VLOGA IN POMEN CISTEINSKIH KATEPSINOV PRI CELIČNI SMRTI INDUCIRANI Z LIGANDOM TRAIL

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Vloga in pomen cisteinskih katepsinov pri celični smrti inducirani z ligandom TRAIL

Doktorska disertacija

Role and significance of cysteine cathepsins in TRAIL induced apoptosis

Doctoral Dissertation

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Kazalo

Povzetek

Apoptoza je proces, s katerim večcelični organizmi varno odstranjujejo odvečne, postarane ali poškodovane celice. Med apoptozo se vsebina celice ne sprosti v medcelični prostor, zato ne pride do vnetnega odziva. Napake v uravnavanju procesa apoptoze lahko privedejo do raznih patoloških stanj, tako zaradi prekomerne kot zaradi nezadostne apoptoze. Apoptoza se lahko sproži po dveh glavnih poteh, zunanji in notranji. Pri notranji poti proapoptotski signali povzročijo sproščanje citokroma c iz mitohondrijev v citosol, ki skupaj s proteinom Apaf-1 oblikuje kompleks apoptosom, na katerem se aktivira kaspaza 9. Zunanja pot potrebuje proapoptotski signal iz zunanjosti celice. Vezava ligandov smrti na membranske receptorje smrti sproži tvorbo kompleksa DISC (death inducing signaling complex), na katerem se aktivirata kaspazi 8 ali 10. Na koncu obeh poti se aktivirata izvršitveni kaspazi 3 in 7.

Cisteinske proteaze kaspaze so ključni encimi pri apoptozi. Vedno več pa je dokazov, ki govorijo o pomembnosti lizosomskih proteaz pri apoptozi, predvsem cisteinskih katepsinov. Destabilizacija lizosomov pri različnih bolezenskih stanjih povzroči sproščanje lizosomske vsebine v citosol. Tako sproščeni katepsini lahko sprožijo apoptozo z aktivacijo notranje poti. S cepitvijo molekule Bid in razgradnjo antiapoptotskih proteinov družine Bcl-2 pripomorejo k permeabilizaciji zunanje mitohondrijske membrane in sproščanja citokroma c v citosol. Poleg tega z razgradnjo antiapototskega proteina XIAP katepsini vplivajo na aktivnost kaspaz tudi v kasnejših fazah apoptoze.

Natančen mehanizem destabilizacije lizosomske membrane pri aktivaciji zunanje poti apoptoze še ni natančno pojasnjen. Nekateri avtorji trdijo, da je poškodba lizosomov zgodnji dogodek v apoptozi in da katepsini že v začetnih fazah pripomorejo pri destabilizaciji mitohondrijske membrane. Naše nedavne študije pa kažejo, da je po sprožitvi apoptoze z ligandoma Fas in TNF-alfa destabilizacija lizosomov pozni dogodek. Namen našega dela je bil raziskati mehanizem destabilizacije lizosomske membrane pri apoptozi sproženi z ligandom TRAIL pri mišjih embrionalnih fibroblastih in humanih tumorskih celičnih linijah.

Pri mišjih embrionalnih fibroblastih smo ugotovili, da je apoptoza sprožena z ligandom TRAIL od kaspaz odvisen proces, pri čemer se najprej poškoduje mitohondrijska membrana in je poškodba lizosomov sekundarni dogodek. Odsotnost katepsina B je vplivala na delež apoptotskih celic, ni pa vplivala na stabilnost mitohondrijske ali lizosomske membrane. Katepsini najverjetneje predstavljajo ojačitveno zanko med obema organeloma, niso pa pomembni pri začetni sprožitvi poškodb mitohondrijev. V naši raziskavi smo kot povezavo med poškodbami obeh organelov identificirali reaktivne kisikove zvrsti (ROS). ROS se sprostijo iz poškodovanih mitohondrijev in so odgovorne za nadaljnje poškodbe tako drugih mitohondrijev kot lizosomov. Z uporabo odstranjevalca ROS Tempola in kelatorja železovih ionov desferoksamina smo potrdili, da je sprostitev citokroma c iz mitohondrijev pogojena tudi z oksidacijo kardiolipina.

Z uporabo inhibitorjev katepsinov smo tudi pri humanih tumorskih celičnih linijah pokazali, da katepsini ne vplivajo na potek apoptoze, ki jo sproži ligand TRAIL. Po poškodbi lizosomov se katepsini sprostijo v citosol, kjer tudi ohranijo svojo aktivnost,

vendar z inhibicijo njihove aktivnosti ne preprečimo poškodb mitohondrijev in tudi ne apoptoze.

Oksidativni stres lahko povzroči poškodbe lizosomske membrane in prehajanje katepsinov v citosol. Med tarčami katepsinov so tudi sirtuini, proteini, ki sodelujejo pri preusmeritvi avtofagije k apoptozi. Z uporabo rekombinantnih katepsinov B, L in S smo dokazali *in vitro* cepitve sirtuinov.

Poznavanje molekularnega mehanizma poteka apoptoze je nepogrešljivo pri načrtovanju uporabe inhibitorjev proteaz pri zdravljenju tumorskih bolezni. Z modelom apoptoze sprožene z ligandom TRAIL pri mišjih embrionalnih fibroblastih in več humanih tumorskih celičnih linijah smo natančneje opisali časovni potek apoptoze s poudarkom na poškodbah mitohondrijev in lizosomov ter vpletenosti cisteinskih proteaz kaspaz in katepsinov.

Abstract

Apoptosis is an innate mechanism by which a multicellular organisms eliminates unwanted, damaged and potentially harmful cells. During apoptosis inflammation of the surrounding tissues is prevented. Defects in an apoptotic signaling can lead to numerous pathological conditions. Apoptosis is mediated through two main pathways, the extrinsic or the death receptor pathway and the intrinsic or mitochondrial pathway. In the intrinsic pathway proapoptotic signals trigger release of cytochrome c from the mitochondria into the cytosol, where it bounds with Apaf-1 protein and forms apoptosome complex and subsequent activation of caspase 9. In the extrinsic pathway proapoptotic molecules bind to the death receptors on the cell surface resulting in a formation of death-inducing signaling complex (DISC) in which capases 8 and 10 are activated. In the end of both pathways effector caspases 3 and 7 are activated.

Caspases are a family of evolutionary conserved cystein proteases mediating initiation and execution of apoptosis. But there is increasing evidence that alternative proteolytic enzymes as lysosomal proteases (cathepsins) can initiate or propagate proapototic signals. As a result of lysosomal membrane permabilization cathepsins are released into the cytosol. Once in the cytosol they can activate apoptotic pathway through cleveage of proapoptotic molecule Bid or inactivation of antiapoptotic Bcl-2 proteins and with cleavage of XIAP cathepsins have effect also in later stages of apoptosis.

The mechanism of lysosomal membrane destabilization in death receptor pathway is still not well understood. It was reported that lysosomal rapture was an early event and it preceded mitochondrial membrane destabilization. However, recent studies with Fas and TNFa induced apoptosis have shown that lysosomes were destabilized after mitochondria.

In our experiments with mouse embryonic fibroblasts (MEF) we showed that caspases are crucial in TRAIL induced apoptosis and that destabilization of mitochondria precede destabilization of lysosomes. Effect of cathepsin B was evaluated using cathepsin B knockout mice. Our result show absence of cathepsin B has no effect on either mitochondrial or lysosomal membrane destabilization process in comparison to wild type cells. However the decrease in apoptosis was observed. We conclude that cathepsin B does not play a role in initial phases of apoptosis, but it may be a factor in later stages of apoptosis in TRAIL induces apoptosis in MEFs. As a possible link between mitochondria and lysosomes we identified reactive oxygen species (ROS). ROS are produced by mitochondria and if mitochondria are damaged ROS can cause irreversible damage in the cell. We also found out that release of cytochrome c depends on cardiolipin oxidation and ROS importance was confirmed with iron chelator DFO in scavenger Tempol.

In the second part we evaluated involvement of cathepsins in apoptosis in human cell lines. Using cathepsins inhibitors E-64d and CA-074Me we could not prevent TRAIL induced apoptosis. Although mitochondria and lysosomes were damaged in the process and the active cathepsins are released into the cytosol, inhibiting cathepsins did not have any effect on organelle stability or level of apoptosis.

Oxidative stress can cause lysosome membrane permeabilization and leakage of cathepsins into the cytosol. Sirtuines, which play important role in subverted autophagy leading to apoptosis, are among possible cathepsin targets. We showed *in vitro* cleavages

of sirtuines by cathepsins B, L and S.

Understanding of the molecular mechanisms of apoptosis is crucial in the application of protease inhibitors in cancer treatment. On a model of TRAIL induced apoptosis in mouse embryonic fibroblasts and several human cancer cell lines we here describe link between mitochondria and lysosomes as well as involvement of lysosomes and lysosomsal proteases, cathepsins, in the process.

Seznam kratic

a.e.	=	arbitrarne enote
Ac-DEVD-AFC	=	N-acetil-Asp-Glu-Val-Asp-7-amino-4-trifluorometil kumarin
AIF	=	dejavnik, ki sproži apoptozo; apoptosis inducing factor
aneksin V-PE	=	aneksin V-pikoeritrin
AO	=	akridin oranž
Apaf-1	=	aktivacijski faktor 1 apoptozne proteaze; apoptotic protease-activating factor
APS	=	amonijev persulfat
Atg	=	gen, povezan z avtofagijo; autophagy-related gene
Bak	=	antagonist Bcl-2 in ubijalec; Bcl-2 antagonist/killer
BANA	=	benzoil arginin naptilamid
Bax	=	z Bcl-2 povezan protein x; Bcl-2 associated x protein
Bcl-2	=	protein 2 iz limfoma celic B; B-cell lymphoma 2
BH	=	regije, homologne Bcl-2; Bcl-2 homology regions
Bid	=	protein smrti, ki reagira z domeno BH-3; BH-3 interacting domain death protein
Bim	=	posrednik celične smrti, ki reagira z Bcl-2; Bcl-2 interacting mediator of cell
BIR	=	bakulovirusno zaporedje IAP; baculoviros IAP repeat
BSA	=	goveji serumski albumin; Bovine serum albumin
Ca-074Me	=	[(2S,3S)-3-Propilkarbamoiloksiran-2-karbonil]-L-isoleucil-L-prolin metil ester
CAD	=	DNaza, ki jo aktivira kaspaza; caspase activated DNase
CARD	=	domena, ki rekrutira kaspaze; caspase recruitment domain
CED	=	protein smrti pri *Caenorhabditis elegans*; *Caenorhabditis elegans* death
cFLIP	=	celični FLIP; cellular FLIP
CHAPS	=	3-[(kloraminopropil)dimetilamonio]-2-hidroksi-1-propan
CHX	=	cikloheksimid
CM-H_2DCFDA	=	fluo-3-acetoksimetilester (fluo-3AM), 5-(and 6)-klorometil-2',7'-diklorodihidrofluorescein diacetat
CrmA	=	modifikator A odgovora na citokine; cytokine response modifier A
DcR	=	receptor vaba, lažni receptor; decoy receptor
DD	=	domena smrti; death domain
DED	=	efektorska domena smrti; death effector domain
DFO	=	desferoksamin, kelator železovih ionov; desferrioxamine
DIABLO	=	protein z nizkim pI, ki neposredno veže IAP; direct IAP binding protein with low pI
DISC	=	signalni kompleks, ki sproži smrt; death inducing signaling complex

DMEM	=	po Dulbeccu spremenjen medij Eagle; Dulbecco's modified Eagle medium
DMSO	=	dimetil sulfoksid; dimethyl sulfoxide
DNA	=	deoksiribonukleinska kislina; deoxyribonucleic acid
DNA-PK	=	od DNA odvisna kinaza; DNA-dependent protein kinase
DR	=	receptor smrti; death receptor
DTT	=	ditiotreitol
E-64d	=	L-trans-epoksisukcinil(OEt)-Leu-3-metilbutilamid
ECL	=	povečana kemoluminiscenca; enhanced chemoluminiscence
EDTA	=	etilendiamin tetraacetat
EGTA	=	etilen glikol-bis(β-aminoetil eter)-N, N, N', N'-tetra acetat
Endo G	=	endonukleaza G
FADD	=	na Fas vezan protein z domeno smrti; Fas-associated protein with death domain
Fas	=	ligand smrti Fas, tudi CD95 in APO-1
FBS	=	zarodkov goveji serum; fetal bovine serum
FBS-HI	=	toplotno inaktiviran zarodkov goveji serum; heat inactivated fetal bovine serum
FLICE	=	ICE, podoben FADDu; Fadd-like ICE
FLIP	=	proteini, ki inhibirajo FLICE; FLICE-inhibitory proteins
GST	=	glutation S-transferaza; glutathione S-transferase
HEPES	=	4-(2-hidroksietil)-1-piperazinetanesulfonična kislina
IAP	=	inhibitor apoptoze; inhibitors of apoptosis
ICAD/DFF45	=	inhibitor CAD/DNA fragmentacijski dejavnik 45; DNA fragmentation factor 45
ICE	=	encim, ki cepi interlevkin 1β; interleukin-1β converting enzyme
JNK	=	C-Jun N-terminalna kinaza; c-Jun N-terminal kinase
kDa	=	kilo Dalton
Lamp	=	membranski protein, povezan lizosomom; lysosome associated membrane protein
LeuLeuOMe	=	L-Levcil-L-levcin metilni ester
M6PR	=	receptor manoze 6-fosfat; mannose 6-phosphate receptor
MAPK	=	kinaza, ki jo aktivira mitogen; mitogen-activated protein kinase
MEF	=	mišji embrionalni firoblasti; mouse embryonic fibroblasts
MHC	=	poglavitni histokompatibilnostni kompleks; major histocompatibility complex
MOPM	=	permeabilizacija zunanje membrane mitohondrija; mitochondrial outer membrane permeabilization
MSDH	=	O-metil-serin dodecilamid hidroklorid; O-methyl-serine dodecylamide
NAD+	=	nikotinamid adenin dinukleotid; nicotinamide adenine dinucleotide
NaDS	=	natrijev dodecil sulfat
NAO	=	10-nonil akridin oranž; 10-nonyl acridine orange
NF-κB	=	jedrni dejavnik κB; nuclear factor-κB
OPG	=	osteoprotegerin
PAGE	=	poliakrilamidna gelska elektroforeza; polyacrylamide gel electrophoresis
PARP	=	poliADP-riboza polimeraza; (poly) ADP-ribose polymerase

PBS	=	s fosfatom zapufrana raztopina soli; phosphate buffer saline
PE	=	fikoeritrin
pH	=	negativni logaritem koncentracije vodikovih ionov
PI	=	propidijev jodid;
PIDD	=	protein z domeno smrti, ki ga inducira p53; p53-induced protein with a death domain
PLA2	=	fosfolipaza A2
PLAD	=	domena, ki se sestavi pred vezavo liganda; preligand assembly domain
RING domena	=	(Really Interesting New Gene)
RIP	=	protein, ki sodeluje z receptorjem; receptor interacting protein
RIPA	=	radioimuno precipitacijski test; RadioImmunoPrecipitation Assay
ROS	=	reaktivne kisikove zvrsti; reactive oxygen species
rpm	=	obrati na minuto; revolutions per minute
RPMI	=	Roswell Park Memorial Institute
Sir	=	dejavnik tihe informacije; silent information factor
SMAC	=	drugi mitohondrijski aktivator kaspaz; second mitohondria-derived activator of caspase
tBid	=	skrajšana oblika molekule Bid; truncated Bid
TEMED	=	N, N, N', N'-tetrametilendiamin
Tempol	=	4-hidroksi-TEMPO; 4-hidroksi-2,2,6,6-tetrametilpiperidin-1-oksil
TNF	=	dejavnik tumorske nekroze; tumor necrosis factor
TNF-R1	=	receptor tipa 1 za TNF; tumor necrosis factor receptor 1
TRADD	=	na receptor TNF vezan protein, ki vsebuje domeno DD; TNF receptor- associated protein with death domain
TRAF-2	=	dejavnik 2, povezan s TNF-receptorjem; TNF-receptor-associated factor-2
TRAIL	=	TNF podoben ligand, ki sproži apoptozo; TNF-related apoptosis-inducing ligand
TRAIL-R	=	TRAIL receptor
VDAC	=	napetostno občutljiv anionski kanal; voltage-dependent anion channel
XIAP	=	na kromosom X vezan inhibitor apoptoze; X-linked inhibitors of apoptosis
Z-Phe-Arg-AMC	=	benziloksikarbonil-Phe-Arg-7-amino-4-metilkoumarin
Z-VAD-FMK	=	z-Val-Ala-Asp-fmk; N-benziloksikarbonil-Val-Ala-Asp-fluorometilketon

1 Uvod

1.1 Apoptoza

Celična delitev in celična smrt sta endogena, evolucijsko ohranjena in natančno kontrolirana procesa, ki v večceličnih organizmih uravnavata število celic (Pereira in Amarante-Mendes, 2011). Celično smrt lahko na podlagi morfoloških značilnosti razdelimo na apoptozo, nekrozo in avtofagijo. Njihove morfološke razlike so prikazane v tabeli 1 (Kroemer in sod., 2009).

Apoptoza je urejen in osnoven biološki proces, nujno potreben za normalno delovanje organizma. Z njim organizem varno odstranjuje odvečne, postarane ali poškodovane celice. S tem zagotavlja pravilen razvoj in delovanje imunskega sistema, embriogeneze, tkivne homeostaze in vnetne odzive (Wyllie in sod., 1980). Celice, ki imajo apoptotske značilnosti se odstranijo v procesu fagocitoze, ko jih požrejo okoliški makrofagi (Kroemer in sod., 2009). Apoptotska celica je fagocitirana v celoti in zato ne prihaja do sproščanja celične vsebine. Nasprotno pa pri nekrozi nekontrolirana sprostitev vsebine celice v okolje povzroči vnetni odziv, ki poškoduje sosednje celice (Alenzi in sod., 2010).

Apoptoza je nujna za razvoj in vzdrževanje večceličnih organizmov ter je zelo natančno uravnavana, saj lahko napake pri njeni regulaciji povzročijo različna patološka obolenja navedena v tabeli 2 (Rastogi in sod., 2009; Tait in Green, 2010).

Tabela 1: *Tipi celične smrti in njihove morfološke značilnosti* (Kroemer in sod., 2009)

Celična smrt	Morfološke značilnosti
Apoptoza	Spremenjena oblika celic
	Krčenje celičnega volumna
	Fragmentacija jedra
	Spremembe citoplazemskih organelov
	Brstenje celične membrane
	Fagocitoza
Avtofagija	Ni kondenzacije kromatina
	Vakuolizacija citoplazme
	Kopičenje avtofagnih vakuol
	Malo ali nič fagocitoze
Nekroza	Nabrekanje citoplazme
	Permeabilizacija plazemske membrane
	Nabrekanje citosolnih organelov

Poleg morfoloških sprememb (Tabela 1) so pomembne tudi biokemijske spremembe v celici. Med apoptozo so najpomembnejše aktivacija proapoptotskih proteinov družine Bcl-2, aktivacija kaspaz, permeabilizacija mitohondrijske membrane, fragmentacija DNA, izpostavitev fosfatidilserina na zunanjo stran celične membrane, prekomerna tvorba reaktivnih kisikovih zvrsti (ROS) ter kopičenje enoverižnih DNA (Kroemer in sod., 2009).

Tabela 2: *Bolezni povezane z motnjami apoptoze* (Rastogi in sod., 2009)

Motnja v apoptozi	
Nezadostna apoptoza	Prekomerna apoptoza
Rak	Nevrodegenerativne bolezni
Avtoimunske bolezni	Kardiovaskularne bolezni
Virusne okužbe	Vnetje
Sepsa	Sladkorna bolezen tipa I

1.2 Sprožitev apoptoze

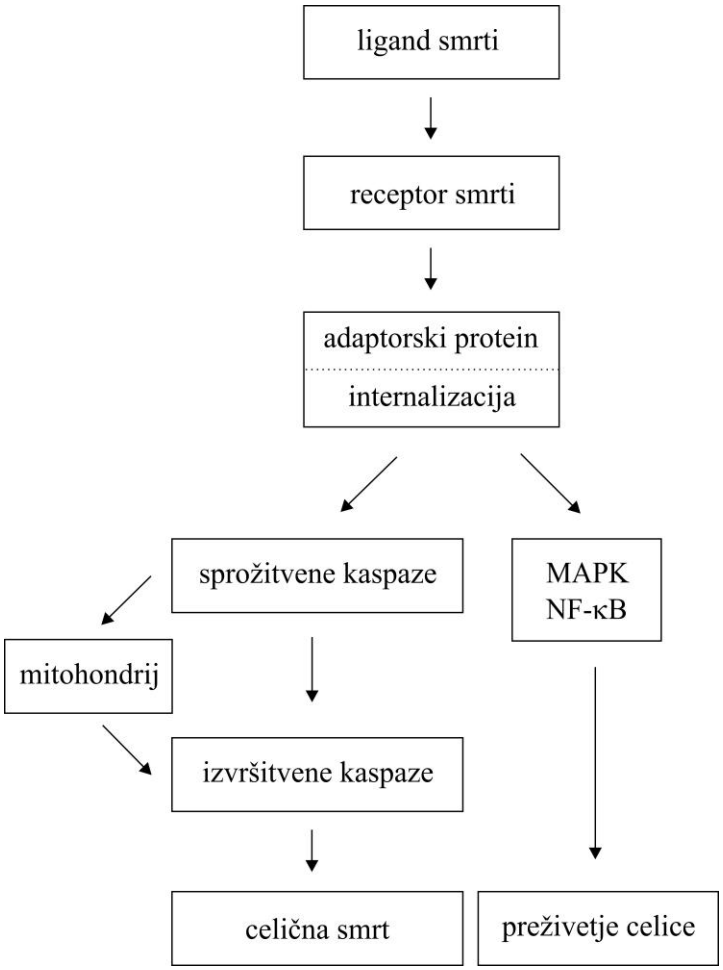
Apoptozo lahko sprožijo različni signali in sicer po dveh različnih poteh, zunanji (ekstrinzični) in notranji (intrinzični). Zunanja pot se aktivira, kadar se na receptorje smrti, ki so na zunanji strani celične površine, vežejo ligandi smrti. Notranjo pot pa lahko sprožijo različni signali s skupnim imenom stresni signali, ki poškodujejo mitohondrije in povzročijo sprostitev proapoptotskih dejavnikov v citosol. V to skupino uvrščamo poškodbe DNA, hipoksijo, inhibicijo sinteze makromolekul, poškodbe citoskeleta itd. Na koncu obeh poti se aktivirajo kaspaze, ki vodijo v nadaljnje korake apoptoze (Pereira in Amarante-Mendes, 2011).

1.2.1 Zunanja (ekstrinzična, receptorska) pot

Zunanjo pot apoptoze sproži vezava ligandov na receptorje smrti. To so člani družine receptorjev dejavnika tumorske nekroze (TNF), transmembranskih receptorjev s C-terminalnim znotrajceličnim delom in N-terminalnim zunajceličnim delom, na katere se vežejo ligandi. Značilnost receptorjev smrti je ohranjeno zaporedje 80 aminokislinskih preostankov v citoplazemski regiji, poimenovano tudi domena smrti (DD), ki je nujno za sprožitev apoptoze (Ashkenazi in Dixit, 1998; Locksley in sod., 2001). Najbolj raziskani receptorji so Fas (CD95/APO-1), TNF receptor 1 (TNF-R1/CD120a), TRAIL receptor 1 (TRAIL-R1/DR4) ter TRAIL receptor 2 (TRAIL-R2/DR5/APO-2). Čeprav imata tudi receptorja DR3 (APO-3, TRAMP) in DR 6 domeno smrti, ju ne uvrščamo med pomembnejše sprožilce apoptoze (Wajant, 2003), TNF receptor 2 (p75/CD120b) znotrajcelične domene smrti niti nima.

Receptorji smrti se aktivirajo z vezavo specifičnih ligandov. To so citokini iz družine TNF. Na Fas receptor se veže ligand FasL, na TNF receptor se veže TNF-α in na TRAIL receptorje se veže TRAIL (Ashkenazi in Dixit, 1998; French in Tschopp, 2003). Za prenos signala je potrebna oligomerizacija receptorjev (Boldin in sod., 1995), zelo verjetno pa je, da se ti lahko preko specifičnih zunajceličnih funkcionalnih regij, ki so

bogate s cisteinskimi preostanki, povežejo v kompleks PLAD (preligand assembly domain) že pred vezavo liganda (Chan in sod., 2000a; Chan in sod., 2000b). Vezava liganda povzroči konformacijske spremembe receptorja zaradi katerih se lahko na receptor vežejo adaptorski proteini (Fas-associated protein with death domain FADD, TNF receptor-associated protein with death domain TRADD). Ti se na receptorju vežejo na domeno smrti. Na drugi strani pa imajo adaptorski proteini efektorsko domeno smrti (death effector domain DED) s katero se povežejo s kaspazami. Nastali kompleks imenujemo DISC (death inducing signaling complex), iz njega se sprosti v citosol aktivna heterotetramerna molekula kaspaze, sestavljena iz dveh velikih in dveh malih podenot, ki lahko sproži apoptotsko signalno kaskado (Guicciardi in Gores, 2009). Splošen mehanizem poteka signaliziranja preko receptorjev smrti je prikazan na sliki 1.



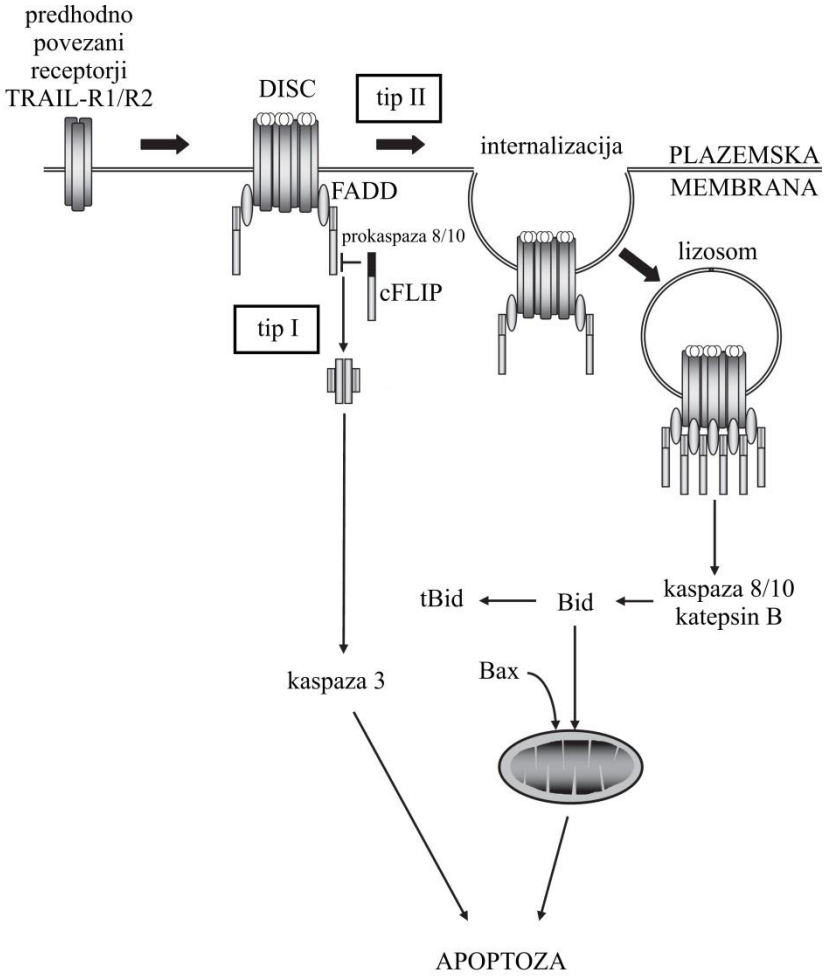
Slika 1: *Splošen mehanizem signaliziranja apoptoze, ki ga sprožijo ligandi smrti* (Guicciardi in Gores, 2009)

1.2.1.1 Ligand TRAIL

Znotraj družine TNF ima pomembno vlogo TRAIL (″TNF-related apoptosis-inducing ligand″ – TNF-ju podoben ligand, ki sproži apoptozo). Zaradi svoje lastnosti, da selektivno sproži apoptozo v tumorskih celicah, medtem ko na normalne celice nima učinka (Walczak in sod., 1999), spada med najbolj obetavne terapevtike za zdravljenje raka.

TRAIL sproži apoptozo z vezavo na receptorja TRAIL-R1 (DR4) in TRAIL-R2 (DR5) (Pan in sod., 1997). TRAIL-R1 se izraža v večini človeških tkiv in nekaterih tumorjih (Golstein, 1997), medtem ko se TRAIL-R2 pojavlja enako pogosto tako v normalnem tkivu kot v tumorjih. TRAIL se lahko veže tudi na dva tako imenovana receptorja vaba (″decoy receptor″) TRAIL-R3 (DcR1/LIT) in TRAIL-R4 (DcR2/TRUNDD) (Degli-Esposti in sod., 1997a; Degli-Esposti in sod., 1997b). Receptorja vaba sta podobna receptorjema TRAIL-R1 in TRAIL-R2. Imata enako zunajcelično in transmembransko regijo, vendar TRAIL-R3 nima celotne domene smrti, pri TRAIL-R4 pa je okrnjena ter posledično nefunkcionalna, zato vezava TRAIL-a na receptorja vaba ne sproži apoptoze. Tako prekomerno izražanje receptorjev vab zaradi antagonističnega delovanja prepreči apoptozo (Riccioni in sod., 2005). Poleg naštetih receptorjev se TRAIL lahko veže še na peti receptor, osteoprotegerin (OPG), toda pri fizioloških pogojih je afiniteta za to vezavo zelo nizka (Truneh in sod., 2000).

TRAIL z vezavo na zunajcelični del receptorja omogoči, da se na znotrajcelični del receptorja lahko veže adaptorski protein FADD. Na FADD se vežeta kaspazi 8 ali 10 in se v avtokatalitičnem procesu aktivirata (Kuang in sod., 2000). Nadaljnja signalizacija lahko poteka po dveh poteh in glede na to delimo celice na dva tipa. V celicah tipa I je aktivacija kaspaze preko kompleksa DISC zadostna za neposredno aktivacijo kaspaze 3. V celicah tipa II je najprej potrebna internalizacija receptorja za prenos signala za aktivacijo kaspaze 8. Kaspaza 8 cepi molekulo Bid, katere skrajšana oblika tBid omogoči aktivacijo in oligomerizacijo proteinov Bax in Bak v zunanji mitohondrijski membrani. Nastali kompleksi omogočajo prehod molekul iz mitohondrija v citosol. Tako sprožena mitohondrijska pot apoptoze aktivira zadostno količino kaspaze 3 in sproži apoptozo (Slika 2) (Guicciardi in Gores, 2009; Newsom-Davis in sod., 2009).



Slika 2: *Signalne poti, ki jih sproži ligand TRAIL* (Guicciardi in Gores, 2009)

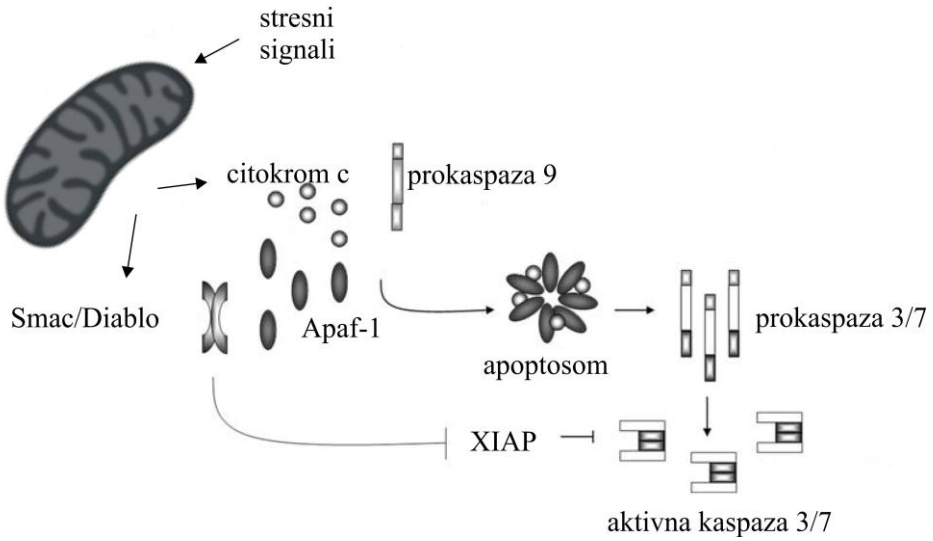
1.2.2 Notranja (intrinzična, mitohondrijska) pot

Notranjo oziroma mitohondrijsko pot apoptoze aktivirajo različni dejavniki, imenovani tudi stresni signali, kot so poškodbe DNA, poškodbe endoplazemskega retikuluma, sevanje UV, citotoksične snovi in pomanjkanje rastnih faktorjev. Osrednji dogodek pri tej poti je permeabilizacija zunanje mitohondrijske membrane (MOMP). Zaradi tega se iz medmembranskega mitohondrijskega prostora v citosol sprostijo proapoptotske molekule, npr. citokrom c in Smac/Diablo (Tait in Green, 2010).

V citosolu se iz mitohondrija sproščeni citokrom c poveže z adaptorskim proteinom Apaf-1 (apoptotic protease activating factor 1), kar povzroči konformacijske spremembe in oligomerizacijo v apoptosom. Apoptosom je struktura, sestavljena iz sedmih molekul Apaf-1 in sedmih citokromov c, na kateri se prokaspaza 9 dimerizira in aktivira v kaspazo 9. Ta s cepitvijo aktivira kaspazi 3 in 7 (Slika 3) (Riedl in Salvesen, 2007).

Poleg citokroma c se iz mitohondrija sprostijo še nekateri drugi pomembni proapoptostki dejavniki. Dejavnika Smac/Diablo in HtrA2/Omi se vežeta na ali razgradita

inhibitorje apoptotskih proteinov in s tem prispevata k sprožitvi kaspazne kaskade (Brenner in Mak, 2009). Dejavnik Smac (second mitochondria-derived activator of caspase) z vezavo na protein XIAP (X-linked inhibitor of apoptosis protein) prepreči inhibicijo aktivnih kaspaz 3, 7 in 9 (Eckelman in sod., 2006; Petersen in sod., 2007).

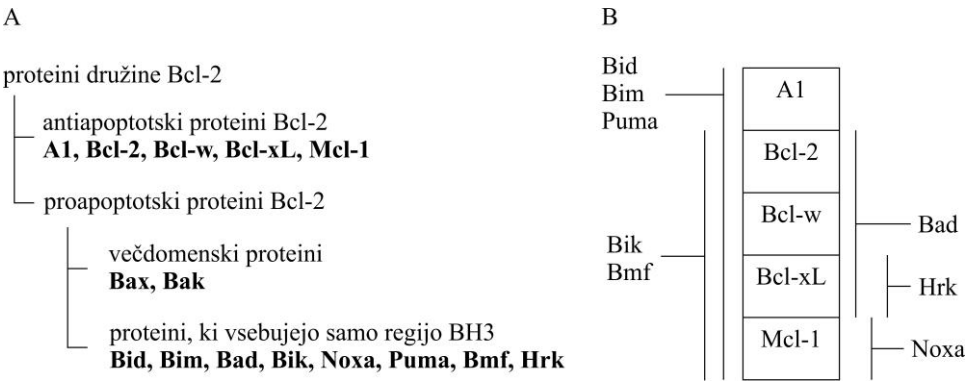


Slika 3: *Mehanizem aktivacije kaspaz pri notranji poti apoptoze* (Newsom-Davis in sod., 2009)

1.3 Proteini Bcl-2

Permeabilizacijo zunanje mitohondrijske membrane nadzirajo proteini in družine Bcl-2 (B-cell lymphoma-2). Glede na funkcijo in strukturo jih razdelimo na antiapoptotske, ki zavirajo apoptozo, in proapoptotske, ki apoptozo pospešujejo (Slika 4).

V družino antiapoptotskih proteinov uvrščamo proteine Bcl-2, Bcl-xL, Bcl-w, Mcl-1 in A1. Najdemo jih v jedrni membrani in membrani endoplazemskega retikuluma ter v citosolu. Družino proapoptotskih proteinov razdelimo na dve podskupini. V prvo uvrščamo proteine z tremi domenami BH (BH1-BH3), to so Bax, Bak, Bok in drugi. Drugo podskupino tvorijo proteini, ki imajo le regijo BH3 (BH3-only); Bid, Puma, Noxa, Bim, Bad in ostali (Youle in Strasser, 2008). Z izjemo Bax-a, ki ga najdemo tudi v mitohondrijski membrani, se vsi proapoptotski proteini nahajajo v citosolu.



Slika 4: *Delitev proteinov družine Bcl-2 (A) in profili povezav med antiapoptotskimi proteini Bcl-2 s proteini, ki vsebujejo samo regijo BH3 (B)* (Chipuk in sod., 2010)

Proteini, ki imajo samo regijo BH3, delujejo kot senzorji apoptotskih signalov, njihova aktivnost pa se uravnava s cepitvami (Bid), defosforilacijo (Bim, Bad) ali na nivoju transkripcije (Puma, Noxa) (Youle in Strasser, 2008). Cepitev proteina Bid v tBid (″truncated″ Bid) omogoči prenos proteina v mitohondrij, kjer sodeluje pri oligomerizaciji proteinov Bax in Bak ter tvorbi por (Korsmeyer in sod., 2000).

Večdomenska proapoptotska proteina Bax in Bak lahko tvorita pore na mitohondrijski membrani. Aktivirata se posredno ali neposredno. Pri neposrednem načinu se nekateri proapoptotski proteini, ki jih imenujemo aktivatorji (Bid, Bim, Puma) vežejo neposredno na Bax in Bak in ju tako aktivirajo. Pri posredni aktivaciji sta Bax in Bak najprej v neaktivnem kompleksu povezana z antiapoptostkimi Bcl-2 proteini. Proapoptotski proteini, ki imajo le BH3 regijo, lahko z vezavo na te Bcl-2 proteine sprostijo proteina Bax in Bak (Brenner in Mak, 2009).

Protein Bak se nenehno prenaša v zunanjo mitohondrijsko membrano, kjer ga inhibira protein VDAC2. Aktivatorji tBid, Bim ali Puma lahko prekinejo povezavo Bak-VDAC2 ter s tem omogočijo oligomerizacijo in tvorbo pore. Bax je citosolen monomeren protein, katerega C-končni heliks α9 preprečuje dimerizacijo. Struktura proteina se spremeni, ko se na N-končni heliks α1 vežejo tBid, Bim ali Puma, s heliksom α9 pa se Bax vgradi v mitohondrijsko membrano (Kim in sod., 2009). Bax lahko tvori pore sam, ali pa v povezavi z Bak-om ali tBid-om (Kuwana in sod., 2002). Pore omogočajo permeabilizacijo membrane in prehod proapoptotskih molekul v citosol.

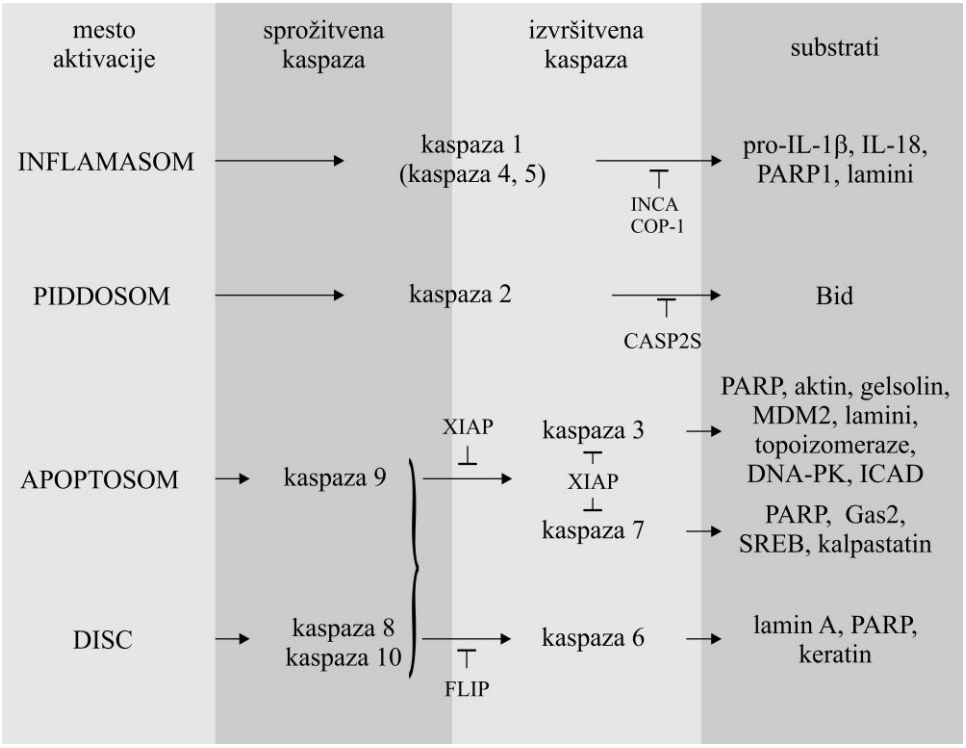
1.4 Kaspaze

Kaspaze so cisteinske proteaze, ki jih uvrščamo v družino C14 in klan CD. So zelo dobro evolucijsko ohranjene in imajo pomembno vlogo v procesu celične smrti. Ime kaspaza (caspase – cysteine-dependent aspartate-specific protease) pove, da pri katalizi sodeluje cisteinski aminokislinski preostanek in da cepijo peptidno vez za aspartatom (Alnemri in sod., 1996). Prva odkritja o vpletenosti kaspaz pri procesu celične smrti so povezana s proučevanjem nematoda *Caenorhabditis elegans* (Ellis in Horvitz, 1986). Kaspaza Ced-3 (″cell death defective″) je edina potrebna za sprožitev apoptoze v tem organizmu, medtem ko je v bolj kompleksnih organizmih potrebnih več kaspaz. Prvi odkrit homolog kaspaze

Ced-3 pri sesalcih je bila kaspaza ICE (″interleukin-1β-converting enzyme) (Cerretti in sod., 1992).

Kaspaze so med najpomembnejšimi udeleženci pri apoptozi (Salvesen in Riedl, 2008). So signalne proteaze, ki cepijo številne celične proteine (Timmer in Salvesen, 2007), vendar zelo specifično, saj navadno na substratu opravijo majhno število cepitev (največkrat celo samo eno). Zato je posledica njihove delovanja največkrat aktivacija ali inaktivacija proteinov, ne pa razgradnja (Bratton in Salvesen, 2010; Gutierrez in Ronai, 2006).

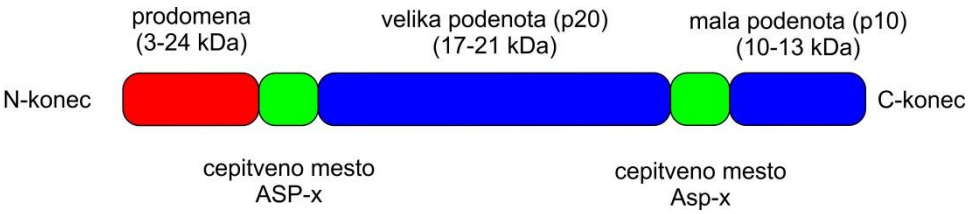
Kaspaze lahko v splošnem delimo na apoptotske in provnetne. Vendar imajo apoptotske kaspaze tudi nekatere neapoptotske funkcije (Bredesen, 2008), prav tako pa so tudi vnetne kaspaze vključene v nekatere vrste celične smrti (Labbe in Saleh, 2008). Med apoptotskimi kaspazami ločimo sprožitvene, to so kaspaze 8, 9 in 10, ter izvršitvene kaspaze 3, 6 in 7. Kaspaza 2 ima značilnosti obeh skupin (Ribe in sod., 2008).



Slika 5: *Delitev kaspaz, njihove aktivacijske poti in substrati kaspaz* (Chowdhury in sod., 2008; Pop in Salvesen, 2009)

1.4.1 Struktura kaspaz

Vse kaspaze imajo podobno strukturno organiziranost. Sintetizirajo se v obliki zimogenov/proencimov ter so enoverižni proteini, ki imajo N-terminalne domene pred katalitičnimi domenami (Slika 6). Sprožitvene kaspaze se sintetizirajo kot monomeri, izvršitvene pa kot dimeri. Pri aktivaciji se katalitična domena cepi na veliko in malo podenoto, ki pa se takoj povežeta. V aktivni obliki so kaspaze dimeri, vsaka molekula pa ima dve aktivni mesti (Pop in Salvesen, 2009).



Slika 6: *Struktura kaspaz* (Chowdhury in sod., 2008; Pop in Salvesen, 2009; Salvesen in Riedl, 2008)

1.4.2 Aktivacija kaspaz

Za razliko od večine ostalih proteaz, se kaspaze ne aktivirajo z odcepitvijo prodomene. V kaskadni verigi kaspaze cepijo in s tem aktivirajo ena drugo, kaspaze, ki so na začetku verige, pa imajo drugačen tip aktivacije (Slika 7).

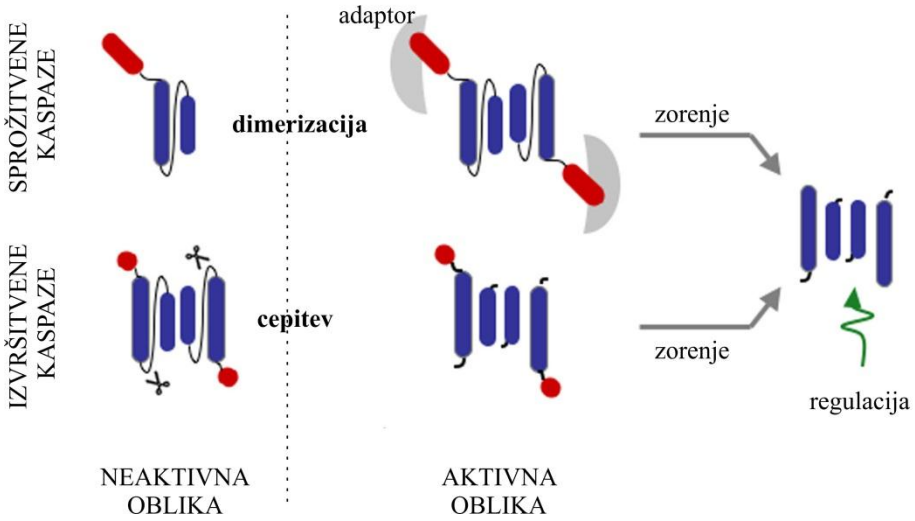
Pri sprožitvenih kaspazah je za aktivacijo potrebna homodimerizacija monomerov. Po apoptotskem signalu se kaspaze vežejo na specifične domene DED (kaspaza 8 in 10) ali CARD (kaspaza 1, 2 in 9) adaptorskih molekul. S tem se doseže visoka lokalna koncentracija kaspaz in aktivacija preko dimerizacije je posledica bližine molekul (″proximity-induced dimerization″) (Riedl in Salvesen, 2007). Vsaka sprožitvena kaspaza ima svoje mesto aktivacije. Kompleks DISC poveže in aktivira kaspazi 8 in 10, apoptosom kaspazo 9 in PIDDosom kaspazo 2. Provnetne kaspaze 1, 4 in 5 imajo podoben model aktivacije, in sicer se z domeno CARD povežejo na inflamasomu (Martinon in Tschopp, 2007). Sprožitvena kaspaza 9 se lahko aktivira tudi na drugačen način. Stres lahko povzroči translokacijo kaspaz v endoplazemski retikulum. Pri miših so pokazali, da interakcija med kaspazama 7 in 12 aktivira kaspazo 12, ki lahko nato aktivira kaspazo 9 (Lamkanfi in sod., 2002). Ta način aktivacije kaspaze 9 ni odvisen od sproščanja citokroma c iz mitohondrija.

V nasprotju s sprožitvenimi kaspazami, ki so v citosolu neaktivne v obliki monomernih zimogenov, so izvršitvene kaspaze pred aktivacijo v obliki dimerov in potrebujejo cepitev katalitične domene za aktivacijo (Alenzi in sod., 2010). Aktivnost že tvorjenega dimera preprečuje povezovalni peptid (linker) med veliko in malo podenoto katalitičnega mesta. Tvorbo aktivnega mesta omogoči cepitev povezovalnega peptida, ki jo izvršijo sprožitvene kaspaze (kaspaza 8, 9, 10) ali pa grancim B (serinska proteaza specifična za limfocite) (Pop in Salvesen, 2009). Aktivacija izvršitvenih kaspaz se razlikuje pri notranji in zunanji poti apopotoze.

Pri zunanji poti obstajata dva različna načina aktivacije kaspaz (Scaffidi in sod., 1998). Celice tipa I tvorijo zadosti kompleksa DISC in aktivne kaspaze 8, da aktivirajo izvršitveni kaspazi 3 in 7. Pri celicah tipa II je število kompleksov DISC premajhno za

tvorbo zadostnega števila aktivne kaspaze 8. Za aktivacijo kaspaz 3 in 7 se v teh celicah sproži ojačitvena zanka. Preko cepitve proteina Bid se sproži sproščanje citokroma c iz mitohondrija (Korsmeyer in sod., 2000; Kuwana in sod., 2002). To omogoči tvorbo apoptosoma in aktivacijo kaspaze 9, ta pa lahko aktivira dodatne količine kaspaz 3 in 7. Pri notranji poti apoptoze poškodbe mitohondijev povzročijo sproščanje citokroma c v citosol in aktivacijo kaspaze 9 na apoptosomu, kar vodi v aktivacijo izvršitvenih kaspaz (Jiang in Wang, 2000).

Aktivaciji kaspaz pogosto sledi (avto)proteolitična cepitev. Tako imenovano zorenje (″maturation″) omogoči odcepitev prodomene in/ali cepitev povezovalnega peptida. Posledica zorenja so novi epitopi in preureditve. Zorenje brez aktivacije sicer ne omogoči encimske aktivnosti (Pop in sod., 2007), toda delovanje encima se lahko tudi razlikuje, če aktivaciji ne sledi zorenje. Aktivirana kaspaza 8 brez zorenja lahko sproži proliferacijo in aktivacijo T celic, ne more pa sprožiti celične smrti, ker je za to potrebna cepljena oblika kaspaze 8 (Kang in sod., 2008; Oberst in sod., 2010). Cepitev povezovalnega peptida pri kaspazi 9 omogoči njeno regulacijo, saj se izpostavijo novi epitopi, na katere se lahko veže protein XIAP (Srinivasula in sod., 2001).



Slika 7: *Mehanizem aktivacije kaspaz* (Pop in Salvesen, 2009)

1.4.3 Specifičnost kaspaz

Kaspaze cepijo substrate za aspartatnim aminokislinskim preostankom, vendar je cepitev peptida odvisna tudi od drugih dejavnikov. Zaporedje P4-P3-P2-P1-P1', pri čemer kaspaza cepi med P1 in P1', je substrat za kaspaze, če je P1 aspartat. Izjema je kaspaza Dronc pri vinski mušici *Drosophila*, sicer sorodna kaspazi 9, ki *in vitro* cepi tudi za glutaminom (Snipas in sod., 2008). Naslednji pogoj je, da je P1' aminokislinski preostanek majhen in nenabit (Gly, Ser in Ala). Interakcija preostankov P4-P2 s katalitično zanko lahko spremeni slab substrat v dobrega in obratno (Pop in Salvesen, 2009).

1.4.3.1 Substrati kaspaz

Kaspaze izvršijo apoptozo s cepitvijo specifičnih substratov. Proteomska analiza je razkrila že preko 400 celičnih proteinov, ki jih med apoptozo cepijo kaspaze (Pop in

Salvesen, 2009). Med njimi so proteini vključeni v celično strukturo, signaliziranje, kontrolo celičnega cikla ter popravila DNA (Alenzi in sod., 2010).

1.4.3.1.1 Cepitve DNA

Ena od značilnosti apoptoze je fragmentacija DNA, pri čemer so prav tako vpletne kapsaze. CAD (″caspase activated DNase″) je encim, ki cepi DNA in je v citosolu povezan z inhibitorjem ICAD/DFF45. Kaspaza 3 s cepitvijo sprosti CAD in sproži razgradnjo kromosomske DNA (Enari in sod., 1998).

1.4.3.1.2 Inaktivacija proteinov, ki so potrebni za popravilo DNA

Prvi odkrit substrat kaspaz je bil encim PARP (″poly ADP-ribose polymerase″). Sodeluje pri popravilu DNA, stabilnosti DNA in regulaciji transkripcije. Kaspaze, posebej kaspazi 3 in 7, odcepita fragment iz DNA vezavnega mesta PARP-a. Cepitev proteina pomembnega za popravljalni mehanizem DNA pospeši apoptozo, ni pa vzrok zanjo. Med popravljane mehanizme, ki jih onemogočijo kaspaze sodi še cepitev popravljalnega proteina DNA-PK (″DNA-dependent protein kinase″) (Alenzi in sod., 2010).

1.4.3.1.3 Razgradnja celičnih struktur

Cepitve celičnih struktur povzročijo značilne morfološke spremembe med apoptozo. Kaspaze lahko direktno cepijo aktin in aktivirajo še druge proteine (gelsolin, Gas-2), ki prav tako cepijo aktin (Earnshaw in sod., 1999; Kothakota in sod., 1997). Tarča proteaz so tudi lamini, ki vzdržujejo obliko jedra in so vpleteni v povezave med kromatinom in jedrno membrano (Alenzi in sod., 2010).

Tabela 3: *Substrati kaspaz* (Alenzi in sod., 2010; Chowdhury in sod., 2008)

Skupina	Proteini
citoplazemski proteini	aktin, gelsolin, adherini, keratin 18, serpini
jedrni proteini	lamin A, lamin B, receptor lamina B, NuMA
metabolni proteini in proteini za popravila DNA	PARP, DNA-PK, topoizomeraze, RNA polimeraza
kinaze	PKC, MAPK, ERK, Akt
prenašalci signalov	citokini, fosfolipaza A2, Bcl2-2
proteini celičnega cikla in delitve	p21, p27, pRB

1.4.4 Regulacija kaspaz

Proteoliza je ireverzibilen proces zato je potrebna njena natančna kontrola. Celica ima tri načine za preprečitev neželene aktivnosti kaspaz: inhibicija, razgradnja in inhibitorje vabe (″decoy inhibitors″).

Različne molekule se lahko vežejo na vezavno mesto za substrat in s tem inhibirajo proteaze. Virusna proteina CrmA in p35/p49 sta nespecifična kaspazna inhibitorja (Stennicke in sod., 2002), človeški protein XIAP pa selektivno inhibira kaspazo 9 (preko domene BIR3) ter kaspazi 3 in 7 (preko domene BIR2).

Ostali člani skupine IAP (cIAP1, cIAP2, ILP-2, survivin…) ne inhibirajo kaspaz direktno (Eckelman in sod., 2006). Udeleženi so pri razgradnji kaspaz, ki je prav tako eden od mehanizmov regulacije. Poleg vezavnih domen BIR, imajo ti proteini tudi domene RING in domene povezane z ubikvitinom. Ubikvitinilacija usmerja proteine v razgradnjo preko proteasoma.

Tretji način regulacije kaspaz vključuje proteine vabe, ki so strukturno podobni kaspaznim prodomenam. Proteini vabe tekmujejo s kaspazami za vezavo na aktivacijska mesta in s povezavo preprečijo vezavo in aktivacijo kaspaz (Pop in Salvesen, 2009).

1.4.5 Pomen kaspaz pri drugih celičnih procesih

Poleg vpletenosti v procese celične smrti so kaspaze vključene tudi v druge celične procese. Pomemben vpliv pri proliferaciji imata kaspazi 3 in kaspaza 8 (delitve in aktivacija limfocitov in celic ubijalk (Chun in sod., 2002), isti kaspazi igrata vlogo pri celični diferenciaciji (Kang in sod., 2004) in migraciji celic (Helfer in sod., 2006), proces metamorfoze pa vključuje kaspazi 3 in 6 (Schoenmann in sod., 2010).

1.5 Lizosomi in apoptoza

Lizosomi so citoplazemski organeli obdani z enojno membrano, pH znotraj njih je med 3,8 in 5,0. Tako nizek pH omogoča ATP-aza, ki črpa protone iz citosola v lizosom (Luzio in sod., 2007). Od endosomov, ki so prat tako citoplazemski organeli obdani z enojno membrano, se lizosomi razlikujejo po nižjem pH in odsotnosti manoza-6-fosfatnega receptorja (Turk in Turk, 2009). V lisozomih poteka razgradnja celičnih makromolekul z več kot 50 različnimi hidrolazami (proteaze, lipaze, nukleaze, glikozidaze, fosfolipaze…). Med pomembnejšimi proteazami znotraj lizosomov so katepsini (Turk in Turk, 2009). Lizosome pred razgradnjo z lastnimi hidrolazami ščitijo glikozilirani membranski proteini, kot sta Lamp-1 in Lamp-2 (Eskelinen, 2006).

Zaradi visoke vsebnosti hidrolitičnih encimov predstavljajo lizosomi potencialno nevarnost za celico. V primeru poškodb lizosomske membrane se njihova vsebina lahko sprosti v citosol in sproži razgradnjo celičnih komponent. Delna permeabilizacija lizosomske membrane inducira apoptozo, popoln razpad lizosomov pa lahko celo povzroči nakisanje citosola in vodi v nekrozo (Boya in Kroemer, 2008).

Dolgo časa so domnevali, da so lizosomi v procesih celične smrti vpleteni le pri nekrozi in avtofagiji, njihova vloga pri apoptozi naj bi bila le razgradnja apoptotskih telesc (Ferri in Kroemer, 2001; Leist in Jaattela, 2001), delovanje lizosomalnih proteaz pa omejeno na nespecifično razgradnjo celičnih proteinov. Z novimi spoznanji, predvsem možnost delne permeabilizacije lizosomske membrane, pa se je pogled na vlogo lizosomov pri apoptozi spremenil (Guicciardi in sod., 2004). Tako pri nekrozi kot pri apoptozi je količina sproščenih encimov v citosol zadostna, da preseže zaščitno delovanje citosolnih inhibitorjev (Berg in sod., 1995; Claus in sod., 1998; Turk in sod., 2002a) in encimi lahko sodelujejo v procesih celične smrti.

1.5.1 Katepsini

Ime katepsini izvira iz grške besede kathepsin (prebaviti). Danes to poimenovanje zajema serinske proteaze (katepsina A in G), aspartatne proteaze (katepsina D in E) in lizosomske cisteinske proteaze. Poznamo 11 človeških cisteinskih katepsinov (B, C, F, H, K, L, O, S, V, X in W) (Turk in sod., 2002b; Turk in sod., 2000; Turk in sod., 2001). Ti za svoje optimalno delovanje potrebujejo reducirajoče, rahlo kislo okolje, ki jim ga nudi lizosom (Turk in sod., 2011). Kisel pH je potreben, ker so zunaj lizosomov katepsini pri nevtralnem pH relativno hitro ireverzibilno inaktivirani. V nevtralnem ali alkalnem pH območju pride do deprotonizacije histidinskega preostanka (His159, papainsko štetje), prekinejo se elektrostatske interakcije in poruši se terciarna struktura (Turk in sod., 1994). Izjema je katepsin S, ki je stabilen pri nevtralnem in rahlo bazičnem pH (Kirschke in sod., 1989).

Večina katepsinov se izraža v najrazličnejših tkivih in so vpleteni v normalno celično razgradnjo proteinov. Med najbolj zastopanimi so katepsini B, H in L (Brix in sod., 2008), medtem ko se katepsini K, W, S in F bolj specifično izražajo le v nekaterih tkivih/celicah in naj bi imeli bolj specifične vloge. Katepsin K sodeluje pri preoblikovanju kosti (Asagiri in Takayanagi, 2007), katepsin W pri delovanju imunskih celic (Linnevers in sod., 1997), katepsin S pa ima pomembno vlogo pri procesiranju invariantne verige MHC razreda II (Hsing in Rudensky, 2005). Pri slednjem procesu sta udeležena še katepsina L in V. Nedavne študije so pokazale, da se katepsini poleg lizosomov nahajajo še v jedru, citoplazmi in plazemski membrani. Pomembno funkcijo naj bi imeli predvsem v jedru, kjer lahko katepsina L in F procesirata histone (Ceru in sod., 2010; Maubach in sod., 2008). Vse več je dokazov, da so specifične funkcije katepsinov povezane z njihovo lokalizacijo v celici in zunaj nje (Zavasnik-Bergant in Turk, 2006) in da katepsini delujejo kot signalne molekule (Stoka in sod., 2007; Turk, 2006; Turk in Stoka, 2007).

1.5.1.1 Procesiranje katepsinov

Lizosomski katepsini se sintetizirajo v obliki preproencimov. Pri prehodu v endoplazemski retikulum pride do cepitve signalnega peptida in hkratne glikolizacije N-konca molekule. Propeptid omogoča pravilno zvijanje proteina in prehod v endosome/lizosome s pomočjo manoza-6-fosfatnega receptorja (M6PR), služi pa tudi kot inhibitor proteolitične aktivnosti proencima (Twining, 1994). V endosomu katepsini postanejo aktivni po odcepitvi M6PR in propeptida.

1.5.1.2 Aktivacija katepsinov

Aktivacija katepsinov se začne z odstranitvijo propeptida iz aktivnega mesta. Kisel pH oslabi interakcije med propeptidom in encimom. Zaradi tega imajo nekateri proencimi, npr. prokatepsin B, nizko katalitično aktivnost, ki je ravno zadostna za avtokatalitično aktivacijo (Pungercar in sod., 2009). V drugem koraku lahko ti proencimi aktivirajo druge prokatepsine in s tem sprožijo verižno reakcijo aktivacije prokatepsinov. Na ta način se lahko aktivirajo endopeptidaze (katepsini B, H, L, S in K), medtem ko eksopeptidazi (katepsina C in X) za aktivacijo potrebujeta cepitev s strani drugih molekul (katepsina L in S) (Dahl in sod., 2001; Turk in sod., 2001).

1.5.1.3 Regulacija aktivnosti katepsinov

V normalnih pogojih imajo celice več mehanizmov za preprečevanje neželene in zato nevarne proteolize katepsinov (Turk in sod., 1997; Turk in sod., 2001). Omenili smo že lokalizacijo katepsinov v lizosomih ali drugih organelih, aktivacijo proencimov ter pH, najbolj pomemben mehanizem pa je regulacija z inhibitorji (Twining, 1994).

1.5.1.3.1 Endogeni inhibitorji cisteinskih katepsinov

Najpomembnejši način regulacije aktivnosti katepsinov so inhibitorji. Večina endogenih inhibitorjev je kompetitivnih, osnova njihovega delovanja je vezava v aktivno mesto, s čimer onemogočijo vezavo substrata. Razdelimo jih v štiri družine. V prve tri uvrščamo inhibitorne proteine stefine, cistatine in kininogene (Barrett in sod., 1986; Turk in Bode, 1991; Turk in sod., 2008). V četrto skupino sodijo neinhibitorni homologi cistatinov kot so fetuini in s histidinom bogati glikoproteini (Jones in sod., 2005; Reynolds in sod., 2005).

Stefini so enoverižni, neglikozilirani proteini brez signalnega peptida in disulfidnih mostičkov, veliki približno 100 aminokislinskih preostankov. Primarno se nahajajo znotraj celice, najdemo pa jih tudi v telesnih tekočinah (Abrahamson in sod., 1986). Cistatini so enoverižni, neglikozilirani proteini, veliki približno 115 aminokislinskih preostankov, za razliko od cistatinov pa imajo dve disulfidni vezi in signalno zaporedje za izločanje iz celice. Kinogeni so zunajcelični večfunkcijski glikoproteini, so enoverižni z več domenami za vezavo cisteinskih proteaz. Med ostalimi inhibitorji cisteinskih proteaz najdemo tiropine (fragment p41 invariantne verige MHC razreda II) in serpine, inhibitorje serinskih proteaz (Turk in sod., 2002a; Turk in sod., 2011).

1.5.1.4 Substrati katepsinov

Katepsini lahko *in vitro* v kislem pH razgradijo različne proteine. Zunaj celice katepsini razgrajujejo komponente ekstracelularnega matriksa kot so elastin, kolagen in proteoglikani (Lutgens in sod., 2007), kar jih postavlja v vlogo pomembnih igralcev pri raku. Tumorske celice izražajo povečane količine lizosomskih encimov, predvsem katepsina B in D. Povečano izločanje teh encimov izven celice naj bi z razgraditvijo zunajceličnega matriksa pospeševalo rast tumorjev, invazivnost, angiogenezo in tvorbo metastaz (Koblinski in sod., 2000). Znotraj lizosoma sodelujejo katepsini predvsem pri nespecifični razgradnji proteinov. Zaradi nestabilnosti in izgube aktivnosti pa ni veliko podatkov o znotrajceličnih citosolnih substratih (Repnik in sod., 2012). Prvi znani substrat je protein Bid, proapoptotski homolog Bcl-2 (Stoka in sod., 2001). Ostali znani *in vitro* substrati so prikazani v tabeli 4.

1.5.1.5 Struktura katepsinov

Katepsini so majhne molekule velikosti približno 30 kDa, z izjemo katepsina C, ki je tetramer, velik približno 200 kDa. Imajo dve domeni, L (left) in R (right). Domena L je sestavljena iz treh alfa vijačnic, domena R pa iz antiparalelne beta ploskve z značilno sodčku podobno strukturo. Vmesna površina obeh domen tvori režo z aktivnim mestom, ki ima obliko črke V. V sredini reže sta aminokislini v aktivnem mestu, Cys25 in His159 (papainsko štetje). Cys25 se nahaja v levi domeni na N-terminalnem delu alfa vijačnice, His159 pa na desni domeni na nasprotni strani žepa (Turk in sod., 2001).

Tabela 4: *Poimenovanje, katalitske lastnosti ter in vitro substrati človeških lizosomskih cisteinskih proteaz* (Conus in Simon, 2008; Turk in sod., 2011)

ime	sinonim	Vrsta peptidazne aktivnosti	Specifični substrati
katepsin L		endopeptidaza	Bid, Bak, BimEL, Bcl-2, Bcl-xL, XIAP, E-kadherin
katepsin V	katepsin L2, U	endopeptidaza	
katepsin S		endopeptidaza	Bid, Bak, BimEL, Bcl-2, Bcl-xL, XIAP, E-kadherin
katepsin K	O, O2, X	endopeptidaza	Bid, Bak, BimEL, Bcl-2, Bcl-xL, XIAP
katepsin F		endopeptidaza	
katepsin B	B1	endopeptidaza in karboksipeptidaza	Bid, Bak, Bcl-2, Bcl-xL, XIAP, PARP-1, kaspaze-1/-2/-11, E-kadherin
katepsin H	katepsin I	endopeptidaza in aminomonopeptidaza	Bid, Bak, BimEL, Bcl-2, Bcl-xL, XIAP
katepsin X	katepsin Z, P, Y	karboksimono(di)peptidaza	
katepsin C	katepsin J, dipeptidil-peptidaza I	aminodipeptidaza	
katepsin W	limfopain	ni znano	
katepsin O		ni znano	

1.5.1.6 Sproščanje katepsinov iz lizosomov

Regulacija sproščanja molekul iz lizosomov je pomemben dejavnih njihove vpletenosti v celične procese. Permeabilizacija lizosomov je povezana z apoptozo sproženo z oksidativnim stresom, lizosomotropiki, sfingozinom ter s TNF in TRAIL tretiranimi celicami (Kagedal in sod., 2001b; Werneburg in sod., 2002; Werneburg in sod., 2007; Zdolsek in sod., 1993). Obstaja več teorij o mehanizmih sproščanja, a dokončnega odgovora o njih še nismo dobili. Nekateri menijo, da so za selektiven prenos odgovorni posebni translokacijski proteini, drugi pa, da se na lizosomski membrani tvorijo pore, ki neselektivno prepuščajo le molekule do določene velikosti (Boya in Kroemer, 2008). Domneva se tudi, da lahko proteina Bax in Bak, ki sicer tvorita pore na zunanji mitohondrijski membrani, tvorita pore tudi na lizosomih. Te pore lahko brez poškodbe membrane prepuščajo tudi molekule večje od 100 kDa (Feldstein in sod., 2006). Ker pa je permeabilizacija veziklov v celici selektivna in ne pride do popolnega razpada vseh membran, verjetno obstajajo regulatorni mehanizmi permeabilizacije (Guicciardi in sod., 2004).

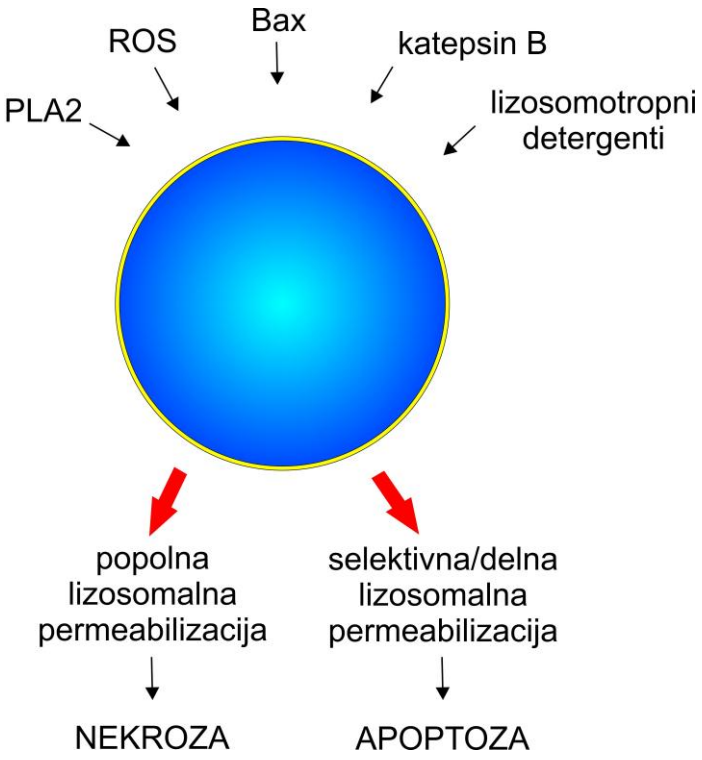
Eden prvih znanih dejavnikov poškodbe lizosomske membrane so bile reaktivne kisikove zvrsti (ROS), ki so zelo reaktivne molekule. Veliko ROS nastane pred poškodbo lizosomov. ROS se v celici primarno tvorijo v mitohondrijih, zato poškodujejo predvsem lizosome v bližini mitohondrijev (Boya in Kroemer, 2008). Nekaj ROS se lahko tvori tudi v samih lizosomih. Prosto železo znotraj lizosomov katalizira pretvorbo vodikovega

peroksida v hidroksilne radikale, ti pa oksidirajo membranske lipide in povzročijo destabilizacijo lizosomskih membran (Blomgran in sod., 2007; Brunk in sod., 2001). ROS lahko pri apoptozi sproženi s TNF-α nastanejo tudi neodvisno od kaspaze 8. Za nastanek ROS pri tej poti je odvisen protein RIP-1, ki se veže na adaptorski protein TRADD (Cauwels in sod., 2003). Poleg neposrednega delovanja lahko ROS na stabilnost membran vplivajo tudi z aktivacijo fosfolipaze A2, ki razgrajuje membranske fosfolipide celičnih organelov (Zhao in sod., 2001).

Sfingolipid sfingozin je lizosomotrop in je prav tako naslednji pomemben dejavnik poškodb lizosomov. Zaradi dolgega lipofilnega repa in polarne glave se lahko po protonaciji akumulira v kislih organelih, kjer deluje kot detergent. V celicah nastaja pri procesiranju ceramida, ki se tvori v lizosomih ob delovanju kisle sfingomielinaze (Heinrich in sod., 2004). Nizke koncentracije sfingozina povzročijo permebilizacijo lizosomske in mitohondrijske membrane ter aktivacijo kaspaz (Kagedal in sod., 2001a), kar privede do apoptoze. Nasprotno pa visoke koncentracije sfingozina povzročijo obsežnejšo poškodbo lizosomov in posledično nekrozo (Kagedal in sod., 2001b). Povečane koncentracije sfingozina so opazili pri apoptozi sproženi s TNF-α, do permebilizacije lizosomske membrane pa je prišlo le pri celicah, ki so izražale katepsin B. Nasprotno pa pri celicah z izbitim genom za katepsin B niso opazili poškod lizosomov (Werneburg in sod., 2002).

Kot že omenjeno, lahko tudi proapoptotska proteina Bax in Bak tvorita pore na lizosomski membrani. Sodita v družino proteinov Bcl-2, ki so pomembni v apoptozi saj regulirajo permeabilizacijo zunanje mitohondrijske membrane (Youle in Strasser, 2008). Obstaja več teorij, kako bi lahko proteina Bax in Bak tvorila pore na lizosomski membrani. Lahko bi prišlo do tvorbe por na podoben način, kot se tvorijo na mitohondriju in bi omogočile sprostitev lizosomske vsebine v citosol (Kagedal in sod., 2005; Werneburg in sod., 2007). Prav tako bi lahko konformacijska sprememba proteina Bax, ki so jo opazili pri apoptozi sproženi s prostimi maščobnimi kislinami, omogočila prenos na lizosomsko membrano (Feldstein in sod., 2004; Feldstein in sod., 2006). Nedavna študija pa je pokazala tudi možnost, da se proteina Bax/Bak preneseta v lizosom kot posledica avtofagnega prenosa poškodovanih mitohondrijev (Oberle in sod., 2010).

Na permeabilizacijo lizosomskih membran vplivajo tudi sami katepsini. Študije na celicah z izbitim genom za katepsin B so pokazale manjšo lizosomsko permeabilizcijo po indukciji apoptoze s TNF-α v primerjavi s celicami divjega tipa. Katepsin B bi lahko na permeabilizacijo deloval znotraj ali zunaj lizosoma (Werneburg in sod., 2002). Novejše raziskave so razkrile, da povečana aktivnost katepsinov razpolovi življensko dobo proteinom Lamp-1 in Lamp-2 in s tem destabilizira lizosomske membrane (Ferri in Kroemer, 2001). Hkrati pa naj bi katepsin B podaljšal življenjsko dobo sfingozina z razgradnjo sfingozin kinaze-1, ki pretvarja proapoptostki sfingozin v antiapoptotski sfingozin-1-fosfat (Guicciardi in sod., 2001).



Slika 8: *Mehanizmi lizosomske permeabilizacije* (Boya in Kroemer, 2008; Guicciardi in sod., 2004)

1.5.1.7 Katepsini in apoptoza

Po sprostitvi v citosol katepsini ohranijo svojo aktivnost od nekaj minut do nekaj ur predno se inaktivirajo (Turk in sod., 1993; Turk in sod., 2000) in tako lahko razgrajujejo proteine tudi zunaj lizosoma. Katepsin B je med najbolj stabilnimi katepsini pri fiziološkem pH in ima pomembno vlogo v različnih modelih apoptoze sprožene z žolčnimi kislinami v hepatocitih, s TNF-α sproženo apoptozo v primarnih hepatocitih in tumorskih celicah, apoptozo nevronov in apoptozo zaradi pomanjkanja hranil (Guicciardi in sod., 2001; Roberts in sod., 1997; Shibata in sod., 1998). Za katepsin D pa je na študijah pokazana pomembna vloga pri apoptozi sproženi z ligandi Fas in TNF-α, oksidativnim stresom, sfingozinom in p53 (Bidere in sod., 2003; Deiss in sod., 1996; Ollinger, 2000). Mehanizem vpletenosti katepsinov v apoptozo še ni popolnoma pojasnjen.

Katepsini so aktivni takoj po sprostitvi v citosol, in za razliko od kaspaz ne potrebujejo aktivacije (Turk in Stoka, 2007). V citosolu s cepljenjem substratov sodelujejo v apoptotski signalizaciji. Študije največkrat kot substrat katepsinov navajajo protein Bid, proapoptotski protein iz družine Bcl-2 (Cirman in sod., 2004; Droga-Mazovec in sod., 2008; Stoka in sod., 2001). Bid ima med dvema alfa vijačnicama zanko, na kateri so cepitvena mesta katepsinov B, K, L, H in S (na mestu Arg65), katepsina H (na mestu Arg71), kaspaze 8 (na mestu Asp59), grancima B (na mestu Asp75) in kalpainov (na mestu Tyr47) (Repnik in sod., 2012). Najpomembnejšo vlogo pri cepitvi proteina Bid naj bi imela katepsina B in L. Sta najbolj zastopana in pri fiziološkem pH stabilna katepsina. Cepljen Bid omogoča tvorbo por v mitohondrijski membrani (Cirman in sod., 2004). Vendar pa so raziskave na miših z izbitim genom za Bid pokazale, da katepsini sodelujejo

tudi v apoptozi neodvisni od proteina Bid (Houseweart in sod., 2003).

Za citosolni katepsin D (aspartatna proteaza) so si podatki o možnosti cepitve Bid nasprotujoči. Nekateri avtorji so pokazali cepitev (Heinrich in sod., 2004), drugi ne (Cirman in sod., 2004). V celicah tretiranih s staurosporinom lahko katepsin D, ne pa tudi katepsina B in L, sproži aktivacijo Bax in njegovo premestitev na zunanjo mitohondrijsko membrano (Bidere in sod., 2003). Prekomerno izražanje katepsina D sproži apoptozo v celicah HeLa (Deiss in sod., 1996), inhibicija katepsina D s pepstatinom A pa inhibira apoptozo sproženo s kisikom (″oxygen-induced apoptosis″) (Roberg in sod., 2002).

Poleg cepitve proteinov Bid in Bax lahko katepsini *in vitro* razgrajujejo še antiapoptotske proteine iz družine Bcl-2 (Bcl-2, Bcl-xL, Mcl-1) in tako vplivajo na destabilizacijo mitohondrijske membrane (Blomgran in sod., 2007; Droga-Mazovec in sod., 2008).

Vse do sedaj omenjene študije pripisujejo pomen katepsinov pri sprožanju mitohondrijske poti apoptoze in aktivaciji kaspaz 3, 7 in 9. Katepsini pa lahko sodelujejo tudi v signalni poti neodvniso od poškodb mitohondrijske membrane. Pri apoptozi inducirani z LLOMe so pokazali katepsinsko cepitev proteina XIAP (Droga-Mazovec in sod., 2008). Prav tako so pri tako sproženi apoptozi katepsini razgradili membransko vezane gvanilatne kinaze Dlg-1, ZO-1 in ZO-3 (Ivanova in sod., 2011), ki regulirajo celične stike.

Tudi same kaspaze so lahko tarče katepsinov. Katepsini lahko neposredno cepijo in aktivirajo kaspaze. Katepsin B lahko cepi kaspazi 1 in 11 (Schotte in sod., 1998). Ostalih kaspaz, ki so vpletene v apoptotsko pot, katepsini B, H, L, K, L, S in X ne cepijo (Stoka in sod., 2001), med apoptozo v nevtrofilcih pa je katepsin D aktiviral kaspazo 8 (Conus in sod., 2008).

Vse omenjene raziskave pripisujejo pomembno vlogo katepsinov pri apoptozi. Na drugi strani pa tudi kar nekaj študij temu nasprotuje. Uporaba celic z izbitimi geni za katepsine je le znižala apoptozo, ni pa je preprečila (Foghsgaard in sod., 2001; Guicciardi in sod., 2000; Nagaraj in sod., 2006; Werneburg in sod., 2007). Spremljanje časovnega poteka dogajanj v apoptozi je tudi ponudilo dokaze, da se lizosomi destabilizirajo kasneje kot mitohondriji (Bojic in sod., 2007; Oberle in sod., 2010), kar tudi ne govori v prid teorijam o ključni vlogi katepsinov. Inhibicija katepsinov ni znatno znižala apoptoze sprožene z ligandi TNF-α ali Fas pri, celicah U-937 in T98G (Klaric in sod., 2009), primarnih kožnih fibroblastih (Bojic in sod., 2007), celicah Jurkat in hepatocitih (Wattiaux in sod., 2007) ali mišjih embrionalnih fibroblastih (Oberle in sod., 2010).

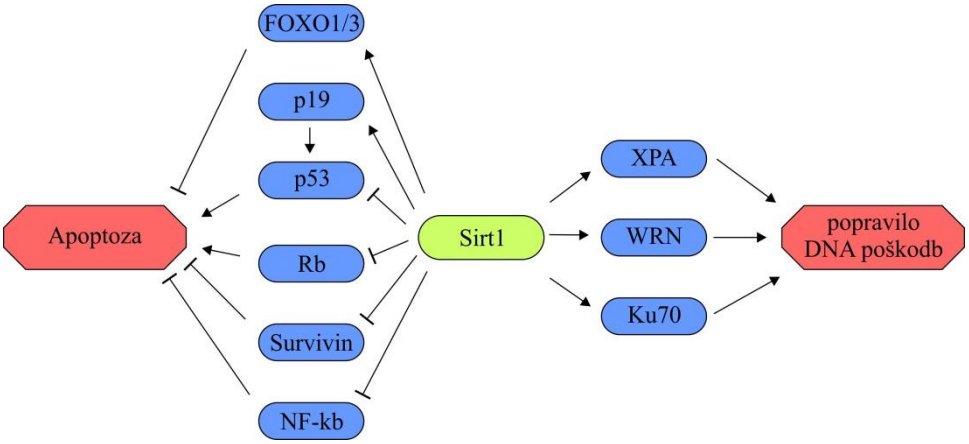
1.6 Sirtuini

Sirtuini so od NAD^+ (″nicotinamide adenine dinucleotide″) odvisne deacetilaze. Njihova glavna naloga naj bi bila zaznava sprememb redoks stanja celice, ki so posledice stresa. Za delovanje potrebujejo kofaktor NAD^+, najdemo pa jih tako v jedru, v citoplazmi in v mitohondrijih (Bosch-Presegue in Vaquero, 2011).

Sirtuinom so največ pozornosti posvečali zaradi študij, ki so povezovale funkcijo sirtuina Sir2 (″silent information regulator 2″) z dolžino življenja. Povečano izražanje sirtuinov pri kvasovkah je podaljšalo življenjsko dobo, sirtuinom podobni proteini pa so podaljšali življenjsko dobo nematodov (Tissenbaum in Guarente, 2001). Pri sesalcih so odkrili sedem homologov kvasnega sirtuina Sir2. Sirt 1 in 2 se nahajata v jedru in citosolu, Sirt 3, 4 in 5 so mitohondrijski proteini, Sirt 6 in 7 pa sta izražena le v jedru (Haigis in Guarente, 2006).

Glede na delovanje lahko sirtuine razdelimo v štiri skupine. Na sirtuine z vlogo pri regulaciji kromatina, stresa in celičnega preživetja, regulacije homeostaze ter celične diferenciacije. Pri odzivu na stres sirtuini z regulacijo različnih preživetvenih poti omogočajo organizmu prilagoditev na oksidativni, metabolni ali genotoksični stres. Z deacetilacijo proteinov p53, Rb, survivina, NF-kβ in transkripcijskih faktorjev uravnavajo celični odziv na stres in celici omogočijo preživetje ali sprožijo apoptozo (Bosch-Presegue in Vaquero, 2011).

Najbolj proučevan človeški sirtuin je protein Sirt1. Njegove tarče so transkripcijski faktorji in kofaktorji, jedrni receptorji, p53 in HSP1 in PARP1, pomembno vlogo pa ima pri boleznih srca in ožilja, neurodegenerativnih boleznih, raku in staranju (Guarente, 2011). Kadar je celica izpostavljena zmernemu stresu ali poškodbam DNA, Sirt1 pripomore k ustavitvi celičnega cikla, popravilu poškodb DNA in posledično preživetju celice. Če pa je celica izpostavljena kroničnemu stresu ali hudim poškodbam DNA, takrat Sirt1 inducira senescenco ali apoptozo (Slika 9) (Bosch-Presegue in Vaquero, 2011).



Slika 9: *Dvojno delovanja sirtuina 1* (Bosch-Presegue in Vaquero, 2011)

Med bolj pogostimi posledicami stresa je tudi senescenca in med njo prihaja do poškodb lizosomov, porušitev gradienta pH znotraj lizosoma in permeabilizacije lizosomske membrane. Pri tem lahko katepsini prehajajo v citosol, razgradijo sirtuine in tako vplivajo na regulacijo preživetja celice (Patschan in sod., 2008a).

2 Namen dela

Apoptoza je dobro organizirana oblika celične smrti, pomembno vlogo igra pri homeostazi in razvoju večceličnih organizmov. Lahko se sproži preko intrinzične (notranje) poti, ki vključuje poškodbe mitohondrijev, ter ekstrinzične (zunanje) poti preko receptorjev smrti na površini celic. Med temi receptorji, ki sodijo v superdružino TNRF (″tumor necrosis factor receptor″), so najbolj znani TNF R1, CD95 (APO-1, Fas) in TRAIL receptor. Liganda TNF-α in Fas sta zelo učinkovita pri ubijanju tumorskih celic, a ima sistemska terapija negativne stranske učinke, saj TNF-α povzroči huda vnetja, ligand Fas ligand pa deluje tudi na zdrave celice.

Pri apoptozi, ki jo sproži ligand TRAIL, se trenutno predpostavlja, da je permeabilizacija lizosomov zgodnji dogodek. Sproščeni katepsini, predvsem katepsin B, lahko cepijo Bid, nastali tBid pa omogoči sprostitev citokroma c iz mitohondrijev, kar vodi do aktivacije kaspaz 3 in 7 in posledično v apoptozo. Nedavno so za liganda Fas in TNF-α pokazali, da poškodba lizosomov nastane šele za poškodbo mitohondrijev. Če podobno velja tudi za apoptozo, ki jo sproži ligand TRAIL, bi to pomenilo nov pogled na vlogo lizosomskih cisteinskih katepsinov pri apoptozi. Poznavanje mehanizma apoptoze je pomemben dejavnik pri načrtovanju zdravljenja rakavih obolenj.

Namen doktorskega dela je bil najprej ugotoviti časovno zaporedje poškodb lizosomov in mitohondrijev pri apoptozi sproženi z ligandom TRAIL v mišjih embrionalnih fibroblastih. V nadaljevanju smo želeli raziskati povezavo med destabilizacijo mitohondrijske in lizosomske membrane pri apoptozi sproženi z ligandom TRAIL in morebitno vlogo katepsinov v tem mehanizmu. Z uporabo inhibitorjev katepsinov pa smo želeli ugotoviti tudi vlogo katepsinov v humanih celičnih linijah.

3 Materiali in metode

3.1 Materiali

3.1.1 Kemikalije

- 0,25 % tripsin (Gibco)
- Ac-DEVD-AFC (N-acetil- aspartat-glutamat-valin-aspartat -7-amino-4-trifluorometilkumarin; Bachem)
- Akridin oranž (Sigma)
- Akrilamid-N,N-metilenenbisakrilamid (Serva)
- Annexin-V-PE kit (BD Pharmigen)
- APS (amonijev persulfat; Serva)
- Bortezomib (Selleck Chemicals)
- Ca-074Me ([L-3-trans-(propilkarbamoil)oksiran-2-karbonil]-L-izoleucin-L-prolin metilni ester; Peptide)
- CHAPS (3-[(3-kolamidopropil)dimetilamonijev]-1-propransulfonat; Fluka)
- Cikloheksimid (Sigma)
- CM-H_2DCFDA (5-(in-6)-klorometil-2',7'-diklorodihidrofluorescin diacetat acetil ester, Invitrogen)
- DFO (deferoksamin mesilat; Sigma)
- DMSO (dimetilsulfoksid; Merck)
- DTT (ditiotreitol; Sigma)
- E-64 (1-L-trans-epoksisukcinil-levcilamido-3-(4-gvanidino) butan; Peptide)
- E-64d (L-trans-epoksisukcinil-(OEt)-leu-3-metilbutilamid; Peptide)
- Fetalni goveji serum FBS (Atlanta Biologicals)
- Glutamin (Sigma)
- Goveji serumski albumin (New England Biolabs)
- Mešanica inhibitorjev proteaz (Sigma)
- Mešanica penicilina in streptomicina (Sigma)
- MitoTracker CMX-Ros (Invitrogen)
- NaDS (natrijev dodecil sulfat; Serva)
- NAO – acridine orange 10-N-nonyl bromide (Invitrogen)
- Označevalec velikosti proteinov: PageRuler Prestained Protein Ladder (MBI Fermentas)
- Posneto mleko v prahu (Pomurske mlekarne)
- Propidijev jodid (Sigma)
- Reagent ECL za kemiluminiscenco (Amersham Pharmacia Biotech)
- Reagent za določanje koncentracije proteinov (Biorad)
- TEMED (N,N,N', N'-tetrametiletilendiamin; Merck)
- TEMPOL (4-hidroksi-2,2,6,6-tetrametilpiperdin 1-oksil; Sigma)

- TRAIL (dejavniku tumorske nekroze (TNF) soroden ligand, ki sproža apoptozo; prof. dr. Henning Walczak, imperial College London, Velika Britanija)
- Tripsin (TrypLE Select) Gibco
- Tris (Serva)
- Tween-20 (Serva)
- Z-Phe-Arg-AMC (N-benziloksikarbonil-L-fenilalanin-L-arginil-4-metil-7-kumarilamid; Bachem)
- Z-VAD-FMK (karbobenzoksi-valil-alanil-aspartil-[O-metil]-fluorometilketon; Bachem)

3.1.2 Protitelesa

- Primarna kozja poliklonska protitelesa proti proteinu BID (R&D Systems)
- Primarna kozja poliklonska protitelesa proti proteinu GST (GE Healthcare)
- Primarna kunčja monoklonska protitelesa proti kaspazi-3 (Cell Signaling)
- Primarna kunčja poliklonska protitelesa proti citokromu C (Cell Signaling)
- Primarna kunčja poliklonska protitelesa proti proti Smac/Diablo (Abcam)
- Primarna mišja monoklonska protitelesa proti aktinu (Sigma)
- Sekundarna kunčja antimišja, kozja antikunčja in oslovska antikozja protitelesa, konjugirana s hrenovo peroksidazo (Abcam)

3.1.3 Gojišča

- Gojišče DMEM z visoko vsebnostjo glukoze in L-glutaminom (PAA, Gibco) obogateno z 10 % FBS, 1 % glutaminom in 1% streptomicinom/penicilinom
- Gojišče RPMI 1640 z L-glutaminom in s HEPES-om (PAA) obogateno z 10 % FBS-HI, 1 % glutaminom in 1% streptomicinom/penicilinom

3.1.4 Celice

- HeLa – celice tumorja materničnega vratu
- HuH-7 – celice hepatocitnega tumorja
- U-937 – celice levkemijskega monocitnega limfoma
- Jurkat – imortalizirane celice limfocitov T
- MEFs – mišji embrionalni fibroblasti

3.1.5 Pufri

Elektroforezni pufer za NaDS-PAGE
25 mM Tris
192 mM L-glicin
0,1 % NaDS

Kaspazni pufer (2X)
100 mM Tris
10 mM DTT
0,1 % CHAPS
10% saharoza
pH 7,4

Nanašalni pufer za NaDS-PAGE (6x)
300 mM Tris
100 mM NaDS
40 mM EDTA
1,2 M DTT
60 % (v/v) glicerol
0,3 % (w/v) bromfenol modro

Prenašalni pufer
25 mM Tris
192 mM L-glicin
20 % metanol

Pufer BANA
15 mM Na_2HPO_4
88 mM KH_2PO_4
1 mM EDTA
0,1 % PEG-6000
pH 6,0

Pufer HEPES
50 mM HEPES
0,25 mM NaCl
0,1 % (v/v) NP-40
pH 7,0

pufer PBS
145 mM NaCl
2,5 mM NaH_2PO_4 x $2H_2O$
7,5 mM Na_2HPO_4 x $2H_2O$
pH 7,4

pufer RIPA
50 mM tris
100 mM NaCl
0,1 % (w/v) NaDS
1 % (v/v) NP-40
0,5 % (w/v) deoksilonska kislina
1 mM EDTA

pufer TBS(T)
20 mM Tris
500 mM NaCl
(0,5 % (v/v) Tween-20)

Pufer z digitoninom
70 μg/ml digitonin
250 mM saharoza
20 mM HEPES
10 mM KCl
1 mM $MgCl_2$
1 mM EDTA
1 mM EGTA
pH 7,5

Pufer za barvanje
0,5 % barvilo Coomassie Blue R-250
50 % metanol
10 % ocetna kislina

Pufer za ekstrakcijo
70 μg/ml digitonin
250 mM saharoza
20 mM HEPES
10 mM KCl
1 mM $MgCl_2$
1 mM EDTA
1 mM EGTA
pH 7,5

Pufer za razbarvanje
50 % metanol
10 % ocetna kislina

3.1.6 Laboratorijska oprema

- Aparatura za prenos western P8DS Penguin (Owl Separation Systems)
- Avtomatska temnica SRX-101A (Konica Minolta)
- Avtomatske pipete (Gilson)
- Centrifuge Eppendorf 5402, 5410, 5417R, 5415R, 5810R
- Centrifugirke (15 ml in 50 ml)
- Filmi Biomax Light fiml (Kodak)
- Fluorimeter za mikrotitrske plošče Safire (Tecan)
- Inkubator za celične linije (Binder)
- Invertni fluorescenčni mikroskop IX71 (Olympus)
- Kaseta za razvijanje filmov X-omatic regular screens (Kodak)
- Komplet za elektroforezo v prisotnosti NaDS (Biorad)
- Laminar (Iskra PIO)
- Magnetna mešala MM-530 (Tehtnica)
- Nitrocelulozna membrana NC45 (Sigma)
- Oprema za delo s celicami (pipete, plošče za gojenje, centrifugirke…) (TPP, Corning, Greiner)
- pH-meter (Metrel)

- Pretočni citometer FACSCalibur (Becton Dickinson)
  Ekscitacijska svetloba: 488 nm
  Emisijska svetloba, filtri : FL1: 515 – 545 nm
  FL2: 564 – 606 nm
  FL3: > 670 nm
- Vakumska črpalka (ABM)
- Vodna kopel RM6 (Lauda)

3.2 Metode

3.2.1 Priprava primarnih celic mišjih embrionalnih fibroblastov

Primarne celice smo pripravili v sterilni pogojih v laminarju. Potreben material (škarje, pincete, nože, steklene kroglice, magnetna mešala in steklene čaše) smo predhodno sterilizirali (suho avtoklaviranje 2 h pri 180 °C) oziroma smo uporabili sterilen material (petrijevke, pipete, pufri, gojišča). Miši breje 12,5 do 13,5 dni smo uspavali s CO_2. Prerezali smo kožo in potrebušnico ter iz maternice prenesli zarodke v pufer PBS. Odstranili smo jim glavo ter srce in jetra (rdeča organa). Zarodke smo razrezali in prenesli v (po 2 ali 3) stekleno čašo s steklenimi kroglicami in magnetnim mešalom. Dodali smo 10 ml 0,25 % tripsina in z mešanjem inkubirali 15 minut pri temperaturi 37 °C. Tripsiniziranje smo ustavili z dodatkom 10 ml medija DMEM, vsebino čaše prenesli v 50-mililitrsko cetrifugirko in centrifugirali 5 minut pri 1500 rpm. Medij smo odstranili, usedlino (celice) pa resuspendirali v 10 ml svežega medija in prenesli na 10-centimetersko ploščo ter gojili v inkubatorju s 5 % CO_2 pri 37 °C. Naslednji dan smo zamenjali medij, celice pa gojili toliko časa, da so dosegle 90 % konfluentnost. Nato smo jih precepili na 15-centimetrsko ploščo in po doseženi konfluentnosti zamrznili celice v štiri vijale za zamrzovanje.

3.2.2 Metode dela s celicami

3.2.2.1 Gojenje celic

Celice smo gojili v inkubatorju v kontrolirani atmosferi (37 °C, 5 % CO_2 in atmosfera nasičena z vodno paro). Za mišje celice smo uporabljali medij DMEM (Gibco) obogaten z 20 % FBS, 1 % glutaminom in 1 % streptomicinom/penicilinom. Celice HeLa in HuH-7 smo gojili v mediju DMEM (Gibco) obogatenim z 10 % FBS, 1 % glutaminom in 1 % streptomicinom/penicilinom, celice U-937 in Jurkat pa v mediju RPMI 1640 obogatenim z 10 % FBS-HI, 1 % glutaminom in 1 % streptomicinom/penicilinom. Mišje celice smo uporabljali do 4. pasaže, humane pa do 10. pasaže.

3.2.2.2 Tripsinizacija in precepljanje celic

Pri precepljanju celic smo najprej odsesali medij, sprali celice z 10 ml pufra PBS in na celice dodali raztopino TrypLE Select (Gibco). Količina TrypLE Select je bila odvisna od velikosti plošče, na kateri smo gojili celice (v 10-centimetrsko ploščo smo dodali 1 ml, v eno vdolbino plošče s 6 vdolbinicami 400 μl, v eno vdolbinico plošče z 12 vdolbinicami pa 200 μl). Celice z raztopino TrypLE Select smo inkubirali v CO_2 inkubatorju pri 37 °C dokler se celice niso odlepile, nato smo jih dodali kDMEM za inaktivacijo tripsina (v razmerju 1:10) in jih nacepili za poskus.

3.2.2.3 Zamrzovanje celic

Celice, ki jih nismo potrebovali v poskusu ali za nadaljne precepljanje smo zamrznili. Po tripsinizaciji smo celice prešteli, centrifugirali 5 minut pri 1300 rpm, odstranili supernatant, celice resuspendirali v mediju za zamrzovanje (90 % FBS, 10 % DMSO) in jih po 10^7 celic/ml alikvotirali v zamrzovalne vijale. V posodi, ki omogoča ohlajanje 1 °C/ na minuto, smo jih zamrznili pri -80 °C.

3.2.2.4 Odmrzovanje celic

Vijale s celicami smo iz tekočega dušika ali zamrzovalnika pri -80 °C odtajali v vodni kopeli pri 37 °C. Odtajane celice smo prenesli v 10 ml gojitvenega medija, centrifugirali 5 minut pri 1300 rpm, medij odstranili, celice pa resuspendirali v 10 ml novega gojitvenega medija in prenesli na ploščo.

3.2.3 Sprožitev apoptoze z ligandom TRAIL

3.2.3.1 Določitev optimalne koncentracije TRAIL

Celice smo nacepili v ploščo s 6 vdolbinicami, tako, da je bilo v vsaki vdolbinici $1x10^6$ celic (U-937, Jurkat) oziroma $1x10^5$ celic (HeLa, HuH-7, MEFs). Celice HeLa, HuH-7 in MEFs smo inkubirali preko noči v inkubatorju CO_2 pri 37 °C, da so se pritrdile. Po menjavi medija smo vanj dodali ligand TRAIL in CHX ali ligand TRAIL in bortezomib v različnih koncentracijah ter po 16 h (MEFs) oziroma po 24 h (ostale celice) določili delež apoptoze.

3.2.3.2 Sprožitev apoptoze

Celicam smo dodali inhibitorje cisteinskih katepsinov E-64d in CA-074Me, inhibitor kaspaz Z-VAD-FMK (vse v končni koncetraciji 10 μM), odstranjevalec TEMPOL in kelator železovih ionov DFO (oba v končni koncetraciji 7 mM). Po 2 urni inkubaciji smo sprožili apoptozo z ligandom TRAIL in CHX ali ligandom TRAIL in bortezomibom in inkubirali nadaljnjih 16 do 24 ur v inkubatorju s 5 % CO_2 pri 37 °C.

3.2.3.3 Določanje deleža apoptotskih celic

Pri zgodnjih fazah apoptoze se membranski fosfolipid fosfatidilserin prenese na zunanjo stran membrane, kar lahko zasledimo z uporabo Annexin-V-PE. Annexin V se veže na fosfatidilserin, fikoeritrin (PE) pa je fluorokrom, ki nam omogoča detekcijo. Med kasnejšimi fazami apoptoze prihaja do kondenzacije in fragmentacije DNA. Propidijev jodid (PI) je interkelator, ki se veže na DNA in nam omogoča detekcijo pozno-apoptotskih oziroma nekrotskih celic. S kombinirano uporabo Annexin –V-PE in PI lahko določamo deleže zgodnje in pozno apoptotskih celic.

Celice smo tripsinizirali (z izjemo suspenzijskih celic U-937 in Jurkat, kjer to ni potrebno) in centrifugirali 5 minut pri 1300 rpm. Medij smo odsesali, celice pa resuspendirali v 1 ml pufra PBS, prešteli in ponovno centrifugirali 5 minut pri 1300 rpm. Odsesali smo pufer ter še drugič sprali celice s pufrom PBS. 10^6 celic/ml smo resuspendirali v 1 ml pufra za vezavo konjugata Annexina-V-PE. Suspenzijo (100 μl) smo prenesli v tubico za pretočno citometrijo, dodali 2 μl konjugata Annexina-V-PE in inkubirali v temi. Po 15 minutah smo dodali še 300 μl pufra za vezavo konjugiranega Annexina-V-PE, 2 μl PI ter pomerili fluorescenco označenega vzorca s pretočnim citometrom.

3.2.3.4 Določitev deleža celic s poškodovanimi mitohondriji

Mitohondrije smo označevali s kemikalijo MitoTracker CMX-Ros. Ta v reducirani obliki difundira preko plazemske membrane in vstopi v mitohodrije. V metabolno aktivnih mitohodrijih se oksidira, pri tem pa se spremeni iz nefluorescentne v fluorescetno obliko. Celice s poškodovanimi mitohodriji tako kažejo zmanjšano intenziteto rdeče fluorescence v primerjavi s celicami z nepoškodovanimi mitohondriji.

Celice smo tripsinizirali (z izjemo suspenzijskih celic U-937 in Jurkat, kjer to ni potrebno) in centrifugirali 5 minut pri 1300 rpm. Medij smo odsesali, celice pa resuspendirali v 1 ml pufra PBS, prešteli in ponovno centrifugirali 5 minut pri 1300 rpm. Odsesali smo pufer ter še drugič sprali celice s pufrom PBS. 10^6 celic/ml smo resuspendirali v 1 ml gojišča DMEM s končno koncetracijo barvila MitoTracker CMX-Ros 50 nM. Celice smo inkubirali 30 minut pri 37 °C, nato smo jih centrifugirali 5 minut pri 1300 rpm, odsesali medij in resuspendirali v 1 ml pufra PBS. Na pretočnem citometru smo pomerili rdečo fluorescenco (filter FL3).

3.2.3.5 Določitev deleža celic s poškodovanimi lizosomi

Delež celic s poškodovanimi lizosomi smo določali z uporabo kemikalije akridin oranž (AO). AO je lizosomotrop, ki se kopiči v kislih organelih in oddaja fluorescenco rdeče barve. Pri celicah s poškodovanimi lizosomi se v lizosomih nakopiči manj AO, zato je intenziteta fluorescence nižja.

Celice smo tripsinizirali (z izjemo suspenzijskih celic U-937 in Jurkat, kjer to ni potrebno) in centrifugirali 5 minut pri 1300 rpm. Medij smo odsesali, celice pa resuspendirali v 1 ml pufra PBS, prešteli in ponovno centrifugirali 5 minut pri 1300 rpm. Odsesali smo pufer ter še drugič sprali celice s pufrom PBS. 10^6 celic/ml smo resuspendirali v 1 ml gojišča DMEM s končno koncetracijo AO 5 μg/ml. Celice smo inkubirali 15 minut pri 37 °C, nato smo jih centrifugirali 5 minut pri 1300 rpm, odsesali medij in resuspendirali v 1 ml pufra PBS. Na pretočnem citometru smo pomerili rdečo fluorescenco (filter FL3).

3.2.3.6 Spremljanje nastanka reaktivnih kisikovih zvrsti

Nastanek reaktivnih kisikovih zvrsti (ROS) smo spremljali z indikatorjem CM-H_2DCFDA. Ta prehaja v celico v reducirani obliki, ki je nefluorescentna. Zaradi deacetilacije indikatorja s celičnimi esterazami ta ne more iz celice, oksidacija pa spremeni indikator v fluorescirajočo molekulo.

Indikator smo dodali celicam 30 minut pred dodatkom liganda TRAIL in eno uro pred koncem poskusa, tako da je bila njegova koncentracija 5 μM. Po koncu poskusa smo celice tripsinizirali, centrifugirali 5 minut pri 1300 rpm, odstranili medij in celice resuspendirali v 1 ml PBS. Na pretočnem citometru smo pomerili zeleno fluorescenco (filter FL1).

3.2.3.7 Spremljanje oksidacije kardiolipina

Oksidacijo kardiolipina smo spremljali z indikatorjem 10-N-nonil akridin oranžem (″10-N-nonyl-acridine orange″ – NAO). NAO se veže le na neoksidirano obliko kardiolipina in oddaja fluorescenco rdeče barve. Oksidirana oblika kardiolipina ne veže NAO in zato je intenziteta oddane fluorescence pri tej obliki nižja.

NAO smo dodali v medij v koncentraciji 50 nM in inkubirali 30 minut pri 37 °C. Nato smo celice tripsinizirali, resuspendirali v mediju, centrifugirali 5 minut pri 350g in 4 °C, sprali s PBS, ponovno centrifugirali in resusupendirali v PBS. Na pretočnem citometru smo pomerili rdečo fluorescenco (filter FL3).

3.2.3.8 Priprava celotnega celičnega lizata

Celice smo (če so bile pritrjene najprej tripsinizirali in) centrifugirali 5 minut pri 3000 rpm. Odstranili smo medij ter celice dvakrat sprali s hladnim pufrom PBS. Za določanje katepsinske aktivnosti smo nato celice resuspendirali v 50 µl pufra HEPES, za določanje kaspazne aktivnosti ter imunodetekcijo pa v 50 µl pufra RIPA. Na ledu smo inkubirali 10 minut, nato smo celice centrifugirali 10 minut pri 14000 rpm in 4 °C, prenesli supernatant v novo epico in shranili pri -70 °C oziroma takoj uporabili v analizah.

3.2.3.9 Priprava citosolnega lizata

Celice smo po potrebi tripsinizirali in centrifugirali 5 minut pri 3000 rpm. Odstranili smo medij ter celice dvakrat sprali s hladnim pufrom PBS. Resuspendirali smo jih v 200 µl hladnega pufra za ekstrakcijo, za potrebe imunodetekcije pa smo dodali še mešanico inhibitorjev proteaz v razmerju 1:100 (v/v). Inkubirali smo na ledu 10 minut, pri tem pa smo vsako minuto 5 sekund vorteksirali. Celice smo centrifugirali 3 minute pri 3000 rpm, supernatant prenesli v novo epico in shranili pri -70 °C.

3.2.3.10 Določanje celotne koncetracije proteinov v vzorcu

Vsebnost proteinov v celičnih lizatih smo določili z metodo po Bradfordu. Poskus smo naredili na ploščah s 96 vdolbinicami, vrednosti absorbance pa odčitali s fluorimetrom za mikrotitrske plošče. Najprej smo pripravili umeritveno krivuljo z BSA (0-100 µg/ml). 160 µl ustrezne redčitve BSA smo dodali 40 µl Bradforovega reagenta, premešali in izmerili absorbanco pri 595 nm. Vzorce smo redčili v razmerju 1:100-1:500, od tega 160 µl zmešali s 40 µl Bradfordovega reaganta ter izmerili absorbanco. Koncentracijo proteinov v vzorcu smo nato določili iz umeritvene krivulje.

3.2.3.11 Merjenje encimske aktivnosti

Aktivnost izbranih encimov smo določali z uporabo različnih sintetičnih fluorogenih substratov. Ustrezen volumen vzorca (50 µg proteinov) smo odpipetirali v črno mikrotitrsko ploščo ter dodali do 40 µl dH_2O. Nato smo dodali še 50 µl ustreznega pufra ter inkubirali vzorce 10 minut pri 37 °C, dodali 10 µl izbranega substrata tako da je bila končna koncentracija substrata 20 µM in izmerili fluorescenco. Sprememba fluorescence v časovni enoti (naklon) je sorazmerna encimski aktivnosti.

Tabela 5: *Substrati in njihove značilnosti za določanje encimske aktivnosti*

Encim	Pufer	Substrat	Vzbujevalna svetloba	Emisijska svetloba
Kaspaza-3	kaspazni pufer	Ac-DEVD-AFC	400 nm	505 nm
Katepsini	Fosfatni pufer pH 6,0	Z-Phe-Arg-AMC	370 nm	460 nm

3.2.3.12 Poliakrilamidna gelska elektroforeza v prisotnosti NaDS

Poliakrilamidno gelsko elektroforezo z dodanim denaturantom natrijevim dodecilsulfatom (NaDS-PAGE) smo uporabili pod redukcijskimi pogoji (v prisotnosti DTT) za ločevanje proteinov iz celičnih lizatov. Poliakrilamidni gel je sestavljen iz ločevalnega in koncentracijskega dela. Vzorcem smo pred nanosom na gel dodali nanašalni pufer z NaDS, DTT ter jih termično obdelali (5-minutna inkubacija pri 100 °C). Za referenčne velikosti proteinov smo nanesli proteinske standarde. Elektroforeza je potekala v elektroforeznem pufru v aparaturi za elektroforezo pri konstantnem toku 30 mA/gel. Za obarvanje vseh proteinov v vzorcih smo uporabili 0,2 % raztopino Coomassie Brilliant Blue, za potrebe imunodetekcije pa smo uporabili prenos western.

Tabela 6: *Sestava poliakrilamidnih gelov*

Komponenta	Ločevalni gel		Koncentracijski gel
	12,5 %	15 %	5 %
40 % akrilamid-bisakrilamid (37,5:1)	3,13 ml	3,75 ml	1,25 ml
4x separacijski pufer (1,5 M Tris, pH 8,8)	2,5 ml	2,5 ml	/
4x koncentracijski pufer (0,5 M Tris, pH 6,8)	/	/	2,5 ml
dH2O	4,27 ml	3,65 ml	6,15 ml
10 % NaDS	100 µl	100 µl	100 µl
TEMED	15 µl	15 µl	15 µl
10 % APS	30 µl	30 µl	30 µl

3.2.3.13 Detekcija proteinov s prenosom western

Prenos western je metoda prenosa proteinov iz poliakrilamidnega gela na nitrocelulozno membrano s pomočjo električnega toka. Prenos je potekal v aparaturi za prenos western dve uri pri konstantnem toku 200 mA. Prosta mesta na membrani smo blokirali z enourno inkubacijo v blokirni raztopini (5 % posneto mleko v prahu v pufru TBS-T) pri sobni temperaturi z rahlim mešanjem. Membrano smo nato inkubirali s primarnimi protitelesi preko noči pri 4 °C. Spiranje nevezanih protiteles je potekalo pri sobni temperaturi 1 uro z rednim menjavanjem raztopine za spiranje TBS-T (vsakih 10 minut). Sledila je 1,5 urna inkubacija pri sobni temperaturi s sekundarnimi protitelesi. Ponovno smo spirali membrano 1 uro z rednim menjanjem raztopine za spiranje pri sobni temperaturi. Za detekcijo proteinov smo uporabili sistem ECL po navodilih proizvajalca.

3.2.4 Cepitve sirtuinov s cisteinskimi katepsini B, S in L

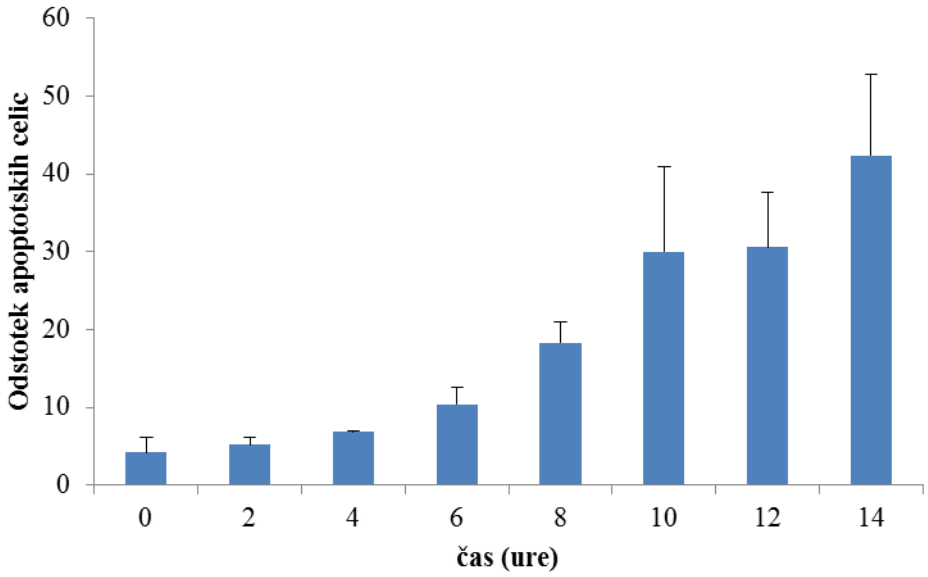
Analize smo opravljali z rekombinantnimi sirtuini Sirt1, Sirt2 in Sirt5, ki so imeli vezan označevalec GST. Za teste proteolitskih cepitev proteina sirtuin s cisteinskimi katepsini smo najprej aktivirali rekombinantne katepsine v pufru BANA pH 6,0 z dodatkom 1 mM DTT. Po 5 minutni inkubaciji pri 37 °C smo dodali sirtuin (v molskem razmerju sirtuin:katepsin=3:1) v pufru BANA pH 7,2. Reakcija je potekala 1 uro pri 37 °C nato pa smo jo ustavili z dodatkom 5 µl 1 M DTT in 5 µl nanašalnega pufra z NaDS ter inkubirali 10 minut pri 100 °C. Produkte reakcije smo analizirali z NaDS-PAGE in Coomassie barvanjem, posamezne proteine pa s prenosom western.

4 Rezultati

4.1 Sprožitev apoptoze z ligandom smrti TRAIL v celicah mišjih embrionalnih fibroblastov (MEFs)

4.1.1 Določitev časa inkubacije z ligandom TRAIL

Ligand TRAIL lahko sproži apoptozo v različnih primarnih celicah in celičnih linijah (Finnberg in sod., 2005; Nagaraj in sod., 2006). Pri našem delu smo uporabili mišje embrionalne fibroblaste (MEF), saj smo imeli na voljo tudi miši z izbitim genom za katepsin B. Najprej smo testirali ali lahko v primarnih celicah mišjih embrionalnih fibroblastov sprožimo apoptozo z ligandom TRAIL. Različne celice so različno občutljive na TRAIL, nekatere so celo neobčutljive in pri njih TRAIL ne more sprožiti apoptoze. Za senzibilizacijo celic na ligand TRAIL smo pri delu z MEFi uporabili inhibitor proteinske sinteze CHX. CHX prepreči aktivacijo preživetvene poti preko NF-κB in omogoči sprožitev apoptoze. Za izhodišče smo vzeli tretiranje z ligandom TNF-α, ki je na teh celicah sprožil zadostno apoptozo (Petelin, 2009). Celice smo tretirali z ligandom TRAIL v koncentraciji 100 ng/ml ob prisotnosti CHX 1 μg/ml 14 ur. Da bi ugotovili časovni potek apoptoze, smo meritve odstotka apoptotskih celic opravljali vsaki 2 uri (Slika 10).



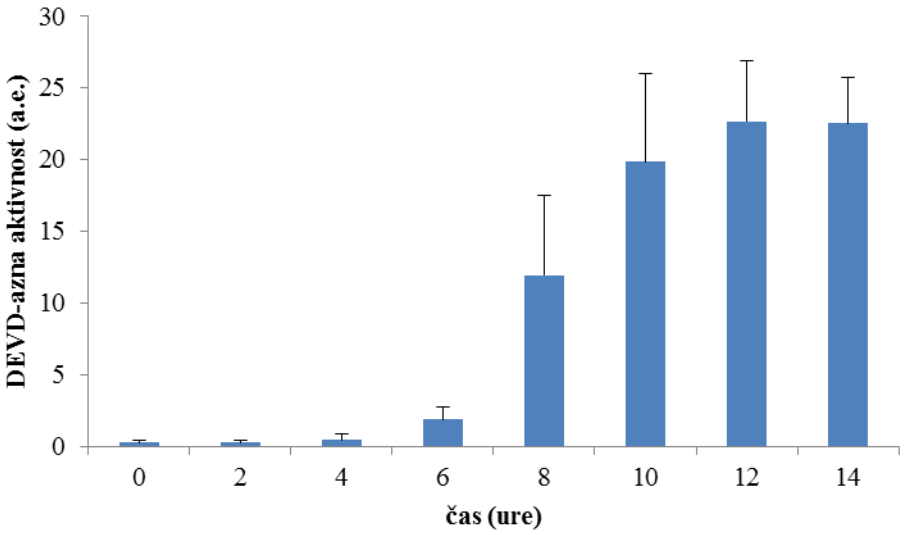
Slika 10: *Odstotek apoptotskih celic v mišjih embrionalnih fibroblastih divjega tipa po sprožitvi apoptoze z ligandom TRAIL*

Ugotovili smo, da so celice mišjih embrionalnih fibroblastov občutljive na ligand

TRAIL in da so primeren model za nadaljnje študije. Tekom eksperimenta je delež apoptotskih celic naraščal in po 14 urah dosegel 40 odstotkov (Slika 10).

4.1.2 DEVD-azna aktivnost

Da bi potrdili, da je pri mišjih embrionalnih fibroblastih apoptoza sprožena z ligandom TRAL odvisna od kaspaz, smo v dveurnih časovnih intervalih po dodatku liganda TRAIL pripravili celotne celične lizate in izmerili DEVD-azno aktivnost.

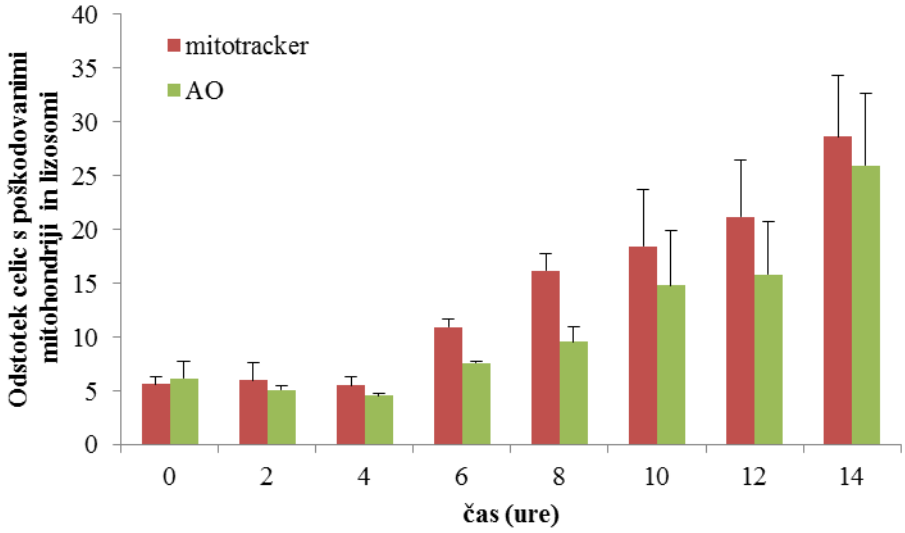


Slika 11: *DEVD-azna aktivnost v celotnih celičnih lizatih mišjih embrionalnih fibroblastov po sprožitvi apoptoze z ligandom TRAIL*

Kot vidimo na sliki 11, pride med potekom eksperimenta po dodatku liganad TRAIL do znatnega povečanja kaspazne aktivnosti. Kaspazna aktivnost se začne povečevati po 8 urah, kar nakazuje na od kaspaz odvisno celično smrt v izbranem modelu apoptoze.

4.1.3 Določitev deleža celic s poškodovanimi lizosomi in mitohondriji

Večina študij apoptoze sprožene po zunanji poti zagovarja, da naj bi najprej prišlo do poškodb lizosomov, šele nato pa do poškodb mitohondrijev, deloma tudi zaradi proapoptotskih faktorjev, ki se sprostijo iz lizosomov in pripomorejo k poškodbi mitohondrijske membrane. Preverili smo kateri organeli, mitohondriji ali lizosomi, se prej poškodujejo v izbranem modelu sprožitve apoptoze z ligandom TRAIL v mišjih embrionalnih fibroblastih. V ta namen smo spremljali stabilnost mitohondrijev in lizosomov v odvisnosti od časa. Specifična barvila se kopičijo v zdravih organelih in oddajajo fluorescenco. V poškodovanih organelih je količina barvil v njih manjša in posledično intenziteta oddane svetlobe nižja, tako da lahko z uporabo specifičnih barvil določimo odstotek celic s poškodovanimi mitohondriji in lizosomi.

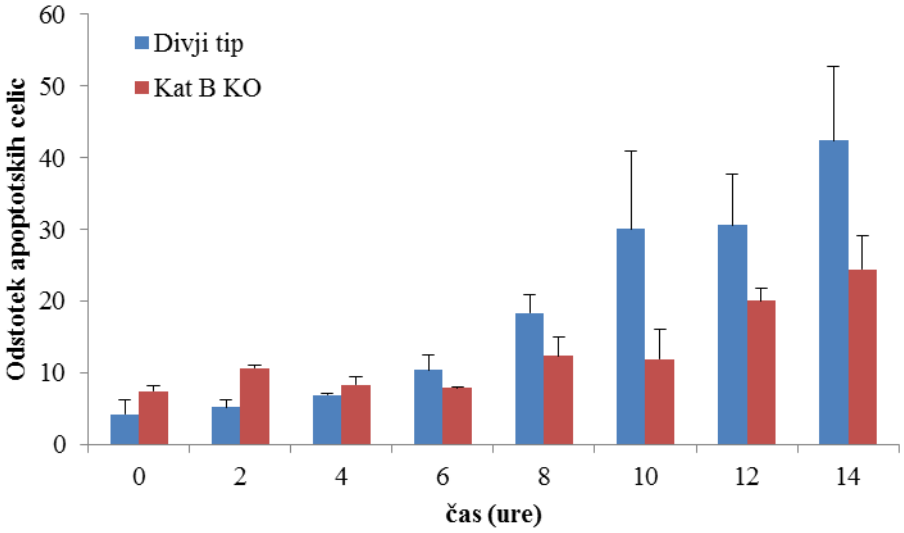


Slika 12: *Odstotek celic s poškodovanimi mitohondrij (mitotracker) in lizosomi (AO) v odvisnosti od časa po sprožitvi apoptoze z ligandom TRAIL*

Na sliki 12 vidimo, da je odstotek poškodovanih mitohondrijev in lizosomov do štirih ur po sprožitvi apoptoze z ligandom TRAIL na mišjih embrionalnih fibroblastih enak. Po šestih urah od sprožitve apoptoze pa je delež poškodovanih mitohondrijev v celicah večji od deleža poškodovanih lizosomov v celicah. Tako lahko sklepamo, da je v izbranem modelu najprej prišlo do poškodb mitohondrijev v celicah in šele nato do poškodb lizosomov.

4.1.4 Delež apoptotskih celic v celicah z izbitim genom za katepsin B

Pokazano je bilo, da imajo katepsini, še posebej katepsin B, pomembno vlogo pri apoptozi sproženi z ligandom TRAIL (Nagaraj in sod., 2006; Werneburg in sod., 2007). Do sedaj smo pokazali, da v izbranem modelu poškodba lizosomov ni primarni dogodek pri apoptozi sproženi z ligandom TRAIL. Zanimalo nas je, kakšno vlogo ima katepsin B, ki mu pripisujejo pomembno vlogo pri apoptozi v izbranem modelu. V ta namen smo z ligandom TRAIL sprožili apoptozo v mišjih embrionalnih fibroblastih z izbitom genom za katepsin B in spremljali časovni potek apoptoze.

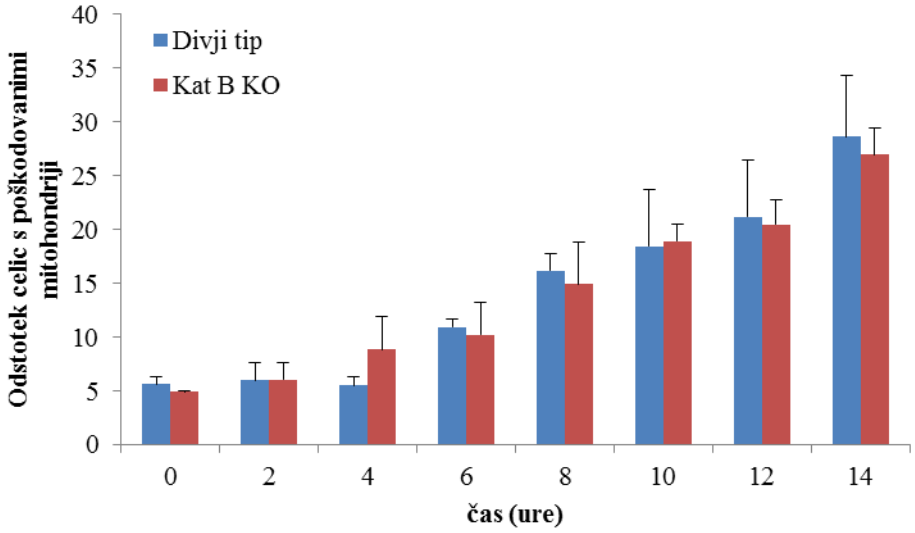


Slika 13: *Primerjava odstotka apoptotskih celic v mišjih embrionalnih fibroblastih divjega tipa (Divji tip) in mišjih embrionalnih fibroblastih z izbitim genom za katepsin B (Kat B KO) po sprožitvi apoptoze z ligandom TRAIL*

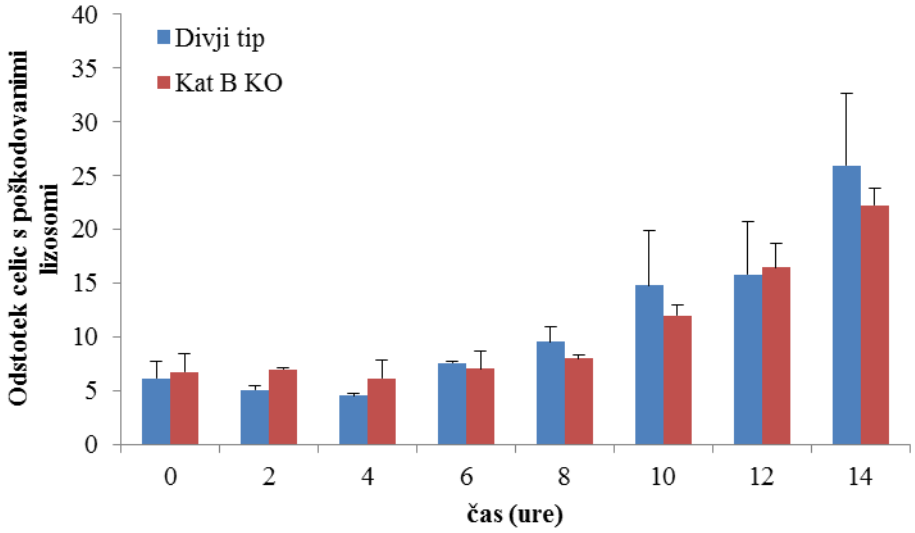
Tako kot v divjem tipu smo tudi v mišjih embrionalnih celicah z izbitim genom za katepsin B uspešno sprožili apoptozo. Vendar je pri celicah z izbitim genom za katepsin B delež apoptotskih celic manjši kot pri divjem tipu. V začetnih urah sta deleža primerljiva, proti koncu pa delež apoptotskih celic pri celicah z izbitim genom za katepsin B ne raste tako hitro kot delež apoptoze pri divjem tipu. Tako v celicah z izbitim genom za katepsin B delež apoptotskih celic po 14. urah doseže 25 % v primerjavi z 42 % deležem apoptotskih celic med celicami divjega tipa.

4.1.5 Celice s poškodovanimi mitohondriji in lizosomi v celicah z izbitim genom za katepsin B

Ugotovili smo, da odsotnost katepsina B pri mišjih celicah z izbitim genom za katepsin B vpliva na delež apoptoze. Da bi preverili, ali ima odsotnost katepsina B tudi vlogo pri poškodbah mitohondrijev in lizosomov, smo primerjali delež poškodovanih organelov pri celiceh divjega tipa in celicah z izbitom genom za katepsin B. S tem bi tudi ugotovili, na kateri stopnji sodeluje katepsin B v procesu apoptoze.

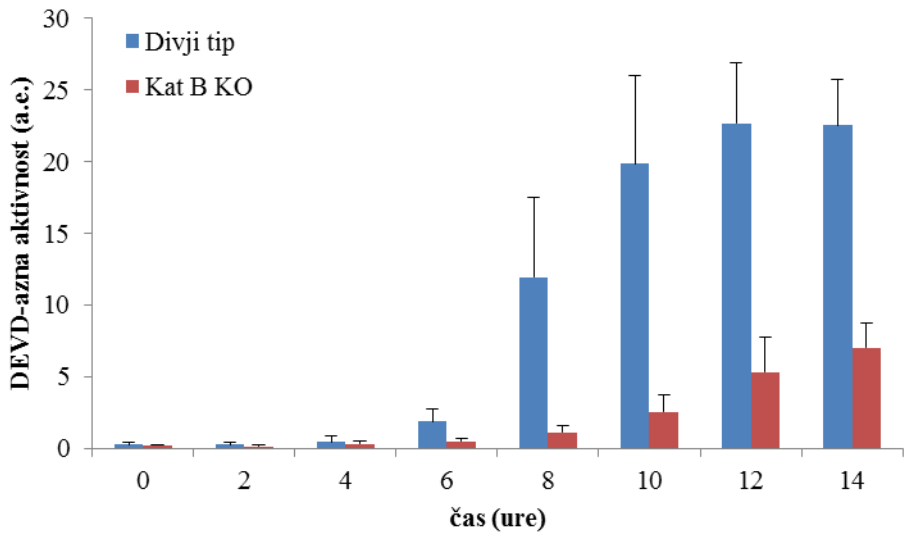


Slika 14: *Primerjava deleža poškodovanih mitohondrijev v celicah mišjih embrionalnih fibroblastih divjega tipa (Divji tip) in v mišjih embrionalnih fibroblastih z izbitim genom za katepsin B (Kat B KO) po sprožitvi apoptoze z ligandom TRAIL*



Slika 15: *Primerjava deleža poškodovanih lizosomov v celicah mišjih embrionalnih fibroblastih divjega tipa (Divji tip) in v mišjih embrionalnih fibroblastih z izbitim genom za katepsin B (Kat B KO) po sprožitvi apoptoze z ligandom TRAIL*

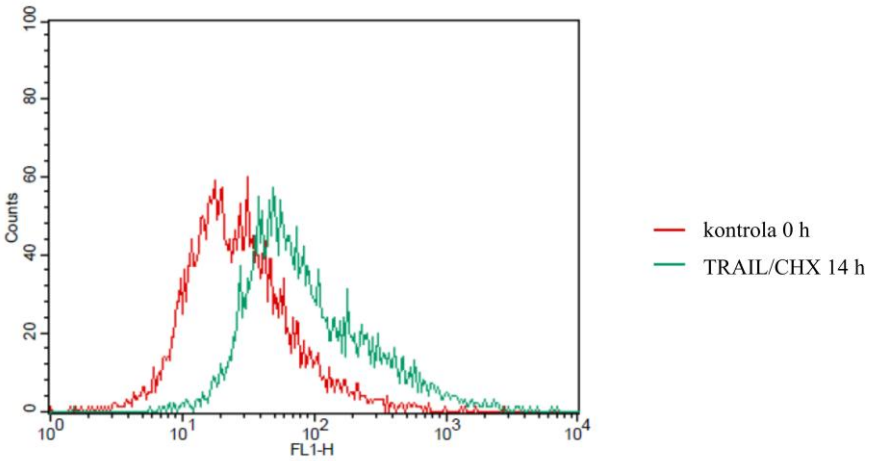
Stabilnost mitohondrijev in lizosomov je pri celicah divjega tipa in pri celicah z izbitim genom za katepsin B enaka. Pri obeh celicah prihaja do časovne odvisnosti poškodb mitohondrijev in lizosomov po sprožitvi apoptoze z ligandom TRAIL in za razliko od vpliva na apoptozo, kjer ima odsotnost katepsina B pomemben učinek (Slika 13), na stabilnost mitohondrijev (Slika 14) oziroma lizosomov (Slika 15) odsotnost lizosomskega encima nima vpliva. Se pa ob odsotnosti katepsina B zniža aktivnost kaspaz (Slika 16).



Slika 16: *Primerjava DEVD-azne aktivnosti v celicah mišjih embrionalnih fibroblastih divjega tipa (Divji tip) in v mišjih embrionalnih fibroblastih z izbitim genom za katepsin B (Kat B KO) po sprožitvi apoptoze z ligandom TRAIL*

4.1.6 Nastanek reaktivnih kisikovih zvrsti med apoptozo

V celicah z izbitim genom za katepsin B smo pokazali, da odsotnost katepsina B sicer vpliva na delež apoptotskih celic, ne vpliva pa na stabilnost mitohondrijske in lizosomske membrane. Zanimalo nas je, kateri dejavnik pri apoptozi sproženi z ligandom TRAIL povzroči destabilizacijo teh organelov. V literaturi se pri apoptozi sproženi z ligandom TNF-α kot možni kandidati omenjajo reaktivne kisikove zvrsti (ROS). Te naj bi nastajale predvsem kot posledica poškodovanih mitohondrijev in bi posledično lahko vplivale na stabilnost lizosomske membrane. Ker smo že pokazali, da se lizosomi poškodujejo kasneje kot mitohondriji pri izbranem modelu apoptoze, smo preverili, ali so lahko morda ROS tisti dejavnik, ki imajo pomembno vlogo pri poškodbi celičnih organelov. Za spremljanje nastanka ROS smo uporabili barvilo CM-H_2DCFDA. Prisotnost ROS oksidira barvilo in ga spremeni v fluorescirajočo molekulo, ki oddaja višjo intenziteto zelene svetlobe kot nefluorescirajoča molekula.

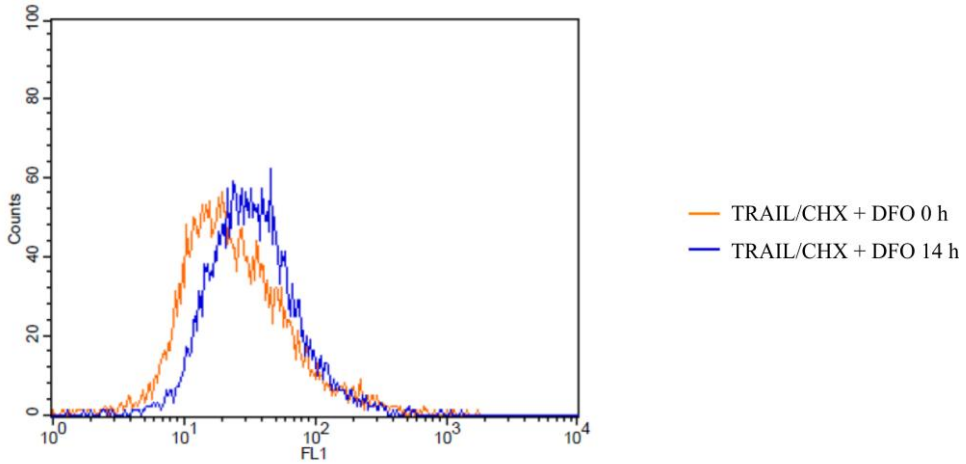


Slika 17: *Nastanek reaktivnih kisikovih zvrsti v mišjih embrionalnih fibroblastih divjega tipa 14 ur po sprožitvi apoptoze z ligandom TRAIL*

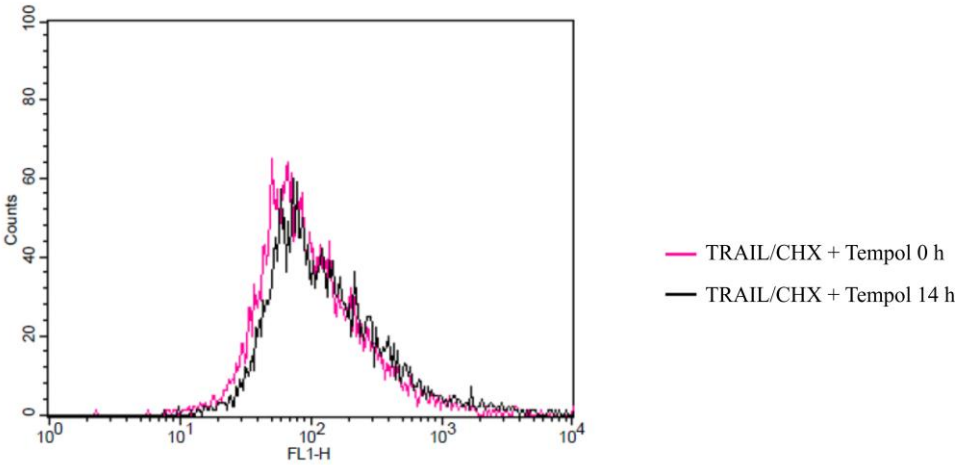
Potrdili smo, da lahko spremljamo nastajanje ROS in da pri apoptozi sproženi z ligandom TRAIL pride do povečanja ROS v celici. Na sliki 17 je prikazana sprememba fluorescence barvila pri tretiranih in netretiranih celicah.

4.1.6.1 Vpliv desferoksamina (DFO) in Tempola na tvorbo ROS med apotozo

Po potrditvi nastanka ROS smo njihov vpliv na potek apoptoze preverili tako, da smo uporabili desferoksamin (DFO), ki je kelator železovih ionov in prepreči nastanek ROS v lizosomu, z uporabo Tempola pa smo odstranili ROS predvsem v citosolu.



Slika 18: *Nastanek reaktivnih kisikovih zvrsti v mišjih embrionalnih fibroblastih divjega tipa 14 ur po sprožitvi apoptoze z ligandom TRAIL ob prisotnosti DFO-ja*

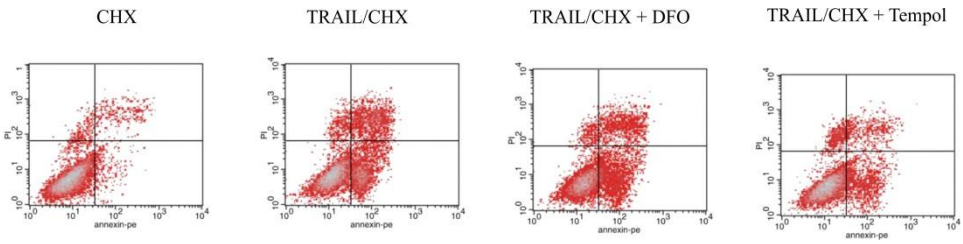


Slika 19: *Nastanek reaktivnih kisikovih zvrsti v mišjih embrionalnih fibroblastih divjega tipa 14 ur po sprožitvi apoptoze z ligandom TRAIL ob prisotnosti Tempola*

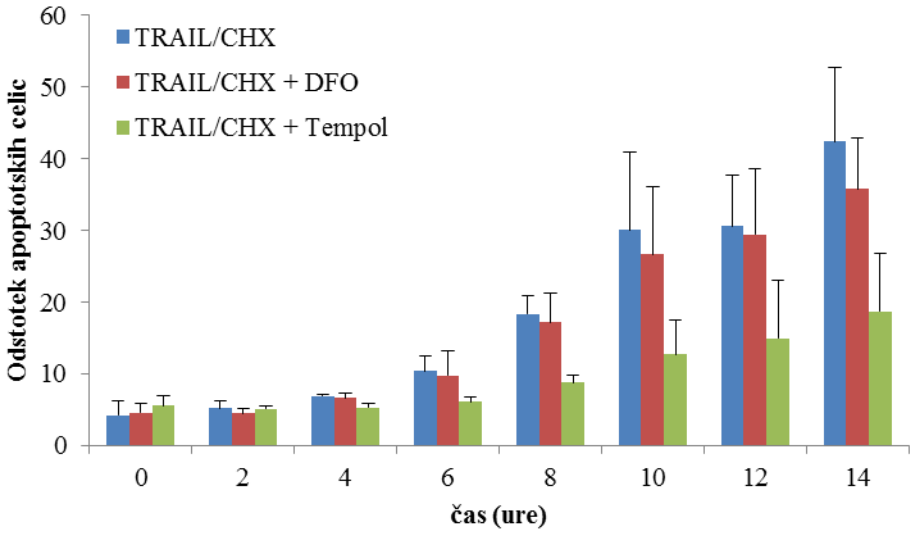
Primerjava slike 17 in slike 18 pokaže, da je uporaba DFO-ja zmanjšala nastanek ROS, ni pa jih povsem preprečila. Ker je delovanje DFO-ja omejeno le na lizosom, predvidevamo, da so preostale ROS, ki so nastale pri uporabi DFO-ja posledica ROS iz citosola in drugih organelov. Odstranjevalec Tempol pa je s svojim delovanjem po citosolu skoraj popolnoma preprečil tvorbo ROS (Slika 19).

4.1.7 Vpliv DFO-ja in Tempola na delež apototskih celic, delež celic s poškodovanimi mitohondriji in delež celic s poškodovanimi lizosomi

Pokazali smo, da lahko z dodatkom DFO-ja ali Tempola učinkovito zmanjšamo ROS v celici. Nato smo želeli preveriti, kako zmanjšanje ROS vpliva na apoptozo ter stabilnost mitohodrijske in lizosomske membrane.



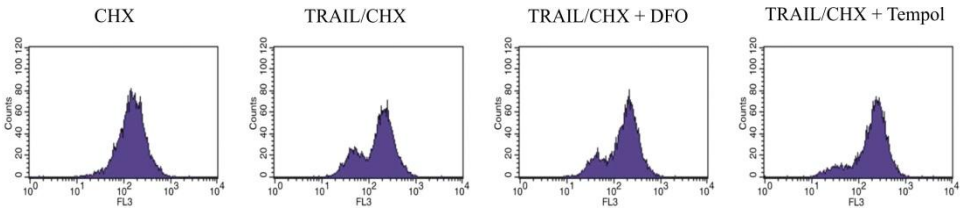
Slika 20: *Pikčasti diagram dvojnega barvanja celic z Annexin-V-PE in PI celic mišjih embrionalnih fibroblastov divjega tipa po 14 urni inkubaciji z ligandom TRAIL v prisotnosti DFO-ja ali Tempola*



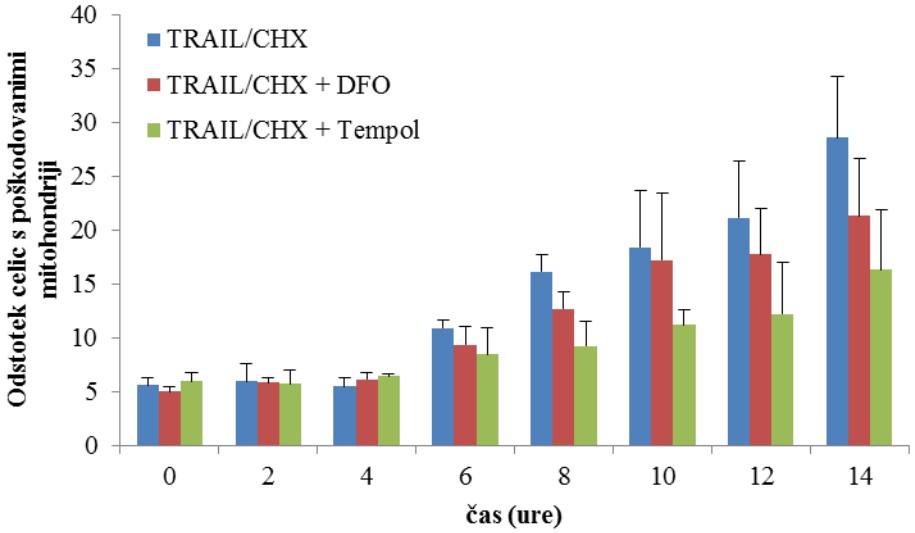
Slika 21: *Odstotek apoptoze pri celicah mišjih embrionalnih fibroblastov divjega tipa z ligandom TRAIL v prisotnosti DFO-ja ali Tempola v odvisnosti od časa*

Dodatek DFO-ja ne vpliva bistveno na delež apoptotskih celic pri apoptozi sproženi z ligandom TRAIL, odstotek apoptotskih celic pri uporabi DFO-ja narašča podobno kot pri tretiranju s samim TRAIL. Nasprotno pa uporaba odstranjevalca reaktivnih kisikovih spojin Tempola zniža delež apoptotskih celic za polovico (Slika 21) in sicer na delež apoptotskih celic vpliva vse od začetka tretiranja z ligandom TRAIL.

Po potrditvi vpliva ROS na apoptozo sproženo z ligandom TRAIL pri mišjih embrionalnih celicah divjega tipa smo preverili, kakšen je mehanizem delovanja ROS, oziroma v kateri fazi mehanizma apoptoze imajo ROS največji vpliv. Tako kot pri proučevanju vpliva katepsina B smo preverili vpliv na stabilnost mitohodrijske in lizosomske membrane.

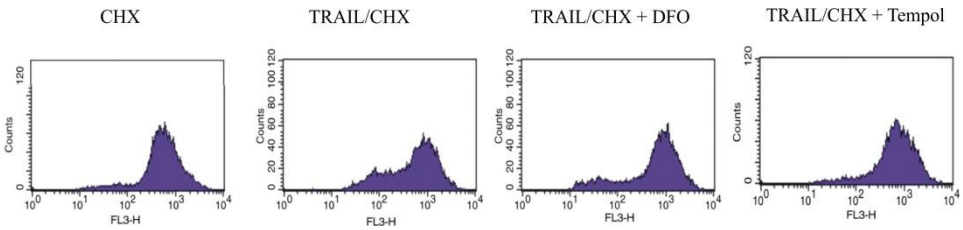


Slika 22: *Histogram barvanja celic mišjih embrionalnih fibroblastov divjega tipa s poškodovanimi mitohondriji z barvilom MitoTracker CMX-Ros po 14 urni inkubaciji z ligandom TRAIL v prisotnosti DFO-ja ali Tempola*

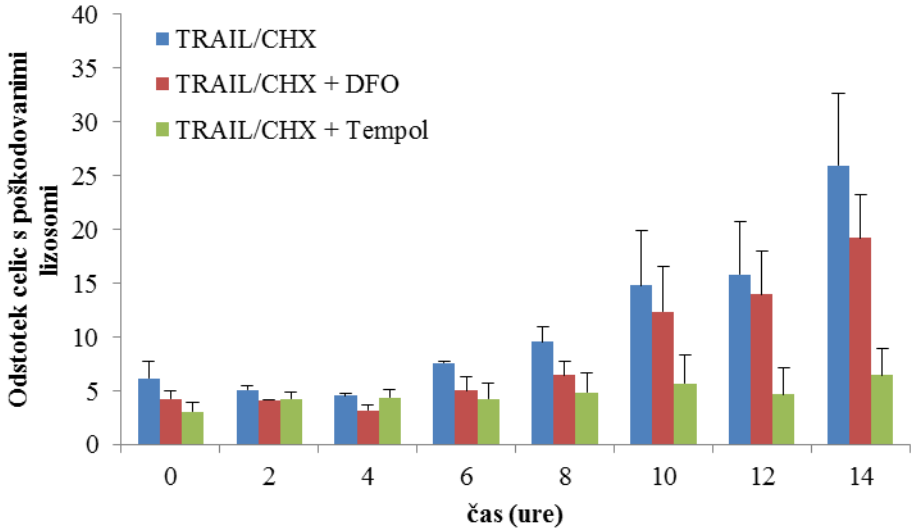


Slika 23: *Odstotek celic mišjih embrionalnih fibroblastov divjega tipa s poškodovanimi mitohondriji po tretiranju z ligandom TRAIL v prisotnosti DFO-ja ali Tempola v odvisnosti od časa*

DFO in Tempol vplivata na stabilnost mitohondrijske membrane v izbranem modelu apoptoze. V prvih 4. urah ni sprememb, poškodbe mitohondrijev se začnejo po 6. urah po sprožitvi apoptoze in naraščajo vse do konca poskusa. Pri tem tako DFO kot Tempol ne preprečita poškodb mitohondrijev, jih pa zmanjšata. Tempol ima pri tem še enkrat večji učinek kot DFO.



Slika 24: *Histogram barvanja celic mišjih embrionalnih fibroblastov divjega tipa s poškodovanimi lizosomi z barvilom AO po 14 urni inkubaciji z ligandom TRAIL v prisotnosti DFO-ja ali Tempola*

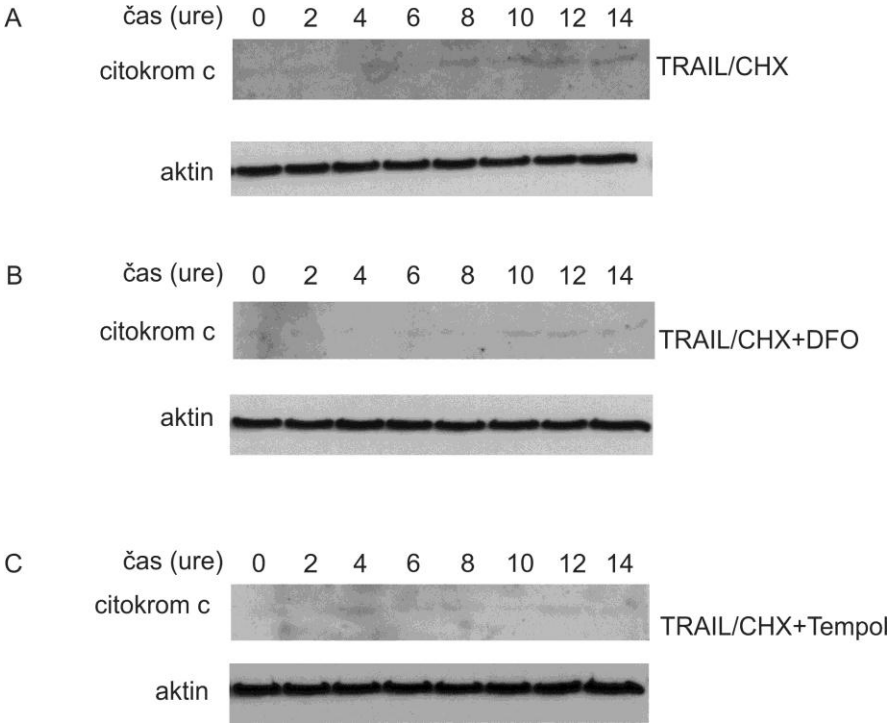


Slika 25: *Odstotek celic mišjih embrionalnih fibroblastov divjega tipa s poškodovanimi lizosomi po tretiranju z ligandom TRAIL v prisotnosti DFO-ja ali Tempola v odvisnosti od časa*

ROS s svojim delovanjem vplivajo tudi na stabilnost lizosomske membrane. Z njihovo odstranitvijo v citosolu Tempol bistveno pripomore k zmanjšanju poškodb lizosomov pri apoptozi sproženi z ligandom TRAIL. Tudi uporaba DFO-ja pripomore k zmanšanju deleža celic s poškodovanimi lizosomi, vendar je učinek delovanja DFO-ja na lizosome primerljiv z delovanjem DFO-ja na mitohondrije. Zaščita lizosomov z DFO-jem je bistveno manjša od zaščite s Tempolom.

4.1.8 Imunodetekcija sproščenega citokroma c

Po poškodbi mitohondrija se sprosti pomemben proapoptotski dejavnik citokrom c. Citokrom c je namreč sestavni del apoptosoma, na katerem se aktivira kaspaza 9, ki nato aktivira kaspazi 3 in 7. Aktivni kaspazi 3 in 7 pa sta izvršitveni kaspazi, ki sprožita apoptozo. Preverili smo, ali zmanjšanje poškodb mitohondrijev, ki smo ga dosegli z DFO-jem in Tempolom vpliva tudi da sproščanje citokroma C iz mitohondrijev.

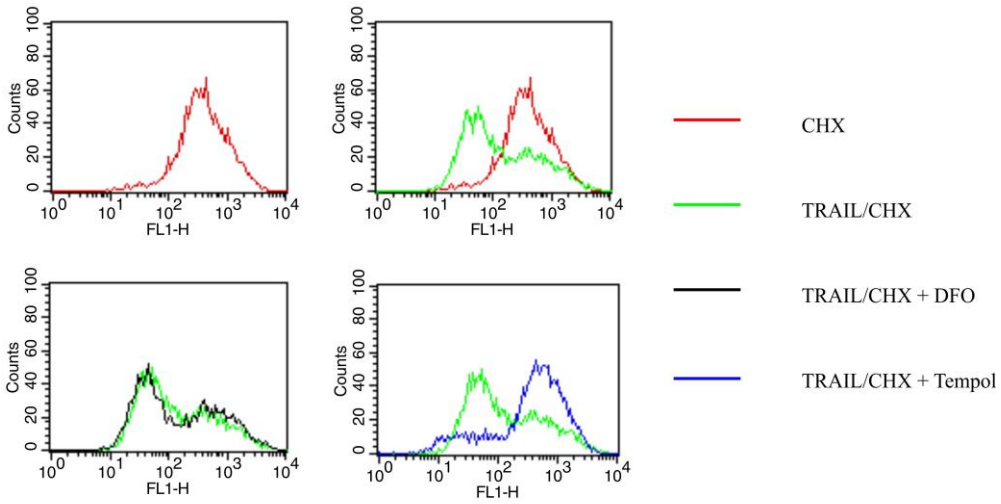


Slika 26: *Imunodetekcija citokroma c v mišjih embrionalnih fibroblatih divjega tipa po tretiranju z ligandom TRAIL (A) v prisotnosti DFO-ja (B) in Tempola (C) v odvisnosti od časa*

Uporaba DFO-ja in Tempola zadrži, a ne prepreči sproščanja citokroma c iz mitohondrijev. Z metodo imunodetekcije lahko pri apoptozi, sproženi z ligandom TRAIL, opazimo sproščanje citokroma iz mitohondrijev med 6. in 8. uro pa sprožitvi apoptoze. Uporaba odstranjevalcev reaktivnih kisikovih zvrsti pa zadrži sproščanje citokroma C in ga lahko opazimo šele po 10. urah pri uporabi DFO-ja oziroma po 12. urah pri uporabi Tempola (Slika 26).

4.1.9 Oksidacija kardiolipina

Sproščanje citokroma c iz mitohondrijev je posledica mitohondrijskih poškodb. V mitohondriju je citokrom c vezan na kardiolipin in za sprostitev citokroma c je potrebna oksidacija kardiolipina. Le-to lahko določimo z uporabo 10-N-nonil-akridin oranža (10-N-nonyl-acridine orange – NAO). NAO se veže le na neoksidirano obliko kardiolipina, vezavo pa določamo s pomočjo pretočnega citometra.

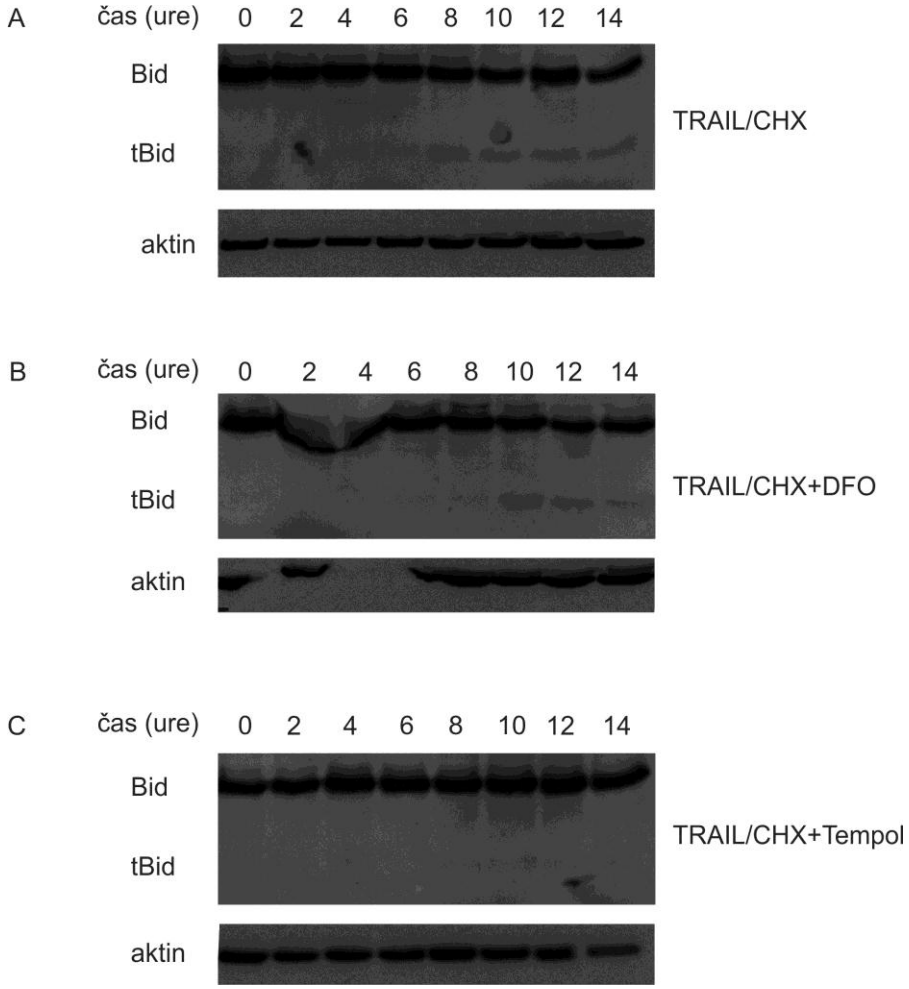


Slika 27: *Histogram barvanja celic mišjih embrionalnih fibroblastov divjega tipa z barvilom NAO po 14 urni inkubaciji z ligandom TRAIL v prisotnosti DFO-ja in Tempola*

Tretiranje mišjih embrionalnih fibroblastov divjega tipa z ligandom TRAIL povzroči oksidacijo kardiolipina. Dodatek DFO-ja ne prepreči oksidacije kardiolipina, medtem ko jo dodatek Tempola bistveno, a ne popolnoma, prepreči.

4.1.10 Imunodetekcija cepljene oblike proteina Bid

Poškodbe lizosomov in mitohondrijev so med seboj povezane preko proteina Bid. Iz poškodovanih lizosomov se sprostijo katepsini, ki lahko v citosolu cepijo protein Bid. Cepljena oblika tBid povzroči oligomerizacijo proteinov Bax in Bak v zunanji mitohondrijski membrani in tvorbo por. Preverili smo, ali v izbranem modelu apoptoze pride do cepitve proteina Bid.

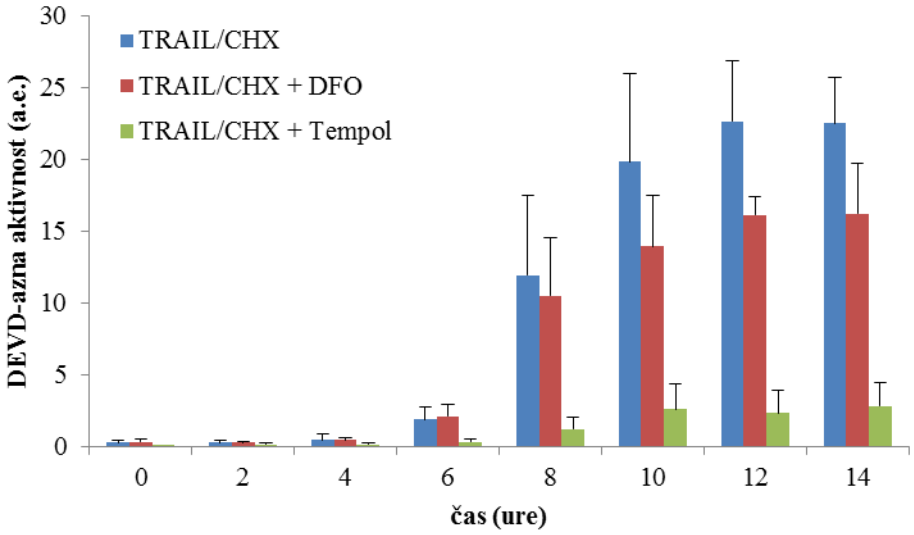


Slika 28: *Imunodetekcija cepljene oblike proteina Bid (tBid) v mišjih embrionalnih fibroblastih divjega tipa po tretiranju z ligandom TRAIL (A) v prisotnosti DFO-ja (B) in Tempola (C) v odvisnosti od časa*

Pri apoptozi sproženi z ligandom TRAIL se cepljena oblika proteina Bid, tBid, pojavi med 6. do 8. urami po sprožitvi apoptoze. Dodatek DFO-ja povzroči zakasnitev cepitve proteina Bid med 8. in 10. ure po začetku apoptoze. Podobno pri dodatku Tempola vidimo sicer šibkejše lise, ki pa se prav tako pojavijo med 8. in 10. uro po sprožitvi apoptoze z ligandom TRAIL.

4.1.11 Vpliv DFO-ja in Tempola na kaspazno aktivnost med apoptozo

Izvršitvene kaspaze so ključni encimi pri apoptozi. Procesi, ki jih sproži ligand TRAIL po začetku apoptoze vodijo do njihove aktivacije. Tudi katepsini iz poškodovanih lizosomov imajo vlogo pri njihovi aktivaciji, saj inaktivirajo XIAP, protein, ki inhibira kaspazo 9. Preverili smo, če in kako DFO in Tempol vplivata na aktivnost izvršitvenih kaspaz 3 in 7.



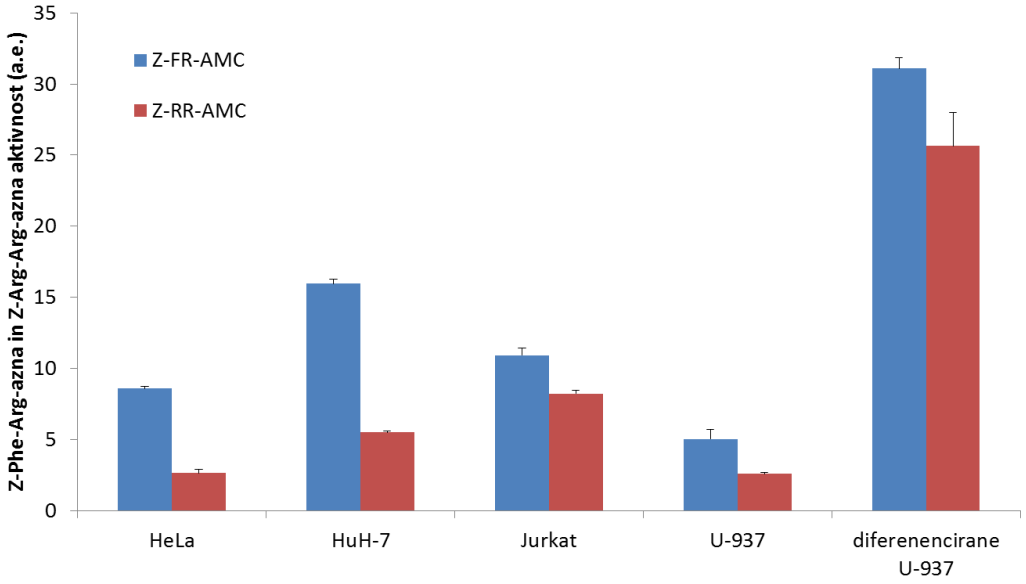
Slika 29: *DEVD-azna aktivnost v celotnih celičnih lizatih mišjih embrionalnih fibroblastov po sprožitvi apoptoze z ligandom TRAIL v prisotnosti DFO-ja ali Tempola v odvisnosti od časa*

V celicah MEF po apotozi sproženi z ligandom TRAIL DEVD-azna aktivnost narašča enako prvih 6 ur v odsotnosti odstranjevalcev ROS in ob prisotnosti DFO-ja. Nato pride do zmanjšanja DEVD-azne aktivnosti ob prisotnosti DFO-ja, ki je po 14 urah za 30 % nižja. Uporaba Tempola že od začetka zelo vpliva na DEVD-azno aktivnost in le-ta tudi ob koncu doseže le 12 % vrednosti kaspazne aktivnosti pri apoptozi brez prisotnosti Tempola (Slika 29).

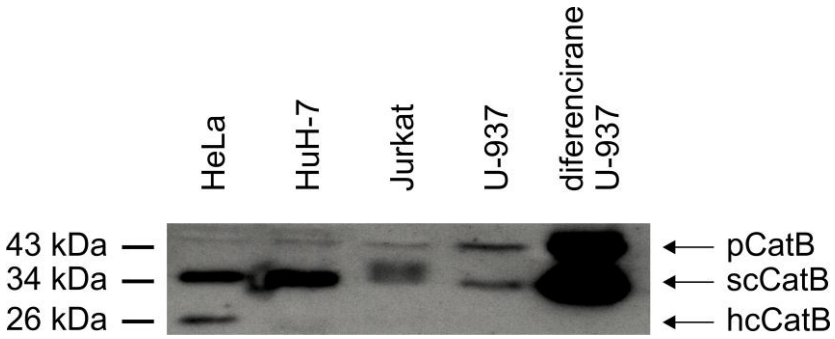
4.2 Sprožitev apoptoze z ligandom smrti TRAIL v humanih celičnih linijah

4.2.1 Določitev koncentracije liganda TRAIL in časa inkubacije za sprožitev apoptoze

Vlogo katepsinov pri celični smrti sproženi z ligandom smrti TRAIL smo testirali z uporabo štirih različnih humanih celičnih linij. Izbrali smo linije z različnimi aktivnostmi katepsinov (Slika 30), rezultate smo potrdili tudi z uporabo prenosa po Westernu (Slika 31).



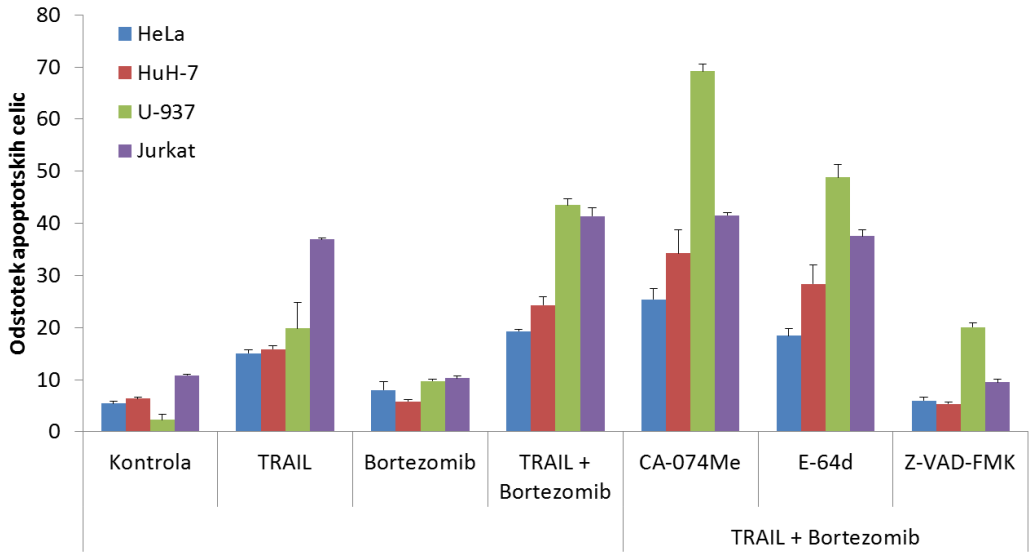
Slika 30: *Katepsinska aktivnost v celotnih celičnih ekstraktih pri izbranih celičnih linijah*



Slika 31: *Imunodetekcija katepsina B v celotnih celičnih ekstraktih pri izbranih celičnih linijah (pCatB – prokatepsin B, scCatB – enojna veriga katepsina B, hcCatB – težka veriga katepsina B)*

Humane celične linije smo senzibilizirali na ligand TRAIL z uporabo bortezomiba, proteaosomskega inhibitorja. Ker pri humanih linijah nimamo na voljo celic z izbitimi

geni za katepsine, smo v kontrolnih pogojih uporabili splošni inhibitor katepsinov E-64d in specifični inhibitor za katepsin B CA-074Me. Kot kontrolo smo uporabili še inhibitor kaspaz Z-VAD-FMK. Vse inhibitorje smo dodali v končni koncetraciji 10 µM. Najprej smo določili pri katerih pogojih (koncentracija in čas inkubacije) ligand TRAIL sproži apoptozo pri izbranih humanih celičnih linijah. Z določenimi eksperimentalnimi pogoji smo nato z uporabo inhibitorjev testirali vpliv katepsinov na potek apoptoze sprožene z ligandom TRAIL.



Slika 32: *Delež apoptotskih celic pri apoptozi sproženi z ligandom TRAIL ob prisotnosti različnih inhibitorjev v humanih celičnih linijah*

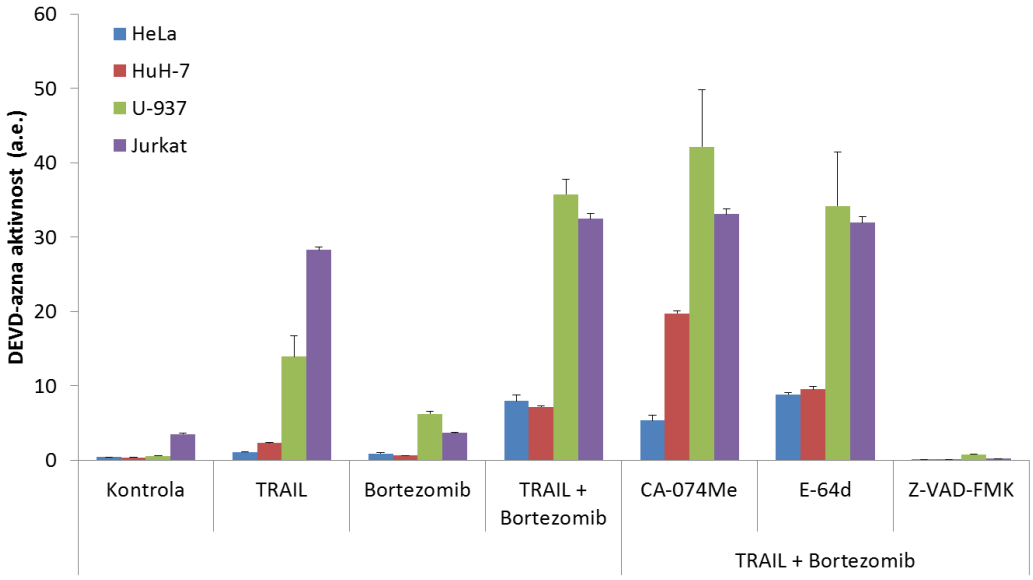
Ligand TRAIL je spožil apoptozo v vseh izbranih humanih celičnih linijah. Uporabljene celične linije so različno občutljive za ligand TRAIL in potrebna je bila optimizacija, da smo pri vseh linijah dobili podoben delež apoptotskih celic. Za sprožitev apoptoze v humanih linijah HeLa, HuH-7 in U-937 smo uporabili koncentracijo liganda TRAIL 100 ng/ml in koncentracijo bortezomiba 10 nM, čas inkubacije je bil 24 ur. Za celice Jurkat je bila potrebna koncentracija liganda TRAIL 1 ng/ml in 10 nM bortezomiba v času 18 ur. Uporaba bortezomiba ja povečala delež apoptotskih celic, povečanje pa je bilo večje, kot bi bil le skupen učinek liganda TRAIL in bortezomiba.

Sami inhibitorji proteaz niso bili toksični za celice. Inhibitor kaspaz Z-VAD-FMK je znatno zmanjšal apoptozo pri celicah U-937, pri ostalih treh linijah pa jo celo popolnoma preprečil. Splošni katepsinski inhibitor E-64d ni imel vpliva na delež apoptotskih celic. Specifični inhibitor za katepsin B CA-074Me pri celicah Jurkat ni imel vpliva na apoptozo, pri ostalih treh linijah pa je njegov dodatek celo prispeval k povečanju deleža apoptotskih celic (Slika 32).

4.2.2 DEVD-azna aktivnost

Pri apoptozi imajo pomembno vlogo kaspaze in v naslednjem poskusu smo želeli preveriti, kako ligand TRAIL in inhibitorji vplivajo na kaspazno aktivnost v celotnih

celičnih lizatih. Hkrati je bil to tudi kontrolni test za učinkovitost inhbicije kaspaz z inhibitorjem Z-VAD-FMK.

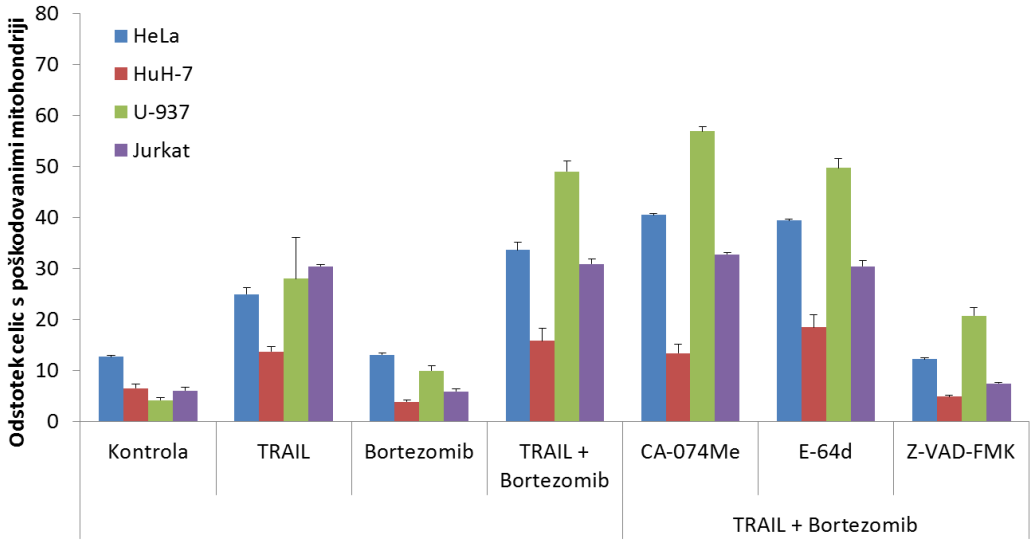


Slika 33: *DEVD-azna aktivnost v celotnem celičnem ekstraktu po apoptozi sproženi z ligandom TRAIL ob prisotnosti različnih inhibitorjev v humanih celičnih linijah*

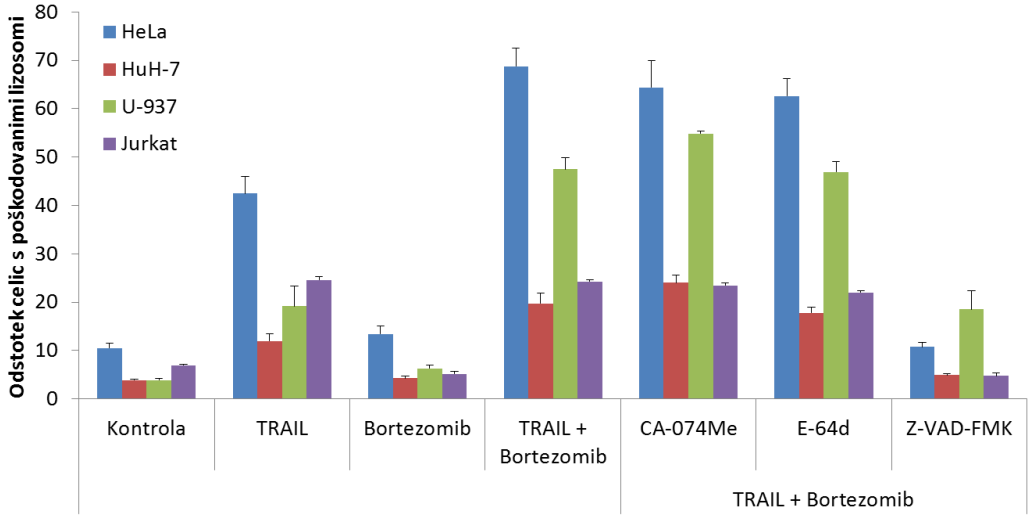
Pri kontrolnih poskusih je DEVD-azna aktivnost zelo nizka, saj v normalnem delovanju kaspaze v celici niso aktivne, se pa aktivirajo tekom apoptoze, kar smo z našimi testi tudi pokazali. DEVD-azna aktivnost je bila popolnoma preprečena z uporabo inhibitorja kaspaz Z-VAD-FMK pri vseh celičnih linijah. Prav tako pri vseh celičnih linijah na aktivnost kaspaz ni vplival splošen inhibitor katepsinov E-64d. Drugače pa je z rezultati s specifičnem inhibitorjem katepsina B CA-074Me. V celicah Jurkat in U-937 ni imel vpliva na DEVD-azno aktivnost, v celicah HeLa je CA-074Me znižal DEVD-azno aktivnost, v celicah HuH-7 pa je bila DEVD-azna aktivnost nekoliko višja (Slika 33).

4.2.3 Delež poškodovanih mitohondrijev in lizosomov pri apoptozi

Tako kot pri mišjih embrionalnih fibroblastih smo tudi pri humanih linijah želeli preveriti, ali bi z inhibicijo aktivnosti katepsinov lahko vplivali na poškodbe mitohondrijev in lizosomov. Vendar tokrat nismo spremljali časovnega poteka stabilnosti celičnih organelov, ampak smo le določili delež celic s poškodovanimi mitohondriji in lizosomi v končni točki. Tako ne moremo oceniti ali se prej poškodujejo mitohondriji ali lizosomi, lahko pa ocenimo sam vpliv inhibicije katepsinov na stabilnost organelov.



Slika 34: *Delež celic s poškodovanimi mitohondriji pri apoptozi sproženi z ligandom TRAIL ob prisotnosti različnih inhibitorjev v humanih celičnih linijah*

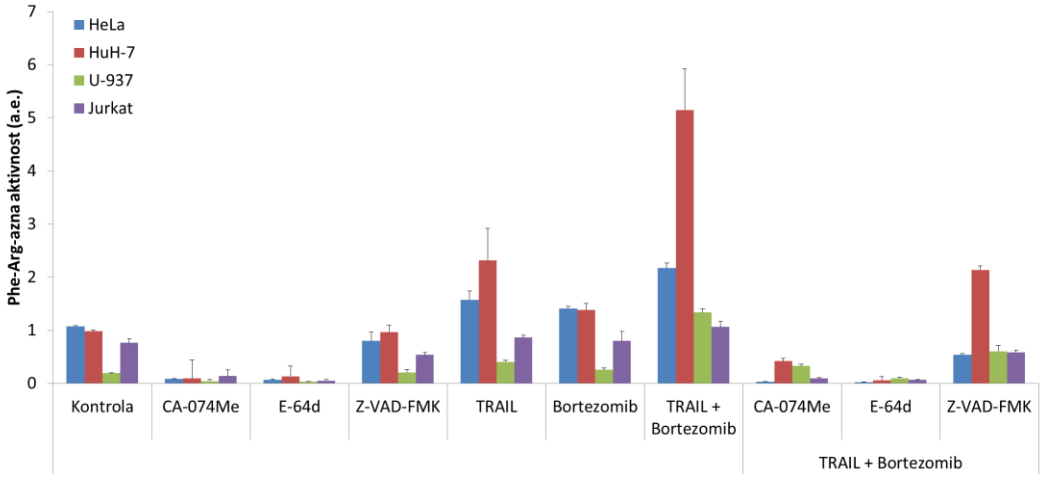


Slika 35: *Delež celic s poškodovanimi lizosomi pri apoptozi sproženi z ligandom TRAIL ob prisotnosti različnih inhibitorjev v humanih celičnih linijah*

Po pričakovanjih smo ugotovili, da se med apoptozo sproženo z ligandom TRAIL v izbranih humanih celičnih linijah poškodujejo tako lizosomi kot mitohondriji. Oba inhibitorja katepsinov, E-64d in CA-074Me, pri vseh celičnih linijah nimata vpliva na delež poškodovanih mitohondrijev in lizosomov. Inhibitor kaspaz Z-VAD-FMK je pri celicah U-937 znatno zmanjšal delež poškodovanih organelov, ni popolnoma preprečil poškod mitohondrijev in lizosomov. Pri ostalih celičnih linijah pa je bil pri dodatku Z-VAD-FMK delež poškodovanih organelov enak kot v kontroli (Slika 34 in Slika 35). Vsi trije inhibitorji proteaz sami niso vplivali na poškodbe mitohondrijev ali lizosomov v poteku poskusa.

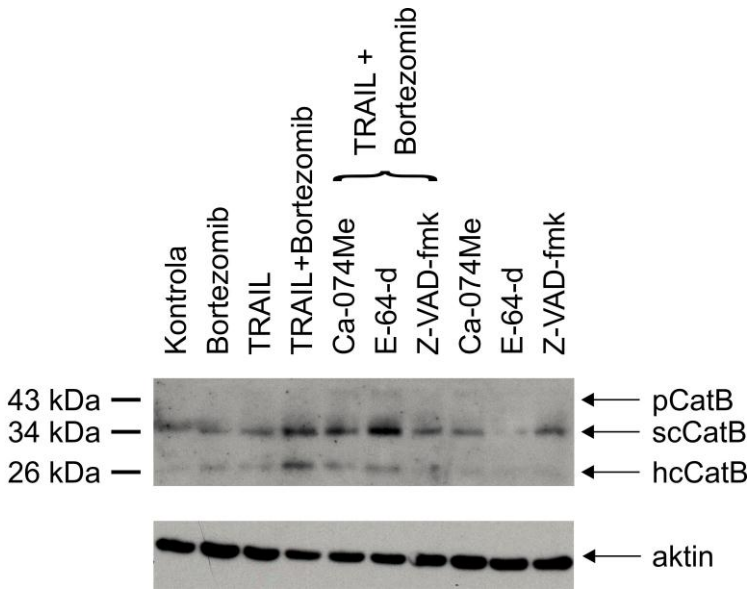
4.2.3.1 Katepsinska aktivnost

Potrdili smo, da pri apoptozi sproženi z ligandom TRAIL prihaja do poškodb mitohondrijev in lizosomov. Iz poškodovanih lizosomov se lahko sproščajo katepsini, ki s svojim delovanjem v citosolu pripomorejo k poteku apoptoze. Vendar se katepsini po sprostitvi iz lizosoma v citosolu ireverzibilno inaktivirajo. Za njihov prispevek pri mehanizmu apoptoze pa je pomembna ravno njihova aktivnost v citosolu. Tako smo preverili, kakšna je aktivnost katepsinov v citosolu celice po sprožitvi apoptoze z ligandom TRAIL in preverili učinkovitost inhibicije katepsinov z inhibitorji tudi v celotnih celičnih ekstraktih.

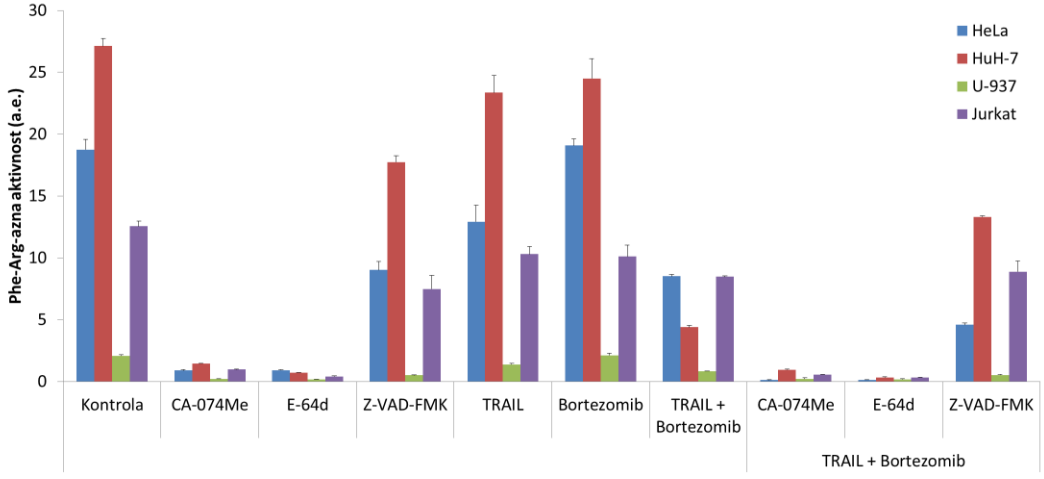


Slika 36: *Katepsinska aktivnost v celičnem citosolu po apoptozi sproženi z ligandom TRAIL ob prisotnosti različnih inhibitorjev v humanih celičnih linijah*

Med apoptozo se aktivnost katepsinov v citosolu poveča pri vseh izbranih humanih celičnih linijah. Inhibitorja katepsinov CA-074Me in E-64d skoraj popolnoma preprečita aktivnost katepsinov v citosolu, medtem ko jo kaspazni inhibitor Z-VAD-FMK delno prepreči. S tem smo dokazali, da so katepsini po sprostitvi v citosol aktivni in lahko pripomorejo pri mehanizmu apoptoze, ter da kaspazni inhibitor Z-VAD-FMK tudi delno vpliva na aktivnost katepsinov (Slika 36). Na celicah Jurkat smo še dodatno potrdili sprostitev katepsinov v citosol z imunodetekcijo katepsina B v citosolu (Slika 37).



Slika 37: *Imunodetekcija katepsina B v celičnem citosolu po apoptozi sproženi z ligandom TRAIL ob prisotnosti različnih inhibitorjev v celicah Jurkat (pCatB – prokatepsin B, scCatB – enojna veriga katepsina B, hcCatB – težka veriga katepsina B)*

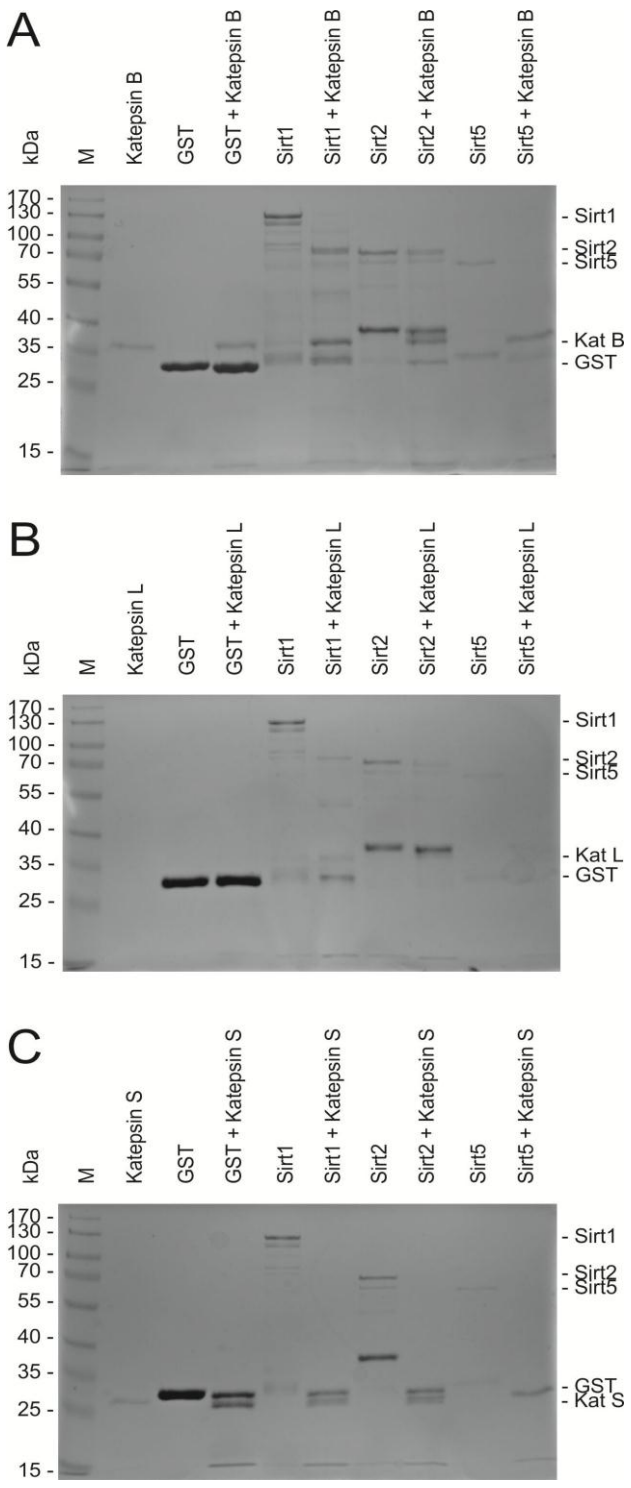


Slika 38: *Katepsinska aktivnost v celotnem celičnem ekstraktu po apoptozi sproženi z ligandom TRAIL ob prisotnosti različnih inhibitorjev v humanih celičnih linijah*

Meritev celokupne katepsinske aktivnosti v celici smo opravili na celotnih celičnih lizatih. Za razliko od aktivnosti katepsinov v citoslu, se katepsinska aktivnost v totalnih celičnih lizatih tekom apoptoze zmanjša pri vseh izbranih celičnih linijah. Inhibitorja katepsinov sta popolnoma preprečila katepsinsko aktivnost, delno pa je na katepsinsko aktivnost imel vpliv tudi inhibitor kaspaz Z-VAD-FMK (Slika 38).

4.3 Cepitve sirtuinov s katepsini

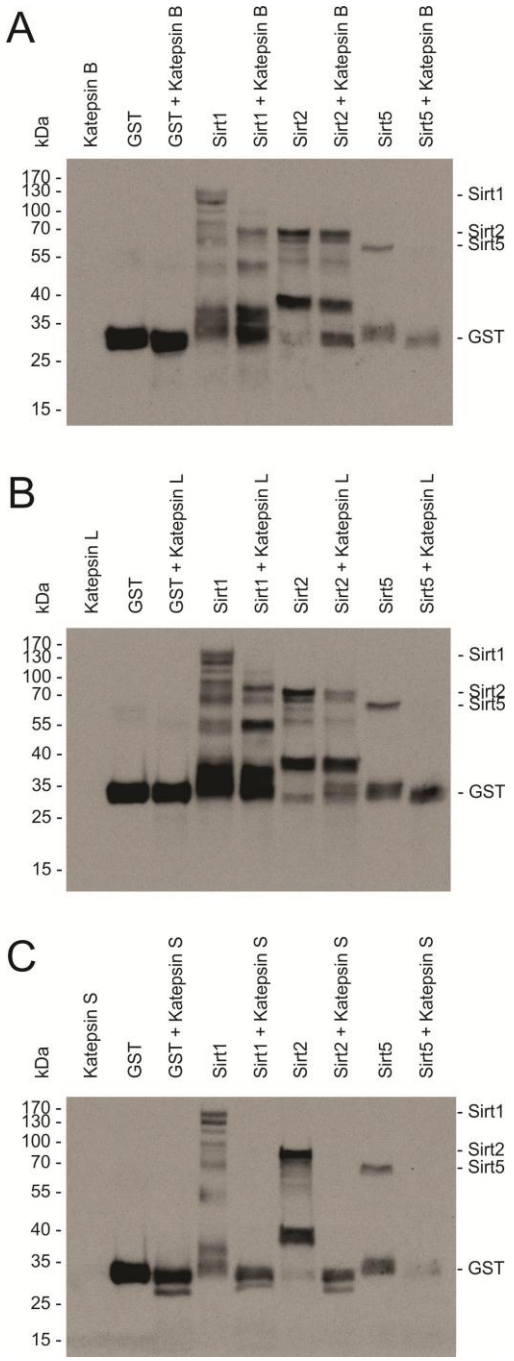
Sirtuini, še posebno sirtuin 1 (Sirt1), imajo pomembno vlogo pri odpornosti celice proti različnim stresnim dejavnikom, med drugim tudi ščitijo celico pred ROS (Mantel in Broxmeyer, 2008). Med študijami mehanizma delovanja proteina Sirt1 so opazili indukcijo permeabilizacije lizosomske membrane, zato smo želeli preveriti, ali sproščeni katepsini v citosolu lahko cepijo sirtuine. Cepitve rekombinantnih sirtuinov smo preverjali *in vitro* pri nevtralnem pH s katepsini B, L in S. Katepsina B in L sta najbolj zastopana katepsina v celicah, katepsin S pa je med vsemi cisteinskimi katepsini najbolj stabilen pri nevtralnem pH.



Slika 39: *Analiza cepitev rekombinantnih sirtuinov s katepsinom B (A), L (B) in S (C) pri pH 7,2 in 37 °C z gelsko elektroforezo v prisotnosti NaDS*

Rekombinantni sirtuini so imeli pripet označevalec GST, zato smo kot kontroli imeli dodatno še protein GST. Na sliki 39 vidimo, da vsi trije izbrani katepsini cepijo sirtuine Sirt1, Sirt2 ter Sirt5. Vendar jih ne cepijo enako učinkovito. Največjo razgradnjo vidimo pri delovanju katepsina S, manj pri katepsinu L in najmanj pri katepsinu B. Poleg razlik v učinkovitosti cepitve z različnimi katepsini vidimo tudi razlike med velikostjo novo nastalih fragmentov cepitve.

Vsi sirtuini so imeli pripet označevalec GST. Da bi potrdili, ali je pri cepitvah prišlo do razgradnje v polipeptidni verigi sirtuinov in ne le do odcepitve GST, smo cepitvene reakcije analizirali še s pomočjo detekcije GST s prenosom western.



Slika 40: *Analiza cepitev rekombinantnih sirtuinov s katepsinom B (A), L (B) in S (C) pri pH 7,2 in 37 °C s prenosom western*

Potrdili smo, da pri inkubaciji sirtuinov v prisotnosti izbranih katepsinov pride do cepitve znotraj polipeptidne verige sirtuinov in ne le do odcepitve GST (Slika 40). Predvsem pri cepitvi s katepsinom S je vidno, da po delovanju katepsina ostane le GST sonda, preostanek proteinov je popolnoma razgrajen. Katepsin S popolnoma razgradi vse tri modelne sirtuine, deloma tudi GST. Katepsina B in L nista tako učinkovita pri razgradnji sirtuinov. Kaže, da gre za bolj specifično cepitev oziroma cepitve.

5 Razprava

Ligand TRAIL lahko selektivno sproži apoptozo v različnih tumorskih celicah, medtem ko na normalne celice nima vpliva. Razlogi za to še niso povsem pojasnjeni, med najbolj verjetnimi pa so število receptorjev vab, aktivacija preživetja preko NF-κB poti, inhibicija aktivacije kaspaz s proteinom cFLIP, regulacija s proteini družine Bcl-2 (Walczak in sod., 1999). V naši študiji smo uporabili mišje embrionalne fibroblaste (MEFs) in ugotovili, da ligand TRAIL pri njih sproži apoptozo, kar je tudi v skladu z drugimi študijami (Finnberg in sod., 2005; Johnson in sod., 2003). Na več načinov lahko povečamo občutljivost celic za ligand TRAIL. Pri našem delu z MEFs smo uporabili inhibitor proteinske sinteze cikloheksimid (CHX). Za sprožitev apoptoze je namreč pomembno, da ne pride do aktivacije preživetvene poti preko NF-κB, ki jo torej z uporabo CHX preprečimo. Koncentracijo dodanega CHX (1 μg/ml), ki sam ni toksičen za celice, smo določili v ločenem poskusu. Inhibicija sinteze proteinov namreč pomeni, da celica ne more sintetizirati novih proteinov za svoje normalno delovanje in posledično umre zaradi stresa. Koncentracijo liganda TRAIL (100 ng/ml) in čas inhkubacije smo določili s spremljanjem deleža apoptostkih celic z uporabo pretočnega citometra pri različnih koncentracijah liganda TRAIL na vsaki dve uri. Po 14. urah inkubacije s kombinacijo liganda TRAIL/CHX je bil odstotek apoptotskih celic okoli 40 % (Slika 10). V poskusih smo želeli doseči delež apoptotskih celic med 30 in 50 %, da bi bili gotovi, da gre za proces apoptoze in ne za umiranje celic zaradi drugih razlogov. Ko smo se prepričali, da gre za apoptozo, smo želeli potrditi, ali gre za proces apoptoze odvisen od kaspaz. Pri nekaterih oblikah celične smrti se kaspaze ne aktivirajo, temveč imajo pomembno vlogo druge proteaze, kot so grancimi, lizosomski katepsini, kalpaini, serinske proteaze idr. (Broker in sod., 2005). Iz slike 11 vidimo, da začne aktivnost kaspaz rasti po 8. urah od začetka tretiranja celic in vse do konca poskusa narašča. Tako smo v izbranem modelu potrdili, da z ligandom TRAIL v prisotnosti CHX v mišjih embrionalnih fibroblastih sprožimo od kaspaz odvisno apoptozo. Prav tako lahko zaključimo, da v poteku našega poskusa (14 ur) še ne prihaja do umiranja celic z nekrozo, ker aktivnost kaspaz vseskozi narašča. V tem primeru bi namreč DEVD-azna aktivnost začela upadati.

Predhodne študije so pokazale, da pri apoptozi sproženi preko receptorjev smrti prihaja do destabilizacije membran lizosomov (Guicciardi in sod., 2000; Werneburg in sod., 2002). Mehanizmi poškodb lizosomske membrane še niso povsem pojasnjeni, več avtorjev pa trdi, da gre za zgodnji dogodek v apoptozi. Zgodnje sproščanje katepsinov iz poškodovanih lizosomov bi lahko pomembno prispevalo k poškodbi mitohondrijske membrane. Katepsini namreč lahko cepijo molekulo Bid (Droga-Mazovec in sod., 2008), ki omogoča vgradnjo por Bax/Bak v zunanjo mitohondrijsko membrano in s tem permebilizacijo mitohondrijske membrane. Glede na rezultate, prikazane na sliki 12 pa lahko sklepamo, da se naša opažanja ne skladajo s temi zaključki. Naši rezultati kažejo, da je poškodba lizosomske membrane pozen dogodek v apoptozi, torej da pride najprej do poškodb mitohondrijev in šele nato do poškodb lizosomske membrane. Nekatere nedavne študije z ligandom TNF-α in ligandom Fas so prišle do podobnih ugotovitev. Predhodne študije, kjer so prišli do nasprotnih zaključkov, so proučevale apoptozo v eni sami končni točki in zato so težko določili zaporedje dogodkov, ko je celica že zelo poškodovana. Mi

pa smo spremljali časovni potek poškodb mitohondrijev in lizosomov od začetka sprožitve apoptoze in tako natančno ugotovili, kdaj pride do poškodb. V primeru poznih poškodb lizosomov katepsini niso pomembni za začetne stopnje pri poškodbah mitohondrijev, vsekakor pa lahko sproščeni katepsini iz poškodovanih lizosomov v nadaljnjih korakih apoptoze dodatno prispevajo k poškodbam mitohondrijev s cepitvijo proteina Bid.

V izbranem modelu smo pokazali, da se najprej poškodujejo mitohondriji in šele nato lizosomi. Naslednja naloga je bila ugotoviti ali lahko molekule sproščene iz mitohondrijev vplivajo na destabilizacijo lizosomske membrane in ustvarijo nekakšno povratno zanko med tema dvema organeloma. V literaturi zasledimo različne predlagane mehanizme destabilizacije lizosomske membrane, ki jih delimo v dve skupini. V prvo skupino uvrščamo spojine, ki direktno destabilizirajo lizosomsko membrano, sproščena lizosomska vsebina pa sproži apoptozo. Sem sodijo lizosomotropni detergenti (LeuLeuOMe, MSDH), proste maščobne kisline, inhibitorji protonskih črpalk in celo nekateri virusni proteini. V naši študiji je bolj pomembna druga skupina molekul, ki nastanejo ali se aktivirajo med apoptozo sproženo preko zunanje poti apoptoze. Med apoptozo sproženo z ligandom TNF-α so opazili povečano nastajanje sfingozina, ki lahko poškoduje lizosome (Guicciardi in sod., 2001). Predlagano je bilo tudi, da je destabilizacija lizosomov odvisna od kaspaze 8 ali kaspaze 9 (Guicciardi in sod., 2001; Gyrd-Hansen in sod., 2006), čeprav mehanizem, kako bi proteaze cepile neproteinske komponente membrane ni bil pojasnjen. Nekateri raziskovalci so predlagali, da bi pore v lizosomu lahko nastale po podobnem mehanizmu kot pore v mitohondrijih. Proteini družine Bcl-2 bi se lahko po sprožitvi apoptoze premestili iz citosola v lizosomsko membrano (Kagedal in sod., 2005; Werneburg in sod., 2007). Natančen mehanizem tudi tukaj ni bil pojsnjen, saj se te proteini preferenčno vežejo na zunanjo mitohondrijsko membrano. Nedavna študija pa je pokazala, da se proteini Bax/Bak sicer lahko prenesejo v lizosom, vendar zgolj kot posledica avtofagične razgradnje mitohondrijev (Oberle in sod., 2010). Iz poškodovanih mitohondrijev tudi uhajajo reaktivne kisikove zvrsti (ROS), ki lahko med drugim povzročijo oksidacijo lipidov v lizosomski membrani in tako destabilizirajo membrane organelov (Repnik in sod., 2012; Repnik in Turk, 2010).

V nekaterih študijah so pokazali, da lahko molekule znotraj lizosoma prispevajo k destabilizaciji membran. Rezultati poskusov s celicami, ki ne izražajo katepsina B, (Baskin-Bey in sod., 2005) in z uporabo inhibitorjev katepsinov (Liu in sod., 2003) kažejo na vpletenost katepsina B pri ohranjanju stabilnosti lizosomske membrane pri procesu apoptoze. V naši raziskavi smo najprej raziskali, ali odsotnost katepsina B v mišjih embrionalnih fibroblastih (celice z izbitim genom za katepsin B) vpliva na delež apoptotskih celic pri apoptozi, ki jo sprožimo z ligandim TRAIL. Na delež apoptotskih celic ob odsotnosti katepsina B je kar za 40 % manjši kot v celicah divjega tipa (Slika 13), kar nakazuje pomembno vlogo katepsina B pri poteku apoptoze. Toda ali katepsin B vpliva tudi na stabilnost mitohondrijske ali lizosomske membrane, morda celo na stabilnost obeh? Rezultati presenetljivo kažejo, da ni razlik med stabilnostjo mitohondrijske membrane pri celicah z izbitim genom za katepsin B in celicami divjega tipa (Slika 14). Prav tako se zaradi odsotnosti katepsina B stabilnost lizosomske membrane ne spremeni (Slika 15). Zato lahko sklepamo, da ima v izbranem modelu katepsin B pomembno vlogo pri apoptozi šele po poškodbi obeh omenjenih organelov. Tarča katepsinov v apoptotskem mehanizmu po poškodbi mitohondrijev je med drugim tudi protein XIAP, ki inhibira izvršitvene kaspaze. Z njegovo razgradnjo bi lahko katepsini preprečili inhibicijo apoptoze. V primeru celic z izbitim genom za katepsin B pa te razgradnje verjetno ne bi bilo in XIAP bi lahko zavrl apoptozo, kar se sklada z našimi

ugotovitvami, da je v odsotnosti katepsina B delež apoptotskih celin manjši. S svojo nespecifično razgradnjo lahko katepsini cepijo tudi druge molekule in znano je, da katepsini sodelujejo pri od kaspaz neodvisni celični smrti in razgrajujejo komponente celice. Vse omenjene možnosti se skladajo z našimi ugotovitvami, vendar nadaljnjega mehanizma delovanja katepsina B nismo proučevali, saj nas je predvsem zanimal mehanizem povezave med mitohondriji in lizosomi pri apoptozi sproženi z liganom TRAIL. Ne moremo pa tudi povsem izključiti možnosti, da je pri pripravi celic z izbitim genom za katepsin B prišlo še do kakšne druge spremembe v genskem zapisu celice, ki bi lahko vplivale na potek apoptoze.

Katepsin B se torej ni izkazal za ključno molekulo, ki povezuje destabilizacijo mitohondrijske in lizosomske membrane. Kot naslednjo možnost smo proučili vpliv reaktivnih kisikovih zvrsti. V mitohondrijih poteka dihalna veriga in med oksidoredukcijskimi reakcijami tvorbe energije se lahko pojavijo zelo reaktivni prosti radikali, ROS. Mitohondriji imajo učinkovite sisteme nevtralizacije pobeglih ROS, vendar kadar pride do poškodbe mitohondrijske membrane lahko ROS nenadzorovano uidejo iz mitohondrija. V citosolu so vključene v proces peroksidacije lipidov, v katerem oksidirajo nenasičene maščobne kisline membran. Z uporabo barvila CM-H_2DCFDA smo spremljali nastanek ROS v celici. Med apoptozo sproženo z ligandom TRAIL pride do nastanka ROS (Slika 17). Za nadaljnje raziskave smo izbrali dve spojini, ki naj bi zmanjšali ROS v celici. Desferoksamin (DFO) je kelator železovih ionov, ki se endocitira v lizosome, tam pa deluje kot past za omenjene ione (Tenopoulou in sod., 2005). Dodatek DFO-ja ne prepreči popolnoma povečanja ROS tekom apoptoze, jih pa opazno zmanjša (Slika 18). Tempol je prav tako odstranjevalec ROS, ki pa predvsem odstranjuje superoksidne anione na podoben način kot superoksid dismutaza (Khattab, 2006). Z dodatkom Tempola smo skoraj povsem preprečili tvorbo ROS v izbranem modelu apoptoze (Slika 19). Z obema omenjenima reagentoma smo uspešno znižali tvorbo ROS med apoptozo. Zanimalo nas je tudi, ali bi z odstranitvijo ROS lahko vplivali na delež apototskih celic in delež poškodovanih mitohondrijev ter lizosomov. Najprej smo preverili vpliv zmanjšanja ROS v celici na delež apoptotskih celic. Dodatek DFO-ja ima majhen učinek na delež apoptotskih celic (20 %), medtem ko uporaba Tempola kar za 60 % zniža njihov delež (Slika 21). Ti rezultati nakazujejo, da ROS v citosolu pomembno prispevajo k apoptozi. Zelo reaktivni ROS v citosolu hitro poškodujejo molekule, s katerimi pridejo v stik in tudi membrane organelov v bližini. Ravno zaradi visoke reaktivnosti lahko preprečimo delovanje le tistih ROS, ki so v bližini Tempola. Če ROS prej kot s Tempolom reagirajo z drugimi molekulami, to sproži nastanek novih radikalov in posledično kaskadno reakcijo. Zelo verjetno je tudi, da lahko iz mitohondrija sproščeni ROS dodatno poškodujejo isti ali sosednje mitohondrije in povzročijo dodatno uhajanje ROS in ostalih proapoptotskih dejavnikov iz mitohondrijev. Ta trditev je dodatno potrjena z analizami poškodb celic s poškodovanimi mitohondriji in lizosomi pri apoptozi sproženi z ligandom TRAIL in uporabo Tempola. Tempol je zaščitil 40 % celic s poškodovanimi mitohondriji (Slika 23) in kar 75 % celic s poškodovanimi lizosomi (Slika 25). Po drugi strani DFO deluje znotraj lizosoma in preprečuje tvorbo radikalov preko Fentonove reakcije. Uporaba DFO-ja zaščiti 75 % celic s poškodovanimi lizosomi in ravno toliko celic s poškodovanimi mitohondriji (Slika 23 in Slika 25). Celotno gledano lahko sklepamo, da preprečitev nastanka radikalov znotraj lizosoma delno zaščiti tako lizosome kot mitohondrije, vendar se učinek ne prenese popolnoma tudi na zaščito pred apoptotskim mehanizmom. Tempol zelo zaščiti lizosome pred poškodbami, učinek na mitohondrije ni tako izrazit, učinek na apoptozo pa je nekje med obema. Iz vsega opisanega lahko zaključimo, da sama zaščita mitohondrijev še ne pomeni enakovredne preprečitve apoptotske kaskade. Zelo verjetno imajo lizosomi pri procesu apoptoze vlogo

nekakšne povratne zanke za ojačitev apoptotskega signaliziranja. Po poškodbi mitohondrijev se iz njih sproščajo ROS, ki z nadaljnjimi delovanjem poškodujejo tako mitohondrije kot lizosome ter verjetno ostale citosolne komponente. Iz poškodovanih lizosomov pa se sprostijo katepsini, ki delno vplivajo s povratno zanko na poškodbe mitohondrijev, delno pa delujejo na molekule, ki so vpletene v apoptotsko signalizacijo po poškodbi mitohondrijev (npr. razgradnja XIAP). Slednje se tudi ujema z našimi rezultati dela s celicami z izbitim genom s katepsinom B. Tukaj odsotnost katepsina ni vplivala na stabilnost mitohondrijev in lizosomov, imela pa je znaten vpliv na apoptozo (Slika 13). Poleg splošnih poškodb mitohondrijev, ki smo jih določali z barvilom MitoTracker CMX-Ros, nas je zanimalo tudi sproščanje citokroma c iz mitohondrija. Citokrom je namreč sestavni del apoptosoma, kompleksa, ki aktivira kaspazo 9. Za določitev sproščenega citokroma c v citosolu smo uporabili metodo imunodetekcije. Na sliki 26 vidimo, da se pri apoptozi citokrom c sprosti med 6. in 8 uro. Uporaba DFO-ja je zavrla sproščanje in citokrom c je opazen šele po 10. urah, z uporabo Tempola pa ga detektiramo po 12. urah od sprožitve apoptoze. Rezultati detekcije sproščenega citokroma C v citosolu se ujemajo z rezultati poškodb mitohondrijev določenih z barvilom MitoTracker CMX-Ros, vendar je zaradi povprečja meritev z barvilom MitoTracker CMX-Ros težko natančno oceniti, pri katerem deležu poškodovanih mitohondrijev opazimo sproščanje citokroma c z imunodetekcijo. Da bi se citokrom c lahko povezal v apoptosom se mora prekiniti tudi vez s kardiolipinom, na katerega je citokrom c vezan. Oksidacija kardiolipina, prikazana na sliki 27 pa pokaže, da DFO ne vpliva na stopnjo njegove oksidacije. Rezultat je v skladu s našimi pričakovanji, saj DFO deluje v lizosomih in nima posebnega vpliva na mitohondrije. Ima pa očiten vpliv na oksidacijo kardiolipina Tempol, saj kot je razvidno s slike znatno prepreči oksidacijo kardiolipina. Popolnoma oksidacija ni preprečena, ampak tudi nastanka vseh ROS z uporabo Tempola nismo preprečili (Slika 19). Z določitvijo oksidacije kardiolipina lahko pojasnimo dejstvo, da se delež poškodb mitohondrijev ni ujemal s končnim deležem apoptoze. Namreč tudi če so bili mitohondriji poškodovani, a se kardiolipin ni oksidiral in s tem prekinil povezavo s citokromom c, se ta ni mogel povezati v apoptosom in aktivirati kaspaze 9. Preverili smo tudi aktivnost izvršitvenih kaspaz v prisotnosti DFO-ja in Tempola (Slika 29). Učinek DFO-ja je primerjiv z njegovim učinkom na mitohondrije in lizosome tako v skladu z našimi pričakovanji. Povsem drugače pa je z delovanjem Tempola na kaspaze, saj prepreči kar 88 % kaspazne aktivnosti. Čeprav se ta rezultat sklada z rezultati o oksidaciji kardiolipina in posledično aktivacijo kaspaz, pa ne pojasni nesorazmerja med kaspazno aktivnostjo in deležem apoptotskih celic. Možna razlaga je ta, da se zaradi poškodb mitohondrijev sprožijo alternativni mehanizmi celične smrti, ki so od kaspaz neodvisni. Celica ima varovalne mehanizme, ki lahko sprožijo apoptozo tudi kadar kaspaze odpovedo. Največkrat se kot mehanizmi od kaspaz neodvisne poti omenja katepsine, kalpaine, druge proteaze in proapoptotske faktorje sproščene iz mitohondrijev (Endo G, AIF, Omi), ki sprožijo od kaspaz neodvisno celično smrt. Proučevanje podrobnega mehanizma apoptoze ob prisotnosti Tempola se tekom te študije nismo lotili, vsekakor pa se ponujajo nadaljnje možnosti raziskav v tej tematiki.

V drugem delu smo preverjali pomen katepsinov pri apoptozi v človeških celičnih linijah. Za primerjavo smo izbrali celične linije, pri katerih smo določili različen nivo katepsinske aktivnosti (Slika 30). V vseh izbranih celičnih linijah lahko sprožimo apoptozo z ligandom TRAIL. S sočasno uporabo bortezomiba, ki je inhibitor proteasoma, pa smo občutljivost celic še povečali. Za normalno delovanje celice je potrebna homeostaza, pri čemer igra proteasom pomembno vlogo, zato lahko z inhibicijo njegovega delovanja vplivamo na delovanje celice. Med drugim pride do sprememb v

celičnem ciklu, aktivaciji kinaz in celo v neposredni sprožitvi apoptoze (Adams in sod., 1999). Poleg tega se po tretiranju celic z bortezomibom poveča količina receptorjev za ligand TRAIL (DR4 in DR5) na površini celice (Liu in sod., 2007). Večje število receptorjev bi lahko imelo močan vpliv na jakost apoptotskega signala v celici. Raziskave so pokazale tudi povišano koncentracijo kaspaze 8 pri apoptozi sproženi z ligandom TRAIL, če so bile celice predhodno tretirane z bortezomibom (Ganten in sod., 2005). Verjetno bortezomib vpliva na apoptotsko signaliziranje na več nivojih in ravno zato poveča občutljivost rakavih celic za ligand TRAIL. Bortezomib smo dodali v koncentracijah, v katerih sam ni imel vpliva na proces apoptoze, hkrati pa je povečal občutljivost celic za ligand TRAIL.

Vpliv katepsinov na potek apoptoze v izbranih celičnih linijah smo ugotavljali z uporabo njihovih inhibitorjev, in sicer E-64d, ki je splošni inhibitor cisteinskih katepsinov, in CA-074Me, ki je specifičen inhibitor katepsina B. Poleg tega smo kot kontrolo za inhibicijo apoptoze uporabili inhibitor kaspaz Z-VAD-FMK. Inhibicija katepsinov ni znižala nivoja apoptoze pri izbranih celičnih linijah, medtem ko smo z inhibicijo kaspaz apoptozo preprečili (Slika 32). Iz tega lahko sklepamo, da katepsini ne igrajo pomembne vloge pri sprožitvi apoptoze z ligandom TRAIL. Več raziskav sicer pripisuje katepsinom vlogo v procesu apoptoze (Foghsgaard in sod., 2001; Nagaraj in sod., 2006), toda verjetno so katepsini vpleteni v ojačitev apoptoznega signala, saj naši rezultati kažejo, da katepsini ne vplivajo na samo sprožitev apoptoze. Internalizacija receptorjev v endosomih/lizosomih bi lahko značilno vplivala na potek znotrajceličnega signaliziranja pri apoptozi (Repnik in sod., 2012). Pri apoptozi sproženi z ligandoma Fas in TNFα so ugotovili, da je za učinkovito tvorbo kompleksa DISC potrebna internalizacija receptorjev Fas in TNFR1 (Schutze in sod., 2008), medtem ko njihova internalizacija ni bila potrebna za sprožitev apoptoze z ligandom TRAIL (Kohlhaas in sod., 2007). Pri našem delu nismo preverili ali pride do internalizacije receptorjev, vendar če bi katepsini igrali pomembno vlogo pri sprožitvi apoptoze z ligandom TRAIL, bi inhibicija njihove aktivnosti imela vpliv na proces apoptoze. Toda inhibitorji katepsinov v vseh izbranih celičnih linijah z različnimi nivoji aktivnosti katepsinov niso preprečili sprožitve apoptoze. Zaradi tesne povezave katepsinov z lizosomi smo preverili tudi kako inhibicija aktivnosti katepsinov v teh celičnih linijah vpliva na aktivnost kaspaz in stabilnost mitohondrijske in lizosomske membrane (Slike 33, 34 in 35). Potrdili smo vpletenost kaspaz v apoptotske procese v izbranih celičnih linijah, saj smo z njihovo inhibicijo preprečili poškodbe mitohondrijev in lizosomov. Inhibicija katepsinske aktivnosti ni imela vpliva na stabilnost mitohondrijev in lizosomov, zato je verjetno, da katepsini tudi pri teh procesih ne igrajo ključne vloge. Z meritvami katepsinske aktivnosti v citosolu (Slika 36) smo potrdili, da se pri apoptozi sproženi z ligandom TRAIL v izbranih človeških linijah po poškodbah lizosomov iz njih v citosol sprostijo katepsini in so v citosolu tudi aktivni. Proteolitična aktivnost katepsinov v citosolu ni ključno za apoptozo, saj njena inhibicija ne spremeni poteka apoptoze. Tako lahko zaključimo, da katepsini niso ključni v nobeni stopnji procesa apoptoze, ki jo sproži ligand TRAIL pri izbranih človeških celicah.

Sirtuini imajo pomembno vlogo pri celičnem odzivu na stres. Zaznavajo spremembe v celici in uravnavajo celični odziv nanje. Med tarčami sirtuinov sta tudi proteina pomembna pri avtofagiji Atg 5 in Atg 7 (Lee in sod., 2008). Pokazano je bilo, da pri različnih mehanizmih, v katerih sodelujejo sirtuini, pride sočasno tudi do poškodb lizosomske membrane (Patschan in sod., 2008a; Patschan in sod., 2008b). V študiji so

sodelavci pokazali, da permeabilizacija lizosomske membrane povzroči znižanje količine proteina Sirt1, z uporabo inhibitorja katepsinov E-64d pa so močno znižali senescenco in občutljivost celic na oksidativni stres (Chen in sod., 2012). Nas je zanimalo, ali so sirtuini lahko substrati katepsinov in do upada količine sirtuinov kot posledice permeabilizacije lizosomske membrane pride zaradi njihove razgradnje s katepsini . Izbrali smo katepsina B in L, ker sta najbolj zastopana katepsina v celicah, ter katepsin S, ki je najbolj stabilen pri nevtralnem pH. Vsi trije izbrani katepsini *in vitro* cepijo sirtuine (Slika 39). Po pričakovanjih smo najintenzivnejšo razgradnjo opazili pri katepsinu S, ki ima sicer podoben optimalen pH delovanja kot katepsin L (pH 6), vendar je katepsin S bolj stabilen pri nevtralnem pH. Katepsina B in L sta v našem primeru slabše cepila sirtuine, tudi to je pričakovano, saj sta njuna optimalna pH delovanja nižja kot pri katepsinu S. V cepitvenih analizah smo uporabili rekombinantne sirtuine v fuziji s GST. Da bi potrdili, da katepsini niso le cepili povezave med sirtuini in sondo, smo opravili še analize s prenosom western in uporabo protiteles proti GST. Zgolj cepitev na mestu med proteinom in sondo bi se pokazala kot nastanek novega GST fragmenta in ustrezno velikega fragmenta sirtuina. Lise, ki so višje od samega GST pomenijo na protein vezan GST in vsaka lisa nižja od celega proteina z GST pomeni vezan GST na delno razgrajen protein. Pojav novih lis pri teh nepopolnih cepitvah je le še dodaten dokaz, da gre za cepitve v predelu proteina, in ne le odcepitev sonde GST. Pri delovanju katepsina S je jasno, da je prišlo do popolne razgradnje proteina, deloma pa je katepsin S razgradil tudi samo sondo GST (Slika 40 C). Katepsina B in L popolnoma razgradita Sirt5, medtem ko je cepitev Sirt1 in Sirt2 nepopolna. Zmanjšanje količine sirtuinov ob permeabilizaciji lizosomske membrane je torej lahko posledica razgradnje s sproščenimi katepsini.

Poškodbe lizosomov so lahko posledica delovanje stresnih dejavnikov nanje, med njimi reaktivnih kisikovih spojin. Okvarjeno delovanje lizosomov vpliva tudi na avtofagijo, saj je onemogočen zadnji korak pri recikliranju avtofagosomov. Če pri tem pride še do sproščanja katepsinov v citosol, kjer lahko katepsini aktivirajo Bid ali razgradijo antiapoptotske dejavnike družine Bcl-2, se lahko v celici namesto avtofagije sproži apoptoza. Dokaz, da je v celicah razgradnja posledica delovanja katepsinov in ne drugih proteaz, je bil dodatek inhibitorja katepsinov E-64d, ki je preprečil razgradnjo sirtuinov. V študiji smo dodatno *in vitro* neposredno pokazali, da so tudi sirtuini lahko substrati katepsinov. Poleg tega so poskusi na celicah z izbitim genom za sirtuin 1 pokazali, da imajo sirtuini vlogo pri avtofagiji. Te celice so imele namreč več aktiviranih kaspaz od celic divjega tipa. Tako lahko poškodba lizosomov in sproščanje katepsinov v citosol privedeta do cepitev sirtuinov, ti ne morejo deacetilirati svojih tarč (npr. Atg5 in Atg 7) in v celici se namesto avtofagije sproži apoptoza.

6 Zaključki

Doktorska disertacija doprinaša nova spoznanja k razumevanju mehanizma poškod lizomov pri apoptozi, sproženi z ligandom TRAIL. Pokazali smo, da lahko z ligandom TRAIL sprožimo apoptozo v celicah mišjih embrionalnih fibroblastov. Pri tem pride najprej do poškodbe mitohondrijske membrane, poškodba lizosomske membrane pa je pozni dogodek. Večina raziskav je dosedaj predvidevala obraten vrstni red. Poškodbe obeh organelov povezujejo reaktivne kisikove zvrsti (ROS), ki se sprostijo ob poškodbi mitohondrijev in posledično poškodujejo lizosomsko membrano. Zaradi te poškodbe se v citosol sprostijo katepsini, ki lahko preko cepitve proteina Bid še dodatno vplivajo na poškodbo mitohondrijske membrane. Hkrati imajo katepsini pomemben učinek na potek apoptoze po poškodbi mitohondrijev, kar smo pokazali pri celicah z izbitom genom za katepsin B. Povezava med mitohondriji in lizosomi predstavlja ojačitveno zanko v mehanizmu apoptoze.

Pri človeških celičnih linijah smo z uporabo inhibitorjev proteaz ovrgli ključno vlogo katepsinov pri sprožitvi apoptoze, saj je inhibicija katepsinov ni preprečila. Njihove vloge pri ojačitvi apoptotskega signala ne gre izključiti.

Oksidativni stres lahko povzroči sproščanje katepsinov iz lizosomov. V citosolu celice bi lahko katepsini s cepitvami sirtuinov povzročili, da bi lahko namesto avtofagije v celici potekla apoptoza. *In vitro* smo dokazali, da lahko katepsini B, L in S cepijo sirtuine.

7 Zahvale

Prvi odstavek tekočega (pod)poglavja – prikaz oblike. / First paragraph in current heading – format presentation. (Format: Paragraph_First)

Naslednji odstavek tekočega (pod)poglavja – prikaz oblike. / Next paragraph in current heading – format presentation. (Format: Paragraph_Next)

8 Literatura in viri

Abrahamson, M.; Barrett, A. J.; Salvesen, G.; Grubb, A. Isolation of six cysteine proteinase inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids. *The Journal of Biological Chemistry* **261**, 11282–11289 (1986).

Adams, J.; Palombella, V. J.; Sausville, E. A.; Johnson, J.; Destree, A.; Lazarus, D. D.; Maas, J.; Pien, C. S.; Prakash, S.; Elliott, P. J. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Research* **59**, 2615–2622 (1999).

Alenzi, F. Q.; Lotfy, M.; Wyse, R. Swords of cell death: caspase activation and regulation. *Asian Pacific Journal of Cancer Prevention : APJCP* **11**, 271–280 (2010).

Alnemri, E. S.; Livingston, D. J.; Nicholson, D. W.; Salvesen, G.; Thornberry, N. A.; Wong, W. W.; Yuan, J. Human ICE/CED-3 protease nomenclature. *Cell* **87**, 171 (1996).

Asagiri, M.; Takayanagi, H. The molecular understanding of osteoclast differentiation. *Bone* **40**, 251–264 (2007).

Ashkenazi, A.; Dixit, V. M. Death receptors: signaling and modulation. *Science* **281**, 1305–1308 (1998).

Barrett, A. J.; Fritz, H.; Grubb, A.; Isemura, S.; Jarvinen, M.; Katunuma, N.; Machleidt, W.; Muller-Esterl, W.; Sasaki, M.; Turk, V. Nomenclature and classification of the proteins homologous with the cysteine-proteinase inhibitor chicken cystatin. *The Biochemical Journal* **236**, 312 (1986).

Baskin-Bey, E. S.; Canbay, A.; Bronk, S. F.; Werneburg, N.; Guicciardi, M. E.; Nyberg, S. L.; Gores, G. J. Cathepsin B inactivation attenuates hepatocyte apoptosis and liver damage in steatotic livers after cold ischemia-warm reperfusion injury. *American Journal of Physiology Gastrointestinal and Liver Physiology* **288**, G396–402 (2005).

Berg, T.; Gjoen, T.; Bakke, O. Physiological functions of endosomal proteolysis. *The Biochemical Journal* **307** (Pt 2), 313–326 (1995).

Bidere, N.; Lorenzo, H. K.; Carmona, S.; Laforge, M.; Harper, F.; Dumont, C.; Senik, A. Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis. *The Journal of Biological Chemistry* **278**, 31401–31411 (2003).

Blomgran, R.; Zheng, L.; Stendahl, O. Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. *Journal of Leukocyte Biology* **81**, 1213–1223 (2007).

Bojic, L.; Petelin, A.; Stoka, V.; Reinheckel, T.; Peters, C.; Turk, V.; Turk, B. Cysteine cathepsins are not involved in Fas/CD95 signalling in primary skin fibroblasts. *FEBS Letters* **581**, 5185–5190 (2007).

Boldin, M. P.; Mett, I. L.; Varfolomeev, E. E.; Chumakov, I.; Shemer-Avni, Y.; Camonis, J. H.; Wallach, D. Self-association of the "death domains" of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. *The Journal of Biological Chemistry* **270**, 387–391 (1995).

Bosch-Presegue, L.; Vaquero, A. The dual role of sirtuins in cancer. *Genes & Cancer* **2**, 648–662 (2011).

Boya, P.; Kroemer, G. Lysosomal membrane permeabilization in cell death. *Oncogene* **27**, 6434–6451 (2008).

Bratton, S. B.; Salvesen, G. S. Regulation of the Apaf-1-caspase-9 apoptosome. *Journal of Cell Science* **123**, 3209–3214 (2010).

Bredesen, D. E. Programmed cell death mechanisms in neurological disease. *Current Molecular Medicine* **8**, 173–186 (2008).

Brenner, D.; Mak, T. W. Mitochondrial cell death effectors. *Current Opinion in Cell Biology* **21**, 871–877 (2009).

Brix, K.; Dunkhorst, A.; Mayer, K.; Jordans, S. Cysteine cathepsins: cellular roadmap to different functions. *Biochimie* **90**, 194–207 (2008).

Broker, L. E.; Kruyt, F. A.; Giaccone, G. Cell death independent of caspases: a review. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* **11**, 3155–3162 (2005).

Brunk, U. T.; Neuzil, J.; Eaton, J. W. Lysosomal involvement in apoptosis. *Redox Report : communications in free radical research* **6**, 91–97 (2001).

Cauwels, A.; Janssen, B.; Waeytens, A.; Cuvelier, C.; Brouckaert, P. Caspase inhibition causes hyperacute tumor necrosis factor-induced shock via oxidative stress and phospholipase A2. *Nature Immunology* **4**, 387–393 (2003).

Cerretti, D. P.; Kozlosky, C. J.; Mosley, B.; Nelson, N.; Van Ness, K.; Greenstreet, T. A.; March, C. J.; Kronheim, S. R.; Druck, T.; Cannizzaro, L. A.; et al. Molecular cloning of the interleukin-1 beta converting enzyme. *Science* **256**, 97–100 (1992).

Ceru, S.; Konjar, S.; Maher, K.; Repnik, U.; Krizaj, I.; Bencina, M.; Renko, M.; Nepveu, A.; Zerovnik, E.; Turk, B.; Kopitar-Jerala, N. Stefin B interacts with histones and cathepsin L in the nucleus. *The Journal of Biological Chemistry* **285**, 10078–10086 (2010).

Chan, F. K.; Chun, H. J.; Zheng, L.; Siegel, R. M.; Bui, K. L.; Lenardo, M. J. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* **288**, 2351–2354 (2000a).

Chan, K. F.; Siegel, M. R.; Lenardo, J. M. Signaling by the TNF receptor superfamily and T cell homeostasis. *Immunity* **13**, 419–422 (2000b).

Chen, J.; Xavier, S.; Moskowitz-Kassai, E.; Chen, R.; Lu, C. Y.; Sanduski, K.; Spes, A.; Turk, B.; Goligorsky, M. S. Cathepsin Cleavage of Sirtuin-1 in Endothelial Progenitor Cells Mediates Stress-Induced Premature Senescence. *The American journal of pathology* (2012).

Chipuk, J. E.; Moldoveanu, T.; Llambi, F.; Parsons, M. J.; Green, D. R. The BCL-2 family reunion. *Molecular Cell* **37**, 299–310 (2010).

Chowdhury, I.; Tharakan, B.; Bhat, G. K. Caspases - an update. *Comparative Biochemistry and Physiology Part B, Biochemistry & Molecular Biology* **151**, 10–27 (2008).

Chun, H. J.; Zheng, L.; Ahmad, M.; Wang, J.; Speirs, C. K.; Siegel, R. M.; Dale, J. K.; Puck, J.; Davis, J.; Hall, C. G.; Skoda-Smith, S.; Atkinson, T. P.; Straus, S. E.; Lenardo, M. J. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature* **419**, 395–399 (2002).

Cirman, T.; Oresic, K.; Mazovec, G. D.; Turk, V.; Reed, J. C.; Myers, R. M.; Salvesen, G.

S.; Turk, B. Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. *The Journal of Biological Chemistry* **279**, 3578–3587 (2004).

Claus, V.; Jahraus, A.; Tjelle, T.; Berg, T.; Kirschke, H.; Faulstich, H.; Griffiths, G. Lysosomal enzyme trafficking between phagosomes, endosomes, and lysosomes in J774 macrophages. Enrichment of cathepsin H in early endosomes. *The Journal of Biological Chemistry* **273**, 9842–9851 (1998).

Conus, S.; Perozzo, R.; Reinheckel, T.; Peters, C.; Scapozza, L.; Yousefi, S.; Simon, H. U. Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation. *The Journal of Experimental Medicine* **205**, 685–698 (2008).

Conus, S.; Simon, H. U. Cathepsins: key modulators of cell death and inflammatory responses. *Biochemical Pharmacology* **76**, 1374–1382 (2008).

Dahl, S. W.; Halkier, T.; Lauritzen, C.; Dolenc, I.; Pedersen, J.; Turk, V.; Turk, B. Human recombinant pro-dipeptidyl peptidase I (cathepsin C) can be activated by cathepsins L and S but not by autocatalytic processing. *Biochemistry* **40**, 1671–1678 (2001).

Degli-Esposti, M. A.; Dougall, W. C.; Smolak, P. J.; Waugh, J. Y.; Smith, C. A.; Goodwin, R. G. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* **7**, 813–820 (1997a).

Degli-Esposti, M. A.; Smolak, P. J.; Walczak, H.; Waugh, J.; Huang, C. P.; DuBose, R. F.; Goodwin, R. G.; Smith, C. A. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *The Journal of Experimental Medicine* **186**, 1165–1170 (1997b).

Deiss, L. P.; Galinka, H.; Berissi, H.; Cohen, O.; Kimchi, A. Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. *The EMBO Journal* **15**, 3861–3870 (1996).

Droga-Mazovec, G.; Bojic, L.; Petelin, A.; Ivanova, S.; Romih, R.; Repnik, U.; Salvesen, G. S.; Stoka, V.; Turk, V.; Turk, B. Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues. *The Journal of Biological Chemistry* **283**, 19140–19150 (2008).

Earnshaw, W. C.; Martins, L. M.; Kaufmann, S. H. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annual Review of Biochemistry* **68**, 383–424 (1999).

Eckelman, B. P.; Salvesen, G. S.; Scott, F. L. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Reports* **7**, 988–994 (2006).

Ellis, H. M.; Horvitz, H. R. Genetic control of programmed cell death in the nematode C. elegans. *Cell* **44**, 817–829 (1986).

Enari, M.; Sakahira, H.; Yokoyama, H.; Okawa, K.; Iwamatsu, A.; Nagata, S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**, 43–50 (1998).

Eskelinen, E. L. Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. *Molecular Aspects of Medicine* **27**, 495–502 (2006).

Feldstein, A. E.; Werneburg, N. W.; Canbay, A.; Guicciardi, M. E.; Bronk, S. F.; Rydzewski, R.; Burgart, L. J.; Gores, G. J. Free fatty acids promote hepatic lipotoxicity by stimulating TNF-alpha expression via a lysosomal pathway.

Hepatology **40**, 185–194 (2004).

Feldstein, A. E.; Werneburg, N. W.; Li, Z.; Bronk, S. F.; Gores, G. J. Bax inhibition protects against free fatty acid-induced lysosomal permeabilization. *American Journal of Physiology Gastrointestinal and Liver Physiology* **290**, G1339–1346 (2006).

Ferri, K. F.; Kroemer, G. Organelle-specific initiation of cell death pathways. *Nature Cell Biology* **3**, E255–263 (2001).

Finnberg, N.; Gruber, J. J.; Fei, P.; Rudolph, D.; Bric, A.; Kim, S. H.; Burns, T. F.; Ajuha, H.; Page, R.; Wu, G. S.; Chen, Y.; McKenna, W. G.; Bernhard, E.; Lowe, S.; Mak, T.; El-Deiry, W. S. DR5 knockout mice are compromised in radiation-induced apoptosis. *Molecular and Cellular Biology* **25**, 2000–2013 (2005).

Foghsgaard, L.; Wissing, D.; Mauch, D.; Lademann, U.; Bastholm, L.; Boes, M.; Elling, F.; Leist, M.; Jaattela, M. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *The Journal of Cell Biology* **153**, 999–1010 (2001).

French, L. E.; Tschopp, J. Protein-based therapeutic approaches targeting death receptors. *Cell Death and Differentiation* **10**, 117–123 (2003).

Ganten, T. M.; Koschny, R.; Haas, T. L.; Sykora, J.; Li-Weber, M.; Herzer, K.; Walczak, H. Proteasome inhibition sensitizes hepatocellular carcinoma cells, but not human hepatocytes, to TRAIL. *Hepatology* **42**, 588–597 (2005).

Golstein, P. Cell death: TRAIL and its receptors. *Current Biology : CB* **7**, R750–753 (1997).

Guarente, L. Franklin H. Epstein Lecture: Sirtuins, aging, and medicine. *The New England Journal of Medicine* **364**, 2235–2244 (2011).

Guicciardi, M. E.; Deussing, J.; Miyoshi, H.; Bronk, S. F.; Svingen, P. A.; Peters, C.; Kaufmann, S. H.; Gores, G. J. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. *The Journal of Clinical Investigation* **106**, 1127–1137 (2000).

Guicciardi, M. E.; Gores, G. J. Life and death by death receptors. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* **23**, 1625–1637 (2009).

Guicciardi, M. E.; Leist, M.; Gores, G. J. Lysosomes in cell death. *Oncogene* **23**, 2881–2890 (2004).

Guicciardi, M. E.; Miyoshi, H.; Bronk, S. F.; Gores, G. J. Cathepsin B knockout mice are resistant to tumor necrosis factor-alpha-mediated hepatocyte apoptosis and liver injury: implications for therapeutic applications. *The American Journal of Pathology* **159**, 2045–2054 (2001).

Gutierrez, G. J.; Ronai, Z. Ubiquitin and SUMO systems in the regulation of mitotic checkpoints. *Trends in Biochemical Sciences* **31**, 324–332 (2006).

Gyrd-Hansen, M.; Farkas, T.; Fehrenbacher, N.; Bastholm, L.; Hoyer-Hansen, M.; Elling, F.; Wallach, D.; Flavell, R.; Kroemer, G.; Nylandsted, J.; Jaattela, M. Apoptosome-independent activation of the lysosomal cell death pathway by caspase-9. *Molecular and Cellular Biology* **26**, 7880–7891 (2006).

Haigis, M. C.; Guarente, L. P. Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction. *Genes & Development* **20**, 2913–2921 (2006).

Heinrich, M.; Neumeyer, J.; Jakob, M.; Hallas, C.; Tchikov, V.; Winoto-Morbach, S.; Wickel, M.; Schneider-Brachert, W.; Trauzold, A.; Hethke, A.; Schutze, S. Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3

activation. *Cell Death and Differentiation* **11**, 550–563 (2004).

Helfer, B.; Boswell, B. C.; Finlay, D.; Cipres, A.; Vuori, K.; Bong Kang, T.; Wallach, D.; Dorfleutner, A.; Lahti, J. M.; Flynn, D. C.; Frisch, S. M. Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions. *Cancer Research* **66**, 4273–4278 (2006).

Houseweart, M. K.; Vilaythong, A.; Yin, X. M.; Turk, B.; Noebels, J. L.; Myers, R. M. Apoptosis caused by cathepsins does not require Bid signaling in an in vivo model of progressive myoclonus epilepsy (EPM1). *Cell Death and Differentiation* **10**, 1329–1335 (2003).

Hsing, L. C.; Rudensky, A. Y. The lysosomal cysteine proteases in MHC class II antigen presentation. *Immunological Reviews* **207**, 229–241 (2005).

Ivanova, S.; Gregorc, U.; Vidergar, N.; Javier, R.; Bredt, D. S.; Vandenabeele, P.; Pardo, J.; Simon, M. M.; Turk, V.; Banks, L.; Turk, B. MAGUKs, scaffolding proteins at cell junctions, are substrates of different proteases during apoptosis. *Cell Death & Disease* **2**, e116 (2011).

Jiang, X.; Wang, X. Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *The Journal of Biological Chemistry* **275**, 31199–31203 (2000).

Johnson, T. R.; Stone, K.; Nikrad, M.; Yeh, T.; Zong, W. X.; Thompson, C. B.; Nesterov, A.; Kraft, A. S. The proteasome inhibitor PS-341 overcomes TRAIL resistance in Bax and caspase 9-negative or Bcl-xL overexpressing cells. *Oncogene* **22**, 4953–4963 (2003).

Jones, A. L.; Hulett, M. D.; Parish, C. R. Histidine-rich glycoprotein: A novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems. *Immunology and Cell Biology* **83**, 106–118 (2005).

Kagedal, K.; Johansson, A. C.; Johansson, U.; Heimlich, G.; Roberg, K.; Wang, N. S.; Jurgensmeier, J. M.; Ollinger, K. Lysosomal membrane permeabilization during apoptosis--involvement of Bax? *International Journal of Experimental Pathology* **86**, 309–321 (2005).

Kagedal, K.; Johansson, U.; Ollinger, K. The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* **15**, 1592–1594 (2001a).

Kagedal, K.; Zhao, M.; Svensson, I.; Brunk, U. T. Sphingosine-induced apoptosis is dependent on lysosomal proteases. *The Biochemical Journal* **359**, 335–343 (2001b).

Kang, T. B.; Ben-Moshe, T.; Varfolomeev, E. E.; Pewzner-Jung, Y.; Yogev, N.; Jurewicz, A.; Waisman, A.; Brenner, O.; Haffner, R.; Gustafsson, E.; Ramakrishnan, P.; Lapidot, T.; Wallach, D. Caspase-8 serves both apoptotic and nonapoptotic roles. *Journal of Immunology* **173**, 2976–2984 (2004).

Kang, T. B.; Oh, G. S.; Scandella, E.; Bolinger, B.; Ludewig, B.; Kovalenko, A.; Wallach, D. Mutation of a self-processing site in caspase-8 compromises its apoptotic but not its nonapoptotic functions in bacterial artificial chromosome-transgenic mice. *Journal of Immunology* **181**, 2522–2532 (2008).

Khattab, M. M. TEMPOL, a membrane-permeable radical scavenger, attenuates peroxynitrite- and superoxide anion-enhanced carrageenan-induced paw edema and hyperalgesia: a key role for superoxide anion. *European Journal of Pharmacology* **548**, 167–173 (2006).

Kim, H.; Tu, H. C.; Ren, D.; Takeuchi, O.; Jeffers, J. R.; Zambetti, G. P.; Hsieh, J. J.; Cheng, E. H. Stepwise activation of BAX and BAK by tBID, BIM, and PUMA

initiates mitochondrial apoptosis. *Molecular Cell* **36**, 487–499 (2009).

Kirschke, H.; Wiederanders, B.; Bromme, D.; Rinne, A. Cathepsin S from bovine spleen. Purification, distribution, intracellular localization and action on proteins. *The Biochemical Journal* **264**, 467–473 (1989).

Klaric, M.; Tao, S.; Stoka, V.; Turk, B.; Turk, V. Cysteine cathepsins are not critical for TNF-alpha-induced cell death in T98G and U937 cells. *Biochimica et Biophysica Acta* **1794**, 1372–1377 (2009).

Koblinski, J. E.; Ahram, M.; Sloane, B. F. Unraveling the role of proteases in cancer. *Clinica Chimica Acta; International Journal of Clinical Chemistry* **291**, 113–135 (2000).

Kohlhaas, S. L.; Craxton, A.; Sun, X. M.; Pinkoski, M. J.; Cohen, G. M. Receptor-mediated endocytosis is not required for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. *The Journal of Biological Chemistry* **282**, 12831–12841 (2007).

Korsmeyer, S. J.; Wei, M. C.; Saito, M.; Weiler, S.; Oh, K. J.; Schlesinger, P. H. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death and Differentiation* **7**, 1166–1173 (2000).

Kothakota, S.; Azuma, T.; Reinhard, C.; Klippel, A.; Tang, J.; Chu, K.; McGarry, T. J.; Kirschner, M. W.; Koths, K.; Kwiatkowski, D. J.; Williams, L. T. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278**, 294–298 (1997).

Kroemer, G.; Galluzzi, L.; Vandenabeele, P.; Abrams, J.; Alnemri, E. S.; Baehrecke, E. H.; Blagosklonny, M. V.; El-Deiry, W. S.; Golstein, P.; Green, D. R.; Hengartner, M.; Knight, R. A.; Kumar, S.; Lipton, S. A.; Malorni, W.; Nunez, G.; Peter, M. E.; Tschopp, J.; Yuan, J.; Piacentini, M.; Zhivotovsky, B.; Melino, G. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death and Differentiation* **16**, 3–11 (2009).

Kuang, A. A.; Diehl, G. E.; Zhang, J.; Winoto, A. FADD is required for DR4- and DR5-mediated apoptosis: lack of trail-induced apoptosis in FADD-deficient mouse embryonic fibroblasts. *The Journal of Biological Chemistry* **275**, 25065–25068 (2000).

Kuwana, T.; Mackey, M. R.; Perkins, G.; Ellisman, M. H.; Latterich, M.; Schneiter, R.; Green, D. R.; Newmeyer, D. D. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111**, 331–342 (2002).

Labbe, K.; Saleh, M. Cell death in the host response to infection. *Cell Death and Differentiation* **15**, 1339–1349 (2008).

Lamkanfi, M.; Declercq, W.; Kalai, M.; Saelens, X.; Vandenabeele, P. Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death and Differentiation* **9**, 358–361 (2002).

Lee, I. H.; Cao, L.; Mostoslavsky, R.; Lombard, D. B.; Liu, J.; Bruns, N. E.; Tsokos, M.; Alt, F. W.; Finkel, T. A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 3374–3379 (2008).

Leist, M.; Jaattela, M. Four deaths and a funeral: from caspases to alternative mechanisms. *Nature Reviews Molecular Cell Biology* **2**, 589–598 (2001).

Linnevers, C.; Smeekens, S. P.; Bromme, D. Human cathepsin W, a putative cysteine protease predominantly expressed in CD8+ T-lymphocytes. *FEBS Letters* **405**, 253–

259 (1997).

Liu, N.; Raja, S. M.; Zazzeroni, F.; Metkar, S. S.; Shah, R.; Zhang, M.; Wang, Y.; Bromme, D.; Russin, W. A.; Lee, J. C.; Peter, M. E.; Froelich, C. J.; Franzoso, G.; Ashton-Rickardt, P. G. NF-kappaB protects from the lysosomal pathway of cell death. *The EMBO Journal* **22**, 5313–5322 (2003).

Liu, X.; Yue, P.; Chen, S.; Hu, L.; Lonial, S.; Khuri, F. R.; Sun, S. Y. The proteasome inhibitor PS-341 (bortezomib) up-regulates DR5 expression leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. *Cancer Research* **67**, 4981–4988 (2007).

Locksley, R. M.; Killeen, N.; Lenardo, M. J. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**, 487–501 (2001).

Lutgens, S. P.; Cleutjens, K. B.; Daemen, M. J.; Heeneman, S. Cathepsin cysteine proteases in cardiovascular disease. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* **21**, 3029–3041 (2007).

Luzio, J. P.; Pryor, P. R.; Bright, N. A. Lysosomes: fusion and function. *Nature Reviews Molecular Cell Biology* **8**, 622–632 (2007).

Mantel, C.; Broxmeyer, H. E. Sirtuin 1, stem cells, aging, and stem cell aging. *Current Opinion in Hematology* **15**, 326–331 (2008).

Martinon, F.; Tschopp, J. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death and Differentiation* **14**, 10–22 (2007).

Maubach, G.; Lim, M. C.; Zhuo, L. Nuclear cathepsin F regulates activation markers in rat hepatic stellate cells. *Molecular Biology of the Cell* **19**, 4238–4248 (2008).

Nagaraj, N. S.; Vigneswaran, N.; Zacharias, W. Cathepsin B mediates TRAIL-induced apoptosis in oral cancer cells. *Journal of Cancer Research and Clinical Oncology* **132**, 171–183 (2006).

Newsom-Davis, T.; Prieske, S.; Walczak, H. Is TRAIL the holy grail of cancer therapy? *Apoptosis : An International Journal on Programmed Cell Death* **14**, 607–623 (2009).

Oberle, C.; Huai, J.; Reinheckel, T.; Tacke, M.; Rassner, M.; Ekert, P. G.; Buellesbach, J.; Borner, C. Lysosomal membrane permeabilization and cathepsin release is a Bax/Bak-dependent, amplifying event of apoptosis in fibroblasts and monocytes. *Cell Death and Differentiation* **17**, 1167–1178 (2010).

Oberst, A.; Pop, C.; Tremblay, A. G.; Blais, V.; Denault, J. B.; Salvesen, G. S.; Green, D. R. Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. *The Journal of Biological Chemistry* **285**, 16632–16642 (2010).

Ollinger, K. Inhibition of cathepsin D prevents free-radical-induced apoptosis in rat cardiomyocytes. *Archives of Biochemistry and Biophysics* **373**, 346–351 (2000).

Pan, G.; Ni, J.; Wei, Y. F.; Yu, G.; Gentz, R.; Dixit, V. M. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277**, 815–818 (1997).

Patschan, S.; Chen, J.; Gealekman, O.; Krupincza, K.; Wang, M.; Shu, L.; Shayman, J. A.; Goligorsky, M. S. Mapping mechanisms and charting the time course of premature cell senescence and apoptosis: lysosomal dysfunction and ganglioside accumulation in endothelial cells. *American Journal of Physiology Renal Physiology* **294**, F100–109 (2008a).

Patschan, S.; Chen, J.; Polotskaia, A.; Mendelev, N.; Cheng, J.; Patschan, D.; Goligorsky, M. S. Lipid mediators of autophagy in stress–induced premature senescence of

endothelial cells. *American Journal of Physiology Heart and Circulatory Physiology* **294**, H1119–1129 (2008b).

Pereira, W. O.; Amarante-Mendes, G. P. Apoptosis: a programme of cell death or cell disposal? *Scandinavian Journal of Immunology* **73**, 401–407 (2011).

Petelin, A. Vloga in pomen cisteinskih katepsinov pri različnih modelih programirane celične smrti : doktorska disertacija = Role and significance of cysteine cathepsins in different models of apoptosis : doctoral dissertation. (Mednarodna podiplomska šola Jožefa Stefana, Ljubljana, 2009).

Petersen, S. L.; Wang, L.; Yalcin-Chin, A.; Li, L.; Peyton, M.; Minna, J.; Harran, P.; Wang, X. Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. *Cancer Cell* **12**, 445–456 (2007).

Pop, C.; Fitzgerald, P.; Green, D. R.; Salvesen, G. S. Role of proteolysis in caspase-8 activation and stabilization. *Biochemistry* **46**, 4398–4407 (2007).

Pop, C.; Salvesen, G. S. Human caspases: activation, specificity, and regulation. *The Journal of Biological Chemistry* **284**, 21777–21781 (2009).

Pungercar, J. R.; Caglic, D.; Sajid, M.; Dolinar, M.; Vasiljeva, O.; Pozgan, U.; Turk, D.; Bogyo, M.; Turk, V.; Turk, B. Autocatalytic processing of procathepsin B is triggered by proenzyme activity. *The FEBS Journal* **276**, 660–668 (2009).

Rastogi, R. P.; Richa; Sinha, R. P. Apoptosis: Molecular Mechanisms and Pathogenicity. *Excli Journal* **8**, 155–181 (2009).

Repnik, U.; Stoka, V.; Turk, V.; Turk, B. Lysosomes and lysosomal cathepsins in cell death. *Biochimica et Biophysica Acta* **1824**, 22–33 (2012).

Repnik, U.; Turk, B. Lysosomal-mitochondrial cross-talk during cell death. *Mitochondrion* **10**, 662–669 (2010).

Reynolds, J. L.; Skepper, J. N.; McNair, R.; Kasama, T.; Gupta, K.; Weissberg, P. L.; Jahnen-Dechent, W.; Shanahan, C. M. Multifunctional roles for serum protein fetuin-a in inhibition of human vascular smooth muscle cell calcification. *Journal of the American Society of Nephrology : JASN* **16**, 2920–2930 (2005).

Ribe, E. M.; Serrano-Saiz, E.; Akpan, N.; Troy, C. M. Mechanisms of neuronal death in disease: defining the models and the players. *The Biochemical Journal* **415**, 165–182 (2008).

Riccioni, R.; Pasquini, L.; Mariani, G.; Saulle, E.; Rossini, A.; Diverio, D.; Pelosi, E.; Vitale, A.; Chierichini, A.; Cedrone, M.; Foa, R.; Lo Coco, F.; Peschle, C.; Testa, U. TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL. *Haematologica* **90**, 612–624 (2005).

Riedl, S. J.; Salvesen, G. S. The apoptosome: signalling platform of cell death. *Nature Reviews Molecular Cell Biology* **8**, 405–413 (2007).

Roberg, K.; Kagedal, K.; Ollinger, K. Microinjection of cathepsin d induces caspase-dependent apoptosis in fibroblasts. *The American Journal of Pathology* **161**, 89–96 (2002).

Roberts, L. R.; Kurosawa, H.; Bronk, S. F.; Fesmier, P. J.; Agellon, L. B.; Leung, W. Y.; Mao, F.; Gores, G. J. Cathepsin B contributes to bile salt-induced apoptosis of rat hepatocytes. *Gastroenterology* **113**, 1714–1726 (1997).

Salvesen, G. S.; Riedl, S. J. Caspase mechanisms. *Advances in Experimental Medicine and Biology* **615**, 13–23 (2008).

Scaffidi, C.; Fulda, S.; Srinivasan, A.; Friesen, C.; Li, F.; Tomaselli, K. J.; Debatin, K.

M.; Krammer, P. H.; Peter, M. E. Two CD95 (APO-1/Fas) signaling pathways. *The EMBO Journal* **17**, 1675–1687 (1998).

Schoenmann, Z.; Assa-Kunik, E.; Tiomny, S.; Minis, A.; Haklai-Topper, L.; Arama, E.; Yaron, A. Axonal degeneration is regulated by the apoptotic machinery or a NAD+-sensitive pathway in insects and mammals. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience* **30**, 6375–6386 (2010).

Schotte, P.; Van Criekinge, W.; Van de Craen, M.; Van Loo, G.; Desmedt, M.; Grooten, J.; Cornelissen, M.; De Ridder, L.; Vandekerckhove, J.; Fiers, W.; Vandenabeele, P.; Beyaert, R. Cathepsin B-mediated activation of the proinflammatory caspase-11. *Biochemical and Biophysical Research Communications* **251**, 379–387 (1998).

Schutze, S.; Tchikov, V.; Schneider-Brachert, W. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. *Nature Reviews Molecular Cell Biology* **9**, 655–U655 (2008).

Shibata, M.; Kanamori, S.; Isahara, K.; Ohsawa, Y.; Konishi, A.; Kametaka, S.; Watanabe, T.; Ebisu, S.; Ishido, K.; Kominami, E.; Uchiyama, Y. Participation of cathepsins B and D in apoptosis of PC12 cells following serum deprivation. *Biochemical and Biophysical Research Communications* **251**, 199–203 (1998).

Snipas, S. J.; Drag, M.; Stennicke, H. R.; Salvesen, G. S. Activation mechanism and substrate specificity of the Drosophila initiator caspase DRONC. *Cell Death and Differentiation* **15**, 938–945 (2008).

Srinivasula, S. M.; Hegde, R.; Saleh, A.; Datta, P.; Shiozaki, E.; Chai, J.; Lee, R. A.; Robbins, P. D.; Fernandes-Alnemri, T.; Shi, Y.; Alnemri, E. S. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* **410**, 112–116 (2001).

Stennicke, H. R.; Ryan, C. A.; Salvesen, G. S. Reprieval from execution: the molecular basis of caspase inhibition. *Trends in Biochemical Sciences* **27**, 94–101 (2002).

Stoka, V.; Turk, B.; Schendel, S. L.; Kim, T. H.; Cirman, T.; Snipas, S. J.; Ellerby, L. M.; Bredesen, D.; Freeze, H.; Abrahamson, M.; Bromme, D.; Krajewski, S.; Reed, J. C.; Yin, X. M.; Turk, V.; Salvesen, G. S. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *The Journal of Biological Chemistry* **276**, 3149–3157 (2001).

Stoka, V.; Turk, V.; Turk, B. Lysosomal cysteine cathepsins: signaling pathways in apoptosis. *Biological Chemistry* **388**, 555–560 (2007).

Tait, S. W.; Green, D. R. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nature Reviews Molecular Cell Biology* **11**, 621–632 (2010).

Tenopoulou, M.; Doulias, P. T.; Barbouti, A.; Brunk, U.; Galaris, D. Role of compartmentalized redox-active iron in hydrogen peroxide-induced DNA damage and apoptosis. *The Biochemical Journal* **387**, 703–710 (2005).

Timmer, J. C.; Salvesen, G. S. Caspase substrates. *Cell Death and Differentiation* **14**, 66–72 (2007).

Tissenbaum, H. A.; Guarente, L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* **410**, 227–230 (2001).

Truneh, A.; Sharma, S.; Silverman, C.; Khandekar, S.; Reddy, M. P.; Deen, K. C.; McLaughlin, M. M.; Srinivasula, S. M.; Livi, G. P.; Marshall, L. A.; Alnemri, E. S.; Williams, W. V.; Doyle, M. L. Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. *The Journal of Biological Chemistry* **275**, 23319–23325 (2000).

Turk, B. Targeting proteases: successes, failures and future prospects. *Nature Reviews Drug Discovery* **5**, 785–799 (2006).

Turk, B.; Dolenc, I.; Turk, V.; Bieth, J. G. Kinetics of the pH-induced inactivation of human cathepsin L. *Biochemistry* **32**, 375–380 (1993).

Turk, B.; Dolenc, I.; Zerovnik, E.; Turk, D.; Gubensek, F.; Turk, V. Human cathepsin B is a metastable enzyme stabilized by specific ionic interactions associated with the active site. *Biochemistry* **33**, 14800–14806 (1994).

Turk, B.; Stoka, V. Protease signalling in cell death: caspases versus cysteine cathepsins. *FEBS Letters* **581**, 2761–2767 (2007).

Turk, B.; Stoka, V.; Rozman-Pungercar, J.; Cirman, T.; Droga-Mazovec, G.; Oresic, K.; Turk, V. Apoptotic pathways: involvement of lysosomal proteases. *Biological Chemistry* **383**, 1035–1044 (2002a).

Turk, B.; Turk, D.; Salvesen, G. S. Regulating cysteine protease activity: essential role of protease inhibitors as guardians and regulators. *Current Pharmaceutical Design* **8**, 1623–1637 (2002b).

Turk, B.; Turk, D.; Turk, V. Lysosomal cysteine proteases: more than scavengers. *Biochimica et Biophysica Acta* **1477**, 98–111 (2000).

Turk, B.; Turk, V. Lysosomes as "suicide bags" in cell death: myth or reality? *The Journal of Biological Chemistry* **284**, 21783–21787 (2009).

Turk, B.; Turk, V.; Turk, D. Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biological Chemistry* **378**, 141–150 (1997).

Turk, V.; Bode, W. The cystatins: protein inhibitors of cysteine proteinases. *FEBS Letters* **285**, 213–219 (1991).

Turk, V.; Stoka, V.; Turk, D. Cystatins: biochemical and structural properties, and medical relevance. *Frontiers in Bioscience : a journal and virtual library* **13**, 5406–5420 (2008).

Turk, V.; Stoka, V.; Vasiljeva, O.; Renko, M.; Sun, T.; Turk, B.; Turk, D. Cysteine cathepsins: From structure, function and regulation to new frontiers. *Biochimica et Biophysica Acta* (2011).

Turk, V.; Turk, B.; Turk, D. Lysosomal cysteine proteases: facts and opportunities. *The EMBO Journal* **20**, 4629–4633 (2001).

Twining, S. S. Regulation of proteolytic activity in tissues. *Critical Reviews in Biochemistry and Molecular Biology* **29**, 315–383 (1994).

Wajant, H. Death receptors. *Essays in Biochemistry* **39**, 53–71 (2003).

Walczak, H.; Miller, R. E.; Ariail, K.; Gliniak, B.; Griffith, T. S.; Kubin, M.; Chin, W.; Jones, J.; Woodward, A.; Le, T.; Smith, C.; Smolak, P.; Goodwin, R. G.; Rauch, C. T.; Schuh, J. C.; Lynch, D. H. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nature Medicine* **5**, 157–163 (1999).

Wattiaux, R.; Wattiaux-de Coninck, S.; Thirion, J.; Gasingirwa, M. C.; Jadot, M. Lysosomes and Fas-mediated liver cell death. *The Biochemical Journal* **403**, 89–95 (2007).

Werneburg, N. W.; Guicciardi, M. E.; Bronk, S. F.; Gores, G. J. Tumor necrosis factor-alpha-associated lysosomal permeabilization is cathepsin B dependent. *American Journal of Physiology Gastrointestinal and Liver Physiology* **283**, G947–956 (2002).

Werneburg, N. W.; Guicciardi, M. E.; Bronk, S. F.; Kaufmann, S. H.; Gores, G. J. Tumor necrosis factor-related apoptosis-inducing ligand activates a lysosomal pathway of

apoptosis that is regulated by Bcl-2 proteins. *The Journal of Biological Chemistry* **282**, 28960–28970 (2007).

Wyllie, A. H.; Kerr, J. F.; Currie, A. R. Cell death: the significance of apoptosis. *International Review of Cytology* **68**, 251–306 (1980).

Youle, R. J.; Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology* **9**, 47–59 (2008).

Zavasnik-Bergant, T.; Turk, B. Cysteine cathepsins in the immune response. *Tissue Antigens* **67**, 349–355 (2006).

Zdolsek, J.; Zhang, H.; Roberg, K.; Brunk, U. H2O2-mediated damage to lysosomal membranes of J-774 cells. *Free Radical Research Communications* **18**, 71–85 (1993).

Zhao, M.; Brunk, U. T.; Eaton, J. W. Delayed oxidant-induced cell death involves activation of phospholipase A2. *FEBS Letters* **509**, 399–404 (2001).

Kazalo slik

Kazalo tabel

Priloge

V času doktorskega dela je bil objavljen en raziskovalni članek in en pregledni članek.

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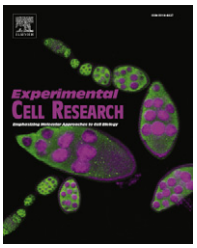

Review Article

Lysosomal pathways to cell death and their therapeutic applications

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ABSTRACT

Lysosomes are the major cell digestive organelles that were discovered over 50 years ago. They contain a number of hydrolases that help them to degrade intracellular and extracellular material delivered. Among the hydrolases, the cathepsins, a group of proteases enclosed in the lysosomes, have a major role. About a decade ago, the cathepsins were found to participate in apoptosis. Following their release into the cytosol, they cleave Bid and degrade antiapoptotic Bcl-2 proteins, thereby triggering the mitochondrial pathway of apoptosis, with the lysosomal membrane permeabilization being the critical step in this pathway. Lysosomal dysfunction is linked with several diseases, including cancer and neurodegenerative disorders, thereby providing a potential for therapeutic applications. In this review lysosomes and lysosomal proteases involvement in apoptosis and their possible pharmaceutical targeting are discussed.

Contents

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Lysosomes and lysosomal proteases

Lysosomes (Greek: digestive body) are cellular organelles critically involved in basic cellular processes including endocytosis, autophagy, phagocytosis and exocytosis [1]. Due to the complex endocytic network, it is hard to distinguish the lysosomes from other related vesicles, such as early/late endosomes and lysosome-related organelles (LROs, secretory lysosomes). Morphological differences could be observed at the ultra-structural level by electron microscopy, while biochemical traits that distinguish them from other vesicles are the lack of the mannose-6-phosphate receptors and their internal acidity. Lysosomal membrane proton pumps create an acidic environment (pH 4.6–5.0) that helps in loosening the structures of macromolecules including proteins, and is optimal for the activity of the rich assortment of lysosomal acid hydrolases that have adapted well to these conditions. The newly synthesized hydrolases are transferred to the lysosomes through the trans-Golgi network which prevents activation of inactive proenzymes before they reach their final destination, thereby protecting cellular components from unwanted damage. Degradation of biological macromolecules within the lysosome is catalyzed by more than 50 hydrolases, including phosphatases, glycosidases, sulphatases, lipases, nucleases and proteases [2,3]. Among the proteases, the best known are the cathepsins (Greek: to digest) belonging to serine, cysteine or aspartic proteases. A number of cathepsins, such as the aspartic cathepsin D and some of the cysteine cathepsins, including cathepsins B, L, C and H, are ubiquitous and among the most abundant lysosomal proteases [4]. However, the expression and distribution of some cathepsins, such as cathepsins S and K, is tissue- and cell type-specific. Cysteine cathepsins constitute the largest group among the cathepsins with 11 members found in humans: B, C, F, H, K, L, O, S, V, X and W [5]. They are predominantly endopeptidases and relatively non-specific enzymes, cleaving their substrates after hydrophobic or basic residues, often at the same sites within the proteins. Cathepsins B and H can act as both endo- and exopeptidases, while cathepsins C and X are exopeptidases only. This redundancy seems to contribute to the maintenance of cell homeostasis in different physiological situations by the conversion, recycling and degradation of cellular and extracellular proteins [6,7].

Cell death basics

There are several ways how cells die. Based on the observation of morphological differences, there are at least two major types of cell death, apoptosis and necrosis, whereas the role of autophagy seems to be primarily protective. Lysosomes and lysosomal proteases have been demonstrated to participate in all three processes. Whereas they are absolutely indispensable for autophagy progression and their breakdown seems to be the irreversible step just prior to the plasma membrane breakdown during necrosis, their role in apoptosis is more controversial and enigmatic. As the role of lysosomes in necrosis and in autophagy has been a subject of numerous reviews [4,8–11], the focus of this review will be on apoptosis.

Depending on the initial signal, apoptosis can be accomplished by involving changes in the function of mitochondria (intracellular, intrinsic stimulus) or by the binding of a death ligand (extracellular, extrinsic stimulus) to its specific transmembrane receptor on the cell surface. Independently of the starting point, a molecular cascade is triggered involving activation of caspases, the key proapoptotic proteases. In the caspase cascade the initiator or apical caspases (caspase-8, or -10 in the death receptor pathway; caspase-9 in the mitochondrial pathway) activate the effector or executioner caspases (caspase-3, -6, -7) by proteolytic cleavage. Activated executioner caspases then cleave numerous cellular proteins, leading to cell death. At the end of the process the cellular content ends up segregated in the apoptotic bodies that are phagocytosed by the surrounding cells thereby preventing inflammation [12].

Activation of the initiator caspases is thus the critical step in apoptosis. In the intrinsic pathway, which is more ancient, stimuli such as cellular damage or lack of essential factors cause mitochondrial outer membrane permeabilization (MOMP), a step that is controlled by the proapoptotic and antiapoptotic proteins of the Bcl-2 family. The critical molecules are Bax and Bak, which, following activation, assemble in the lipid pore, forming MOMP [13,14]. Once the MOMP is functional, the released cytochrome c binds to the cytosolic protease-activating factor-1 (Apaf-1), forming the apoptosome, the caspase-9 activating complex, that recruits and activates the initiator caspase-9, whereas Smac/Diablo antagonizes the X-chromosome-linked inhibitor of apoptosis (XIAP) thereby ensuring propagation of apoptosis [15].

In the extrinsic apoptotic pathway activation of the initiator caspases-8 and -10 is mediated by the extracellular ligands that bind to death receptors on the cell surface. The death receptors are transmembrane proteins belonging to the tumor necrosis factor (TNF) receptor superfamily. Ligand binding triggers a conformational change in the cytoplasmic domain of the receptor exposing the death domain (DD) that recruits the adaptor proteins via their own DD. In addition to the DD, some adaptor proteins contain a death effector domain (DED). The complex between the death receptor and the adaptor protein's DED is called the death-inducing signaling complex (DISC), which recruits the initiator caspase-8 to DISC, thereby enabling its activation [16]. The execution phase downstream of caspase-8 activation is cell-type specific. In type I cells, the amount of DISC-processed caspase-8 allows for the direct activation of sufficient amounts of the executioner caspases-3 and -7 to finalize apoptosis. However, in type II cells the DISC-mediated caspase-8 activation is insufficient for efficient apoptosis triggering and caspase-8, rather than directly activating the executioner caspases activates Bid, thereby creating an amplification loop through recruitment of the mitochondrial pathway to help finishing up dysfunctional or superfluous cells. The next step of both, the intrinsic and the extrinsic pathways, is activation of the executioner caspases, where the two pathways converge, leading to cell death (Fig. 1; [15]).

And where are the lysosomes and how they participate in apoptosis? It is clear that the executioners are not the lysosomes themselves, but their hydrolases and among them the cathepsins. However, in order to become actively involved in cell death, the cathepsins should be released into the cytosol. Lysosomal membrane permeabilization (LMP) is thus the critical step for the lysosomal pathway, similarly to the role of MOMP in the case of mitochondria [17].

Lysosomal membrane permeabilization—the key step in lysosomal apoptosis

A survey of literature revealed that numerous agents and molecules of endogenous or synthetic origin can induce LMP [9,18].

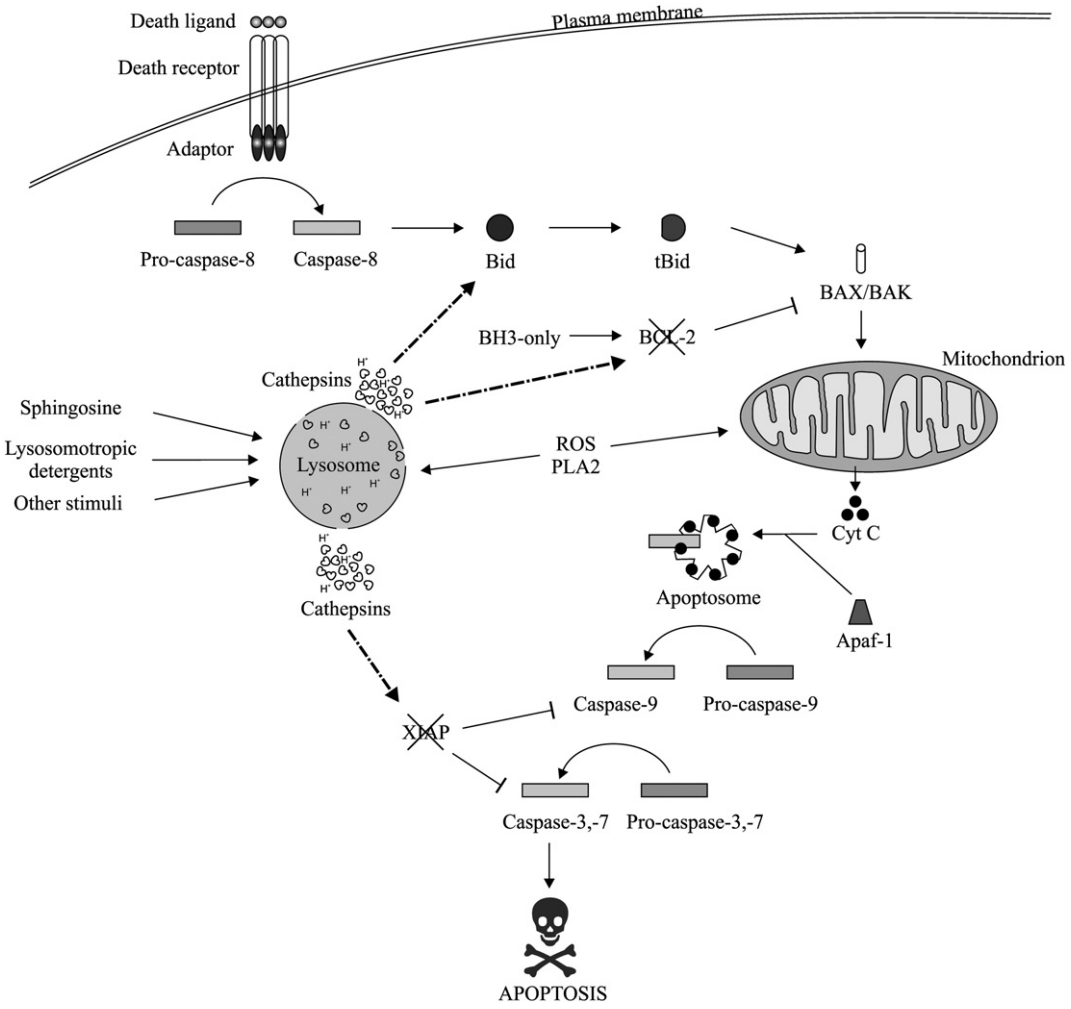

Fig. 1 – The role of lysosomes and lysosomal cathepsins in cell death. A schematic representation of the extrinsic and the intrinsic apoptosis pathways is shown. Pathways leading to lysosomal membrane permeabilization and its consequences are marked, as well as the major stimuli that result in LMP. Cyt C, cytochrome C; BH-3 only, proteins from the BH-3 only family of Bcl-2 family proteins; tBid, cleaved/truncated Bid. Other details are explained in the text.

Among them the reactive oxygen species (ROS) are possibly the most important endogenous LMP inducers [19]. As a result of oxidative stress, large amounts of hydrogen peroxide are produced in mitochondria, which cannot be completely detoxified by the cell. Such hydrogen peroxide is then able to diffuse in the cell and into the lysosomes, where ferruginous material delivered by autophagy is accumulated. This in turn results in production of highly reactive hydroxyl radicals in a Fenton-type reaction. The hydroxyl radicals cause peroxidation of membrane lipids with subsequent release of the lysosomal content into the cytosol. In this way the oxidative stress from mitochondria is amplified through the help of redox-active iron rich lysosomes [19,20]. In addition, lysosomal enzymes have been found to act on mitochondria to promote ROS generation, thereby creating a feedback loop that leads to additional lysosomal damage [21]. Several studies suggested that ROS stimulation can activate phospholipases A2 (PLA2), which trigger destabilization of the membranes of intracellular organelles by degradation of the membrane phospholipids [22]. Another compound suggested to participate in generating ROS that may lead to LMP is the photosensitizer N-aspartyl chlorin e6 (NPe6), which triggers cell death by lysosomal photodamage [23]. Finally, siramesine, a sigma-2 receptor antagonist, was linked with increased ROS production, leading to subsequent LMP and cell death [24].

LMP can also be triggered directly by the compounds termed lysosomotropics already by de Duve in 1974 [25]. After protonation lysosomotropics become trapped in the lysosomes, where they accumulate. When concentration of a lysosomotropic agent reaches the critical level, the compound evolves membranolytic properties, resulting in a release of the lysosomal constituents into the cytosol [26]. Lysosomotropic agents include a variety of compounds of endogenous, as well as synthetic origin. Among the endogenous factors the most important is sphingolipid sphingomyelin. In the lysosomal membranes it can be converted to ceramide and further to sphingosine by acid sphingomyelinase (ASMase) and ceramidase, respectively. Generation of sphingosine is increased in TNF-α-induced apoptosis, and accumulation of sphingosine as well as ceramide in lysosomes can induce LMP. Moreover, ceramide is capable of regulating cathepsin D activity [27], which depends on the functional ASMase, resulting in

engagement of apoptosis by the cleavage of Bid, while sphingosine contributes to the LMP by potentially stimulating the activity of cathepsin B [28]. On the other hand, there are numerous synthetic agents, which act as lysosomotropic detergents. Probably the most studied and characterized is the amino acid methyl ester Leu-LeuOMe, which entered clinical trials for the treatment of graft-versus-host disease during allogeneic bone marrow transplantation. Among other lysosomotropic detergents, known to trigger apoptosis are N-dodecylimidazole and o-methyl-serine dodecylamide hydrochloride (MSDH) [4,9].

Several other stimuli such as quinolone antibiotics, free fatty acids, bile salts, certain viral proteins, drugs that affect microtubule function, proton pump inhibitors and cytotoxins from cobra venom were also suggested to be involved in LMP [4,9]. In addition, Bax and/or Bak have been also suggested to directly insert into the lysosomal membrane during staurosporine-mediated apoptosis, or following c-Jun-N-terminal kinase (JNK)-dependent Bim activation, resulting in LMP [29,30]. However, this suggestion has been recently disputed based on the work in several cell death models [31].

In addition, there are several molecules known to protect lysosomal membranes against permeabilization, implying their possible role as endogenous regulators of LMP. Such molecules include Hsp70, lysosome-associated membrane proteins 1 and 2 (LAMP-1, -2), cholesterol, glycosaminoglycans, as well as endogenous cathepsin inhibitors that prevent the deadly role of the cathepsins in the wrong place at the wrong time [4]. However, despite the considerable knowledge about lysosomal apoptotic pathways, there is still a significant gap in our understanding of the molecular mechanisms leading to lysosomal membrane rupture and the subsequent release of the cathepsins into the cytosol.

The other major issue about LMP and the subsequent cathepsin release is about the timing of LMP. So when in the apoptotic cascade LMP occurs? Is it an early event or a late event? A further important question linked with that issue is whether LMP is a triggering or an amplifying event. In order to properly address this question, we have to discriminate between two situations: the first in which LMP is triggered directly by the lysosomotropic reagents and the second, where LMP is triggered indirectly. In the first group it is clear that LMP is the critical step, as shown for example for the LeuLeuOMe-mediated apoptosis [32,33]. The second group is definitely more ambiguous. Initial studies on TNF-α- and TRAIL-induced apoptosis that have looked into the role of lysosomes and the cathepsins in the extrinsic apoptotic pathway, placed LMP upstream of MOMP. The proposed mechanisms were quite different, varying from caspase-8 mediated cleavage of caspase-9 [34] or Bid [35], to sphingosine formation [28] and c-Jun-N-terminal kinase (JNK)-dependent Bim activation (see above) [30,35]. However, most of these studies only analysed lysosomal leakage at a single time point during a late stage of apoptosis, when both lysosomes and mitochondria are usually disrupted, which precludes determination of the route. Moreover, these studies which involved gene ablation, silencing or inhibition of the individual cathepsins, only attenuated, but not prevented apoptosis, not really supporting the idea of LMP being the real driver of the process. Other more recent data based on kinetics of the process, however, demonstrated that MOMP actually precedes LMP in several different cell death models including death receptor mediated apoptosis, consistent with the role of lysosomes as the amplifiers of the death signals [4,36].

In addition, a completely different mechanism that places lysosomes, but not LMP, at the very beginning of the apoptotic cascade has been suggested. In an elegant study Vince and colleagues have shown that the lysosomal degradation of the complex between TNF receptor-associated factor and cellular inhibitor of apoptosis 1 (TRAF2-cIAP1), which prevents the assembly of DISC through the TNFR/TNF-α pathway, is induced by the TNF-like weak inducer of apoptosis (TWEAK) binding to its receptor FN14 and subsequent recruitment of the TRAF2-cIAP1 complex. Following internalization of the TRAF2-cIAP1 complex in the receptosome, its degradation was suggested to be achieved by the cathepsins in an autophagic fashion [37]. However, this seems to be a more special case and remains to be validated.

Molecular mechanisms downstream of LMP: the execution phase

The next question is how the cathepsins can trigger apoptosis when released into the cytosol. Contrary to the caspases that reside as inactive zymogens in the cytosol, cathepsins are already active upon their arrival. However, they are, with the exception of cathepsin S, relatively quickly inactivated at neutral pH in the cytosol due to the irreversible unfolding (cysteine cathepsins) or the reversible deprotonation of the active site aspartates (cathepsin D). Anyhow, they can still survive long enough to cleave several cellular substrates. Moreover, their cytosolic lifetime can be prolonged due to the lowering of pH in the proximity of the lysosomal leakage by the escaping protons. Of course, the major question is which are the cellular substrates of the cathepsins that are involved in cell death triggering? Surprisingly, very few cytosolic substrates of cathepsins have been identified so far. The idea of activation of the executioner caspases was disputed already a long time ago [38]. Although caspase-8 was found to be activated by cathepsin D in the neutrophils [39], this is probably a very special situation due to the extreme abundance of cathepsin D in these cells [4]. The major cathepsin substrate identified in a number of cellular models is Bid, implying that cathepsins are not the direct executioners of apoptosis, but rather recruit the mitochondrial pathway [8]. Bid is, however, not the critical *in vivo* cathepsin substrate [40]. Later studies revealed that, in addition to processing Bid, cathepsins also degrade the antiapoptotic Bcl-2 proteins, thereby triggering the mitochondrial pathway in a synergistic manner [32]. There are only a few more cathepsin substrates identified but, with the exception of XIAP, they do not seem to be critically involved in apoptosis progression. Moreover, by cleaving the same substrates cathepsins not only trigger apoptosis, but also amplify the mitochondrial pathway following LMP induction by another stimulus [4].

Lysosomes and cathepsins in disease

Lysosomes and related vesicles and lysosomal proteases have numerous important physiological functions, including a critical role in intracellular protein turnover and in MHC class II-mediated immune response, both linked also with autophagy. If uncontrolled, cathepsin activity contributes to various pathologies including cancer, neurodegenerative diseases, atherosclerosis, rheumatoid arthritis and others [41,42].

Especially well documented is the role of cathepsins in cancer progression [27,42,43]. Blocking cathepsins by small molecule inhibitors has been shown to significantly delay cancer progression in a number of mouse cancer models as well as to sensitize tumor cells to other chemotherapeutic agents [43–45]. However, also releasing the cathepsins by disrupting the lysosomes have a potential in anticancer treatment, as they can participate in killing of cancer cells through degradation of antiapoptotic Bcl-2 homologues and inhibitor of apoptosis proteins (IAPs), which are often up-regulated in cancer [4,11]. This apparently contrasting role of the cathepsins in cancer can be, however, explained. As has been shown recently, the majority of extracellular cathepsins are secreted from the infiltrated immune cells, primarily macrophages [46]. In contrast, the major targets of lysosomotropic compounds are intracellular cathepsins, residing primarily in the tumor cells. Therefore, blocking the cathepsins is believed to mainly interfere with cancer progression, whereas disabling the lysosomes interferes with cell death-related processes. In addition to mediating or amplifying apoptosis, lysosomes are indispensable also for autophagy, which is one of the most important survival mechanisms of cancer cells due to hypoxia and metabolic stress in the tumors and their microenvironment [47]. Targeting lysosomes therefore has a great therapeutic potential, because it does not only trigger apoptosis, but also suppresses the autophagic flux, which disables cancer cell from getting metabolic substrates and thereby sensitizes them to other types of chemotherapy [4,11,48]. And this is exactly the option also offered by inhibiting both extra- and intracellular cathepsins using appropriate cathepsin inhibitors. Extracellular inhibition would contribute to blocking extracellular signaling pathways mediated by the cathepsins, whereas blocking intracellular cathepsins would block the autophagic flux, resulting in an apparent autophagosome accumulation and subsequent sensitization to standard chemotherapy. This dual mode of cathepsin inhibition may give a more comprehensive explanation also for the recent results in the mammary gland cancer model, where the effect of taxol has been significantly potentiated by the simultaneous inhibition of macrophage-derived cathepsins [44], although autophagy was not specifically monitored in this study.

In addition, lysosomes and lysosomal proteases have an important role also in neurodegenerative diseases, although this is more linked with autophagy. The hallmark of many late-onset neurodegenerative diseases, such as Parkinson's disease, Huntington's disease and Alzheimer's disease, is the formation of toxic intracellular protein aggregates. The ubiquitin-proteasome system and autophagy are the major degradation pathways for clearance of such aggregate-prone cytosolic proteins. The prevalent clearance route for these proteins is the ubiquitin-proteasome system due to its greater efficiency. However, if the cytosolic proteins are aggregate-prone or poor proteasome substrates, then autophagy becomes the predominant degradation pathway and can be even more efficient than the proteasome. It has been shown that various proteins with polyglutamine expansions, including huntingtin and ataxin 3, mutant forms of α-synuclein and different forms of tau protein are degraded by autophagy. Pharmacological and genetic interruption of autophagy results in slower clearance of these proteins and increases the amount of aggregates and toxicity. In addition, ablation of autophagy provokes the accumulation of ubiquitinated aggregates in various tissues, indicating a great therapeutic potential for autophagy and an important role for lysosomes and lysosomal proteases in the field of neurodegenerative disorders, although different from that in cancer [49].

Another group of lysosome-associated diseases predominantly affecting the central nervous system are the lysosomal storage diseases (LSDs). LSDs comprise a group of more than 50 metabolic disorders. They are caused by dysfunction of the lysosomal proteins, including lysosomal enzymes, lysosomal membrane proteins, and proteins involved in the posttranslational modification and trafficking of lysosomal proteins, which are leading to accumulation of non-degraded metabolites in the lysosomes [3,50]. However, with the exception of some lipofuscinoses that may be linked with malfunction of the cathepsin function judged on the basis of cathepsin ablation studies, others are not linked with the cathepsins [4]. Among the better studied LSDs are the Niemann–Pick (NP) diseases of types A, B and C, all belonging to the group of lipidoses. In type A and B NP disease the enzyme ASMase, which hydrolyzes sphingomyelin to ceramide, is mutated, while ASMase is functional in NP-C. NP-C is characterized by accumulation of unesterified cholesterol and glycolipids in the endosomal/lysosomal pathway. Cholesterol accumulates in the spleen, liver, bone marrow and brain, where it can contribute to progressive neurological damage [50,51]. Recently, it has been shown that in the acidic environment Hsp70 stabilizes lysosomes by binding to an essential co-factor for lysosomal sphingomyelin metabolism, endolysosomal anionic phospholipid bis(monoacylglycero)phosphate (BMP), and thereby enabling the activity of ASMase. In addition, NP-A and NP-B are connected with decreased lysosomal stability, which was suggested to be regulated with recombinant Hsp70 treatment [52].

Conclusions

For a long time lysosomes were believed to be very sturdy organelles that do not decay until the whole cell falls apart, therefore being involved only in the necrotic cell death. Today it is clear that lysosomes and lysosomal proteases can assist caspases in apoptosis, although largely only as signal amplifiers. Despite considerable progress in understanding the role of lysosomes and lysosomal proteases in the apoptotic pathways, the exact mechanism of LMP and its consequences are still not well understood. However, exploiting LMP shows a great potential especially in cancer treatment, where targeting lysosomes could provoke cell death as a consequence of cathepsin-mediated cytotoxicity as well as by abolishing the autophagic flux, which sensitizes them to other chemotherapeutic agents. In addition, blocking the cathepsins in cancer may also be of major importance, especially as the combined therapy, as it can block the extracellular signaling pathways mediated by the cathepsins, as well as interfere with the autophagic flux. Another potentially interesting area for pharmaceutical industry linked with regulation of the lysosome function is neurodegenerative disorders. However, additional work is needed in order to be able to fully exploit the therapeutic potential of lysosomes and lysosomal proteases.

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REFERENCES

[1] J.P. Luzio, P.R. Pryor, N.A. Bright, Lysosomes: fusion and function, Nat. Rev. Mol. Cell Biol. 8 (2007) 622–632.
[2] J. Huotari, A. Helenius, Endosome maturation, EMBO J. 30 (2011) 3481–3500.
[3] P. Saftig, J. Klumperman, Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function, Nat. Rev. Mol. Cell Biol. 10 (2009) 623–635.
[4] U. Repnik, V. Stoka, V. Turk, B. Turk, Lysosomes and lysosomal cathepsins in cell death, Biochim. Biophys. Acta 1824 (2012) 22–33.
[5] A. Rossi, Q. Deveraux, B. Turk, A. Sali, Comprehensive search for cysteine cathepsins in the human genome, Biol. Chem. 385 (2004) 363–372.
[6] V. Turk, V. Stoka, O. Vasiljeva, M. Renko, T. Sun, B. Turk, D. Turk, Cysteine cathepsins: from structure, function and regulation to new frontiers, Biochim. Biophys. Acta 1824 (2012) 68–88.
[7] V. Turk, B. Turk, D. Turk, Lysosomal cysteine proteases: facts and opportunities, EMBO J. 20 (2001) 4629–4633.
[8] B. Turk, V. Turk, Lysosomes as "suicide bags" in cell death: myth or reality? J. Biol. Chem. 284 (2009) 21783–21787.
[9] P. Boya, G. Kroemer, Lysosomal membrane permeabilization in cell death, Oncogene 27 (2008) 6434–6451.
[10] P. Golstein, G. Kroemer, Cell death by necrosis: towards a molecular definition, Trends Biochem. Sci. 32 (2007) 37–43.
[11] T. Kirkegaard, M. Jaattela, Lysosomal involvement in cell death and cancer, Biochim. Biophys. Acta 1793 (2009) 746–754.
[12] M.O. Hengartner, The biochemistry of apoptosis, Nature 407 (2000) 770–776.
[13] J.E. Chipuk, L. Bouchier-Hayes, D.R. Green, Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario, Cell Death Differ. 13 (2006) 1396–1402.
[14] R.J. Youle, A. Strasser, The BCL-2 protein family: opposing activities that mediate cell death, Nat. Rev. Mol. Cell Biol. 9 (2008) 47–59.
[15] C. Pop, G.S. Salvesen, Human caspases: activation, specificity, and regulation, J. Biol. Chem. 284 (2009) 21777–21781.
[16] A. Oberst, C. Pop, A.G. Tremblay, V. Blais, J.B. Denault, G.S. Salvesen, D.R. Green, Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation, J. Biol. Chem. 285 (2010) 16632–16642.
[17] B. Turk, D. Turk, V. Turk, Protease signalling: the cutting edge, EMBO J. 31 (2012) 1630–1643.
[18] V. Stoka, V. Turk, B. Turk, Lysosomal cysteine cathepsins: signaling pathways in apoptosis, Biol. Chem. 388 (2007) 555–560.
[19] T. Kurz, A. Terman, B. Gustafsson, U.T. Brunk, Lysosomes and oxidative stress in aging and apoptosis, Biochim. Biophys. Acta 1780 (2008) 1291–1303.
[20] A. Terman, T. Kurz, B. Gustafsson, U.T. Brunk, Lysosomal labilization, IUBMB Life 58 (2006) 531–539.
[21] M. Zhao, F. Antunes, J.W. Eaton, U.T. Brunk, Lysosomal enzymes promote mitochondrial oxidant production, cytochrome c release and apoptosis, Eur. J. Biochem. 270 (2003) 3778–3786.
[22] C.E. Chwieralski, T. Welte, F. Buhling, Cathepsin-regulated apoptosis, Apoptosis 11 (2006) 143–149.
[23] L. Liu, Z. Zhang, D. Xing, Cell death via mitochondrial apoptotic pathway due to activation of Bax by lysosomal photodamage, Free Radic. Biol. Med. 51 (2011) 53–68.
[24] M.S. Ostenfeld, N. Fehrenbacher, M. Hoyer-Hansen, C. Thomsen, T. Farkas, M. Jaattela, Effective tumor cell death by sigma-2 receptor ligand siramesine involves lysosomal leakage and oxidative stress, Cancer Res. 65 (2005) 8975–8983.
[25] C. de Duve, T. de Barsy, B. Poole, A. Trouet, P. Tulkens, F. Van Hoof, Commentary. Lysosomotropic agents, Biochem. Pharmacol. 23 (1974) 2495–2531.
[26] D.K. Miller, E. Griffiths, J. Lenard, R.A. Firestone, Cell killing by lysosomotropic detergents, J. Cell Biol. 97 (1983) 1841–1851.
[27] M.M. Mohamed, B.F. Sloane, Cysteine cathepsins: multifunctional enzymes in cancer, Nat. Rev. Cancer 6 (2006) 764–775.
[28] N.W. Werneburg, M.E. Guicciardi, S.F. Bronk, G.J. Gores, Tumor necrosis factor-alpha-associated lysosomal permeabilization is cathepsin B dependent, Am. J. Physiol. Gastrointest. Liver Physiol. 283 (2002) G947–G956.
[29] A.C. Johansson, H. Appelqvist, C. Nilsson, K. Kagedal, K. Roberg, K. Ollinger, Regulation of apoptosis-associated lysosomal membrane permeabilization, Apoptosis 15 (2010) 527–540.
[30] N.W. Werneburg, M.E. Guicciardi, S.F. Bronk, S.H. Kaufmann, G.J. Gores, Tumor necrosis factor-related apoptosis-inducing ligand activates a lysosomal pathway of apoptosis that is regulated by Bcl-2 proteins, J. Biol. Chem. 282 (2007) 28960–28970.
[31] C. Oberle, J. Huai, T. Reinheckel, M. Tacke, M. Rassner, P.G. Ekert, J. Buellesbach, C. Borner, Lysosomal membrane permeabilization and cathepsin release is a Bax/Bak-dependent, amplifying event of apoptosis in fibroblasts and monocytes, Cell Death Differ. 17 (2010) 1167–1178.
[32] G. Droga-Mazovec, L. Bojič, A. Petelin, S. Ivanova, R. Romih, U. Repnik, G.S. Salvesen, V. Stoka, V. Turk, B. Turk, Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues, J. Biol. Chem. 283 (2008) 19140–19150.
[33] T. Cirman, K. Orešić, G. Droga-Mazovec, V. Turk, J.C. Reed, R.M. Myers, G.S. Salvesen, B. Turk, Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins, J. Biol. Chem. 279 (2004) 3578–3587.
[34] M. Gyrd-Hansen, T. Farkas, N. Fehrenbacher, L. Bastholm, M. Hoyer-Hansen, F. Elling, D. Wallach, R. Flavell, G. Kroemer, J. Nylandsted, M. Jaattela, Apoptosome-independent activation of the lysosomal cell death pathway by caspase-9, Mol. Cell. Biol. 26 (2006) 7880–7891.
[35] N. Werneburg, M.E. Guicciardi, X.M. Yin, G.J. Gores, TNF-alpha-mediated lysosomal permeabilization is FAN and caspase 8/Bid dependent, Am. J. Physiol. Gastrointest. Liver Physiol. 287 (2004) G436–G443.
[36] K. Schrader, J. Huai, L. Jockel, C. Oberle, C. Borner, Non-caspase proteases: triggers or amplifiers of apoptosis? Cell. Mol. Life Sci. 67 (2010) 1607–1618.
[37] J.E. Vince, D. Chau, B. Callus, W.W. Wong, C.J. Hawkins, P. Schneider, M. McKinlay, C.A. Benetatos, S.M. Condon, S.K. Chunduru, G. Yeoh, R. Brink, D.L. Vaux, J. Silke, TWEAK-FN14 signaling induces lysosomal degradation of a cIAP1-TRAF2 complex to sensitize tumor cells to TNFalpha, J. Cell Biol. 182 (2008) 171–184.
[38] V. Stoka, B. Turk, S.L. Schendel, T.H. Kim, T. Cirman, S.J. Snipas, L.M. Ellerby, D. Bredesen, H. Freeze, M. Abrahamson, D. Bromme, S. Krajewski, J.C. Reed, X.M. Yin, V. Turk, G.S. Salvesen, Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route, J. Biol. Chem. 276 (2001) 3149–3157.
[39] S. Conus, R. Perozzo, T. Reinheckel, C. Peters, L. Scapozza, S. Yousefi, H.U. Simon, Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation, J. Exp. Med. 205 (2008) 685–698.
[40] M.K. Houseweart, A. Vilaythong, X.M. Yin, B. Turk, J.L. Noebels, R.M. Myers, Apoptosis caused by cathepsins does not require Bid signaling in an in vivo model of progressive myoclonus epilepsy (EPM1), Cell Death Differ. 10 (2003) 1329–1335.

[41] J. Reiser, B. Adair, T. Reinheckel, Specialized roles for cysteine cathepsins in health and disease, J. Clin. Invest. 120 (2010) 3421–3431.
[42] O. Vasiljeva, T. Reinheckel, C. Peters, D. Turk, V. Turk, B. Turk, Emerging roles of cysteine cathepsins in disease and their potential as drug targets, Curr. Pharm. Des. 13 (2007) 387–403.
[43] C. Palermo, J.A. Joyce, Cysteine cathepsin proteases as pharmacological targets in cancer, Trends Pharmacol. Sci. 29 (2008) 22–28.
[44] T. Shree, O.C. Olson, B.T. Elie, J.C. Kester, A.L. Garfall, K. Simpson, K.M. Bell-McGuinn, E.C. Zabor, E. Brogi, J.A. Joyce, Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer, Genes Dev. 25 (2011) 2465–2479.
[45] G. Mikhaylov, U. Mikac, A.A. Magaeva, V.I. Itin, E.P. Naiden, I. Psakhye, L. Babes, T. Reinheckel, C. Peters, R. Zeiser, M. Bogyo, V. Turk, S.G. Psakhye, B. Turk, O. Vasiljeva, Ferri-liposomes as an MRI-visible drug-delivery system for targeting tumours and their microenvironment, Nat. Nanotechnol. 6 (2011) 594–602.
[46] V. Gocheva, H.W. Wang, B.B. Gadea, T. Shree, K.E. Hunter, A.L. Garfall, T. Berman, J.A. Joyce, IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion, Genes Dev. 24 (2010) 241–255.
[47] R. Mathew, V. Karantza-Wadsworth, E. White, Role of autophagy in cancer, Nat. Rev. Cancer 7 (2007) 961–967.
[48] U. Repnik, B. Turk, Lysosomal-mitochondrial cross-talk during cell death, Mitochondrion 10 (2010) 662–669.
[49] D.C. Rubinsztein, The roles of intracellular protein-degradation pathways in neurodegeneration, Nature 443 (2006) 780–786.
[50] M.L. Schultz, L. Tecedor, M. Chang, B.L. Davidson, Clarifying lysosomal storage diseases, Trends Neurosci. 34 (2011) 401–410.
[51] L. Zhang, R. Sheng, Z. Qin, The lysosome and neurodegenerative diseases, Acta Biochim. Biophys. Sin. (Shanghai) 41 (2009) 437–445.
[52] T. Kirkegaard, A.G. Roth, N.H. Petersen, A.K. Mahalka, O.D. Olsen, I. Moilanen, A. Zylicz, J. Knudsen, K. Sandhoff, C. Arenz, P.K. Kinnunen, J. Nylandsted, M. Jaattela, Hsp70 stabilizes lysosomes and reverts Niemann–Pick disease-associated lysosomal pathology, Nature 463 (2010) 549–553.

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Cell Injury, Repair, Aging and Apoptosis

Cathepsin Cleavage of Sirtuin 1 in Endothelial Progenitor Cells Mediates Stress-Induced Premature Senescence

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Stress-induced premature senescence (SIPS) of endothelial cells (ECs) has emerged as a contributor to global EC dysfunction. One of the cellular abnormalities mechanistically linked to SIPS is lysosomal dysfunction. In this study, we examined the impact of a range of cardiovascular risk factors on the expression of sirtuin 1 (SIRT1), SIPS, and apoptosis, and we documented the role of SIRT1 in reduced EC and endothelial progenitor cell (EPC) viability. These findings were confirmed in mice with selective endothelial SIRT1 knockout. The effects of stressors could be partially mimicked by inducing lysosomal membrane permeabilization or inhibiting autophagy, and were reversed by a cathepsin inhibitor. We provide evidence that SIRT1 is an important substrate of cysteine cathepsins B, S, and L. An antioxidant/peroxynitrite scavenger, ebselen, prevented stress-induced SIRT1 depletion and subversion of autophagy by mitigating lysosomal dysfunction. In conclusion, our data advance the concept of "stem cell aging" by establishing the critical role of lysosomal dysfunction in the development of SIPS through the cathepsin-induced proteolytic cleavage of SIRT1, a mechanism linking cell stress to apoptosis and SIPS. Ebselen potently protects lysosomal membrane integrity, preventing cathepsin-induced cleavage of SIRT 1 in EPCs and blunting SIPS and apoptotic cell death induced by relevant cardiovascular stressors. The proposed mechanism of SIRT1 depletion in stress has all of the attributes of being a paradigm of SIPS of EPCs. ***(Am J Pathol 2012, 180:973-983; DOI: 10.1016/j.ajpath.2011.11.033)***

Stress-induced premature senescence (SIPS) of endothelial cells has emerged as a notable contributor to global endothelial cell dysfunction (ECD) in diseases as diverse as diabetes, hypertension, chronic kidney disease, and atherosclerosis, to name a few.[1] We have previously demonstrated that critical cellular abnormalities preceding and mechanistically intimately linked to development of SIPS are characterized by lysosomal dysfunction manifesting in collapse of lysosomal pH gradient and lysosomal membrane permeabilization, and by subverted autophagy presenting as accumulation of giant autolysosomal vacuoles choking endothelial cells.[2,3]

Identification of the silent information regulator 2 (Sir2) in the yeast[4] and a subsequent demonstration of its role in delaying aging in *Saccharomyces cerevisiae*[5] have ignited interest in the role of members of this family in SIPS-related processes. Seven mammalian homologues of this NAD-dependent histone deacetylase have been identified with SIRT 1 and 2 expressed in the nucleus and the cytosol, SIRT 3–5 being mitochondrial proteins and SIRT 6 and 7 expressed in the nucleus.[6] It has been demonstrated that SIRT 1, abundant in embryonic stem cells, plays an essential role in regulating their self-renewal and resistance to stressors, like reactive oxygen species.[7–9] In addition, SIRT1 expression is one of the regulators of autophagy,[10] thus rais-

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ing a question of potential SIRT1 contribution to subverted autophagy in stressed endothelial and endothelial progenitor cells.

Endothelial progenitor cells (EPC) are not impervious to developing SIPS. Studies in db/db type 2 diabetic mice with the metabolic syndrome revealed premature senescence of bone marrow–derived EPC.[11] Chronic treatment with an antioxidant and peroxynitrite scavenger ebselen reversed SIPS *in vivo* and *in vitro*.[11–13] These data are in accord with the concept of "stem cell aging" as a basis for organismal aging.[14]

In the present study, we attempted to integrate the previous findings on endothelial SIPS with the exponentially growing field of SIRT1 effects on aging processes as applied to senescence of EPC. Specifically, we examined the impact of a range of relevant cardiovascular risk factors on the expression of SIRT1, along with development of SIPS and apoptosis, and documented the role of SIRT1 in the changes of cell viability. These findings were confirmed in mice with selective endothelial SIRT1 knockout. Furthermore, studies showed reciprocal relations between SIRT1 and p62 expression in stressed EPC, thus demonstrating an attendant abnormality of autophagy. The described effects of stressors could be partially mimicked by inducing lysosomal membrane permeabilization or by inhibiting autophagy and reversed by a cell-permeable inhibitor of cathepsins. Incubation of cathepsins B, S, and L with SIRT1 resulted in its proteolytic cleavage. We also inquired whether an antioxidant therapy combined with peroxynitrite scavenging (ebselen), shown in previous studies to protect endothelial cells and EPC from SIPS, exerts its effect via SIRT1 and, if it does so, what is/are the possible mechanism(s). The data presented here not only demonstrate the critical role of lysosomal membrane permeabilization followed by the release of cathepsins, cleavage, and depletion of SIRT-1 with the ensuing development of SIPS, but also establish ebselen as a potent protector of lysosomal membrane integrity that prevents cathepsin-induced cleavage of SIRT 1 in murine EPC, and we document its efficacy in preventing SIPS and apoptotic cell death induced by several relevant cardiovascular stressors.

Materials and Methods

Cell Culture

Murine EPCs were isolated from embryos at E7.5 to E7.8 of development and kindly provided by Dr. A Hatzopoulos[15]; these are referred to as eEPC to distinguish them from primary cultures of EPC. eEPC were repeatedly analyzed in the course of the study for an array of markers including FITC- or PE-conjugated anti-mouse CD117 (c-kit), CD150, Sca-1, CD34, CD31, CD44, CD45, Flk,−1, Tie2, Ulex Europaeus lectin, as well as antibodies to von Willebrand factor (vWF) and endothelial nitric oxide synthase (eNOS) followed by corresponding fluorescent secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) to ensure stability of their phenotype. EPC showed robust staining with Ulex Europaeus lectin and antibodies to vWF, but expressed low levels of Flk-1, CD31, CD34, and Sca-1, suggesting that the cells retained their nondifferentiated phenotype. All primary antibodies were produced by BD Biosciences (Rockville, MD) except for antibody to vWF (Dako North America, Carpinteria, CA) and to eNOS (Santa Cruz Biotechnology, Santa Cruz, CA). Data were acquired using a FACScan cytometer equipped with a 488-nm argon laser and a 620-nm red diode laser and analyzed using CellQuest software (Becton Dickinson, San Jose, CA). The setup of FACScan was performed using unstained and single antibody–stained cells. EPC were plated on 0.1% gelatin-coated plates and maintained at 37°C and 5% CO_2 in DMEM culture medium containing 20% heat-inactivated serum (55°C 30 minutes; Invitrogen, Carlsbad, CA), 0.1 mmol/L 2-mercaptoethanol, 1 mmol/L MEM nonessential amino acids (Invitrogen, Grand Island, NY), 100 U/mL penicillin and 100 μg/mL streptomycin, 2 mmol/L L-glutamine (Invitrogen), and 2 mmol/L HEPES, pH 7.5.

Mice with Endothelial SIRT1 Deletion

An endothelial SIRT1-deleted mouse model ($Sirt^{endo-/-}$ or $Sirt^{endo+/-}$) was developed by cross-breeding of CBy.129(B6)-*Sirt1*^{tm1Ygu}/J (homozygous for targeted allele SirT1co/co, viable and fertile, containing a loxP-flanked neomycin cassette upstream and downstream of exon 4 of the targeted gene) with the Tie2-Cre transgenic mice (both from Jackson Laboratories, Bar Harbor, ME).[16] These mice thrive similar to their wild type and heterozygote littermates (Mendelian distribution of offspring), have normal blood pressure at age of 8 weeks, but exhibit a mild impairment in vasorelaxation and moderate impairment in *ex vivo* angiogenesis (manuscript in preparation).

Bone marrow–derived mononuclear cells (MNCs) were obtained according to previously described procedure using Histopaque 1077 (ICN, Costa Mesa, CA) gradient centrifugation.[11] Isolated bone marrow MNCs were washed three times with EGM-2 medium (Cambrex, Walkersville, MD) supplemented with 2% penicillin/streptomycin (Invitrogen), and 0.25 μg/mL amphotericin B (Invitrogen). MNCs were resuspended in 3 mL complete EGM-2 medium and seeded onto a 35-mm tissue culture dishes precoated with pronectin (Sigma-Aldrich, St. Louis, MO) at 37°C, 5% CO_2, in a humidified incubator. After 24 hours in culture, nonadherent cells and debris were aspirated, adherent cells were washed once with complete EGM-2 medium, and complete EGM-2 medium was added to each well. Medium was changed daily for 7 days and then every other day until the first passage. EPC were assayed by co-staining with acetylated LDL (acLDL)-DiI (Biomedical Technologies, Stoughton, MA) for 3 hours at 37°C and FITC-conjugated *Ulex europaeus* lectin (Sigma-Aldrich) for 30 minutes at 37°C, both of which are features characteristic of endothelial lineage.

Detection of Senescent and Apoptotic Cells

Senescence-associated β-galactosidase (SA β-gal)–positive cells were detected by cytochemical staining

at pH 6.[17] Stained cells were viewed under an inverted microscope at ×200 magnification. The number of SA-β-gal–positive cells per total number of cells in the same field was determined by counting at least eight random fields for each sample under bright-field illumination. Detection of SA β-gal in the *en face* aortic preparations was performed using a previously described protocol.[18]

A Caspase Detection Kit (Calbiochem, La Jolla, CA) was used to detect activated caspases in cultured cells. The FITC-labeled caspase inhibitor benzyloxycarbonyl Val-Ala-aspartic acid fluoromethyl ketone (VAD-FMK) irreversibly binds to activated caspases 1 to 9. In brief, cells were incubated in FITC-VAD-FMK containing culture medium (1:300, vol:vol) for 0.5 to 1 hour in 37°C incubator with 5% CO_2. To detect apoptosis by fluorescence microscopy, cells were co-stained with Hoechst 33342 (Sigma, St. Louis, MO). The data presented were obtained by counting the proportion of apoptotic cells per 2000 cells using fluorescence microscopy (in each experiment, at least 15 to 20 randomly chosen fields were examined). To detect apoptosis by flow cytometry, cells were re-suspended in PBS containing 1 μg/mL propidium iodide (PI, Molecular Probes, Eugene, OR). FAM-VAD-FMK and PI fluorescence was measured by FACScan (Becton Dickinson, San Jose, CA).

Western Blot Analysis

Cells were treated with H_2O_2, asymmetric dimethylarginine (ADMA) and nonenzymatically glycated long-lived protein, collagen I (GC), or their respective controls, symmetric dimethylarginine and native collagen I (SDMA and NC, respectively) 3 days after the last change of the culture medium. Cell lysates were prepared in a buffer containing 50 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, and protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were assayed for total protein concentration (BCA assay, Pierce, Rockford, IL). SDS-PAGE was performed using 4% to 20% Tris-glycine gels (Invitrogen) under reducing conditions with 10 to 30 μg of total protein used from each sample for electrophoresis followed by electrotransfer to Immobilon-P (Millipore, Billerica, MA) membrane. Secondary HRP-conjugated antibody was used at a dilution of 1:2000. The membranes were incubated with primary antibodies overnight in a cold room, followed by HRP-conjugated IgG, and were visualized with an enhanced chemiluminescence detection system (Pierce). Primary anti-mouse SIRT1 polyclonal antibody (Millipore, Billerica, MA), anti-mouse Atg5 monoclonal antibody (R&D Systems, Minneapolis, MN), anti–acetyl lysine polyclonal antibody (Abcam, Cambridge, MA), and anti–β-tubulin monoclonal antibody (Sigma) was used at the dilution of 1:1000. Primary anti-p62/SQSTM-1 antibody (Abnova, Walnut, CA) was used at 1:10,000, whereas primary anti-GST antibody (GE Healthcare, Piscataway, NJ) at 1:5000. Secondary HRP-conjugated antibodies were used at a dilution of 1:2000. Protein A/G PLUS-Agarose beads for immunoprecipitation was ordered from Santa Cruz Biotechnology (Santa Cruz, CA).

SIRT1 Expression Plasmid and Short Hairpin RNA

The plasmid pUSeAmp-Sir2α was obtained from Millipore. Four short hairpin RNA (shRNA) constructs against Mus musculus SIRT1 (#TR505485), and the 29-mer scrambled shRNA cassette in pRS Vector were purchased from Origene Technologies (Rockville, MD) and used according to the manufacturer's recommendations. To ensure efficiency and specificity, all constructs were tested by transfecting EPC and the endogenous SIRT1 level monitored by Western blot analysis. Cells were suspended in mouse ES Cell Nucleofector Solution (VPH-1001; Lonza, Cologen, Germany) to a final concentration of 1 to 5×10^6 cells/100 μl. The cells were transfected using 2 to 10 μg of shRNA by electroporation in Amaxa certified cuvettes, program A-023. The cells were transferred to the prewarmed medium and incubated for indicated periods of time at 37°C with 5% CO_2.

Lysosomal pH and Intracellular Cathepsin Detection

Lysosomal pH was monitored using a metachromatic fluorophore acridine orange, as previously reported.[3] The activity of intracellular cathepsin B in eEPC was monitored using Magic Red (MR) Cathepsin detection kit using the fluorophore cresyl violet (Immunochemistry Technologies, Bloomington, IN). EPC were loaded with this cathepsin substrate for 1 hour at 37°C in 5% CO_2 in the culture medium containing MR-Cathepsin solution at 1:260 dilution.[19] After loading, cells were washed twice with PBS, and intracellular localization of the hydrolyzed (fluorescent) MR product was examined using fluorescence microscopy (Nikon Eclipse E800) at an excitation 540 nm and emission 590 nm at ×600 magnification. The subcellular MR fluorescence intensity was analyzed using line-scan function routines of MetaVue software. The average signal intensity in cytosolic and lysosomal compartments was normalized against the background and their ratio was calculated. At least 20 images were analyzed for each treatment and each experiment was repeated at least three times.

SIRT1 Cleavage by Cysteine Cathepsins

Recombinant human cysteine cathepsins B, L, and S were prepared as described previously,[20–22] and active site titrated,[23] whereas recombinant SIRT1 was prepared as a fusion protein with GST as described above. Cleavage of SIRT1 (1.47 μmol/L final concentration) by cathepsins B, L, and S (0.57 μmol/L final concentration) was analyzed essentially as described previously.[24] As a positive control GST (3.8 μmol/L) was included.

Statistical Analysis

All experiments were repeated at least three times. Values are given as mean ± SE. Data were analyzed using analysis of variance with *post hoc* analysis for multiple group comparisons using Bonferroni method. A P value of <0.05 was considered statistically significant.

Results

Effects of Cardiovascular Stressors on EPC Viability and SIRT1 Expression

Previous studies have demonstrated that cardiovascular risk factors used herein promoted SIPS and/or apoptosis in mature endothelial cells[12] eventuating in vasculopathy. Therefore, we inquired whether similar phenomena are observed in endothelial progenitor cells. A range of relevant cardiovascular stressors encompassing an inhibitor and uncoupler of eNOS, asymmetric dimethylarginine (ADMA, 5 μg/mL), an advanced glycation endproduct (AGE)–modified long-lived protein, collagen I (GC, 100 μmol/L), and a pro-oxidant hydrogen peroxide (H_2O_2, 50 to 100 μmol/L) were applied to embryonic EPC for 24 to 72 hours. As summarized in Figure 1, all stressors significantly increased the proportion of apoptotic and prematurely senescent EPC.

The existing evidence linking senescence to the expression of sirtuins and, in particular, SIRT1, prompted us to examine the expression of this protein deacetylase in embryonic EPC. Application of each of three stressors was associated with a significant decline in the expression of SIRT1 (Figure 2). The similar decline in SIRT1 expression was observed in human umbilical vein endothelial cells and in mouse hemangioendothelioma endothelial cells (not shown).

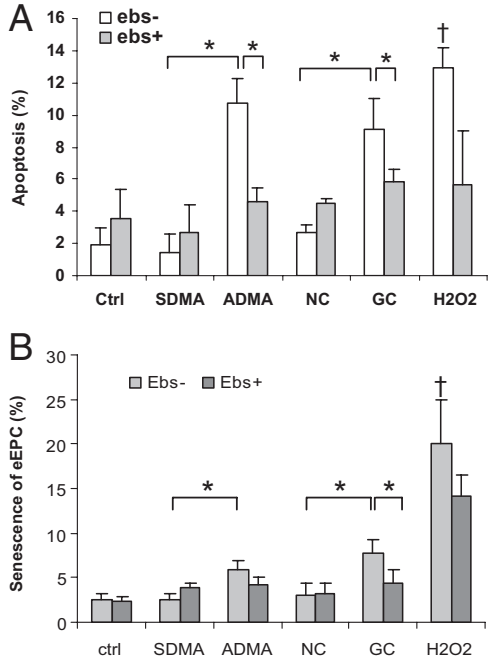

Figure 1. Treatment with GC, ADMA, or H_2O_2 induces apoptosis and senescence in eEPC. After 3 days of treatment with GC, ADMA, or H_2O_2, significant increases in proportion of apoptotic (**A**) and senescent (**B**) cells occurred compared with their individual control groups: native collagen (NC), symmetric dimethylarginine (SDMA), and control untreated cells (Ctrl). Co-application of ebselen (ebs) with GC, ADMA, or H_2O_2 (ebs+) prevents apoptosis. Ebselen also prevents premature senescence induced by GC (**B**). Data are mean ± SD of five independent experiments. $^*P < 0.05$; $^\dagger P < 0.05$ compared with Ctrl/ebs−.

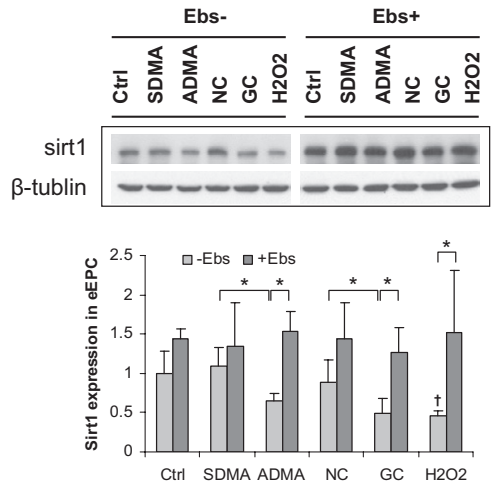

Figure 2. SIRT1 expression in eEPC after co-application of GC, ADMA, or H_2O_2 with ebselen (ebs). Western blot analysis demonstrated reduced SIRT1 level in EPC after 3 days of treatment with GC, ADMA, or H_2O_2. Co-treatment with ebselen prevented the observed effect. The results of densitometric analysis are shown in relative units compared with Ctrl/ebs−. Data are means ± SD of six independent experiments. $^*P < 0.05$; $^\dagger P < 0.05$ compared with Ctrl/ebs−.

These data raise a question of causality between changes in SIRT1 expression and EPC viability: Namely, is SIRT1 depletion sufficient and necessary to account for increased SIPS and apoptosis and could its overexpression prevent these outcomes of cell stress? To address this question, we manipulated SIRT1 expression genetically or pharmacologically, alone or in combination with the stressors, and monitored cell viability. Overexpression of SIRT1 did not affect basal rate of apoptosis or SIPS in embryonic EPC; however, the proportion of apoptotic cells was significantly reduced in SIRT1-transfected cells subjected to each of the stressors, whereas the proportion of SIPS in embryonic EPC was significantly decreased in ADMA-treated cells (Figure 3, A and B). In contrast, repression of SIRT1 with shRNA construct resulted in a significant increase in SIPS cells under basal conditions, enhanced apoptosis even under control conditions (Ctrl, SDMA, and NC groups), and further enhanced apoptosis in GC group (Figure 3C). An increase in SIPS was seen in Ctrl and NC groups following shRNA/SIRT1 transfection (Figure 3D). Results obtained with an inhibitor of SIRT1, sirtinol are summarized in Figure 3F. Sirtinol, 100 μmol/L, resulted in increased proportion of senescent eEPC under all basal conditions. Thus, both the SIRT1-targeted "gain-of-function" and "loss-of-function" experiments supported its role in protection and destruction, respectively, of embryonic EPC cultured in the presence and even absence of noxious stimuli.

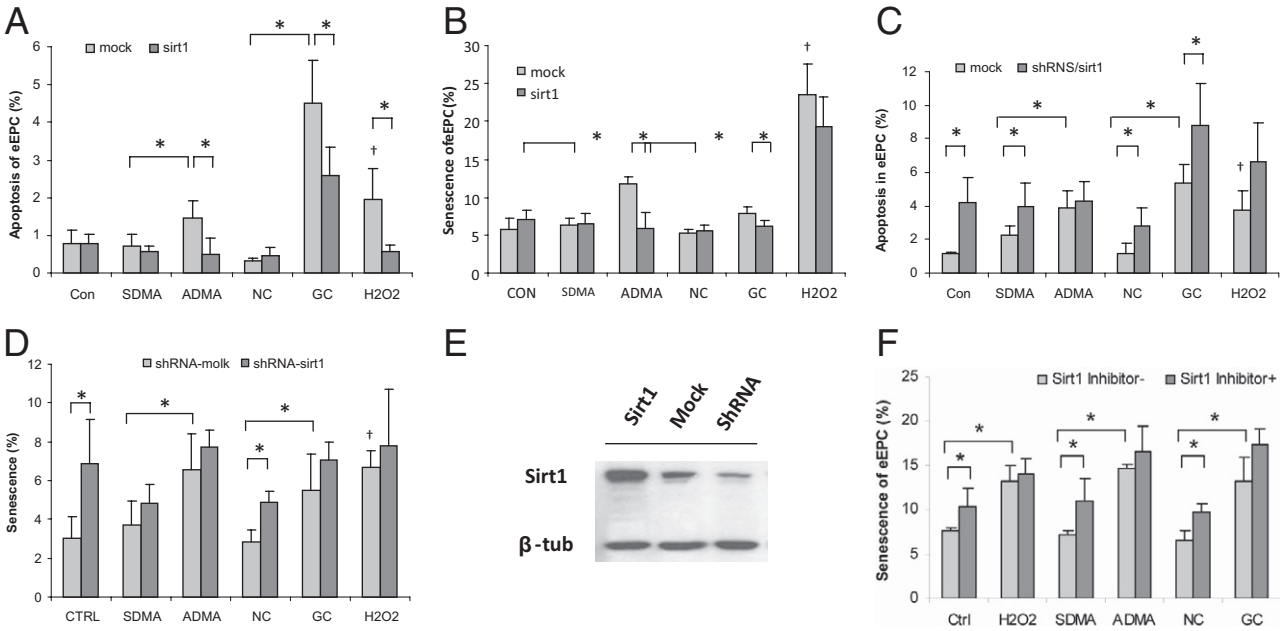

Figure 3. Apoptosis and senescence of eEPC after transfection of SIRT1 plasmid or shRNA/SIRT1 construct. **A** and **B:** Overexpression of SIRT1 prevented apoptosis induced by ADMA, GC, or H_2O_2 (**A**) and SIPS induced by ADMA or GC (**B**). **C** and **D:** In contrast, decreasing SIRT1 level with shRNA (shRNA/SIRT1) enhanced apoptosis even under control conditions as show in Ctrl, SDMA, and NC groups, and further enhanced apoptosis in GC group (**C**). An increase in SIPS was seen in Ctrl and NC groups after shRNA/SIRT1 transfection (**D**). **E:** Representative Western blot analysis of SIRT1 expression after its overexpression (SIRT1), inhibition (shRNA), and control transfection, mock-control (Mock). **F:** Alternatively, cells were treated with the inhibitor Sirtinol at 100 μmol/L, which resulted in increased proportion of senescent eEPC under basal conditions. Data are mean ± SD of five independent experiments. $^*P < 0.05$; $^\dagger P < 0.05$ compared with Ctrl/ebs−.

Senescence of Endothelial and Endothelial Progenitor Cells Obtained from Mice with Endothelial SIRT1 Knockout

To test the validity of above cell culture findings in a whole-animal system, we generated mice with endothelial deletion of SIRT1. Mice with Tie-2–driven endothelial and EPC SIRT1 deletion were used at age 3 to 4 months to obtain EPC and examine progenitor and mature endothelial cells for the possible premature senescence. Bone marrow–derived EPC, after a 3-week-long *in vitro* expansion, exhibited *in vitro* an almost twofold increase in the frequency of prematurely senescent cells under basal conditions in heterozygote and homozygote mice ($Sirt^{endo+/-}$ and $Sirt^{endo-/-}$), whereas the frequency of activated caspase-positive cells was elevated only in homozygote SIRT1-deletion mice ($Sirt^{endo-/-}$) (Figure 4A). Whole-mount *en face* thoracic aortic preparations, obtained from mice at age 3 to 4 months, were stained for SA-β-gal expression. Endothelial SIRT1-deleted aortas exhibited a robust staining for SA-β-gal in endothelial cells located in the plane of aorta and at the orifices of intercostal arteries, whereas neither wild-type nor heterozygote littermates showed SA-β-gal positivity at these locations (Figure 4, B and C). These findings confirm *in vitro* observations made in the established progenitor cell line on the role of SIRT1 depletion in the acquisition of senescent phenotype.

Ebselen Prevents Down-Regulation of SIRT1 by Cardiovascular Stressors

Pursuant to the previous finding that a selenoorganic peroxynitrite scavenger, ebselen, improves viability of stressed HUVEC and embryonic EPC,[11] we next examined the possibility that the observed effect is related to modulation of SIRT1. Indeed, EPC subjected to ADMA, GC, or H_2O_2 in the presence of ebselen showed a significant attenuation of apoptosis, whereas SIPS was significantly reduced in embryonic EPC subjected to GC or H_2O_2 (Figure 1). Co-application of ebselen with ADMA, GC, or H_2O_2 was associated with normalization of SIRT1 expression, otherwise profoundly suppressed by these stressors (Figure 2).

Effects of Cardiovascular Stressors Are Mediated via Induction of Lysosomal Membrane Permeabilization

One of the targets of deacetylase activity of SIRT1 is represented by members of autophagic proteins Atg 5 and 7.[10] Therefore, it could be expected that one of the consequences of SIRT1 depletion in stressed EPC is manifested as impaired autophagy. Indeed, subverted autophagy was found in our previous work in stressed HUVEC.[5] Because the abundance of Sequestosome-1 protein, p62/SQSTM-1, which targets proteins destined for degradation to form aggregates and sequester in autophagosomes to be later degraded in autolysosomes,[25] reflects the intensity of autophagic flux, we next

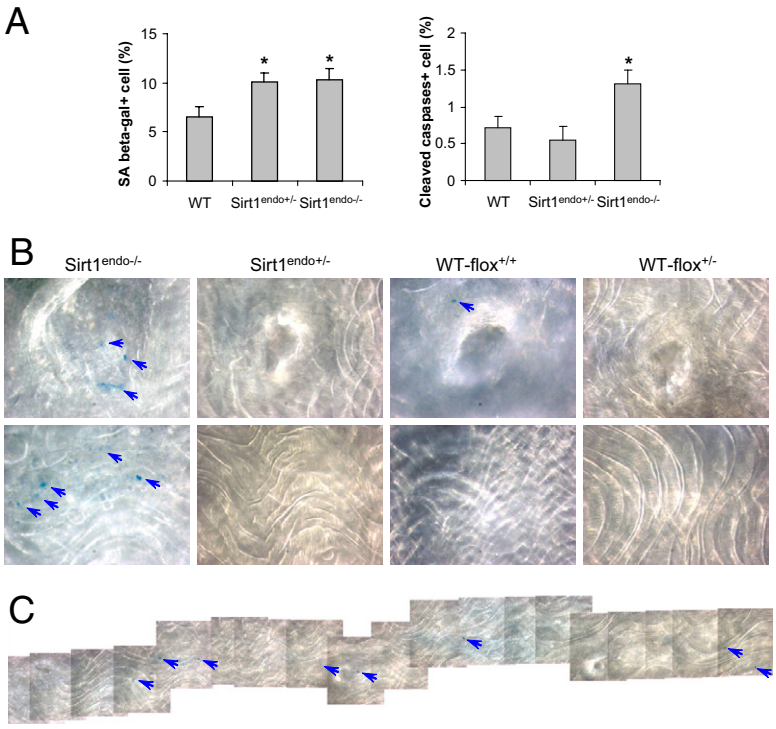

Figure 4. Cleaved caspases and SA-β-galactosidase staining of bone marrow–derived EPC and SA-β-galactosidase staining of aortic endothelial cells in endothelial SIRT1-deleted and control mice. **A:** Proportion of SA-β-galactosidase–positive and activated caspase–positive EPC isolated from the bone marrow of $Sirt^{endo-/-}$, $Sirt^{endo+/-}$, and wild-type mice ($WT\text{-}flox^{+/+}$ and $WT\text{-}flox^{+/-}$). **B:** *En face* aortic staining for SA-β-galactosidase reveals minimal staining in control and heterozygotic mice and massive clusters of stained cells in endothelial SIRT1 knockout mice. Representative images of orifices to intercostals arteries and the plain portion of the thoracic aorta of controls and endothelial SIRT1-deleted mice. Original magnification, ×60. **C:** Representative composite of consecutive images reconstructing the length of *en face* thoracic aorta obtained from a 4-month-old endothelial SIRT1 knockout mouse, depicting frequency of clusters of SA-β-gal–positive cells. Original magnification, ×60. **Arrows** indicate cells stained for SA-beta-galactosidase.

examined the possibility of its accumulation in stressed embryonic EPC. The endogenous p62/SQSTM-1 expression level was elevated in eEPC after 24 hours of ADMA and H_2O_2 treatment (Figure 5A), and after 72 hours of GC treatment (Figure 5A). Co-application of ebselen prevented changes in p62/SQSTM-1 in all treatment groups and at all time points, thus suggesting that stress to embryonic EPC is associated with the impaired autophagy and this impairment is mitigated by ebselen. Indeed, testing the ability of stress-induced SIRT1 depletion to lead toward impaired Atg5 deacetylation, we next examined the abundance of acetylated Atg5. As shown in Figure 5B, immunoblotting of immunoprecipitated Atg5 with the antibodies against acetylated lysine demonstrated that one of the stressors used, GC, shown to deplete SIRT1, led to the increase in the level of acetylated Atg5, whereas co-application of ebselen prevented it.

Lysosomal membrane permeabilization and dysfunction induced by diverse stressors has been identified as

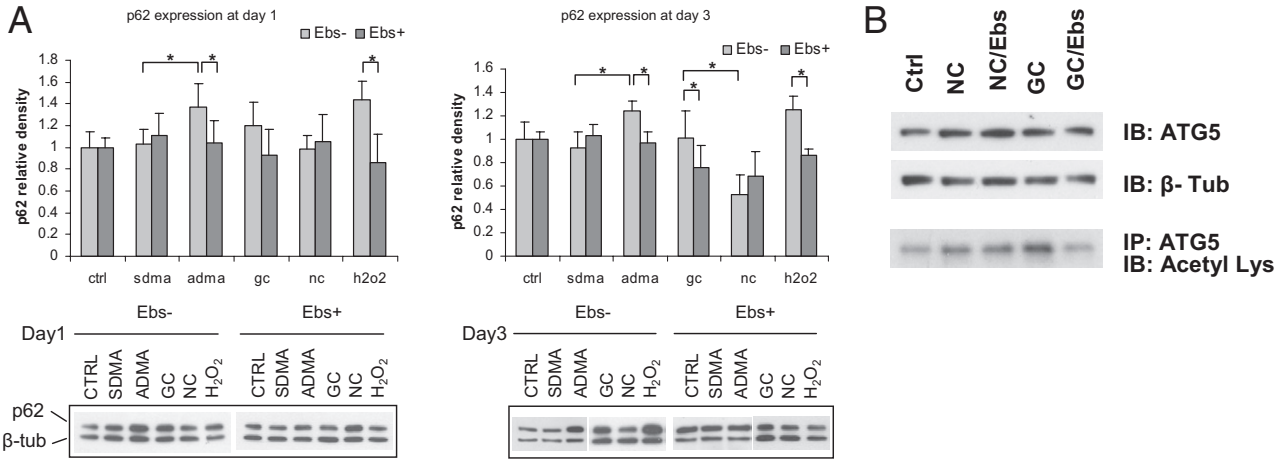

Figure 5. Dysfunctional autophagy of eEPC after GC, ADMA, or H_2O_2 treatment. **A:** Levels of p62. Endogenous p62 expression level was elevated in eEPC after 24 hours ADMA and H_2O_2 treatment (**A**, **left panel**) and after 72 hours of GC treatment (**A**, **right panel**). Co-application of ebselen (ebs) prevented changes in p62 in all treatment groups and at all time points. Results of densitometry analysis are shown in relative units compared with Ctrl/ebs−. Data are mean ± SD of four independent experiments. $^{*}P < 0.05$; $^{\dagger}P < 0.05$ compared with Ctrl/ebs−. **B:** Analysis of acetylated Atg5 in eEPC by immunoprecipitation and immunoblotting. Atg5-Atg12 protein complex (56 KDa) was precipitated from eEPC cell lysates using monoclonal anti-mouse Atg5 antibody and protein A/G PLUS-Agarose beads. Acetylation was analyzed by immunoblotting using polyclonal anti–acetyl lysine antibody. The amounts of cell lysate used for immunoprecipitation in each group were balanced first by protein concentration followed for confirmation by immunoblotting using β-tubulin (55 KDa) and Atg5-Atg12 antibodies. Data are representative of two separate experiments.

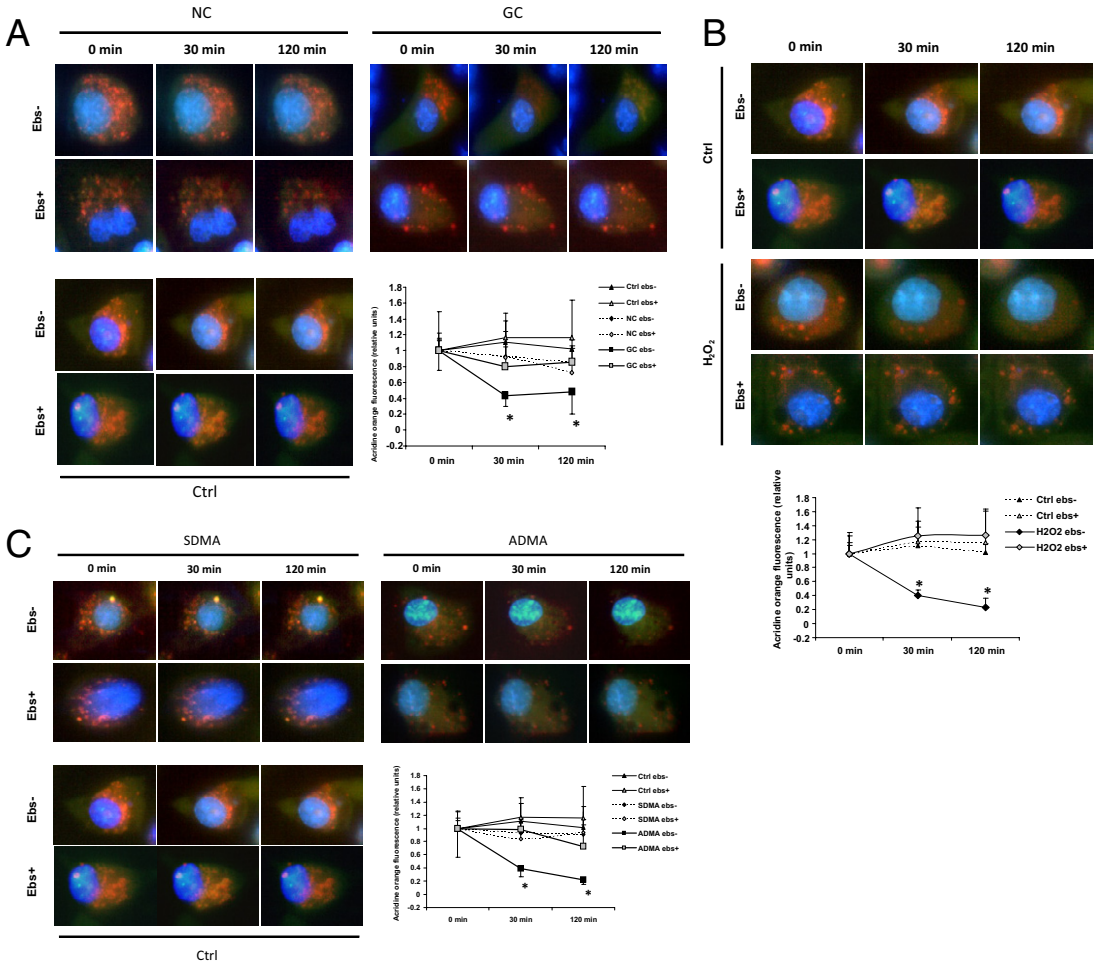

Figure 6. Lysosomal membrane permeabilization in EPC exposed to stressors. **A:** GC triggers lysosomal pH collapse. After incubation in 1 μmol/L acridine orange for 15 minutes, the fluoroprobe was detected as a red-punctuated fluorescence signal in intact cells, as shown in control. When the cells were exposed to GC, the punctuated fluorescence pattern nearly disappeared in the span of 2 hours, whereas it was preserved in cells treated with NC. Treatment with ebselen prevented changes induced by GC. **Lower right panel** shows quantification (ratio) of red acridine orange fluorescence signal intensity in lysosomes against cytosolic green fluorescence. The relative fluorescence intensity was adjusted to the intensity at 0 minute for each individual treatment group. Data are mean ± SD of three independent experiments. $^*P < 0.05$ compared with GC/ebs− and NC groups. **B:** H_2O_2 triggers lysosomal pH collapse. Experimental conditions as in **A**. Relative fluorescence intensity was adjusted to the intensity at 0 minutes for each individual treatment group. Data are mean ± SD of three independent experiments. $^*P < 0.05$ compared with H_2O_2/ebs−. **C:** ADMA triggers lysosomal pH collapse. Experimental conditions as in **A**. $^*P < 0.05$ compared with ADMA/ebs− and SDMA groups.

one of the causes for subversion of autophagy[26] and Patschan et al[2,3] observed subverted autophagy in HUVEC exposed to GC. To assess the possibility that impaired autophagy in embryonic EPC could be related to stressor-induced lysosomal membrane permeabilization, we next performed time-lapse microscopy of acridine orange-labeled embryonic EPC, as previously detailed.[3] Using this metachromatic fluorophore that emits red/orange at low pH values and green at a neutral pH, we demonstrated that all three stressors induced collapse of lysosomal pH gradient and lysosomal membrane permeabilization (Figure 6, A–C) manifesting in the reduction of punctated orange fluorescence pattern and acquisition of diffuse green cytosolic fluorescence. Coapplication of ebselen with the stressors resulted in a remarkable preservation of lysosomal membrane integrity, consistent with the prevention of oxidant-induced lysosomal membrane permeabilization.

Lysosomal Membrane Permeabilization Results in SIRT1 Depletion

Therefore, the question we asked next was: Does a primary abnormality in lysosomal membrane permeability or a subversion of autophagy affect the level of SIRT1? Lysosomal pH gradient collapse and membrane permeability was induced by treating embryonic EPC with a lysosomotropic alkaline proton pump inhibitor, chloroquine (30 μmol/L). This treatment did not result in the accumulation of p62/SQSTM-1 within 24 hours and at this time SIRT1 expression was unchanged from control (Figure 7). However, by 72 hours, chloroquine resulted in accumulation of p62/SQSTM-1 and significant reduction of SIRT1. In parallel experiments, 3 methyladenine (4 mmol/L), an inhibitor of class III phosphatidylinositol-3-kinase necessary for induction of autophagy,[27] was used as an inhibitor of autophagy. This

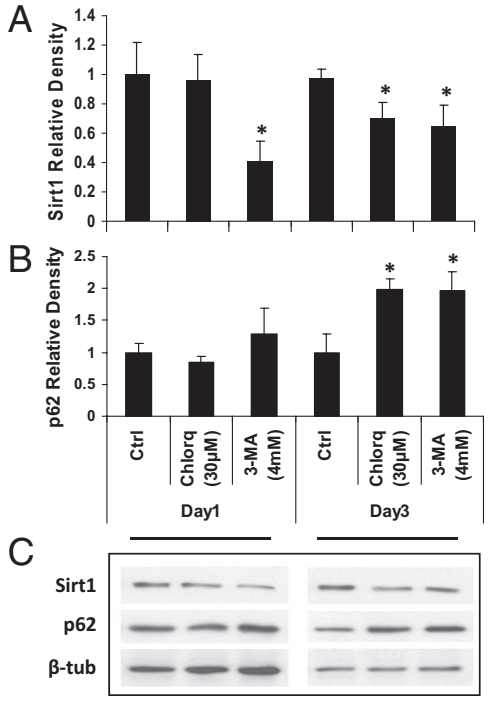

Figure 7. Changes of lysosomal permeability influence the endogenous level of SIRT1 and p62. Treatment of eEPC for 24 to 72 hours with chloroquine (chlor, 30 μmol/L) or 3-MA (4 mmol/L), suppressed SIRT1 expression level (**A** and **C**). In contrast, p62 showed significant accumulation after 72 hours of treatment with chloroquine or 3-MA (**B** and **C**). Results of densitometry analysis (**A** and **B**) are shown in relative units compared with control at day 1 or day 3. Data are mean ± SD of four independent experiments. $^*P < 0.05$ compared with their corresponding controls on day 1 or 3.

treatment resulted in the suppression of SIRT1 levels on days 1 and 3, whereas significant accumulation of p62/SQSTM-1 was detectable at day 3. Hence, these studies are supportive of the idea that lysosomal dysfunction is at least in part responsible for SIRT1 depletion eventuating in subverted autophagy in stressed embryonic EPC.

Role of Cathepsins in SIRT1 Degradation

The above findings are suggestive of the leaked lysosomal contents, including cathepsins, that could affect SIRT1 levels, either directly or indirectly. To test this possibility, embryonic EPC were subjected to the above cardiovascular stressors in the presence of E64d, a broad-spectrum cell-permeable inhibitor of cysteine cathepsins.[28] As shown in Figure 8A, this treatment resulted in a significant blunting of SIRT-1 depletion associated with exposure to ADMA, GC, and H_2O_2. Moreover, E64d pretreatment of embryonic EPC significantly reduced SIPS and apoptosis induced by oxidative stress (Figure 8B). These findings, in conjunction with the demonstration of stress-induced lysosomal dysfunction and subversion of autophagy, argue in favor of the involvement of lysosomal membrane permeabilization[26] in triggering SIPS or apoptotic cell death.

To test further the idea of lysosomal membrane permeabilization, we used a cathepsin B substrate that acquires fluorescence on cleavage, Magic Red,[19] to visualize cathepsin B translocation to the cytoplasm (Figure 9). Image analysis data demonstrated elevated level of cytoplasmic fluorescence intensity, with the ratio of cytoplasmic:lysosomal fluorescence intensities consistently increased after induction of cell stress, but blunted by co-application of ebselen. These finding further confirm lysosomal membrane permeabilization on stress and demonstrate the release of cathepsin B from the lysosomal compartment to the cytosol. In addition, the data support the argument that the observed SIRT1 preservation by ebselen may be due to its prevention of stress-induced lysosomal membrane permeabilization.

To directly verify the possibility of cathepsin cleavage of SIRT1, degradation of recombinant SIRT1 by cysteine cathepsins B, L, and S was investigated *in vitro* at neutral pH. The major reason to select these three cathepsins was that cathepsins B and L are the most abundant and ubiquitously expressed cysteine cathepsins, whereas cathepsin S is also found in endothelial cells, in addition to

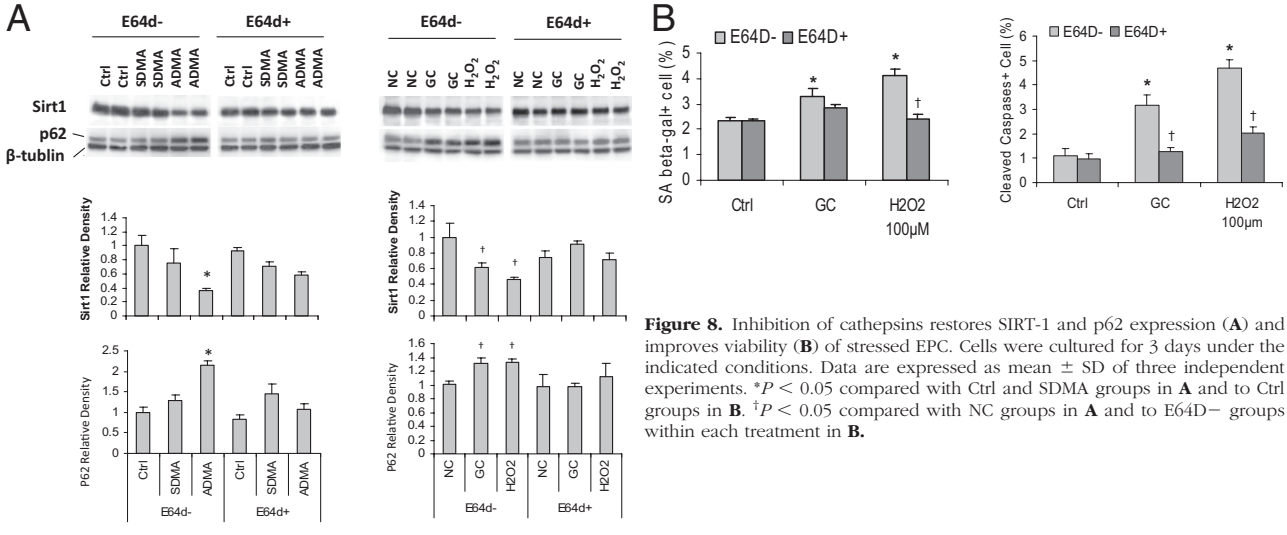

Figure 8. Inhibition of cathepsins restores SIRT-1 and p62 expression (**A**) and improves viability (**B**) of stressed EPC. Cells were cultured for 3 days under the indicated conditions. Data are expressed as mean ± SD of three independent experiments. $^*P < 0.05$ compared with Ctrl and SDMA groups in **A** and to Ctrl groups in **B**. $^\dagger P < 0.05$ compared with NC groups in **A** and to E64D− groups within each treatment in **B.**

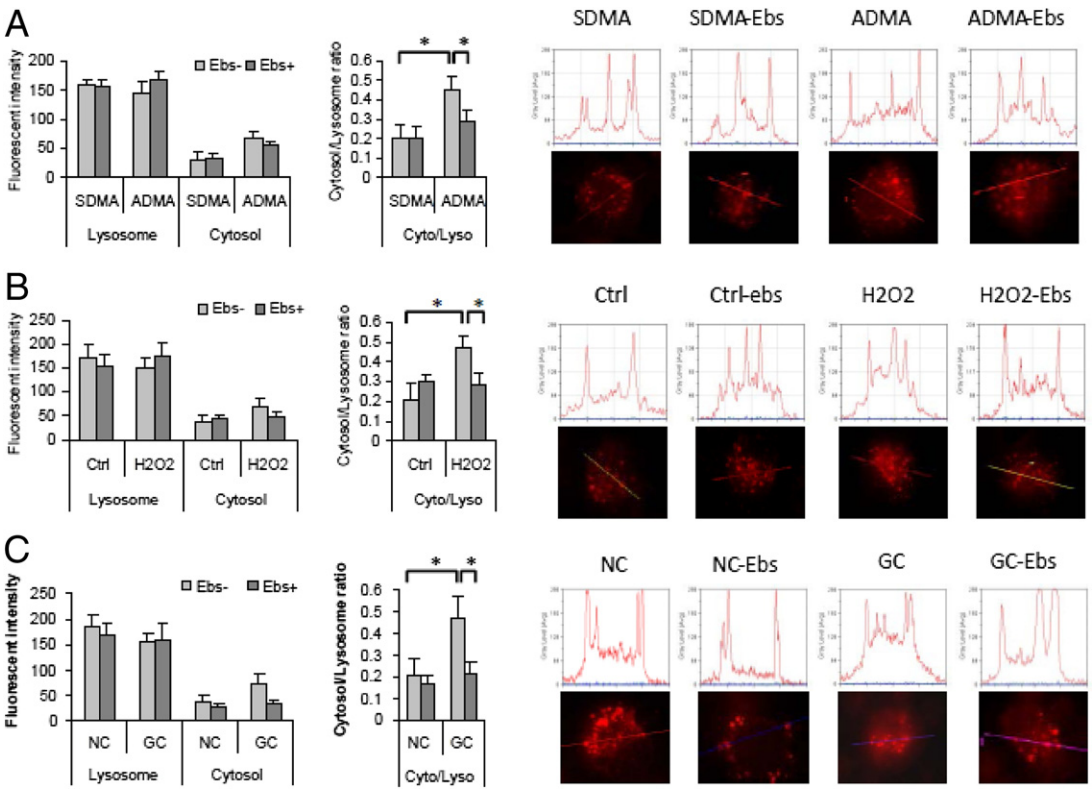

Figure 9. Leakage of cathepsin B from the lysosomal compartment to the cytosol and preventive effect of co-application of ebselen with the stressors; **A:** ADMA; **B:** hydrogen peroxide; **C:** glycated collagen). **Upper panels** in **A** and **B**, and **left panel** in **C**, summarize relative fluorescence intensities. Corresponding **lower** and **right panels** of **A**, **B**, and **C** illustrate representative cells (of 20 to 50 each) and line scanning of fluorescence intensity over lysosomes and cytosol. Data are mean ± SD of three independent experiments. $^*P < 0.05$.

being the most stable at neutral pH among all of the cathepsins. As shown in Figure 10, all three cathepsins cleaved and degraded SIRT1, supporting the hypothesis that cysteine cathepsins are directly responsible for SIRT1 degradation in EPC. Notably, the size of SIRT1 fragments differed with the use of different cathepsins, in accord with their specific cleavage profiles. The cleavage within SIRT1 protein and not within the GST moiety was additionally confirmed by Western blotting against GST, which helped to distinguish the fragmentation pattern of SIRT1 from that of GST.

Discussion

Data presented herein demonstrated that SIRT1 depletion occurring in stressed embryonic EPC is causatively linked to induction of SIPS and apoptosis of these cells. These findings buttress the concept of "stem cell aging" as a prerequisite for organismal aging.[14] Because the goal of the present study was to define protracted consequences of cell stress rather than its acute effects, which are known to induce SIRT1 system, the timing of the studies performed here was postponed 1 to 3 days after the application of stressors. Reciprocal relations between SIRT1 expression and the level of p62/SQSTM-1, a marker of autophagic flux, in combination with lysosomal membrane permeabilization implied that autophagy is subverted in stressed embryonic EPC, similar to what previously has been observed in cultured endothelial cells.[3] The key role of SIRT1 deficiency in premature senescence of endothelial and endothelial progenitor cells was confirmed *in vivo* and *in vitro* in mice

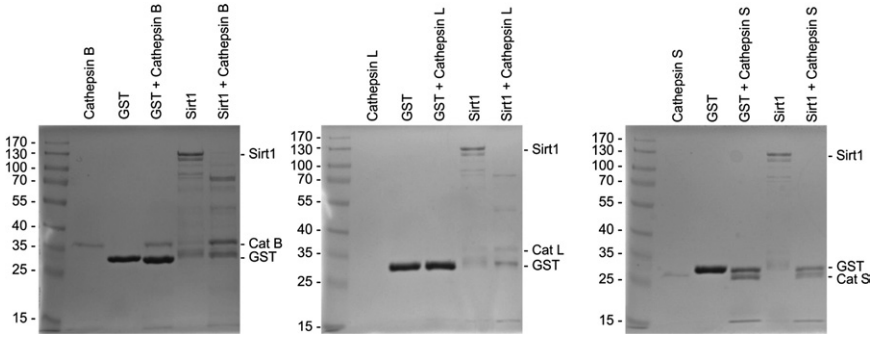

Figure 10. *In vitro* cleavages of recombinant SIRT1 by cathepsins at pH 7.2 and 37°C. Experimental details are given in *Materials and Methods*. Recombinant GST, SIRT1-GST, and cathepsins B, L, and S were used as negative controls and are marked with lines. Cathepsin-treated GST was used as a positive control. Partial degradation of Sirt1 was observed with cathepsin B, whereas cathepsin S was found to completely degrade Sirt1-GST, leaving only partially processed GST. Cathepsin L was slightly less efficient than cathepsin S.

with endothelial SIRT1 deletion: EPC obtained from these animals showed increased frequency of SA-β-gal–positive and activated caspase-positive cells under basal conditions; and aortic endothelium in whole-mount *en face* preparations showed increased SA-β-gal positivity. One of the proximal causes for subverted autophagy was identified as lysosomal membrane permeabilization. In fact, a direct permeabilization of lysosomes *per se* mimics the stress-induced reciprocal relations between SIRT1 and p62/SQSTM-1. Moreover, blockade of cathepsins activity with E64d prevented stress-induced depletion of SIRT1 and SIPS (induced by hydrogen peroxide or GC), further supporting the role of lysosomal membrane permeabilization in SIRT1 depletion. Elevated cytoplasmic activity of cathepsin B, as judged from the imaging data obtained with the fluorescent substrate, Magic Red, further supported the possibility of the involvement of cathepsin in SIRT1 depletion. This mode of action was directly confirmed in the *in vitro* co-incubation of SIRT1 with cathepsins B, L, and S. Furthermore, considering the role of oxidative stress in lysosomal membrane permeabilization, we applied a selenoorganic antioxidant and peroxynitrite scavenger, ebselen, which protected SIRT1 from stress-induced depletion by preventing the loss of lysosomal membrane integrity and, therefore, reducing cytoplasmic shift of cathepsins. Collectively, the data outline a pathway from stressed embryonic EPC via oxidative stress to lysosomal membrane permeabilization, followed by SIRT1 depletion secondary to leaked cathepsins (Figure 11). This pathway may be linked to subverted autophagy, which serves as an amplifier for SIPS. Ebselen, by preventing oxidative stress-induced loss of lysosomal membrane integrity, blocks this pathway. Although each step of the pathway may have multiple mechanistic branching, the outline rendered here appears to provide the best fit of the data.

It has been established that lysosomal dysfunction is a frequent accompaniment of cell stress.[29,30] Moreover, lysosomal destabilization is caused by reactive oxygen species,[31,32] known to be induced by all three stressors used in the present studies.[3,12,33] In this context, effects of ebselen, a selenoorganic antioxidant and peroxynitrite scavenger, are conceivably mediated through reduction of free radical–induced lysosomal membrane permeabilization, and data presented herein confirm this prediction.

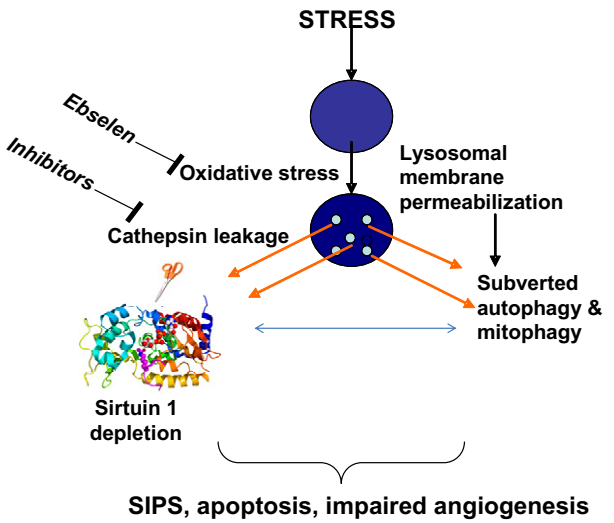

Figure 11. Hypothetical pathway of SIPS induction in EPC by relevant cardiovascular stressors.

Lysosomal dysfunction is known to subvert autophagy leading to accumulation of autophagosomes/autolysosomes, as well as the engulfed material (lipofuscin-loaded lysosomes), because of the lack of the final recycling step.[34] When this loss of function is accompanied by the lysosomal membrane permeabilization, the cathepsins released into the cytosol may trigger apoptosis and redirect the normally compartmentalized autophagy to nonphysiological sets of targets. The molecular mechanisms by which cathepsins trigger apoptosis are not entirely clear; however, there is accumulating evidence that they activate Bid and degrade antiapoptotic members of the Bcl-2 family, resulting in the engagement of mitochondrial pathways in cell death.[24] Here we demonstrate for the first time that SIRT1 is an important substrate of cysteine cathepsins. Its degradation by the cathepsins can thus represent a hitherto hidden mechanism linking cell stress with SIPS or apoptosis. Moreover, lysosomal membrane permeabilization and subversion of autophagy could also be associated with the accumulation of p62/SQSTM-1. It has recently been demonstrated that p62/SQSTM-1 accumulation not only is a marker of perturbed autophagic flux but may also affect cell viability by competing with the kelch-like ECH-associated protein 1 (Keap1) for a stress-response transcription factor Nrf2.[35] It has been shown that p62/SQSTM-1 binds to Keap1, the partner of Nrf2 assuring its degradation, and p62 accumulation stabilizes Nrf2 and hyperactivates its target genes. Although it has not been established whether Nrf2 hyperactivation may instigate cell senescence, one of the conditions leading to p62/SQSTM-1 accumulation, namely subverted autophagy, has been linked to cell aging.[5] Hence, it is possible, albeit not studied here, that the finding of subverted autophagy leading to p62/SQSTM-1 accumulation in and SIPS of stressed EPC can be at least in part attributed to the above Nrf2-Keap1–dependent mechanism.

Induction of autophagy is a part of an adaptive response to stress and is implicated in lifespan extension.[36–38] It has been proposed that disruption of autophagy induces cell senescence.[39] Our findings provide an integrative mechanism linking stress-induced lysosomal membrane permeabilization with cathepsin-mediated cleavage of SIRT1 and subverted autophagy in embryonic EPC, leading to their SIPS or apoptosis. Ebselen prevents SIRT1 depletion by improving lysosomal membrane integrity. This is not a trivial finding because, from the therapeutic standpoint, administration of ebselen, the clinical safety of which has been demonstrated, could represent a convenient attempt to pharmacologically manipulate the level of SIRT1. The proposed mechanism of SIRT1 depletion in stress has all of the attributes of being a paradigm of premature cell senescence: a broad range of stressors inducing it, the

commonality of lysosomal permeabilization, and universality of sirtuin action.

References

1. Erusalimsky JD, Kurz DJ: Endothelial cell senescence. Handb Exp Pharmacol 2006, 213–248
2. Patschan S, Chen J, Polotskaia A, Mendelev N, Cheng J, Patschan D, Goligorsky MS: Lipid mediators of autophagy in stress-induced premature senescence of endothelial cells. Am J Physiol Heart Circ Physiol 2008, 294:H1119–H1129
3. Patschan S, Chen J, Gealekman O, Krupincza K, Wang M, Shu L, Shayman JA, Goligorsky MS: Mapping mechanisms and charting the time course of premature cell senescence and apoptosis: lysosomal dysfunction and ganglioside accumulation in endothelial cells. Am J Physiol Renal Physiol 2008, 294:F100–F109
4. Rine J, Strathern JN, Hicks JB, Herskowitz I: A suppressor of mating-type locus mutations in Saccharomyces cerevisiae: evidence for and identification of cryptic mating-type loci. Genetics 1979, 93:877–901
5. Kennedy BK, Austriaco NR, Jr., Zhang J, Guarente L: Mutation in the silencing gene SIR4 can delay aging in S. cerevisiae. Cell 1995, 80:485–496
6. Haigis MC, Guarente LP: Mammalian sirtuins—emerging roles in physiology, aging, and calorie restriction. Genes Dev 2006, 20:2913–2921
7. Han MK, Song EK, Guo Y, Ou X, Mantel C, Broxmeyer HE: SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. Cell Stem Cell 2008, 2:241–251
8. Narala SR, Allsopp RC, Wells TB, Zhang G, Prasad P, Coussens MJ, Rossi DJ, Weissman IL, Vaziri H: SIRT1 acts as a nutrient-sensitive growth suppressor and its loss is associated with increased AMPK and telomerase activity. Mol Biol Cell 2008, 19:1210–1219
9. Mantel C, Broxmeyer HE: Sirtuin 1, stem cells, aging, and stem cell aging. Curr Opin Hematol 2008, 15:326–331
10. Lee IH, Cao L, Mostoslavsky R, Lombard DB, Liu J, Bruns NE, Tsokos M, Alt FW, Finkel T: A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. Proc Natl Acad Sci USA 2008, 105:3374–3379
11. Chen J, Li H, Addabbo F, Zhang F, Pelger E, Patschan D, Park HC, Kuo MC, Ni J, Gobe G, Chander PN, Nasjletti A, Goligorsky MS: Adoptive transfer of syngeneic bone marrow-derived cells in mice with obesity-induced diabetes: selenoorganic antioxidant ebselen restores stem cell competence. Am J Pathol 2009, 174:701–711
12. Chen J, Brodsky SV, Goligorsky DM, Hampel DJ, Li H, Gross SS, Goligorsky MS: Glycated collagen I induces premature senescence-like phenotypic changes in endothelial cells. Circ Res 2002, 90:1290–1298
13. Brodsky SV, Gealekman O, Chen J, Zhang F, Togashi N, Crabtree M, Gross SS, Nasjletti A, Goligorsky MS: Prevention and reversal of premature endothelial cell senescence and vasculopathy in obesity-induced diabetes by ebselen. Circ Res 2004, 94:377–384
14. Sharpless NE, DePinho RA: How stem cells age and why this makes us grow old. Nat Rev Mol Cell Biol 2007, 8:703–713
15. Hatzopoulos AK, Folkman J, Vasile E, Eiselen GK, Rosenberg RD: Isolation and characterization of endothelial progenitor cells from mouse embryos. Development 1998, 125:1457–1468
16. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M: Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol 2001, 230:230–242
17. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi J: A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 1995, 92:9363–9367
18. van der Loo B, Fenton MJ, Erusalimsky JD: Cytochemical detection of a senescence-associated beta-galactosidase in endothelial and smooth muscle cells from human and rabbit blood vessels. Exp Cell Res 1998, 241:309–315
19. Erwig LP, McPhilips KA, Wynes MW, Ivetic A, Ridley AJ, Henson PM: Differential regulation of phagosome maturation in macrophages and dendritic cells mediated by Rho GTPases and ezrin-radixin-moesin (ERM) proteins. Proc Natl Acad Sci USA 2006, 103:12825–12830
20. Rozman J, Stojan J, Kuhelj R, Turk V, Turk B: Autocatalytic processing of recombinant human procathepsin B is a bimolecular process. FEBS Lett 1999, 459:358–362
21. Bromme D, Nallaseth FS, Turk B: Production and activation of recombinant papain-like cysteine proteases. Methods 2004, 32:199–206
22. Mihelic M, Dobersek A, Guncar G, Turk D: Inhibitory fragment from the p41 form of invariant chain can regulate activity of cysteine cathepsins in antigen presentation. J Biol Chem 2008, 283:14453–14460
23. Turk B, Krizaj I, Kralj B, Dolenc I, Popovic T, Bieth JG, Turk V: Bovine stefin C, a new member of the stefin family. J Biol Chem 1993, 268:7323–7329
24. Cirman T, Oresic K, Mazovec GD, Turk V, Reed JC, Myers RM, Salvesen GS, Turk B: Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. J Biol Chem 2004, 279:3578–3587
25. Lamark T, Kirkin V, Dikic I, Johansen T: NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. Cell Cycle 2009, 8:1986–1990
26. Boya P, Kroemer G: Lysosomal membrane permeabilization in cell death. Oncogene 2008, 27:6434–6451
27. Petiot A, Ogier-Denis E, Blommaart EF, Meijer AJ, Codogno P: Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J Biol Chem 2000, 275:992–998
28. Tamai M, Matsumoto K, Omura S, Koyama I, Ozawa Y, Hanada K: In vitro and in vivo inhibition of cysteine proteinases by EST, a new analog of E-64. J Pharmacobiodyn 1986, 9:672–677
29. Roberg K, Ollinger K: Oxidative stress causes relocation of the lysosomal enzyme cathepsin D with ensuing apoptosis in neonatal rat cardiomyocytes. Am J Pathol 1998, 152:1151–1156
30. Yuan XM, Li W, Dalen H, Lotem J, Kama R, Sachs L, Brunk UT: Lysosomal destabilization in p53-induced apoptosis. Proc Natl Acad Sci USA 2002, 99:6286–6291
31. Repnik U, Turk B: Lysosomal-mitochondrial cross-talk during cell death. Mitochondrion 2010, 10:662–669
32. Terman A, Kurz T, Gustafsson B, Brunk UT: Lysosomal labilization. IUBMB Life 2006, 58:531–539
33. Addabbo F, Ratliff B, Park HC, Kuo MC, Ungvari Z, Csiszar A, Krasnikov B, Sodhi K, Zhang F, Nasjletti A, Goligorsky MS: The Krebs cycle and mitochondrial mass are early victims of endothelial dysfunction: proteomic approach. Am J Pathol 2009, 174:34–43
34. Kurz T, Eaton JW, Brunk UT: Redox activity within the lysosomal compartment: implications for aging and apoptosis. Antioxidants 13:511–523
35. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, Sou YS, Ueno I, Sakamoto A, Tong KI, Kim M, Nishito Y, Iemura S, Natsume T, Ueno T, Kominami E, Motohashi H, Tanaka K, Yamamoto M: The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nature 12:213–223
36. Terman A, Brunk UT: Autophagy in cardiac myocyte homeostasis, aging, and pathology. Cardiovasc Res 2005, 68:355–365
37. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ: Potential therapeutic applications of autophagy. Nat Rev Drug Discov 2007, 6:304–312
38. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA: Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 2009, 460:392–395
39. Levine B, Kroemer G: Autophagy in the pathogenesis of disease. Cell 2008, 132:27–42

Cysteine cathepsins are not critical for TRAIL- and CD95-induced apoptosis in several human cancer cell lines

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Running Title: Cysteine cathepsins in death receptor pathway

Abstract

The potential role of cysteine cathepsins in TRAIL (Apo2L)- and CD95 (Fas/APO-1)-induced apoptosis was investigated using four different cell lines, HeLa, HuH-7, Jurkat and U-937. All four cell lines exhibited different levels of cathepsins and responded differently to apoptosis triggering with Jurkat cells being the most sensitive and the only ones that were sensitive to the agonistic anti-APO-1 antibody. Apoptosis was accompanied by caspase activation, loss of mitochondria and lysosome integrity, and by the release of cathepsins into the cytosol, as judged on the basis of the hydrolysis of the cathepsin substrate Z-FR-AMC and by the immunological detection of cathepsin B. Inhibition of caspases by the broad spectrum inhibitor Z-VAD-FMK prevented apoptosis, including mitochondrial and lysosomal membrane permeabilisation, as well as cathepsin release into the cytosol, consistent with caspases playing a crucial role in the process. Conversely, however, whilst the broad spectrum cathepsin inhibitor E-64d and the more cathepsin B-selective inhibitor CA-074Me completely blocked cathepsin activity, these inhibitors neither prevented apoptosis and its progression nor mitochondrial and lysosomal membrane permeabilisation associated with this type of cell death. Consequently, cathepsin release into the cytosol was also not prevented. Together, these data indicate that cysteine cathepsins are not required for TRAIL- and CD95-mediated apoptosis in various human cancer cell lines. This does not, however, rule out that lysosomes and cathepsins may amplify the signal in certain cell lines or under different stimulation conditions than the ones employed here.

Keywords

Lysosomes, Cathepsins, TRAIL (Apo2L), CD95 (Fas/APO-1), apoptosis, death receptor

Abbreviations: anti-APO-1, monoclonal antibody to human CD95 of the murine IgG3 isotype; AFC, 7-amino-4-trifluoromethylcoumarin; AMC, 7-amino-4-methylcoumarin; CA-074Me, [(2S,3S)-3-Propylcarbamoyloxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester; CHX, cycloheximide; E-64d, L-trans-epoxysuccinyl(OEt) -Leu-3-methylbutylamide; FMK, fluoromethylketone; TRAIL, TNF-related apoptosis-inducing ligand; Z, benzyloxycarbonyl.

Introduction

Members of the tumor necrosis factor (TNF) superfamily of cytokines, in particular TNF itself, the CD95 (Fas/APO-1) ligand (CD95L), and the TNF-related apoptosis-inducing ligand (TRAIL/Apo2L), trigger the extrinsic apoptosis pathway by binding to their corresponding receptors on the surface of the target cells. These receptors form part of death receptor subfamily of the TNF receptor (TNF-R) superfamily, which includes, amongst others CD95, TRAIL-R1 (DR4) and TRAIL-R2 (KILLER/TRICK2/DR5/Apo2). Once activated, these receptors recruit the adapter molecule Fas-associated death domain (FADD), thereby inducing the formation of the death-inducing signal complex (DISC), which enables recruitment and subsequent activation of the initiator caspases 8/10. Active caspase-8 then activates the executioner caspases-3 and -7, leading to apoptosis. Based on the level of DISC and caspase-8 activity, cells can be divided into the type I cells, which are capable of forming high DISC levels and/or contain less of the X-linked inhibitor of apoptosis protein (XIAP), and the type II cells that are less well capable of forming the DISC and/or contain high XIAP levels. Consequently, in the type I cells the DISC-generated caspase-8 activity is sufficient to overcome the inhibitory effect of XIAP at a given concentration on caspase activation whereas in the type II cells the ration between DISC-generated caspase-8 activity and XIAP expression is such that direct activation of effector caspases is not sufficient to induce apoptosis so that an amplification of the signal through the mitochondria is required for the induction of apoptosis. The engagement of the mitochondrial pathway is accomplished by the caspase-8-mediated cleavage of the proapoptotic Bcl-2 homologue Bid (Ashkenazi and Dixit, 1999; Gonzalvez and Ashkenazi, 2010; Newsom-Davis et al., 2009; Walczak and Krammer, 2000).

Although caspases have a major role in death receptor-induced apoptosis, there have been numerous reports that cysteine cathepsins, in particular cathepsin B, may be involved in the process downstream of caspase-8 activation (Foghsgaard et al., 2001; Groth-Pedersen and Jaattela, 2010; Guicciardi and Gores, 2009; Guicciardi et al., 2004; Kirkegaard and Jaattela, 2009). Under normal conditions, cysteine cathepsins are localized in the vesicles of the endolysosomal system, where they are, in addition to some more specific functions, also involved in the bulk protein degradation (Brix et al., 2008; Reiser et al., 2010; Turk et al., 2002; Turk and Turk, 2009; Turk et al., 2012b). However, when released into the cytosol the cathepsins were found to trigger apoptosis through the cleavage of Bid and degradation of the antiapoptotic Bcl-2 homologs and XIAP, thereby engaging the mitochondrial pathway (Blomgran et al., 2007; Cirman et al., 2004; Droga-Mazovec et al., 2008; Stoka et al., 2001). They were also suggested to act independently of the caspases, although their molecular targets were never identified (Foghsgaard et al., 2001). Among the factors that were suggested to destabilize the lysosomal membranes, resulting in a release of the cathepsins into the cytosol are also members of the TNF family (Fehrenbacher and Jaattela, 2005; Guicciardi et al., 2007; Guicciardi and Gores, 2009; Kirkegaard and Jaattela, 2009; Repnik et al., 2012; Stoka et al., 2005; Stoka et al., 2007; Turk and Turk, 2009; Werneburg et al., 2007).

However, the exact role of cathepsins in the death receptor signaling is still unclear, as conflicting results have being reported (Bojic et al., 2007; Brunk and Svensson, 1999; Fehrenbacher et al., 2004; Foghsgaard et al., 2001; Guicciardi et al., 2007; Guicciardi et al., 2000; Klaric et al., 2009; Oberle et al., 2010; Vasiljeva et al., 2008; Yang et al., 2010b).

CD95, TRAIL-R1 and TRAIL-R2, and TNF-R1 are all expressed by immune cells, but they are also widely expressed by a number of tumor cells, making them attractive anticancer targets (Ashkenazi, 2008; Ashkenazi and Herbst, 2008; Gerspach et al., 2011; Papenfuss et al., 2008). In particular, agonists targeting the TRAIL pathway, including soluble recombinant TRAIL and the agonistic antibodies to TRAIL-R1 and TRAIL-R2, are now in advanced clinical trials showing at least partially encouraging results with few adverse effects (Gerspach et al., 2011; Newsom-Davis et al., 2009; Yang et al., 2010a). Understanding the molecular mechanisms leading to death receptor-induced apoptosis and the killing of tumor cells is, therefore, of major importance. In order to address the potential role of lysosomes and cysteine cathepsins in CD95- and TRAIL-induced apoptosis, we have followed apoptosis, mitochondrial and lysosomal integrity, as well as the activity of cathepsins, using four different cancer cell lines containing different levels of cathepsins. Blocking cathepsin activity during CD95- and TRAIL-induced apoptosis was found not to affect apoptosis progression, even though lysosomal membrane integrity was lost and cathepsins were released into the cytosol, both in a caspase-dependent manner. These results indicate that, while caspases are, cathepsins are not required for CD95- and TRAIL-induced apoptosis.

Results

Triggering of TRAIL death receptors or CD95 induces caspase-dependent apoptosis that is independent of cathepsin activity

In order to evaluate the possible role of cysteine cathepsins in TRAIL- and Fas-induced apoptosis, four different cell lines, HeLa, HuH-7, Jurkat and U-937, were selected. In the preliminary experiments, the levels of cysteine cathepsins in individual cell lines were estimated on the basis of the hydrolysis of Z-FR-AMC substrate, which is hydrolysed well by most cathepsins, including cathepsins B, S, L, V, F and K, and of the cathepsin B substrate Z-RR-AMC. In addition, U-937 cells differentiated into the macrophages were tested, as they are known to contain high levels of cathepsins, especially cathepsin B (Klaric et al., 2009; Kos et al., 2005). As can be seen in Figure 1a, differentiated U-937 cells exhibited the highest Z-FR-AMC hydrolysing activity and a comparable Z-RR-AMC hydrolysing activity, in agreement with previous results. HuH-7 cells exhibited the second highest Z-FR-AMC-hydrolysing activity, followed by Jurkat and HeLa cells, whereas undifferentiated U-937 cells exhibited an almost 10-fold lower activity as compared to the differentiated U-937 cells, and a more than 3-fold lower activity than the HuH-7 cells, suggesting a significantly lower cathepsin expression. When these cells were compared on the basis of their Z-RR-AMC hydrolysing activity, it can be seen that the majority of cathepsin activity could be attributed to

cathepsin B in the immune type cells, especially in differentiated U-937 cells. This result was confirmed by western blotting (Figure 1b).

In the next set of experiments HeLa, HuH-7, Jurkat and undifferentiated U-937 cells were tested for their sensitivity to apoptosis induction by TRAIL and an agonistic antibody to CD95. As shown in Figure 2a, all four cell lines responded to TRAIL with the Jurkat cells being by far most sensitive to the treatment (1 ng/ml TRAIL vs. 100 ng/ml in the other three cell lines), based on Annexin V/PI staining. Simultaneous inhibition of proteasome activity significantly enhanced apoptosis in HeLa, HuH-7 and particularly in the U-937 cells, but not in the Jurkat cells, in agreement with the previous findings (Brooks et al., 2005; Koschny et al., 2010). We next performed the same set of experiments in the presence of E-64d, a broad-spectrum cell-permeable inhibitor of cysteine cathepsins, CA-074Me, a cell-permeable cathepsin B inhibitor, or Z-VAD-FMK, a broad-spectrum caspase inhibitor which served as positive control. As can be seen in Figure 2a, none of the cathepsin inhibitors prevented apoptosis, whereas Z-VAD-FMK conferred significant, albeit not complete protection, suggesting that cysteine cathepsins are not critical for the induction or progression of TRAIL-induced apoptosis in human cells. In a related experiment, we evaluated the role of cathepsins in CD95-mediated apoptosis using the agonistic antibody anti-APO-1. In contrast to TRAIL, anti-APO-1 induced apoptosis in combination with protein A (0.3 ng/ml of anti-APO-1 and protein A, 18 hours) only in Jurkat cells, whereas no apoptosis was induced in the other three cell lines tested even at concentrations of 1000 ng/ml (Figure 2b). Therefore, all the subsequent studies with the CD95 system were performed only in Jurkat cells. Similar to the results with TRAIL, cathepsin inhibitors did not prevent CD95-mediated apoptosis in Jurkat cells, in agreement with a marginal, if any, role of cathepsins in this apoptosis pathway (Figure 2c). None of the inhibitors by themselves exhibited cytotoxicity in any of the cell lines used (not shown).

As Z-VAD-FMK did not completely rescue apoptosis, we next investigated the activity of caspases in the total cell extracts following TRAIL and anti-APO-1 treatment. In agreement with a major role of caspases in death receptor-induced apoptosis, caspase activity, as measured by the hydrolysis of the Ac-DEVD-AMC substrate, was significantly increased in all cell lines investigated, with the highest levels in U-937 and Jurkat cells (Figure 3). None of the cathepsin inhibitors blocked the DEVD-ase activity, again suggesting that cysteine cathepsins are not involved in death receptor-mediated apoptosis. In contrast, Z-VAD-FMK efficiently blocked all the DEVD-ase activity, in agreement with its caspase-blocking properties. The small increase in TRAIL-induced apoptosis observed in U-937 cells in the presence of the inhibitor can, therefore, be attributed to either a non-caspase component (caspase-independent) or to an incomplete caspase inhibition as a very small DEVD-ase activity was still seen in U-937 cells in the presence of Z-VAD-FMK.

Mitochondria and lysosomes are disrupted in TRAIL- and anti-APO-1-induced apoptosis

In the next step the stability of mitochondria and lysosomes, and the effect of cathepsin and caspase inhibitors on these organelles' stability, were evaluated in both TRAIL and anti-APO-1-induced apoptosis. Again, mitochondrial membrane permeabilization (MMP) and lysosomal membrane permeabilization (LMP) were monitored at the 24 hour (HeLa, HuH-7 and U-937 cells) or 18 hour (Jurkat cells) time point, where significant apoptosis was observed in the previous set of experiments (see above). As can be seen in Figure 4, the percentage of cells with damaged mitochondria increased in all cell lines investigated in both TRAIL and anti-APO-1-induced apoptosis, being the highest in U-937 cells (around 50 %), followed by Jurkat cells, which is consistent with the higher sensitivity of these two cell lines to TRAIL and anti-APO-1, respectively (Figure 2). Both cathepsin inhibitors, E-64d and CA-074Me, did not prevent MMP, whereas Z-VAD-FMK conferred complete protection in HeLa, HuH-7 and Jurkat cells, in agreement with the major role of caspases in the process. However, Z-VAD-FMK did not completely protect the mitochondria in U-937 cells, similar to its incomplete protection against apoptosis in the same cell line. Interestingly, TRAIL induced major LMP in all cell lines, with the highest being observed in HeLa cells, where almost 70% of the cells had damaged lysosomes (Figure 5a). Anti-APO-1 induced LMP in about 20% of Jurkat cells, although a direct comparison with TRAIL-induced apoptosis is not possible. However, a very similar picture was observed with the inhibitors. E-64d and CA-074Me had no influence on the induction of LMP, whereas Z-VAD-FMK completely protected Jurkat cells against LMP (Figure 5b).

Cathepsin activity in the cytosol is increased during TRAIL- and CD95-mediated apoptosis

As both TRAIL- and anti-APO-1-induced apoptosis were found to be accompanied by LMP, the next questions were whether cathepsins were released into the cytosol, how quantitative this release was and how active they were? To address these questions, the activity of the escaped cysteine cathepsins in the cytosol was measured as well as the total cathepsin activity. In addition to measuring the activity of released cathepsins in the absence of any chemical inhibitors of cathepsins or caspases, the effect of all three inhibitors on release and activity of cathepsins was evaluated. In the control experiment in untreated cells, the activity of the cathepsins was completely blocked by 10 μM E-64d and CA-074Me in all four cell lines (Figure 6a). Although this implies that the predominant cathepsin activity in all cell lines examined was cathepsin B activity, one has to be aware that in contrast to the nonesterified CA-074, CA-074Me is not really selective for cathepsin B (Bogyo et al., 2000), suggesting that other cathepsins could contribute to the Z-FR-AMC-hydrolysing activity. This is supported by the finding that in HeLa cells the cathepsin L activity is at least comparable to the cathepsin B activity, based on the measurements employing the broad-spectrum cathepsin activity-based probe CM-282 (U. Repnik & B. Turk, unpublished). However, Z-VAD-

FMK also reduced the cathepsin activity by 20-30% in all four cell lines already at 10 μM concentration, in agreement with the known cross-reactivity of the inhibitor (Rozman-Pungercar et al., 2003). Next, the total activity of the cathepsins in cells was measured following the addition of TRAIL +/- bortezomib and anti-APO-1 +/- protein A. As can be seen in Figure 6, the total cathepsin activity was only slightly decreased upon TRAIL, bortezomib or anti-APO-1 treatment. However, the combination of TRAIL and bortezomib resulted in a significantly reduced cathepsin activity in all four cell lines. As this combination was the most active with respect to apoptosis induction on all cells, it further suggested that cathepsins slowly inactivate following their release into the cytosol. To test this hypothesis, we next determined the cytosolic cathepsin activity. As shown in Figures 7a and c, only a minor cathepsin activity was detected in the cytosol of untreated cells as compared to the total cathepsin activity. Following TRAIL/bortezomib treatment, a significant increase of the cytosolic cathepsin activity was observed. Moreover, this cytosolic cathepsin activity represented the majority of the total cathepsin activity in the cells, suggesting that most of the cathepsins were released into the cytosol. In contrast, the release of cathepsins into the cytosol was much lower under all other conditions (TRAIL only, anti-APO-1). Again, E-64d and CA-074Me completely blocked cathepsin activity, whereas the cytosolic activity of the cathepsins was, at least in HuH-7 cells, significantly higher than the cytosolic activity of the untreated cells. However, this activity represented only a minor portion of the total cathepsin activity in HuH-7 cells as judged on the basis of the total cell extracts (10-15%; Figure 6a), in agreement with the major protection role of Z-VAD-FMK and the efficient caspase inhibition. The release of cathepsins into the cytosol was additionally confirmed on the protein level by western blotting of cathepsin B following treatment of Jurkat cells with TRAIL or anti-APO-1 (Figures 7b and 7d).

Discussion

Whereas the role of caspases as the main players in the death receptor pathway has been clearly established, there is much dispute about the potential role of lysosomal cathepsins in this pathway. Using four different cancer cell lines we here show that the activity of cysteine cathepsins is not critical for induction or progression of apoptosis induced by recombinant TRAIL or the agonistic antibody anti-APO-1. This is despite the fact that apoptosis induction by these two agents was found to be accompanied by significant damage to mitochondria and lysosomes, and the release of cathepsins into the cytosol of apoptotic cells. All four cell lines used belonged to type II cells that require an amplification of the signal through engagement of the mitochondrial pathway (Kim et al., 2004; Lee et al., 2005; Scaffidi et al., 1998; Terrisse et al., 2002) in order to exclude the possibility of a direct activation of caspase-3 by activated caspase-8. In addition, they were of different origin, including immune (Jurkat and U-937), hepatocyte carcinoma (HuH-7) and cervical cancer (HeLa), and were found to contain different levels and compositions of cathepsins (Figure 1). This suggests that the bystander role of cathepsins in the death receptor apoptosis pathway is a

more general phenomenon, rather than one limited to a single cell line. Moreover, both immune-type cell lines, Jurkat and U-937, were more sensitive to TRAIL than HuH-7 and HeLa cells, although at least U-937 cells exhibited substantially lower cathepsin activity than the other cell lines, in agreement with the nonessential role of cathepsins in TRAIL-induced apoptosis. Although only Jurkat cells responded well to the agonistic antibody anti-APO-1, the conclusions were essentially the same as for TRAIL-induced apoptosis (Figure 2). This is in agreement with several recent studies which demonstrated that cysteine cathepsins are not critical for the progression of CD95-mediated apoptosis in several types of mouse cells (Bojic et al., 2007; Oberle et al., 2010) and *in vivo* in mouse liver or in Jurkat cells (Wattiaux et al., 2007), and neither for the progression of TNF-induced apoptosis in primary mouse mammary gland cancer cells (Vasiljeva et al., 2008) or in several human cancer cell lines (Klaric et al., 2009). This is further supported by the finding that even a complete inhibition of the cathepsins' activity (Figures 6 and 7) had no influence on apoptosis progression (Figures 2 and 3). Moreover, the lack of the effect of cathepsin inhibition on apoptosis progression also disputed the possibility that cathepsins would have an important role in the endolysosomal degradation of signaling complexes during CD95 or TRAIL receptor internalization, as observed earlier for the related TWEAK system, where cathepsins were responsible for the degradation of cIAP1–TRAF2 complex (Vince et al., 2008). Our results, however, neither preclude nor do they support the possibility that receptor internalization may serve as an important step during CD95- or TRAIL-mediated signaling as suggested previously (Akazawa et al., 2009; Guicciardi and Gores, 2009; Kohlhaas et al., 2007; Schutze et al., 2008).

In apparent contrast to these results, several earlier studies suggested that cathepsins, especially cathepsin B, are important mediators of apoptosis upstream of MMP in many different cell types in TNF- and TRAIL-induced apoptosis when released into the cytosol (Fehrenbacher et al., 2004; Foghsgaard et al., 2001; Guicciardi et al., 2007; Guicciardi et al., 2000; Nagaraj et al., 2006, 2007). However, in these studies apoptosis was not abrogated, but only delayed, arguing against a critical role of cathepsins in the pathway. In addition, in most of these studies the cytosolic and total activities of cathepsins were not monitored. Furthermore, all these studies only looked at apoptosis at a single time point and rather late stages, when the organelles are already disrupted and the lysosomes become leaky, making assumptions about the pathway more speculative. As seen in both TRAIL and CD95-mediated apoptosis, lysosomes were damaged in the majority of cells at a late point in time, with the highest percentage observed in HeLa cells (ca. 70 %; Figure 5a). However, HeLa cells were also the least sensitive to apoptosis induction, a result that clearly contrasts with the idea of a critical role of LMP in death receptor-induced apoptosis. Moreover, if cathepsins were indeed critical for MMP, cathepsin inhibition should have blocked it, which was, however, not the case (Figure 4). In addition, the cytosolic activity of cathepsins was found to be considerably lower than the total cathepsin activity following apoptosis induction (Figures 6 and 7) and significantly lower than the proportion of cells with damaged lysosomes (Figure 5), suggesting that only a minor portion of the total cathepsins were

released into the cytosol. Furthermore, the cytosolic activity of cathepsins in the cells treated with a combination of bortezomib and TRAIL was higher than in the cells treated with TRAIL alone (Figure 6), consistent with the more efficient induction of apoptosis than TRAIL alone. However, the total cathepsin activity was decreased in these cells in agreement with the idea that cathepsins once in the cytosol, become inactivated due to the unfavorable pH conditions (Turk and Turk, 2009).

This raises the question of whether cathepsins are involved in death receptor apoptosis at all, and what is/are the potential mechanism(s)? The critical issue seems to be the lysosomal membrane permeabilization. It has been recently shown that in fibroblasts LMP is a Bax/Bak-dependent event in Fas apoptosis, placing lysosomes downstream of the mitochondria in the death receptor pathway (Oberle et al., 2010). Moreover, lysosomal destabilization was also found to be a very late event in CD95 apoptosis in hepatocytes *in vivo* and in skin fibroblasts, consistent with their role downstream of the mitochondria in this pathway (Bojic et al., 2007; Wattiaux et al., 2007). This could also explain why the inhibition of cathepsins did not affect apoptosis progression nor prevent MMP in these cells. Moreover, blocking the caspases prevented both MMP and LMP, suggesting that LMP occurs downstream of caspase-8 activation and, possibly, LMP. Based on these and previous findings it can be suggested that LMP is an MMP-dependent step. Potential links could be associated with sphingolipid, arachidonic acid and fatty acid metabolism, oxidative stress, as well as with some other pathways (Repnik et al., 2012; Schrader et al., 2010). The pathways downstream of LMP are probably linked with cathepsin-mediated Bid cleavage and subsequent recruitment of the mitochondrial pathway (Repnik et al., 2012; Turk et al., 2012a). Although Bid is not a critical *in vivo* apoptotic substrate of the cathepsins (Houseweart et al., 2003), a diminished Bid cleavage was observed in cathepsin B-deficient primary mouse skin fibroblasts during CD95-induced apoptosis (Bojic et al., 2007), supporting this idea. This further suggests that the cathepsins are possibly involved in an amplification loop involving mitochondria and lysosomes (Repnik et al., 2012; Schrader et al., 2010). The level of amplification may depend on the cell type and the stimulus, ranging from essentially zero to a significant amount that can be detected.

In conclusion, our results suggest that cysteine cathepsins are not critically involved in TRAIL- and CD95-induced apoptosis. Although lysosomes were found to be damaged and cysteine cathepsin activity in the cytosol was increased, inhibition of cysteine cathepsins in the selected cell lines had no effect on apoptosis progression. However, the possibility that cysteine cathepsins are involved in the amplification, but not in the initiation, of death receptor-mediated apoptosis cannot be excluded.

Materials and methods

Materials

Cell culture media DMEM, RPMI 1640, fetal bovine serum (FBS), glutamine and penicillin/streptomycin were purchased from PAA (Austria). Trypsin was from Gibco (USA), CHX, Protein A, propidium iodide and acridine orange were obtained from Sigma (USA), CHAPS, polyethyleneglycol, Protein A, propidium iodide and acridine orange were obtained from Sigma (USA), HEPES and EDTA were from Serva (Germany), sucrose was from Fluka (Germany) and dithiothreitol was from Fermentas (Canada). Cathepsin inhibitors (2S,3S)-trans-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester (E-64d) and [(2S,3S)-3-Propylcarbamoyloxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA-074Me) were from the Peptide Research Institute (Osaka, Japan). Caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK), 7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) and benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin (Z-FR-AMC) were from Bachem AG (Switzerland). Annexin V-PE was from BD Biosciences Inc. (USA), Mitotracker Red CMXRos from Invitrogen (USA) and Bradford reagent was from Bio-Rad (Germany). Recombinant human TRAIL was prepared as previously described (Ganten et al., 2004) and generously provided by Henning Walczak (Imperial College London, London, UK); the agonistic monoclonal antibody anti-APO-1 of the murine isotype IgG3 (Trauth et al., 1989) was a generous gift from Peter H. Krammer (Deutsches Krebsforschungszentrum, Heidelberg, Germany).

Cells

All cell lines used (U-937 - Human Caucasian histiocytic lymphoma, HeLa - Human cervix carcinoma, HuH-7 – Human hepatocellular carcinoma (HuH-7D12) and Jurkat - Human leukemic T-cell lymphoblasts) were purchased from the European Collection of Cell Cultures (ECACC).

U-937 and Jurkat cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 1% glutamine and 1% penicillin/streptomycin. For the experiment $1x10^6$ cells were plated into a 12-well plate. For the differentiation of U-937 into macrophages $1.4x10^6$ cells were plated into one 6-well with 30 nM phorbol 12-myristate 13-acetate (PMA) (Sigma) for 48 hours, followed by 24 hours of regeneration without PMA. HeLa and HuH-7 cells were grown in DMEM medium supplemented with 10% FBS, 1 % glutamine and 1% penicillin/streptomycin. For the experiment $8x10^4$ cells were plated into 12-well plate 16 hours prior to treatment. All cells were cultured at 37 °C in a humidified air atmosphere with 5% CO_2.

Induction of apoptosis

Cathepsin (E-64d and CA-074Me) or caspase (Z-VAD-FMK) inhibitors were added at a final concentration of 10 μM two hours before the induction of apoptosis with TRAIL/Bortezomib or anti-APO-1/protein A. Twenty-four hours incubation of cells with 100 ng/ml TRAIL in combination with 10 nM Bortezomib was used to induce apoptosis in HeLa, U-937 and HuH-7 cells, whereas 18 hours incubation with 1 ng/ml TRAIL and 1 ng/ml Bortezomib was used to induce apoptosis in Jurkat cells. Twenty-four hours incubation of cells with up to 1000 ng/ml anti-APO-1, in combination with 1000 ng/ml protein A, was used to induce apoptosis in HeLa, U-937 and HuH-7 cells, whereas 18 hours incubation with 0.3 ng/ml anti-APO-1 and 0.3 ng/ml protein A was sufficient to induce apoptosis in Jurkat cells. Total and cytosolic cellular extracts were prepared as described previously (Droga-Mazovec et al., 2008; Ivanova et al., 2008; Klaric et al., 2009).

Flow cytometry

Quantification of cell death

Annexin-V-Pe and propidium iodide were used to determine phosphatidylserine (PS) exposure and the loss of membrane integrity according to the manufacturer's instructions. Analysis was made with a FACScalibur flow cytometer (Becton Dickinson, USA) and the CellQuest software.

Fluorescent dyes MitoTracker Red CMXRos and acridine orange (AO) were used to assess the integrity of mitochondria and lysosomes, as described previously (Bojic et al., 2007; Ivanova et al., 2008).

Activity measurements

Proteins from total extracts were tested for DEVD-ase activity by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AFC. Extracts were mixed in the 96-well plate with caspase buffer (100 mM HEPES, 200 mM NaCl, 0.2 % (w/v) CHAPS, 20 % (w/v) sucrose, 2 mM EDTA, 20 mM dithiothreitol, pH 7.0) to the final volume of 90 μl. After 15 minutes incubation at 37 °C the substrate was added to a final concentration of 10 μM, and substrate hydrolysis continuously measured in a 96-well plate reader (Safire Tecan) at excitation and emission wavelengths of 400 and 505 nm, respectively.

The cathepsin activity in total and cytosolic extracts was monitored using the fluorogenic substrates Z-FR-AMC (general cathepsin substrate) or Z-RR-AMC (cathepsin B substrate) as follows. Forty μl of extracts were mixed in the 96-well plate with buffer (100 mM phosphate buffer, 1 mM EDTA, 1 mM dithiothreitole and 0.1 % (w/v) polyethyleneglycol, pH 6.0) to the final volume of 90 μl. After 15 minutes of incubation at 37 °C, 10 μl of the substrate was added to a final concentration of 10 μM, followed by the continuous monitoring of substrate

hydrolysis in a 96-well plate reader (Tecan Safire) at excitation and emission wavelengths of 370 and 460 nm, respectively.

Immunoblotting

Equal amounts (50 μg) of protein, as determined by the BioRad assay, were loaded and resolved in 15% SDS–PAGE gels and electro-transferred to the nitrocellulose membranes. Blots were probed with cathepsin B specific mouse monoclonal antibodies at a concentration of 5 μg/ml. Goat antimouse horseradish peroxidase-conjugated secondary antibodies (Abcam) (1:3000 dilution) were added, followed by visualization with ECL according to the manufacturer's instructions (GE Healthcare Bio-Sciences Corp., USA). Actin probed with rabbit anti-actin antibodies (Sigma, USA) was used as the loading control for the cytosolic extracts.

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References

Akazawa, Y., Mott, J.L., Bronk, S.F., Werneburg, N.W., Kahraman, A., Guicciardi, M.E., Meng, X.W., Kohno, S., Shah, V.H., Kaufmann, S.H.*, et al.* (2009). Death receptor 5 internalization is required for lysosomal permeabilization by TRAIL in malignant liver cell lines. Gastroenterology *136*, 2365-2376 e2361-2367.

Ashkenazi, A. (2008). Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. Nat Rev Drug Discov *7*, 1001-1012.

Ashkenazi, A., and Dixit, V.M. (1999). Apoptosis control by death and decoy receptors. Curr Opin Cell Biol *11*, 255-260.

Ashkenazi, A., and Herbst, R.S. (2008). To kill a tumor cell: the potential of proapoptotic receptor agonists. J Clin Invest *118*, 1979-1990.

Blomgran, R., Zheng, L., and Stendahl, O. (2007). Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. J Leukoc Biol *81*, 1213-1223.

Bogyo, M., Verhelst, S., Bellingard-Dubouchaud, V., Toba, S., and Greenbaum, D. (2000). Selective targeting of lysosomal cysteine proteases with radiolabeled electrophilic substrate analogs. Chem Biol *7*, 27-38.

Bojic, L., Petelin, A., Stoka, V., Reinheckel, T., Peters, C., Turk, V., and Turk, B. (2007). Cysteine cathepsins are not involved in Fas/CD95 signalling in primary skin fibroblasts. FEBS Lett *581*, 5185-5190.

Brix, K., Dunkhorst, A., Mayer, K., and Jordans, S. (2008). Cysteine cathepsins: cellular roadmap to different functions. Biochimie *90*, 194-207.

Brooks, A.D., Ramirez, T., Toh, U., Onksen, J., Elliott, P.J., Murphy, W.J., and Sayers, T.J. (2005). The proteasome inhibitor bortezomib (Velcade) sensitizes some human tumor cells to Apo2L/TRAIL-mediated apoptosis. Annals of the New York Academy of Sciences *1059*, 160-167.

Brunk, U.T., and Svensson, I. (1999). Oxidative stress, growth factor starvation and Fas activation may all cause apoptosis through lysosomal leak. Redox report : communications in free radical research *4*, 3-11.

Cirman, T., Oresic, K., Mazovec, G.D., Turk, V., Reed, J.C., Myers, R.M., Salvesen, G.S., and Turk, B. (2004). Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. J Biol Chem *279*, 3578-3587.

Droga-Mazovec, G., Bojic, L., Petelin, A., Ivanova, S., Romih, R., Repnik, U., Salvesen, G.S., Stoka, V., Turk, V., and Turk, B. (2008). Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues. J Biol Chem *283*, 19140-19150.

Fehrenbacher, N., Gyrd-Hansen, M., Poulsen, B., Felbor, U., Kallunki, T., Boes, M., Weber, E., Leist, M., and Jaattela, M. (2004). Sensitization to the lysosomal cell death pathway upon immortalization and transformation. Cancer Res *64*, 5301-5310.

Fehrenbacher, N., and Jaattela, M. (2005). Lysosomes as targets for cancer therapy. Cancer Res *65*, 2993-2995.

Foghsgaard, L., Wissing, D., Mauch, D., Lademann, U., Bastholm, L., Boes, M., Elling, F., Leist, M., and Jaattela, M. (2001). Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. J Cell Biol *153*, 999-1010.

Ganten, T.M., Haas, T.L., Sykora, J., Stahl, H., Sprick, M.R., Fas, S.C., Krueger, A., Weigand, M.A., Grosse-Wilde, A., Stremmel, W*., et al.* (2004). Enhanced caspase-8 recruitment to and activation at the DISC is critical for sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by chemotherapeutic drugs. Cell Death Differ *11 Suppl 1*, S86-96.

Gerspach, J., Pfizenmaier, K., and Wajant, H. (2011). Therapeutic targeting of CD95 and the TRAIL death receptors. Recent Pat Anticancer Drug Discov *6*, 294-310.

Gonzalvez, F., and Ashkenazi, A. (2010). New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene *29*, 4752-4765.

Groth-Pedersen, L., and Jaattela, M. (2010). Combating apoptosis and multidrug resistant cancers by targeting lysosomes. Cancer letters.

Guicciardi, M.E., Bronk, S.F., Werneburg, N.W., and Gores, G.J. (2007). cFLIPL prevents TRAIL-induced apoptosis of hepatocellular carcinoma cells by inhibiting the lysosomal pathway of apoptosis. American journal of physiology Gastrointestinal and liver physiology *292*, G1337-1346.

Guicciardi, M.E., Deussing, J., Miyoshi, H., Bronk, S.F., Svingen, P.A., Peters, C., Kaufmann, S.H., and Gores, G.J. (2000). Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. J Clin Invest *106*, 1127-1137.

Guicciardi, M.E., and Gores, G.J. (2009). Life and death by death receptors. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *23*, 1625-1637.

Guicciardi, M.E., Leist, M., and Gores, G.J. (2004). Lysosomes in cell death. Oncogene *23*, 2881-2890.

Houseweart, M.K., Vilaythong, A., Yin, X.M., Turk, B., Noebels, J.L., and Myers, R.M. (2003). Apoptosis caused by cathepsins does not require Bid signaling in an in vivo model of progressive myoclonus epilepsy (EPM1). Cell Death Differ *10*, 1329-1335.

Ivanova, S., Repnik, U., Bojic, L., Petelin, A., Turk, V., and Turk, B. (2008). Lysosomes in apoptosis. Methods Enzymol *442*, 183-199.

Kim, E.C., Yun, B.S., Ryoo, I.J., Min, J.K., Won, M.H., Lee, K.S., Kim, Y.M., Yoo, I.D., and Kwon, Y.G. (2004). Complestatin prevents apoptotic cell death: inhibition of a mitochondrial caspase pathway through AKT/PKB activation. Biochemical and biophysical research communications *313*, 193-204.

Kirkegaard, T., and Jaattela, M. (2009). Lysosomal involvement in cell death and cancer. Biochim Biophys Acta *1793*, 746-754.

Klaric, M., Tao, S., Stoka, V., Turk, B., and Turk, V. (2009). Cysteine cathepsins are not critical for TNF-alpha-induced cell death in T98G and U937 cells. Biochim Biophys Acta *1794*, 1372-1377.

Kohlhaas, S.L., Craxton, A., Sun, X.M., Pinkoski, M.J., and Cohen, G.M. (2007). Receptor-mediated endocytosis is not required for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. J Biol Chem *282*, 12831-12841.

Koschny, R., Holland, H., Sykora, J., Erdal, H., Krupp, W., Bauer, M., Bockmuehl, U., Ahnert, P., Meixensberger, J., Stremmel, W*., et al.* (2010). Bortezomib sensitizes primary human esthesioneuroblastoma cells to TRAIL-induced apoptosis. J Neurooncol *97*, 171-185.

Lee, S.H., Kim, Y.K., Kim, C.S., Seol, S.K., Kim, J., Cho, S., Song, Y.L., Bartenschlager, R., and Jang, S.K. (2005). E2 of hepatitis C virus inhibits apoptosis. Journal of immunology *175*, 8226-8235.

Nagaraj, N.S., Vigneswaran, N., and Zacharias, W. (2006). Cathepsin B mediates TRAIL-induced apoptosis in oral cancer cells. Journal of cancer research and clinical oncology *132*, 171-183.

Nagaraj, N.S., Vigneswaran, N., and Zacharias, W. (2007). Hypoxia inhibits TRAIL-induced tumor cell apoptosis: involvement of lysosomal cathepsins. Apoptosis *12*, 125-139.

Newsom-Davis, T., Prieske, S., and Walczak, H. (2009). Is TRAIL the holy grail of cancer therapy? Apoptosis *14*, 607-623.

Oberle, C., Huai, J., Reinheckel, T., Tacke, M., Rassner, M., Ekert, P.G., Buellesbach, J., and Borner, C. (2010). Lysosomal membrane permeabilization and cathepsin release is a Bax/Bak-dependent, amplifying event of apoptosis in fibroblasts and monocytes. Cell Death Differ *17*, 1167-1178.

Papenfuss, K., Cordier, S.M., and Walczak, H. (2008). Death receptors as targets for anti-cancer therapy. J Cell Mol Med *12*, 2566-2585.

Reiser, J., Adair, B., and Reinheckel, T. (2010). Specialized roles for cysteine cathepsins in health and disease. J Clin Invest *120*, 3421-3431.

Repnik, U., Stoka, V., Turk, V., and Turk, B. (2012). Lysosomes and lysosomal cathepsins in cell death. Biochim Biophys Acta *1824*, 22-33.

Rozman-Pungercar, J., Kopitar-Jerala, N., Bogyo, M., Turk, D., Vasiljeva, O., Stefe, I., Vandenabeele, P., Bromme, D., Puizdar, V., Fonovic, M*., et al.* (2003). Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity. Cell Death Differ *10*, 881-888.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., and Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. Embo J *17*, 1675-1687.

Schrader, K., Huai, J., Jockel, L., Oberle, C., and Borner, C. (2010). Non-caspase proteases: triggers or amplifiers of apoptosis? Cell Mol Life Sci *67*, 1607-1618.

Schutze, S., Tchikov, V., and Schneider-Brachert, W. (2008). Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nature reviews Molecular cell biology *9*, 655-662.

Stoka, V., Turk, B., Schendel, S.L., Kim, T.H., Cirman, T., Snipas, S.J., Ellerby, L.M., Bredesen, D., Freeze, H., Abrahamson, M*., et al.* (2001). Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. J Biol Chem *276*, 3149-3157.

Stoka, V., Turk, B., and Turk, V. (2005). Lysosomal cysteine proteases: structural features and their role in apoptosis. IUBMB Life *57*, 347-353.

Stoka, V., Turk, V., and Turk, B. (2007). Lysosomal cysteine cathepsins: signaling pathways in apoptosis. Biol Chem *388*, 555-560.

Terrisse, A.D., Bezombes, C., Lerouge, S., Laurent, G., and Jaffrezou, J.P. (2002). Daunorubicin- and Ara-C-induced interphasic apoptosis of human type II leukemia cells is caspase-8-independent. Biochim Biophys Acta *1584*, 99-103.

Trauth, B.C., Klas, C., Peters, A.M., Matzku, S., Moller, P., Falk, W., Debatin, K.M., and Krammer, P.H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science *245*, 301-305.

Turk, B., Stoka, V., Rozman-Pungercar, J., Cirman, T., Droga-Mazovec, G., Oresic, K., and Turk, V. (2002). Apoptotic pathways: involvement of lysosomal proteases. Biol Chem *383*, 1035-1044.

Turk, B., Turk, D., and Turk, V. (2012a). Protease signalling: the cutting edge. Embo J *31*, 1630-1643.

Turk, B., and Turk, V. (2009). Lysosomes as "suicide bags" in cell death: myth or reality? J Biol Chem *284*, 21783-21787.

Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B., and Turk, D. (2012b). Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochim Biophys Acta *1824*, 68-88.

Vasiljeva, O., Korovin, M., Gajda, M., Brodoefel, H., Bojic, L., Kruger, A., Schurigt, U., Sevenich, L., Turk, B., Peters, C*., et al.* (2008). Reduced tumour cell proliferation and delayed development of high-grade mammary carcinomas in cathepsin B-deficient mice. Oncogene *27*, 4191-4199.

Vince, J.E., Chau, D., Callus, B., Wong, W.W., Hawkins, C.J., Schneider, P., McKinlay, M., Benetatos, C.A., Condon, S.M., Chunduru, S.K*., et al.* (2008). TWEAK-FN14 signaling induces lysosomal degradation of a cIAP1-TRAF2 complex to sensitize tumor cells to TNFalpha. J Cell Biol *182*, 171-184.

Walczak, H., and Krammer, P.H. (2000). The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res *256*, 58-66.

Wattiaux, R., Wattiaux-de Coninck, S., Thirion, J., Gasingirwa, M.C., and Jadot, M. (2007). Lysosomes and Fas-mediated liver cell death. The Biochemical journal *403*, 89-95.

Werneburg, N.W., Guicciardi, M.E., Bronk, S.F., Kaufmann, S.H., and Gores, G.J. (2007). Tumor necrosis factor-related apoptosis-inducing ligand activates a lysosomal pathway of apoptosis that is regulated by Bcl-2 proteins. J Biol Chem *282*, 28960-28970.

Yang, A., Wilson, N.S., and Ashkenazi, A. (2010a). Proapoptotic DR4 and DR5 signaling in cancer cells: toward clinical translation. Curr Opin Cell Biol *22*, 837-844.

Yang, F., Tay, K.H., Dong, L., Thorne, R.F., Jiang, C.C., Yang, E., Tseng, H.Y., Liu, H., Christopherson, R., Hersey, P*., et al.* (2010b). Cystatin B inhibition of TRAIL-induced apoptosis is associated with the protection of FLIP(L) from degradation by the E3 ligase itch in human melanoma cells. Cell Death Differ *17*, 1354-1367.

Figure legends

Figure 1

(A) Cathepsin activity in different cell lines. Activity in the total cell extract was determined using the fluorogenic substrate Z-FR-AMC. The results are means ± SD of three independent experiments.

(B) Immunodetection of cathepsin B in total cell extracts of different cell lines. pCatB, procathepsin B, scCatB, single chain mature cathepsin B; hcCatB, heavy chain of mature cathepsin B.

Figure 2

(A) TRAIL-induced apoptosis shown as a percentage of Annexin-V-PE positive cells. HeLa, HuH-7 and U-937 cells lines were treated with 100 ng/ml TRAIL and 10 nM bortezomib for 24 h, Jurkat cells were treated with 1 ng/ml TRAIL and 1 nM bortezomib for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration 10 μM. The results are means ± SD of three independent experiments.

(B) CD95-mediated apoptosis shown as a percentage of Annexin-V-PE positive cells. HeLa, HuH-7 and U-937 cells lines were treated with 1000 ng/ml anti-APO-1 and 1000 ng/ml protein A for 24 h, Jurkat cells were treated with 0.3 ng/ml anti-APO-1 and 0.3 ng/ml protein A for 18 h. The results are means ± SD of three independent experiments.

(C) CD95-mediated apoptosis shown as a percentage of Annexin-V-PE positive cells in Jurkat cells. Cells were treated with 0.3 ng/ml anti-APO-1 and 0.3 ng/ml protein A for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration 10 μM. The results are means ± SD of three independent experiments.

Figure 3

(A) Caspase activity after TRAIL-induced apoptosis. HeLa, HuH-7 and U-937 cells lines were treated with 100 ng/ml TRAIL and 10 nM bortezomib for 24 h, Jurkat cells were treated with 1 ng/ml TRAIL and 1 nM bortezomib for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration 10 μM. Caspase activity was determined using the fluorogenic substrate Ac-DEVD-AFC. The results are means ± SD of three independent experiments and are expressed in RFU.

(B) Caspase activity after CD95-mediated apoptosis. Jurkat cells were treated with 0.3 ng/ml anti-APO-1 and 0.3 ng/ml protein A for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration of 10 μM. Caspase activity was determined using the fluorogenic substrate Ac-DEVD-AFC. The results are means ± SD of three independent experiments and are expressed in RFU.

Figure 4

(A) Mitochondrial integrity during TRAIL-induced apoptosis. HeLa, HuH-7 and U-937 cells lines were treated with 100 ng/ml TRAIL and 10 nM bortezomib for 24 h, Jurkat cells were treated with 1 ng/ml TRAIL and 1 nM bortezomib for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration of 10 μM. Mitotracker Red CMX-Ros uptake indicates the percentage of cells with decreased fluorescence. The results are means ± SD of three independent experiments.

(B) Mitochondrial integrity during CD95-mediated apoptosis. Jurkat cells were treated with 0.3 ng/ml anti-APO-1 and 0.3 ng/ml protein A for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration of 10 μM. Mitotracker Red CMX-Ros uptake indicates the percentage of cells with decreased fluorescence. The results are means ± SD of three independent experiments.

Figure 5

(A) Lysosomal integrity during TRAIL-induced apoptosis. HeLa, HuH-7 and U-937 cells lines were treated with 100 ng/ml TRAIL and 10 nM bortezomib for 24 h, Jurkat cells were treated with 1 ng/ml TRAIL and 1 nM bortezomib for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration of 10 μM. Acridine orange uptake indicates the percentage of cells with decreased fluorescence. The results are means ± SD of three independent experiments.

(B) Lysosomal integrity during CD95-mediated apoptosis. Jurkat cells were treated with 0.3 ng/ml anti-APO-1 and 0.3 ng/ml protein A for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration of 10 μM. Acridine orange uptake indicates the percentage of cells with decreased fluorescence. The results are means ± SD of three independent experiments.

Figure 6

(A) Cathepsin activity in total extracts obtained from cells that underwent TRAIL-induced apoptosis. Cathepsin activity was determined using Z-FR-AMC fluorogenic substrate. Other experimental conditions were as described in the Materials and Methods. The results are means ± SD of three independent experiments and are expressed in RFU.

(B) Cathepsin activity in total extracts of cells that underwent CD95-mediated apoptosis. Other experimental conditions were as described in the Materials and Methods. Cathepsin

activity was determined using Z-FR-AMC fluorogenic substrate. The results are means ± SD of three independent experiments and are expressed in RFU.

Figure 7

(A) Cathepsin activity in the cytosol of cells that underwent TRAIL-induced apoptosis. HeLa, HuH-7 and U-937 cells lines were treated with 100 ng/ml TRAIL and 10 nM bortezomib for 24 h, Jurkat cells were treated with 1 ng/ml TRAIL and 1 nM bortezomib for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration of 10 μM. Cathepsin activity was determined using the fluorogenic substrate Z-FR-AMC. The results are means ± SD of three independent experiments and are expressed in RFU.

(B) Immunodetection of cathepsin B in cytosolic cell extracts of Jurkat cells following TRAIL-induced apoptosis. pCatB, procathepsin B, scCatB, single chain mature cathepsin B; hcCatB, heavy chain of mature cathepsin B. Other experimental conditions were as described in the Materials and Methods.

(C) Cathepsin activity in the cytosol of cells that underwent CD95-mediated apoptosis. Jurkat cells were treated with 0.3 ng/ml anti-APO-1 and 0.3 ng/ml protein A for 18 h. Inhibitors (CA-074Me, E-64d and Z-VAD-FMK) were added at a final concentration of 10 μM. Cathepsin activity was determined using the fluorogenic substrate Z-FR-AMC. The results are means ± SD of three independent experiments and are expressed in RFU.

(D) Immunodetection of cathepsin B in cytosolic extracts of Jurkat cells following induction of apoptosis by anti-APO-1. pCatB, procathepsin B, scCatB, single chain mature cathepsin B; hcCatB, heavy chain of mature cathepsin B. Experimental conditions were as described in Materials and Methods.

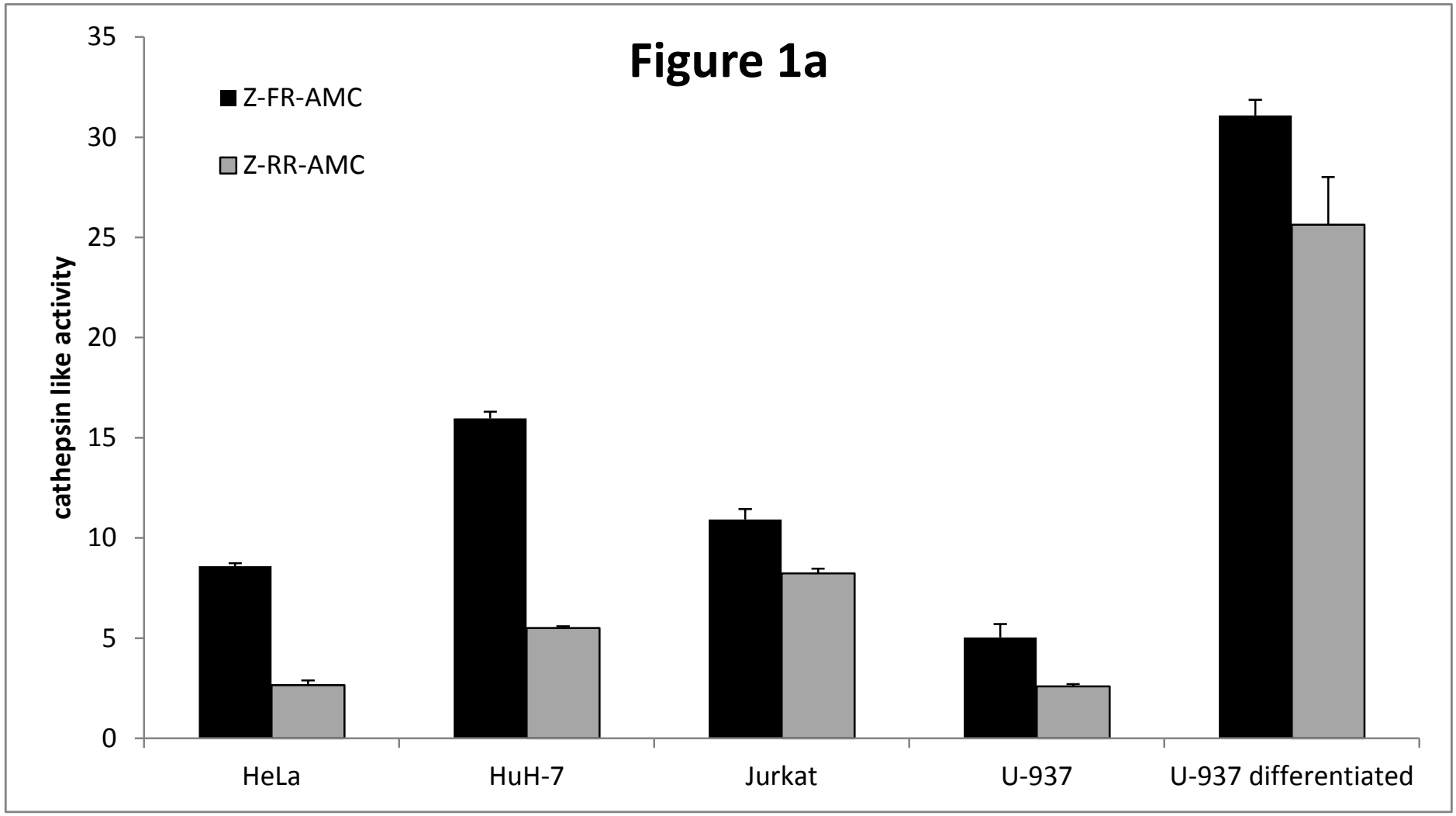
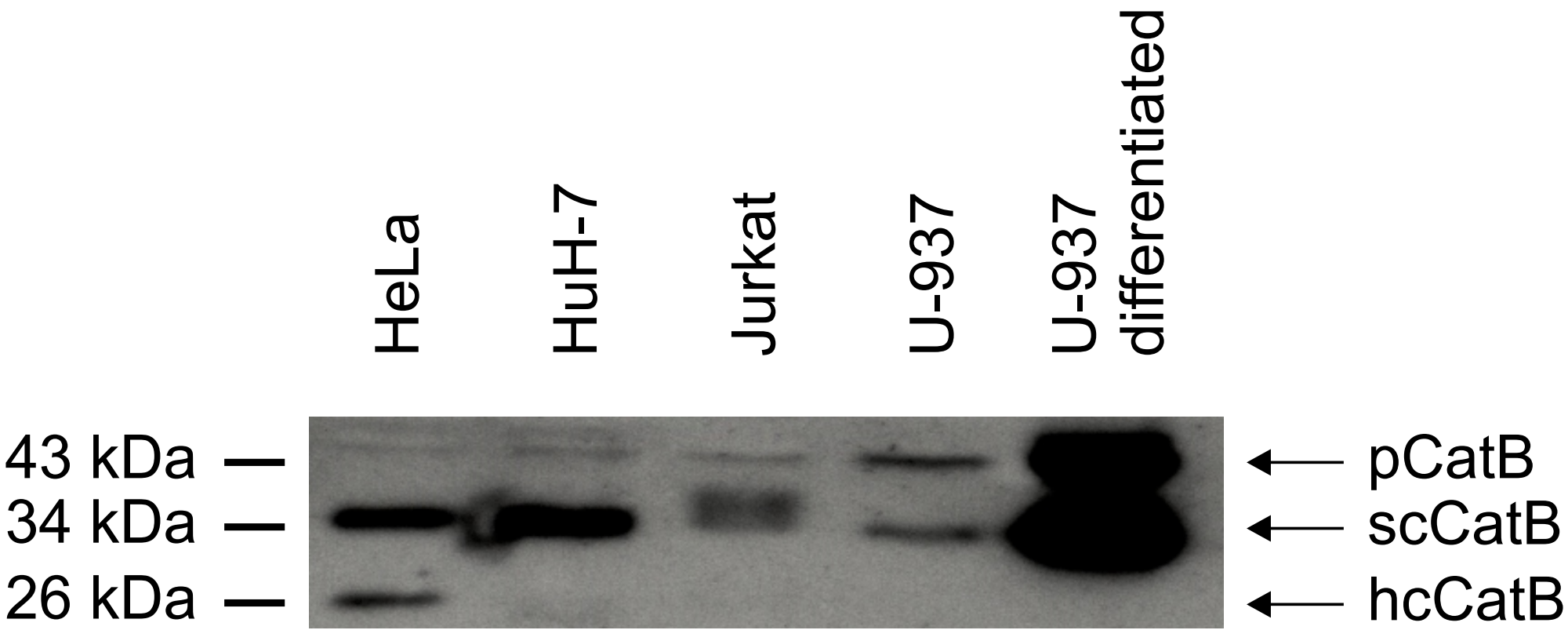


Figure 1a
Z-FR-AMC
Z-RR-AMC
cathepsin like activity
35
30
25
20
15
10
5
0
HeLa
HuH-7
Jurkat
U-937
U-937 differentiated

Figure 1b


HeLa
HuH-7
Jurkat
U-937
U-937 differentiated
43 kDa
34 kDa
26 kDa
pCatB
scCatB
hcCatB

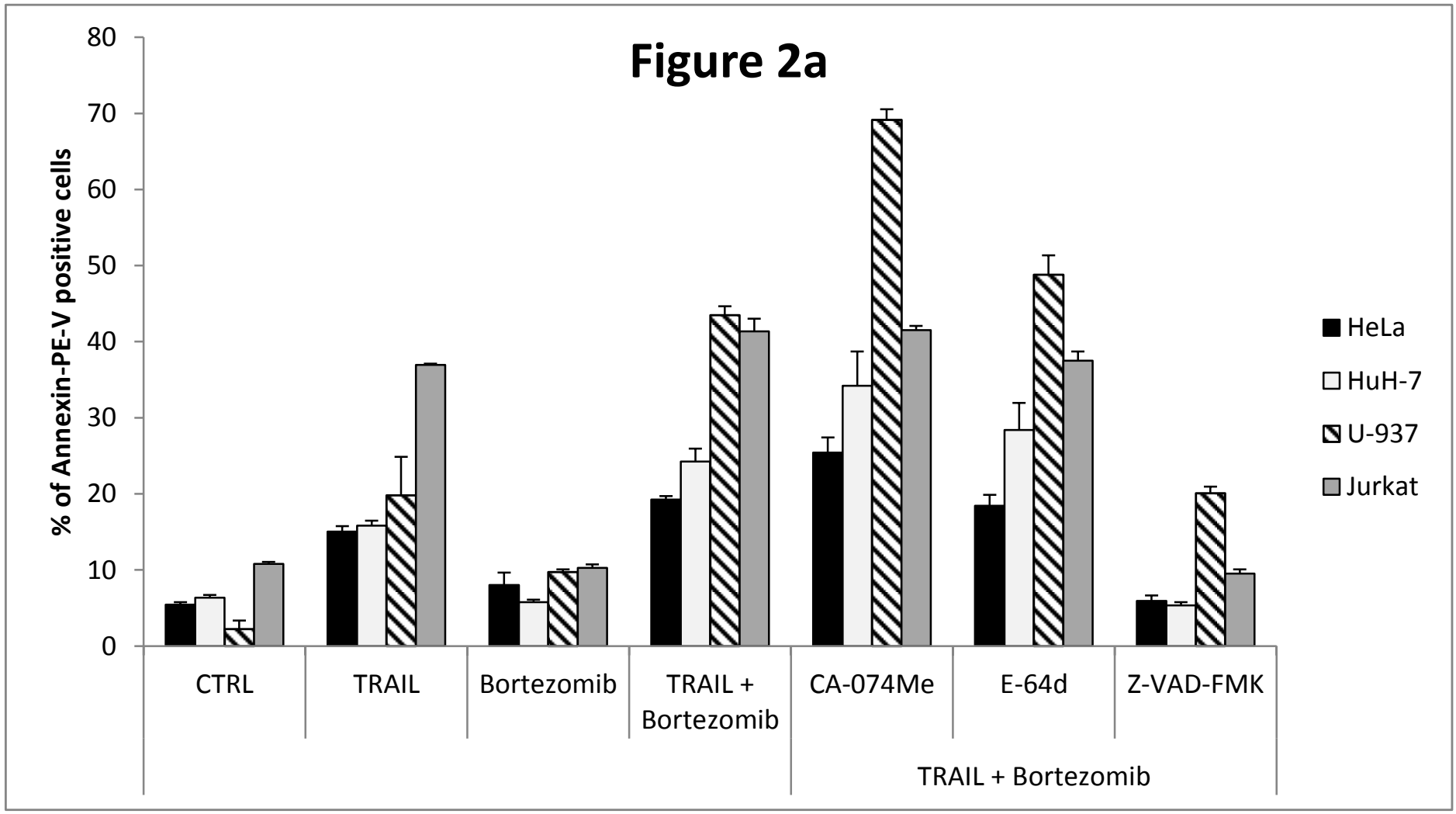
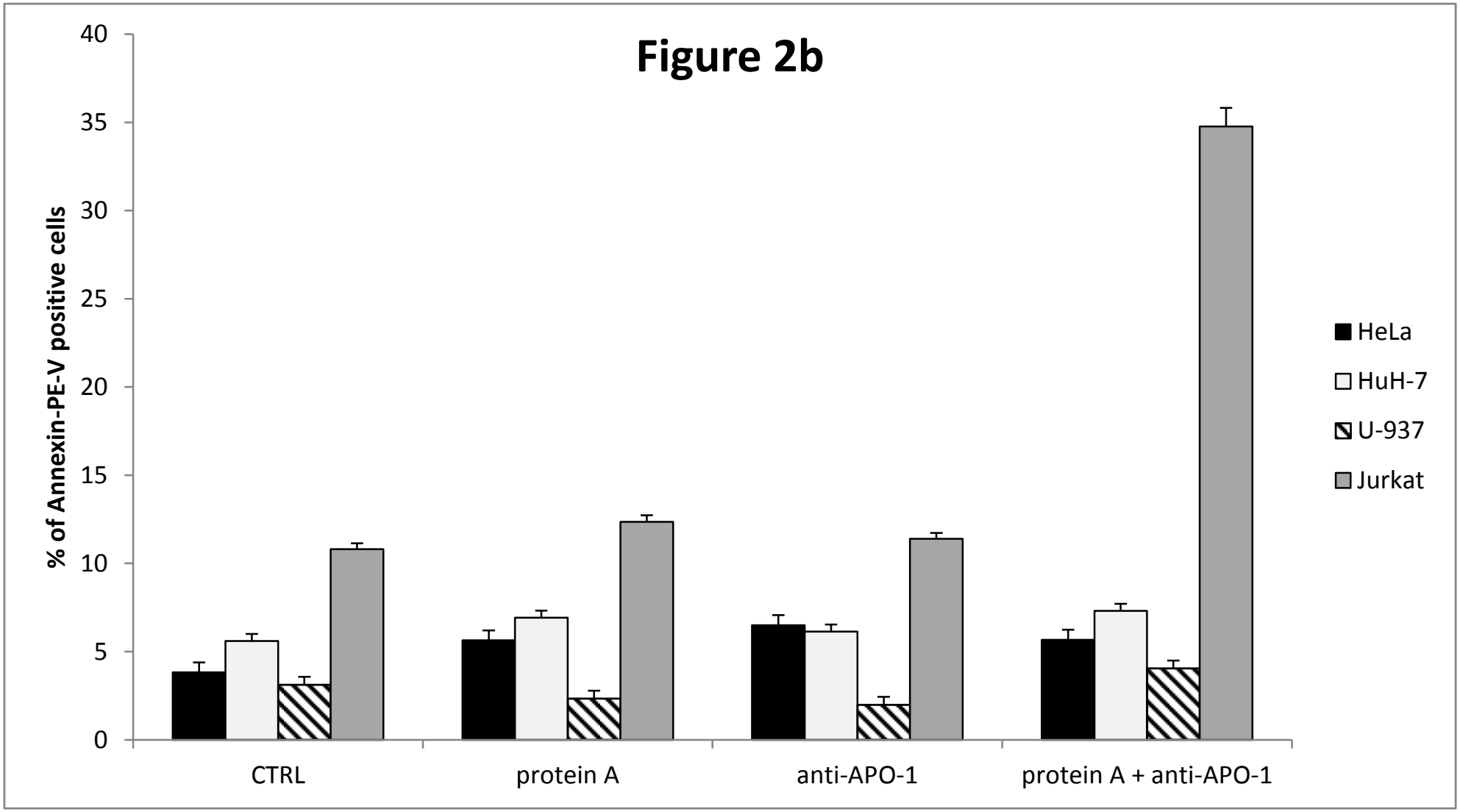


Figure 2a
% of Annexin-PE-V positive cells
80
70
60
50
40
30
20
10
0
CTRL
TRAIL
Bortezomib
TRAIL + Bortezomib
CA-074Me
E-64d
Z-VAD-FMK
TRAIL + Bortezomib
HeLa
HuH-7
U-937
Jurkat

Figure 2b


% of Annexin-PE-V positive cells
40
35
30
25
20
15
10
5
0
CTRL
protein A
anti-APO-1
protein A + anti-APO-1
HeLa
HuH-7
U-937
Jurkat

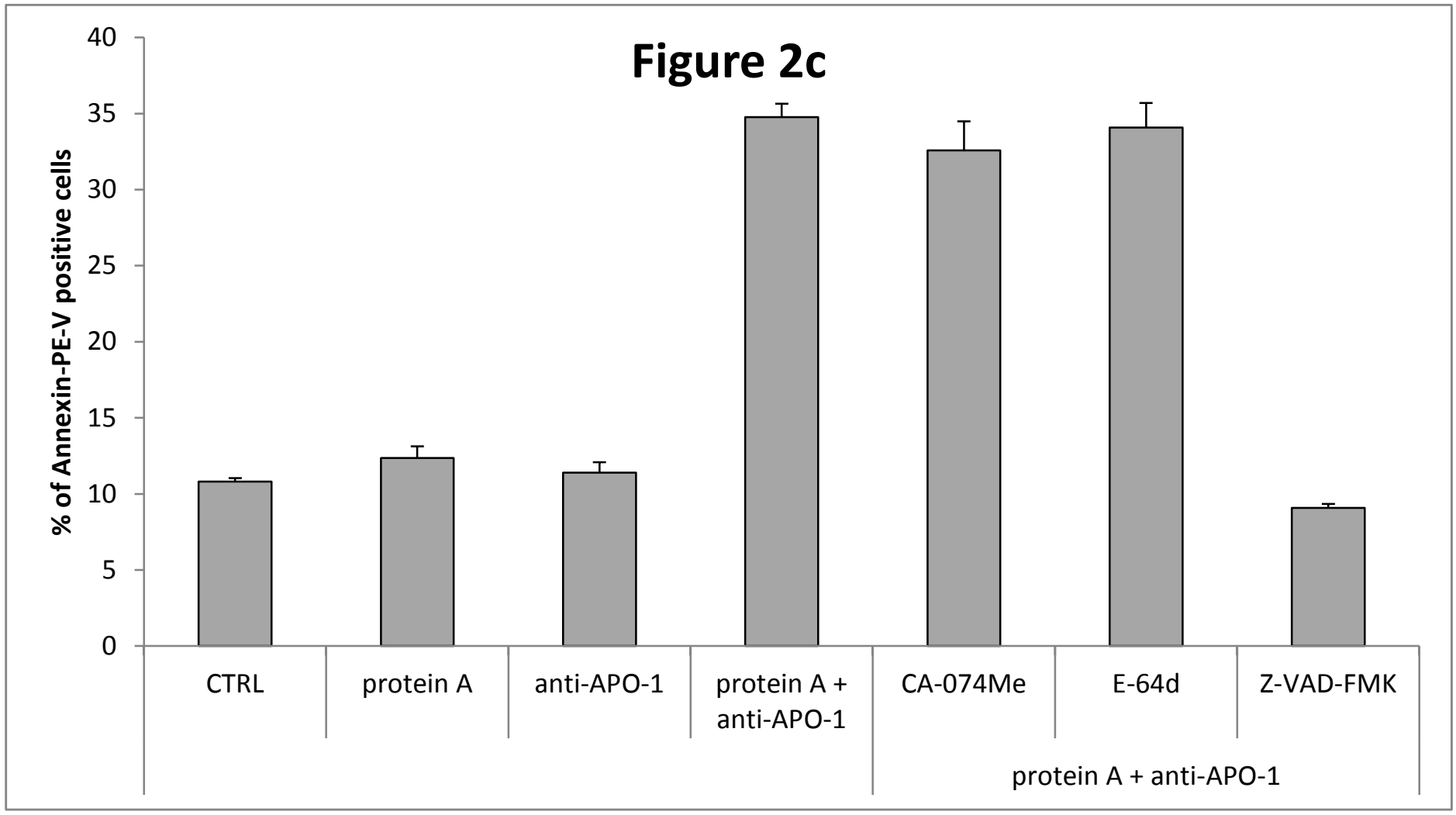


Figure 2c
% of Annexin-PE-V positive cells
40
35
30
25
20
15
10
5
0
CTRL
protein A
anti-APO-1
protein A + anti-APO-1
CA-074Me
E-64d
Z-VAD-FMK
protein A + anti-APO-1

Figure 3a

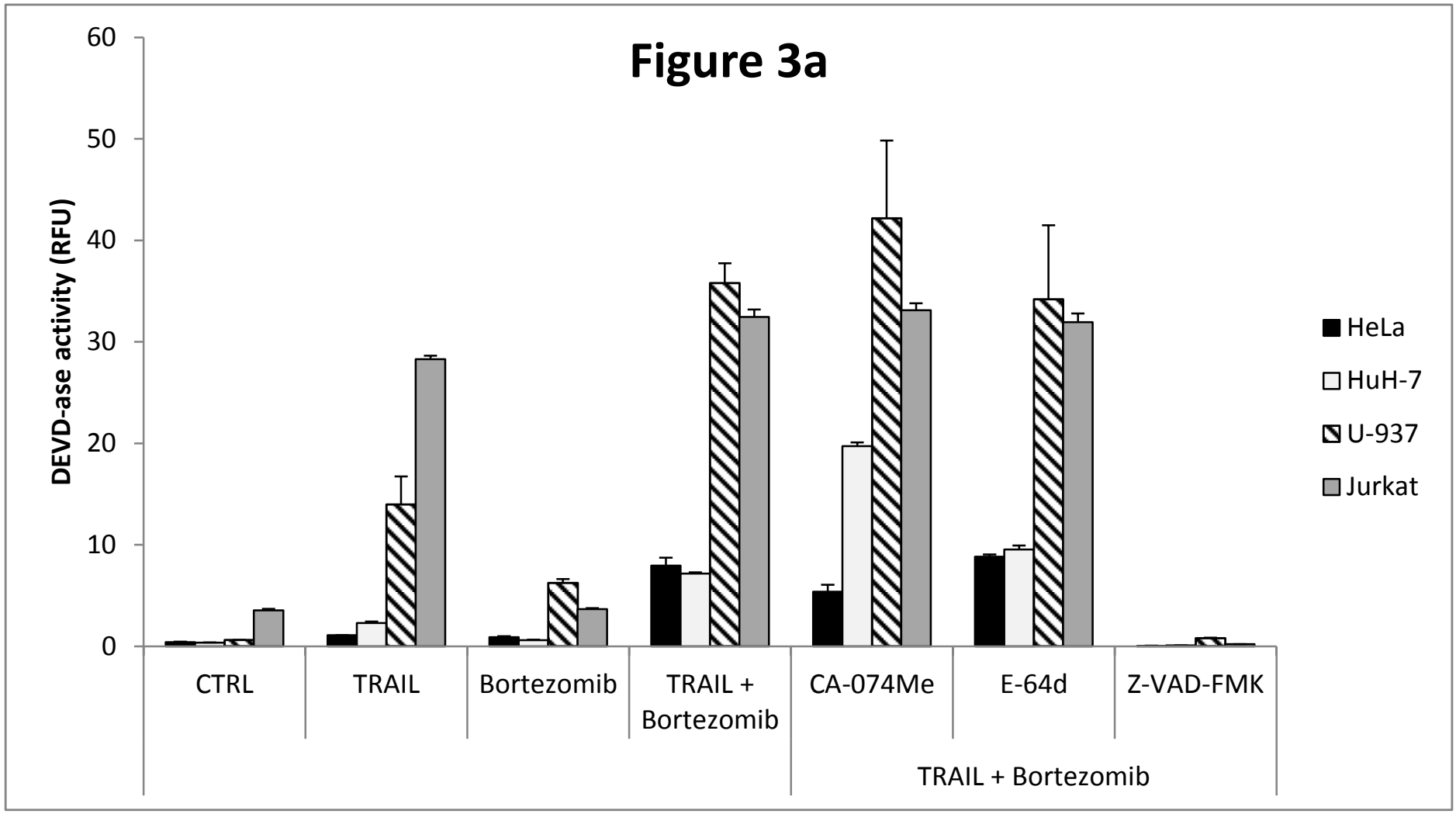
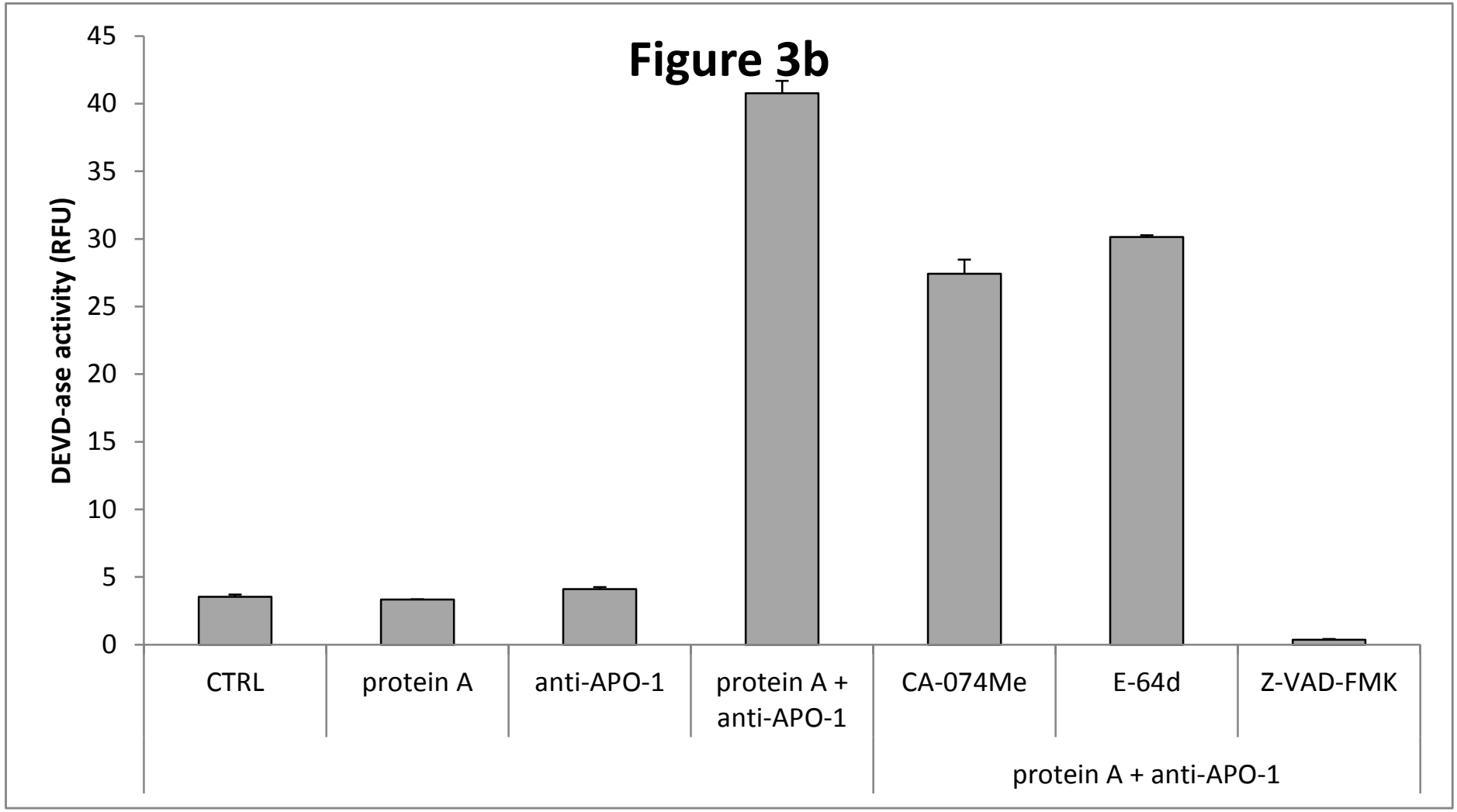

DEVD-ase activity (RFU)
60
50
40
30
20
10
0
CTRL
TRAIL
Bortezomib
TRAIL + Bortezomib
CA-074Me
E-64d
Z-VAD-FMK
TRAIL + Bortezomib
HeLa
HuH-7
U-937
Jurkat

Figure 3b


DEVD-ase activity (RFU)
45
40
35
30
25
20
15
10
5
0
CTRL
protein A
anti-APO-1
protein A + anti-APO-1
CA-074Me
E-64d
Z-VAD-FMK
protein A + anti-APO-1

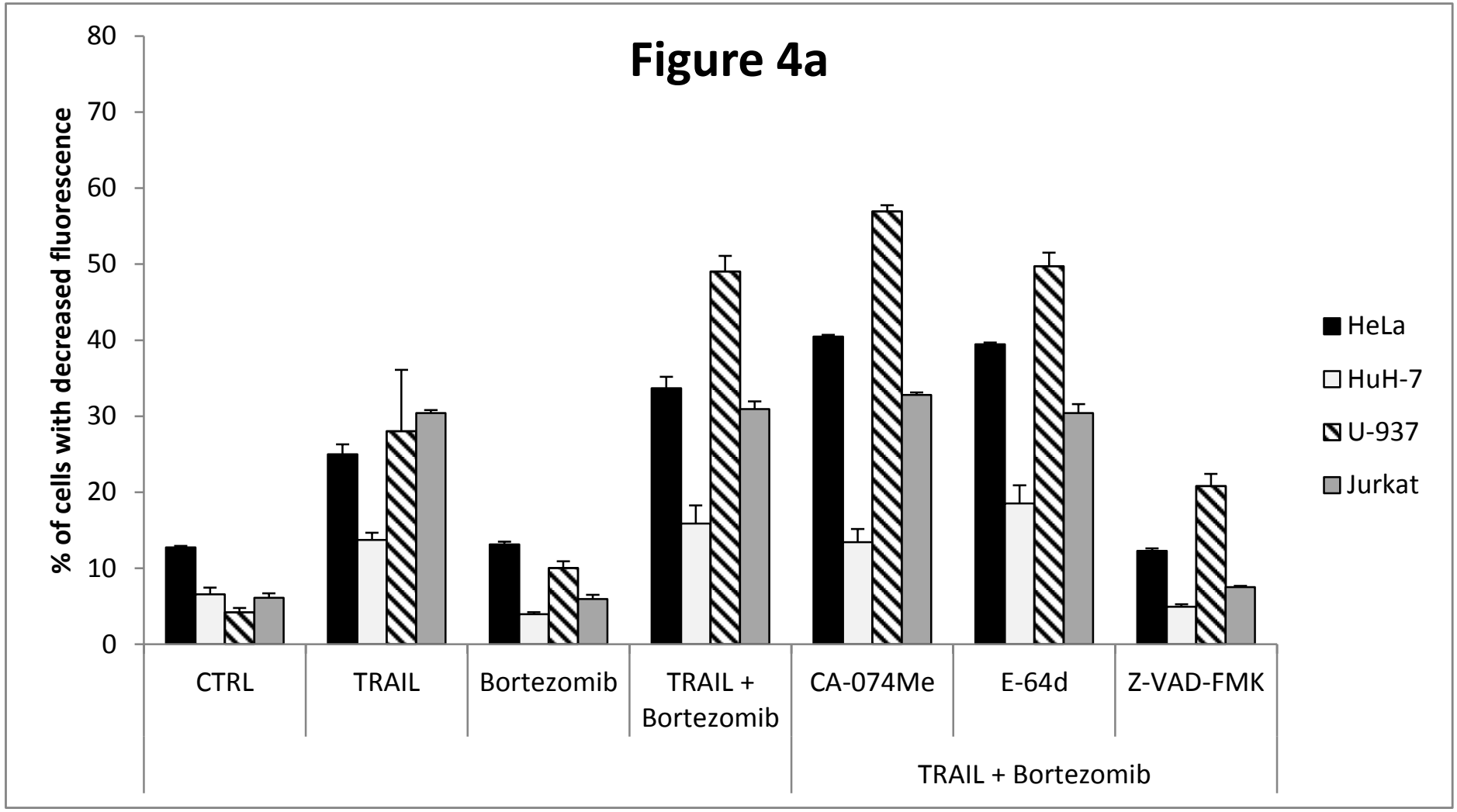
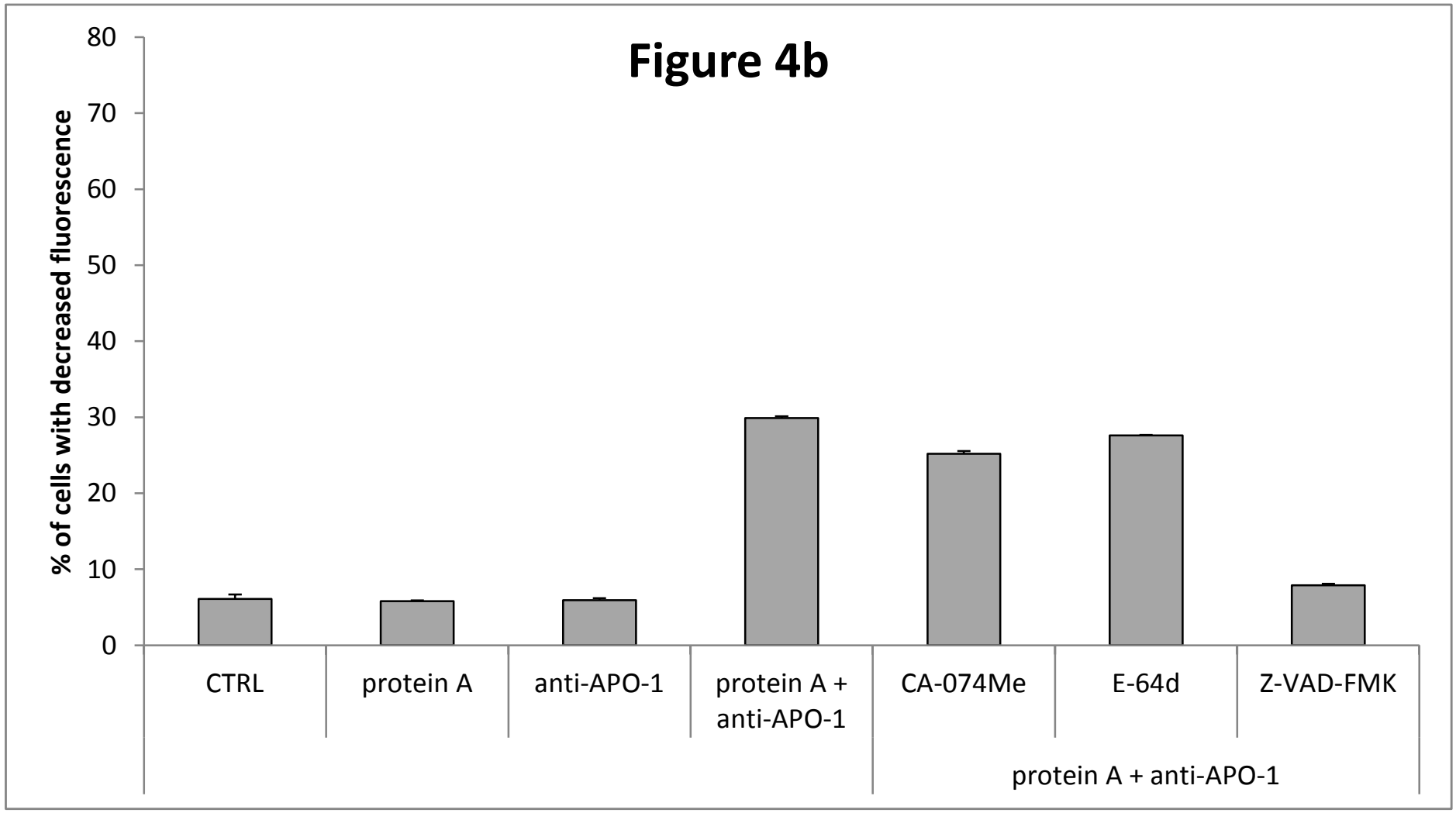


Figure 4a
% of cells with decreased fluorescence
80
70
60
50
40
30
20
10
0
HeLa
HuH-7
U-937
Jurkat
CTRL
TRAIL
Bortezomib
TRAIL + Bortezomib
CA-074Me
E-64d
Z-VAD-FMK
TRAIL + Bortezomib

Figure 4b


% of cells with decreased fluorescence
80
70
60
50
40
30
20
10
0
CTRL
protein A
anti-APO-1
protein A + anti-APO-1
CA-074Me
E-64d
Z-VAD-FMK
protein A + anti-APO-1

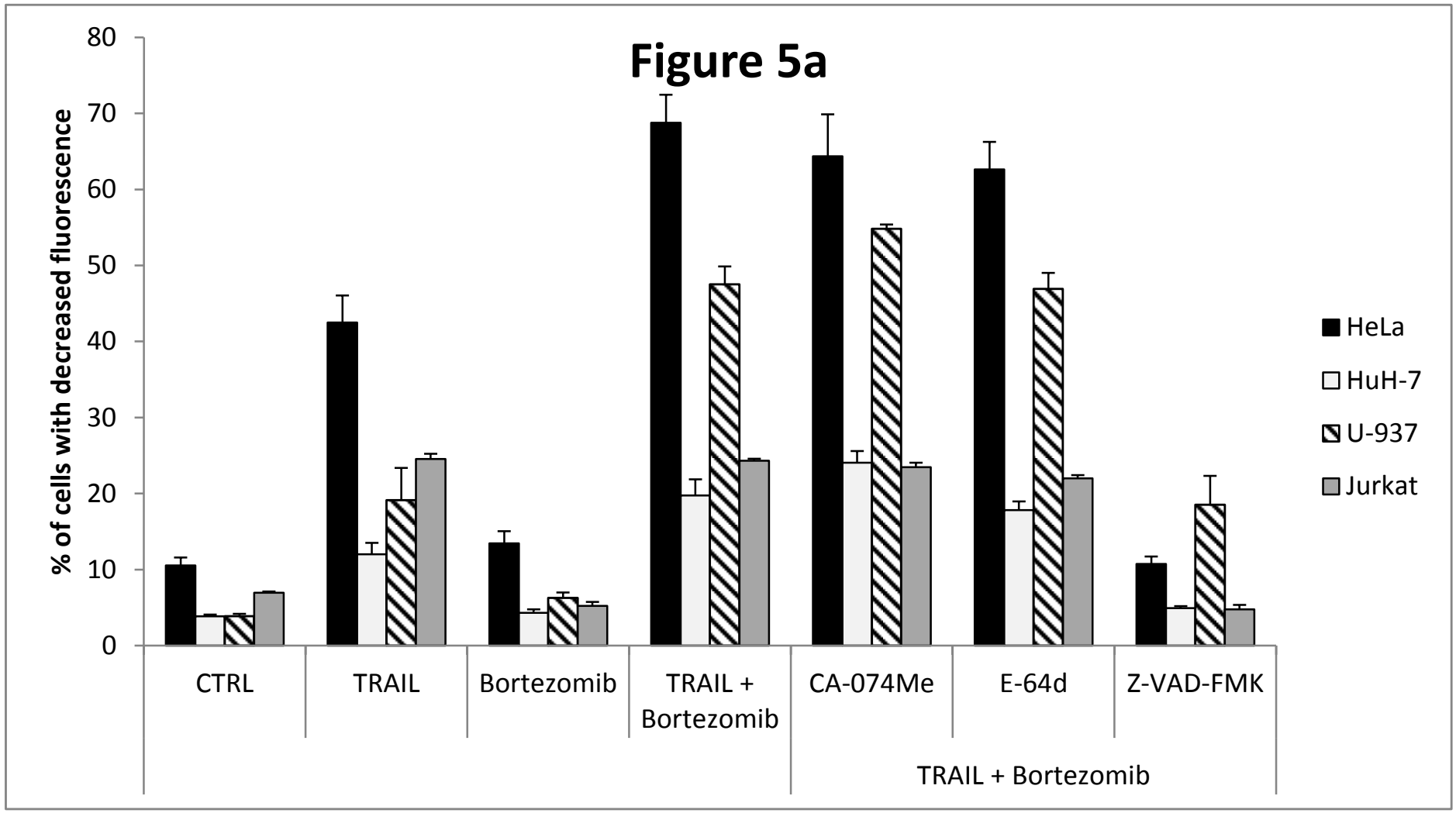


Figure 5a
% of cells with decreased fluorescence
80
70
60
50
40
30
20
10
0
HeLa
HuH-7
U-937
Jurkat
CTRL
TRAIL
Bortezomib
TRAIL + Bortezomib
CA-074Me
E-64d
Z-VAD-FMK
TRAIL + Bortezomib

Figure 5b

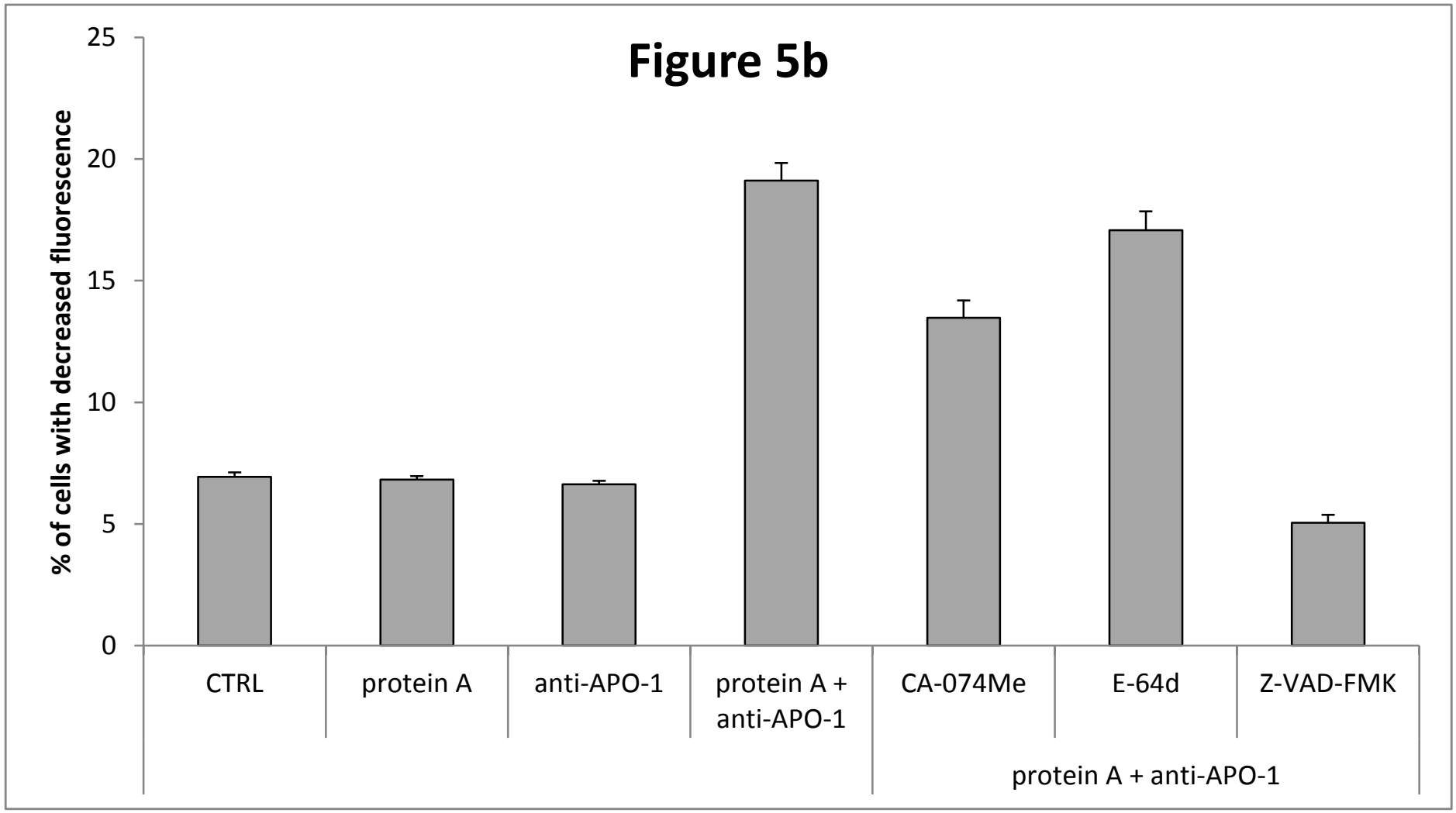
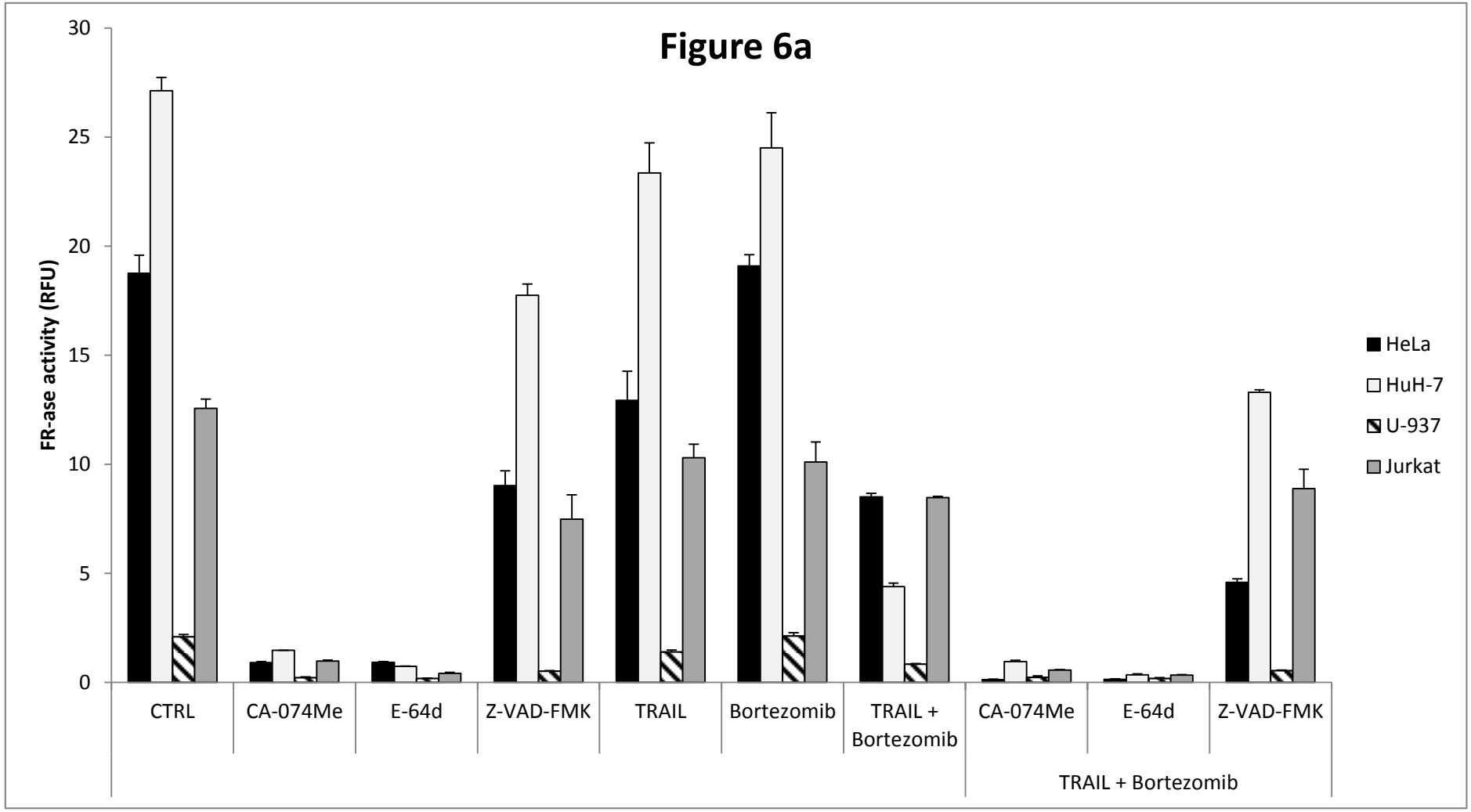

% of cells with decreased fluorescence
25
20
15
10
5
0
CTRL
protein A
anti-APO-1
protein A + anti-APO-1
CA-074Me
E-64d
Z-VAD-FMK
protein A + anti-APO-1

Figure 6a


FR-ase activity (RFU)
30
25
20
15
10
5
0
HeLa
HuH-7
U-937
Jurkat
CTRL
CA-074Me
E-64d
Z-VAD-FMK
TRAIL
Bortezomib
TRAIL + Bortezomib
CA-074Me
E-64d
Z-VAD-FMK
TRAIL + Bortezomib

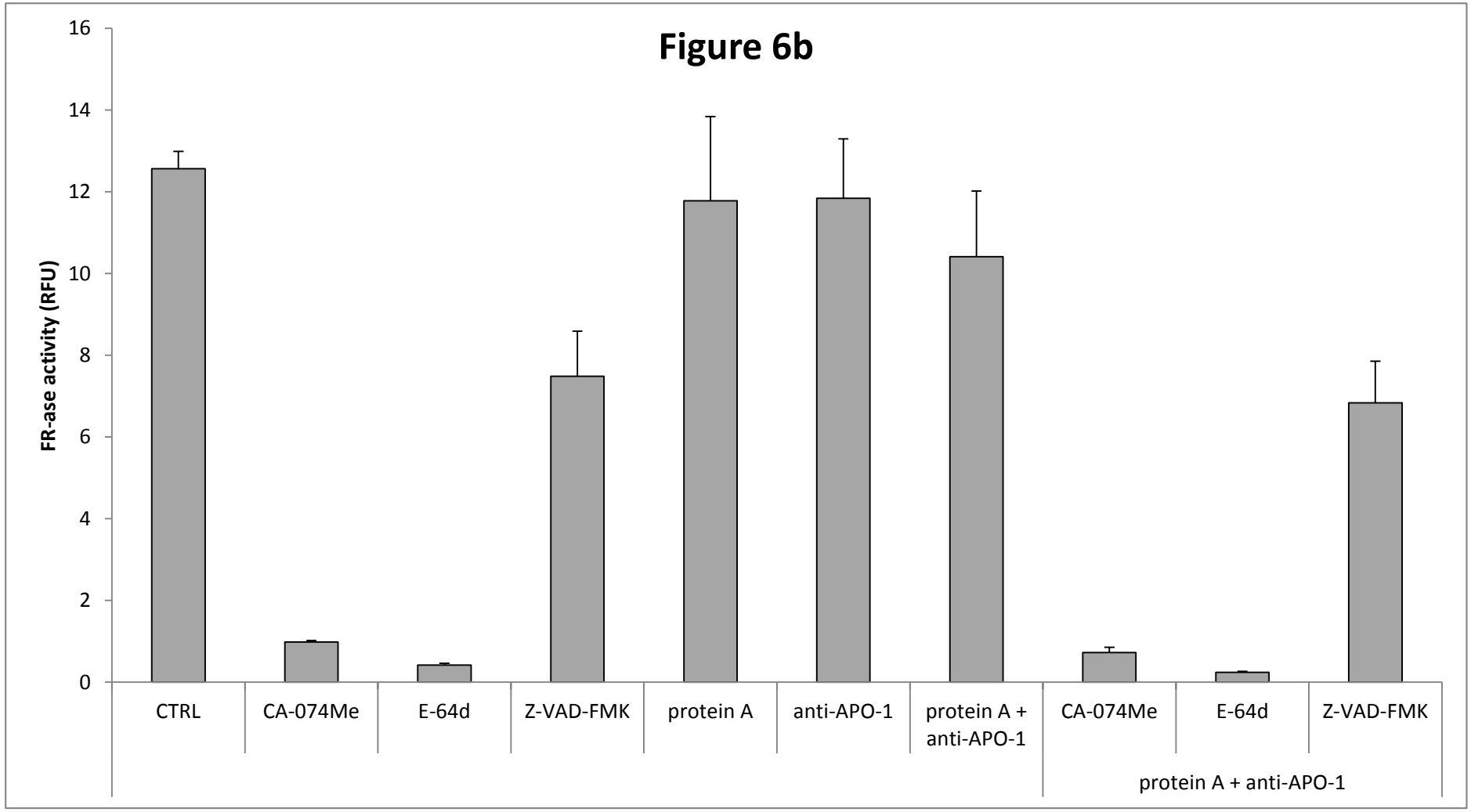


Figure 6b
FR-ase activity (RFU)
16
14
12
10
8
6
4
2
0
CTRL
CA-074Me
E-64d
Z-VAD-FMK
protein A
anti-APO-1
protein A + anti-APO-1
CA-074Me
E-64d
Z-VAD-FMK
protein A + anti-APO-1

Figure 7a

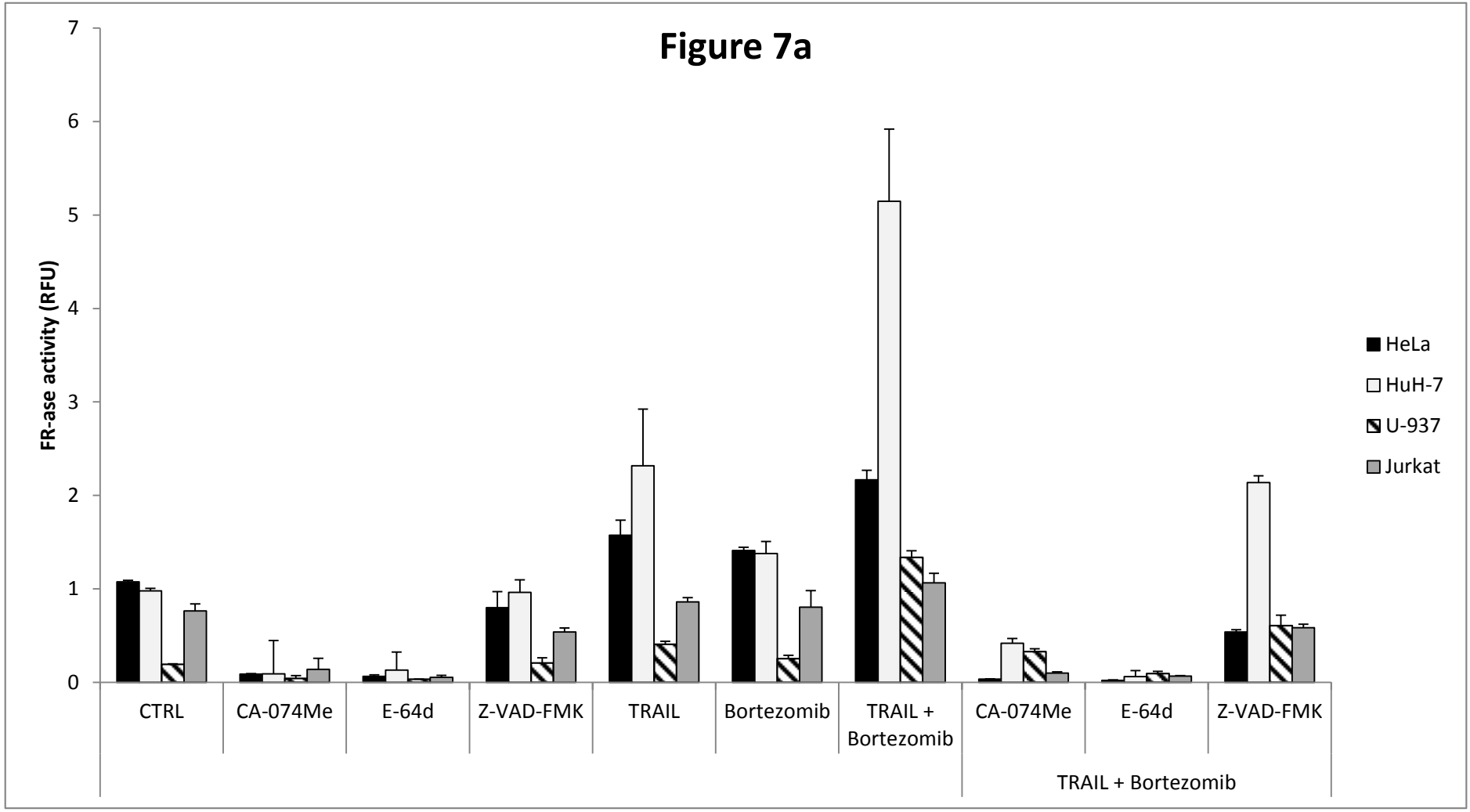
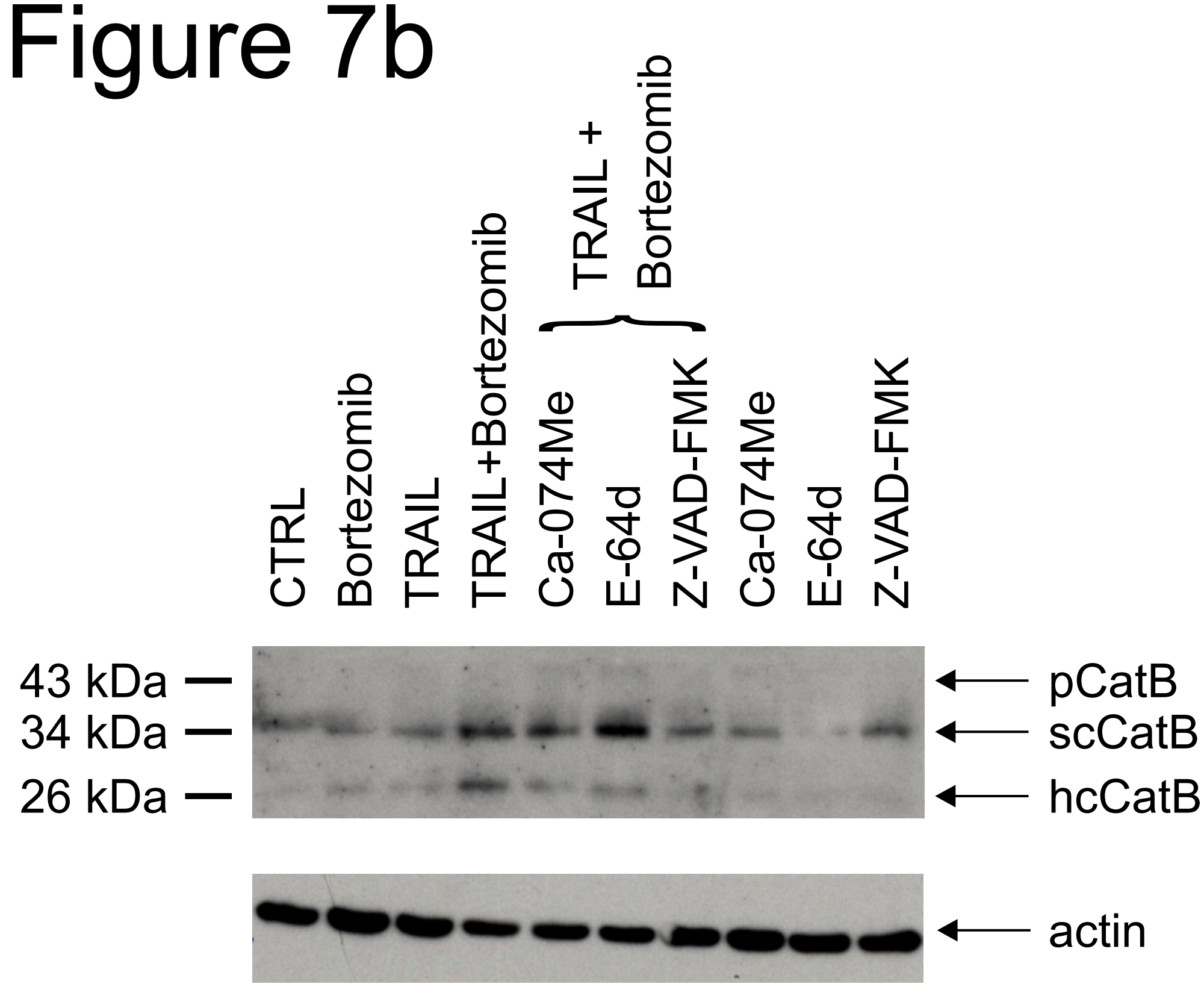

FR-ase activity (RFU)
7
6
5
4
3
2
1
0
HeLa
HuH-7
U-937
Jurkat
CTRL
CA-074Me
E-64d
Z-VAD-FMK
TRAIL
Bortezomib
TRAIL + Bortezomib
CA-074Me
E-64d
Z-VAD-FMK
TRAIL + Bortezomib

Figure 7b


CTRL
Bortezomib
TRAIL
TRAIL+Bortezomib
Ca-074Me
E-64d
Z-VAD-FMK
TRAIL + Bortezomib
Ca-074Me
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Z-VAD-FMK
43 kDa
34 kDa
26 kDa
pCatB
scCatB
hcCatB
actin

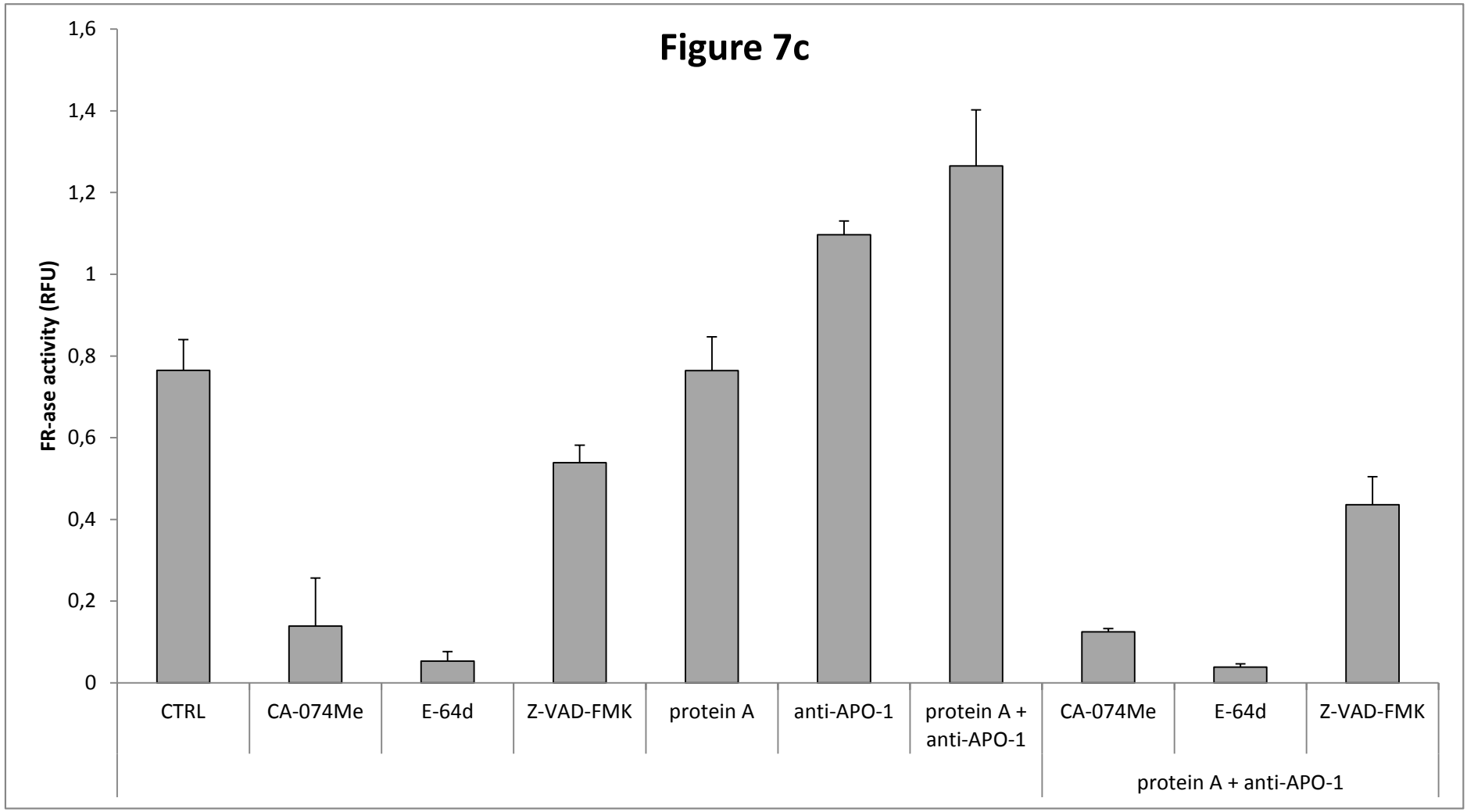
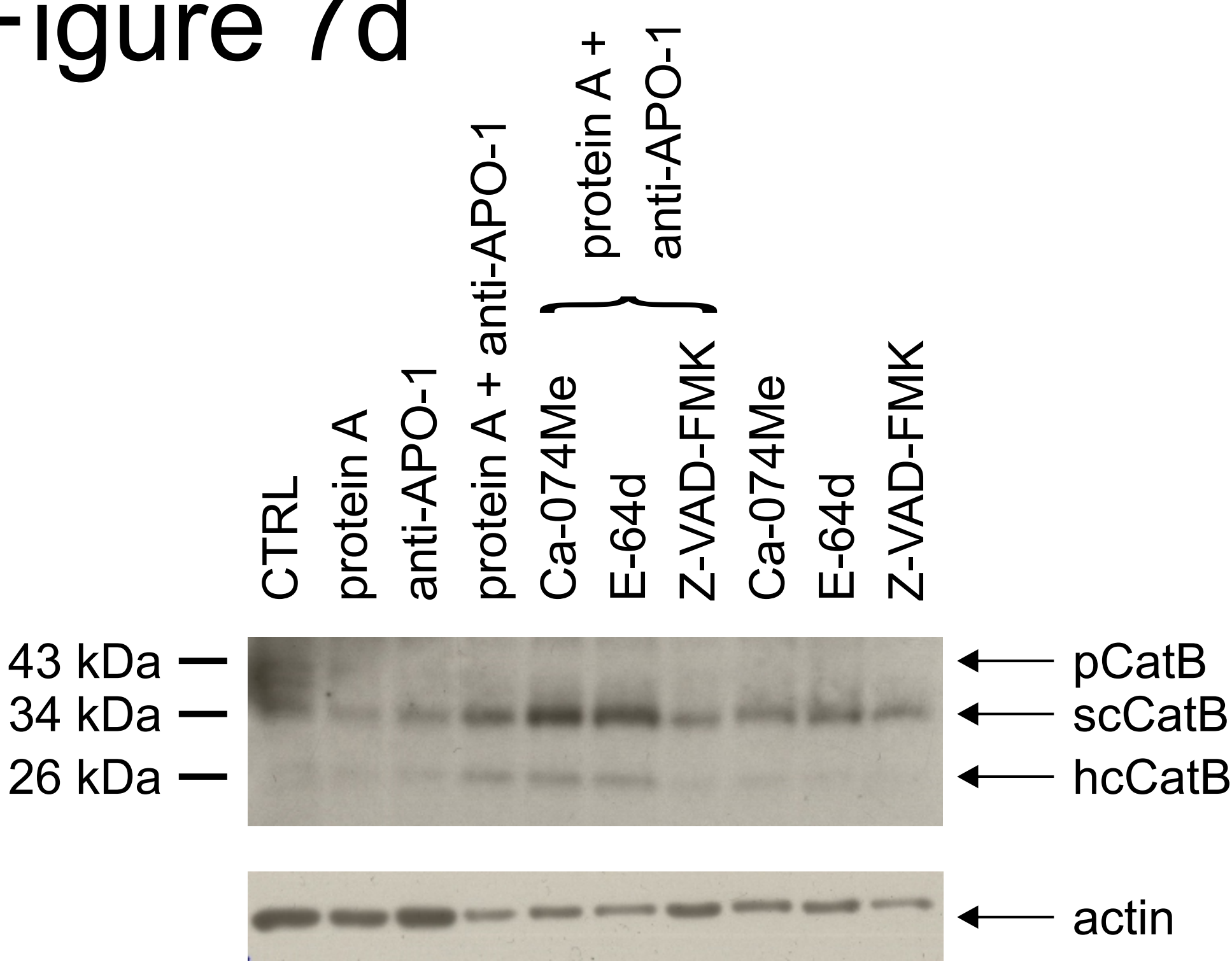


Figure 7c
FR-ase activity (RFU)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
CTRL
CA-074Me
E-64d
Z-VAD-FMK
protein A
anti-APO-1
protein A + anti-APO-1
CA-074Me
E-64d
Z-VAD-FMK
protein A + anti-APO-1

Figure 7d


CTRL
protein A
anti-APO-1
protein A + anti-APO-1
Ca-074Me
E-64d
Z-VAD-FMK
protein A + anti-APO-1
Ca-074Me
E-64d
Z-VAD-FMK
43 kDa
34 kDa
26 kDa
pCatB
scCatB
hcCatB
actin