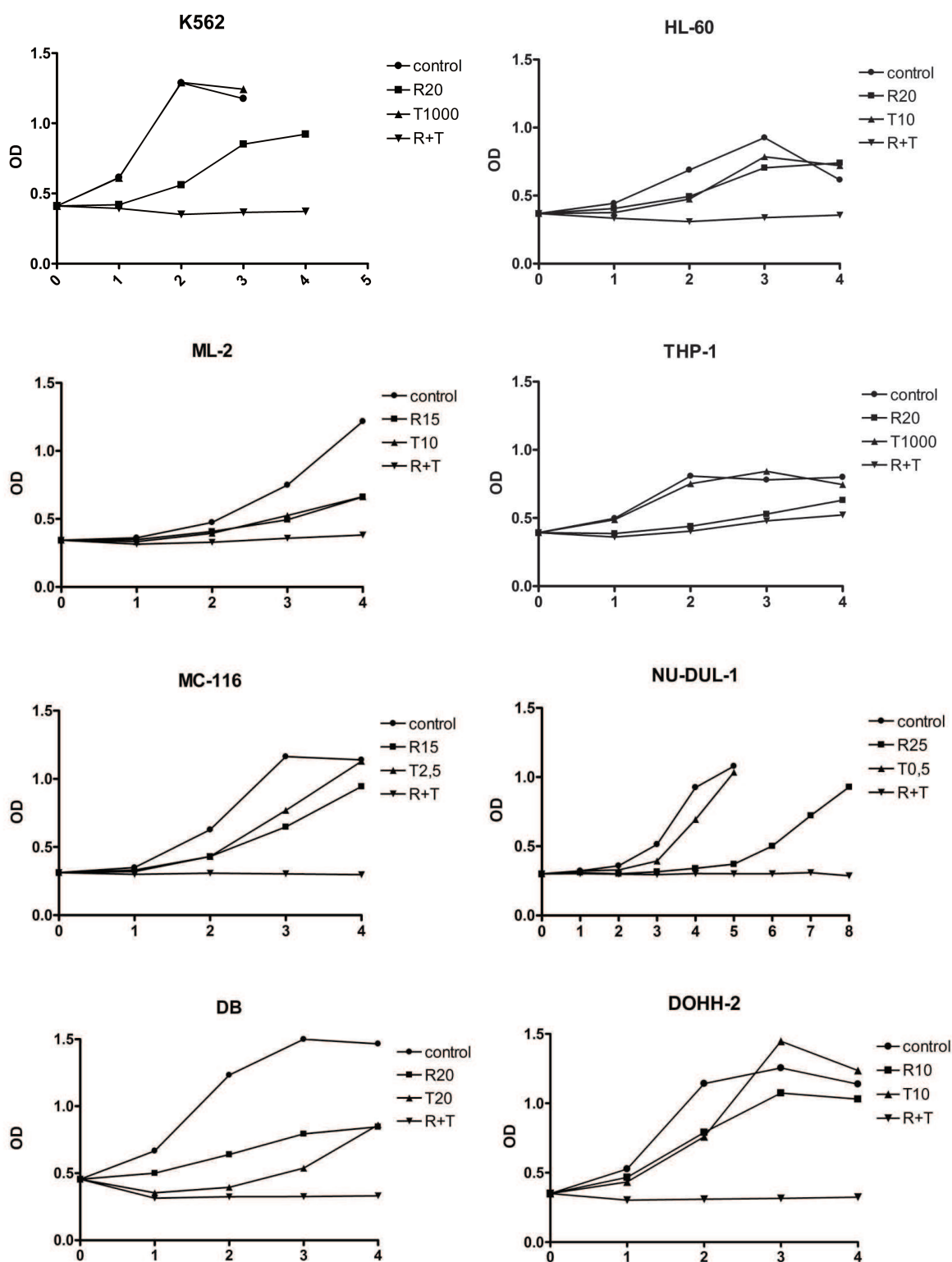
1. **Molinsky J**, Klanova M, Koc M, et al. Roscovitine sensitizes leukemia and lymphoma cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Leukemia & Lymphoma*. 2013;54(2):372-380. **IF = 2,580**
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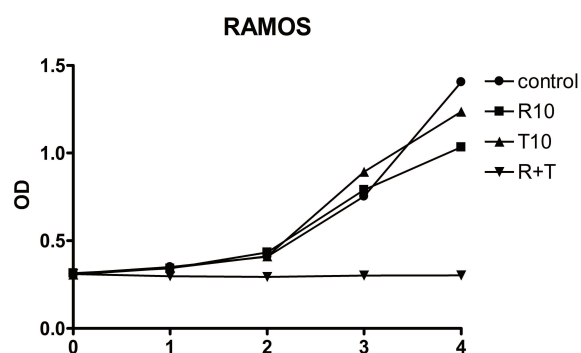
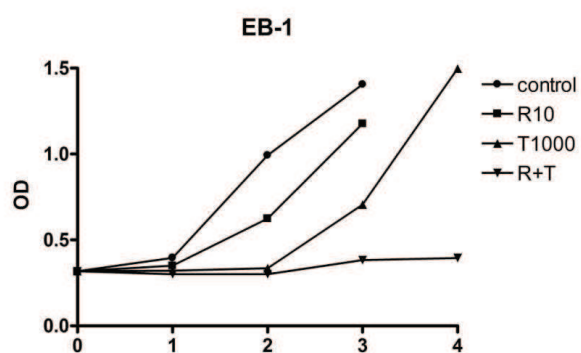
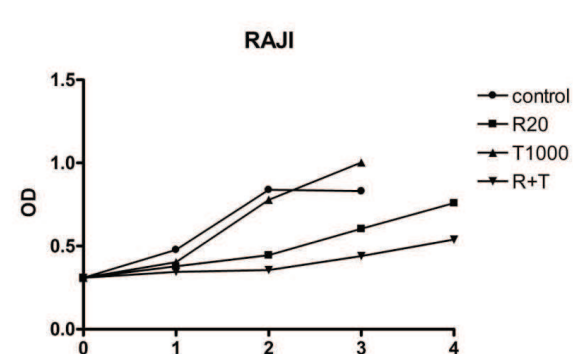
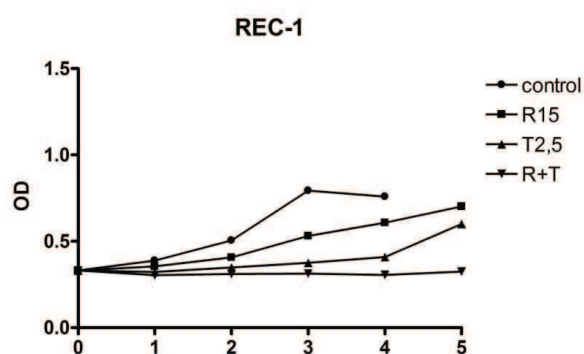
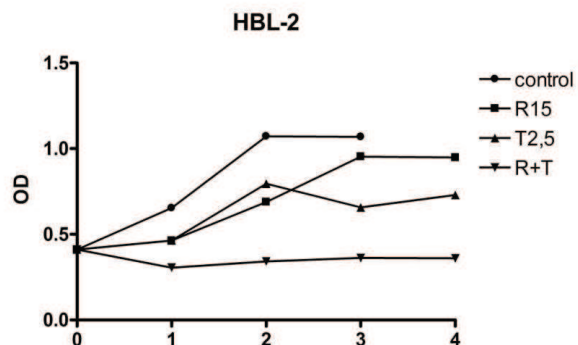
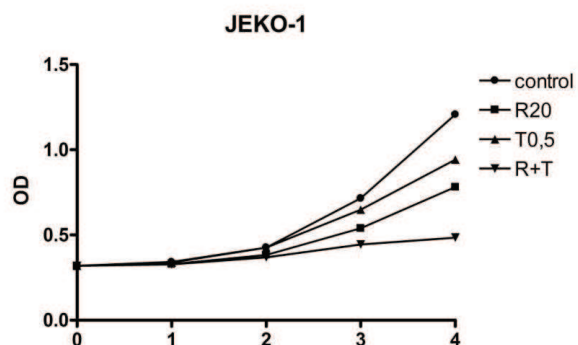
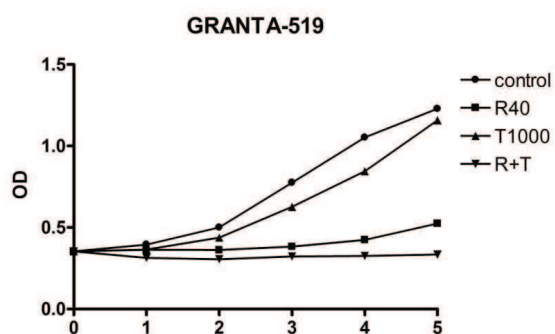
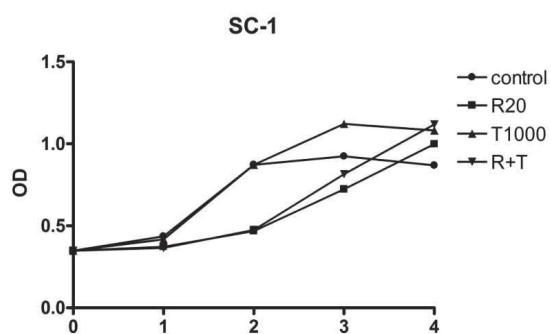
Others:

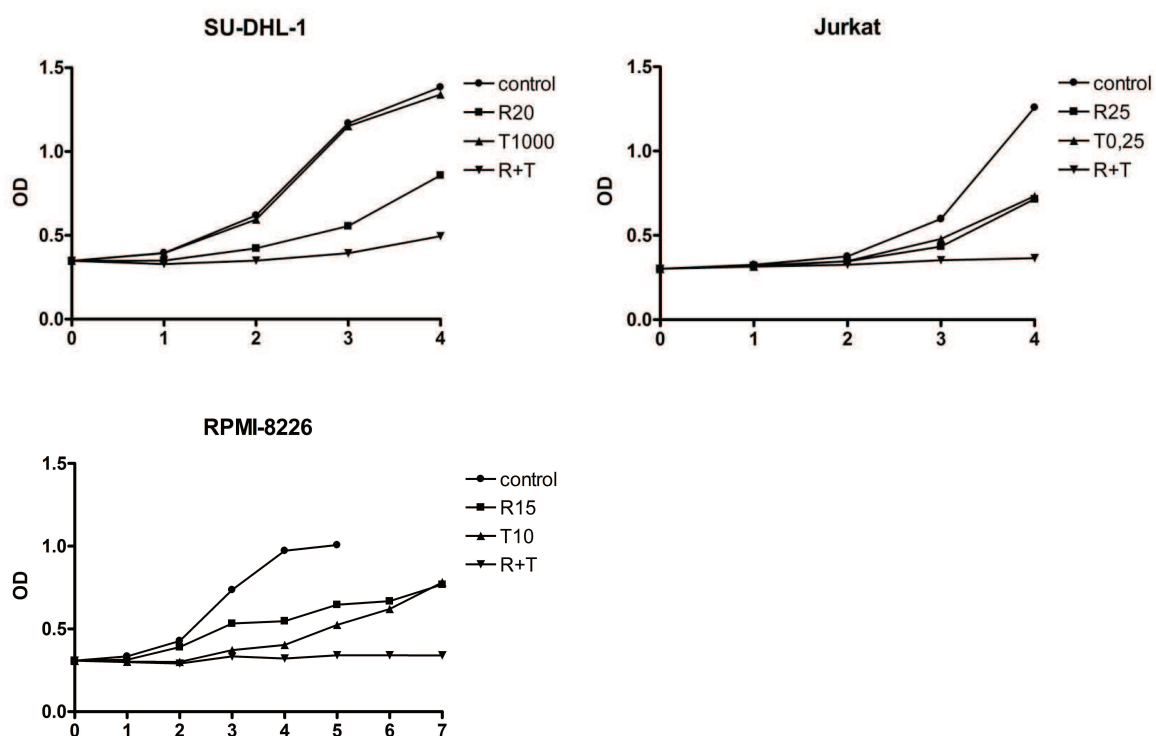
5. **Molinsky J**, Klanova M, Maswabi B, et al. In Vivo Growth of Mantle Cell Lymphoma Xenografts in Immunodeficient Mice Is Positively Regulated by VEGF and Associated with Significant Up-regulation of CD31/PECAM1. *Folia Biologica (Praha)*. 2013;59(1):26-31. **IF = 1,151**

9 APÊNDICES

9.1 Supplementary figure 1

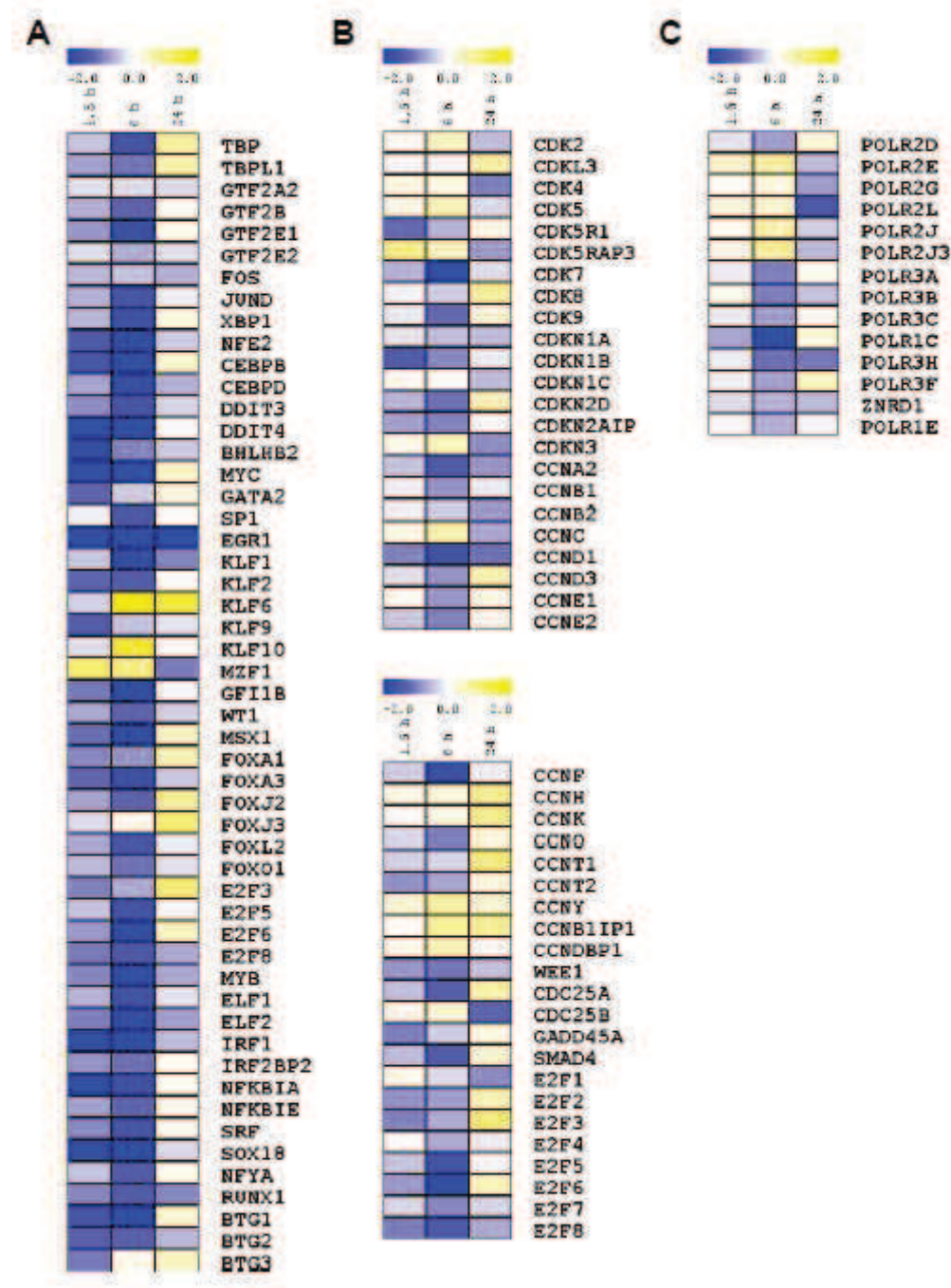






Supplementary figure 1. Cell lines were treated with roscovitine (R), TRAIL (T) and combination (R+T) of both (concentrations as indicated for TRAIL in ng/ml and for roscovitine in μ M) and proliferation measured as described in Material and Methods.

9.2 Supplementary figure 2



Supplementary figure 2. K-562 cells were treated with roscovitine for 1.5, 6 and 24 hours and global gene expression was assessed by Illumina chips. Figure shows expression patterns of selected gene functional groups compared to untreated controls: **(A)** transcription factors, **(B)** cell cycle genes, **(C)** subunits of RNA polymerases. Columns represent timepoints and rows correspond to genes. Color changes within a row indicate expression levels relative to the untreated controls (data analysed using MeV4 software, color scale limits: fold change = ± 2 , blue downregulation, yellow upregulation).

9.3 Publications related to the dissertation topic

ORIGINAL ARTICLE: RESEARCH

Roscovotine 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 roscovotine, a cyclin-dependent kinase inhibitor, sensitized various solid cancer cells to TRAIL. We show that roscovotine and TRAIL demonstrate synergistic cytotoxicity in hematologic malignant cell lines and primary cells. Pretreatment of TRAIL-resistant leukemia cells with roscovotine 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 roscovotine. These results suggest that roscovotine 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 roscovotine-induced sensitization to TRAIL in leukemia/lymphoma cells.

Keywords: Roscovotine, 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 roscovotine [9–11]. R-roscovotine (seliciclib) 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]. Roscovotine-induced blockage 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]. Roscovotine 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. Peripheral 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 TNF α 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 (MBL International), anti-caspase-8, FLIP (Alexis Biochemicals), anti-Mcl-1, XIAP (BioVision), anti-Bax, Bak, Bcl-XL, COX IV, phospho-eIF2 α , 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 TNF α , 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 μ M 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 μ M CFSE. K-562 and RAMOS cells were treated for 24 h with 40 μ M or 10 μ M 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 CFSE-negative population using annexin V-phycoerythrin (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 μ M roscovitine for 12 h and 24 h. After incubation with 1 μ g/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 μ M 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 20 s, 72 °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 μ M 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 μ M 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 μ M roscovitine for 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 $MgCl_2$, 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 $-80^{\circ}C$.

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 $-80^{\circ}C$. 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 μ g/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^{scid} Il2rg^{tm1Wjl}/SzJ* mice (Jackson Laboratory) were maintained in individually ventilated cages. RAMOS and HBL-2 cells were harvested, suspended in phosphate buffered saline (PBS) and injected (10×10^6 /mouse) subcutaneously into the left dorsal flank of 8- to 12-week-old mice. When tumors reached 500–1000 mm³, 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 μ L, TRAIL (250 μ g/mouse) was given intraperitoneally in a volume of 300 μ L, 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 $\pm$ 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: $SF_{R+T} < SF_R \times SF_T$: synergism; $SF_{R+T} > SF_R \times SF_T$: antagonism; $SF_{R+T} = SF_R \times SF_T$: 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,

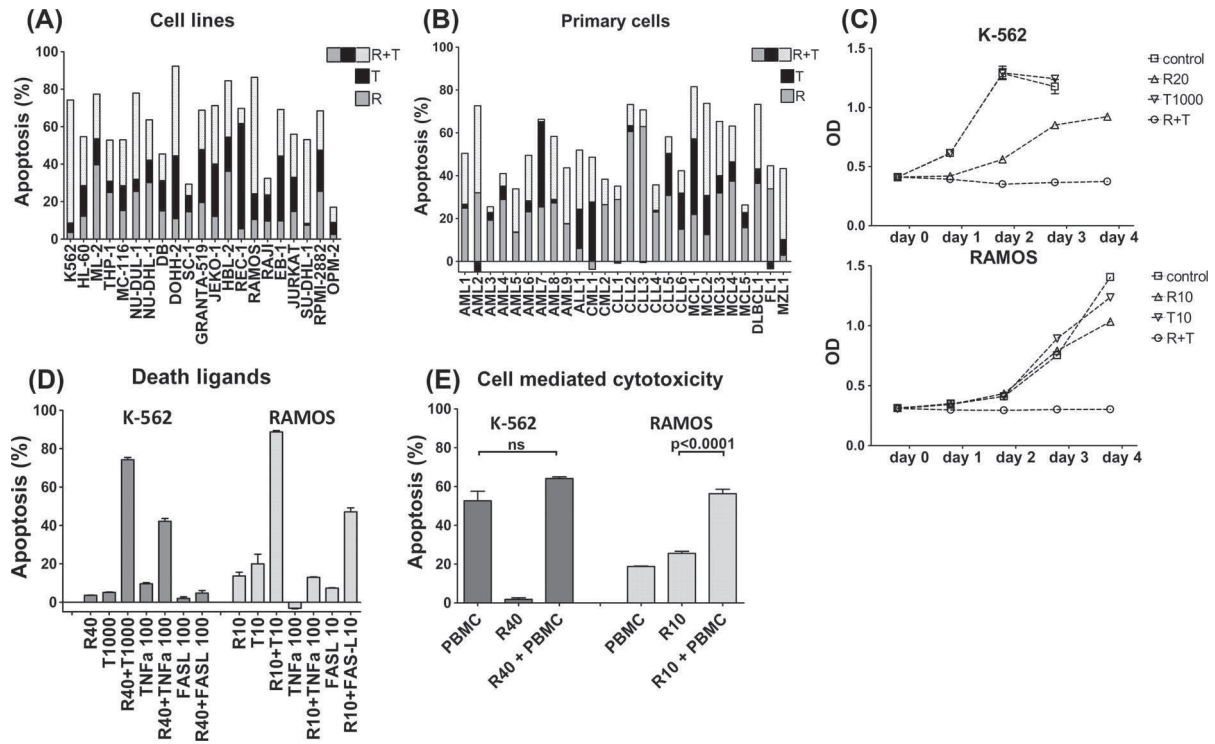


Figure 1. Roscovitine sensitizes leukemia and lymphoma cells to TRAIL, TNF α and FASL-induced apoptosis and enhances cell-mediated cytotoxicity. (A, B) Percentage of annexin V-FITC-positive, apoptotic cells induced by 24 h exposure of cell lines (A) or primary cells (B) to roscovitine (R), TRAIL (T) 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), TNF α , 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 μ M). 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 TNF α - 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 TNF α -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 decoy receptors was unchanged (data not shown). Immunoprecipitation of DISC

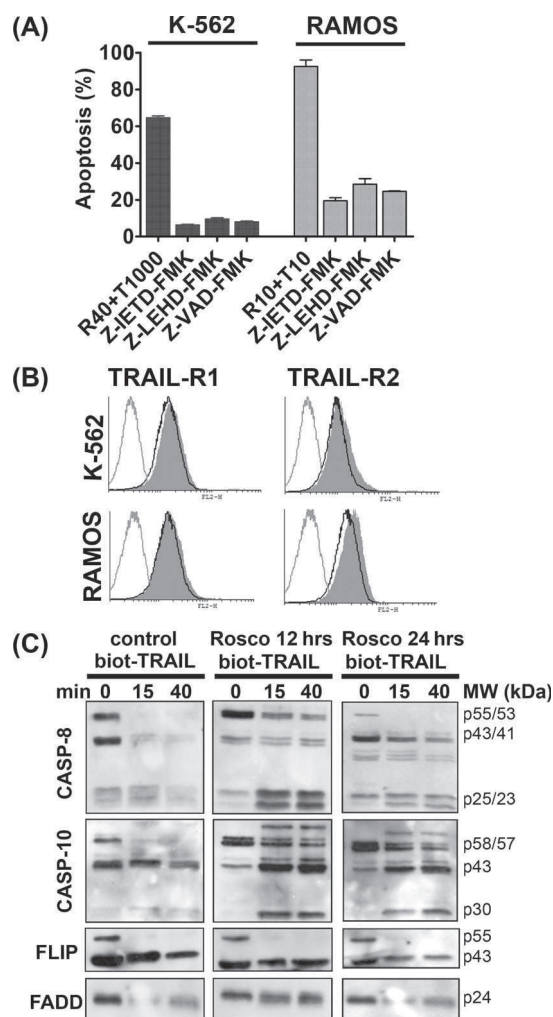


Figure 2. Roscovitine enhances DISC-mediated activation of initiator caspases 8/10 without affecting cell surface expression of TRAIL receptors. (A) K-562 and RAMOS cells were exposed to the combination of roscovitine and TRAIL (concentrations as indicated) 1 h after pretreatment with 100 μ M Z-VAD-FMK or Z-IETD-FMK and Z-LEHD-FMK inhibitors. The level of apoptosis was determined after 24 h. Columns represent means and error bars standard deviations of three independent experiments. (B) Flow cytometry analysis of TRAIL-R1 and TRAIL-R2 expression of K-562 and RAMOS cells before (black line, empty histograms) and after 24 h treatment with roscovitine (filled histograms). Gray line, empty histograms represent cells stained with control isotype antibody. Histograms shown are representative examples from three independent experiments. (C) Western blots of DISC proteins from biotin-TRAIL-treated K-562 cells (0, 15 and 40 min after TRAIL addition). K-562 cell line was either untreated (control) or pretreated with 40 μ M roscovitine for 12 and 24 h prior to addition of biotin-TRAIL.

proteins from the lysates of K-562 cells treated with biotinylated TRAIL and pre-exposed to roscovitine for 12 and 24 h revealed enhanced processing of both CASP8 (increased p25/23) and CASP10 (increased p30) proteins at the DISC [Figure 2(C)]. We also noted 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 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,

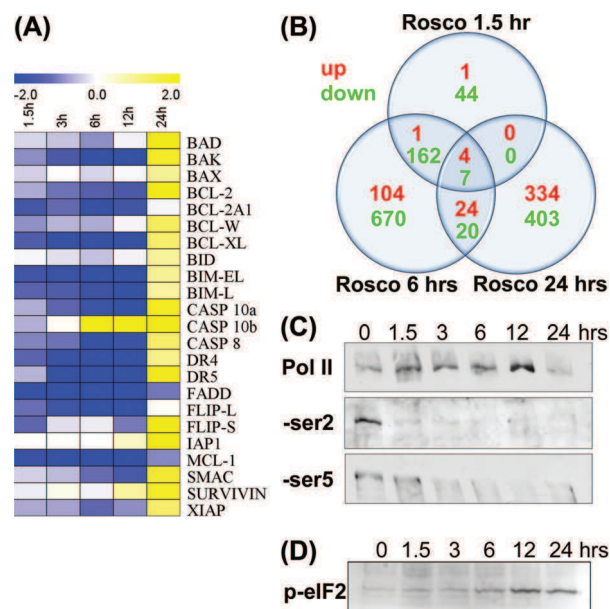


Figure 3. Treatment with roscovitine induces inhibition of transcription and translation. (A) Expression of several pro- and antiapoptotic genes was assessed by real-time RT-PCR 1.5, 3, 6, 12 and 24 h after treatment with roscovitine. The majority of genes were down-regulated during 12 h followed by up-regulation after 24 h. Columns represent timepoints and rows correspond to genes. Color changes within a row indicate expression levels relative to untreated controls (data analyzed using MeV4 software, color scale limits: logFC = ± 2 , blue down-regulation, yellow up-regulation). (B) Global gene expression of K-562 cells was assessed by Illumina chips 1.5, 6 and 24 h after treatment with roscovitine compared to untreated controls. Figure shows Venn diagram with number of genes up-regulated or down-regulated by more than two-fold. (C) Western blot analysis of protein expression of RNA polymerase II and its phosphorylation on serine 2 and serine 5 of CTD 1.5, 3, 6, 12 and 24 h after treatment with roscovitine. (D) Phosphorylation status of translation initiation factor eIF-2 α was assessed by Western blotting of cell lysates prepared 1.5, 3, 6, 12 and 24 h after treatment with roscovitine.

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-2 α) in K-562 cells after treatment with roscovitine.

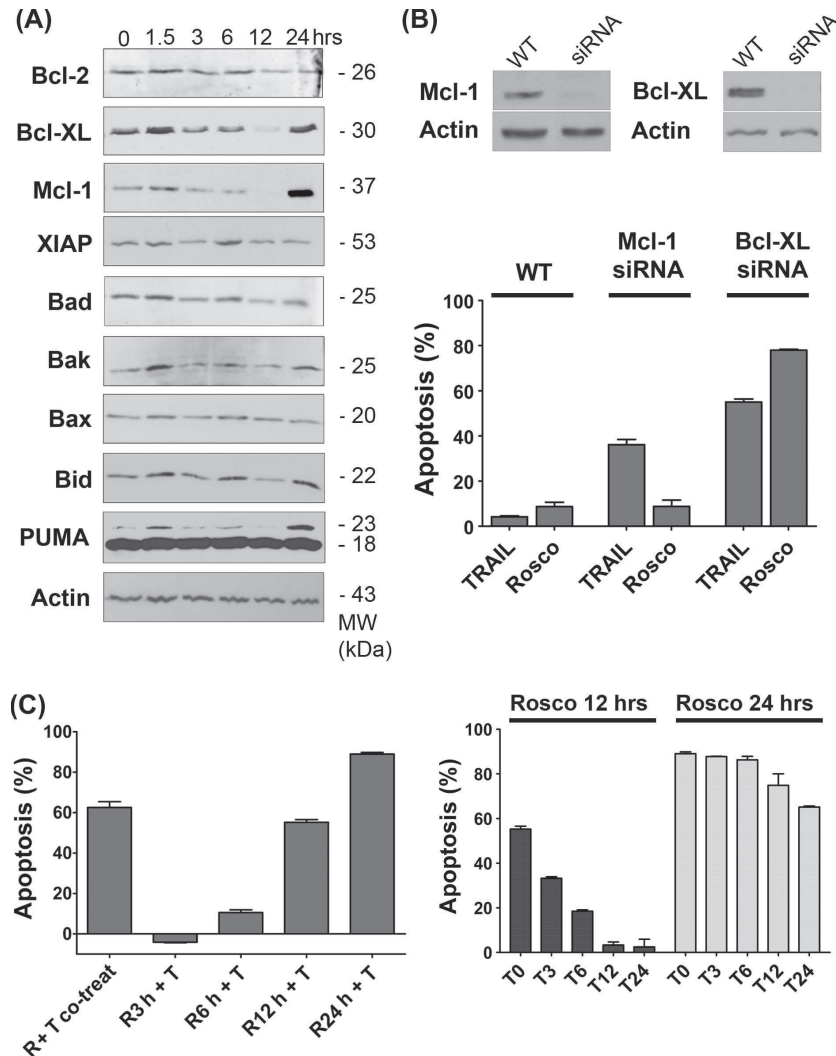


Figure 4. BCL-2 family proteins and mitochondria play an important role in proapoptotic effects of roscovitine in K-562 cells. (A) Western blotting showing protein expression of several pro- and antiapoptotic regulators 1.5, 3, 6, 12 and 24 h after treatment with roscovitine. (B) K-562 cells with down-regulated 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 μ M 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-2 α 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 roscovitine

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 α at 1.5 h, and up-regulation of PUMA α at 24 h.

Roscovitrine-induced sensitization to TRAIL is dependent on length of cell pre-incubation with roscovitrine

Co-treatment of malignant hematopoietic cells with low-toxic doses of roscovitrine 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 roscovitrine-induced sensitization to TRAIL, we studied the dependence of TRAIL sensitization on the length of exposure of K-562 cells to roscovitrine. We found that at least 12 h pretreatment with roscovitrine 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 roscovitrine for 24 h remained sensitive to TRAIL even 24 h after roscovitrine removal, indicating strong establishment of long-lasting changes in cell physiology in favor of TRAIL-induced pro-death signaling [Figure 4(C)].

Roscovitrine 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 roscovitrine for 3, 6, 12 and 24 h. Interestingly, unlike the data obtained from the whole cell lysates, treatment with roscovitrine resulted in a gradual increase in mitochondrial levels of all tested pro- and antiapoptotic proteins, with the only exception of PUMA β [Figure 5(A)]. These results imply that antiapoptotic BCL-2 proteins might sequester proapoptotic BCL-2 proteins. K-562 cells treated with roscovitrine were more sensitive to ABT-737, an inhibitor of BCL-2, BCL-XL and BCL-W [Figure 5(B)].

Roscovitrine 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 roscovitrine and TRAIL, as single agents or in combination. Combined treatment of roscovitrine 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 roscovitrine and TRAIL. Exposure of K-562 and RAMOS cells to roscovitrine did not alter the cell surface expression of TRAIL receptors,

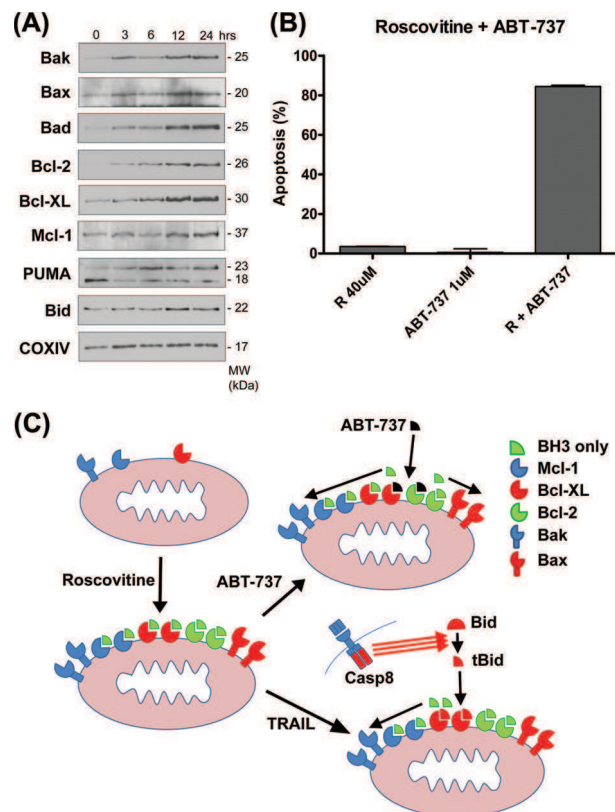


Figure 5. Roscovitrine induces up-regulation of both pro- and antiapoptotic BCL-2 family proteins at the mitochondria. (A) Western blotting showing mitochondrial expression of several pro- and antiapoptotic regulators from the BCL-2 family 3, 6, 12 and 24 h after treatment with roscovitrine. (B) K-562 cells were treated with roscovitrine (R), ABT-737 and combination of both (concentrations as indicated) and apoptosis determined by annexin V-FITC staining and flow cytometry. Columns represent means and error bars standard deviations of three independent experiments. (C) Proposed mechanism of roscovitrine-mediated sensitization to TRAIL-induced apoptosis. Treatment with TRAIL induces increased binding of PUMA and BIM to MCL-1 and tBid to BCL-XL in K-562 cells without activation of apoptotic cascade [28]. Roscovitrine induces up-regulation of pro- and antiapoptotic BCL-2 family members on mitochondria. Antiapoptotic BCL-2 family proteins sequester proapoptotic BH3-only proteins. Exposure of roscovitrine-treated K-562 cells to ABT-737, inhibitor of BCL-2, BCL-XL and BCL-W, displaces proapoptotic BH3-only proteins from antiapoptotic BCL-2 family proteins and these activate Bak/Bax. Similarly, treatment of primed cells with TRAIL activates apoptotic cascade probably due to TRAIL-induced redistribution of BCL-2 family members in roscovitrine-primed cells.

suggesting that roscovitrine-induced proapoptotic changes should be localized downstream. We have demonstrated that activation of the initiator caspases CASP8/10 was significantly more effective in roscovitrine-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 roscovitrine 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 roscovitrine also increased apoptosis induced by TNF α and FASL, respectively. As cytotoxic T and natural killer (NK) cells have

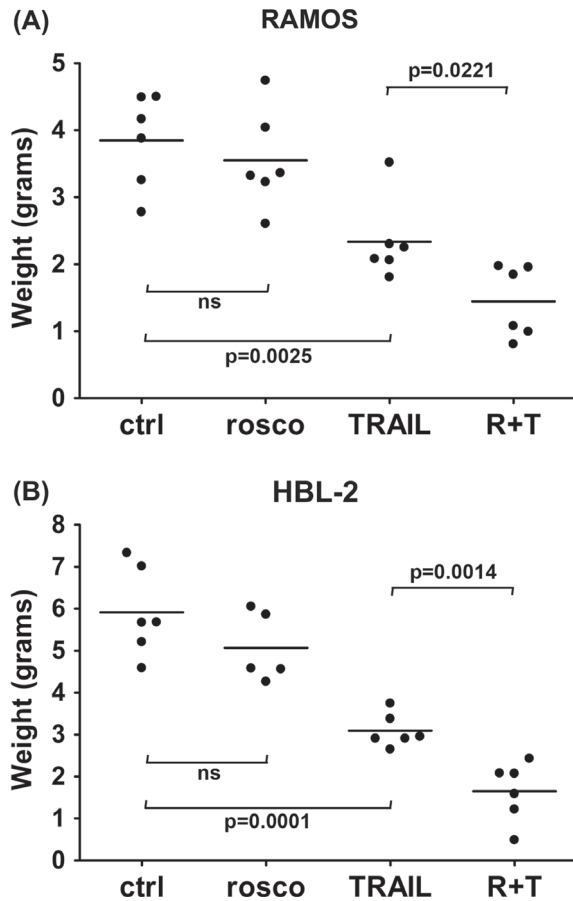


Figure 6. Roscovitine and TRAIL synergistically inhibit growth of subcutaneous lymphoma xenografts. Immunodeficient NOD.Cg-Prkdc^{scid} Il2r^{tm1Wjl}/SzJ mice were subcutaneously injected with RAMOS or HBL-2 cell lines. After tumors developed, mice were randomized and treated with two cycles of roscovitine (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.

been reported to use death ligands to kill target cells, we 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 PUMA β . 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.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

This work was supported by a grant from the Ministry of Health of the Czech Republic (IGA MZ NS/10287-3).

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Supplementary material available online

Supplementary Figures 1–3 showing additional results to be found online at <http://informahealthcare.com/doi/abs/10.3109/10428194.2012.710331>).

Multi-level disruption of the extrinsic apoptotic pathway mediates resistance of leukemia cells to TNF-related apoptosis-inducing ligand (TRAIL)

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Received October 4, 2012/ Accepted November 14, 2012

Disruption of apoptotic pathways belongs to commonly reported molecular mechanisms that underlie cancer drug resistance. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, Apo2L) is a cytokine of the TNF family with selective anti-tumor activity and minimal toxicity toward healthy tissues. Primary leukemia cells are, however, largely intrinsically resistant to TRAIL-induced apoptosis. In this study we analyzed molecular differences between TRAIL-resistant K562 cell line and TRAIL-sensitive K562 clones. We demonstrate that TRAIL-sensitive K562 cells differ from the TRAIL-resistant cell line by cell surface downregulation of TRAIL decoy receptor 1, upregulation of both TRAIL death receptors, enhanced assembly and improved functioning of the death-inducing signaling complex, and increased cytoplasmic protein expression of CASP8 and key proapoptotic BCL2 members BID, BIM, BAD and BAK. The molecular basis of the intrinsic leukemia cell TRAIL resistance thus appears a consequence of the multi-level disruption of the extrinsic apoptotic pathway. The results of this study also suggest that the leukemia TRAIL-resistance is functional, leaving a possibility of overcoming the resistance by preexposure of the leukemia cells to potent TRAIL sensitizers, e.g. BH3-mimetics.

Key words: leukemia, drug-resistance, TRAIL, apoptosis, BCL2 family

Intrinsic drug resistance represents one of the main obstacles in cancer therapy. Evading apoptosis through impairment of apoptotic pathways belongs to the commonly reported molecular mechanisms that underlie drug resistance. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, Apo2L) is a cytokine of the TNF family with selective anti-tumor activity and minimal toxicity toward healthy tissues. TRAIL binds to two death receptors (TRAIL-R1/DR4, TRAIL-R2/DR5) and two decoy receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR4) [1,2]. Ligation of TRAIL death receptors leads to formation of the death-inducing signaling complex (DISC) composed of the TRAIL, death receptors, core adapter protein FADD, initiator caspases CASP8 and CASP10, and FLIP, a competitive inhibitor of CASP8/10. Activated initiator caspases cleave and activate effector caspases CASP3, 6 and 7, as well as the proapoptotic BCL2 member BID. Truncated BID (tBID) in turn amplifies the proapoptotic signal through the mitochondria [3]. This amplification of the apoptotic signaling from TRAIL death receptors is required in the

majority of tumor cells (type II cells). Expression of TRAIL receptors was shown to correlate with prognosis in a variety of tumors, including hematologic malignancies [4-13]. In the last decade, TRAIL-induced apoptosis gained much attention as a potentially targeted anti-cancer therapy [14]. Unfortunately, the anti-tumor activity of both recombinant human TRAIL and anti-TRAIL-death receptor monoclonal antibodies proved to be limited in most reported clinical trials [15-18]. Despite the disappointing clinical results published so far TRAIL might re-emerge as a promising treatment approach in the future, mainly because TRAIL death receptor-targeted therapy possesses favorable toxic profile, it can induce apoptosis in diverse premalignant cell subsets, and it can be largely augmented by pre- or co-treatment with various molecules called TRAIL sensitizers [14,19-26]. Moreover, bioengineered TRAIL molecules or monoclonal antibodies selectively targeting individual TRAIL death receptors with much greater efficacy might demonstrate more potent anti-tumor activity compared to recombinant TRAIL [27,28].

Primary myeloid leukemia cells are largely intrinsically resistant to TRAIL-induced apoptosis. Several molecular mechanisms responsible for the leukemia TRAIL resistance were reported up to now, including overexpression of TRAIL decoy receptors, overexpression of FLIP or altered expression of apoptosis regulators including BCL2 family members, heat-shock proteins or inhibitor of apoptosis (IAP) proteins [29–37].

In the present study we analyzed the molecular nature of two TRAIL-sensitive K562 clones derived by limiting dilutions from the original TRAIL-resistant K562 cell line. We demonstrated that the TRAIL-sensitive clones differed from the TRAIL-resistant cell line by decreased expression of TRAIL decoy receptor 1, increased expression of both TRAIL death receptors, increased assembly and enhanced functioning of the DISC, and increased cytoplasmic expression of CASP8 and key proapoptotic BCL2 members BID, BIM, BAD and BAK. The molecular basis of leukemia cell TRAIL resistance thus appears a consequence of a multi-level disruption of the extrinsic apoptotic pathway.

Materials and methods

Cell culture. K-562 cell line was cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Peripheral blood samples were collected from patients with *de novo* diagnosed acute myeloid leukemia (AML, $n = 5$) and chronic myeloid leukemia (CML, $n = 5$) before therapy after obtaining informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated by Ficoll-Paque (GE Healthcare) density-gradient centrifugation and cultured in IMDM supplemented with 30% FBS.

Establishment of TRAIL-sensitive cell clones. TRAIL-sensitive K562 clones were established by limiting dilution from the original TRAIL-resistant K562 cells. K562 cells were diluted to a final concentration of 5 cells per 1 ml, distributed onto 96-well plates (100 μ L per well) and examined under light microscope. Only wells that contained a single cell were used for clone selection. In 14 days the wells with grown K562 clones were split into two wells. While the former well of each K562 clone was exposed to 1000 ng/mL TRAIL, the latter well was maintained in fresh medium. The percentage of apoptotic cells was measured after 24 hours. Two clones with the highest sensitivity to TRAIL (designated S1, S2) were expanded and used in further experiments described in detail in this current report.

Flow cytometry and detection of apoptosis. Cell surface expression of TRAIL receptors was analyzed by flow cytometry (FACS Canto, Becton Dickinson, California, USA) using primary anti-TRAIL receptor antibodies (R&D Systems, Minnesota, USA) and secondary PE-conjugated antibodies (Jackson ImmunoResearch Laboratories Inc., USA). Apoptosis was measured by flow cytometry using Annexin-V-FITC (Apronex Biotechnologies, Czech Republic) staining. The

percentage of apoptotic cells was evaluated using the DIVA software.

Cell proliferation assay. Cells were distributed onto 96-well plate (5.000 cells per well) on day 1. On day 2 increasing concentrations of cytotoxic drugs imatinib, etoposide, doxorubicin, cytarabine, and bortezomib were added to particular wells. Cell proliferation was measured in triplicates using WST-8 Cell Proliferation Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol.

DISC precipitation. Cell lysates for immunoprecipitation were prepared from 5×10^8 cells. Cells were precooled prior the addition of biotinylated TRAIL (final concentration 1000 ng/ml). After 15 minute incubation on ice the cells were divided into three equal fractions and quickly warmed to 37°C. Each fraction was incubated at 37°C and analyzed at 0, 10 and 30 minutes after warming (i.e. 0 minutes = precipitation after 15 minute exposure to biotinylated TRAIL on ice). Cells were lysed, and protein content of each sample was assessed using Bradford method. After equilibration of protein content, streptavidin-agarose beads were added to each sample and precipitated in cold room at 4°C for one hour. Beads were then separated by centrifugation and washed 5x. Finally, protein complexes were eluted by incubating samples in a heating block at 95°C for 15 minutes and agarose beads were removed by centrifugation. Protein precipitates were used for subsequent immunoblotting.

Western blot analysis. Cell lysates were resolved by electrophoresis using Novex® NuPAGE® 10% Bis-Tris Mini Gels (Life Technologies™, Carlsbad, CA, USA) and transferred to PVDF membranes using iBlot® Dry Blotting System (Life Technologies™, Carlsbad, CA, USA). Blocking of non-specific binding sites was performed by incubation in 0.1% PBS-Tween 20 and fat-free dry milk (5%). Membranes were incubated with specific primary antibody overnight at 4°C, followed by washing in PBS and incubation with HRP-conjugated secondary antibody at room temperature for 1 hour. Obtained immunoblots were developed by the enhanced chemiluminescence (ECL) reagent kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) according to the manufacturer's protocol.

Primary antibodies used in this study were as follows (with dilutions used in the experiments): Beta-actin (I19) Santa Cruz, 43kDa, goat (1:1000); Caspase 2 (C20), Santa Cruz, 51kDa, rabbit (1:200); Caspase 3 (31A1067) Alexis, 32kDa, mouse (1:1000); Caspase 8 (12F5), Alexis, 55/54kDa, mouse (1:1000); Caspase 9 (5B4), MBL, 45/37/35kDa, mouse (1:500); Caspase 10 (4C1), MBL, 58/57kDa, mouse (1:500); BAD (C7), Santa Cruz, 25kDa, mouse (1:500); BAK (3814S), Cell Signaling, 25kDa, rabbit (1:1000); BID (5C9) Santa Cruz, 22kDa, mouse (1:200); XIAP (2F1), MBL, 55kDa, mouse (1:1000); BCL-XL (M125), Santa Cruz, 30/18kDa, rabbit (1:200); BIM (H191), Santa Cruz, 24/21/19kDa, rabbit (1:200); MCL-1 (3035-100), BioVision, 43/37kDa, rabbit (1:200); BAX (610983), BD, 21kDa, mouse (1:250); BCL-2 (610539), BD,

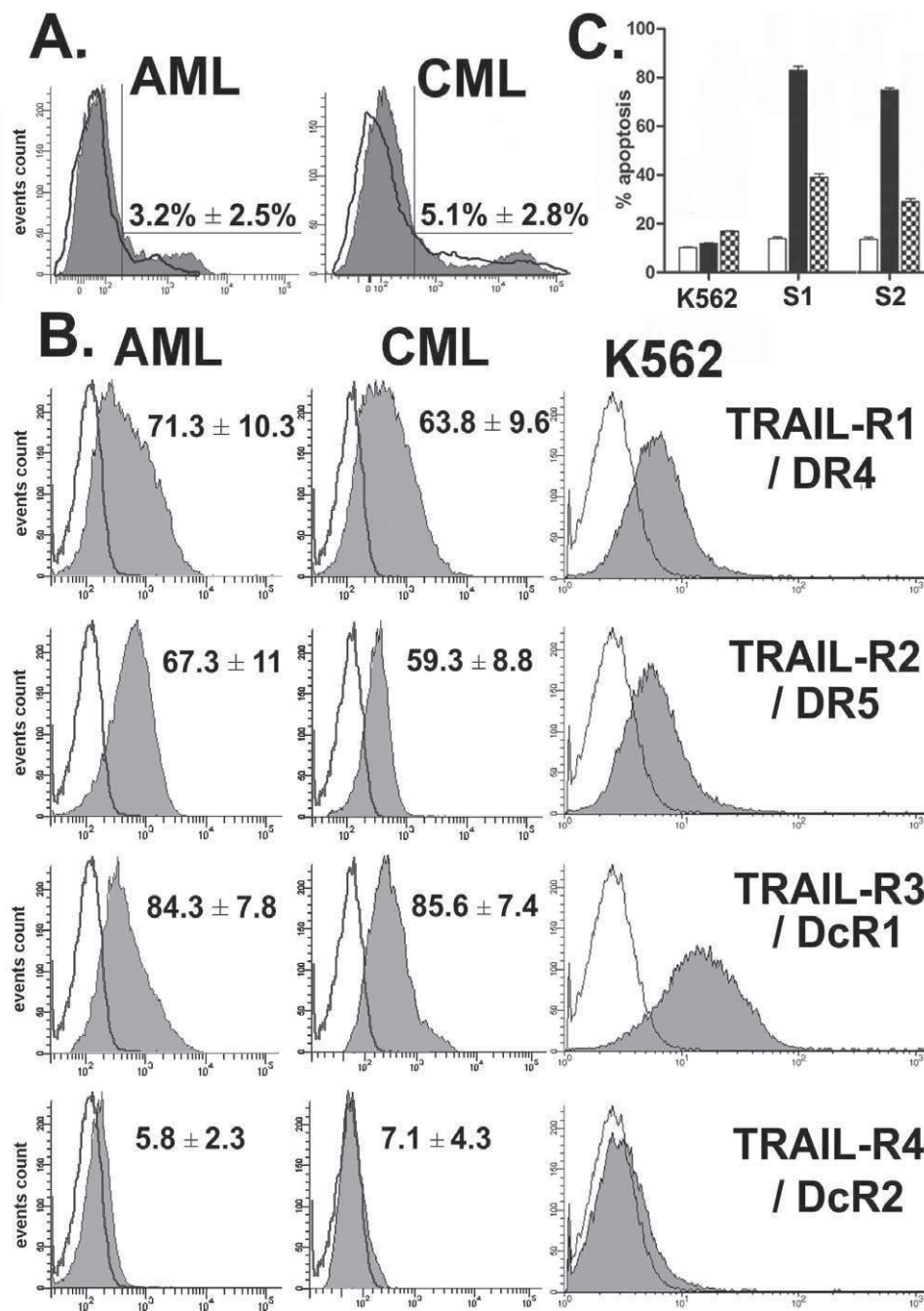


Figure 1. A. Primary AML (n=5) and CML (n=5) cells are inherently resistant to TRAIL-induced apoptosis. Percentage of Annexin-V-positive cells was analyzed by flow cytometry 24 hours after exposure of the cells to TRAIL (1000 ng/ml). The histograms indicate representative examples of each group (AML, CML), and numbers represent means $\pm$ SDs of the percentages of apoptotic cells calculated from the entire groups (AML, CML). Specifically, the numbers represent differences between the TRAIL-exposed and TRAIL-unexposed cells. B. Flow cytometry analysis of cell surface expression of TRAIL receptors 1-4 on primary AML cells (n=5), CML cells (n=5) and K562 cell line (K562). The histograms indicate representative examples of each group (AML, CML, K562). Full histograms are stains with primary and secondary antibodies, while open histograms correspond to the control stains of the cells with secondary antibody only. The numbers represent differences between the mean fluorescence intensities (MFIs) $\pm$ SDs calculated for stains with primary and secondary antibodies, and MFIs calculated for stains with secondary antibodies only. C. S1 and S2 K562 clones are more sensitive to TRAIL and TNF-alpha compared to the original K562 cell line. The percentage of Annexin-V-positive cells 24 hours after exposure to TRAIL (1000 ng/ml) and TNF-alpha (100 ng/ml) was measured using flow cytometry. Columns represent mean percentages of Annexin-V-positive apoptotic cells of untreated cells (open columns), cells after the exposure to TRAIL (black columns), and TNF-alpha (checkered columns). Error bars represent standard deviations of two independent experiments. The treatment with TRAIL and TNF-alpha induced significantly greater apoptosis of S1 ($p < 0.01$) and S2 ($p < 0.01$) clones compared to similarly treated original K562 cells. The background apoptosis of untreated S1, S2, and original K562 cells was not significantly different.

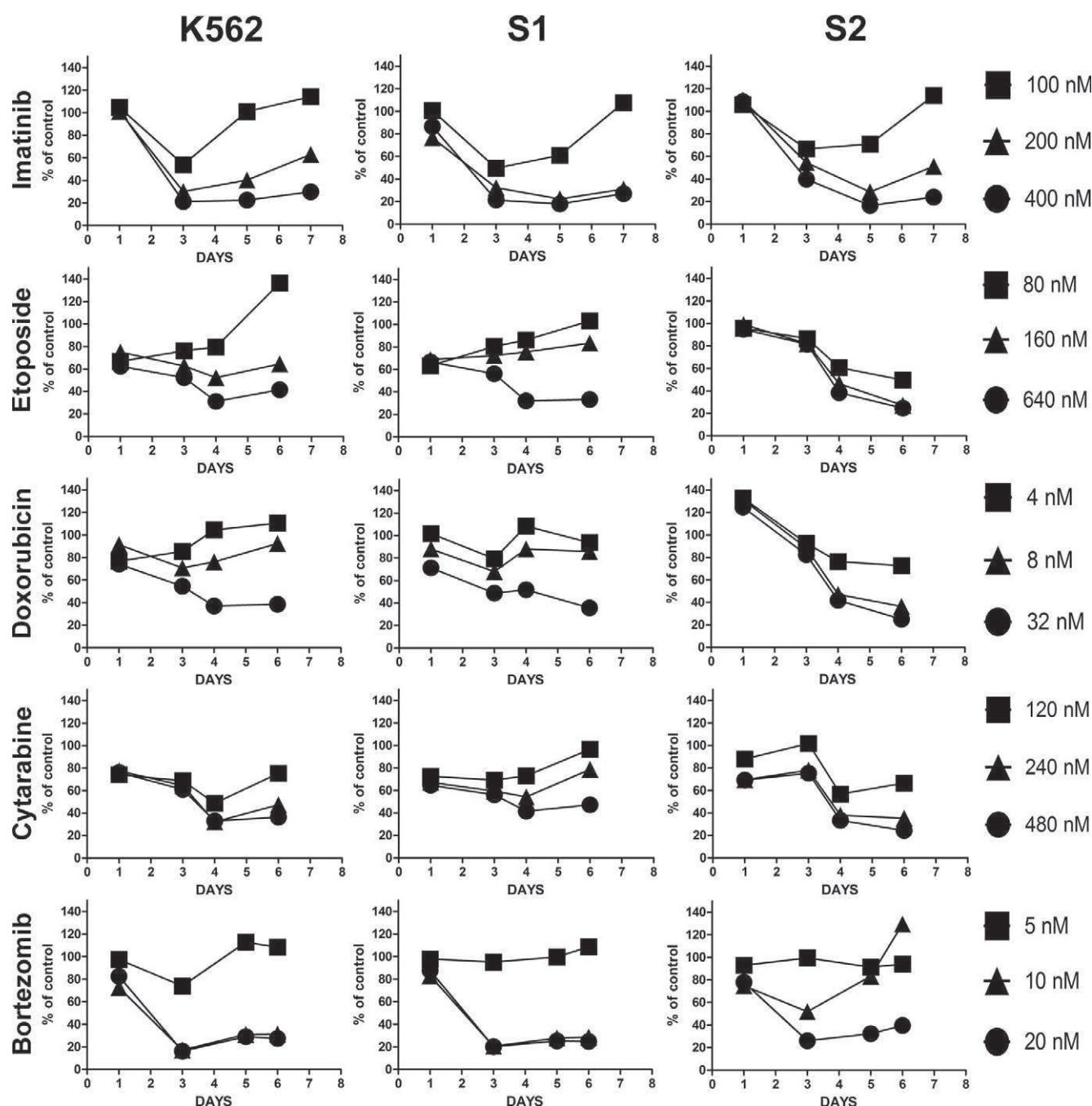


Figure 2. A. K562 cells and TRAIL-sensitive clones S1 and S2 are sensitive to selected anti-tumor drugs. WST-8-based cell-proliferation assay was used to assess impact of the cytotoxic agents on proliferation rate. Measurements were performed in triplicates over several days (X axis). On Y axis, ratios between the drug-exposed and unexposed cells are shown ("% control").

26kDa, mouse (1:1000), Survivin (3161-100), BioVision, 17 kDa, rabbit (1:1000).

Statistical analysis. Student's t-test was applied to evaluate statistical significance of the observed differences between TNF-alpha- and TRAIL-induced apoptosis in TRAIL-sensitive clones S1 and S2 compared to the original K562 cell line.

Results

Primary AML and CML blasts express TRAIL receptors 1-3, and are inherently resistant to TRAIL-induced apoptosis. We analyzed proapoptotic activity of TRAIL using peripheral blood mononuclear cells obtained from

patients with newly diagnosed acute myeloid leukemia (AML, $n = 5$) and chronic myeloid leukemia (CML, $n = 5$). We confirmed that all primary AML and CML blasts were indeed resistant to TRAIL-induced apoptosis showing less than 10% apoptosis after 24 hour exposure to 1000 ng/mL TRAIL (Fig. 1a). We also demonstrated that the primary AML/CML blasts expressed both TRAIL death-receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5), as well as the decoy receptor 1 (TRAIL-R3/DcR1), but not the decoy receptor 2 (TRAIL-R4/DcR2) (Fig. 1b).

TRAIL-sensitive K562 clones S1 and S2 show increased sensitivity to TNF-alpha, but not to cytotoxic agents imatinib, cytarabine, etoposide, doxorubicin, and bortezomib. To analyze the molecular basis of the observed TRAIL-resistance of myeloid leukemia blasts, we used K562 cell line, established from a patient with erythroleukemia progressed from CML, which is characterized by TRAIL-resistance and similar pattern of expression of TRAIL receptors compared to primary AML/CML blasts (Fig. 1b, 1c). By limiting dilution approach we derived two TRAIL-sensitive K562 clones designated S1 and S2. In concordance with our previous study on HL60 cells [37] we demonstrated that the TRAIL-sensitive clones S1 and S2 were also more sensitive to TNF-alpha compared to the original K562 cell line (Fig. 1c), but have retained sensitivity to selected anti-tumor agents imatinib, cytarabine, etoposide, doxorubicin or bortezomib (Fig. 2).

TRAIL-sensitive K562 clones show marked cell surface downregulation of decoy receptor 1, slight upregulation of both death receptors, and increased recruitment and cleavage of CASP8 at the DISC after exposure to TRAIL. Flow cytometry analysis of TRAIL-sensitive clones S1 and S2 revealed marked downregulation of the cell surface decoy receptor 1, and slight upregulation of both TRAIL death receptors, while expression of both TNF receptors remained unchanged (Fig. 3a). Analysis of death-inducing signaling complex (DISC) revealed comparable amounts of precipitated TRAIL-receptor 2 (DR5) in TRAIL-resistant K562 cells and TRAIL-sensitive clones S1 and S2 after exposure to biotinylated TRAIL (at all time-points) (Fig. 3b). S1 and S2 clones, however, showed markedly increased binding of CASP8 to the DISC (at 0 minutes), as well as enhanced cleavage of CASP8 at 10 and 30 minutes after exposure to biotinylated TRAIL (Fig. 3b). As the amounts of cleaved FLIP-L at 10 and 30 minutes appeared similar in all tested samples, CASP8 to FLIP ratio was significantly increased in TRAIL sensitive clones compared to the original K562 cell line (Fig. 3b).

TRAIL-sensitive K562 clones show increased protein expression of several key proapoptotic BCL2 family members. In our recently published study by Molinsky et al. (20) we demonstrated that downregulation of antiapoptotic BCL2 molecules MCL1 and BCL-XL strongly sensitized K562 cells to TRAIL-induced apoptosis. We thus decided to analyze expression profile of key BCL2 members and other selected apoptosis regulators in TRAIL-sensitive K562 clones com-

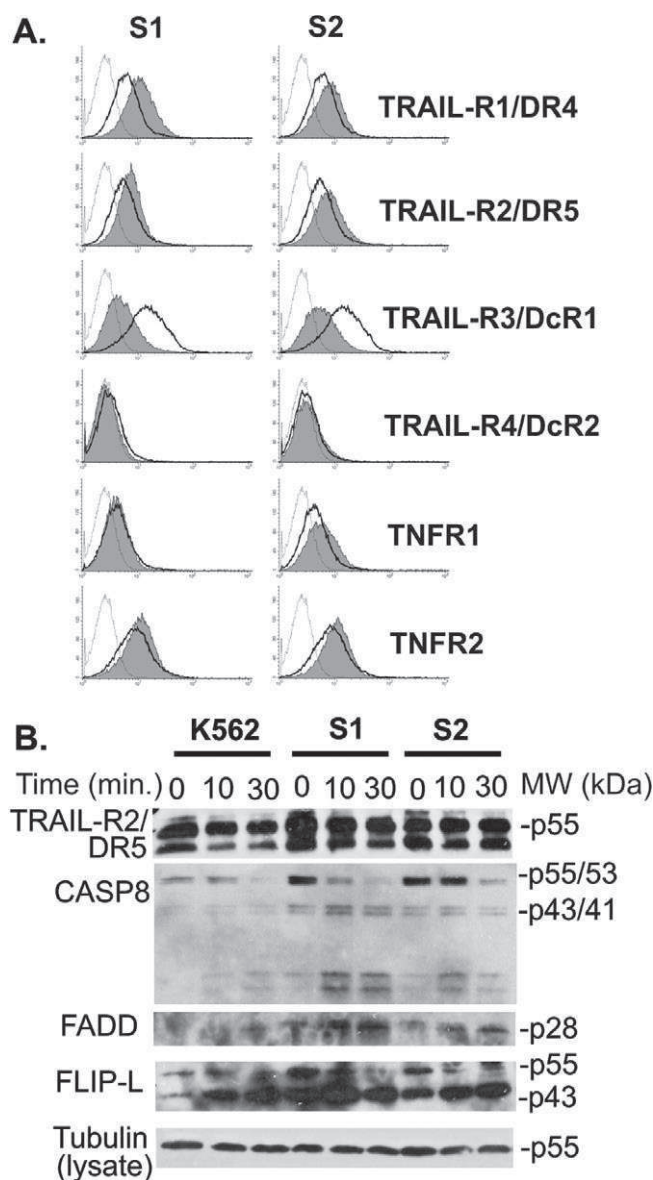


Figure 3. A. Flow cytometry analysis of cell surface expression of TRAIL and TNF receptors on TRAIL-sensitive K562 clones S1 and S2 (full histograms) compared to the original TRAIL-resistant K562 cell line (thick open histograms). Open thin histograms represent cells stained with secondary antibody only. **B.** Analysis of death-inducing signaling complex (DISC) by precipitation with biotinylated TRAIL. Tubulin immunoblots from whole cell lysates (placed at the bottom) were used as a control of protein load.

pared to TRAIL-resistant K562 cell line by western blotting. In both TRAIL-sensitive clones we found protein upregulation of proapoptotic proteins CASP8, BAD, BID, BIM, BAK, and upregulation of antiapoptotic BCL-XL (Fig. 4). Downregulation of BCL2 and upregulation of XIAP was detected in clone S1, while downregulation of MCL1 was detected in S2 clone (Fig. 4).

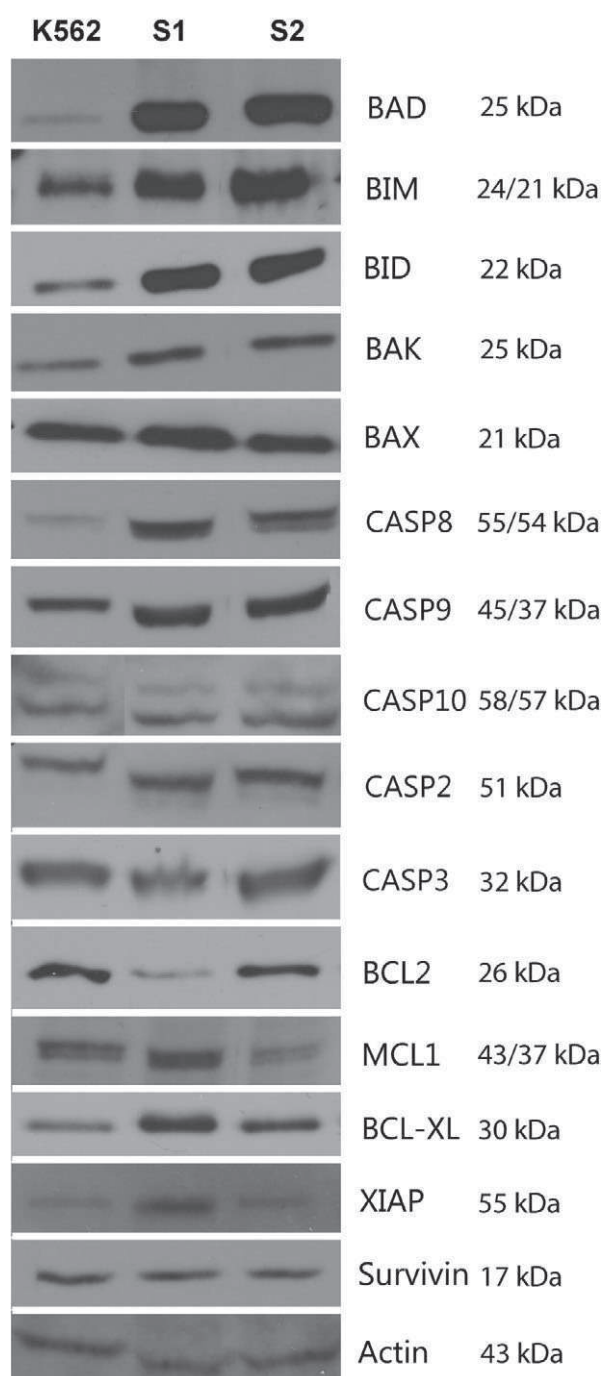


Figure 4. Western blot analysis of whole cell lysates of TRAIL-sensitive K562 clones (S1, S2) and the original K562 cell line (K562). Western blots were performed as described in Materials and Methods. Beta-actin immunoblots were used as a control of protein load.

Discussion

Up to the present time a wide array of molecules and signaling pathways were reported as pivotal players mediating

TRAIL-resistance of leukemia cells [6,35,37,38]. In this study we analyzed molecular mechanisms responsible for TRAIL-resistance of K562 cell line and the nature of TRAIL-sensitivity of two TRAIL-sensitive K562 clones (S1, S2) established by limiting-dilution approach from the original K562 cell line. Both TRAIL-sensitive K562 clones showed almost identical molecular changes compared to the original K562 cells, namely 1. marked downregulation of the decoy receptor 1 together with slight upregulation of both death receptors, 2. increased expression of cytosolic CASP8, enhanced recruitment of CASP8 to the DISC, and increased cleavage of CASP8 at the DISC, and 3. protein overexpression of key proapoptotic BCL2 members BID, BAD, BIM, and BAK, together with upregulation of BCL-XL (Fig. 3-4). Each of these molecular changes, with the exception of BCL-XL, might contribute to the observed enhanced sensitivity of S1 and S2 clones to TRAIL. The molecular differences between TRAIL-resistant and TRAIL-sensitive K562 cells thus appeared to involve the extrinsic apoptotic pathway at three different levels: 1. the receptor level, 2. the DISC level, and 3. the mitochondria level.

Despite the fact that the precise role of the decoy receptors 1 and 2 in TRAIL-triggered apoptosis remains incompletely understood, most studies published so far supported their role in mediating TRAIL-resistance [9,10,10,29,30,39-44]. In this study, we observed cell surface downregulation of the decoy receptor 1 in both TRAIL-sensitive clones (Fig. 3a). Surprisingly, analysis of the DISC after exposure to biotinylated TRAIL revealed that the amounts of precipitated death receptor 5 were comparable in TRAIL-resistant and TRAIL-sensitive K562 cells (Fig. 3b). Recruitments of other key DISC components CASP8, FADD and FLIP, however, displayed significantly decreased levels in TRAIL-resistant K562 cells (Fig. 3b). While cleavage of CASP8 was significantly decreased in TRAIL-resistant K562 cells, the amounts of cleaved FLIP-L appeared similar in all analyzed samples (Fig. 3b). We thus assume that the generation of large amounts of cleaved FLIP-L together with small amounts of cleaved CASP8 led to the functional blockage of propagation of apoptotic signal from the ligated TRAILR2/DR5 in K562 cell line.

In our previous study by Molinsky et al. [20] we demonstrated that targeted downregulation of a single anti-apoptotic protein MCL1 or BCL-XL, but not BCL2, was sufficient to fully sensitize K562 cells to TRAIL. We also showed that pre-treatment with BH3-mimetic agent ABT-737 sensitized K562 cells to TRAIL. In this current study we reported significant upregulation of key proapoptotic BCL2 family proteins BID, BAD, BIM and BAK in TRAIL-sensitive K562 clones compared to TRAIL-resistant K562 cells (Fig. 4). We assume that the consequences of either MCL1/BCL-XL downregulation/sequestration or BID/BAD/BIM/BAK upregulation are similar in that these molecular events induce permissive state of TRAIL-sensitivity by effectively amplifying proapoptotic signals from the TRAIL-ligated death receptors via the mitochondria. Based on the results of both our studies we suppose that changed expressions of BCL2 family members represent major molecular

events that mediate TRAIL-sensitivity of K562 clones, while overexpression of cytoplasmic CASP8 and downregulation of cell surface decoy receptor 1 might contribute to the extent of TRAIL-sensitivity. We suppose that the increased DISC assembly and enhanced cleavage of CASP8 represent direct consequences of the changed expression of BCL2 proteins, CASP8 and DcR1.

In concordance with our previous study by Klener et al. [37], restoration of the extrinsic apoptotic cascade in TRAIL-sensitive clones S1 and S2 was associated with increased sensitivity to TNF- α , another important proapoptotic cytokine (Fig. 1c). In contrast, susceptibility of the clones remained unchanged to all of the tested anti-tumor drugs including imatinib (targeted agent: inhibitor of BCR-ABL tyrosine kinase), cytarabine (genotoxic agent: antinucleotide), etoposide (genotoxic agent: inhibitor of topoisomerase), doxorubicin (genotoxic agent: intercalating agent) and bortezomib (targeted agent: inhibitor of proteasome) (Fig. 2). The reason, why the observed proapoptotic changes in the protein expression of several key BCL2 molecules were not associated with increased sensitivity to tested cytotoxic or targeted agents remains a conundrum. In the study by Molinsky et al. [20] we demonstrated that roscovitine, a cyclin-dependent kinase inhibitor, sensitized K562 cells (as well as primary AML/CML blasts) to TRAIL-induced apoptosis, and that this sensitization was associated with changed profile of BCL2 proteins. Despite the fact that pretreatment with roscovitine restored sensitivity of K562 cells to TRAIL and TNF- α , it did not increase sensitivity of the cells to commonly used cytotoxic agents. This finding was very similar compared to the results observed in this report. Precise molecular mechanisms, by which changed profile of BCL2 proteins at the mitochondria might selectively augment receptor-triggered apoptosis, remain to be elucidated.

In conclusion, inherent TRAIL-resistance of K562 cells (and potentially of AML and CML blasts) appears a consequence of multi-level disruption of the extrinsic apoptotic pathway. The results of this study also suggest that the leukemia TRAIL-resistance is functional (not structural), leaving a possibility of overcoming the resistance by preexposure of the leukemia cells to potent TRAIL sensitizers, e.g. BH3-mimetics.

Acknowledgements: Financial Support: GAUK 259153/86309, PRVOUK P24/LF1/3, UNCE 204021, SVV-2012-254260507. Special thanks to Monika Hulova.

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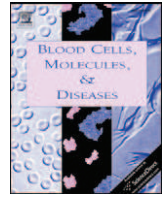
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Contents lists available at ScienceDirect

Blood Cells, Molecules, and Diseases

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TRAIL-induced apoptosis of HL60 leukemia cells: Two distinct phenotypes of acquired TRAIL resistance that are accompanied with resistance to TNF α but not to idarubicin and cytarabine[☆]

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ARTICLE INFO

Article history:

Submitted 2 October 2008

Available online 26 November 2008

(Communicated by M. Lichtman, M.D., 06 October 2008)

Keywords:

Apoptosis

TNF-related apoptosis-inducing ligand

Tumor necrosis factor- α

Neoplasm drug resistance

Leukemia

ABSTRACT

TNF-related apoptosis-inducing ligand (TRAIL) is a proapoptotic cytokine implicated in cancer cell surveillance. A potential of TRAIL as a cancer-specific therapeutic agent has been proposed, either as a single agent or in combination with chemotherapy. Prolonged exposure of TRAIL-sensitive leukemia cell line, wild-type (WT) HL60 cells to recombinant soluble TRAIL or to cytostatic agents, cytarabine and idarubicin, resulted in the establishment of resistant subclones with distinct phenotypic features. The TRAIL resistant HL60 subclones were characterized by decreased expression of TRAIL and TNF α death receptors. These resistant subclones had impaired activation of caspases 8 and 10 in response to TRAIL and TNF α , decreased TRAIL-induced nuclear translocation of NF κ B RelA/p65, and dysregulation of the expression of several apoptosis regulators. Among the TRAIL resistant HL60 subclones we identified two separate phenotypes that differed in the expression of CD14, osteoprotegerin, and several apoptosis regulators. Both these TRAIL resistant HL60 subclones were resistant to TNF α , suggesting disruption of the extrinsic apoptotic pathway, but not to cytostatic agents, cytarabine and idarubicin. The concurrently derived HL60 subclones were cytarabine and idarubicin-resistant but remained sensitive to TRAIL-induced apoptosis. We identified distinct pathways for the development of HL60 leukemia cell resistance to apoptosis induction. These findings are relevant for the design of more effective strategies for leukemia therapy.

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Introduction

Chemotherapy of leukemia is often associated with the development of drug-resistant subclones that cause progression and/or relapse of the disease; thus, targeted therapy of leukemia would improve outcome and decrease toxicity of chemotherapy. TRAIL belongs to the tumor necrosis factor (TNF) family of death ligands [1,2] and induces apoptosis in target cells by its receptor-mediated interaction [3]. Receptors for TRAIL include “death” receptors DR4/TRAIL-R1 and DR5/TRAIL-R2 and “decoy” receptors DcR1/TRAIL-R3, DcR2/TRAIL-R4, and osteoprotegerin/OPG/TRAIL-R5. TRAIL “death” and “decoy” receptors differ based on their ability to transduce or inhibit TRAIL-induced apoptotic signaling [4–9].

While normal tissues, including hematopoietic progenitor cells, are resistant to TRAIL-induced apoptosis, TRAIL triggers programmed death in many malignancies and cell lines [10–13]. In some cell lines, TRAIL synergizes with chemotherapeutic agents and/or radiation by several cell-type specific mechanisms [14].

Selection of TRAIL-resistant clones in the previously TRAIL-sensitive tumor cell population would have significant implications for TRAIL-based cancer therapy. Several molecular mechanisms responsible for TRAIL resistance have been described [15]. Resistance of tumor cells to TRAIL was reported to be associated with the deregulation of diverse signaling molecules including TRAIL-receptors, apical caspase 8, c-FLIP, and various apoptosis regulators including BCL-2, IAP and HSP family proteins [16]. In HL60 cells, resistance to TRAIL was recently attributed to the mutation of caspase 8 as well as to the down-regulation of cell surface TRAIL death receptors, DR4 and DR5 [17]. Most of the studies have focused on constitutive TRAIL resistance, while molecular mechanisms responsible for acquired TRAIL resistance of malignant cells remain largely uncharacterized [17].

[☆] Financial support: IGAMZ 8317-4, MSM 0021620806, MSM 0021620808, LC 06044, 1M6837805001, and GAUK 50/2004/c.

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In the present study, we derived TRAIL-resistant subclones of myeloid leukemia HL60 cell line by prolonged exposure to TRAIL and characterized their mechanism of resistance.

Materials and methods

Cell culture conditions, antibody staining, and apoptosis measurement

Cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, MD, USA) in the presence of 10% fetal bovine serum (FBS) in 37 °C humidified atmosphere with 5% CO₂. The expression of TRAIL receptors was measured by flow cytometry (FACS Calibur; Becton Dickinson, CA, USA) using anti-TRAIL receptor antibodies (R and D systems, MN, USA) and corresponding secondary antibody (Jackson Immuno Research Laboratories, Inc., USA). TRAIL death receptors were blocked with rabbit polyclonal antibodies to TRAIL-R1/DR4 (B5/B4), TRAIL-R2/DR5 (5CII) (Apronex Biotechnologies, Czech Republic). TRAIL decoy receptors were blocked with goat polyclonal antibodies (R and D systems, MN, USA). Apoptosis was analyzed by flow cytometry (FACS Calibur) using Annexin-V-FITC staining (Apronex Biotechnologies, Czech Republic). The pharmacological blockage of apical caspases 8 and 10 was achieved by preincubation of the cells with caspase-specific inhibitors Z-IETD-FMK, Z-AEVD-FMK, and Z-VAD-FMK at concentrations ranging from 0.64 to 80 μM (R and D systems, MN, USA).

Establishment of TRAIL or cytostatic-resistant HL60 cells

TRAIL-resistant HL60 cells were derived by selective pressure of TRAIL (200 ng/ml, Apronex Biotechnologies, Czech Republic). Cytostatic-resistant HL60 cells were selected by cultivating cells with increasing doses of idarubicin (Zavedos, Pharmacia, Italy, 2 nM to 30 nM) and cytarabine (Cytosar, Pharmacia and Upjohn, Belgium, 0.1 μM to 2 mM). Proportions of TRAIL-resistant cells in the WT HL60 population were determined by limiting dilution assays.

Real-time RT-PCR analysis

Real-time PCR analysis was performed using the IQTM5 multicolor real-time PCR detection system (Bio-Rad, USA). The results were normalized to β-actin mRNA expression. The inter-assay variability in β-actin expression was less than 0.5 cycles.

Measurement of caspases 8 and 10 activity

For the induction of apoptosis, one million cells were incubated either with TRAIL 200 ng/ml or TNFα 100 ng/ml for 0, 1, 2, and 4 h. Caspases 8 and 10 activation was measured by Caspase8/FLICE and Caspase10/FLICE fluorometric assays (R and D systems, MN, USA) according to the manufacturer's protocol. Briefly, cells were washed with PBS, centrifuged and lysed in a cell lysis buffer. After the addition of DTT (1 mM final), 2× reaction buffer, and caspase-specific fluorometric substance (IETD-AFC or AEVD-AFC) the lysates were incubated at 37 °C. The fluorescence intensity was measured by Plate CHAMELEON fluorescence reader (Hidex, Finland) after the 90 minute incubation period.

Detection of NFκB translocation

NFκB RelA/p65 translocation was measured using TransAmTM ELISA-based NFκB RelA/p65 activation assay (Active Motif, Belgium) according to the manufacturer's instructions. Briefly, nuclear lysates were isolated at time 0, 20, 40, 60, 90, 120 and 180 min after the exposure of cells to TRAIL (200 ng/ml). Positive control for NFκB RelA/p65 translocation assay, Jurkat nuclear lysate (Active Motif, Belgium), was used to generate a standard curve. The relative concentrations (relative units

per milligram of total lysate protein) of HL60 cell lysates were extrapolated from the standard curve. The optical density of Jurkat nuclear lysate 5 μg/ml was assigned the value of 100 relative units.

Xenotransplantations of HL60 cells into immunodeficient NOD/LtSz-Rag1^{null} mice

NOD/LtSz-Rag1^{null} female mice (breeding pairs purchased from The Jackson Laboratory, Maine, USA), age 8 to 12 weeks, were 4 Gy γ-irradiated on day -1. TRAIL-resistant or WT HL60 cells, 10 million in 300 μL PBS, were tail vein injected on day 0 (6 mice each group). The mice were observed daily and were sacrificed when they developed hind-leg paralysis or exhibited generalized failure to thrive.

Results

Development of TRAIL-resistant HL60 subclones

The HL60 acute myeloid leukemia cell line is sensitive to TRAIL-induced apoptosis. The incubation of wild-type (WT) HL60 cells with TRAIL for at least 1 h was critical for apoptosis induction, as shorter incubations reduced, and longer incubations did not materially increase the proportion of apoptotic cells. The proportion of cells that escaped TRAIL-induced apoptosis was dependent on the TRAIL dose. We then determined the effect of the TRAIL dose on the generation of TRAIL-resistant cells. A limiting dilution assay revealed the following ratio of TRAIL-resistant cells to TRAIL-sensitive WT HL60 cells: 1/28±4 for 20 ng/ml TRAIL, 1/2088±264 for 200 ng/ml TRAIL, and 1/19,100±1995 for 2000 ng/ml TRAIL.

We then studied the effect of antibody-induced blocking of TRAIL receptors on TRAIL-induced apoptosis. Antibody-mediated inhibition of TRAIL-R1/DR4 receptor more potently suppressed TRAIL-induced apoptosis of WT HL60 cells than did antibody-mediated blockage of TRAIL-R2/DR5 (Figs. 1A, B); however, similar treatment of TRAIL decoy receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR2, TRAIL-R5/OPG) did not significantly influence TRAIL-induced apoptosis (Fig. 1B).

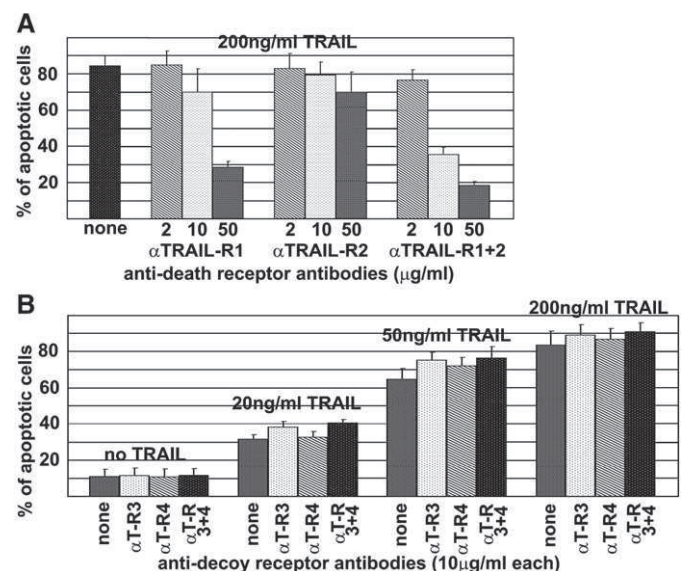


Fig. 1. (A, B) TRAIL-induced apoptosis of HL-60 cells proceeds mainly through TRAIL-R1 receptor. The level of WT HL60 cell line apoptosis induced by TRAIL (200 ng/ml) after the one hour pre-incubation with 2, 10, and 50 μg/ml of blocking antibodies against TRAIL death receptors, αTRAIL-R1, αTRAIL-R2, and αTRAIL-R1+2 (A) or 10 μg/ml of blocking antibodies against TRAIL decoy receptors, αTRAIL-R3, αTRAIL-R4, and αTRAIL-R3+4 (B). The concurrent incubation of the cells with antibodies only (without addition of TRAIL) did not significantly change the number of apoptotic cells (data not shown). Columns represent means and error bars standard deviations of three independent experiments.

HL60 TRAIL-resistant cells are resistant to $\text{TNF}\alpha$, but remain sensitive to idarubicin and cytarabine; HL60 cells resistant to cytarabine or idarubicin remain sensitive to TRAIL and $\text{TNF}\alpha$

By prolonged exposure of 3 to 4 weeks of WT HL60 cells to TRAIL, we selected twenty TRAIL-resistant HL60 subclones. We then analyzed the susceptibility of the selected TRAIL-resistant subclones to another apoptosis-inducing agent, $\text{TNF}\alpha$. Interestingly, the $\text{TNF}\alpha$ -induced apoptosis of TRAIL-resistant HL60 subclones was suppressed to a similar

extent as the TRAIL-induced apoptosis (Figs. 2A–C). The cell surface expression of $\text{TNF}\alpha$ receptors, TNF-R1 and TNF-R2 , was significantly down-regulated in TRAIL-resistant subclones compared to WT HL60 cells (Figs. 3A, B). TRAIL-resistant HL60 subclones remained sensitive to the chemotherapy agents, idarubicin and cytarabine (Figs. 2A–C). Selective exposure of cytarabine and idarubicin was then used to derive cytarabine- or idarubicin-resistant HL60 cell lines. Both cytarabine- and idarubicin-resistant HL60 subclones remained susceptible to TRAIL- and $\text{TNF}\alpha$ -induced apoptosis (Figs. 2D, E).

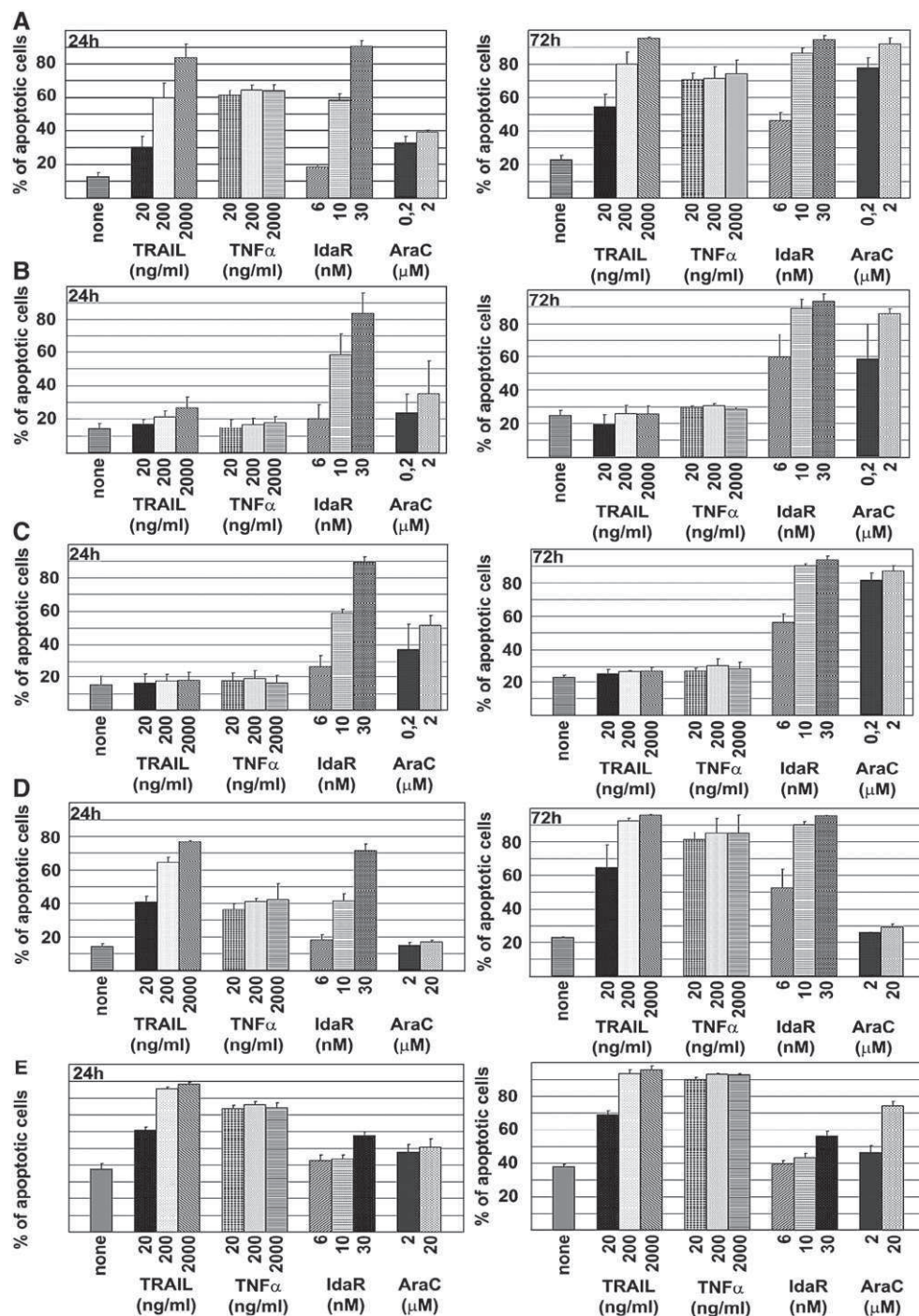


Fig. 2. (A–E) TRAIL-resistant HL60 subclones are resistant to $\text{TNF}\alpha$, but remain sensitive to idarubicin and cytarabine. The level of apoptosis induced in (A) WT HL60 cells, (B) TRAIL-resistant P1 subclones, (C) TRAIL-resistant P2 subclones, (D) cytarabine (AraC)-resistant HL60 subclones, and (E) idarubicin (IdaR)-resistant subclones at 24 h and 72 h after the addition of selected apoptotic inducers at given concentrations. Flow cytometry analysis of Annexin-V stained cells. Columns represent means and error bars standard deviations of three independent experiments.

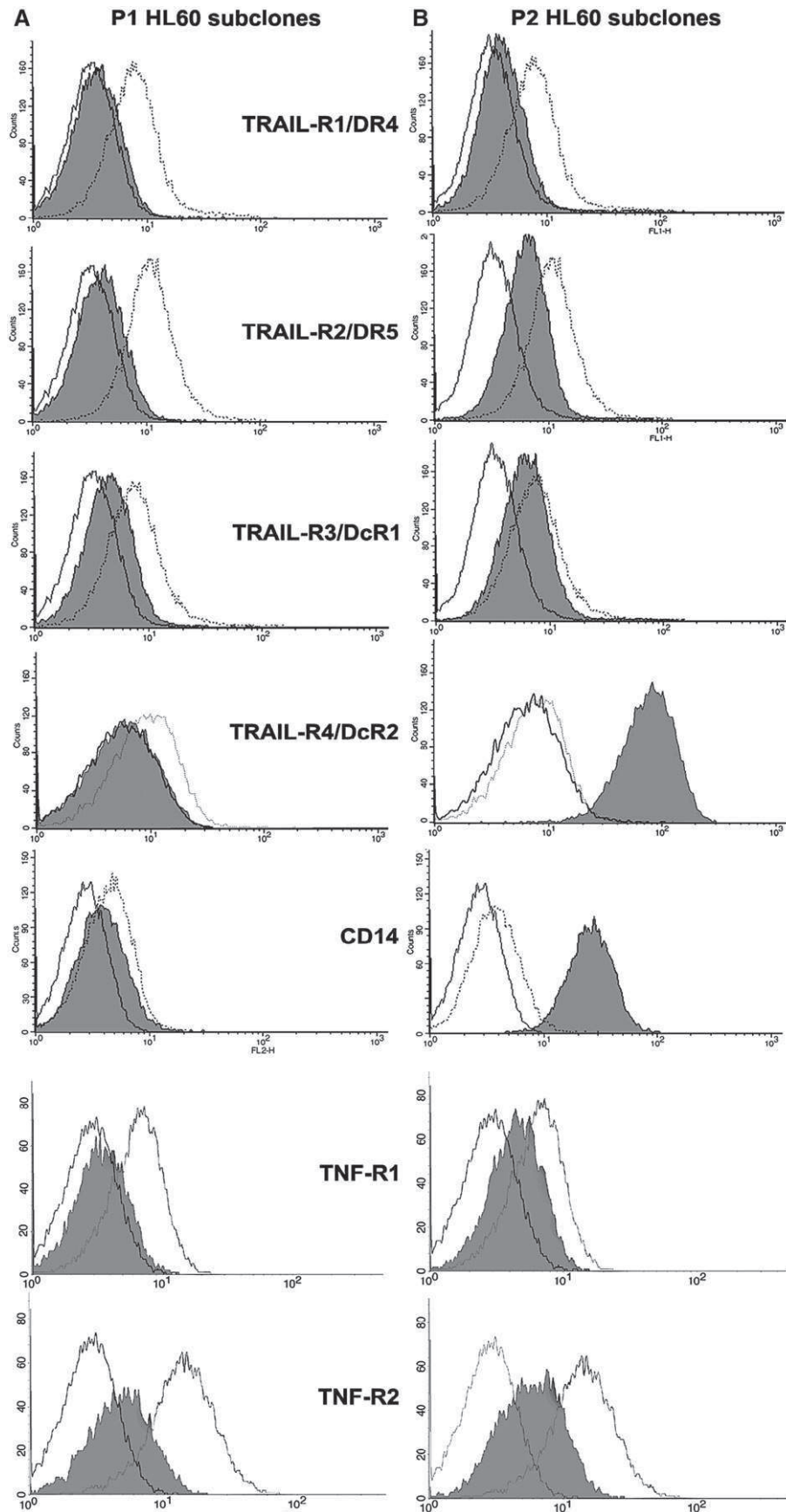


Fig. 3. (A, B) Flow cytometry analysis of TRAIL and TNF α receptors and CD14 expression of TRAIL-resistant P1 (A) and P2 (B) HL60 subclones. Filled histograms represent stains of TRAIL-resistant HL60 subclones P1 (A) and P2 (B). Empty histograms bounded by continuous line represent control stains with secondary antibody or isotype antibody. Empty histograms bounded by dashed line represent corresponding stains of WT HL60 cells. Results shown are representative examples of phenotype changes associated with acquired TRAIL resistance of HL60 subclones.

Two distinct phenotypic changes of TRAIL-resistant HL60 subclones

Based on the expression pattern of individual TRAIL receptors, TRAIL, and CD14, we identified HL60 TRAIL-resistant subclones with two distinct phenotypes, designated as P1 and P2. Subclones P1, detected in 17 of 20 (85%), of generated TRAIL-resistant HL60 subclones were characterized by suppressed expression of both TRAIL death receptors and of decoy receptor DcR1/TRAIL-R3 (Fig. 3A). Subclones P2, found in 3 of 20 (15%) of TRAIL-resistant HL60 subclones were also characterized by suppressed expression of both TRAIL death receptors and decoy receptor DcR1/TRAIL-R3. The surface protein expressions of DR5/TRAIL-R2 and DcR1/TRAIL-R3 were, however, less affected in P2 cells than P1 cells (Figs. 3A, B). In contrast to P1, P2 subclones had up-regulated cell surface DcR2/TRAIL-R4 and surface myeloid differentiation antigen CD14 (Fig. 3B).

The P1 subclones had significant (>2-fold) up-regulation of DcR2/TRAIL-R4 receptor mRNA (2.5-fold) (Fig. 6A). The DR4/TRAIL-R1, DR5/TRAIL-R2 and DcR1/TRAIL-R3 mRNA of P1 cells did not significantly change, which did not correlate with the decrease of cell surface protein expression of these receptors (Figs. 3A and 6A). In P2 subclones, the mRNAs of DR5/TRAIL-R2, DcR1/TRAIL-R3, and TRAIL were significantly down-regulated (3, 14.3, and 11.1 fold, respectively). The DcR2/TRAIL-R4 mRNA was not significantly changed despite the observed cell surface DcR2 protein up-regulation. The mRNA expression of osteoprotegerin (OPG) in P2 subclones was not detectable by the real-time RT-PCR (Fig. 6A).

The prolonged *in vitro* culture of P1 cells without TRAIL (4 weeks) led to partial restoration of P1 cell sensitivity to TRAIL, 40% ± 5% of apoptotic cells (*n*=3), in contrast to freshly derived P1 subclones 8% ± 4% of apoptotic cells (24 h after 200 ng/ml of TRAIL). The TRAIL resistance of P2 subclone cells remained unchanged after 4 weeks of *in vitro* culture without TRAIL.

Using a WST-8 cell proliferation assay in medium without TRAIL, we detected decreased proliferation of P1 cells and increased proliferation of P2 cells compared to WT HL60 cells (Fig. 6E).

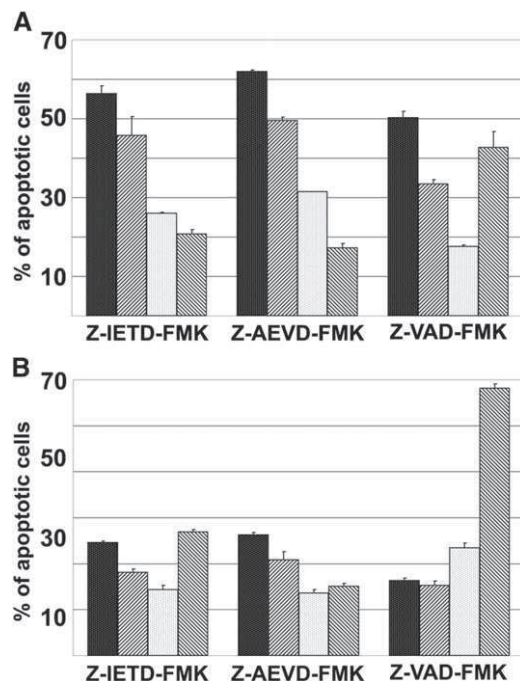


Fig. 4. (A, B) Inhibition of both caspase 8 and caspase 10 significantly reduces TRAIL and TNFα-induced apoptosis. WT HL60 cells were pre-incubated with specific caspase inhibitors (caspase 8 inhibitor Z-IETD-FMK, caspase 10 inhibitor Z-AEVD-FMK, and pan-caspase inhibitor Z-VAD-FMK) for 60 min before exposure to TRAIL or TNFα. For each caspase inhibitor the final concentrations were from left (black) column to right 0.64 μM, 3.2 μM, 16 μM and 80 μM. The level of apoptosis was determined at 24 h after the addition of TRAIL (A) or TNFα (B). The level of apoptosis without caspase inhibition induced by TRAIL and TNFα was 66.83 ± 1.8% and 31.43 ± 0.3%, respectively.

Activation of caspases 8 and 10 is suppressed in TRAIL-resistant HL60 subclones compared to WT HL60 cells

The pharmacological blockage of apical caspases 8 and 10 achieved by preincubating the cells with caspase-specific inhibitors (Z-IETD-FMK, Z-AEVD-FMK, and Z-VAD-FMK) significantly abrogated the proapoptotic effects of TRAIL and TNFα (Figs. 4A, B).

To test the possibility that deregulated activation of apical caspases, caspase 8 and/or 10, contributed to resistance of TRAIL-resistant HL60 subclones to TRAIL and TNFα, we measured the relative activation of caspases 8 and 10 after the TRAIL and TNFα stimulation of WT HL60 cells in P1, and P2 TRAIL-resistant subclones. After the exposure to TRAIL, the activation of both caspase 8 and 10 was significantly decreased in TRAIL-resistant subclones (Figs. 5A, B). While in P1 subclones the caspase 8 activation was lower compared to HL60 WT cells, in P2 subclones we did not detect any caspase 8 activation during the first 4 h after the exposure to TRAIL. Similarly, the increase of relative caspase 10 activity after the challenge to TNFα was lower in P1 and virtually undetectable in P2 subclone cells (Fig. 5D). We detected only minimal activation of caspase 8 in WT HL60 cells during the first 4 h after the exposure to TNFα (Fig. 5C). Interestingly, in WT HL60 cells, the relative increase of caspase 10 activity after both TRAIL and TNFα was higher compared to the increase of the activity of caspase 8.

Distinct expression patterns of antiapoptotic molecules are associated with P1 and P2 TRAIL-resistant HL60 subclones

In search for possible molecular mechanisms that might contribute to acquired TRAIL resistance of HL60 cells, we analyzed gene expressions of selected death-inducing signaling complex (DISC) components and of several essential anti-apoptotic regulators. P1 HL60 subclones had up-regulation of mRNA of the following anti-apoptotic genes: cIAP2 (9.5-fold), MCL1 (4.9-fold), cIAP1 (4.7-fold), XIAP (4.1-fold), BCL2 (3.4-fold), BCL-W (3.1-fold), and BCL-XL (2.3-fold). Both apical caspases, CASP8 (2.1-fold), and CASP10 (3.8-fold) and BID (7-fold) were upregulated in P1 cells (Figs. 6B–D).

P2 cells had significant overexpression of a single measured gene: BID (5.7-fold). None of the measured anti-apoptotic regulator mRNAs, i.e., cIAP1, cIAP2, survivin, NAIP, XIAP, MCL1, BCL2, BCL-W, and BCL-XL, was increased more than two-fold in P2 subclones compared to WT HL60 cells. The following transcripts were down-regulated in P2 subclones: cIAP2 (5.9-fold), MCL1 (2.2-fold), cFLIP-L (2.1-fold), and FADD (4.5-fold) – an adaptor molecule for TRAIL receptor transduction of apoptotic signal and anti-apoptotic regulator (Figs. 6B–D).

HL60 TRAIL-resistant subclones have diminished TRAIL-induced NFκB nuclear translocation

Translocation of NFκB to the nucleus and activation of NFκB target gene expression are an important anti-apoptotic mechanism of malignant cells [18]. Aberrant NFκB activation and nuclear translocation have been described in a wide range of tumors [19,20]. TRAIL induces NFκB nuclear translocation as a result of non-apoptotic signaling pathway activation [21]. RelA/p65, a member NFκB family, was reported to be an essential survival factor of diverse malignant cells by down-regulation of TRAIL death receptors and up-regulation of BCL-XL or cIAP1, cIAP2, members of the IAP family [22,23]. To analyze whether the detected up-regulation of Bcl-XL and IAP family members in P1 HL60 cells and up-regulation of DcR2/TRAIL-R2 in P2 cells is associated with changes in NFκB activation, we measured RelA/p65 translocation to the nucleus after exposure of the WT, P1, and P2 HL60 cells to TRAIL.

The nuclear RelA/p65 level was similar in non-stimulated WT HL60 cells (optical density, OD=0.148 ± 0.022) and TRAIL-resistant cells (OD=0.160 ± 0.014). The increase of nuclear concentration of RelA/p65

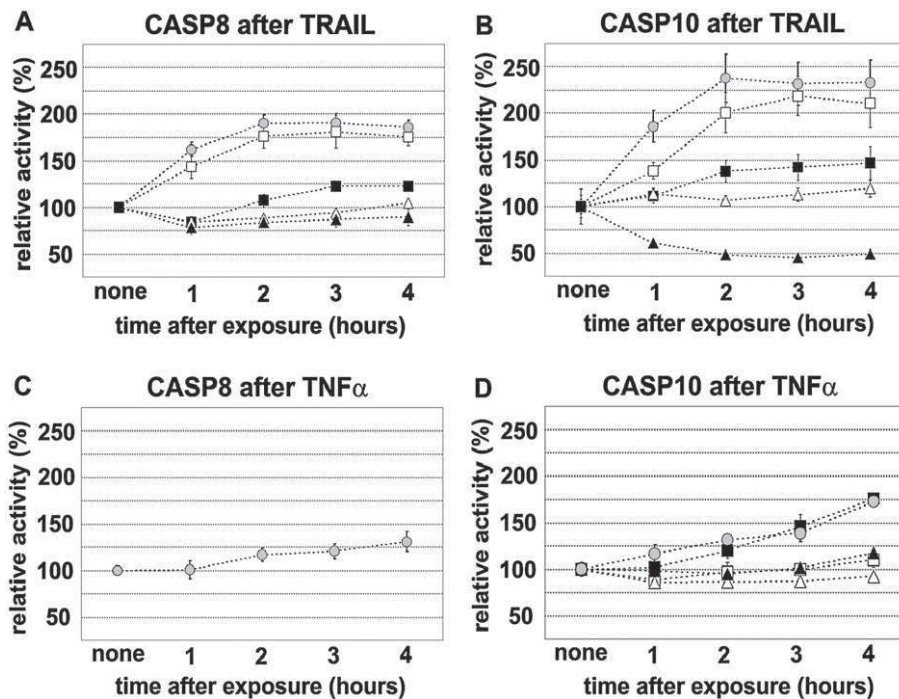


Fig. 5. (A–D) TRAIL and TNF α induced caspases 8 and 10 activation. Decreased TRAIL-induced caspases 8 and 10 activation in TRAIL-resistant HL60 subclones (P1: squares, P2: triangles) compared to WT HL60 cells (WT: circles) (A, B). Similarly, caspase 10 activation was diminished after the challenge with TNF α in TRAIL-resistant HL60 subclones compared to WT HL60 cells (D). Interestingly minimal caspase 8 activation was detected in WT HL60 cells during first 4 h after exposure to TNF α (C). Results are expressed as percent increase of caspase activity. 100% corresponds to caspase activity of unstimulated cells. Results represent means $\pm$ SD of triplicate experiments.

in response to TRAIL was detectable after 20 minute exposure of the cells to TRAIL. WT HL60 cells had earlier onset and significantly higher peak of the RelA/p65 translocation level compared to TRAIL-resistant

subclones of either phenotype. The peak of nuclear RelA/p65 concentration was delayed in P2 subclones compared to both P1 and WT HL60 cells (Fig. 6F).

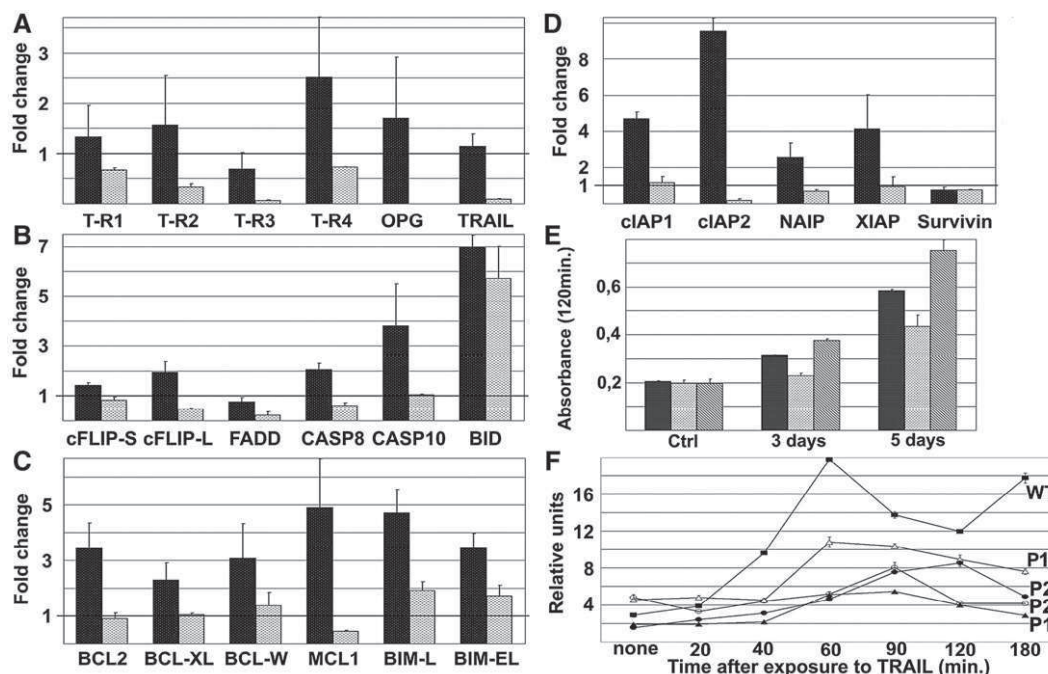


Fig. 6. (A–E) mRNA expression of apoptosis regulator molecules, proliferation rate, and nucleus translocation of RelA/p65 NF κ B in TRAIL-resistant P1 and P2 subclones compared to WT HL60 cells. Real-time RT-PCR analysis of relative mRNA expression changes in TRAIL-resistant HL60 subclones P1 (dark columns) and P2 (light columns) compared to WT HL60 cells (normalized mRNA expression of WT cells = 1). Expression of TRAIL receptors and apoptotic regulator mRNAs were measured in triplicates for each cell population and presented as means (columns) and standard deviations (error bars) from two TRAIL-resistant HL60 cell lines of each subclone phenotype, P1 and P2 (A–D). Proliferation rates of TRAIL-resistant HL60 subclones P1 and P2 is decreased and increased, respectively, compared to the proliferation rate of WT HL60 cells. Representative example of WST-8 cell proliferation assay of three independent experiments performed in quadruplicates is shown (E). TRAIL-induced NF κ B RelA/p65 translocation to the nucleus was suppressed in TRAIL-resistant HL60 subclones compared to WT HL60 cells. WT HL60 cells (WT), two representative TRAIL-resistant HL60 subclones P1 (P1a, P1b) and two representative TRAIL-resistant HL60 subclones P2 (P2a, P2b) were analyzed at given time points after the exposure to 200 ng/ml of TRAIL (F).

The survival of immunodeficient mice xenotransplanted with P2 HL60 cells is shorter compared to mice xenotransplanted with WT or P1 HL60 cells

Overall survival represents a key parameter of tumor aggressiveness and treatment efficacy of human malignancies. We have shown that acquired TRAIL resistance of HL60 cells is associated not only with down-regulation of death receptors for TRAIL but also with down-regulation of receptors for TNF α and with other phenotypic changes, including up-regulation of several antiapoptotic genes in P1 cells, up-regulation of DcR2 and CD14, absence of OPG expression, and the increase in proliferation rate in P2 cells. These changes, while silent *in vitro*, might prove critical during growth and/or metastasizing of the tumor cells *in vivo*. To analyze whether the *in vitro*-detected phenotypic changes of TRAIL-resistant HL60 subclones are associated with *in vivo* changed biologic behavior and/or overall survival, we intravenously xenotransplanted WT, P1 and P2 HL60 cells into immunodeficient NOD/LtSz-Rag1^{null} mice. Animals xenotransplanted with WT HL60 cells ($n=6$) showed diffuse leukemia infiltration of the brain with $25.2\pm6.4\%$ of human CD45+ cells. The leukemia cell infiltration of the bone marrow was higher ($4.8\pm5.1\%$) than the infiltration of the liver ($0.8\pm1.1\%$; $p=0.04$). About half of the animals developed subcutaneous tumors in the areas of lymph node distribution. All the tumors were round-shaped, non-invasive, with the mean weight of 0.8 ± 0.2 g. The tumors consisted of $25\pm4.9\%$ of the CD45+ cells of human origin. The overall survival of the WT cells-injected mice was 41.3 ± 2.3 days.

Transplantation with P1 cells ($n=6$; 2 different P1 subclones) was associated with larger leukemia cell infiltration of the brain ($38.5\pm9.6\%$ of human CD45+ cells) compared to WT HL60 cells ($25.2\pm6.4\%$; $p=0.014$) and P2 cells ($22\pm8.4\%$; $p=0.008$). The P1 cells infiltrated the bone marrow ($7.3\pm6.6\%$) and liver ($1.7\pm1.7\%$). P1 cells-transplanted mice developed occasional non-invasive subcutaneous tumors of similar shape, location, weight (0.4 ± 0.1 g) and composition, of human CD45+ cells $19\pm3.9\%$, as did WT HL60 cells-transplanted mice. The overall survival of P1 cells-injected mice was 40.5 ± 1.5 days.

The animals transplanted with P2 cells ($n=6$; 2 different P2 subclones) had leukemic infiltration of the brain, ($22\pm8.4\%$), bone marrow ($8.6\pm9.9\%$) and liver ($1.4\pm1.3\%$). In sharp contrast, however, the P2-transplanted mice developed also multiple intraperitoneal tissue- and organ-invasive tumors. The mean intraperitoneal tumor weight was 2.6 ± 1.0 g and consisted of a significantly higher percentage of the cells of human origin ($69.8\pm9.8\%$ of human CD45+ cells compared to $25\pm4.9\%$ and $19\pm3.9\%$ in the tumors of WT HL60 cells- and P1 cells-injected mice, respectively; $p\leq0.0001$). Moreover, the P2-injected mice frequently developed hind-leg paralysis (3 of 6 animals) that was followed by their demise. Overall survival of the mice xenotransplanted with P2 cells was significantly shorter (30.8 ± 3.4 days) compared to both WT HL60 cells- and P1 cells-transplanted mice (41.3 ± 2.1 and 40.5 ± 1.5 , respectively; $p\leq0.0001$).

Human cells derived from tumors of WT-, P1-, and P2-xenotransplanted mice were subjected to the measurement of apoptosis in response to TRAIL. Tumor-derived P1 cells showed substantial restoration of TRAIL sensitivity, $48.0\pm5.0\%$ apoptotic cells 24 h after the exposure to 200 ng/ml. In contrast tumor derived P2 cells, preserve TRAIL-resistance, $3\pm1\%$ apoptotic cells 24 h after the exposure to 200 ng/ml of TRAIL.

Discussion

TRAIL induces apoptosis of diverse acute myeloid leukemia cell lines as well as primary leukemic cells [10–13]. The role of individual death receptors in triggering apoptotic pathway in leukemia cells, however, remains largely unexplored. In the present study, antibody-mediated blockage of TRAIL-R1/DR4 receptor-suppressed TRAIL-

induced apoptosis of WT HL60 cells more than did the antibody-mediated blockage of TRAIL-R2/DR5 receptor.

By prolonged exposure of WT HL60 cells to TRAIL, we derived several TRAIL-resistant HL60 subclones. All subclones had down-regulated DR4 and DR5 cell surface TRAIL death receptors, suggesting a mechanism by which these cells escape TRAIL-induced apoptosis.

The TRAIL-resistant subclones were also resistant to TNF α . Resistance to TNF α -induced apoptosis was associated with down-regulation of its receptors, TNF-R1 and TNF-R2. Recently, acquired resistance of a human ovarian carcinoma cell line to TRAIL was associated with cross-resistance to Fas-ligand, another TNF α family death ligand [24]. Together, these results suggest that acquired resistance to TRAIL might involve common regulatory mechanisms including other members of the TNF receptor family. As a consequence, acquired TRAIL resistance of tumors following TRAIL treatment might be associated with resistance to other death ligand family members, TNF α and FasL, suggesting increased ability of the tumor cells to escape tumor immune surveillance and anti-tumor immune responses.

The mRNA profiles of the TRAIL-resistant HL60 subclones suggest that the detected changes of the TRAIL death receptors expression are at least partially regulated post-translationally.

TRAIL-resistant HL60 subclones retained sensitivity to the cytotoxic chemotherapy drugs, cytarabine and idarubicin, compared to WT HL60 cells. The data imply disrupted extrinsic apoptotic pathways, but functionally intact intrinsic apoptotic pathways. In a similar way, the cytarabine- and idarubicin-resistant HL60 subclones retained sensitivity to TRAIL-induced apoptosis, suggesting disrupted intrinsic but functionally intact extrinsic apoptotic pathways. These data suggest that development of novel therapeutic strategies targeting both intrinsic and extrinsic apoptotic pathways might minimize the potential risks of cancer drug-resistance development.

Analysis of the TRAIL-resistant HL60 subclones revealed two distinct phenotypic changes, designated as P1 and P2. The P1 cells had down-regulation of cell surface TRAIL death receptors, DR4 and DR5, and of the decoy receptor DcR1. At the mRNA level, P1 cells exhibited up-regulation of several anti-apoptotic genes. The P2 cells had down-regulation of both TRAIL death receptors, DR4 and DR5. In contrast to P1 cells, P2 cells had unchanged expression of antiapoptotic molecules, but exhibited down-regulation of the Fas-associated death domain adaptor protein (FADD), an important regulator of receptor-mediated apoptosis [25]. In addition, P2 cells had an increase of cell surface decoy receptor 2 (DcR2) and undetectable osteoprotegerin (OPG) mRNA compared to P1 and WT HL60 cells. Moreover, the CD14 differentiation antigen was upregulated in a P2 cells. Recently, Secchiero et al. reported that TRAIL was able to induce myeloid differentiation of both normal and leukemic human myeloid cells [26]. A maturation marker of monocytic differentiation, CD14, is expressed during terminal myeloid differentiation and the tissue specific monocytic commitment. The gain of CD14 expression in P2 cells implies that TRAIL induced differentiation. The proliferation of P2 cells compared to both WT and P1 cells was significantly increased. The increased proliferation of P2 cells suggest that the selective pressure of TRAIL on HL60 cells resulted in genetic or epigenetic changes resulting in the significant growth advantage to these cells that was not present in the original HL60 WT cells. Increased DcR2 expression on the cell surface of P2 HL60 cells suggested a possibility of prosurvival signaling through this receptor, mediated by activation of NF κ B [18,19,22,23]. In the experiments we report here, however, the TRAIL-induced NF κ B translocation to the nucleus was decreased in both TRAIL-resistant P1 and P2 cells. The exact molecular mechanisms of this TRAIL-induced non-apoptotic signaling in HL60 cells remain elusive.

Recently, several studies reported caspase 8 mutation and its accelerated degradation as a mechanism of TRAIL resistance in several

tumor cell lines [17,27]. The role for caspase 10 in mediating TRAIL-induced apoptosis in leukemia remains largely unexplored. In the present study, inhibition of either caspase 8 or caspase 10 led to the significant abrogation of TRAIL or TNF α -induced apoptosis of TRAIL-sensitive WT HL60 cells. Both TRAIL-resistant subclones had decreased activity of caspases 8 and 10 after the challenge with TRAIL or TNF α , which is in concordance with decreased cell surface expression of TRAIL and TNF α receptors. While in P2 cells we did not detect any caspase 8 activation during the first 4 h after the exposure to TRAIL, in P1 cells the caspase 8 activation was significantly lower compared to HL60 WT cells. The partial caspase 8 activation in P1 subclones compared to the complete caspase 8 switch-off in P2 cells is in concordance with the finding that P1 cells partially restored TRAIL sensitivity during long-term culture without TRAIL, while P2 cells remained TRAIL-resistant. Interestingly, in WT HL60 cells the increase of caspase 10 activity after both TRAIL and TNF α was higher compared to the increase of the activity of caspase 8. These data demonstrate that both apical caspases 8 and 10 play important roles in death receptor-mediated apoptosis of HL60 cells.

TRAIL has been reported to induce growth of a subset of leukemia cells with extrinsic apoptotic pathway defects and with the progression of TRAIL-resistant cancer metastasis and growth [28–30]. TRAIL did not stimulate *in vitro* growth of the TRAIL-resistant HL60 subclones (data not shown).

We propose common regulatory mechanisms for TRAIL receptors and other TNF-R family proteins during the development of acquired resistance to TRAIL. In addition, we suggest that the phenotypic changes of P2 cells were at least partially induced by non-apoptotic TRAIL signaling. To test the hypothesis that such changes, while silent *in vitro* might be important *in vivo*, we performed xenotransplantation experiments using immunodeficient mice model. We show that xenotransplanted P2 HL60 cells display more aggressive biologic behavior with different growth patterns and that P2 transplanted mice had significantly shorter overall survival. The shorter overall survival of P2 cells-xenotransplanted mice might be explained by higher proliferation rate of P2 cells, but also by the TRAIL-resistant phenotype of P2 cells compared to TRAIL-sensitive WT HL60 cells and “leaky” TRAIL-resistant P1 cells. Murine TRAIL (mTRAIL) is able to trigger apoptosis of TRAIL-sensitive human cancer cells including HL60 cells (45 $\pm$ 5% apoptotic cells 24 h after the exposure to 200 ng/ml mTRAIL). Stable TRAIL resistance might thus represent a substantial advantage for P1 cells during *in vivo* growth in mice. These results suggest that acquired TRAIL resistance may be associated with more aggressive behavior and the resulting worse clinical outcome.

The data from this study imply that targeting of both intrinsic and extrinsic apoptotic pathways is relevant for the development of effective TRAIL-based therapeutic armamentarium for the treatment of leukemia and other malignancies.

Acknowledgments

We thank Monika Hulova for technical assistance. We are grateful to Josef T. Prchal (University of Utah) for critical reading of the manuscript.

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The plant alkaloid and anti-leukemia drug homoharringtonine sensitizes resistant human colorectal carcinoma cells to TRAIL-induced apoptosis via multiple mechanisms

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Abstract TNF-related apoptosis-inducing ligand (TRAIL) is a pro-apoptotic ligand from the TNF-alpha family that is under consideration, along with agonistic anti-TRAIL receptor antibodies, as a potential anti-tumor agent. However, most primary human tumors are resistant to monotherapy with TRAIL apoptogens, and thus the potential applicability of TRAIL in anti-tumor therapy ultimately depends on its rational combination with drugs targeting these resistances. In our high-throughput screening for novel agents/drugs that could sensitize TRAIL-resistant colorectal cancer cells to TRAIL-induced apoptosis, we found homoharringtonine (HHT), a cephalotaxus alkaloid and tested anti-leukemia drug, to be a very effective, low nanomolar enhancer of TRAIL-mediated apoptosis/growth suppression of these resistant cells. Co-treatment of TRAIL-resistant RKO or HT-29 cells with HHT and TRAIL led to the effective

induction of apoptosis and the complete elimination of the treated cells. HHT suppressed the expression of the anti-apoptotic proteins Mcl-1 and cFLIP and enhanced the TRAIL-triggered activation of JNK and p38 kinases. The shRNA-mediated down-regulation of cFLIP or Mcl-1 in HT-29 or RKO cells variably enhanced their TRAIL-induced apoptosis but it did not markedly sensitize them to TRAIL-mediated growth suppression. However, with the notable exception of RKO/sh cFLIP cells, the downregulation of cFLIP or Mcl-1 significantly lowered the effective concentration of HHT in HHT + TRAIL co-treatment. Combined HHT + TRAIL therapy also led to the strong suppression of HT-29 tumors implanted into immunodeficient mice. Thus, HHT represents a very efficient enhancer of TRAIL-induced apoptosis with potential application in TRAIL-based, anti-cancer combination therapy.

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Electronic supplementary material The online version of this article (doi:10.1007/s10495-013-0823-9) contains supplementary material, which is available to authorized users.

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Keywords Harringtonine · Apoptosis · Death receptor · cFLIP · Mcl-1

Introduction

New natural and rationally designed drugs are being developed and tested in the never-ending battle against cancer [1–5]. Among these agents is the pro-apoptotic ligand TNF-related apoptosis-inducing ligand (TRAIL), which can, along with agonistic anti-TRAIL receptor antibodies, selectively induce the apoptosis of cancer cells. These TRAIL-related apoptogens are currently being evaluated in phase I/II clinical trials (recently reviewed in [6–8]). TRAIL binding to its pro-apoptotic receptors TRAIL-R1/DR4 or TRAIL-R2/DR5 leads to the rapid formation of the intracellular multiprotein death-inducing signaling complex (DISC), which, in addition to the ligand/receptor cluster, is

composed of the adapter protein FADD, the initiator procaspases-8/-10 and the caspases-8/-10 antagonist cFLIP [9, 10]. In addition to these core DISC components, other proteins such as PEA-15/PED, DDX-3, TRADD, DJ-1 or MADD can, in a cell- and conditionally-specific manner, associate with the TRAIL receptosome and negatively affect either DISC formation or caspase-8 processing [11–15].

Though TRAIL induces the apoptosis of a number of cultured cancer cells [16, 17], many primary cancer cells are resistant to TRAIL, either intrinsically and/or through anti-apoptotic signals from the tumor microenvironment. Among these protective signals are the activation of the PI3K/Akt pathway, the increased expression of anti-apoptotic proteins such as cFLIP, Mcl-1, Bcl-XL or XIAP and the suppressed expression/modification of pro-apoptotic molecules (caspases, Bid, pro-apoptotic TRAIL receptors) [8, 18]. Moreover, the TRAIL-induced activation of NF- κ B signaling can enhance the survival and proliferation of these resistant cancer cells [19]. For these reasons, “smart” therapy combining TRAIL apoptogens with current tested anti-tumor drugs seems to be a preferred choice. Indeed, many drugs such as DNA damaging agents, histone deacetylase or proteasome inhibitors, Bcl-2 and IAP antagonists, natural products as well as therapeutic antibodies can synergize with TRAIL, effectively eliminating resistant cancer cells of various origin [20–22]. The sensitizing mechanisms, though not fully understood, include the increased expression of TRAIL receptors or initiator/effector caspases, the production of reactive oxygen species (ROS), the downregulation of anti-apoptotic proteins (cFLIP, Mcl-1), the suppression of anti-apoptotic signaling (NF- κ B or PI3K inhibitors) and the stabilization of pro-apoptotic proteins such as active caspases or tBid (recently reviewed in [20]).

Currently, many natural products such as wogonin, nimbolide and others have emerged as potent sensitizers of resistant cancer cells to TRAIL-induced apoptosis [23–25]. Using a high-throughput screening (HTS) approach, we uncovered homoharringtonine (HHT), an alkaloid originally isolated from *Cephalotaxus harringtonia*, and found it to be one of the most potent sensitizers of resistant colorectal cancer cells to TRAIL-induced apoptosis. HHT has been used in traditional Chinese medicine for the treatment of diverse malignancies. Recently, its semisynthetic variant Omacetaxine mepesuccinate (marketed under the name Omapro) has been considered as an alternative therapeutic option in the treatment of imatinib-resistant chronic myeloid leukemias [26, 27]. HHT can inhibit proteosynthesis, which in affected cells leads to the proteolytic degradation of short-lived anti-apoptotic proteins such as Mcl-1, cFLIP, survivin and others [28]. HHT also suppresses the growth of non-leukemia cancer cells [29, 30], and in our model of TRAIL-resistant RKO or HT-29 colorectal cancer cells,

HHT treatment led to the downregulation of Mcl-1 and cFLIP expression. However, the shRNA-mediated downregulation of either Mcl-1 or cFLIP expression in these cells did not fully sensitize them to TRAIL-induced growth suppression. HHT also very robustly cooperated with TRAIL in the growth suppression of HT-29 tumors in immunodeficient mice. Thus, HHT in combination with TRAIL or with agonistic anti-TRAIL receptor antibodies could represent an interesting option in anticancer therapy.

Materials and methods

Cell cultures and chemicals

The human colon carcinoma cell lines RKO and HT-29 and normal colon epithelial cells CCD 841 were purchased from ATCC. RKO and HT-29 cells were maintained in DMEM + 10 % FCS at 37 °C and 5 % CO₂. CCD 841 cells were cultivated in collagen-coated dishes in the recommended medium (ATCC, CRL-1790) supplemented with 10 % FCS. All chemicals used were purchased from Sigma-Aldrich unless stated otherwise.

High-throughput screening

HT-29 cells were plated in 384-well plates (Corning) at a density of 2,500 cells/25 μ l/well using a Multidrop Combi dispenser (Thermo Scientific) and cultivated overnight. Then, library compounds were added using pintool (V&P Scientific) coupled to a JANUS Automated Workstation (PerkinElmer) to a final concentration of 1 μ M in the absence or in combination with 100 ng/ml of recombinant TRAIL (Killer TRAIL, ENZO LS). The compound library included the Library of Pharmacologically Active Compounds (LOPAC1280, Sigma-Aldrich), the Prestwick Chemical Library (Illkirch, France) and the NIH Clinical Trial Collection (NIH, USA). In total 2,448 various unique compounds were used in the course of HTS screening. The cells were cultivated for 48 h, and then their viability was determined by the CellTiter-Blue assay (Promega) and an EnVision microplate reader (PerkinElmer).

Analysis of cell proliferation/survival and activation of effector caspases

The long-term survival of treated cells was determined by the WST-1 (Roche) and CellTiter-Blue (Promega) assays using a 96- or 384-well format, respectively, according to the manufacturers' recommendations. The real-time kinetic xCELLigence-based cell survival assay was performed according to the manufacturer's recommendations. Briefly,

3,000–5,000 cells were plated in triplicate into individual wells of an E-plate (Roche), placed into the monitoring unit in a CO₂ incubator and monitored for growth. When the cells reached approximately 40–50 % confluency, HHT and/or TRAIL were added and the on-line monitoring continued for an additional 60–70 h. The activity of the effector caspases-3/7 was quantified by the Caspase-Glo 3/7 assay (Promega) in 384-well plates, according to the manufacturer's instructions.

DISC precipitation

Cells grown to 80 % confluence in 140 mm cell culture dishes were pretreated with 50 nM HHT for 3 h, then the medium was replaced with ice-cold medium and the cells were incubated on ice for 10 min. Where applicable, biotin-labeled TRAIL (bio-TRAIL) was added to a final concentration of 1 µg/ml for 20 min. After this pre-incubation, the cold medium containing bio-TRAIL was replaced with warm medium at 37 °C (without bio-TRAIL), and the cells were incubated at 37 °C for 0–40 min. At selected time points, the culture dishes were placed on ice, and the cultivation medium was replaced with 20 ml of ice-cold phosphate-buffered saline (PBS). After washing with ice-cold PBS, the cells were scraped and centrifuged (800×g, 4 °C, 5 min). The cell pellets were lysed in ice-cold lysis buffer (1 % NP-40, 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 1 mM Na₃VO₄, 10 % glycerol) supplemented with protease inhibitors (complete protease inhibitor cocktail, Roche) on ice for 30 min, and the lysates were then centrifuged (15,000×g, 4 °C, 30 min). The cleared lysates were diluted to the same protein concentration (usually 3 mg/ml) and incubated with streptavidin agarose beads (Thermo Scientific) at 4 °C for 2 h. After five washes with 10 volumes of the lysis buffer, the DISC precipitates were directly eluted with 2× SDS sample buffer (95 °C, 5 min) and analyzed by Western blotting.

Cell lysis and western blotting

Cells were washed with ice-cold PBS, scraped, centrifuged, and the cell pellets lysed in ice-cold lysis buffer (1 % Triton X-100, 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM Na₃VO₄) supplemented with protease inhibitors (Complete, Roche) on ice for 30 min. The lysates were centrifuged (15,000×g, 4 °C, 30 min), and the amount of total protein in the supernatants was quantified by the BCA assay (Pierce). The supernatants were diluted 1:1 with 2× SDS sample buffer, heated to 95 °C for 5 min and analyzed by Western blotting (usually 50 µg of total protein was loaded per well). The antibodies against following proteins were used: Bcl-XL (2764), Bid (2002), caspase-9 (9502), FADD (2782), IκBα (8414), P-IκBα

(2859), NF-κB/p65 (4764), P-NF-κB/p65 (3033), SAPK/JNK (9252), P-SAPK/JNK Thr183/Tyr185 (9251), p-38 MAPK (9212) and P-p38MAPK (9211) from Cell Signaling; Mcl-1 (559027) and Caspase-8 (559932) from BD Biosciences; β-actin (SC-1615) and Bak (SC-832) from Santa Cruz Biotechnology; cFLIP (ALX-804-428) and Bim (ALX-804-351) from Enzo Life Science; caspase-10 (M059-3) from MBL and DR5 (SAB3500427) from Sigma-Aldrich.

Flow cytometry analysis of apoptosis and receptor expression

For the Annexin V-FITC assay of apoptotic cells, the cell cultures (typically 1.5×10^5 cells in each well of a 24-well plate) were harvested by trypsinization, washed once with PBS and once with ice-cold Annexin binding buffer. Then, Annexin V-FITC (Apronex Biotechnologies) was added to a final concentration of 2 µg/ml and the samples incubated on ice for 20 min. After adding Hoechst 33258 to a final concentration of 0.5 µg/ml, the samples were analyzed by flow cytometry on a LSRII (BD Biosciences).

For flow cytometry analysis of death receptor expression, cells were harvested by trypsinization and incubated in a blocking buffer (PBS + 0.2 % gelatin and 0.1 % sodium azide—PBS-GA) containing 20 % heat-inactivated human serum for 10 min. Then, the samples were incubated on ice for 30 min with antibodies against DR4, DR5 (10-403, 11-461, EXBIO) or FAS/CD95 (DX2, R&D System Inc.) diluted in PBS-GA to a final concentration of 2–5 µg/ml. The cells were then washed twice with ice-cold PBS-GA and incubated with phycoerythrin-conjugated goat anti-mouse IgG1 (Southern Biotech, diluted in PBS-GA to 4 µg/ml) on ice for 30 min. After two final washes, the cells were resuspended in PBS-GA containing 0.5 µg/ml Hoechst 33258, and the cell surface expression of receptors on living cells (Hoechst 33258-negative) was analyzed by flow cytometry on a LSRII.

Preparation of RKO and HT-29 cells with the downregulated expression of cFLIP or Mcl-1 and RKO cells with transiently suppressed expression of p53

HEK293T cells were transfected with the lentivirus packaging plasmids pMD2G, psPAX (Addgene) and pLKO.1 carrying shRNA against cFLIP (4 shRNA), Mcl-1 (5 shRNA) (all from Open Biosystems/Hermo Scientific). Two days later the lentiviral particles were purified from the supernatant using PEG/it Virus Precipitation Solution (SBI, LV810A-1). RKO and HT-29 cells were transduced at MOI 5 and selected in medium containing puromycin (3 µg/ml) for 4 days. Cells displaying the effective (80–90 %) downregulation of cFLIP (TRCN0000007229—shFLIP1, TRCN0000007230—shFLIP2) or Mcl-1 (TRCN0000005515—shMcl-1-2,

TRCN0000005517—shMcl-1-4, TRCN0000005518—shMcl-1-5) expression were used for further experiments. For Mcl-1, the effective downregulation of its expression was achieved by co-transduction of the target cells with shMcl-1-5+2 (=shMcl-1 5/2) or shMcl-1-5+4 (=shMcl-1 5/4) expressing lentiviruses.

Expression of p53 in RKO cells was downregulated by their Lipofectamine RNAiMax-mediated transfection with p53 On-TARGET siGENOME (Thermo Scientific/Dharmacon) according to the manufacture's recommendations. Two days post-transfection, the p53 downregulation was confirmed by Western blotting and cells were used within the following day for experiments.

Subcutaneous xenograft studies

Female immunodeficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (Jackson Laboratory) were maintained in individually ventilated cages. HT-29 cells were harvested, suspended in PBS, and injected (10⁷ cells/mouse) subcutaneously into the left dorsal flank of 8–12-week-old mice. When tumors reached 800–1,000 mm³, the animals were randomized into four groups and treated. The control group was injected with vehicle (PBS) intraperitoneally. HHT (1.3 or 2.6 mg/kg; 26 or 52 µg/mouse) and TRAIL (25 mg/kg; 500 µg/mouse) were administered intraperitoneally in a volume of 300 µl. The animals were treated with these agents and their combination on days 1, 3 and 5; when the tumors in the control group reached 2.5–3 cm in diameter, the animals were sacrificed and the tumors weighed.

Statistical analysis

Data are presented as mean ± SD (standard deviation) for the given number of independent experiments. Student's unpaired *t* test or one-way ANOVA for multiple comparisons were used to determine significant differences between the experimental groups. Values of **p* < 0.05 were considered significant, ***p* < 0.01 very significant and ****p* < 0.001 extremely significant.

Results

Homoharringtonine enhances the TRAIL-induced apoptosis and growth suppression of TRAIL-resistant colorectal cancer cells but not normal colon epithelial cells

TRAIL-induced apoptosis/growth suppression of primary tumors is rather inefficient. The new emerging concept based on TRAIL-related apoptogens encompasses tailored selection of TRAIL-induced apoptosis enhancing agents combined

with molecular targeting (e.g., to EGFR via anti-EGFRcs Fv-TRAIL fusion protein) [31, 32]. We were interested in identifying novel TRAIL sensitizers, and thus we screened several chemical libraries for compounds that would significantly enhance TRAIL-mediated growth suppression of resistant colon carcinoma cells (HT-29). Compounds that showed to be at least two times more efficient in decreasing the viability of HT-29 cells in the presence of TRAIL were considered as primary hits. These hits were validated in dose-response for their ability to enhance TRAIL-mediated apoptosis (Suppl. Fig. 1). Among the positive hits were previously identified sensitizers such as camptothecin and thapsigargin [33, 34]. Other validated hits were emetine and its analog cephaeline, the inhibitors of protein synthesis anisomycin, the alcohol dehydrogenase inhibitor disulfiram and HHT. HHT was by far the most potent TRAIL sensitizer and the only compound showing activity at low nanomolar concentrations.

Homoharringtonine consistently and strongly sensitized not only HT-29, but also other TRAIL-resistant carcinoma cell lines such as RKO and SW620 to TRAIL-induced apoptosis (Fig. 1a and data not shown). In contrast, the normal colon epithelial cell line CCD 841 and normal human fibroblasts remained resistant to combined HHT + TRAIL treatment (Fig. 1a and data not shown). HHT also synergized with TRAIL in enhancing the TRAIL-induced activation of effector caspases (Fig. 1b, c). For RKO cells the saturation in caspase-3/7 activity was reached at 35 ng/ml and for HT-29 at 76 ng/ml of recombinant TRAIL (LC50 = 7 and 15 ng/ml, respectively) with a constant 100 nM HHT (Fig. 1b). Vice versa, titrating HHT under a constant LC50 concentration of TRAIL revealed that the saturating concentration of HHT required for the activation of effector caspases is 50–60 nM with a LC at 15 nM (Fig. 1c). More efficient activation of caspases is necessary but might not be sufficient for the reliable elimination of cancer cells. Thus, by various approaches, we analyzed the long-term survival of resistant cancer cells treated with HHT, TRAIL and their combination. Both xCELLigence- and WST-1-based survival assays confirmed that only the combined effect of 50 nM HHT and TRAIL could lead to the effective long-term elimination of cancer cells (Fig. 2 and Suppl. Fig. 2a, c). In agreement with the Annexin-FITC staining, combined HHT + TRAIL treatment of normal colon epithelial cells with sub-lethal and lethal combinations of HHT + TRAIL did not markedly change their viability (Fig. 2 and Suppl. Fig. 2b).

Homoharringtonine modifies TRAIL-induced proximal signaling in colorectal cancer cells and the expression of some apoptosis-regulating proteins

For the efficient induction of TRAIL-induced apoptosis, the essential requirements are (a) the presence and

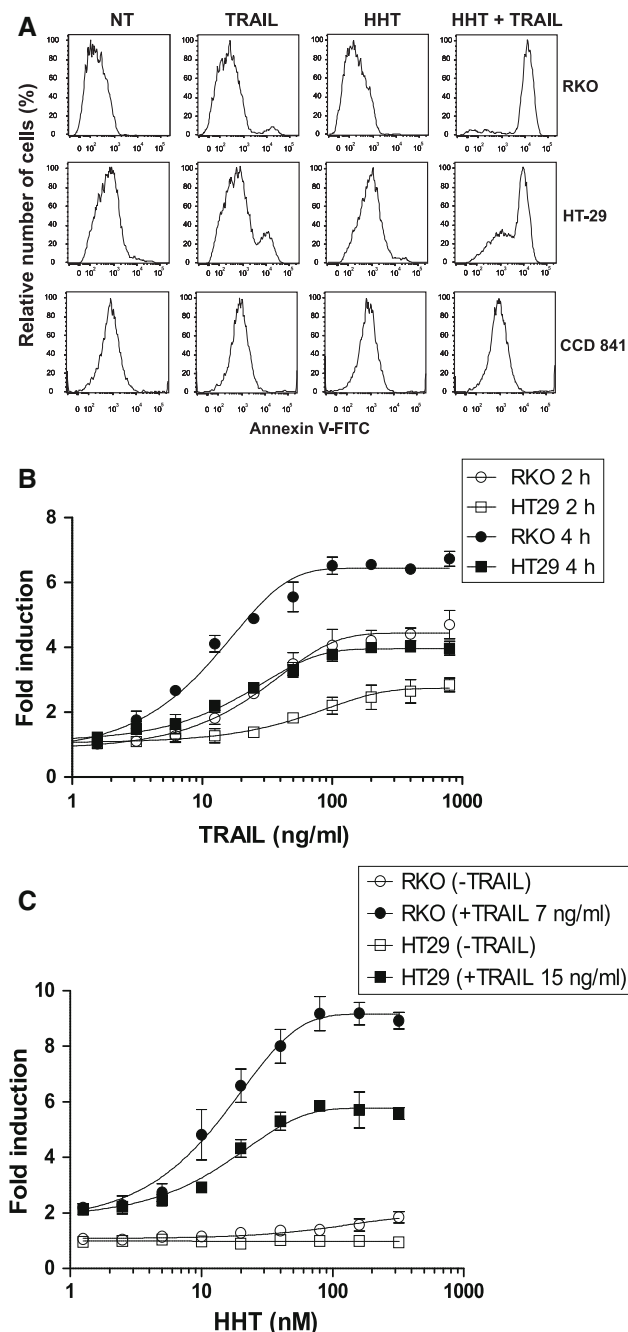


Fig. 1 Homoharringtonine enhances TRAIL-induced pro-apoptotic signaling in resistant colorectal cancer cells. HT-29 or RKO cells were treated with TRAIL, HHT or their combination and assayed for the manifestation of apoptosis by Annexin V-FITC staining and the activity of caspase-3. **a** Cells were incubated with 50 nM HHT, 20 ng/ml TRAIL or their combination for 5 h, stained with Annexin V-FITC and analyzed by flow cytometry. **b** The concentration-dependent activation of caspase-3 in cells co-treated with 100 nM HHT and TRAIL for 2 and 4 h was quantified by the Caspase 3/7 GLO assay. **c** Titration of HHT at a fixed concentration of TRAIL (7 ng/ml for RKO and 15 ng/ml for HT-29 cells) quantified by the caspase 3/7 GLO assay. The experiments were run in triplicate in 384-well plates and were repeated three times. Means and standard deviations are shown

accessibility of pro-apoptotic TRAIL receptors and (b) the efficient formation of the DISC platform leading to rapid self-processing and the activation of caspase-8. However, short or long-term treatment (4 or 15 h) of RKO or HT-29 cells with 50 nM HHT did not significantly affect the cell surface expression of TRAIL receptors (we even noticed a small but reproducible decrease in TRAIL-R2/DR5 expression in RKO cells; Suppl. Fig. 3 and Fig. 3—lower staining of DR5 in the cell lysates).

Next, we aimed to assess the efficacy of DISC formation and the processing of the initiator caspases. In RKO cells, TRAIL engagement led to the rapid formation of the DISC and the processing of both initiator caspases-8 and -10 (Fig. 3a, left side of the left panel). In RKO cells pre-treated with HHT for 3 h, TRAIL addition resulted in a lesser quantity of DISC proteins in the precipitates (Fig. 3a, right side of the left panel); however, the processing of the initiator caspases and cFLIP cleavage in the DISC were not significantly affected. A decrease of both DR5 and also cFLIP levels (both L and S forms) in HHT-pretreated RKO cells was also observable in the cell-free lysates used for DISC precipitation in response to TRAIL addition (Fig. 3a, right panel). Similarly in HT-29 cells we also observed a drop in cFLIP levels in precipitated DISC complexes and no observable differences in the efficacy of activation of the initiator caspases (data not shown).

In addition to the activation of caspases, TRAIL-induced proximal signaling also feeds into MAP kinase and NF- κ B pathways. TRAIL-treatment of RKO cells resulted in the strong but transient activation of NF- κ B signaling (phosphorylation of I κ B and p65/RelA, transient degradation of I κ B, Fig. 3b). I κ B phosphorylation and degradation were evident at 30 and 60 min post-TRAIL treatment respectively, also in cells co-treated with TRAIL + HHT. However, after co-treatment, in contrast to TRAIL-only-treated cells, there was no recovery of I κ B expression at 2 h post-treatment (p.t.), and we even observed the stronger phosphorylation of p65/RelA at this time point. HHT alone did not have any effect on canonical NF- κ B signaling (Fig. 3b, upper panels). Treatment with either HHT or TRAIL alone also activated the JNK signaling pathway and induced JNK1/2 phosphorylation as early as 30–60 min p.t. (Fig. 3b, middle panels). Interestingly, there was a strong synergism in JNK1/2 phosphorylation in RKO cells co-treated with HHT + TRAIL. Similarly, the TRAIL-induced phosphorylation of p38 MAP kinase was significantly enhanced at 60 min post-treatment in cells co-treated with TRAIL + HHT (Fig. 3b, lower panels). Published reports have connected the increased activation of JNK and p38 kinases with the apoptosis-sensitizing effects of HHT [35, 36], and these authors used MAP kinase inhibitors for the suppression of HHT-mediated apoptosis. However, neither the JNK inhibitor SP600125 nor the p38 inhibitor SB202190 had any effect

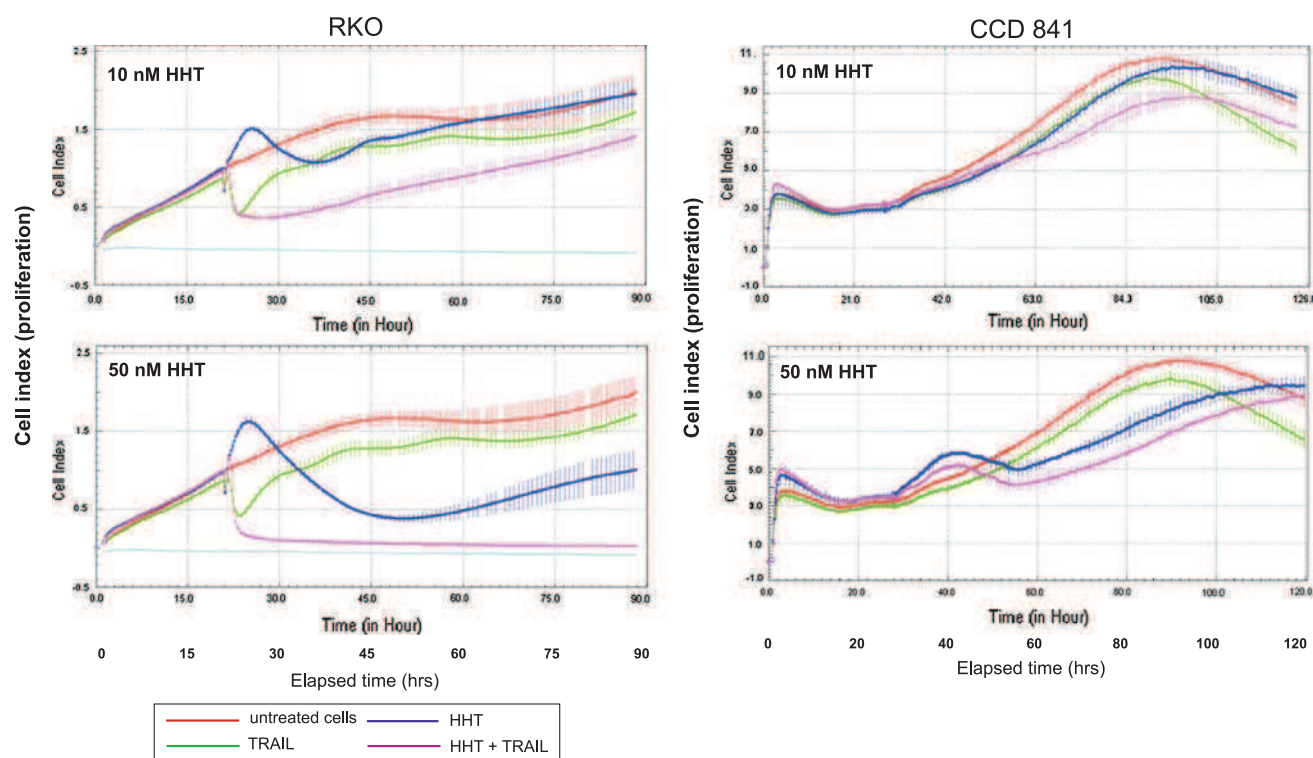


Fig. 2 HHT collaborates with TRAIL in the growth suppression of resistant cancer cells. 5,000 cfu of cancer RKO or normal CCD 841 cells were seeded in triplicate into a 96-well E-plate and treated with 100 ng/ml TRAIL, 10 or 50 nM HHT or their combination in the

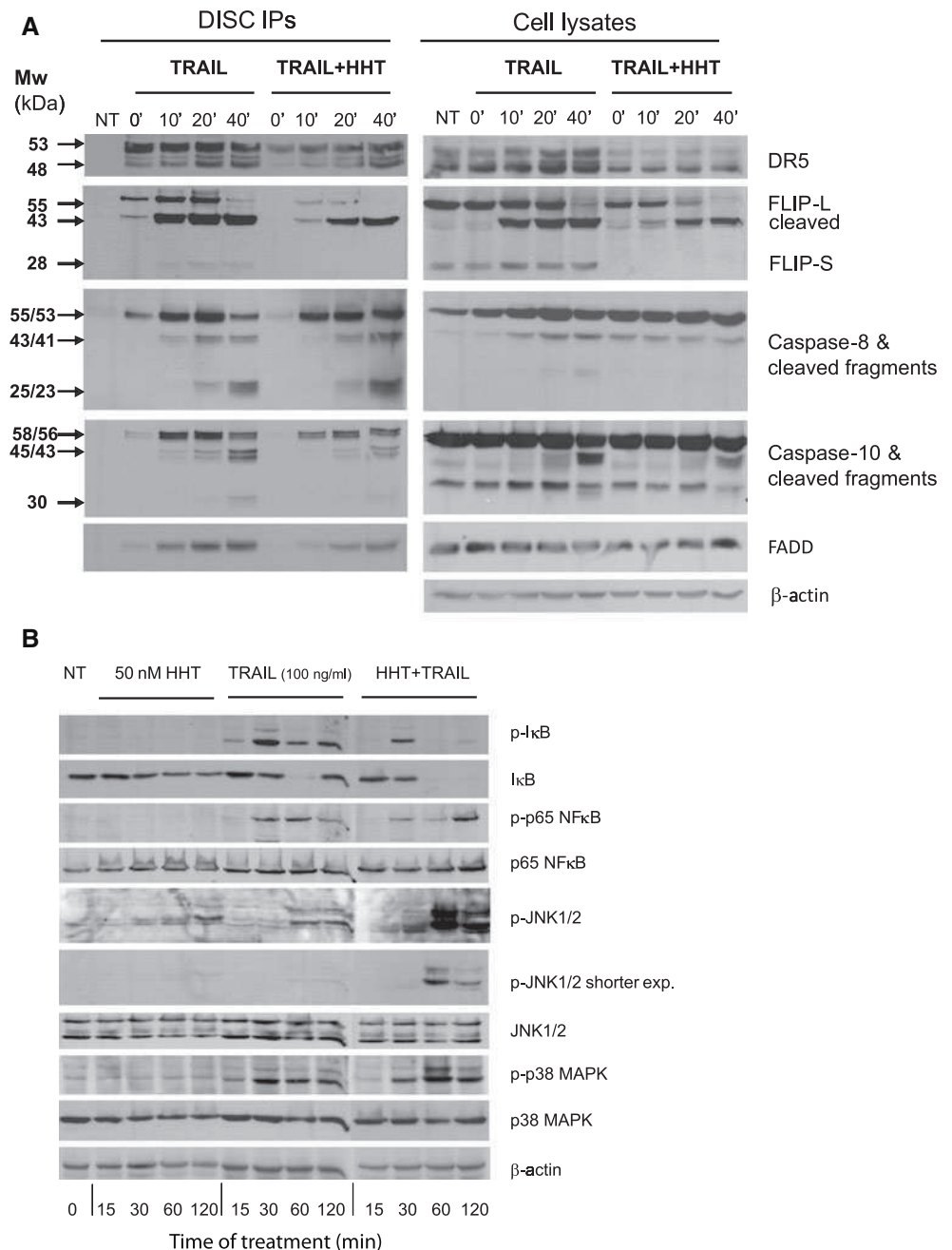
xCELLigence RTCA analyzer (Roche). The cell index representing cell proliferation was measured every 15 min. The data represent one out of four experiments with similar outcomes

(positive or negative) on the HHT-induced sensitization of either RKO or HT-29 cells to TRAIL-induced apoptosis (data not shown), indicating none or minor role of these kinases in the HHT-mediated sensitization of these cells to TRAIL. In contrast to MAP/stress kinases inhibitors blocking NF- κ B signaling with IKK α inhibitors Bay 11-7082 surprisingly resulted in blunting the HHT-mediated sensitization of RKO cells to TRAIL-induced apoptosis (Suppl. Fig. 4 and not shown). Especially at higher 20 μ M concentration that effectively suppressed I κ B phosphorylation, Bay 11-7082 reproducibly attenuated TRAIL-triggered externalization of phosphatidyl choline (Suppl. Fig. 4a) and activation of caspases (Suppl. Fig. 4b). However, this concentration was also significantly toxic for RKO cells as documented by over 25 % dead, Hoechst-positive cells after their 5 h treatment with Bay 11-7082 alone or in combination with HHT/TRAIL (Suppl. Fig. 4A, right panel). Likely also for Bay's toxic side effects, long-term survival of Bay 11-7082/HHT/TRAIL-treated cells was very low and similarly as for HHT + TRAIL-treated cells virtually no cells survived this combined treatment.

The significant down-regulation of cFLIP expression in HHT + TRAIL-treated cells (see Fig. 3a) as well as published data on HHT as a translation inhibitor [37] prompted

us to assess the effect of HHT on the stability of apoptosis-regulating proteins. Western blots of RKO or HT-29 lysates of cells treated with 50 nM HHT for up to 6 h documented a significant, time-dependent decrease of both cFLIP-L and, even more strongly, of cFLIP-S levels starting between 1 and 2 h p.t. We did not see any effect of HHT on the cellular levels of Bak, Bax, Bcl-XL, Bim or XIAP (Fig. 4a and data not shown), but the stability of the short-lived protein Mcl-1 was strongly affected by HHT treatment and its levels dramatically dropped within 2–4 h post-treatment (Fig. 4a). Kinetics of caspase processing and cleavage of some of their substrate was then analyzed in HHT + TRAIL-treated RKO cells. The cells were either treated with 50 nM HHT and 100 ng/ml TRAIL alone for up to 4 h or they were co-treated for the same time periods. In contrast to HHT- or TRAIL-only treated cells, cells co-treated with the combination of HHT + TRAIL efficiently within the first 2 h processed both initiator caspases- and -10. After the second hour of the treatment also caspase-8 substrate tBid and mitochondrial-activated caspase-9 were fully or strongly processed/activated only in HHT + TRAIL-treated cells (Fig. 4b). In addition the combined treatment even accelerated HHT-mediated downregulation of cFLIP-S or Mcl-1.

Fig. 3 Homoharringtonine affects TRAIL-induced proximal signaling in RKO cells. **a** Precipitation of TRAIL receptor DISC from Biot-TRAIL- and Biot-TRAIL + HHT-treated RKO cells (Biot-TRAIL was used at a concentration of 1 μ g/ml and HHT at 50 nM). The precipitated proteins and cell lysates were analyzed by Western blotting using the indicated antibodies. **b** RKO cells were treated with HHT, TRAIL or their combination for up to 120 min, and the expression and phosphorylation status of the proteins shown were determined by Western blotting. The data represent typical blots from two (a) or three (b) independent experiments



Downregulation of cFLIP or Mcl-1 enhances TRAIL-induced apoptosis but is not sufficient for TRAIL-mediated growth suppression of resistant cells

As HHT treatment of TRAIL-resistant cells led to a time-dependent downregulation of cFLIP and Mcl-1, two major inhibitors of TRAIL-induced apoptosis, we aimed to assess the individual contribution of these proteins to the TRAIL-resistant phenotype of RKO and HT-29 cells. Using two different shRNA-expressing lentiviruses for each gene, we established both RKO and HT-29 cells with the downregulated expression of either cFLIP or Mcl-1. In both cell

lines we achieved at least an 80–90 % decrease in the expression of either cFLIP or Mcl-1 (Fig. 5a). The downregulation of Mcl-1 enhanced TRAIL-induced pro-apoptotic signaling in both cell lines (Fig. 5b and Suppl. Fig. 5a, b, right graphs). However, to our surprise downregulating cFLIP augmented TRAIL-induced apoptosis only in HT-29 cells, but it had no or even an inhibitory effect on the TRAIL-induced apoptosis of RKO cells. Similarly, using a sub-optimal, 25 nM concentration of HHT enhanced the TRAIL-induced apoptosis of both RKO and HT-29 cells with the downregulated expression of Mcl-1 and of HT-29 cells with the downregulated expression

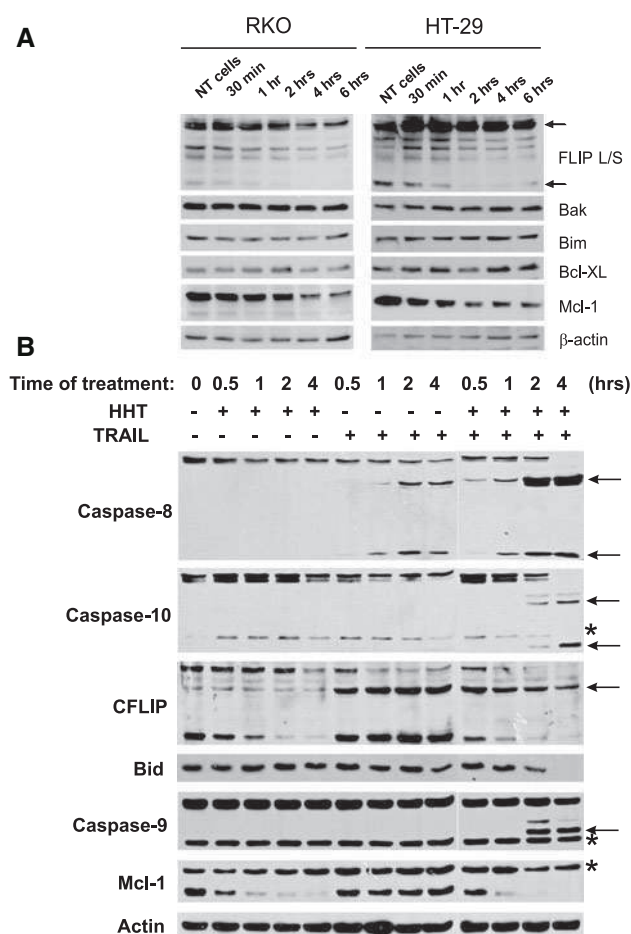


Fig. 4 Homoharringtonine suppresses the expression of some anti-apoptotic proteins and enhances processing of caspases in TRAIL-resistant cancer cells. **a** RKO or HT-29 cells were treated with 50 nM HHT for up to 6 h, and the expression of selected apoptosis-regulating proteins was analyzed by Western blotting. Representative Western blots from three independent experiments are shown. *Arrows* point to cFLIP-L and cFLIP-S, respectively. **b** RKO cells were treated with HHT (50 nM), TRAIL (100 ng/ml) and their combination in time-dependent manner and the expression/processing of selected, TRAIL-mediated apoptosis-related caspases and proteins was analyzed by Western blotting. *Arrows* indicate processed caspases and cleaved cFLIP-L, and *asterisk* then non-specifically stained proteins

of FLIP, but such treatment led to much lower pro-apoptotic responses in RKO cells with the downregulated expression of cFLIP (Fig. 5b and Suppl. Fig. 5).

The results of viability/proliferation assays of these cells with suppressed cFLIP or Mcl-1 expression followed a quite similar pattern. Both CellTiter-Blue and xCELLigence viability tests showed that despite the significant increase of apoptosis in TRAIL-treated cells with the downregulated expression of Mcl-1 or cFLIP (only for HT-29), knocking-down either of these anti-apoptotic proteins had only a marginal effect on the long-term survival of TRAIL-treated cells (Suppl. Fig. 5, left panels and Suppl. Fig. 6). However, especially the suppressed expression of

Mcl-1 markedly boosted the HHT-mediated sensitization of both RKO and HT-29 cells to TRAIL-induced apoptosis, as documented by more than a twofold lower viability in comparison to control cells in the CellTiter-Blue assay (Suppl. Fig. 5) and virtually no survival of treated cells in the xCELLigence test (compare none and shMcl-1; Suppl. Fig. 6). Strangely, RKO cells with the downregulated expression of cFLIP virtually became resistant to the sensitizing effect of HHT and TRAIL + HHT (Suppl. Fig. 5, left panels and Suppl. Fig. 6). These cells basically reflected just the anti-proliferative effects of HHT itself.

RKO cells in contrast to HT-29 contain wt p53 and thus it might be possible that p53 presence/activity could make RKO cells even more vulnerable to the combined effect of HHT + TRAIL. But this premise was not fulfilled, as RKO cells with by siGENOME downregulated expression of p53 responded to either TRAIL alone or TRAIL + HHT with no difference in the efficacy or kinetics of their apoptosis (data not shown).

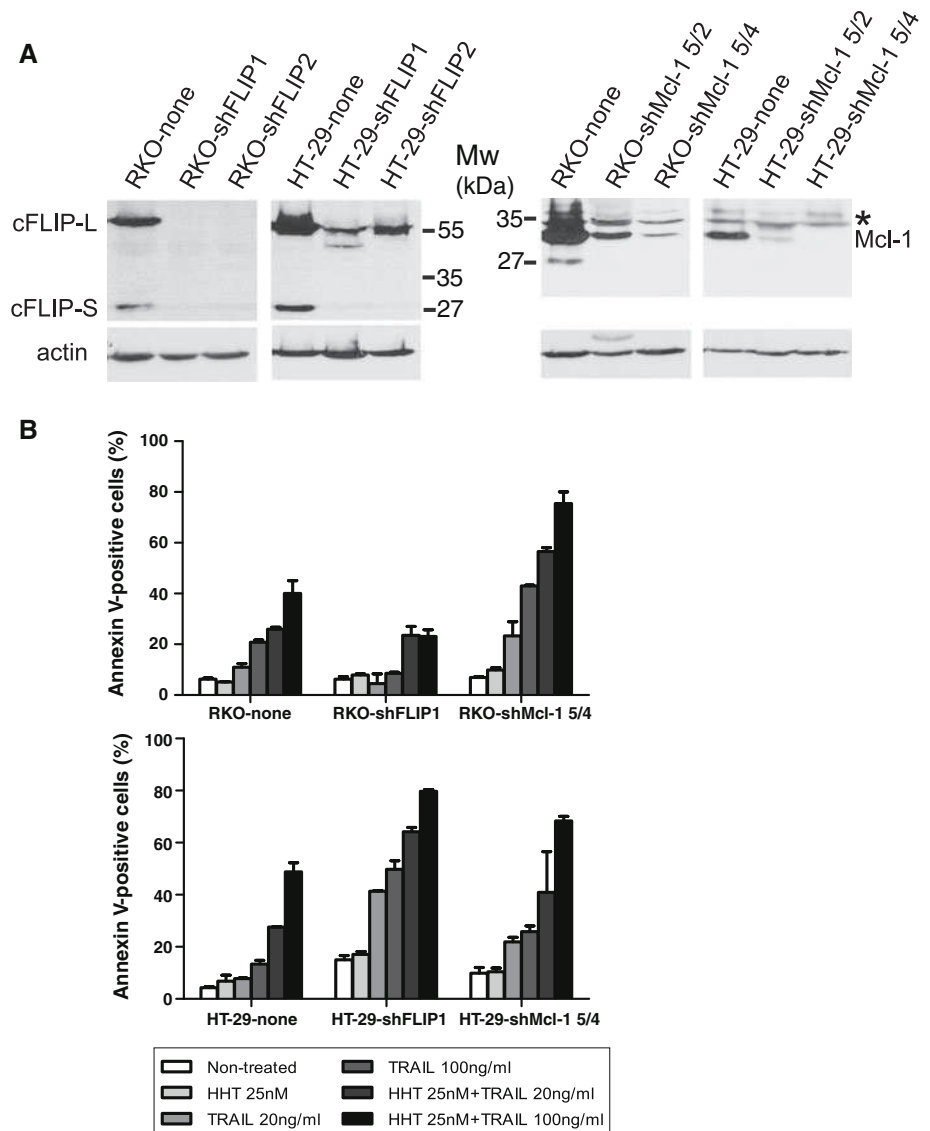
The combined action of homoharringtonine and TRAIL efficiently suppressed the growth of TRAIL-resistant cancer cells in vivo

To validate our in vitro findings documenting the very strong sensitizing impact of HHT on the TRAIL-induced apoptosis/growth suppression of resistant cancer cells, we used an in vivo model with HT-29 cells subcutaneously xenotransplanted into immunodeficient NOD/SCID mice. A cohort of 36 mice was injected with 10^7 cells, and when the resulting tumors reached approximately 1 cm^3 , the mice were divided into six groups and treated three times (every second day) with intraperitoneal injections of TRAIL (25 mg/kg), HHT (1.3 mg/kg) or a combination of TRAIL with HHT. Within 18 days of tumor growth, the tumor mass in untreated mice increased approximately 3.5-fold; both individual treatments had only a marginal inhibitory effect on tumor growth, but the tumors in animals co-treated with HHT + TRAIL ceased growing, and the animals did not suffer any adverse effects (Fig. 6). All mice in the HHT + TRAIL-treated cohort survived the treatment until their sacrifice at the end of the experiment.

Discussion

Primary tumors only weakly react to mono-therapy with TRAIL apoptogens, and even binary or ternary combinations of TRAIL with sensitizing agents were only partly effective in a number of phase I/II trials. Thus, if TRAIL apoptogens are still to be considered as potential anti-tumor drugs, tailored combination treatment that would overcome

Fig. 5 Downregulation of Mcl-1 or cFLIP predominantly leads to the increased sensitivity of resistant cancer cells to TRAIL-induced apoptosis. RKO or HT-29 cells were transduced with cFLIP- or Mcl-1-expressing lentiviruses and analyzed for their sensitivity to TRAIL- or HHT + TRAIL-induced apoptosis. **a** Western blots of mixed populations of RKO and HT-29 cells stably transduced with the pLKO1 lentivirus (none) and two different cFLIP or Mcl-1 shRNAs. Asterisk at Mcl-1 signal indicates non-specific staining. **b** The activation of TRAIL-induced apoptosis in these cells treated for 5 h with the indicated reagents was analyzed by Annexin-V-FITC staining and flow cytometry. The data represent means and standard deviations of three independent experiments from shFLIP and shMcl-1 expressing RKO cells



the acquired resistance of cancer cells and have few, or better, no side effects would be an optimal therapeutic option [31].

Here we show that HHT, an inhibitor of translation and a tested anti-leukemia drug, efficiently sensitizes resistant cancer cells to their TRAIL-mediated elimination both in vitro and in vivo and, within the therapeutic window, is not harmful to normal cells or the organism. In agreement with the published data we also found that in our model of TRAIL-resistant colorectal cancer cell lines, HHT caused, in a time- and concentration-dependent manner, a fairly rapid (within 2–4 h) drop in the cellular levels of two important regulators of TRAIL-induced apoptosis—the anti-apoptotic proteins Mcl-1 and cFLIP [38, 39]. Also as expected, the shRNA-mediated downregulation of either of these proteins significantly enhanced TRAIL-induced apoptosis but surprisingly had only a marginal effect on

TRAIL-mediated growth suppression. However, shRNA treatment lowered the effective sensitizing concentration of HHT by at least two-fold.

In contrast to HT-29 cells, downregulation in RKO cells provided quite ambiguous results. While the downregulation of Mcl-1 strongly boosted both TRAIL-induced apoptosis and HHT-mediated sensitization, cells with down-regulated cFLIP expression became more resistant not only to TRAIL, but also to their HHT-mediated sensitization to TRAIL-induced apoptosis. This was quite an unexpected finding as the downregulation of cFLIP in various cancer cell lines has generally been connected with the strong enhancement of TRAIL- or FasL-induced apoptosis [40–42]. However, cFLIP-L, in sharp contrast to cFLIP-S or -R, is likely not just a competitive inhibitor of caspase-8 activation, but also, due to its high affinity for caspase-8, either a platform in a cytoplasmic caspase-8-activating complex

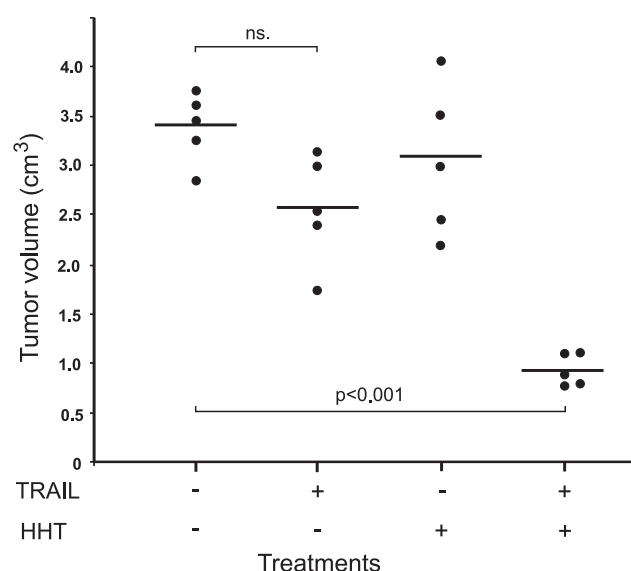


Fig. 6 Homoharringtonine assists TRAIL in suppressing the growth of colon cancer cells in immunodeficient mice. HT-29 cells (10^6 cfu) were subcutaneously injected into a cohort of NOD/SCID mice, and when the tumors reached approximately 1 cm^3 in size, these mice (at least five per group) received three intraperitoneal injections (every second day) of TRAIL (25 mg/kg), HHT (1.3 mg/kg) or their combination (25 mg/kg TRAIL plus 1.3 mg/kg HHT). At day 10 post-treatment the mice were sacrificed, and the size of their tumors was determined. The average volumes and standard deviations are shown with *marked* statistical significance

[43] or even an enhancer of caspase-8 processing in the DISC at low (sub) stoichiometric concentrations [44, 45]. RKO cells express significantly less cFLIP than HT-29 cells, and thus shRNA-mediated downregulation basically eliminated cFLIP expression in these cells. This apparently made RKO cells more resistant even to the combined effects of HHT + TRAIL, indicating a positive role for low levels cFLIP-L in the fine tuning of TRAIL-induced apoptotic signaling in these cells. This finding might be in apparent contradiction with the HHT-mediated downregulation of cFLIP in RKO cells. However, in HHT-treated RKO cells the more unstable cFLIP-S is rapidly downregulated, but the decrease in cFLIP-L levels is only modest, and cFLIP-L is still detectable in the DISC precipitates (and lysates) of HHT + TRAIL-treated RKO cells.

Intracellular levels of Mcl-1 were many times shown as important decision nodes for various apoptotic stimuli including e.g. BH3 analog ABT-737 [46], and Mcl-1 expression alone or together with cFLIP was targeted and suppressed by a number of existing and novel drugs such as roscovitine, quercetin or aspirin plus sorafenib, enhancing thus (not exclusively) TRAIL-induced apoptosis of cancer cells [47–49]. Multikinase inhibitor sorafenib or ectopic expression of cMyc blocked TRAIL-induced, NF- κ B-mediated expression of Mcl-1 and thus sensitized TRAIL-resistant cells to TRAIL-induced apoptosis [50]. In contrast

to this generally accepted anti-apoptotic role of NF- κ B signaling we found out that blocking NF- κ B signaling by BAY 11-7082, a well-known inhibitor of IKK α also attenuated sensitizing effect of HHT and suppressed TRAIL-induced apoptosis of RKO cell. This unexpected finding is however supported by recent publication from Simone Fulda's group. In this communication the authors for the first time document that in glioblastoma cell lines, blocking NF- κ B signaling pathway by overexpression of dominant-negative non-phosphorylated I κ B also suppressed TRAIL-induced apoptosis likely via for yet unknown reasons less efficient caspase-8 processing in DISC [51].

Being a potent inhibitor of translation, HHT could be potentially harmful to normal cells in combination with TRAIL, similarly as another anti-cancer drug and proteasome inhibitor, bortezomib (Velcade) [52]. On the other hand, bortezomib has proven to be a very efficient enhancer of the TRAIL-induced apoptosis of therapy-resistant glioblastoma or melanoma cells and Lexatimumab-resistant BJAB cells [53–55] and within the therapeutic window also safe to normal cells. Similarly, combining HHT with TRAIL was not only effective in vitro, but also suppressed the growth of xenotransplanted TRAIL-resistant HT-29 cells in immunodeficient mice, thus proving its efficacy and safety within the therapeutic window. As an already tested drug in cancer treatment, HHT could, after thorough testing, represent a potentially interesting option for sensitizing TRAIL-resistant cancer cells to apoptosis.

Acknowledgments The work on this project was mainly supported by grants from the Czech Science Foundation No. P301-10-1971 and the Ministry of Education No. LH12202 to LA, then by IGA-MZ: NT13201-4/2012, PRVOUK-P24/LF1/3, and UNCE 204021 to PK, GA-UK 259211/110709 to JM, the Ministry of Education grants No. LC06077 and LO2011022 to PB and by the institutional grant RVO 68378050. We are grateful to James Dutt for proofreading the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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