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Kanonické a nekanonické signální dráhy aktivované receptory pro ligand TRAIL v lidskych buňkách

Canonical and non-canonical signalling triggered by activated TRAIL receptors in human cells

Doctoral Thesis

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Declaration of honour

This work was elaborated in the Laboratory of Cell Signalling and Apoptosis at the Institute of Molecular Biology and in the Laboratory of Molecular Therapy at the Biotechnological Institute, Academy of Sciences, Prague, Czech Republic.

I hereby declare that I have worked on the Thesis independently and that I have reported all the information, sources and literature used. This work, or a substantial part of it, has not been submitted to obtain another or the same academic title.

Author of the Thesis contributed to the experimental part as following:

In Results 4.1 TRAIL induces apoptosis but not necroptosis in colorectal and pancreatic cancer cells preferentially via the TRAIL-R2/DR5 receptor, author contributed by most of the experiments with exception of following: cloning of vectors for receptor specific TRAIL ligands was done by Martin Peterka for TST-TRI-TRAIL variants and Jan Švadlenka for His-TRI-TRAIL ligands, qPCR in Fig. 4.14 was done by Jan Švadlenka. Simona Benešová and Marie Ksandrová partially contributed with Western blots in Fig. 4.6 A-B and in Fig. 4.13 A. Jan Švadlenka partially contributed to Western blots showed in Fig. 4.8 C. Data in Fig. 4.14 were not included in the published paper Nahacka et al., 2017.

Author contributed to the second part of Results 4.2 Human embryonic and induced pluripotent stem cells express TRAIL receptors and can be sensitized to TRAIL-induced apoptosis project by preparing cell line with downregulated cFLIP, which were subsequently used in experiments as shown in Fig. 4.15-4.16. Experiments in Fig. 4.15-4.16 were done in prof. Ales Hampl laboratory, Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno, Czech Republic by Vladimir Vinarsky and his colleagues.

To the last part of Results 4.3 Streptomycetes metabolites of the Manumycin family can via enhanced production of reactive oxygen species sensitize colon cancer cells to TRAIL-induced apoptosis, author participated by measuring apoptosis in RKO and SW-620 cell lines and by measuring production of mitochondrial ROS after cell treatment with Manumycin A, Manumycin B and Asukamycin in Fig. 4.17 B and 4.19 respectively. Western blots and detection of caspase activation in Fig. 4.18 was performed by Martin Klíma.

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FOREWORD

Regulated cell death (RCD) is an essential phenomenon occurring inside every living organism from a single prokaryote to a complex human body. RCD is participating in a broad spectrum of important physiological and pathological processes. Removing the damaged, dysfunctional or infected cells is essential for embryogenesis and maintains the fragile balance/homeostasis in an adult organism. Disruption of this balance leads to a number of pathological states and diseases. Unsubstantiated cell death during brain or heart ischemia and in drugs/alcohol-damaged liver leads to impaired function of these organs and often ends with death of a patient. On the other hand attenuation of the cell death by viruses protects their host from premature cell death thus allowing viral replication to proceed. Additionally and importantly, regulated cell death serves as a natural barrier to tumour formation and cancer progression and became a target of many anti-cancer therapies. TRAIL ligand, a member of TNFα ligand family inducing extrinsic apoptosis, was showed to induce apoptosis *in vitro* and *in vivo* in various cancer cells while sparing the normal ones. Its ability to selectively kill tumour cells without systemic toxic side effects has raised initial expectations that regrettably led to disappointments in clinical trials. Recombinant TRAIL ligands or apoptosis-inducing TRAIL-receptor monoclonal antibodies did not manifest a satisfying outcome in clinical trials with no or minimal effect on suppression and elimination of various tumours. Though unsuccessful, the outcome from these trials led to the formulation of better and possibly patient-tailored therapeutic protocols, which include the use of novel sensitizers and the discovery and preparation of modified TRAIL-based agents, thus opening a new window of opportunity for its use in cancer therapy. Importantly, new discoveries concerning the possible involvement of non-canonical TRAIL signalling in cancer progression, invasion and metastasis raised new questions, which now need to be examined and answered.

In my thesis I was mainly focused on analysis of canonical and non-canonical TRAIL-induced signalling in human cells. In the Literature Review I summarized the recent knowledge about two main types of regulated cell death: canonical apoptosis and non-canonical necroptosis and involvement of TRAIL ligand in both of these processes. In addition I also examined other types of non-canonical TRAIL-induced signalling such as activation of NFκB, JNK and p38 kinase pathways. The following Result section in the Thesis is divided into three parts. The first one comprises the major piece of my Thesis work recently published in BBA Molecular Cell Research

with my first authorship. In this project I prepared a new set of TRAIL ligands and addressed TRAIL receptor (DR4 and DR5) selective canonical and non-canonical signalling. Searching for the new types of recombinant proteins and the possible differences in the signalling of the two receptors DR4 and DR5 in different cancer cell lines can lead to more specific patient customized treatment and better clinical outcome. The second part presents the data from the published article on TRAIL-induced signalling in human embryonic stem cells, a collaborative project between our and Ales Hampl (MU Brno) laboratory. In this study we examined a role of TRAIL ligand and extrinsic apoptosis in human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC). The outcome of this study could be a useful material for further research and use in various biomedical applications from cell therapy to disease modelling or drug development. The final third part presents the data from yet unpublished work about Manumycin A and its mechanism of sensitizing colorectal cancer cells to not only TRAIL-induced apoptosis. Searching for the new sensitizers to overcome resistance to TRAIL-induced cell death is important part of cancer research. Finally, in the Discussion part, I compared our data with other published studies and discussed them especially in the context of cancer research.

Zuzana Nahácka, Prague, 2019

ABSTRACT

TRAIL ligand can trigger apoptosis of permissive human cells via engagement of its two pro-apoptotic receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5). Its ability to induce apoptosis independently on p53 status and to selectively kill cancer cells *in vitro* and *in vivo* made this ligand an attractive target in cancer research. However, acquired resistance of primary cancer cells, unsatisfactory outcome of clinical trials and recent studies arguing that TRAIL might under specific conditions promote cancer progression, opened new plethora of questions, which need to be addressed.

Though both receptors DR4 and DR5 are ubiquitously expressed, different types of tumours show preference for either of the receptors. The relative participation of DR4 and DR5 in TRAIL-induced signalling is still largely unknown. To analyse TRAIL receptor-specific signalling, I prepared Strep-tagged, trimerised variants of recombinant human TRAIL ligands with high affinity for either DR4 or DR5 receptor. Using these receptor-specific ligands, I examined a contribution of individual pro-apoptotic receptors to TRAIL-induced signalling pathways. I found that in TRAIL resistant colorectal HT-29 cells but not in pancreatic PANC-1 cancer cells, DISC formation and initial caspase-8 processing proceeded comparably in both DR4- and DR5-activated receptor complexes. However, TRAIL-induced apoptosis proceeded in both cell lines predominantly via DR5 receptor. ShRNA-mediated downregulation of DR4 or DR5 receptors in HT-29 cells also pointed to a stronger contribution of DR5 in TRAIL-induced apoptotic signalling. In contrast to TRAIL-induced apoptosis, I did not observed significant differences in necroptotic signalling activated either by DR4- or DR5-specific ligands. Activation of auxiliary signalling pathways involving NF-κB and stress kinases p38 and JNK proceeded under apoptotic conditions mainly in a DR5-dependent manner, while these kinase pathways were during necroptosis similarly activated.

In addition to TRAIL receptor-specific signalling this Thesis introduces Manumycin A (Man A) as an effective sensitizer of TRAIL-induced apoptosis in colorectal cancer cell lines RKO and SW-620. I documented that treatment of RKO and SW-620 with Manumycin A in combination with TRAIL ligand or ABT-199/737 led to statistical increased apoptosis and co-treatment of these cells with Man A and TRAIL WT led to stronger activation of caspases -8/ -9 and caspase-3 as

well as increased processing of caspase-3 targets PARP and Bid proteins. Importantly I found out that this sensitizing effect is most probably related to the enhanced production of likely mitochondrial ROS in ManA-treated cells.

Additionally, data from the collaborative project on human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC) showed that despite expression of both DR4 and DR5 receptors in these cells, they were naturally resistant to TRAIL-induced apoptosis. However, their pre-treatment with homoharringtonine led to decrease of expression of two anti-apoptotic proteins Mcl-1 and cFLIP and to their sensitization to TRAIL-induced apoptosis. Similarly shRNA-mediated downregulation of cFLIP led to their enhanced apoptotic response to TRAIL and thus cFLIP likely represents the important regulatory node in TRAIL-induced apoptosis of human pluripotent stem cells.

In summary, this study provided several new options how to overcome cellular resistance to TRAIL-triggered apoptosis and proposed answers to several questions raised in the field of TRAIL-induced signalling. It also provided first systematic insight into DR4-/DR5-specific signalling in colorectal and pancreatic cancer cells, where using a number of approaches documented that apoptotic and auxiliary but not necroptotic signalling in these cells largely relies on DR5 receptor. Moreover, I proposed secondary metabolite of Streptomyces Manumycin A as a novel potent sensitizer of TRAIL-induced apoptosis and I provided the first evidence that, irrespective of their origin, human stem cells express canonical components of the extrinsic apoptotic system and upon stress can activate death receptor-mediated apoptosis.

ABSTRAKT

TRAIL ligand spouští apoptózu interakcí se svými dvěma pro-apoptotickými receptory TRAIL-R1 (DR4) a TRAIL-R2 (DR5). Jeho schopnost indukovat apoptózu nezávisle na statusu p53 a selektivně zabíjet rakovinné buňky *in vitro* a *in vivo* zapříčinila, že tento ligand patřící do rodiny cytokinů TNF se jevil atraktivním cílem výzkumu terapie nádorových onemocnění. Avšak rezistence mnoha primárních nádorových buněk, neuspokojivý výsledek klinických testů a nedávné studie, které ukazují protichůdné výsledky a to konkrétně, že TRAIL může za specifických podmínek podporovat místo toho, aby inhiboval progresi nádorů, vyvolaly v oblasti výzkumu signalizace indukované ligandem TRAIL nové množství otázek, které je potřeba zodpovědet.

Ačkoliv jsou oba receptory DR4 a DR5 rovnoměrne exprimovány, různé typy nádorů vykazují preferenci pro jeden nebo druhý z receptorů. Relativní účast signalizace DR4 a DR5 indukované TRAIL ligandem je málo známá. Abychom analyzovali signalizaci ligandu TRAIL specifickou pro jednotlivé receptory, připravili jsme trimerizované varianty rekombinantního lidského ligandu TRAIL, značené pomocí Strep-tag, s vysokou afinitou pro receptory DR4 nebo DR5. Zjistila jsem, že narozdíl od pankreatických rakovinových buňek PANC-1 rezistentních k apoptóze indukované ligandem TRAIL, probíhá tvorba komplexu DISC a počáteční autoaktivace kaspázy-8 v rezistentních kolorektálních buňkách HT-29 srovnatelně jak v buňkách aktivovaných DR4, tak i DR5 ligandem. Apoptóza indukovaná ligandem TRAIL probíhala v obou buněčných liniích mnohem efektivněji prostřednictvím receptoru DR5. Potlačení exprese receptorů DR4 a DR5 pomocí shRNA v buňkách HT-29 také ukázalo na významnější přínos receptoru DR5 v apoptóze indukované ligandem TRAIL. Na rozdíl od apoptotické buněčné smrti jsem nezaznamenala statisticky významné rozdíly v nekroptotické signalizaci, která byla srovnatelně aktivována jak ligandy specifickými pro DR4, tak i DR5 receptor. Aktivace dalších signálních drah, zahrnujících NF-κB a stresové kinázy p38 a JNK, probíhala za apoptotických podmínek hlavně DR5-dependentním způsobem, zatímco tyto signalizační cesty byly v průběhu nekroptózy srovnatelně aktivovány.

V dizertační práci jsem dále také ukázala, že sekundární metabolit streptomycét Manumycin A je ve srovnání s příbuzným Manumycinem B nebo Asukamycinem účinným senzitizátorem

apoptózy indukované ligandem TRAIL v buněčných liniích RKO a SW-620 kolorektálního karcinomu. Dokázala jsem, že inkubace RKO a SW-620 s Manumycinem A v kombinaci s TRAIL ligandem nebo ABT199/737, vedla ke statisticky významnému zvýšení apoptózy. Také jsem ukázala, že společná inkubace Manumycinu A s ligandem TRAIL vedla k silnější aktivaci kaspáz -8 / -9 a kaspázy-3, stejně jako k zvýšenému šťepení proteinů PARP a tBid. Důležité bylo zjištění, že tento ko-aktivační efekt je pravděpodobně způsoben produkcí ROS generovaných mitochondriemi.

Závěrem, údaje ze spolupráce s Vladimírem Vinarským a kol. ukazují, že lidské embryonální kmenové buňky (hESC) a lidské pluripotentní kmenové buňky (hiPSC) exprimují receptory DR4 i DR5, ale jsou rezistentní vůči apoptóze indukované ligandem TRAIL. Souběžná inkubace s inhibitorem translace homoharringtoninem a ligandem TRAIL vedla k senzitizaci buňek k apoptóze indukované ligandem TRAIL a ke snížení exprese dvou antiapoptotických proteinů Mcl-1 a cFLIP. Ukázala jsem, že potlačení exprese cFLIP pomocí shRNA vedla ke zvýšené citlivosti hESC, a že cFLIP pravděpodobně představuje důležitý regulační uzel ve vnější dráze apoptózy indukované ligandem TRAIL v lidských pluripotentních kmenových buňkách.

Souhrnně vzato, představila jsem první systematický pohled na DR4-/DR5-specifickou signalizaci v kolorektálních a pankreatických rakovinných buňkách, kde jsem použitím několika přístupů prokázala, že pro-apoptotická, ale ne nekroptotická, receptor-specifická signalizace ligandu TRAIL může v kontrastu s posledními studiemi do značné míry probíhat prostřednictvím receptoru DR5. Také jsem ukázala, že Manumycin A je efektivním senzitizátorem apoptózy indukované ligandem TRAIL a poskytla jsem první důkaz, že pluripotentní kmenové buňky, nezávisle na jejich původu, exprimují kanonické složky vnějšího apoptotického systému a vlivem stresových faktorů mohou aktivovat apoptózu zprostředkovanou receptory indukující buňečnou smrt.

LIST OF ABBREVIATIONS

4 HB	four helical bundle domain
ADAM10/17	metallo-proteinase domain-containing protein 10/17
AIF	apoptosis inducing factor
APAF-1	apoptotic protease activating factor 1
BH	Bcl-2 homology domain
BIRC	baculovirus IAP repeat BIR containing domain
BR	brace region
CAD	caspase activated DNAse
CAMK2D	calcium/calmodulin dependent protein kinase type II subunit delta
CARD	caspase activation and recruitment domain
CB domain	calmodulin-binding domain
CRD	cysteine rich domain
DD	death domain
DED	death effector domain
DISC	death inducing signalling complex
DNA	deoxyribonucleic acid
FADD	Fas associated death domain protein
FLICE	Fas-associated death-domain-like interleukin-1beta-converting enzyme
cFLIP	FLICE inhibitory protein
IAP	inhibitor of apoptosis
ID	intermediate domain
IKK complex	inhibitor κB of kinase complex
KD	kinase domain
MLKL	mixed lineage kinase domain-like protein
MOMP	outer mitochondrial membrane permeabilization
OMM	outer mitochondrial membrane

PAMPS	pathogen-associated molecular patterns
PCD	programmed cell death
PIP	phosphatidylinositol phosphate
PKC	protein-kinase C
PLAD	pre-ligand assembly domain
PRR family	pathogen recognition receptor family
PS	phosphatidyl-serine
RCD	regulated cell death
RHIM	RIP homotypic interaction motif
RING	C-terminal Ring zinc-finger, RING- Really Interesting New Gene
RIPK1	receptor-interacting protein kinase 1
RIPK3	receptor-interacting protein kinase-3
RNA	ribonuclease acid
tBid	truncated Bid
TIR	Toll/interleukin receptor domain
TNFα	tumour necrosis factor α
TNFSF	TNF super-family
TNFRSF	TNF receptor super-family
TRADD	TNF receptor-associated death domain protein
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis inducing ligand
TRIF	TIR-domain containing adaptor-inducing IFN-beta
ZBP1- Z-DNA	binding protein 1 (DAI - DNA dependent activator)

1 LITERATURE REVIEW

1.1 Programmed cell death

Programmed cell death (PCD) is referred as a process of genetically-regulated physiological cell death, a self-suicidal cell mechanism proceeding at specific time and place during the development of an organism, occurring predominantly during embryogenesis. The term programmed cell death was firstly used by Lockshin in 1963 to refer to cell death of intersegmental muscles of *Bombyx Mori* (Lockshin, 1963). Later Kerr, Wyllie and Currie proposed term apoptosis for the process of controlled cell deletion, which plays an opposite role to mitosis in the regulation of cell populations (Kerr et al., 1972). Currently among the regulated modes of cell death belong in addition to caspase-dependent apoptosis also other morphological distinct types of cell death such as regulated necrosis/necroptosis, autophagic cell death, pyroptosis, ferroptosis and others (Vanden Berghe et al., 2014; Galluzzi et al., 2018).

1.1.1 Apoptosis

Apoptosis is physiological, genetically controlled and evolutionary conserved mode of cell death. It is a major mode of elimination of unnecessary or damaged cells in multicellular organisms (Green and Llambi 2015). This process is indispensable for the normal development of an organism, maintenance of homeostasis and proper functioning of the immune response. Dysregulated apoptosis can lead to developmental disorders, autoimmune and neurodegenerative diseases or tumorigenesis (Kerr et al., 1972; Baehrecke et al., 2002). Although there is a wide range of physiological and pathological conditions which trigger apoptosis, not all cells will necessarily die in response to the same stimulus. Apoptosis could be triggered by internal as well as external stimuli. Internal stimuli are mainly related to cellular stress, which can be induced by heat, radiation, nutrient deprivation, viral infection, hypoxia, misfolded proteins, damaged chromosomal DNA or increased calcium concentration. External stimuli cover mainly interaction of death ligands with their cognate receptors and dependance receptor triggered apoptosis. Apoptosis is accompanied by specific morphological and biochemical features, which are summarised in Table 1. In early phases occurs cell shrinkage, pyknosis, condensation and

fragmentation of chromatin in the nucleus, cytoplasm becomes more dense and organelles are tightly packed, cells become round and detached from the cell surface (Kerr et al., 1972; Grootjans et al., 2017). Cell shrinkage is accompanied by changes at the plasma membrane where transient blebbing and budding of the membrane results in the formation of apoptotic bodies - structures with intact plasma membrane, maintained organelle integrity with or without nuclear fragments. One of the well-known features of caspase-dependent apoptosis, exploited for its detection and quantification, is plasma membrane externalisation of phosphatidyl-serine (PS) by a flip-flop mechanism. Externalized PS serves as an eat-me signal for phagocytosis of apoptotic bodies by macrophages, parenchymal cells or neoplastic cells. Apoptosis is not accompanied with an inflammatory immune response as apoptotic cells do not release the cell content into surrounding area and are very rapidly phagocyted preferably by macrophages in a process called efferocytosis, preventing the secondary necrosis and production of pro-inflammatory cytokines (Savill et al., 2000; Kurosaka et al., 2003). Besides typical morphological changes on the cellular level, apoptosis is also accompanied by distinct biochemical events. For most of the biochemical events are responsible specific cysteine proteases called caspases and specific DNAses. Caspases cleave a number of structural and functional proteins including the nuclear laminin and contribute to the disruption of the nuclear envelope and activation of Caspase Activated DNAse (CAD) that eventually can cleave chromosomal DNA into small pieces of 180 to 200 base pairs (Bortner et al., 1995; Enari et al., 1998).

Morphological changes	**Biochemical/functional changes**
cell volume shrinkage	activation of initiator and effector caspases
cytoplasm condensation	changes in the mitochondrial membrane potential
nuclear envelope fragmentation	cleavage of distinct structural and functional proteins
nucleus fragmentation	cleavage of chromosomal DNA
formation of apoptotic bodies	externalization of PS
plasma membrane blebbing	increased free calcium levels
cell detachment	secretion of find-me molecules increased concentration of free calcium
rapid phagocytosis	

Table 1: Morphological and biochemical changes occurring during apoptosis (Kerr et al., 1972; Grootjans et al., 2017).

1.1.1.1 **Caspases, the key molecules in apoptosis**

The core of the apoptotic machinery is formed by caspases, enzymes of the family of cysteine-dependent proteases, which cleave the substrate behind the aspartic acid. Caspases are expressed as an inactive zymogens that need to be activated by (auto)processing at certain time and place. Caspases were identified in all multicellular animal organisms and their relatives metacaspases and paracaspases were found in plants, funghi and even prokaryots (Boyce et al., 2004). Caspases as executioners of apoptosis were initially discovered in the nematode *Caenorhabditis elegans*, where caspase CED-3, which possesses a substantial homology to human caspase-3 and caspase-8, was found to be essential for the elimination of distinct cells during embryogenesis (Horvitz et al., 1999). Mammalian caspases can be sub-divided according to their function, the length of their pro-domain, their substrate preference or phylogenetic relationship. Caspases in addition to their major role in apoptotic cell death also participate in other cellular processes such as proliferation, differentiation, development, inflammation and aging. Functionally are caspases divided into two major groups – pro-apoptotic and pro-inflammatory (Fig. 1.1, Shalini et al., 2015). The apoptotic caspases are sub-divided into a long prodomain-containing initiator caspases and short prodomain effector caspases. The activation and activity of the initiator caspases is usually required for the cleavage and thus activation of the effector caspases. Initiator caspases exist as inactive monomers and can be activated by proximity-catalysed self-processing in specific multimeric complexes (Oberst et al. 2010). Among the mammalian initiator caspases can be distinguished two sub-groups with different N-terminal prodomains. These domains typically mediate homotypic protein-protein interactions with other proteins participating in the caspase-activation platforms (Kersse et al., 2011). CARD (caspase activation and recruitment domain) domain-containing caspase-9 binds through this domain to the apoptosome forming protein Apaf-1 and caspase-2 interacts via CARD domain with the PIDDosome adapter protein RAIDD. DED domain (death effector domain) in caspases-8 and -10 serves to mediate their interaction with another adapter protein FADD (Fas associated death domain protein). The different type of prodomain predicts the different kind of apoptotic pathway involvement. The first two caspases - caspase-9 or caspase-2 have function in the intrinsic/mitochondrial apoptosis and are activated in the Apaf-1 forming apoptosome or in PIDD-containing PIDDosome, respectively. The latter two caspase - caspase-8 and caspase-10

have function in the ligand induced/extrinsic apoptosis and are activated at the DISC complex (death inducing signalling complex) – especially caspase-8 (Shalini et al., 2015).

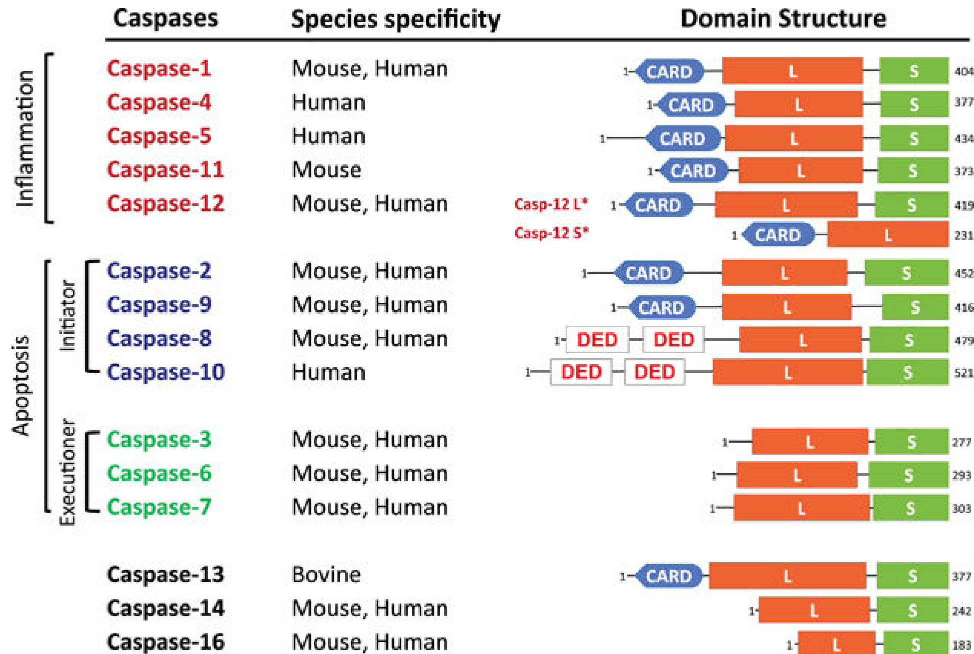


Figure 1.1: The structure and classification of mammalian caspases. CARD (caspase recruitment domain), DED (death effector domain), L stands for large domain, S stands for small domain. Adapted from Shalini et al., 2015.

The effector caspases-3/6/7 do not contain N-terminal protein-protein interaction domains and are expressed as inactive dimers. They are activated by their processing with the initiator caspases (3/7) or some other proteases (e.g. granzyme B). Caspase-6 is cleaved by caspase 3 and -7 (Green and Llambi, 2015). Cleavage between large (L in Fig. 1.1) and small (S in Fig. 1.1) domain and cleaving off the short N-terminal prodomain leads to forming the tetramer consisting of two small and two large subunits. Thus activated effector caspases cleave crucial regulatory and structural proteins, causing disassembly of the cell with the typical morphological features (Table 1) (Salvesen and Riedl, 2008; Crawford and Wells, 2011). Activation and activity of both initiator and effector caspases can be regulated by post-translational modifications (largely by phosphorylation), their stability and through the interaction with regulatory proteins. There are

many regulatory proteins which act upstream of the caspase activation and decide whether the cell survive or commit suicide. Among them the most important caspase regulators are IAP proteins (more in chapter 1.1.1.3 Negative regulation of apoptosis by IAP proteins and cFLIP). They can regulate not only activation of the caspases but can also directly compete for the binding of the caspases and regulate their activity by ubiquitin-ligase dependent manner (Dubrez-Daloz et al., 2008).

In addition to the essential role of caspases in apoptosis, these proteases participate also in other cellular events. They play and important role in regulation of dynamic of actin filaments, cell proliferation, differentiation, cell survival, inflammation or innate immunity. In these processes the activation of caspases is time-and space-limited and usually occurs without concomitant induction of cell death. Activation and activity of caspases is a very complex process and there were identified many cell and viral regulatory proteins which control their activity in apoptotic as well as non-apoptotic processes (Green and Llambi, 2015).

1.1.1.2 **Mechanism of apoptotic signalling pathway**

Apoptosis is energy-dependent process leading to the efficient dismantling of the cell with the minimal pro-inflammatory leakage of intracellular content and effective removal of apoptotic corpses. Apoptotic cascade is in mammals induced by two general pathways: the extrinsic and intrinsic pathway (Fig. 1.3) (Ashkenazi et al., 1998; Igney et al., 2002). In addition, the cytotoxic T cells exploit so-called perforin/granzyme signalling that can induce apoptosis-like cell death via injection of granzyme serine proteases (A, B, etc.). The extrinsic, intrinsic and granzyme B pathways have different initiation steps but leads to the activation of same group of effector caspases resulting in morphological and biochemical features of caspase-dependent apoptosis (Martinvalet et al., 2005).

The extrinsic apoptotic signalling mainly relies on ligand-receptor mediated interaction. The most studied and well known are pro-apoptotic ligands of the tumour necrosis family or TNF ligand family (TNFα, FasL, Apo2L/TRAIL), which bind to their cognate receptors of TNFR receptor family (TNFR1/2, FasR/CD95, TRAIL receptors). Members of the TNFR family share homology in the extracellular cysteine-rich domains (the ligand binding pocket) and some of them

so-called "death receptors" also in their cytoplasmic domain containing α-helical protein-protein interaction region called death domain (DD) (Ashkenazi et al., 1998).

One of the best characterized death ligand-receptor pair inducing extrinsic apoptosis is TNFα/TNFR1. TNFα is a trimeric cytokine produced by number of cells though mainly by activated macrophages in response to cellular stresses such as inflammation, infection or injury. After binding to the receptor TNFR1, TNFα triggers the conformational changes, which lead to the recruitment of the adaptor protein TNF receptor-associated death domain (TRADD) via its own death domain. TRADD serves as a platform for recruiting other proteins as receptor-interacting protein 1 (RIP1), TNF-receptor-associated factor 2 (TRAF2) and others (Grimm et al., 1996; Wajant et al., 2002). Signalling from the activated TNFR1 receptor can proceed through the membrane-associated complex and multifunctional cytoplasmic complexes (Fig. 1.2, Lafont et al., 2018). The first, membrane bound complex I consisting mainly of TNFR1, RIP1, TRADD, TRAF2, cIAP1/2, TAK1, HOIP-HOIL, SHARPIN and other proteins, induce activation of NFκB and JNK pathways. The intracellular cytoplasmic complexes can trigger both apoptosis and necroptosis (see also 1.1.2 Necroptosis) (Micheau and Tschopp, 2003). The core of apoptosis-inducing complex consists of RIP1, TRADD, FADD and caspase-8. Caspase-8 is recruited to the complex by the interaction with the adaptor protein FADD via their DED domain. Proximity-induced activation of caspase-8 leads to the autocatalytic cleavage of caspase and its full activation (Dickens et al., 2012). Activated caspase-8 cleaves and activates the downstream substrates, among them the most important BH3-only protein Bid and effector caspase-3/7. Truncated Bid (tBid) serves as an interconnection between extrinsic and intrinsic apoptosis in type II cells, which in contrast with type I cells, require mitochondrial amplification of apoptotic signalling for efficient induction of apoptosis. tBid blocks anti-apoptotic proteins such as Bcl-2 or Bcl-XL and co-activates pro-apoptotic Bcl-2 proteins Bax and Bak, resulting in the mitochondria-outer membrane permeabilization (MOMP) and downstream activation of intrinsic apoptotic signalling. Caspase-8 can activate effector caspases directly by their cleavage or indirectly through tBid and mitochondria-mediated cell death. Activity of caspase-8 could be inhibited by cFLIP protein that resembles caspase-8 but does not possess full catalytic domain and can compete with caspase-8 for binding of adaptor protein FADD (Green and Llambi, 2015).

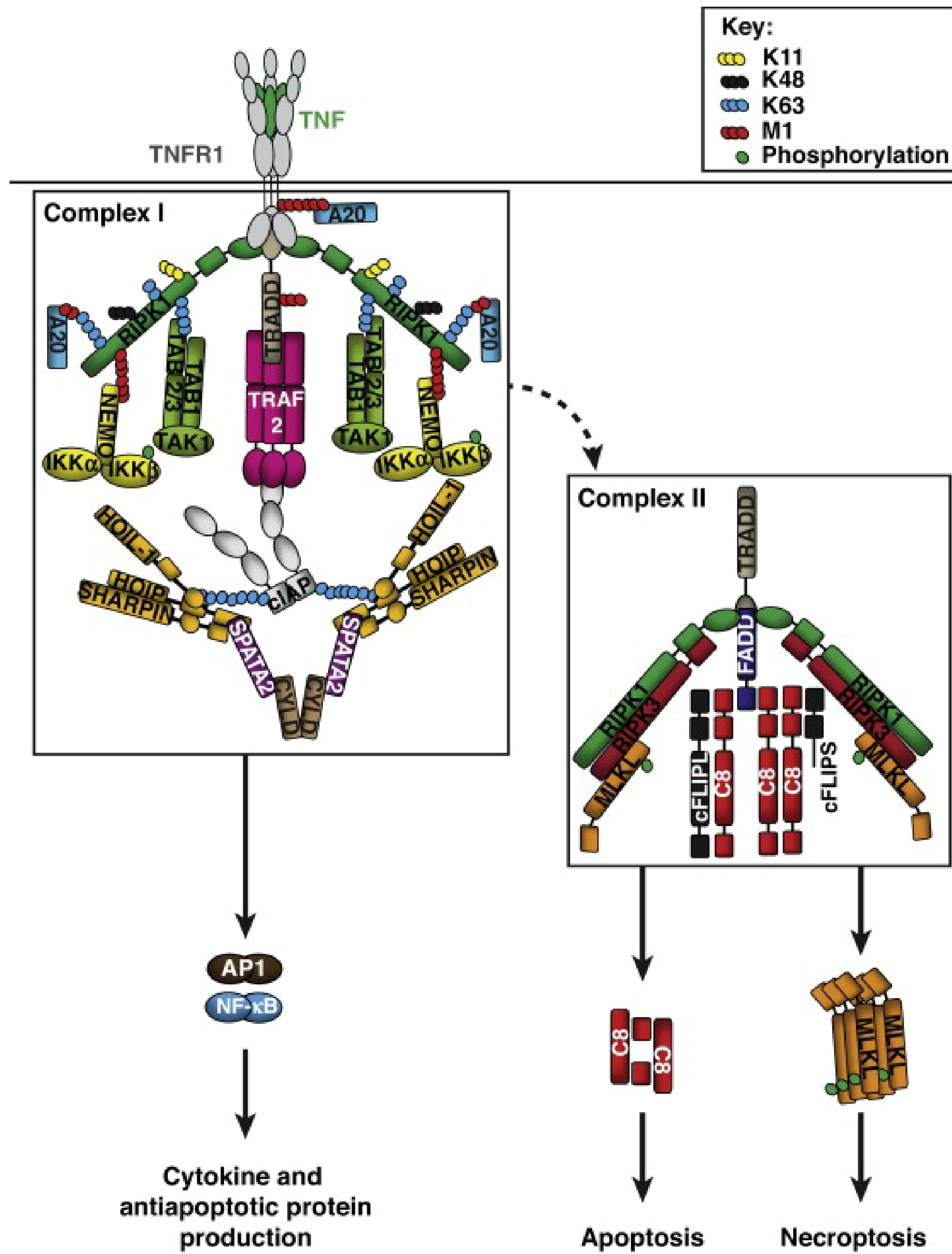


Figure 1.2: Different protein complexes formed in TNFα induced signalling. Membrane-associated Complex I leads to activation of pro-survival pathways while cytoplasmic Complex II can lead to apoptosis or necroptosis. Adapted from Lafont et al., 2018.

Mitochondrial or intrinsic pathway could be activated without receptor-mediated stimuli and is triggered by diverse signals that directly activate intracellular targets involved in the mitochondria-initiated events. Among the signals inducing intrinsic apoptosis belong negative: the absence of growth factors, hormones or cytokines, as well as positive signals: radiation, hypoxia, thermal stress, viral infections or free radicals. These stress signals activate or induce expression of pro-apoptotic BH3-only Bcl-2 proteins such as Bid, Bim, Puma and others which directly and/or indirectly (through inactivating anti-apoptotic Bcl-2 proteins) trigger Bax and Bak homo- and hetero-oligomerization in the mitochondrial outer membrane and its pore-assisted permeabilisation (Kale et al., 2018). The permeabilisation of the outer mitochondrial membrane releases two groups of apoptosis promoting regulatory proteins from the inter-membrane space of mitochondria into the cytosol. First group is consisting of cytochrome *c*, SMAC/DIABLO and protease HtrA2/Omi. In the cytosol, cytochrome *c* binds and activates Apaf-1 protein leading to its oligomerization and forming of the apoptosome complex (Du et al., 2000; van Loo et al., 2002). This arrangement is formed by seven monomers of Apaf-1 set in one ring (Acehan et al., 2002). Apoptosome forming is dATP-dependent process. The apoptosome serves as a docking site for activation of initiator caspase-9. Apaf-1 protein contains in addition to the interaction CARD domain also WD40 repeats, which in mammals have the auto-inhibitory function of blocking CARD/CARD domain interaction of APAF-1 and caspase-9 in non-apoptotic condition. In case of pro-apoptotic stimuli and cytochrome *c* release from the inter-membrane space of mitochondria, cytochrome *c* binds to WD40 domain and causes the conformational change that leads to opening of CARD domain for the interaction with caspase-9 (Hill et al., 2004). Smac/DIABLO and HtrA2/Omi are inhibitors of IAP proteins (inhibitors of apoptosis), thus clearing out a way for full unhampered activity of caspase-9 as well as effector caspases-3 and 7 (van Loo et al., 2002; Schimmer et al., 2004). The second group of pro-apoptotic proteins released in late phase of mitochondria mediated cell death are AIF (apoptosis inducing factor) and endonuclease G. These proteins translocate to the nucleus where firstly AIF cause DNA fragmentation into 50~300 bp and condensation of chromatin and endonuclease G cleave chromatin and produce oligonucleosomal DNA fragments (Joza et al. 2001; Li et al. 2001). Concurrently nuclease CAD (caspase-activated DNase) is activated by caspase-3 through cleavage of its inhibitor ICAD and also cleaves DNA and induces higher chromatin condensation in the nucleus (Enari et al, 1998; Joza et al., 2001; Li et al., 2001).

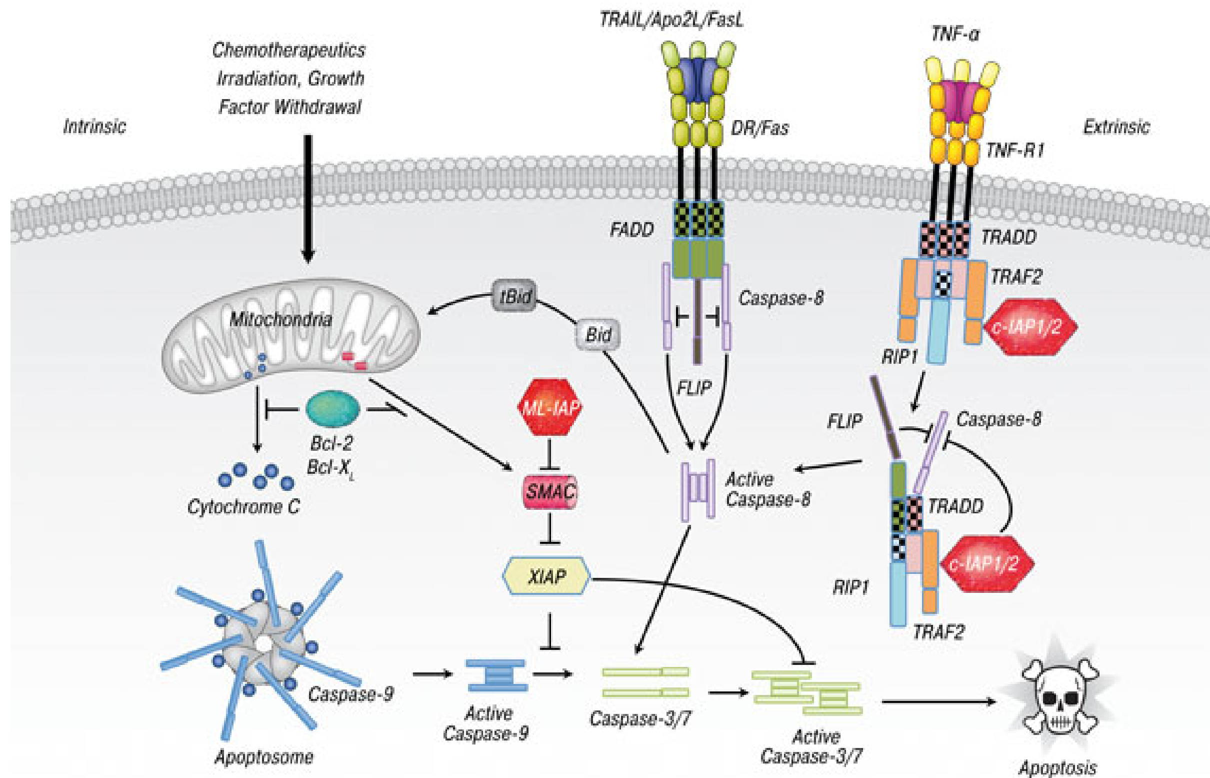


Figure 1.3: Basic model of extrinsic and intrinsic apoptotic pathway. Adapted from de Almagro and Vucic, 2012.

1.1.1.3 Negative regulation of apoptosis by IAP proteins and cFLIP

Among the negative regulators of apoptosis belongs the family of IAP proteins (inhibitors of apoptosis) or also called BIRC (baculovirus IAP repeat BIR containing) proteins. This group of evolutionary conserved proteins was initially identified in genome of baculoviruses as their protection against host-defence apoptotic signalling triggered by viral infection. The first and one of the most important IAP proteins identified in human cells was XIAP, discovered by searching for the BIR domain homology sequence. The function of the c-IAP (cellular IAP) proteins in the human cells is very diverse and involves not only cell death as apoptosis or necroptosis, but also regulate cell migration and cell survival through cell cycle, immunity and inflammation (NAIP, survivin, Apollon/Bruce, ILP-2) (de Almagro and Vucic, 2012).

IAPs share several well conserved protein domains which determine their activity such as N-terminal BIR domains. Their function is to mediate protein-protein interactions and more copies of this domain enable the robust binding character and functions of IAPs in the cell (Eckelman et al., 2006). The specific sequence of BIR1 domain in c-IAP1 and c-IAP2 enables them to bind TRAF1/2 adaptor protein in case of TNF ligand family induced signalling. The second conserved domain that is present in some IAPs, e.g. in cIAP1/2 or XIAP, is a RING domain (C-terminal Ring zinc-finger, RING- Really Interesting New Gene), which acts as E3 ubiquitin ligase and could target IAPs itself and IAP-interacting proteins to the proteosomal degradation. Also the IAP-binding proteins possess N-terminal IAP binding motif, which could be found in plethora of proteins involved in apoptosis as for example caspase-9, Smac/DIABLO or Omi/HtrA2 (Palaga et al., 2002; Vaux et al., 2003).

There are two main ways how IAP proteins regulate cell signalling, either in case of XIAP by direct binding-mediated inhibition of active caspases-3/7/9 or for cIAP-1 and cIAP-2 by the E3 ligase activity and modulation of signalling induced by ligands from the TNF family. The most important substrates for the polyubiquitination by c-IAPs is RIP1 kinase and NIK, both acting in the activation of canonical and non-canonical NFκB signalling. The ubiquitination of other targets as TRAF2, TRAF3 and Smac/DIABLO could have a direct impact on cell survival. XIAP E3 ligase activity is not essential for its anti-apoptotic function but can modulate the fate of the cell. IAPs anti-apoptotic function could be blocked by binding of Smac or Omi/HtrA2 which are released during mitochondria induced apoptosis. Omi/HtrA2 has a dual function because it not only compete with caspases for the binding of XIAP, but also has a serine-protease activity and cleaves XIAP to non-functional protein. As far as the main function of IAP proteins is to maintain cell survival not only by inhibiting the apoptosis but also inducing NFκB and MAPK pro-survival pathway, these proteins are overexpressed in many tumours, e.g. colon and cervical tumours (cIAP1/2), breast cancer (XIAP) and myeloma (cIAP, XIAP, survivin) (Ali et al., 2018; Lalaoui et al., 2018).

Very important anti-apoptotic protein specific for the receptor or extrinsic apoptosis is an „inactive caspase“ cFLIP protein. FLIP was firstly identified in viruses as a viral escape mechanism against the host cell-induced apoptosis (Thome et al., 1997). Later it was mapped in the human genome as the *CFLAR* gene and its product cellular FLIP or cFLIP has three major

isoforms: cFLIP-L (55 kDa), cFLIP-R (25kDa) and cFLIP-S (27 kDa) (Fig. 1.4) (Micheau et al., 2001). Although cFLIP-L possess the greatest similarity to the sequence of caspase-8, it is proteolytically inactive due to the absence of catalytically important residues. The other two forms do not contain the caspase-like domain (C-terminal domain) but all three isoforms have two DED domains and interact with FADD and caspase-8 at the receptor level (Hu et al., 1997; Rasper et al., 1998). While cFLIP-R/S simply inhibit caspase-8 activation by heterodimerization with the molecules of caspase at the receptor level, cFLIP-L seems to have also other than just inhibitory role in DR-triggered signalling. Indeed, cFLIP-L can at high levels inhibit apoptosis in similar way as short splice variants of cFLIP, but as far as it can form heterodimers with caspase-8, in low ratio to caspase-8 it can enhance recruitment of pro-caspase-8 molecules to DISC (Boatright et al., 2004). Heterodimerization of caspase-8 with cFLIP is additionally advanced by caspase-8-mediated cleavage in the cFLIP-L interdomain (Krueger et al., 2001; Chang et al., 2002; Micheau et al., 2002; Pop et al., 2011). Interestingly, the N-terminal prodomain generated by full cleavage of caspase-8 and structurally similar to cFLIP-R/S, remains at DISC and terminates procaspase-8 activation in a feedback loop (Schleich et al., 2016).

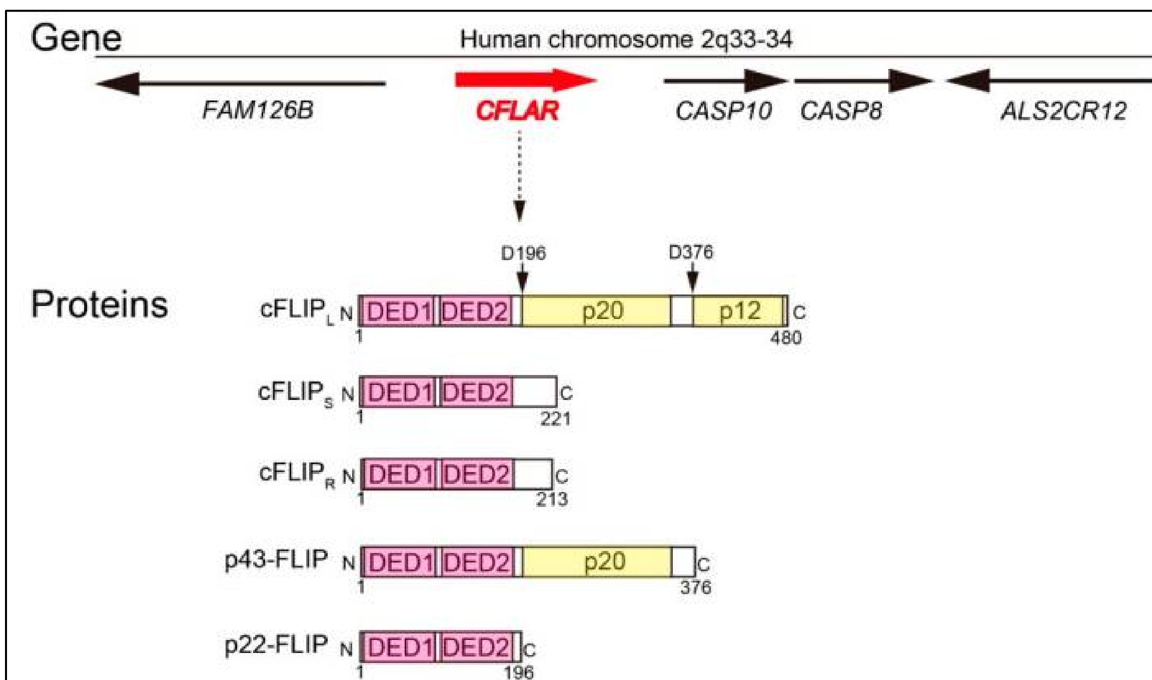


Figure 1.4: Structure of human *CFLAR* gene and protein cFLIP. The red arrow indicates *CFLAR* gene, the black arrows indicate nearby genes on human chromosome 2q33-34. The yellow box indicates caspase-8 like domain, the magenta box is DED interaction domain. Top three depicted proteins are different splice variants of *CFLAR* gene and two bottom ones are products of caspase-8 cleavage. Adapted from Tsuchiya et al., 2015.

1.1.1.4 Regulation of apoptosis by proteins from the Bcl-2 family

In mammalian cells, the members of this protein group form a large family. They are best known as both regulators and inducers of apoptosis with pro- as well as anti-apoptotic functions. Human Bcl-2 (B-cell lymphoma gene 2) was discovered as a proto-oncogene in B-cell lymphoma cells (Tsujimoto et al., 1985). The first identified pro-apoptotic member of this family was protein Bax (Bcl-2 associated protein X) (Oltvai et al., 1993). Although the sequence homology in this protein family is not obvious, the Bcl-2 proteins do have four conserved domains, which are known as Bcl-2 Homology- BH (1-4) domains (Fig. 1.5). These domains are essential not only for the protein-protein interaction, but also for the activity of Bcl-2 proteins (Yin et al., 1994). In addition, most members of this protein family contain also a C-terminal hydrophobic domain (membrane anchor domain), important for their targeting to intracellular lipid membranes (mainly mitochondria and endoplasmatic reticulum) (Antonsson et al., 2004).

All anti-apoptotic proteins from Bcl-2 family have all four BH domains but differ in their cellular localisation. While Bcl-2 itself is predominantly localised in the membranes, including the outer mitochondrial membrane (OMM), endoplasmatic reticulum and nuclear membrane, Bcl-X_L and Mcl-1 do localize both in the cytoplasm and OMM or ER (Bcl-X_L) membranes (Kale et al., 2018). The pro-apoptotic Bcl-2 family proteins can be divided into two main groups. The first one contains multidomain proteins Bax, Bak and Bok. The intracellular localisation of Bax and Bak is different. While Bak is mostly associated with the outer mitochondrial membrane, Bax can be also found in cytoplasm interacting e.g. with 14-3-3 proteins (Goping et al., 1998; Eskes et al., 2000). The multi-domain pro-apoptotic proteins Bax and Bak are in normal condition in inactive form and became activated upon various stimuli including posttranslational modifications. Upon these stimuli they undergo conformational change in the OMM where they can form large protein-

permeable pores (Goping et al., 1998). Through these pores a number of pro-apoptotic proteins is released from the mitochondrial intermembrane space including cytochrome *c*, Smac, HtrA2, endonucleases G and AIF (Li et al., 1998; Lassus et al., 2002; Li et al., 2002). The second more numerous group of pro-apoptotic Bcl-2 proteins are BH-3 only proteins, which are activated by pro-apoptotic (stress) signals at multiple levels. Upon their activation they translocate to mitochondria and interact with anti-apoptotic Bcl-2 proteins attenuating or blocking their function. A small group of BH3-only proteins notably Bim, tBid and likely Puma can in addition to inhibiting anti-apoptotic Bcl-2 proteins also interact with pro-apoptotic Bax and Bak proteins and enhance their oligomerization and pore formation in the OMM (Eskes et al., 2000; Adams and Cory, 2018; Kale et al., 2018; Pentimalli et al., 2018).

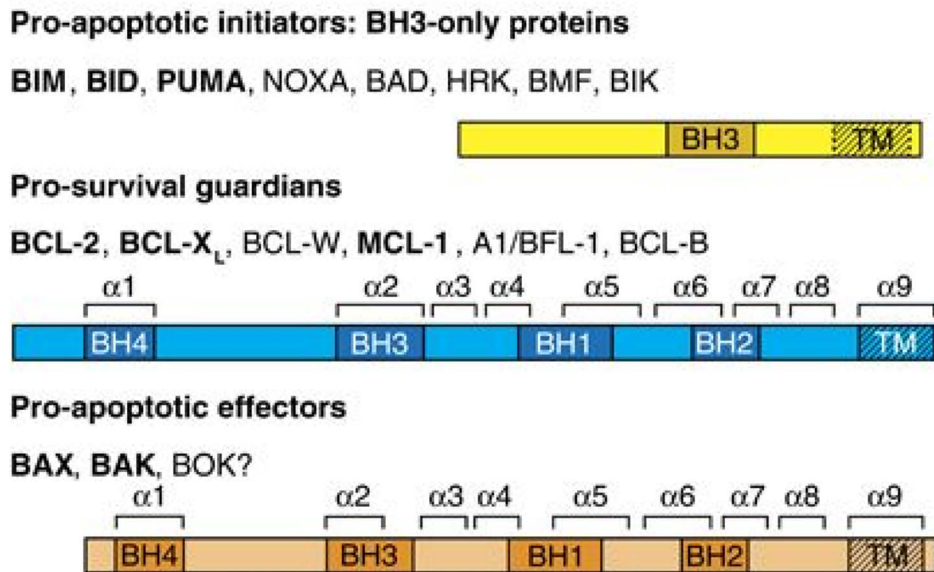


Figure 1.5: Bcl-2 protein family. Different groups of Bcl-2 proteins and their BH domains divided according to their function in apoptosis. Adapted from Adams and Cory, 2018.

1.1.2 Necroptosis

Necrosis was recognized as a mode of non-programmed accidental cell death from the mid of the 19th century. In last decades it has been revealed that necrosis could be under some circumstances induced by defined signalling pathways and thus recognized as another regulated mode of cell death. One of the best characterized pathways of regulated necrosis is so-called necroptosis. According to the recent nomenclature is the term necroptosis defined as a necrotic type of cell death dependent on receptor-interacting protein kinase-3 (RIPK3) (Galluzzi et al. 2018). Necroptosis triggered by activated receptor-interacting protein kinase 1 (RIPK1) (upstream of RIPK3) could be inhibited by chemical inhibitors (e. g. necrostatin-1, Nec-1) or at the level of gene expression of key signalling molecules (such as RIPK3) involved in necroptotic machinery (Vercammen et al., 1998; Degterev et al., 2005; Degterev et al. 2008; Zhang et al., 2009; Sun et al., 2012). It has the morphological features of necrosis with the rounded and swelling cell (oncosis), cytoplasmic vacuolisation, rupture of mitochondria and lysosomal organelles, breakdown of plasma membrane, release of the intracellular content and induction of typical inflammatory response (Krysko et al., 2008).

While apoptosis has a clearly important role during embryonic development, the physiological role of regulated necrosis is more obscure. However, it looks like that this specific mode of regulated necrosis-necroptosis can participate in cell death induction in various pathophysiological conditions. Necroptosis could be triggered on the cellular level by plethora of signals as: TNFα, FasL, TRAIL ligand, lipopolysaccharide, dsRNA, DNA damage, endoplasmatic reticulum stress, viral infection and anti-cancer drugs (Vercammen et al., 1998; Holler et al., 2000; Cho et al., 2009; He et al., 2011; Tenev et al., 2011; Jouan-Lanhouet et al., 2012; Thapa et al., 2013; Saveljeva et al., 2015).

TNFα-induced signalling from the TNFR1 receptor was instrumental for the discovery and further research of necroptosis. As is shown in Fig. 1.2 and also in Fig. 1.6, the ligation of TNFα ligand to its cognate receptor TNFR1 could induce several signalling pathways, where different types of protein complexes are formed depending on the upstream pro-survival or pro-death signal (Grootjans et al., 2017). At the molecular signalling level the TNFα interaction with TNFR1 receptor induces recruitment of RIPK1 and adaptor protein TRADD via their death domains with two main possible complexes: complex I and complex II. In “closed conformation” the RIPK1 is

ubiquitinated by cIAP1/2 and phosphorylated by IKK complex, which prevents its dissociation from the receptor forming complex I (the core of NFκB signalling), or RIPK1 is not ubiquitinated ("opened conformation") or is de-ubiquitinated (for example by A20 or CYLD enzyme or by using the inhibitors of Smac mimetics) and the cytosolic complex II a/b consisting of RIPK1, FADD, caspase-8 with or without the adapter protein TRADD forms. Both of the complexes II a/b lead to the activation of caspase-8-dependent apoptosis. In first case, complex IIa leads to the RIPK1-independent apoptosis. The second complex IIb is formed in case when cIAPs, TAK1 or IKK complex is inactive or absent and the RIPK1 kinase activity becomes inevitable for proceeding of further apoptosis. Activated caspase-8 cleaves in addition to other proteins also both RIP kinases and thus suppresses induction of necroptosis. However, when protease activity of caspase-8 is impaired, the necrosome complex containing RIPK1/3 is formed. The kinase activity and the opened form of RIPK1 is essential for the activation and auto-phosphorylation of RIPK3. Although the RIPK3 is phosphorylated at Ser227 in a Nec-1 inhibitable manner, it was shown *in vitro* that RIPK1 is unable to phosphorylate RIPK3. Therefore, the phosphorylation of this kinase is not the trans-phosphorylation as was once thought, but most probably it is the auto-phosphorylation of the RIPK3 kinase itself, which is at least for TNFα-induced necroptosis, dependent on its interaction with RIPK1. Nevertheless necrosome formation promotes further activation of RIPK3, forming of oligo-amyloid like structures of RHIM domain-containing protein aggregates and RIPK3-dependent phosphorylation and subsequent activation of the mixed linage kinase-like protein (MLKL). p-MLKL translocates to cellular membranes via its interaction with phosphatidyl inositol phosphates (PIP) and either directly by forming pores into the plasma membrane or indirectly through the activation of ion channels induce permeabilization of the plasma membrane and activate the execution phase of necroptosis (Micheau et al., 2003; Zheng et al., 2006; Wang et al., 2008; Pasparakis et al., 2015).

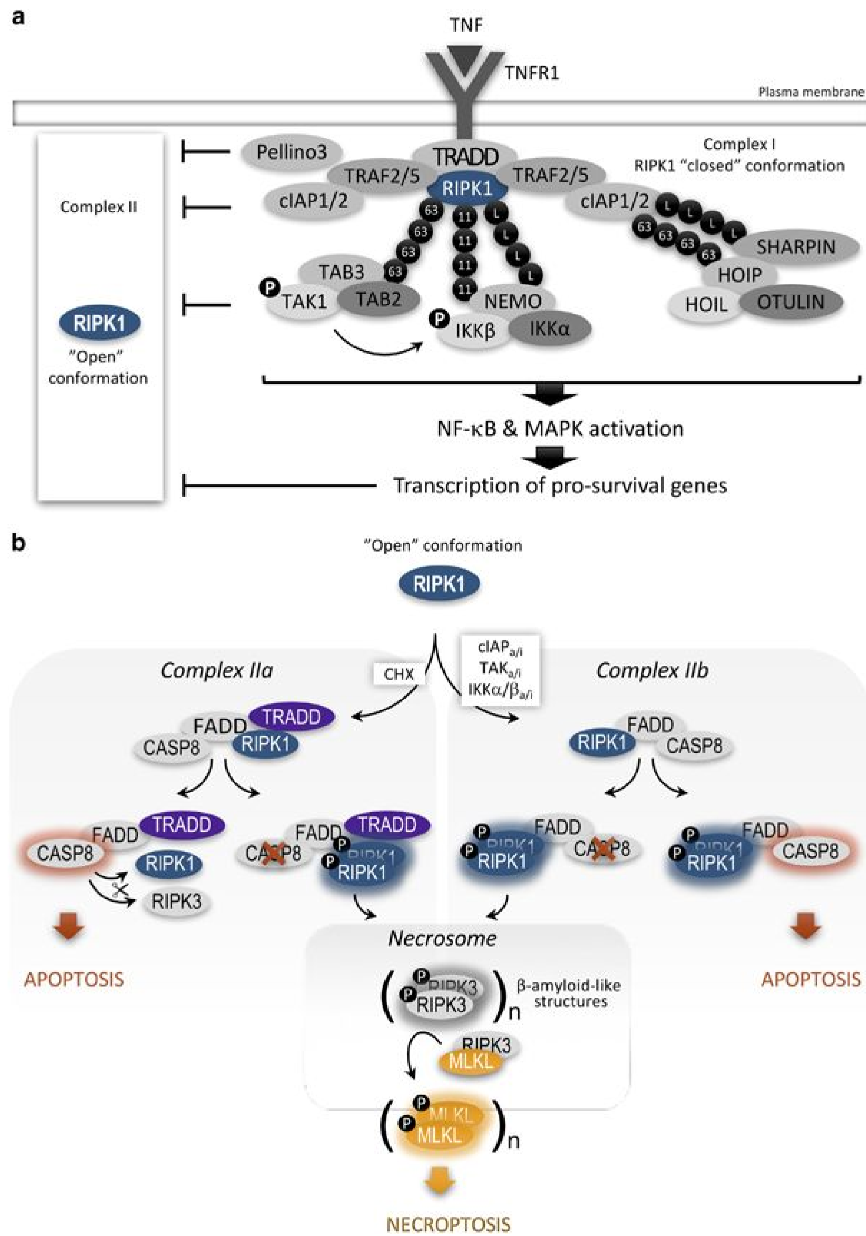


Figure 1.6: Necroptosis and other signalling pathways induced by TNFα ligand. (a) TNFα stimulation leads to forming of pro-survival Complex I. Ubiquitinated RIPK1 recruits the proteins of TAK1 kinase cascade and IKK complex resulting in the activation of MAPK and NFκB pathways. **(b)** RIPK1 dissociates from the TNFR1 receptor and forms two types of apoptotic complexes IIa and IIb or in case of caspase-8 inhibition/inactivation or absence forms the necrosome complex involved in the process of necroptosis. Downstream the phosphorylated protein RIPK1 induces auto-phosphorylation of RIPK3 which phosphorylates MLKL protein leading to execution part of the necroptosis. Adapted from Grootjans et al., 2017.

As shown in Fig. 1.6, there are several signalling molecules shared in different signalling complexes. One of the most important is the RIPK1 kinase, which could represent a switch point of the TNFα ligand signalling pathway of necroptosis and plays similar role also in TRAIL and FasL- induced necroptotic signalling (Holler et al., 2000). This kinase has several very important domains involved in different signalling pathways thus deciding the cell fate (Fig. 1.7). The N-terminal kinase domain is important for the induction of cell death in both RIPK1 dependent apoptosis and necroptosis. The intermediary domain ID plays role in post-translational modification of the RIPK1 where it serves as a scaffold for further K63 and M1 ubiquitination resulting in the activation of JNK, p38, ERK and NF-κB pathway (for more information see chapter: 1.2.4 Non-canonical TRAIL induced signalling). The C-terminal death domain DD is a classical protein-protein interaction domain involved in the induction of apoptosis, serving for the recruitment of other components of the apoptotic machinery, namely FADD and caspase-8. The last one is the C-terminal RHIM domain (RIP homotypic interaction motif), which could be found also in other necroptosis-involved proteins and it serves as a protein-protein interaction domain with not only downstream target kinase RIPK3 but also with other RHIM-containing proteins (Chen et al., 2002; Sun et al., 2002; Festjens et al., 2007; Dondelinger et al., 2013; Pasparakis et al., 2015). RIPK1 shares 33% similarity with the kinase RIPK3, which also contains N-terminal kinase domain, intermediate domain and important C-terminal RHIM domain interacting with RIPK1. According to the most studies it seems that RIPK3 kinase activity is indispensable for the induction of necroptosis (Festjens et al., 2007; Dondelinger et al., 2013). The last key mediator of necroptosis is a pseudo kinase, mixed lineage kinase like, MLKL protein. This protein is likely the main effector of necroptosis (Zhao et al., 2012). MLKL also possess inactive kinase domain, thus the main function part of this protein is the N-terminal 4HB domain, which when phosphorylated by RIPK3 brings MLKL to the plasma membrane through PIP binding, causing pore formation and plasma membrane permeabilisation. RIPK3 and MLKL bind through their kinase domains and presumably they exist in the pre-assembled non-active complexes (Xie et al., 2013). Phosphorylation of MLKL by RIPK3 is an essential step for the next conformational change and opening of HB domain (Murphy et al., 2013). Interestingly, RIPK3 can phosphorylate also other substrate, the calcium-dependent protein kinase II delta or CAMK2D and it looks like that this kinase is able in some special cases through the direct or indirect activation of calcium, potassium and natrium ion channels induce necroptosis independently on MLKL protein (Ashpole et al.,

2012; Zhang et al., 2016; Zhang et al., 2018). The canonical RIPK1/RIPK3-containing necrosome is associated with necroptosis induced by several types of stimuli: ligation of death receptors (TNFR 1/2, FasR, TRAILR 1/2); stimulation of the pathogen recognition receptor family (PRR) expressed by cells of innate immune system to sense pathogen-associated molecular patterns (PAMPS), which includes membrane bound Toll-like receptors (TLR 2, 4, 5 and 9), cytosolic NOD-like receptors and retinoic acid induced gene 1-like receptors; activation of T-cell receptor; using anti-cancer drugs (Smac mimetics), vaccinia infection; IFN, DNA damage and many others (Holler et al., 2000; Cho et al., 2009; He et al., 2011; Tenev et al., 2011; Basit et al., 2013; Thapa et al., 2013). In some cases the autocrine TNFα secretion can boost necroptotic cell death (McNamara et al., 2013). In RIPK1 kinase-independent formation of so-called non-canonical necrosome, RHIM-containing adapter proteins TRIF (CMV infection) or the sensor ZBP1/DAI participate in RIPK3 activation and the induction of necroptosis (Kaiser et al., 2005; Kaiser et al., 2013). It is possible that these proteins serves as a scaffold for the RIPK3 molecules recruitment, activation and auto-phosphorylation (Rebsamen et al., 2009).

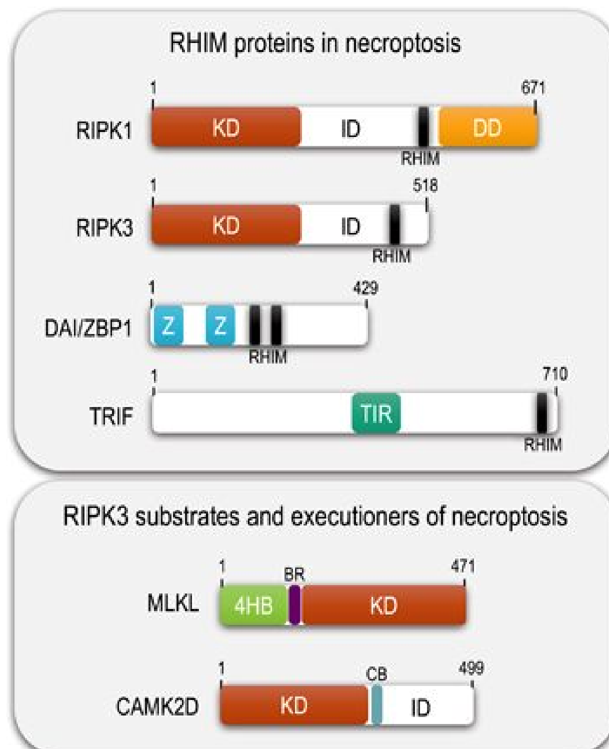


Figure 1.7: The structure of RHIM domain containing proteins involved in the process of necroptosis. Length is indicated in number of amino acids. KD - kinase domain, ID - intermediate domain, DD- death domain, RHIM- RIP homotypic interaction motif, TIR- Toll/interleukin receptor domain, 4 HB- four helical bundle domain, BR- brace region, CB- calmodulin-binding domain, RIPK - receptor interacting kinase, ZBP1- Z-DNA-binding protein 1, TRIF - TIR-domain containing adaptor-inducing IFN-beta, MLKL - mixed lineage kinase domain-like protein, CAMK2D- calcium/calmodulin-dependent protein kinase type II subunit delta. Adapted and adjusted from Grootjans et al., 2017.

1.2 TRAIL induced signalling

1.2.1 TRAIL and the ligands from TNF family

TNFα or tumour necrosis factor is a canonical "death ligand" of the TNF super-family (TNFSF), where also TRAIL (Apo2L) and CD95 ligand (FasL or Apo1L) belong. These prominent executors of extrinsic apoptosis can in addition induce proliferation and survival of normal as well as cancer cells (von Karstedt et al., 2017). They are expressed mainly in the cells of immune system and are associated with the protective function of these cells. Tumour killing activity induced by bacterial infection was known from the beginning of 20th century and later was TNFα ligand identified as the one responsible for this process (Carswell et al., 1975). However, systemic treatment with TNFα ligand led to the lethal inflammatory shock syndrome (Tracey et al., 1988). Similarly also use of other member of TNF family, Fas ligand (or its agonists), which has the same robust anti-tumour killing capability, was severely hepatotoxic in mice (Ogasawara et al., 1993). Interestingly the third member of this group TRAIL ligand or TNF-related apoptosis inducing ligand (Apo2L, TNFSF10) was shown to induce apoptosis selectively in cancer cells *in vitro* and *in vivo* and didn´t manifest any lethal adverse effects encountered with TNFα or Fas ligand (Ashkenazi et al., 1999; Walczak et al., 1999). TRAIL was firstly identified in 1995 based on the homology sequence to the other members of the TNFSF. It has 23% homology to TNFα ligand and 28% homology to FasL (Wiley et al., 1995). Members of TNFSF can be expressed as soluble proteins as lymphotoxin-α and vascular endothelial growth inhibitor (TNFSF15) or as all other members are expressed as type II transmembrane proteins with one transmembrane domain, N-terminus facing to the cytosol and extracellular C-terminus of the protein. TNFα, CD95 and TRAIL ligand could also exist in a soluble form, which results from their cleavage from the cell surface in the process called shedding. In contrast with FasL and TNFα, where a disintegrin and metallo-proteinase domain-containing protein 10 (ADAM10) and ADAM17 were identified to cause the shedding of the ligands, in case of TRAIL is the identity of the responsible proteases unknown (von Karstedt et al., 2017). It was also shown that production of soluble TRAIL is dependent on the activity of cysteine proteases (Mariani and Krammer, 1998). TRAIL is present in the plasma of a healthy adult at app. 100 pg/ml, which is too low to induce any apoptosis in most cells in vitro. However liposome-bound TRAIL can kill cancer cells more efficiently than a soluble protein (Gibellini et al., 2007; De Miguel et al., 2013; Nair et al., 2015). Unlike other TNF family members, which

expression is tightly regulated and are often transiently expressed on activated cells, TRAIL is constitutively expressed mostly by the cells of the immune system. In these cells TRAIL participates in homeostasis of specific T cells, in NK and T-cell mediated killing of virus-infected or transformed cells (Martinez-Lorenzo et al., 1998; Kayagaki et al., 1999; Smyth et al., 2003). Human TRAIL contains of 281 amino acids (114-281 in cleaved form) and harbours 65% of the sequence identity with its 291 amino acids long murine homolog. According to the crystal structure for TRAIL/TRAIL-R2 complex, TRAIL likely as other TNF family ligands appears as a trimer. Homotrimeric TRAIL ligand is stabilised by an intern zinc ion in a non-covalent interaction with cysteine residues from each TRAIL monomer. This interaction was shown to be essential for its stability, solubility and biological activity (Hymowitz et al., 1999; Hymowitz et al., 2000). TNFR receptors signal as trimers as well and it was originally thought that the receptor trimerisation was induced by ligand binding. This presumption was challenged when a specific ligand-independent domain called pre-ligand assembly domain (PLAD) has been identified (Chan et al., 2000). Thus some of the ligand trimers don´t induce the trimerisation but they can already bind to the existing receptor trimeric complexes, clustering into higher multimeric complexes (Lewis et al., 2012; Valley et al., 2012).

1.2.2 TRAIL receptors

Human TRAIL interacts with five receptors: four membrane receptors TRAIL-R1, -R2, -R3, -R4 and one soluble receptor OPG. TRAIL-R1 (DR4, TNFRSF10A) and -R2 (DR5, TNFRSF10B) are considered as pro-apoptotic and are receptor type I transmembrane proteins, homologous to other members of TNFR family. They share significant sequence homology with TNFR1 and CD95 in their death (DD) domain- containing cytoplasmic part. Other common features are the cysteine-rich domains (CRDs) in the extracellular portion of these receptors that form the ligand binding pocket. TRAIL-R4 (DcR2, TNFRSF10D) is also a receptor type I transmembrane protein but unlikely to DR4 and DR5 has a truncated death domain and thus is unable to transduce the apoptotic signal into the cell. TRAIL-R3 (DcR1, TNFRSF10C) lacks the cytoplasmic domain at all and is bound to the cell surface by GPI anchor (Fig. 1.8, von Karstedt et al., 2017). The exact physiological role of DcR1 and DcR2 is not yet known, but there are several experimental data-based hypotheses. The obvious one is that they work as decoy receptors and compete with pro-apoptotic receptors for TRAIL binding. However, this squelching activity of both receptors was

only documented in *in vitro* overexpression studies and moreover they do not possess higher affinity for TRAIL ligand comparing to DR4 and DR5 receptors (Merino et al., 2006). At 37°C TRAIL binds to TRAIL-R2 with the highest affinity than other membrane bound receptors, which is likely to be physiologically relevant especially under a limited supply of TRAIL ligand (Truneh et al., 2000). The decoy function of decoy receptors could be also addressed by the forming of mixed receptor complexes with the two pro-apoptotic receptors DR4/5, thus leading to the ineffective DISC formation. This formation of heteromeric TRAIL receptors could be mediated by pre-ligand assembly domain (Chan et al., 2000). In this mechanism the competition for TRAIL binding plays no direct role and could explain difference between decoy activity and their affinity for TRAIL ligand. The second hypothesis is related to their non-apoptotic signalling and it was shown that at least the truncated TRAIL-R4 can activate pro-survival NFκB pathway (Degli-Esposti et al., 1997; Degli-Esposti, 1999). Nevertheless as mentioned above, most of these experiments were done in overexpression systems and the physiological role of decoy receptors has to be established. Interestingly, TRAIL-R3 is highly expressed in primary gastrointestinal cancer and high expression of TRAIL-R4 is correlated with poor prognosis in breast cancer (Sheikh et al., 1999; Ganten et al., 2009). The fifth receptor which binds TRAIL ligand with the lowest affinity is secreted soluble receptor OPG (osteoprotegerin, TNFRSF11B) (Emery et al., 1998). OPG mainly acts as a regulator of bone development and activation of osteoclastogenesis. This activity is, however, not influenced by TRAIL but RANK ligand that binds also to the RANK receptor involved in the activation of NFκB pathway (Kimberley et al., 2004). The role of OPG as a TRAIL receptor is not clear, it was suggested that it could also act as a decoy receptor in competing for TRAIL binding. TRAIL receptors have been mapped as a gene cluster on the chromosome 8p22-21 and their mRNA expression level shows that TRAIL-R1/-R2/-R4 have widespread expression, while TRAIL-R3 transcripts were restricted to the peripheral blood and spleen (Degli-Esposti et al., 1997).

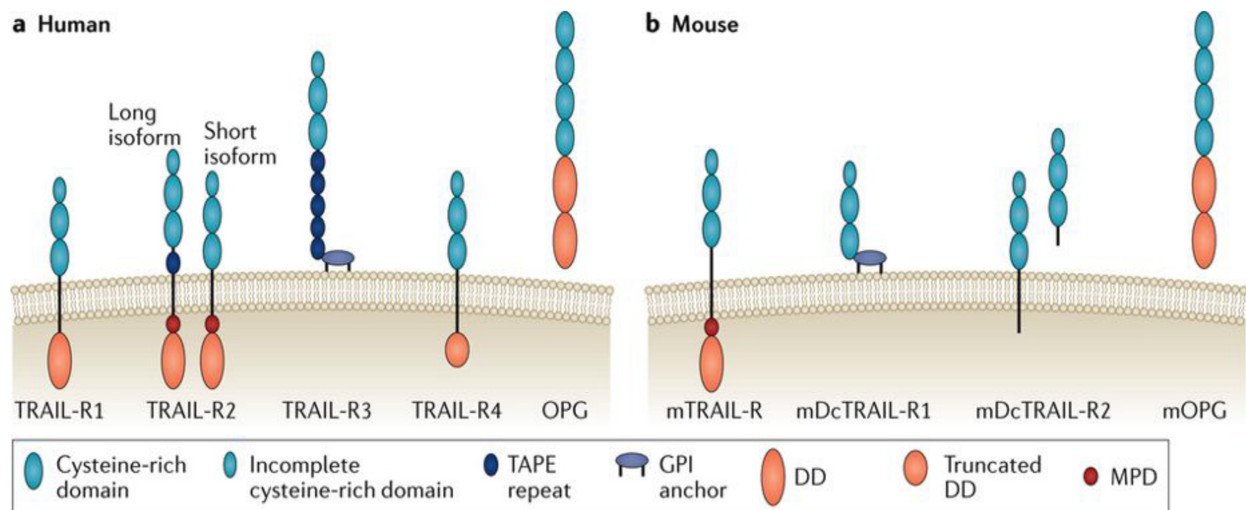


Figure 1.8: Graphic representation of human and mouse TRAIL receptors and their protein domains. Human cells express two pro-apoptotic TRAIL receptors: TRAIL-R1 (DR4) and TRAIL-R2 (DR5) and three non-apoptotic receptors: TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and OPG. On the contrary there is only one pro-apoptotic TRAIL receptor expressed in mice mTRAIL-R, accompanied by two decoy receptors mDcTRAIL-R1 and mDcTRAIL-R2 and mOPG. All of these receptors contain extracellular CRD which forms the binding pocket for TRAIL ligand. Pro-apoptotic TRAIL-R1/R2 and mTRAIL-R contain in their intracellular parts the death domain (DD), which main function is to recruit adaptor protein FADD. TRAIL-R4 contains truncated DD domain. TRAIL-R3 is a glycosylphosphatidylinositol (GPI) anchored protein. TAPE repeat (threonine, alanine, proline, glutamine) domain and MPD (membrane-proximal domain) are present in two isoforms of TRAIL-R2 receptor. TAPE repeat could be also found in TRAIL-R3 and MPD is present in mTRAIL-R receptor. Adapted from Karstedt et al., 2017.

Though there is a significant amount of data reffering to the structure, signalling and function of TRAIL receptors, their precise physiological role and regulation still remains to be determined. It was initially thought that DR4 and DR5 receptor have the same interchangeable function, but nowadays we know that one receptor could be prioritized over the other and this is not always correlated to the expression level of the respective pro-apoptotic receptor. Both receptors share only 46-48 % of sequence homology. There are two different splice variants for DR5 receptor which differ in 29 amino acids located between the cysteine rich domain (CRD) and the

transmembrane domain, but there is no evidence of different functionality between these two variants (Wang and Jeng, 2000). In Ewing´s sarcoma cells was identified also a short DR4 isoform, in which the alternative splice variant lacks 158 amino acids within the extracellular-ligand binding region (CRD domain) and the overexpression of this protein increased efficacy of TRAIL-induced apoptosis in resistant cells by yet unknown mechanism (Picarda et al., 2012; van Roosmalen et al., 2014). Notably, humans have two different pro-apoptotic TRAIL receptors, whereas mice have only one death inducing receptor, which is equally homologous to DR4 (43% of sequence homology) as well as to DR5 (49% of sequence homology) (Kelley et al., 2004; Kelley et al., 2005).

Many factors could imply differences in signalization of DR4 and DR5 receptor. As mentioned above the presence of two death-inducing TRAIL receptors in human cells implicates an evolutionary advantage and low sequence similarity (~50%) suggests structural, functional and regulating differences. Moreover certain types of tumours could have preference in signalling for either of the DR receptors. This apparent preference could be the result of more regulation steps, mostly at the level of transcription, in addition affected by membrane DR4/5 localization, externalisation, post-translational modifications or the endocytosis of DISC upon TRAIL binding (Karstedt et al., 2017). Interestingly, epigenetic silencing of DR4 in melanoma, glioma and ovarian cancer cells caused TRAIL resistance in these tumour cells (Horak et al., 2005; Bae et al., 2008; Elias et al., 2009). Transcription factors as p53, NFκB, CHOP, FOXO3a and AP1, which are correlated to stress-induced apoptosis, can also regulate the expression of both TRAIL receptors (Baritaki et al., 2008; Kurita et al., 2010; Moon et al., 2010; van Roosmalen et al., 2014). Very interesting recent finding marks DR5 as a target of the UPR transcription factor CHOP and one of the pro-apoptotic proteins involved in ER stress-induced apoptosis (Lu et al., 2014). Even though the total protein level of TRAIL receptors can have an impact on TRAIL signalling, it is the cell surface expression which is required for their proper physiological function. The intracellular trafficking mechanisms play also an important role and for example downregulation of components of the signal recognition particle SRP72 and SRP 54 or downregulation of ARAP1 was correlated to the reduction of cell surface levels of DR4 receptor and attenuated TRAIL-triggered apoptosis (Ren et al. 2004; Simova et al., 2008). Moreover, activation of death receptors inhibits clathrin-dependent endocytosis (Austin et al., 2006). Post-translational modifications can also have a significant impact on the outcome of TRAIL-induced signalling. S-palmytoylation of

DR4 on the cysteine triplet (AA 261-263) near transmembrane domain or S-nitrosylation, the covalent coupling of nitrogen monoxide group to a reactive cysteine thiol in DR4, were shown to promote DR4-mediated apoptotic signalling (Tang et al., 2006; Rossin et al., 2009). Similarly O-glycosylation of the extracellular parts of DR4 and DR5 attenuated TRAIL resistance of tumour cells, as also did N-glycosylation in DR4 (Wagner et al., 2007; Yoshida et al., 2007). Another important modification ubiquitination by MARCH-1/8 was found to target DR4 for lysosomal degradation thus regulating its cell surface levels (van de Kooij et al., 2013).

1.2.3 Canonical signalling induced by ligand TRAIL

Upon binding of TRAIL ligand to its receptors, TRAIL induces formation of two signalling complexes: the initial membrane associated complex I or DISC (death inducing signalling complex) and the later, receptor-free cytosolic complex II. Unlike TNFα ligand both of these complexes can induce cell death (Lafont et al., 2017). Notably TRAIL-induced cell death does not include only caspase-dependent apoptosis, but also RIPK1/3-dependent necroptosis. However, when TRAIL-mediated death signalling is suppressed, the activation of auxiliary signalling pathways such as NFkB or MAP kinases can enhance tumour progression through boosting cell migration, proliferation, invasion and production of tumour-supportive cytokines (von Karstedt et al., 2017).

1.2.3.1 Apoptosis

Binding of trimeric TRAIL to its trimeric pro-death receptors DR4 or DR5 induces their clustering and higher oligomerization in the cytoplasmic membrane. The concomitant conformational changes in their intracellular part leads to the recruitment of adaptor protein FADD via the DD homotypic interaction. FADD subsequently recruits the other components of DISC. Binding of FADD to the DISC complex exposes its DED domain for the interaction with DED domains of caspase-8/10 and cFLIP (Lafont et al., 2017; von Karstedt et al., 2017). There are several hypothesis how is the caspase-8 activated at the DISC level but nowadays is mainly accepted caspase-proximity cleavage model. In this model, the molecules of caspase-8 are activated by proximity-induced conformational change and via their DED interactions form clustered chains containing up to eight molecules of pro-caspase-8 per one molecule of FADD. In these chains the activated caspases process themselves initially cleaving of the small p10 subunits

forming p43/p10 dimers. These dimers are already highly active and can further process themselves or other associated proteins such as cFLIP-L or pro-caspase-10. In the final auto-processing step the p18 subunit is cleaved off the p43 peptide and fully active (p18/p10)2 heterotetramer of caspase-8 is released to cytoplasm, where it can cleave other target proteins such as Bid. (Dickens et al., 2012; Fu et al., 2016). Both caspases, caspase-8 and caspase-10 are recruited to the DISC but caspase-10 itself cannot substitute for caspase-8, which is thus essential for inducing of the death receptor-triggered apoptosis (Sprick et al., 2002). cFLIP acts mainly as an inhibitor of apoptosis, though under certain circumstances it can enhance caspase-8 processing (for more information see chapter 1.1.1.3 Negative regulation of apoptosis by IAP proteins and c-FLIP). Recent data suggest a very important role of post-translational modification of caspase-8 such as ubiquitination in the regulation of caspase-8 activity. It was shown that E3 ligase Cullin-3 facilitates to caspase-8 aggregation by non-degradative ubiquitination of caspase-8, leading to enhanced clustering and stabilisation of caspase-8 molecules. In contrast, E3 ligase TRAF2-mediated K48-linked ubiquitination of p18 subunit of active caspase-8 leads to rapid proteasomal degradation of caspase-8 (Jin et al., 2009; Gonzalvez et al., 2012).

TRAIL or FasL-induced apoptosis of permissive cells can be classified into two categories depending on the level of death receptor activation and on certain cellular factors. In so-called type-I cells such as activated T cells massive activation of caspase-8 and absence of caspase inhibitors is sufficient for strong and steady activation of effector caspases and rapid caspase-dependent apoptosis. However, majority of permissive cells (type II cells) does not fulfil these requirements due to low levels of activated caspase-8 and presence of caspase inhibitors such as XIAP and thus a successful progression of apoptosis requires mitochondrial amplification of apoptotic signalling. This amplification is orchestrated by caspase-8-mediated cleavage of BH3 only protein Bid. Cleaved Bid or truncated Bid (tBid) is N-myristoylated and rapidly translocates to mitochondria where it blocks anti-apoptotic Bcl-2 family proteins and also participates in activation of the pro-apoptotic Bax and Bak, which subsequently cause the permeabilisation of the outer mitochondrial membrane and mitochondrial apoptotic signalling as described in part 1.1.1 Apoptosis. Thus the critical factors for the distinction between these two types of cells is their efficiency in DISC formation and caspase-8 activation and also the cellular levels of the caspase-3/9 inhibitor XIAP (Korsmeyer et al., 2000; Jost et al., 2009; Lemke et al., 2014).

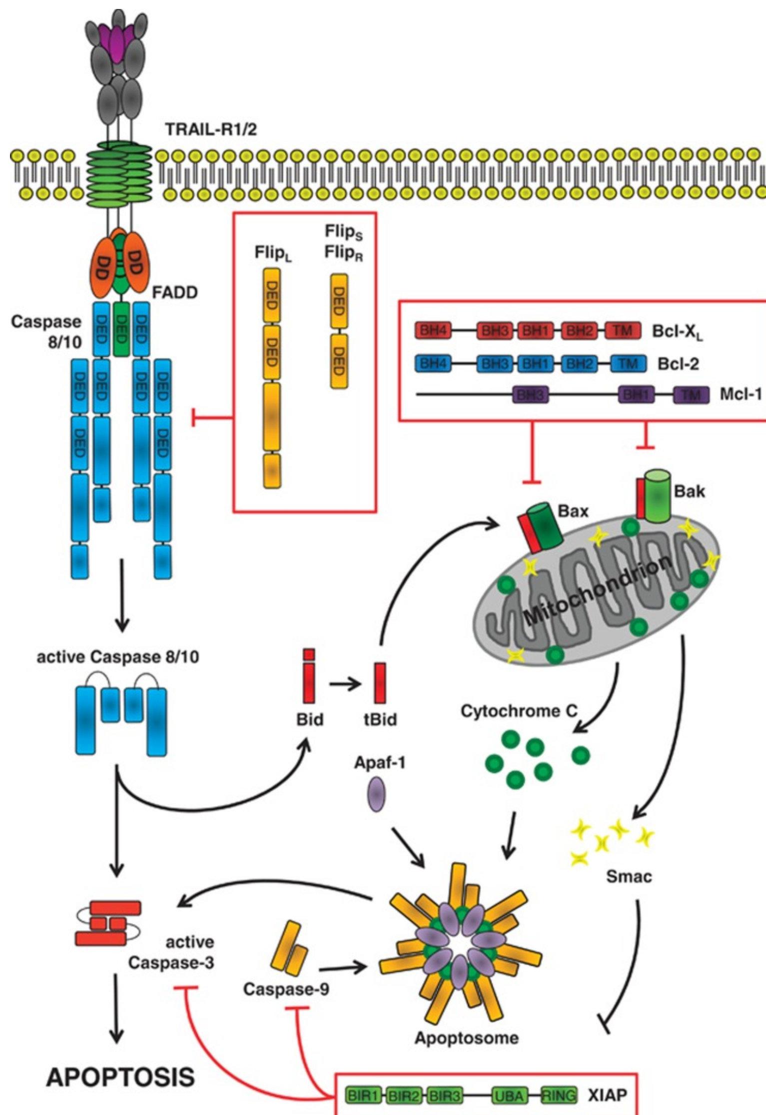


Figure 1.9: TRAIL induced apoptosis in type I and type II cells. In type I cell lines is DISC-activated caspase-8 capable to fully induce apoptosis through direct processing of the effector caspases such as caspase-3. In type II cells, activation of mitochondrial apoptotic signalling mediated by caspase-8-cleaved tBid enhances Bax/Bak-mediated MOMP and subsequent apoptotic signalling. TRAIL-induced apoptosis can be inhibited: (i) at the level of DISC, where cFLIP competes for binding of FADD with caspase-8, (ii) at the level of mitochondria, where anti-apoptotic Bcl-2 proteins can suppress Bax/Bak activation and (iii) at the level of caspases, where XIAP can directly bind and inhibit caspases-3 and -9. Adapted from Lemke et al., 2014.

1.2.4 Non-canonical TRAIL-induced signalling

1.2.4.1 Necroptosis

Necroptosis represents the most general mode of regulated necrosis, which is apparently involved in many pathophysiological processes and emerged as an alternative approach for elimination of cancer cells. TRAIL as one of the still promising anti-cancer agents can in addition to caspase-dependent apoptosis similarly as TNFα also induce necroptosis. TRAIL induced necroptosis is capable to reduce amount of cancer cells, but there are only a few studies concerning the TRAIL-mediated necroptosis comparing to for example TNFα ligand which has been investigated in detail a lot (Philipp et al., 2015). TRAIL-induced necroptosis can be activated under caspase-compromised conditions and enhanced by more stimuli, always by synergistic stimulation of TRAIL ligand with other factors for example acidic extracellular milieu or by blocking protein synthesis by e.g. homoharringtonine or by sirtinol-mediated blocking of histone deacetylation (Jouan-Lanhouet et al., 2012; Pal et al., 2013; Sosna et al., 2016). The basic mechanism of necroptosis proceeds similarly to TNFα-induced necroptosis (see chapter 1.1.2 Necroptosis). The necroptotic signalling triggered by TRAIL is also dependent on RIPK1 and RIPK3 kinases and phosphorylated MLKL-mediated permeabilisation of the cytoplasmic membrane (Jouan-Lanhouet et al., 2012). TRAIL-induced necroptosis is apparently negatively regulated by E3 ligase TRAF2, which in the complex with cIAP1/2 targets RIPK1 for proteosomal degradation (Karl et al., 2014). Interestingly, TRAF2 also suppresses TRAIL-induced apoptosis through targeting p18 subunit of activated caspase-8 for degradation. Apparently PARP-1 might also play a role in TRAIL-induced necroptosis. Jouan-Lanhouet and his colleagues showed that in acidic environment TRAIL-induced necroptosis also involved RIPK1/RIPK3-dependent PARP-1 activation and depletion of ATP (Jouan-Lanhouet et al., 2012). However, PARP-1 participation in TRAIL-induced necroptosis might be just auxilliary as its inhibition or genetic ablation in embryonic MEF cells did not prevent TRAIL-mediated necroptosis (Sosna et al., 2016).

1.2.4.2 Other non-canonical TRAIL-induced signalling pathways

Whereas the induction of apoptosis is a default TRAIL-induced signalling in permissive cells, TRAIL can similarly to other members of TNF ligand family promote cell survival, proliferation, migration, cytokine secretion and immunomodulation and even invasion and metastasis (Karstedt et al., 2015; Karstedt et al., 2017). These non-canonical signalling pathways include activation of canonical NFκB signalling, MAPK kinases (p38, ERK1/2 and JNK) as well as activation of other kinases such as AKT, PKC or TAK-1. These non-canonical or auxiliary signalling pathways mainly negatively affect TRAIL-induced apoptosis and can also promote survival and migration of cancer cells as was recently documented for non-small cell lung cancer cells containing *KRAS* mutation, where the endogenous TRAIL induced cell migration by activating PI3K pathway. Interestingly these TRAIL-triggered pro-invasive behaviour was independent of the DR5 death domain but required the membrane proximal domain (MPD) (deMiguel et al., 2016; Lafont et al., 2017).

The activation of NFκB pathway by TRAIL proceeds very similarly as for TNFα/TNFR1 signalling (Fig. 1.10). NFκB could be activated by TRAIL binding not only to DR4 or DR5 receptor, but additionally also to DcR1 receptor with the truncated intracellular domain (Degli-Esposti et al., 1997; Schneider et al., 1997). TRAIL-induced activation of non-canonical signalling mainly proceeds through the cytosolic complex II, but according to recently published data the membrane-associated complex I is at least partly involved in this signalling (Lalaoui et al., 2011). The core components, presented in both complexes, are among others, FADD, caspase-8, RIPK1 and IKK complex. The essential players of non-cell death signalling are FADD, adaptor protein, which forms a scaffold for the formation of both complexes and caspase-8, which is involved in TRAIL-dependent gene activation and cytokine production (Tang et al., 2003; Kreuz et al., 2004; Varfolomeev et al., 2005; Henry et al., 2017). NFκB pathway is strongly activated in cancer cells resistant to TRAIL-induced apoptosis and can lead to their enhanced proliferation and migration/invasion thus promoting tumorigenesis (Hartwig et al., 2017). Interestingly it was found out that NFκB could mediate the upregulation of TRAIL receptors and recently it was published that in glioma cell lines, the inhibition of NFκB signalling attenuated their TRAIL-induced apoptosis (Liu et al. 2017). Likewise in TNFR1, ubiquitination by E3 ligases as cIAP1/2, HOIP or

LUBAC and de-ubiquitination by DUBs A20 or CYLD are very important regulatory events of TRAIL-induced activation of NFκB pathway (Fig. 1.10; Lafont et al., 2018).

The formation of mainly secondary cellular signalling complex II could in addition to NFkB TRAIL-induced non-canonical signalling lead to activation of MAPK kinases of ERK, JNK and p38 family. These kinases participate in such processes as inflammation, cell proliferation, apoptosis and differentiation. ERK is mostly associated with proliferation and growth factors. TRAIL-induced ERK activation is connected with its anti-apoptotic impact and in several reports was documented that the activation of ERK kinases can inhibit the processing of caspase-8 and Bid or in human melanoma cell lines can inhibit release of Smac/DIABLO from mitochondria. Suppressing activity of ERK kinases by specific inhibitors enhanced apoptosis of human breast cancer cells (Tran et al., 2001; Soderstrom et al., 2002; Zhang et al., 2003; Lee et al., 2006).

On the contrary, p38 and JNK are kinases triggered by stress factors and cytokines and can mediate differentiation and cell death. P38 kinase was shown to either enhance or supress cell death. It was published in HeLa cells that TRAIL-induced ROS production mediated activating of p38 signalling and subsequent cell death. Activity of p38 was essential for the proceeding of apoptosis, however other types of cancer as prostate cancer or breast carcinoma cells were shown to be sensitized to TRAIL induced apoptosis after chemical inhibition of p38 (Azijli et al., 2013).

JNK kinases are in mammals encoded by three genes: JNK1, JNK2 and JNK3 with several splice forms JNKs are activated as classical MAPK kinase in three step cooperative phosphorylation by MAPK3/MAPK2/MAP kinase. These stress kinases are involved in several cellular processes as proliferation, invasive migration, drug resistance and programmed cell death. In addition to apoptosis, JNKs were also shown to have a role in regulating of other types of programmed cell death such as necroptosis, ferroptosis, pyroptosis and autophagy like cell death (Tait et al., 2014; Abe et al., 2016; Xie et al., 2016; Dhanasekaran and Reddy, 2017). JNKs could be activated by death receptors as TNFR1, FasR or TRAIL receptors or by intrinsic stress signals via mitochondria (Dhanasekaran and Reddy, 2017). The phosphorylated JNK translocates to the nucleus where it by phosphorylation of the transcription factor c-Jun triggers formation of heterodimeric AP-1 transcription factor (Holler et al., 2000). In the pro-apoptotic branch of its activity, AP-1 is involved in the expression of several pro-apoptotic genes: TNF-α, Fas-L, TRAIL-R2, Bak, Bax and Bim (Chen et al., 2010). JNKs can also phosphorylate other transcription factors

including JunD, ATF2/3, c-Myc or the most important p53 and p73, enhancing their stabilisation. Activated p53 itself enhances transcription of a number of pro-apoptotic genes including Bcl-2 family memberes Bax, Puma and Noxa, death receptors FasR and DR5 and others (Davis et al., 2000; Dhanasekaran and Reddy, 2017). Modulation of the intrinsic apoptosis by JNKs relies in addition to the increased expression of pro-apoptotic Bcl-2 family proteins also on their JNKs-mediated phosphorylation that affects their function and/or localization. Bax translocation to the mitochondria membrane and subsequent MOMP is increased when JNK phosphorylates Bax cytoplasmic anchor protein (Tsuruta et al., 2004). Bcl-2 family member Bim is phosphorylated in hepatocytes upon TRAIL treatment and this phosphorylation leads to the stabilisation of the protein and helps on its pro-apoptotic activity (Corazza et al., 2006). There is an evidence that JNK can contribute to promoting of apoptosis by direct phosphorylation of anti-apoptotic protein Bcl-2, which suppresses its anti-apoptotic function (Yamamoto et al., 1999). Interestingly, activated JNK kinase could in addition to pro-apoptotic induce also anti-apoptotic signalling. Using RNA interference or specific JNK inhibitors in hepatocellular carcinoma cells surprisingly led to TRAIL-induced apoptosis (Mucha et al., 2009). There might be multiple reasons for this apparently anti-apoptotic effect of JNKs. One of them could be the kinetics of JNKs activation, as it was shown for TNFα-induced signalling where longer activation induced cell death whereas transient activation led to the cell survival. Another reason might be different expression of JNK1 splice variants as the activation of short isoform JNK1 is anti-apoptotic but its long isoforms enhances apoptosis in colorectal cancer cell lines (Ventura et al., 2006; Mahalingam et al., 2009).

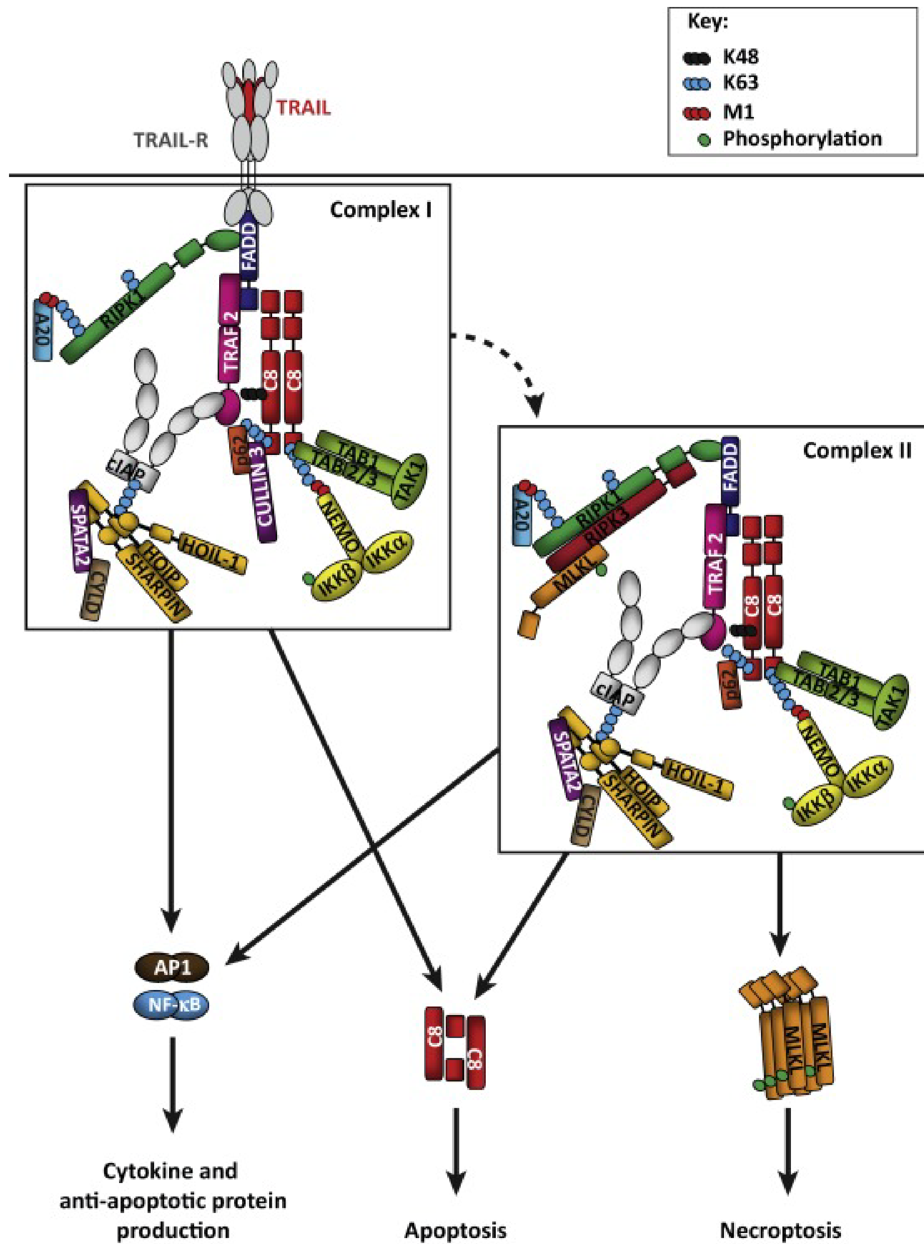


Figure 1.10: TRAIL induced signalling and its regulation. TRAIL binding to its pro-death receptors induces formation of the membrane-bound complex I with a subsequent formation of the cytosolic complex II. Both of these complexes can play a role in the activation of cell death signalling and/or pro-survival pathways. Multiple ubiquitination events play an important role in functional regulation of these complexes. Adapted from Lafont et al., 2018.

2 AIMS OF THE WORK

The main aim of this study was to expand the knowledge in the field of TRAIL-induced signalling and regulated cell death, especially in the context of cancer treatment. This general aim, could be diversified into following goals:

1. To answer the question whether there is a similar or distinct regulation of two pro-apoptotic TRAIL receptors DR4 and DR5 by analysing in detail the canonical and non-canonical signalling from our modified and enhanced TRAIL receptor-specific ligands in a model of colorectal and pancreatic cancer cells.
2. To analyse TRAIL induced signalling in a model of human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC).
3. To analyse the sensitizing effect of Manumycin A itself or in combination with other sensitizers, mainly inhibitors of Bcl-2 proteins in TRAIL resistant colorectal cancer cell lines.

3 MATERIALS AND METHODS

3.1 Bacterial strains, growth media and chemicals

- **TOP10** F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
- **Stbl3** F^-*mcr*B *mrrhsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 λ^-*leumtl*-1
- **BL21-AI™** F^-*omp*T *hsd*S_B (r_B^- m_B^-) *gal dcm ara*B::T7RNAP-*tet*A

All bacterial strains were purchased from Invitrogen (Thermo Fisher). Escherichia coli strains TOP-10 and Stbl-3 were used for preparation of plasmid constructs and BL21-AI™ strain was used for the recombinant protein expression.

E.coli strains were grown in LB medium (LB Broth Miller, Amresco) with ampicillin (100 ug/ml) (preparation of plasmids and recombinant proteins) or on LB agar plates with ampicillin (100 ug/ml) (transformation and sub-cloning). All other further unspecified chemicals were purchased from Sigma-Aldrich.

3.2 DNA constructs and methodological approach used for their preparation

- **Restriction digestion and ligation**

All restriction endonucleases were purchased from Fermentas (Thermo Fisher) and used according to the manufacturer protocol. Upon cleavage, the relevant DNA fragments were separated by agarose electrophoresis and purified from agarose gels by Zymoclean™ Gel DNA Recovery (Zymo Research). The subsequent ligation of digested vector and insert was performed using T4 DNA ligase and ½ of the ligation reaction was used for the transformation of bacteria TOP10 and Stbl3.

- **Polymerase chain reaction- PCR and DNA sequencing**

Amplification of a target gene was carried out by Phusion High Fidelity DNA Polymerase (Finnzymes, now Thermo Fisher) and the resulted PCR product was cleaved with relevant enzyme(s) and cloned into a desired vector. For colony PCR screening was used DreamTaq Green

PCR Master Mix (2X) (Thermo Fisher). The set-up of both PCR reactions (Phusion and DreamTaq) followed their manufacturer's instructions. For the verification of the amplified insert sequence were all PCR products cloned in relevant plasmids sequenced (SeqMe company) and the expression vectors without any mutation or deletion were used in further experiments.

- **TRAIL ligand variants and reagents**

We prepared several forms of recombinant ligands containing in addition to the common bacteriophage trimerisation (TRI) motif and the sequence coding for extracellular part of human TRAIL (amino acids 95-281) also the N-terminal either double- Twin Strep-tag (TST) or the His6 tag. Both TST-TRI cassettes and receptor-specific TRAIL ligand variants were synthetized (GeneScript) and sub-cloned into modified pBSKII vector containing T7 promoter and an upstream sequence from the pET15b plasmid. TRAIL receptor-specific mutants were: DR4.02 (G131R/R149I/S159R/N199R/K201H/S215D, 4C7 mutant; Reis et al., 2010), DR5.01 (E195R/D269H; Van der Sloot et al., 2006) and mutant DR5.02 (Y189N/R191K/E195R/H264R/I266L/D269H; Gasparian et al., 2009). Using this procedure we obtained several constructs:

1. **pBSKII-TST-TRI-TRAIL WT**
2. **pBSKII-TST-TRI-TRAIL 4.2**
3. **pBSKII-TST-TRI-TRAIL 5.1**
4. **pBSKII-TST-TRI-TRAIL 5.2**

His-tagged ligands were prepared using similar approach. All previous Strep-tagged constructs were digested and Twin-Strep-tag was cut out. Then His tag was inserted in frame with trimerisation motif. We obtained following constructs:

1. **pBSKII -His-TRI-TRAIL WT**
2. **pBSKII-His-TRI-TRAIL 4.2**
3. **pBSKII-His-TRI-TRAIL 5.1**
4. **pBSKII-His-TRI-TRAIL 5.2**

- **The list of vectors, primers and shRNA used in experimental work**

Vectors:

- **pUC57-TST-TRI**

The original plasmid ordered from GeneScript company, containing Twin-Strep-tag in-frame with bacteriophage trimerisation (TRI) motif.

- **pBSKII + human TRAIL wild type**
- **pUC57-DR4.2**

The original plasmid with the sequence for mutant TRAIL ligand specific to DR4 receptor. Ordered from GeneScript.

- **pUC57-DR5.1**

The original plasmid with the sequence for mutant TRAIL ligand specific to DR5 receptor. Ordered from GeneScript

- **pUC57-DR5.2**

The original plasmid with the sequence for mutant TRAIL ligand specific to DR5 receptor. Ordered from GeneScript.

- **modified pBSKII+ containing T7 promoter and upstream region from pET15b**

Used for expression of TRAIL constructs in bacteria.

- **psPAX2 (Addgene, #12260)**

Lentiviral packaging plasmid, used for the producing of lentiviruses in HEK293T/17 cells.

- **pMD2.G (Addgene, #12259)**

Lentiviral packaging plasmid, used for the producing of lentiviruses in HEK293T/17 cells.

- **pLKO empty (Addgene, #8453)**

Used for expression of shRNA of target gene in mammalian cells. Serves also as a control for non-specific effect of DNA construct in the cells.

- **pLKO non-targeting shRNA control (#109012)**

Used as a control to pLKO with target shRNA.

shRNA:

- shDR4_#1 TRCN0000005934
- shDR4_#2 TRCN0000005935
- shDR5_#1 TRCN0000005929
- shDR5_#4 TRCN0000005932

- shDR5_#5 TRCN0000005933
- shFLIP_#1 TRCN0000007229
- shFLIP_#2 TRCN0000007230
- shMcl-1_#1 TRCN0000005514
- shMcl-1_#2 TRCN0000005515
- shMcl-1_#3 TRCN0000005516
- shMcl-1_#4 TRCN0000005517
- shMcl-1_#5 TRCN0000005518
- shMcl-1_#6 TRCN0000005519

Primers:

- **Strep-TRI-TRAIL-U (5'Xho)** CGCTCGAGACCTCTGAGGAAACCATTTC
- **StrepTRI-TRAIL-L (3'BamHI)** CGGGATCCTTAGCCAACTAAAAAGGCCC

- **Transformation of chemically competent *E.coli* strain**

50 ul of chemically competent TOP10 or Stbl-3 bacteria strain were transformed by 5 ul of the ligation reaction or 0.5 ug of plasmid DNA. After 30 min of incubation on ice, bacterial suspension was transferred to 42 °C (1 min) to induce heat-shock. Then LB medium (500 ul) was added and bacteria were cultivated at 37 °C, 250 rpm for 30 min. After pelleting (15 s, 13000 rcf) were bacteria ressuspended in 100 ul of LB and seeded onto agar plates containing proper selection antibiotic.

- **Plasmid preparations using alkaline lysis and column-based systems**

For the plasmid alkaline lysis minipreps, the bacterial cultures (1 ml) grown for 12-15 hrs, 250 rpm at 37°C were centrifuged (15-30 s, 13000 rcf) and bacterial pellets were resuspended in 100 ul of the Sol I (50 mM glucose, 25 mM Tris.HCl pH 8.0, 10 mM EDTA pH 8.0). The suspensions were lysed with 200ul of the Sol II (0.2 M NaOH, 1% SDS) and then neutralized using 150 ul of Sol III (5 M sodium acetate, 12 % acetic acid). Heterogenic suspensions containing bacterial chromosomal DNA, lipids and proteins were pelleted 10 min, 13000 rcf. Nucleic acids (plasmid DNA and bacterial RNA) from the supernatants were purified at first by 1 ml of 100 % ethanol

and then 0.5 ml of 70 % ethanol. Plasmid DNAs were diluted in TE buffer (10 mM Tris-HCl pH 8.5, 0.1 mM EDTA) with RNAase (10 ug/ml) and stored at -20°C.

Plasmid miniprep using Zyppy Plasmid Miniprep Kit (Zymo Research) was performed according to the manufacturer protocol. DNA was diluted in TE buffer or in water and stored at -20°C. For isolating of bigger amount of DNA we used Geneaid Plasmid Midi Kit (Geneaid Biotech). Plasmid DNA was isolated according to the manufacturer protocol. DNA was diluted in TE buffer or in water and stored at -20°C.

3.3 Expression of recombinant proteins in E.coli and purification methods

For the expression of recombinant TRAIL ligands was used bacterial strain BL21-AI *E.coli* (Thermo Fisher), which contains a chromosomal insertion of the gene encoding T7 RNA polymerase into *ara*B locus of the *ar*aBAD operon, placing regulation of T7 RNA polymerase gene under the control of *ara*BAD promoter. Expression of mRNA from araBAD operon was regulated by use of L-arabinose in the mid-log phase of growth of bacterial culture.

Transformed BL21-AI *E.coli* bacteria with recombinant expression plasmid were inoculated to 50 ml of LB media with ampicillin (100ng/ml) and culture was grown O/N at 37 °C, 250 rpm. This starting culture was used for the inoculation of 1l of LB media with ampicillin (100ng/ml) and cells were grown till they reached the early-mid log phase of cell growth (OD_{600} = 0.5) at 37 °C, 250 rpm. The culture was then cooled down to 25°C and for the induction of TRAIL expression was used 0.2%w/v L-arabinose. The culture was grown other 4-6 hrs at 25°C, 200 rpm, then centrifuged and pellets were further lysed and purified.

- **Purification protocol for Twin Strep-tagged TRAIL ligands**

Lysis buffer:	**Washing buffer:**
20mM Hepes.Na, pH 7.9	100mM Tris-HCl pH 8.0
300mM NaCl	150 mM NaCl
0.1% Tween-20	1 mM EDTA
5% glycerol	0.1% Tween-20
1x Complete protease inihibitor (Roche)	5% glycerol
1mM mercaptoethanol	1mM mercaptoethanol

Elution buffer:

100mM Tris-HCl pH 8.0

150 mM NaCl

1 mM EDTA

2mM biotin (Sigma)

1x Complete protease inihibitor (Roche)

1mM mercaptoethanol

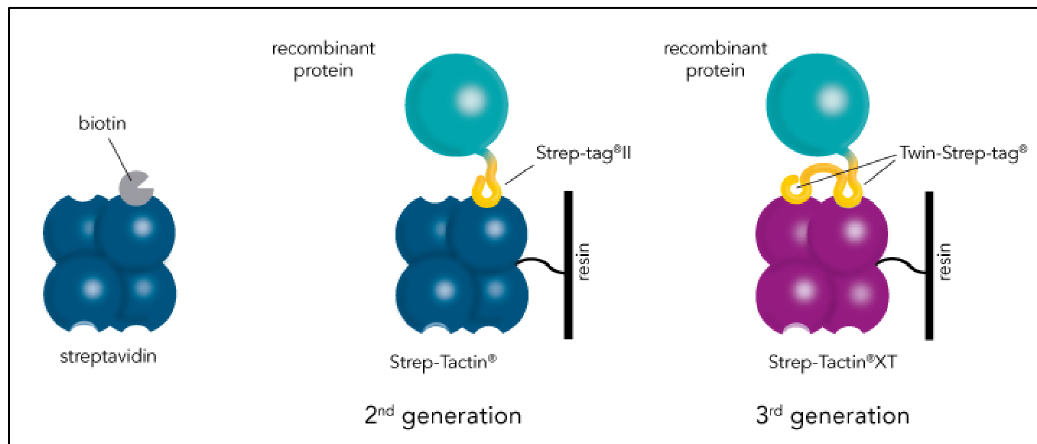


Figure 3.1: Scheme of Strep-tag and Twin Strep-tag purification system (IBA life sciences). System is based on the interaction of biotin with streptavidin, where recombinant peptide analogue of biotin (Strep-tag) has very high binding affinity to Streptactin (analogue of streptavidin). For purification of protein complexes Twin-Strep tag (TST) with two Strep-tags in row was used.

Bacterial cells were resuspended in the lysis buffer and then sonicated using 5 pulses each for 10 seconds (Ultrasonic homogenizer, Cole-Parmer). Sonicated lysate was centrifuged by ultracentrifugation (80,000x g, 4°C, 1h, Beckman Coulter) and supernatant was loaded on 5ml Gravity flow Strep-Tactin Sepharose column (IBA life sciences, Fig. 3.1). Purification of Twin-Strep tagged TRAIL ligands was performed according to the general protocol for Strep-tag recombinant protein purification (Schmidt and Skerra, 2007) and manufacturer instructions (IBA life sciences). Briefly, the matrix (Streptactin Sepharose) with bound TST-TRI-TRAIL was 5x times washed with 1 CV (column bed volume) of the washing buffer. Recombinant TRAIL was eluted in several fractions (6-10) with 0.5 CV of the elution buffer containing biotin. Each washing and elution fraction was collected and 5ul was taken aside for SDS-PAGE electrophoresis and Coomassie blue staining (more in part 3.8 SDS-PAGE electrophoresis and 3.9 CBB-R250 staining). Concentration of the purified proteins was determined using the extinction coefficient and Nanodrop. Aliquotes were diluted approx. to 1 mg/ml and stored at -80°C.

- **Purification protocol for His-tagged TRAIL ligands**

Washing buffer 1:
20 mM Hepes.Na, pH 7.9
300 mM NaCl
0.05% Tween-20
2% glycerol
1mM mercaptoethanol

Washing buffer 2:
20 mM Hepes.Na, pH 7.9
300 mM NaCl
0.05% Tween-20
2% glycerol
1mM mercaptoethanol
5mM imidazole

Elution buffer
20 mM Hepes.Na, pH 7.9
300 mM NaCl
0.05% Tween-20
2% glycerol
1mM mercaptoethanol
100mM imidazole

Dialysis buffer:
20 mM Hepes.Na, pH 7.4
50 mM NaCl
0.01% Tween-20
10 g sucrose
38 mg DTT

His-tagged proteins were purified in a similar way as Strep-tagged. Purification was performed according to the general instructions of manufacturer (Clontech). Supernatant of sonicated and centrifuged bacterial lysate was mixed with equilibrated TALON resin (Clontech) and gently rotated at 4°C for 30 minutes. Then the resin was washed twice with the washing buffer w/o and w/ low concentration of imidazole. The slurry of resin was resuspended in washing buffer 2, poured into TALON column (Clontech) and washed with 10 CV of washing buffer 2. Proteins were eluted with 5 CV of elution buffer and protein content in 0.5 ml fractions (0.5 ml/6-10 fractions) was quantified by Bradford method (Bradford, 1976). Only fractions with highest protein concentration were collected and used in further purifications steps. For removing imidazole and other salts, the pooled fractions were dialyzed O/N against 1l of dialysis buffer in pre-treated dialysis tubing (cut-off 10K) and analysed by SDS-PAGE electrophoresis and Coomassie blue staining (more in part 3.8 SDS-PAGE electrophoresis and 3.9 Coomassie blue staining). Concentration of the purified proteins was determined using the extinction coefficient and Nanodrop photometer, aliquotes were diluted approx. to 1 mg/ml and stored in -80°C.

3.4 Manipulation with mammalian cell lines

Human cell lines and media:

- **HT-29-** human colorectal adenocarcinoma cell line (HTB-38)
- **PANC-1-** human pancreatic adenocarcinoma cell line (CRL-1469)
- **RKO-** human colorectal adenocarcinoma cell line (CRL-2577)
- **SW620-** human colorectal adenocarcinoma cell line (CCL-227)
- **HEK 293T/17-** human kidney epithelial cells (CRL-11268)
- **Ramos-** B-cell lymphoma cell line (CRL-1596)
- **Jurkat-** T-cell leukemia cell line (TIB-152)
- **hESC (CCTL 12 and CCTL 14)-** human embryonic cells (Dolezalova et al., 2012)
- **hiPSC-** human induced pluripotent stem cells (Armstrong et al., 2010)

Human colorectal adenocarcinoma cell lines HT-29, RKO and SW-620, human pancreatic adenocarcinoma cell lines PANC-1 and human kidney epithelial cells HEK 293T/17 were obtained from the ATCC. B-cell lymphoma cell line Ramos and the acute T-cell leukemia cell line Jurkat were kindly provided by Vaclav Horejsi (Simova et al., 2008) and were originally also obtained from ATCC.

For the experiments in part Results 4.2 were used hESC lines (CCTL 12 and CCTL 14) between passages 25-80 and hiPSC cell line (clone 4) between passages 50-80. Colonies of both cell lines were co-cultivated with mitotically inactivated mouse embryonic fibroblasts (MEFs) as described in Barta et al., 2010 or grown on extracellular MEF-derived matrix (ECM). Cultivation on ECM required MEF-conditioned hESC medium which was prepared as described in Vinarsky et al., 2013.

All unspecified media and cell culture additives were purchased from Sigma. Cells were cultured in DMEM (HT-29, PANC-1, RKO, SW-620, HEK 293T/17) or in RPMI (Jurkat, Ramos) supplemented with v/v 10% FCS (Life Technologies), L-glutamine and antibiotics. In the case of PANC-1, non-essential amino acids (Life Technologies) were added to the medium. Cells were kept in a humidified incubator at 20% oxygen, 5% CO_2 and 37°C.

3.5 Preparation of recombinant lentiviruses by calcium phosphate transfection and lenti-viral transduction of target cells

For the production of recombinant lentiviruses we used HEK 293T/17 cells, which constitutively express the simian virus 40 large T antigen and are easily transfectable with plasmid DNA. Day before transfection HEK293/T17 cells were seeded at 20-30% of confluency and 1 hr before the transfection, the cultivation medium was changed for a fresh one in addition containing 25µM chloroquine (inhibitor of lysosomal degradation of plasmid DNA).

Master mix containing 500 ul of 0.25M calcium chloride and two packaging plasmids psPAX2 (Addgene, #12260) and pMD2.G (Addgene, #12259) altogether with our target plasmid (e.g. pLKO1 expressing specific shRNA) was pipetted into eppendorf tubes and then 500 ul of 2xBBS solution pH 6.9 (50mM BES, 280mM NaCl, 1.5mM Na_2HPO_4) was added in a dropwise manner. After 15 minutes was mastermix pipetted on the surface of petri dish. Cells were incubated in a special conditions with 3% of CO_2 and 37°C. After 5-6 hrs were crystals of calcium salt washed away with PBS and fresh media was added. The medium containing lentiviral particles was harvested 36 to 48 hrs post-transfection, and the viral particules were precipitated using PEG-it (System Biosciences) according to manufacturer protocol. Target cells were transduced with viruses at multiplicity of infection MOI 5-10 and selected for puromycine resistance (2 µg/ml, Invivogen).

3.6 Affinity purification of protein complex DISC

DISC lysis buffer:

20 mM Tris-HCl, pH 7.5

150 mM NaCl

1% Tritone X-100

10% glycerol

1x Complete protease inhibitor (Roche)

1 mM EDTA

1mM sodium orthovanadate

10mM NaF

Cells grown to approximately 80% confluence were rapidly cooled by the media exchange for an ice-cold one and then incubated on ice for 20 min (to prevent internalization of the receptors). Twin-Strep-tag labelled TRAIL ligand variants (TST-TRI-TRAIL) were added to the concentration of 1 µg/ml for 15 min, then the cold medium containing TRAIL was replaced with the 37°C warm one without TRAIL, and the cells were incubated at 37°C for specified time periods. At selected time points, the cells were washed with ice-cold PBS and the cell pellets were lysed in ice-cold DISC lysis buffer supplemented with inhibitors of proteases (Complete, Roche) and phosphatases (10 mM NaF and 1 mM Na_3VO_4). Lysates were adjusted to the same protein concentration and the same amount of protein (2 mg) was incubated/gently rotated with Streptactin beads (IBA) overnight at 4°C and then washed 3-4 times with 1 volume of ice-cold lysis buffer. The proteins bound to Streptactin beads were directly eluted with 2 x Laemmli sample buffer (95°C, 5 min) and analysed by Western blotting.

3.7 Preparation of protein lysates

Cells were lysed using SDS-based lysis buffer (2x Laemmli buffer: 50mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS) and samples were subjected to SDS-PAGE.

Sample buffer 2x:
50mM Tris-HCl pH 6.8
20% glycerol
4% SDS
200mM DTT
0.05% w/v Bromphenol blue

Cells were washed twice with cold PBS. Then lysis buffer was added, cells were harvested by scraping and incubated at room temperature, sonicated (4 x 10s at 4 micron amplitude with 15 s cooling intervals, Ultrasonic homogenizer, Cole-Parmer) and centrifuged (10 min, 13 000 rcf, 4°C). Protein concentration in cleared lysates was determined by BCA assay (Thermo Fisher) and the samples with adjusted equal protein concentration were boiled with the equal volume of 2x Laemmli sample buffer and then subjected to SDS-PAGE electrophoresis.

3.8 SDS-PAGE electrophoresis and Western blotting

Chemicals and materials:

- Separating gel in final concentration: different percentage of the final acrylamide: bisacrylamide (Biorad) concentration according to the molecular weight of protein of interest (10-30 kDa protein- 15%, 30-70 kDa- 12%, 70-100 kDa- 10%) (Biorad), 0.1% w/v SDS, 0.1% w/v ammonium persulfate, 375mM Tris pH 8.8, 0.01% TEMED.
- Stacking gel in final concentration: 5% acrylamide-bisacrylamide (Biorad), 0.1% w/v SDS, 0.1% w/v ammonium persulfate, 125mM Tris pH 6.8, 0.01% TEMED.
- PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Fisher)
- 1x Running buffer: 200mM glycine, 25mM Tris-base, 0.1% w/v SDS
- 1x Blotting buffer: 200mM glycine, 25mM Tris-base, 20% methanol
- Blotting membrane- Amersham Protran Premium 0.45 µm NC (GE Healthcare)
- Washing buffer: PBS, 0.05% Tween-20
- Ponceau S solution: 0.1 % (w/v) in 5% acetic acid

- Blocking buffer: 5% w/v milk (Sigma) or 2% w/v bovine serum albumin (Sigma) in Washing buffer (PBS/Tween-20)
- 10% w/v sodium azide
- ECL1 soultion (home-made): 2.5mM luminol (5-amino-2,3-dihydro-1,4-ftalazindion) in 100 mM Tris-HCl pH 8.8
- ECL2 solution (home-made): 5.4 mM H_2O_2 in 100mM Tris-HCl pH 8.8
- Medical X-ray Blue / MXBE Film- Carestream® Kodak® BioMax® XAR Film (Sigma)
- Pierce ECL Western Blotting Substrate (Thermo Fisher)
- Western Bright ECL (Advansta)
- SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher)

SDS-PAGE electrophoresis and Western blotting were performed according to standard protocols (Kurien and Scofield, 2015). The same amount of protein samples were together with the protein standards loaded on polyacrylamide gels. After SDS electrophoresis the gel was either stained with Coomassie Brilliant Blue R250 or the proteins were further transferred onto a nitrocellulose membrane using semi-dry Western blotting. The effectivity of the protein transfer was examined by Ponceau S staining and membrane was then blocked 30 min-1h with blocking buffer (see Chemicals and Materials). The proteins were detected using immuno-staining method with primary antibodies specific to the protein of interest (O/N, 4 °C) and secondary antibodies conjugated with horseradish peroxidase (1h, RT). The protein-antibody complexes were visualized by ECL or Femto solution and luminescent signal was revealed by Medical X-ray Blue films or by Western blot imaging system from Azure Biosystems.

3.9 Coomassie Brilliant Blue staining

- Gel staining buffer: 10% w/v Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid
- Gel de-staining buffer: 40% methanol, 10% acetic acid

After SDS electrophoresis the gel was stained using gel staining buffer for 1h in RT, then was trasferred to gel de-staining buffer, where the backround colour of gel was washed out.

3.10 The list of antibodies used for immuno-detection

If not stated otherwise, antibodies were obtained from Cell Signaling

Primary antibodies:

- anti-caspase-10 (9752)
- anti-caspase-9 (9508)
- anti-caspase-8 1C12 (9746)
- anti-caspase-3 cleaved (9661)
- anti-Bid (2002)
- anti-IκB-α (4814)
- anti-phospho IκB-α (2859, Ser32)
- anti-NF-κB p65 (4764)
- anti-phospho NF-κB (3033, Ser536)
- anti-phospho SAPK/JNK (9255S, Thr183/Tyr185)
- anti-p38/MAPK (9212)
- anti-phospho p38/MAPK (9211S, Thr180/Tyr182)
- anti-phospho MLKL (14516, Thr357/Ser358)
- anti-PARP cleaved (9541)
- anti-XIAP (2042)
- anti-caspase-3 (804-305-C100, Enzo Life Sciences)
- anti-FLIP (NF6, ALX-804-428, Enzo Life Sciences)
- anti-FLIP (7F10, ALX-408-961, Enzo Life Sciences)
- anti-DR4 (8414, Abcam)
- anti-MLKL (184718, Abcam)
- anti-DR5 (D3938, Sigma-Aldrich)
- anti-FADD (610400, Becton Dickenson)
- anti-RIP1 (610458, Becton Dickenson)
- anti-SAPK/JNK (sc-571, Santa Cruz)
- anti-Mcl-1 (sc-819, Santa Cruz)
- anti-actin (1615, Santa Cruz)
- anti-α-tubulin (TU-01, Exbio)
- anti-RIP3 (7604, MAB)

Secondary antibodies:

- GAM-Px- goat anti-mouse secondary antibody, conjugated with horseradish peroxidase (115-035-205, Jackson Immuno Research Laboratories)
- GAR-Px- goat anti-rabbit secondary antibody, conjugated with horseradish peroxidase (111-035-144, Jackson Immuno Research Laboratories)
- RAG-Px- rabbit anti-goat secondary antibody, conjugated with horseradish peroxidase (305-035-003, Jackson Immuno Research Laboratories)

3.11 Flow cytometry analysis

All flow cytometry analyses were run using LSRII and BD LSRFortessa instruments (Becton Dickenson). For all flow cytometry assays, the cells were seeded in 12 or 24 well plates and harvested at 80% confluency. Apoptosis was induced using human recombinant TRAIL ligand variants (TST-TRI-TRAIL WT, 4.2, 5.1, 5.2), His-TRAIL WT (Apronex) either alone or in combination with homoharringtonine (H0635, Sigma-Aldrich) or ABT-737 (S1102, Selleck Chemicals). TRAIL-mediated necroptosis was induced in cancer cells pre-treated with the IAP inhibitor birinapant (S7015) (Selleck Chemicals) and pan-caspase inhibitor Z-VAD-FMK (ALX-260-020-M005, Enzo Life Sciences) or Z-IETD-FMK (550380, Becton Dickenson). Necroptosis inhibitors necrostatin-1 (S8037) and necrosulfonamide (5025) were purchased from Selleck Chemicals and from Tocris, respectively.

- **Assessment of apoptosis - Annexin V binding assay**

TRAIL-treated and control cells were harvested by trypsinisation, washed with ice-cold PBS, then re-suspended in the Annexin-binding buffer (Apronex) and incubated on ice with Annexin V-FITC (Apronex) at the final concentration of 2 µg/ml for 20 min. Prior flow cytometry measurement Hoechst 33258 was added to the final concentration of 0.5 µg/ml. The experiments in Results 4.2 part were done according to the methods in Vinarsky et al., 2013.

- **Analysis of death receptor cell surface expression**

Cells were harvested by trypsinisation, then washed with ice-cold PBS, and re-suspended in PBS containing 0.2 % gelatine and 0.1 % sodium azide (PBS-GA). The samples were incubated on ice with the primary antibodies against DR4 (10-403, EXBIO) and DR5 (11-461, EXBIO) receptors at the final concentration 10 µg/ml for 30 min. After incubation, cells were washed twice with ice-cold PBS-GA and incubated with the secondary goat anti-mouse

IgG1 conjugated with phycoerythrin (final concentration 4 μg/ml; 1070-09, Southern Biotech) on ice for 30 min. Cells were then washed twice with cold PBS-GA, re-suspended in PBS-GA with Hoechst 33258 (0.5 μg/ml) and analysed by flow cytometry.

- **Assessment of necroptosis**

For quantification of necroptosis, cells were harvested with accutase, washed with HBSS with 5uM EDTA and re-suspended in HBSS/EDTA. Propidium iodide was added to the final concentration of 2 μg/ml, and cells were analysed by flow cytometry.

- **Detection of mitochondrial ROS**

Harvested cells were pelleted (300 rcf, 5 min) and washed with PBS. Then MitoSOX™ Red Mitochondrial Superoxide Indicator (Thermo Fisher) was added in the final concentration 5μM, following the manufacturer protocol, and cells were analysed by flow cytometry.

3.12 Real-time quantitative PCR

Total cellular RNA was isolated using RNA Blue (Top-Bio) according to the manufacturer's instructions. Isolated RNA was diluted in RNAase free water and its concentration was quantified by Nanodrop Spectrophotometer (Thermo Fisher). The equal amount of RNA (1-2 ug) was reverse transcripted using RevertAid RT Reverse Transcription Kit (Thermo Fisher) following manufacturer's protocol. cDNA was 5x diluted in RNase free water.

Diluted cDNA was together with relevant primer pair (see below) amplified using Light Cycler 480 SYBR Green I Master kit (Roche) in Light Cycler 480 (Roche) according to the manufacturer's instructions. To calculate the relative amount of mRNA was used formula:

relative concentration of RNA = $2^{Cp\ (control) - Cp\ (sample)}$

All RT-qPCR analyses were normalized to the expression of selected house-keeping genes (GAPDH, SDH, RPL37A, β-actin).

- **The list of primers used in real-time PCR:**

GAPDH fwd	AATTGAGCCCGCAGCCTCCC
GAPDH rev	TGAGCGATGTGGCTCGGCTG
βActin fwd	GGCATCCTCACCCTGAAGTA
βActin rev	AGGTGTGGTGCCAGATTTTC
RPL37A fwd	ATTGAAATCAGCCAGCACGC
RPL37A rev	AGGAACCACAGTGCCAGATCC
SDH fwd	AGATTGGCACCTAGTGGCTG
SDH rev	ACAAAGGTAAGTGCCACGCT
cIAP1 fwd	CAATCAAGGGTACAGTTCGTACA
cIAP1 rev	GACGATGTTTTGGTTCTTCTTCA
cIAP2 fwd	TTGTTGAACACTTGAAGCCATC
cIAP2 rev	ACCAAAGCAGACTAGAACATGAA
DR4 fwd	AACATCGTGCCCTTTGACTC
DR4 rev	TACCAGCTCTGACCACATCG
DR5 fwd	CCAGCAAATGAAGGTGATCC
DR5 rev	GCACCAAGTCTGCAAAGTCA

3.13 Statistical analysis

Statistical analysis was carried out using the GraphPad Prism 6 software. Experiments were performed in at least three biological replicates, data are presented as mean values ± SEM, statistical significance was evaluated using ANOVA with differences at $p \leq 0.05$ considered significant.

4 RESULTS

4.1 TRAIL induces apoptosis but not necroptosis in colorectal and pancreatic cancer cells preferentially via the TRAIL-R2/DR5 receptor

Summary

Human TRAIL is a cytokine that can via engagement of its two pro-apoptotic receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) trigger in addition to apoptosis a number of other signalling pathways such as necroptosis, canonical NF-κB signalling or can activate stress kinases. Though both of these signalling receptors are ubiquitously expressed, their relative participation in TRAIL-induced signalling is still largely unknown. To analyse TRAIL receptor-specific signalling, we prepared Strep-tagged, trimerised variants of recombinant human TRAIL with high affinity for either DR4 or DR5 receptor. Using these receptor-specific ligands, we examined the contribution of individual pro-apoptotic receptors to TRAIL-induced signalling pathways. We found that in TRAIL-resistant colorectal HT-29 cells but not in pancreatic PANC-1 cancer cells, DISC formation and initial caspase-8 processing proceeds comparably via both DR4- and DR5-activated signalling. TRAIL-induced apoptosis, enhanced by the inhibitor of the Bcl-2 family ABT-737, or by the translation inhibitor homoharringtonine, proceeded in both cell lines predominantly via DR5 receptor. ShRNA-mediated downregulation of DR4 and DR5 receptor in HT-29 cells also pointed to a stronger contribution of DR5 in TRAIL-induced apoptosis. In contrast to apoptosis, necroptotic signalling was activated similarly by both DR4- or DR5-specific ligands. Activation of auxiliary signalling pathways involving NF-κB or stress kinases proceeded under apoptotic conditions mainly in a DR5-dependent manner, while these signalling pathways were during necroptosis similarly activated by either of these ligands. Our study provides the first systematic insight into DR4−/DR5-specific signalling in colorectal and pancreatic cancer cells.

Declaration of honour

This is a first-author paper work, where author contributed by most of the experiments with exception of following: cloning of vectors for receptor specific TRAIL ligands was done by Martin Peterka for TST-TRI-TRAIL variants and Jan Švadlenka for His-TRI-TRAIL ligands, qPCR in Fig. 4.14 was done by Jan Švadlenka. Simona Benešová and Marie Ksandrová partially contributed with Western blots in Fig. 4.6 A-B and in Fig. 4.13 A. Jan Švadlenka

partially contributed to Western blots showed in Fig. 4.8 C. Data in Fig. 4.14 were not inlcuded in the published paper Nahacka et al., 2017.

4.1.1 Preparation of TRAIL receptor-specific ligands

For the detail analysis of TRAIL receptor-specific signalling in cancer cells, we designed and prepared recombinant TRAIL ligands specifically binding to either DR4 or DR5 receptor (Fig. 4.1, see also Materials and Methods: TRAIL ligand variants and reagents). Soluble recombinant human TRAIL contains the extracellular region of human TRAIL encompasing amino acids 95-281. To increase its effectivity and to mimic its trimeric formation we introduced T4 phage trimerisation/stabilisation (TRI) motif to the N-terminal sequence of the recombinant ligand. Furthermore, Twin Strep-tag (TST) sequence was included at the N-terminus allowing both its purification from the *E.coli* lysates as well as isolation of specific TRAIL receptor DISCs. DR4 (marked TST-TRI-TRAIL 4.2) and DR5 specific ligands (marked TST-TRI-TRAIL 5.1 and TST-TRI-TRAIL 5.2) were prepared by gene synthesis introducing specific mutations into sequence of human WT TRAIL ligand (marked TST-TRI-TRAIL WT) as showed in Fig. 4.1. For better understanding, TRAIL ligand variants will be in next pages mentioned in shorter name as TST-TRAIL WT/4.2/5.1/5.2.

Recombinant ligands were expressed in E.coli BL21-AI and purified using Streptactin Sepharose system to approx. 95% purity (assessed from SDS-PAGE, data not shown). Biological activity and selectivity of these ligands was assessed using two hematopoietic cell lines: predominantly DR4-signalling Ramos cells (Burkitt lymphoma cells) and DR5-signalling Jurkat cells (T cell leukemia cells). Fig. 4.2 A shows the cell surface expression of both TRAIL receptors and Western blotting analysis in Fig.4.2 B reveals their total cellular levels. Though Ramos cells expressed both receptors, DR4 as well as DR5, it has been documented that they signal just via DR4 receptor (Sung et al., 2009). Jurkat cell line had almost none expression of DR4 receptor and expressed uniquely DR5 both at the cellular and cell surface levels.

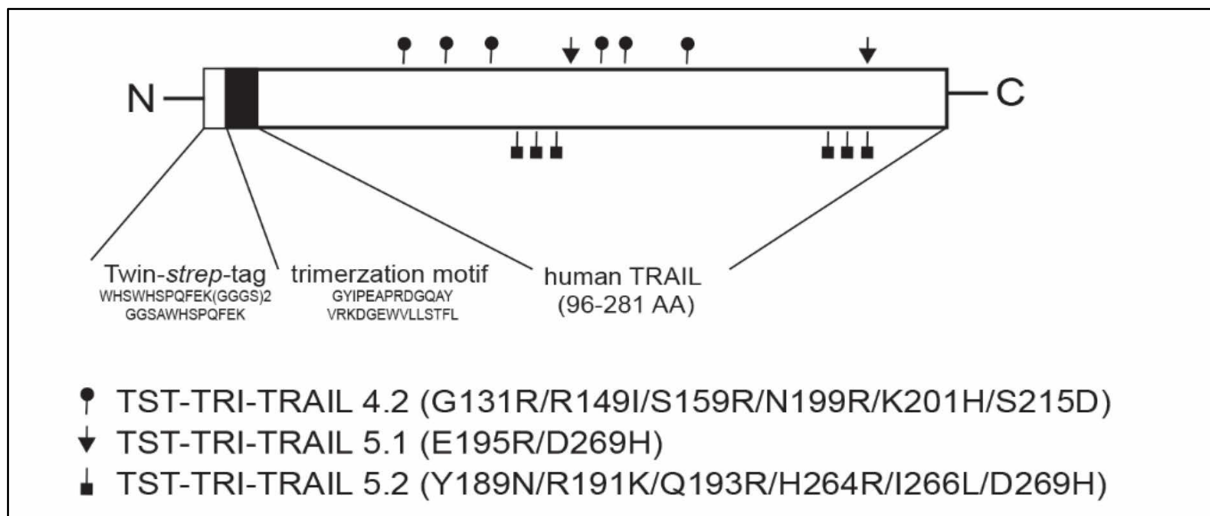


Figure 4.1: Graphical scheme of recombinant DR4- and DR5-specific TST-TRI TRAIL ligands.

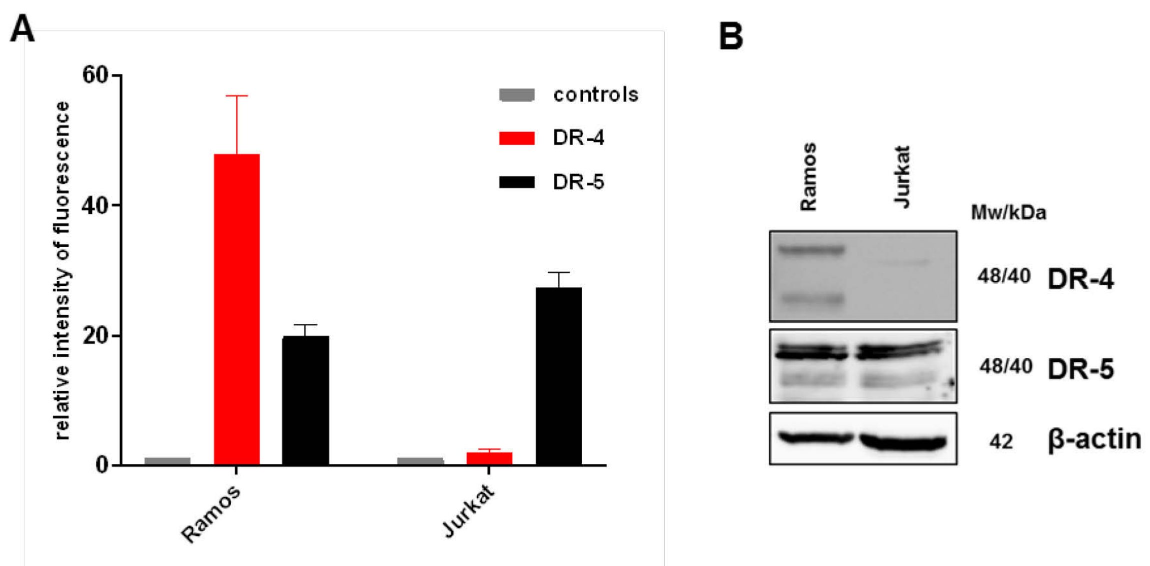
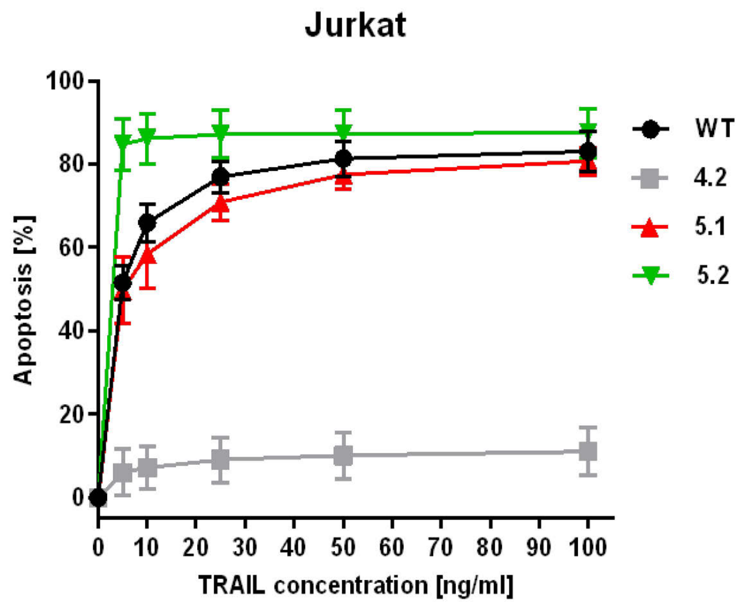


Figure 4.2: Analysis of TRAIL receptors DR4 and DR5 expression in Ramos and Jurkat cells. Cell surface expression of DR4 and DR5 receptor in Jurkat and Ramos cell lines was evaluated by flow cytometry (A). The relative signal is calculated with respect to the control cells stained only with the secondary antibody. Total expression was assessed by Western blotting (B). Data in A were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. Presented data A-B are representative of at least three biological replicates.

A



B

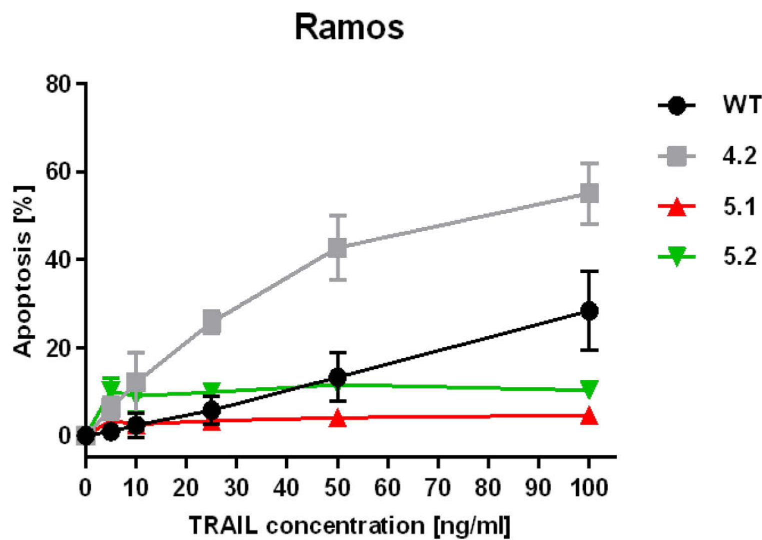


Figure 4.3: Assesment of the physiological activity and selectivity of TRAIL receptor-specific ligands. Jurkat and Ramos cells were treated with 5, 10, 25, 50 and 100 ng/ml of TST-TRAIL WT, 4.2, 5.1, 5.2 for 3hrs (Jurkat) or 4 hrs (Ramos) (A-B). Apoptotic cells were stained with annexin V-FITC and quantified by flow cytometry. Data are presented as mean values ± SEM. Presented data are representative of at least three biological replicates.

The apoptosis assays using the recombinant TST-TRAIL WT and TRAIL receptor-specific mutants in Ramos and Jurkat cells confirmed selectivity and activity of all purified ligands (Fig. 4.3). In agreement with the previous results, Jurkat cell line (Fig. 4.3 A) showed almost none response to DR4-specific ligand, while both DR5-specific ligands were very effective in inducing dose-dependent apoptosis of Jurkat cells (Fig. 4.3 A) (Jang et al., 2003). Interestingly, the ligand 5.2 excelled in inducing Jurkat cells apoptosis and at its concentration of 5 ng/ml induced death of over 80% of Jurkat cells while TST-TRAIL WT at the same concentration induced apoptosis of about 50% of these cells and reached "killing plateau" of 80% of dead/dying cells only at the concentration of 50 ng/ml. DR4-specific ligand TST-TRAIL 4.2 was as documented in Fig. 4.3 B about twice more efficient than TST-TRAIL WT, while DR5-selective ligands were fairly ineffective even at the highest concentrations. It is inevitable to mention here, that during the initial steps we also prepared His-tagged TRAIL ligands WT and specific to DR4 and DR5 receptor (see Materials and Methods, part 3.2). His-tagged TRAIL WT was in several cell lines more efficient in inducing of cell death than Strep-tagged TRAIL WT (similarly His-tagged TRAIL is more effective comparing to leucine zipper TRAIL, see Engels et al., 2005), but when testing the selectivity of His-tagged DR4/5 specific ligands, these turned out to profoundly less selective than TST-tagged TRAIL (data not shown).

4.1.2 DR5 receptor-specific TRAIL ligands effectively induce apoptosis in colorectal and pancreatic cancer cells

Among the most studied types of tumours in the field of TRAIL-induced signalling belong those of colorectal and pancreatic origin. For the experiments addressing efficacy of our TRAIL receptor-specific ligands we selected widely used HT-29 colon colorectal adenocarcinoma cell line and PANC-1- pancreatic epitheloid carcinoma cells. Initially we determined cellular levels as well as relative cell surface expression of both pro-apoptotic TRAIL receptors DR4 and DR5 (Fig. 4.4 A-B). DR4 total protein levels are higher in PANC-1 cells, but its cell surface expression is comparable with the expression on HT-29 cells, which express largely less of total cellular DR4. DR5 cell surface expression, however reflects its total cellular expression and seems to be at least twice higher in HT-29 cells than in PANC-1 cells. As both of these cell lines are being resistant to TRAIL-induced apoptosis (Fig. 4.7 B-C), its effective induction requires using a sensitizer. For this purpose we used homoharringtonine (HHT), an inhibitor of translation and FDA approved anti-CLL drug, which was shown to downregulate mainly FLIP-

1 and Mcl-1 anti-apoptotic proteins, and BH3 analogue ABT-737, that inhibits Bcl-2, Bcl-Xl and Bcl-w (Beranova et al., 2013; Konopleva et al., 2006).

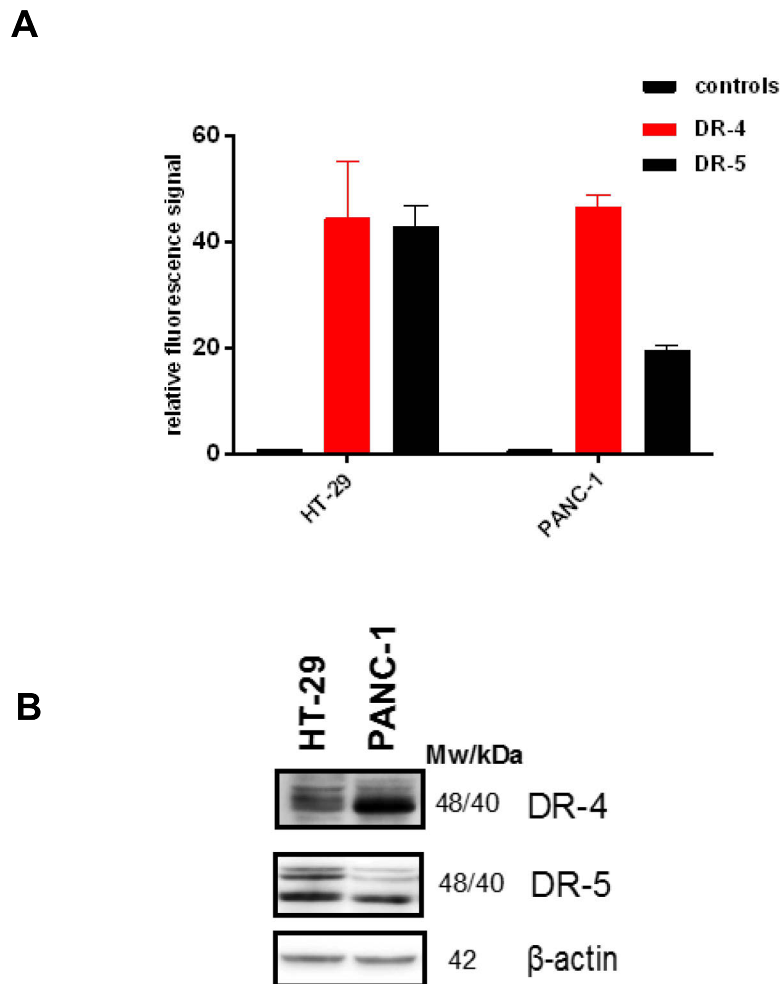
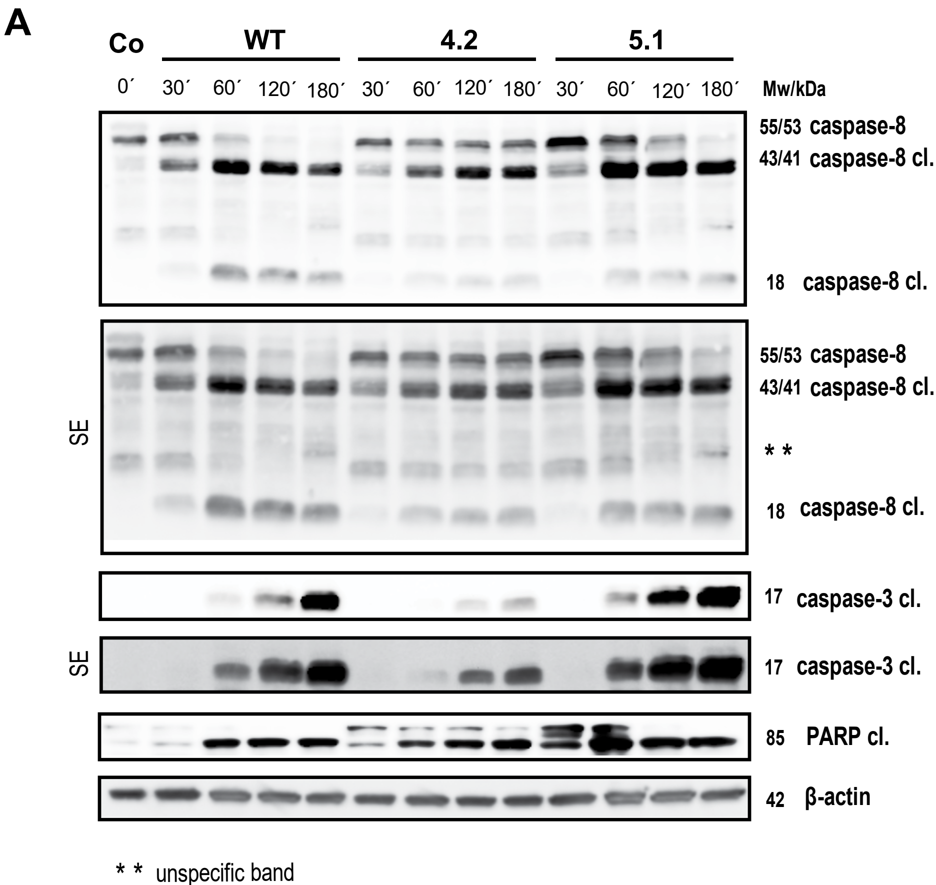


Figure 4.4: Cell surface and total protein expression of DR4 and DR5 in HT-29 and PANC-1 cell lines. Cell surface expression was evaluated by flow cytometry (A). The relative signal is calculated with respect to the control cells stained only with the secondary antibody (A). Total protein expression of DR4 and DR5 receptor in HT-29 and PANC-1 cells was revealed by Western blotting (B). Data in A were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. Presented data A-B are representative of at least three biological replicates.

For the effective induction of TRAIL-triggered apoptosis were cells HT-29 (Fig. 4.5 A-B) or PANC-1 (Fig. 4.6 A-B) pre-incubated with 100 nM HHT for 1 h and then incubated with 100 ng/ml TST-TRAIL variants for 30 min to 3 hrs (HT-29 cells) or 30 min to 5 hrs (PANC-

1). As TRAIL-induced apoptosis depends on an efficient attraction and especially processing of pro-caspase-8, we initially examined kinetics and efficacy of caspase-8 self-processing and cleavage of its downstream target effector caspase-3. In both cell lines was reproducibly observed not only more efficient, but also faster activation of both caspases in DR5-specific ligands-treated cells comparing to DR4-specific and WT ligands. Even more, DR5.2 ligand excelled in the activation of apoptotic signalling in HT-29 as well as in PANC-1 cells as documented by the fastest and efficient self-processing of caspase-8, strongest accumulation of processed caspase-3 and additionally also by cleavage of caspase-3 target, PARP protein (Fig. 4.5 A-B, 4.6 A-B).



B

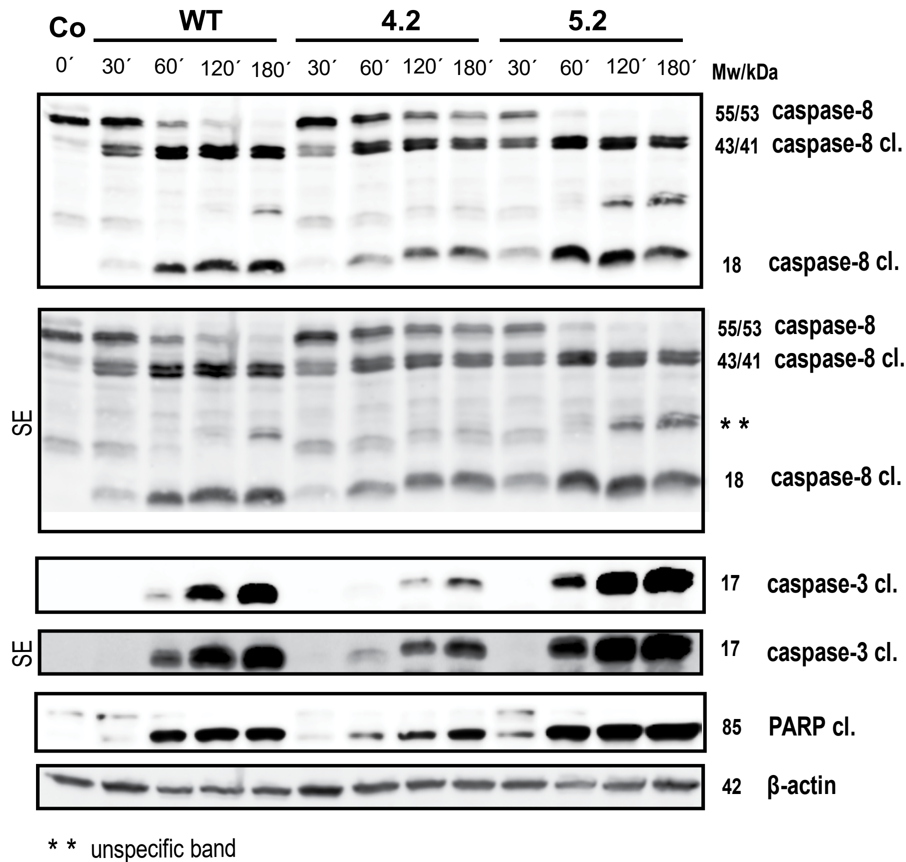
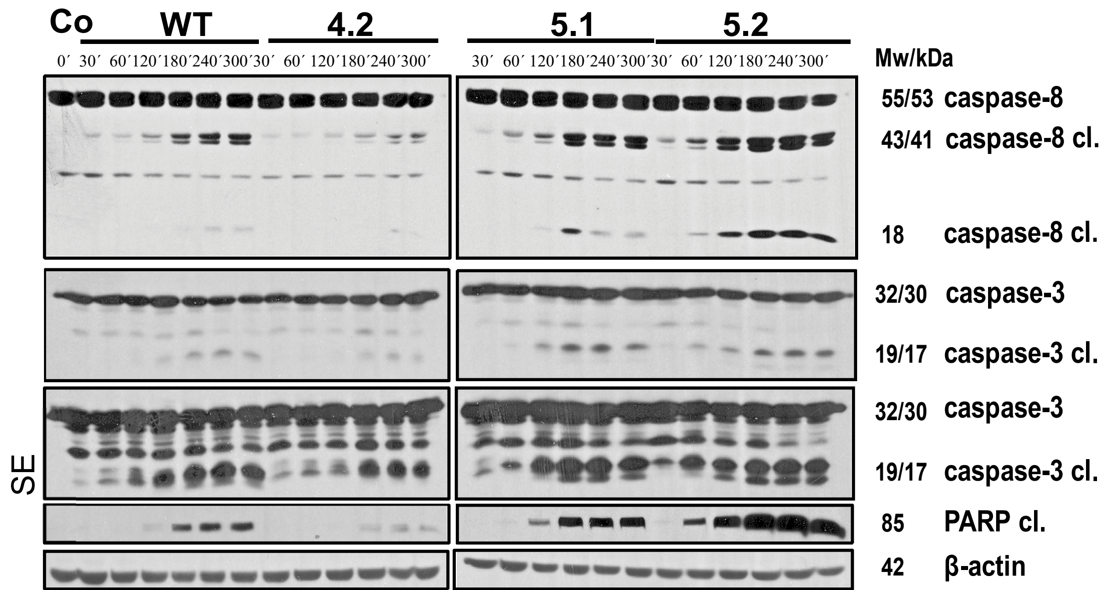


Figure 4.5: TRAIL-induced processing of caspases-8, caspase-3 and caspase-3 target protein PARP in colorectal HT-29 cell line.

Colorectal HT-29 (A-B) were treated with TST-TRAIL variants. Cells were pre-treated with 100 nM HHT for 1 h and then treated with 100 ng/ml of TST-TRAIL WT or 4.2, 5.1 and 5.2 ligands for indicated time periods. The samples were analysed by Western blotting. SE stands for the stronger exposition. The Western blots are representative of at least three biological replicates.


A
PANC-1
Co
WT
4.2
5.1
5.2
0´ 30´ 60´120´180´240´300´30´ 60´ 120´180´240´300´
30´ 60´ 120´180´240´300´30´ 60´ 120´180´240´300´
Mw/kDa
55/53 caspase-8
43/41 caspase-8 cl.
18 caspase-8 cl.
32/30 caspase-3
19/17 caspase-3 cl.
32/30 caspase-3
19/17 caspase-3 cl.
SE
85 PARP cl.
42 β-actin

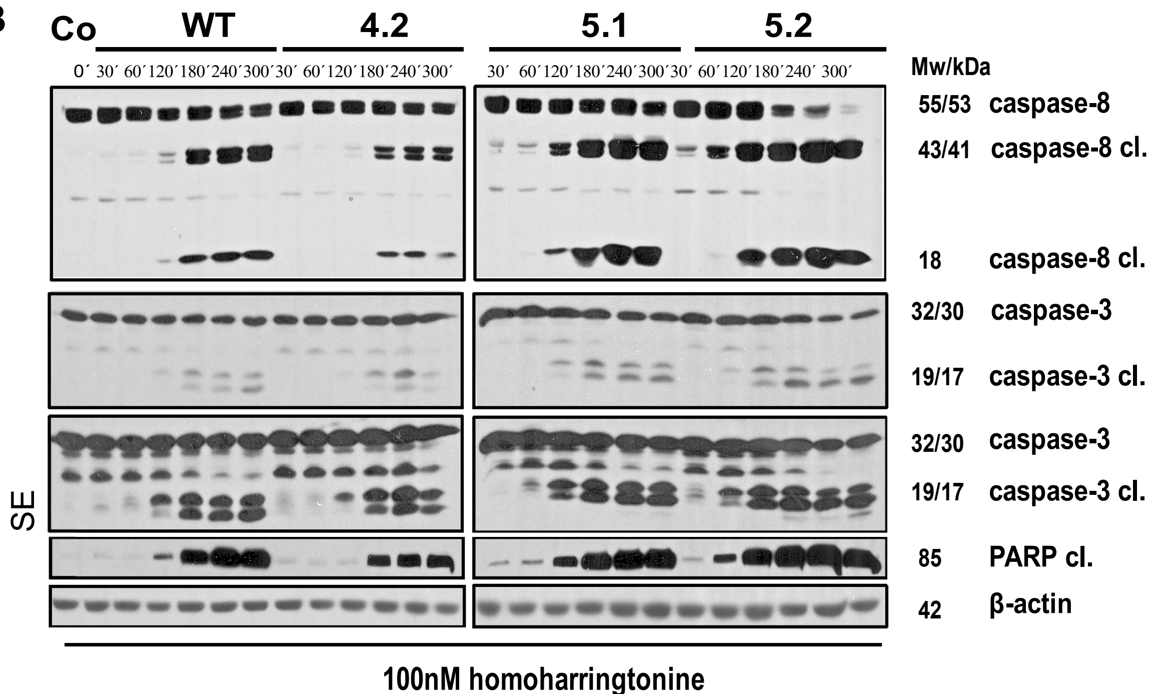

B
PANC-1
Co
WT
4.2
5.1
5.2
0´ 30´ 60´120´180´240´300´30´ 60´120´180´240´300´
30´ 60´120´180´240´300´30´ 60´120´180´240´ 300´
Mw/kDa
55/53 caspase-8
43/41 caspase-8 cl.
18 caspase-8 cl.
32/30 caspase-3
19/17 caspase-3 cl.
32/30 caspase-3
19/17 caspase-3 cl.
SE
85 PARP cl.
42 β-actin
100nM homoharringtonine

Figure 4.6: TRAIL-induced processing of caspases-8, caspase-3 and caspase-3 target protein PARP in pancreatic PANC-1 cell line.

Pancreatic PANC-1 cells (A-B) were treated with TST-TRAIL variants. Cells were either mock-treated (A) or pre-treated with 100 nM HHT for 1 h (B) and then treated with 100 ng/ml of TST-TRAIL WT or 4.2, 5.1 and 5.2 ligands for indicated time periods. The samples were then analysed by Western blotting. SE stands for stronger exposition. The Western blots are representative of at least three biological replicates.

The data from Western blots pointing to faster and more efficient activation of apoptotic signalling by DR5-specific ligands prompted us to compare these apoptotic signalling triggered by receptor-specific ligands using more quantitative flow cytometry analyses. Titration of TRAIL variants revealed that low, limited TRAIL concentration of 10 ng/ml in HT-29 cells pre-treated with 100 nM HHT induced in case of DR5-specific ligands very effective apoptosis (40-60% compare to 15-20% induced by DR4-specific ligand or TST-TRAIL WT). However, at saturating TRAIL ligands concentration of 100 ng/ml both WT and DR5-selective ligands induce in contrast to ligand DR4.2 apoptosis of 70-85% cells (DR4-specific ligands just about 40%) (Fig. 4.7 A-B). When PANC-1 cells were pre-treated with sensitizers HHT (100 nM) or ABT-737 (20 uM), TRAIL-induced apoptotic response was similarly as in HT-29 cells greatly enhanced and also DR5-specific ligands showed a better effectivity than WT or DR4-specific variant (Fig. 4.7 D). Interestingly, while not pre-sensitized, HT-29 as well as PANC-1 were more or less resistant to TRAIL WT-induced apoptosis, but they were both responsive to DR5.2 ligand (Fig. 4.7 B-C).

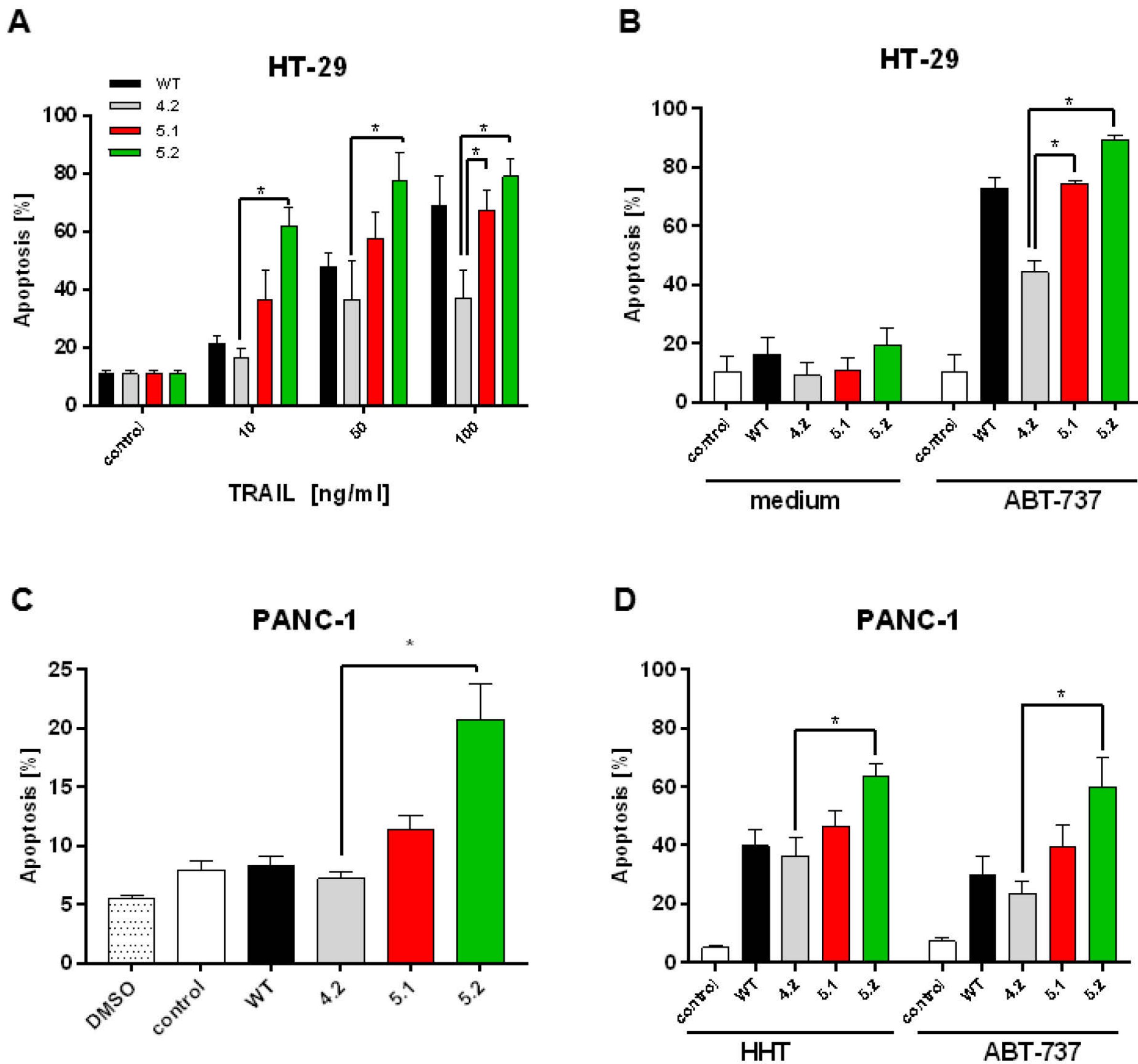


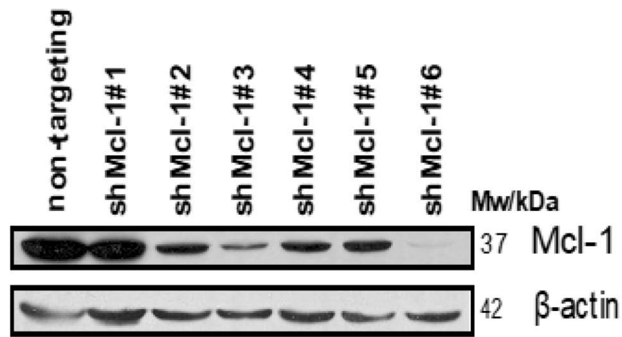
Figure 4.7: DR5 receptor-specific ligands effectively induce apoptosis in colorectal and pancreatic cancer cells.

HT-29 cells were either mock-treated (B) or pre-treated with 100 nM HHT for 1 h (A) or with 20 µM ABT-737 for 4 hrs (B), and then treated with increasing concentration (A) or with 100 ng/ml (B) of TST-TRAIL WT, 4.2, 5.1 and 5.2 for 5 hrs. PANC-1 cells (C-D) were pre-treated with 100 nM HHT for 1h or with 20 µM ABT-737 for 4 hrs and then treated with TRAIL variants at a concentration of 100 ng/ml for 5 hrs. Cells were assessed for apoptosis using annexin V-FITC staining and analysed by flow cytometry (A-D). Data in A-D were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The presented data are representative of at least three biological replicates.

4.1.3 Downregulation of anti-apoptotic protein Mcl-1 sensitizes HT-29 cells for DR5 induced apoptosis

One of the main regulators of apoptosis are members of Bcl-2 protein family, from them Mcl-1 was shown to be highly expressed pro-survival protein in human malignancies (Tong et al., 2017). We previously showed that Mcl-1 is one of the main regulators of TRAIL-induced apoptosis of colorectal cancer cells (Beranova et al., 2013). We examined whether and how could Mcl-1 also affect TRAIL-induced apoptosis in a receptor-specific manner. We prepared several constructs with downregulated Mcl-1 using lentiviral shRNA system (shMcl-1#1-6) and non-targeting shRNA control (NT), which were cloned into pLKO1 plasmid (Fig. 4.8 A). For further experiments were used HT-29 cells transduced with shMcl-1#6 and non-targeting control. Strangely, the lentiviral control transduction using non-targeting shRNA enhanced TRAIL-induced apoptosis of HT-29 cells by about 20-30%, with DR5.2 ligand being the most effective one (Fig. 4.8 B). However, Mcl-1 downregulation even more enhanced TRAIL-induced apoptosis of shMcl-1 HT-29 cells. Interestingly, downregulation of Mcl-1 sensitized HT-29 cells preferentially to WT- and DR5-specific ligands induced apoptosis, while DR4.2 ligand did not trigger enhanced apoptotic signalling at all (Fig. 4.8 B). The flow cytometry data were in a good agreement with the Western blotting analysis of caspase-3 processing and the cleavage of one of its main targets, PARP protein. Both DR5-specific ligands and to a lesser extent also WT-TRAIL induced in time-dependent manner efficient caspase-3 processing (by activated caspase-8) and cleavage of PARP and Bid (Fig. 4.8 C). In contrast, DR4-specific ligand performed poorly and did not induce any notable caspase-3 processing or PARP and Bid cleavage.

A



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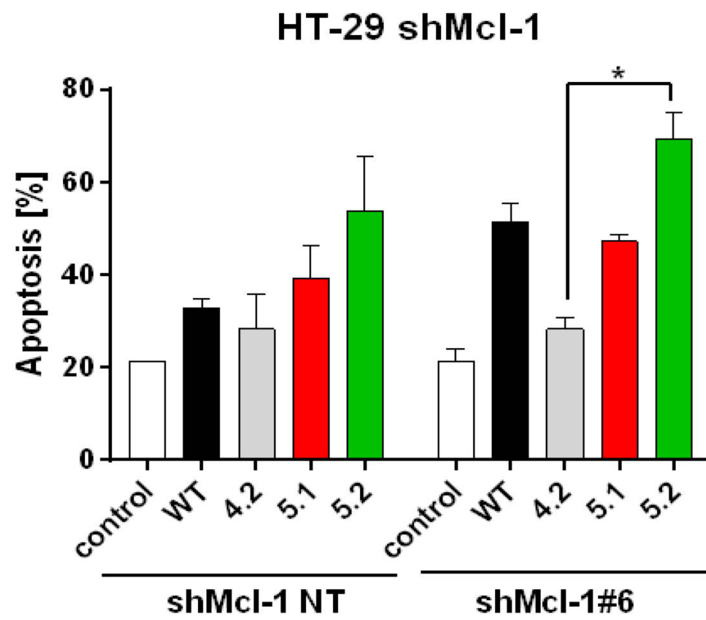


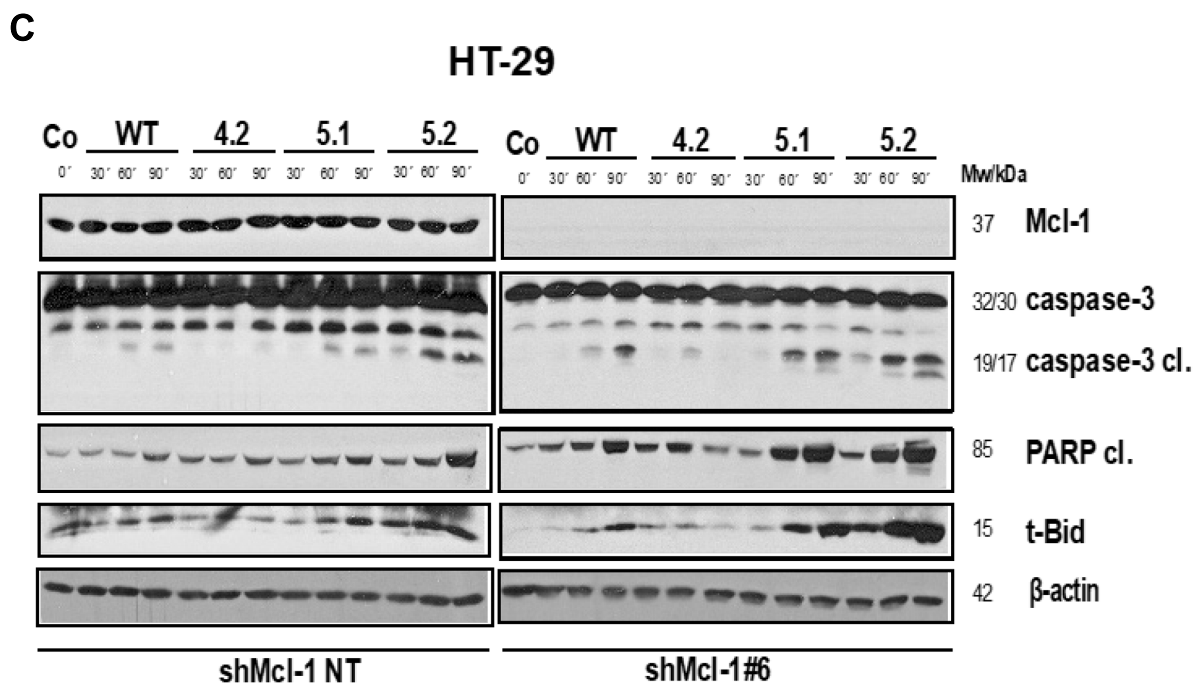


Figure 4.8: Downregulation of anti-apoptotic protein Mcl-1 sensitizes HT-29 cells to DR5-induced apoptosis. HT-29 cells were transduced with lentiviruses containing non-targeting (NT) shRNA or shMcl-1 shRNA #1-6. shRNA-mediated suppression of Mcl-1 protein was evaluated by Western blotting (A). HT-29 cells expressing non-targeting or shMcl-1#6 were treated with 100 ng/ml of TST-TRAIL WT, 4.2, 5.1, 5.2 for 3 hrs (B) or for increasing time periods (C). Cells were analysed either by flow cytometry (B) or by Western blotting (C) Data in Fig. 4.8 B were statistically evaluated by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The presented data are representative of at least three biological replicates.

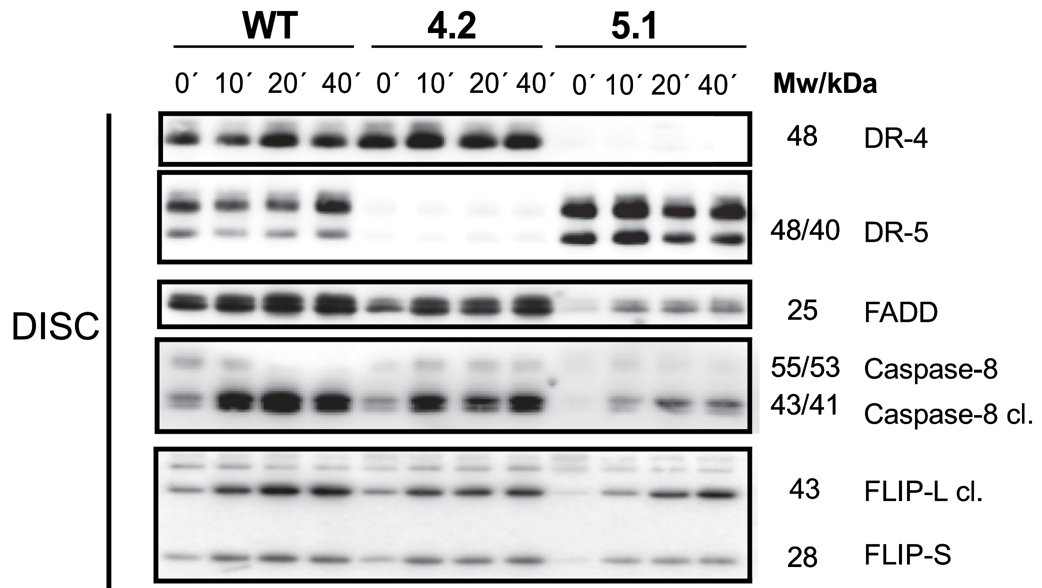
4.1.4 TRAIL receptor-specific variants do not differ in the formation of death-inducing signalling complex in HT-29 and PANC-1 cells

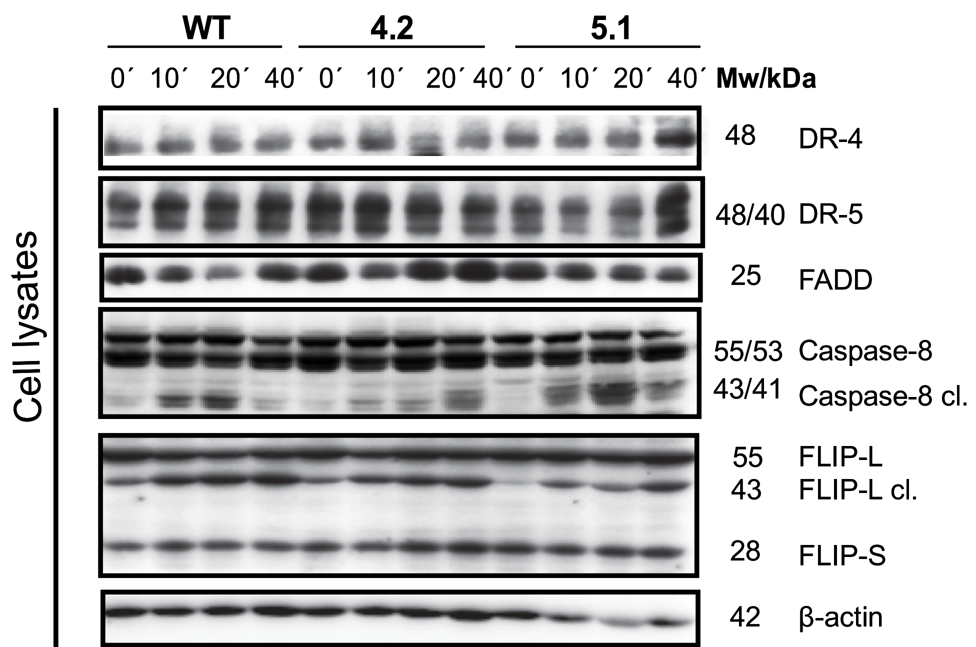
Observed differences in signalling efficacy among the analysed TRAIL receptor specific ligand (caspase processing and induction of apoptosis) prompted us to turn our attention to the formation and composition of the death-inducing signalling complex (DISC). The apoptotic signalling induced by ligand TRAIL could be regulated at many steps and DISC formation is the first and most important one. The dynamics of DISC formation or ratio of individual components could reveal an underlying mechanism behind the more effective induction of

apoptotic signalling by DR5 specific ligands. For DISC analyses we took advantage of the Twin-strep tag that was also used for the purification of recombinant ligands from the *E.coli* clarified lysates. The Twin-strep tag (TST) very strongly and with high affinity binds to Streptactin-Sepharose and thus TST-tagged TRAIL ligands are superior for the efficient pull-down of the TRAIL DISC complexes. HT-29 (Fig. 4.9 A-B) and PANC-1 cells (Fig. 4.9 C-D) were treated with 1 μg/ml of WT, 4.2, 5.1 and 5.2 TST-TRAIL variants and the relevant DISC complexes were isolated using Streptactin Sepharose. Affinity purification of DISC components in HT-29 cells first of all revealed specific interaction of the receptor-specific ligands (in contrast to WT ligand, which precipitated both DR4 and DR5 receptors) with their cognate receptors again confirming their selectivity (Fig. 4.9 A-D, DISC parts). DISC analyses pointed to some noticeable differences in either DISC composition or in the kinetics of their formation (Fig. 4.9 A-D). We noticed that TRAIL WT as well as DR4-specific ligand attracted in contrast to DR5-selective ligands (especially to DR5.1 ligand) in both HT-29 and PANC-1 cells fairly more FADD adapter protein (both during pre-incubation on ice and in the course of DISC formation/processing) (Fig. 4.9 A-D). Interestingly, though both DR5-selective ligands attracted significantly less FADD, their caspase-8 processing in DISC complexes was quite efficient and in case of DR5.2-treated PANC-1 cells even superior to all other ligands (Fig. 4.9 D). More efficient caspase-8 activation by DR5-selective ligands was also reflected in significantly more effective caspase-8 processing in cell lysates of both model cell lines (Figs. 4.9 A-D).

A

HT-29





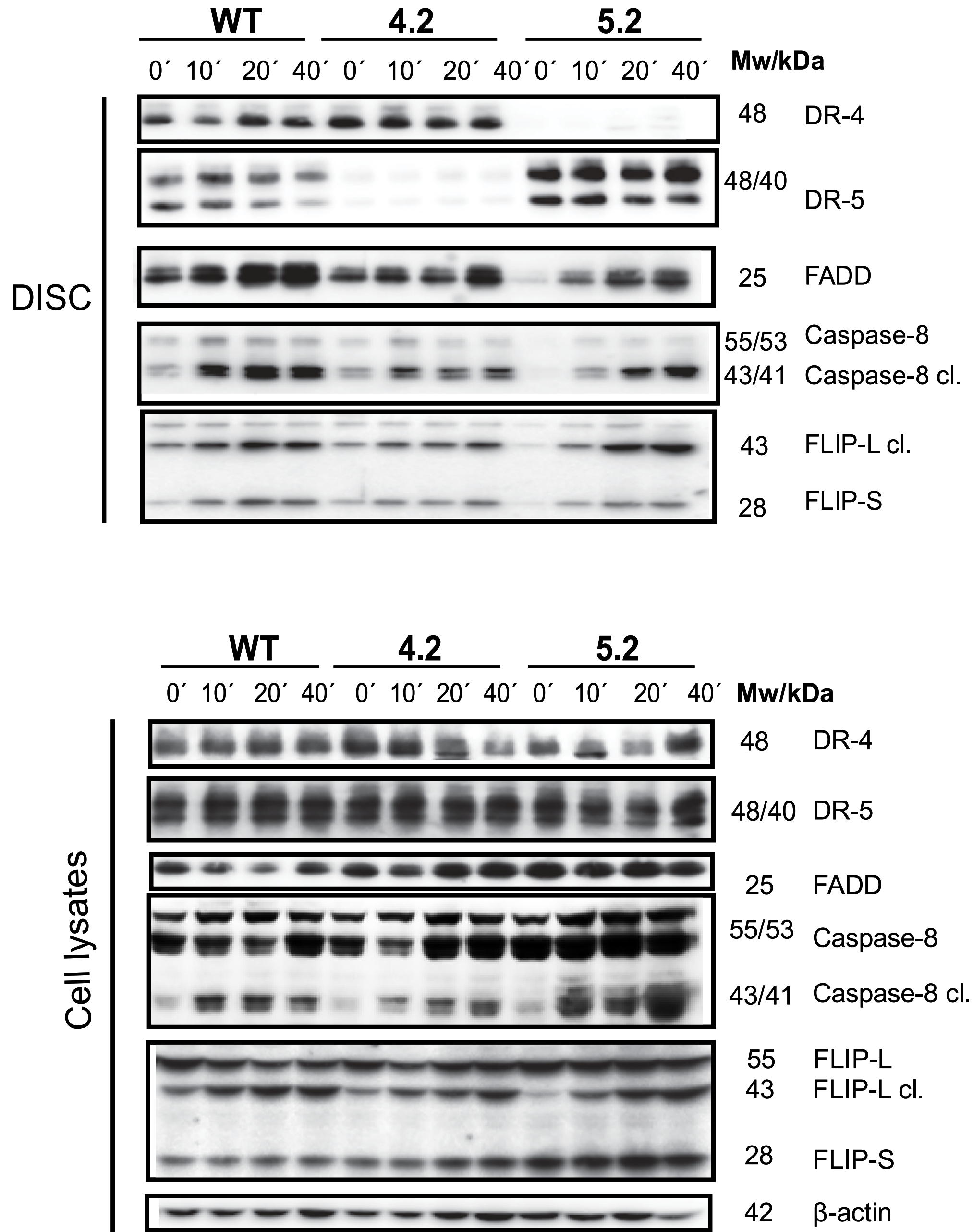
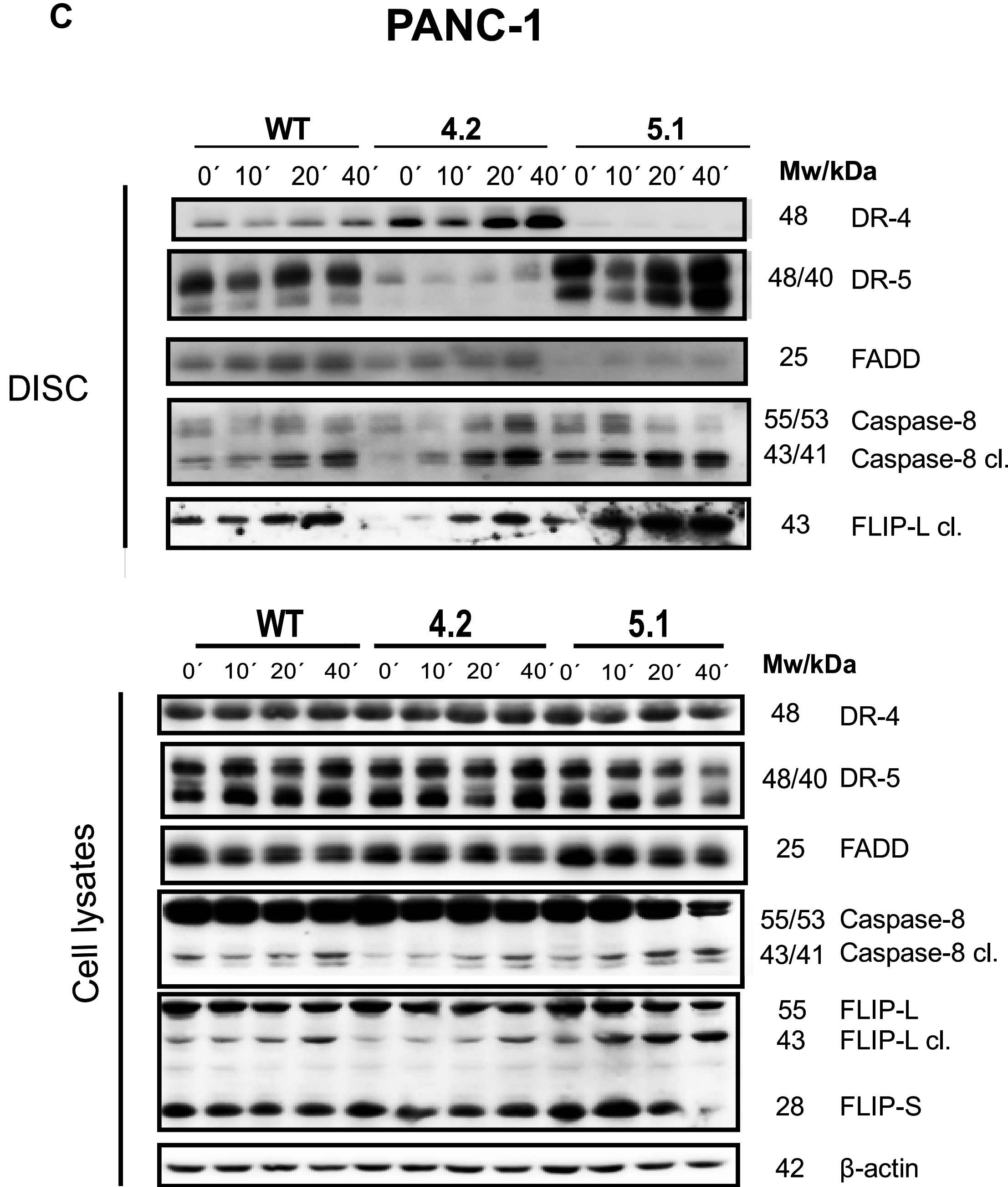


Figure 4.9: TRAIL receptor-specific variants efficiently and selectively induced DISC formation in HT-29 cells.

HT-29 (A-B) cells were pre-incubated with 1 mg/ml of TST-TRAILs: WT, 4.2, 5.1 and 5.2 for 15 min on ice. The cells were then either harvested (time point 0 min) or transferred to 37°C, harvested at specified time periods (10, 20 and 40 min) and lysed. The DISC complexes from

the cleared lysates were isolated on Streptactine agarose and analysed by Western blotting. Presented data are representative of two biological replicates.



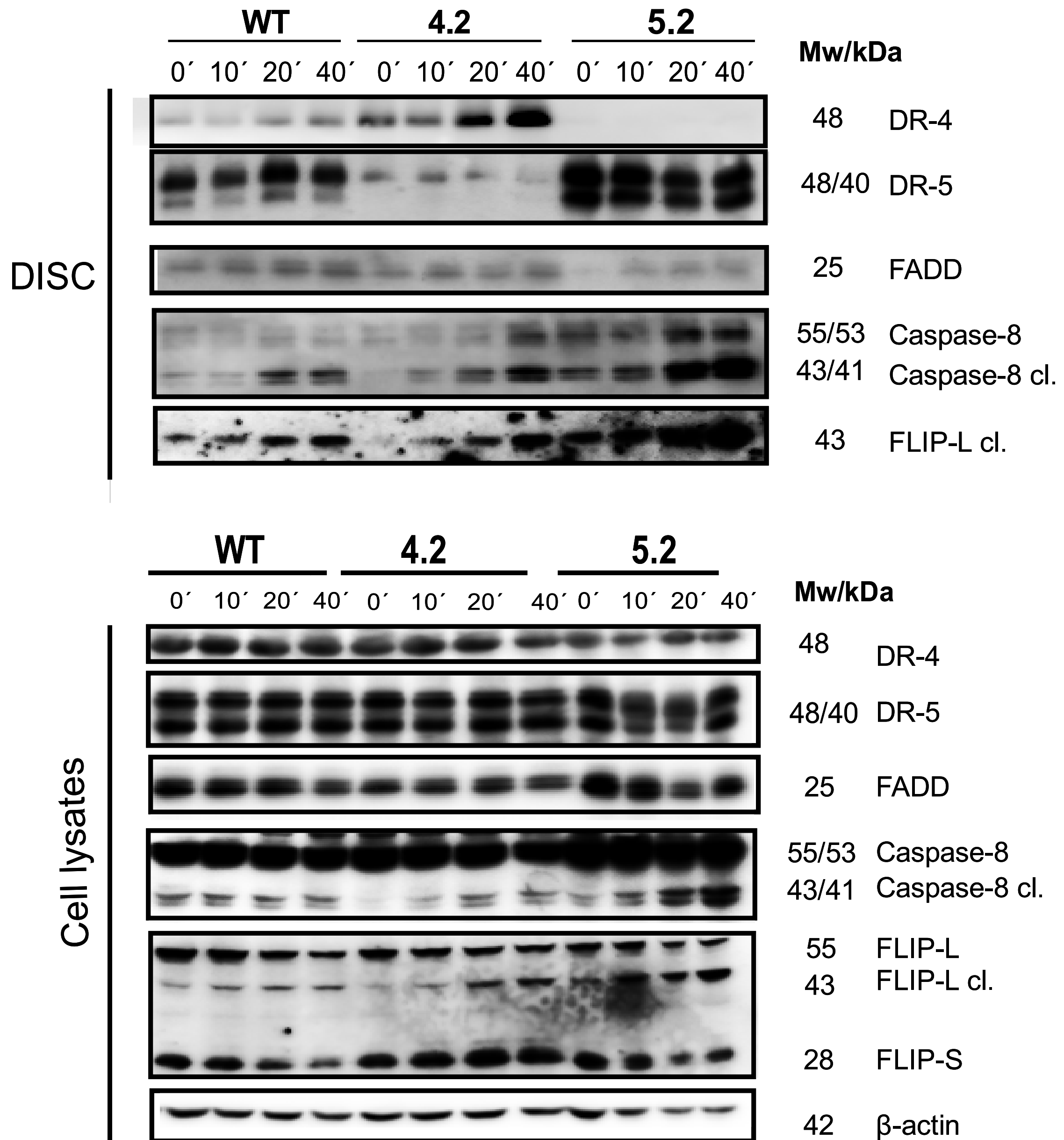


Figure 4.9: TRAIL receptor-specific variants efficiently and selectively induced DISC formation in PANC-1 cells.

PANC-1 (C-D) cells were pre-incubated with 1 mg/ml of TST-TRAILs: WT, 4.2, 5.1 and 5.2 for 15 min on ice. The cells were then either harvested (time point 0 min) or transferred to 37°C, harvested at specified time periods (10, 20 and 40 min) and lysed. The DISC complexes from the cleared lysates were isolated on Streptactine agarose and analysed by Western blotting. Presented data are representative of two biological replicates.

4.1.5 DR5 but not DR4 downregulation results in lower susceptibility of colorectal cancer cells to TRAIL

In order to further decipher and confirm the major role of the DR5 receptor in TRAIL-triggered apoptotic signalling, we employed DR4- or DR5-specific lentiviral shRNA constructs to downregulate their expression in HT-29 cells. We prepared several shRNA constructs sub-cloned into pLKO vector, two different DR4 specific shRNA namely shDR4#01 and shDR4#02 and three different DR5-directed lentiviral shRNAs: shDR5#01, shDR5#04 and shDR5#05. We also included the non-targeting shRNA (NT) and empty pLKO1 (EV) controls and prepared the recombinant lentiviruses for all above-mentioned plasmids. HT-29 cells were then transduced using these lentiviral particles and transduced cells were selected by puromycin. Analysis of the total level of receptors (Fig. 4.10 A) and their cell surface externalisation (Fig. 4.10 B) confirmed that both DR4-targeting shRNAs efficiently suppressed its expression in HT-29 cells. shRNA against DR5 receptor decreased expression of DR5 receptor in all three cases, but mostly in shDR5#05 (Fig. 4.10 B). For further functional analyses were used shDR5#04 and shDR5#05. Interestingly, we also observed that the cell surface expression of DR4 was in DR5-downregulated cell lines slightly elevated and vice versa in DR4-downregulated cell lines DR5 expression was also increased (Fig. 4.10 A-B).

Apoptotic test using DR4- or DR5-selective ligands indeed confirmed that the downregulation of a respective cognate receptor to various degree attenuated apoptotic signalling induced by respective receptor-specific ligands. (Fig. 4.11 B-D). In case of DR4.2 ligand apoptosis of HT-29 transduced with shDR4#01 or shDR4#02 was greatly reduced in both cell lines (Fig. 4.11 B). DR5 receptor selective ligands 5.1 and 5.2 induced apoptosis of HT-29 cells with downregulated expression of DR5 was also attenuated with shDR5#05 being more effective (Fig. 4.11 C-D). Notably, while apoptosis triggered by TST-TRAIL WT (Fig. 4.11 A) was almost unaffected in HT-29 cells with suppressed expression of DR4, downregulation of DR5 by shRNA#04 and #05 significantly attenuated TST-TRAIL WT-induced apoptosis in these cells, thus supporting the major role of DR5 receptor in apoptotic signalling in HT-29 cells.

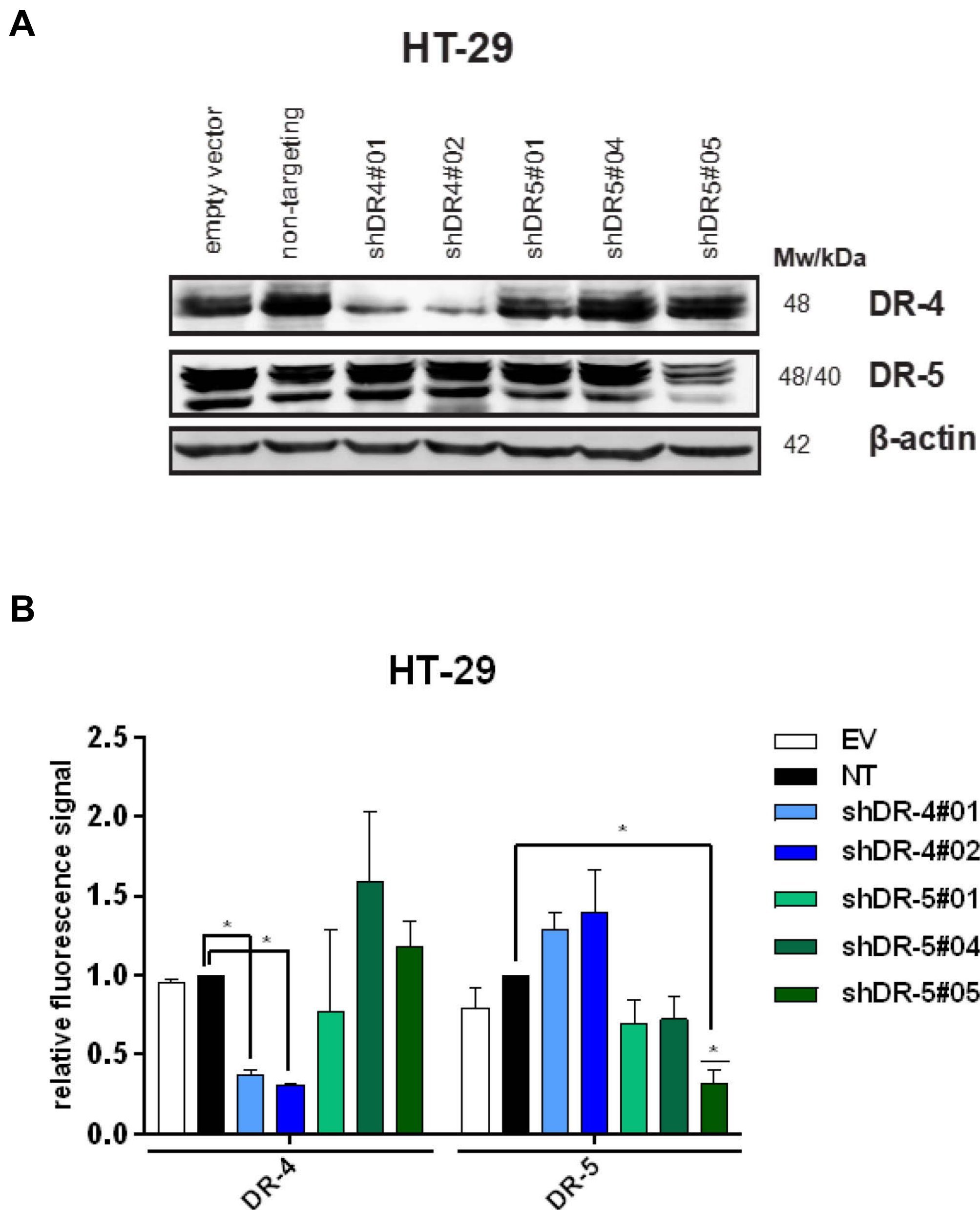
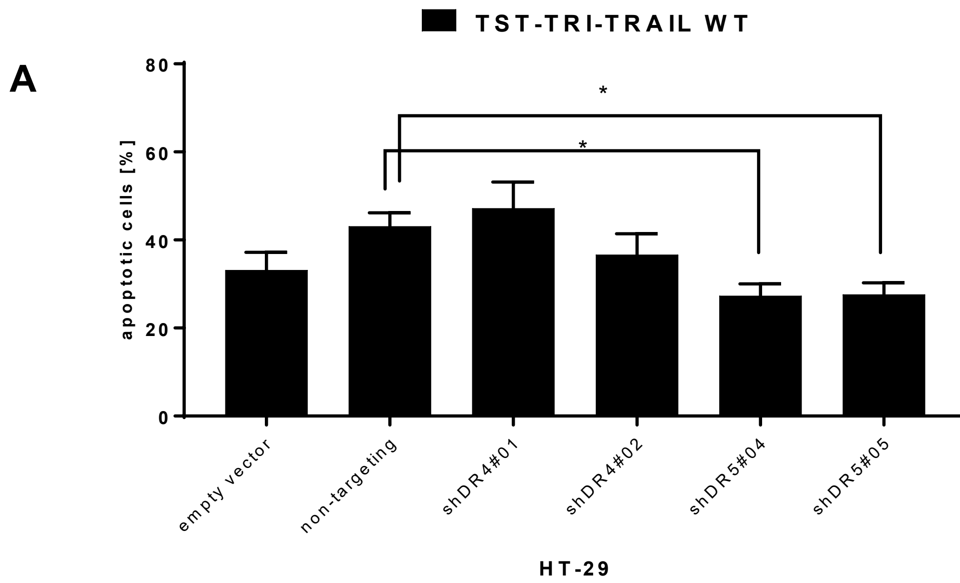


Figure 4.10: shRNA mediated downregulation of DR4 and DR5 receptor. Total cellular (A, Western blotting) and the cell surface (B, flow cytometry) protein expression. Relative fluorescence signal B was normalised to the level of the signal after staining with secondary antibody only and then against the receptor expression in cells expressing non-targeting (NT) control shRNA. Data from three biological replicates were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant.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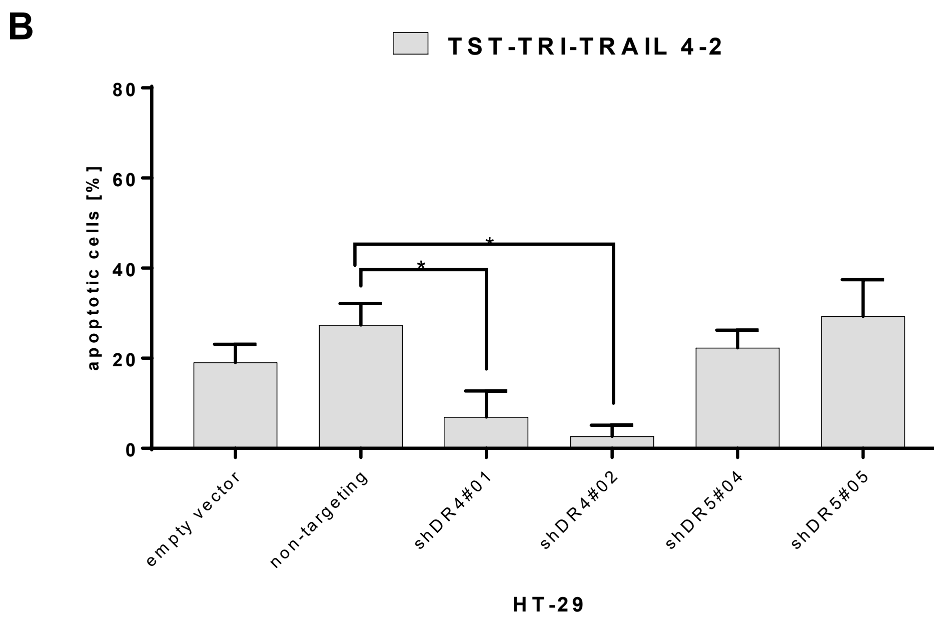
TST-TRI-TRAIL WT
apoptotic cells [%]
80
60
40
20
0
*
*
empty vector
non-targeting
shDR4#01
shDR4#02
shDR5#04
shDR5#05
HT-29


B
TST-TRI-TRAIL 4-2
apoptotic cells [%]
80
60
40
20
0
*
*
empty vector
non-targeting
shDR4#01
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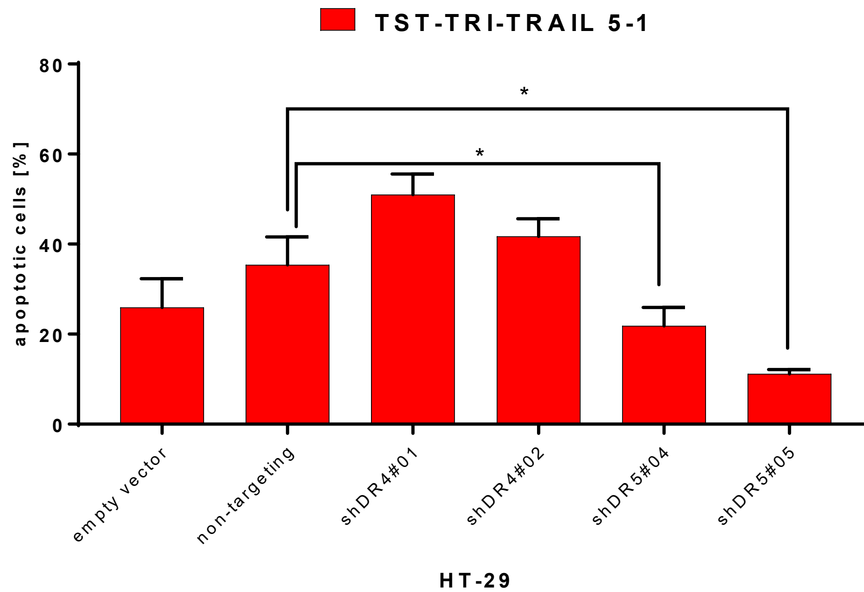
shDR5#05
HT-29

C



D

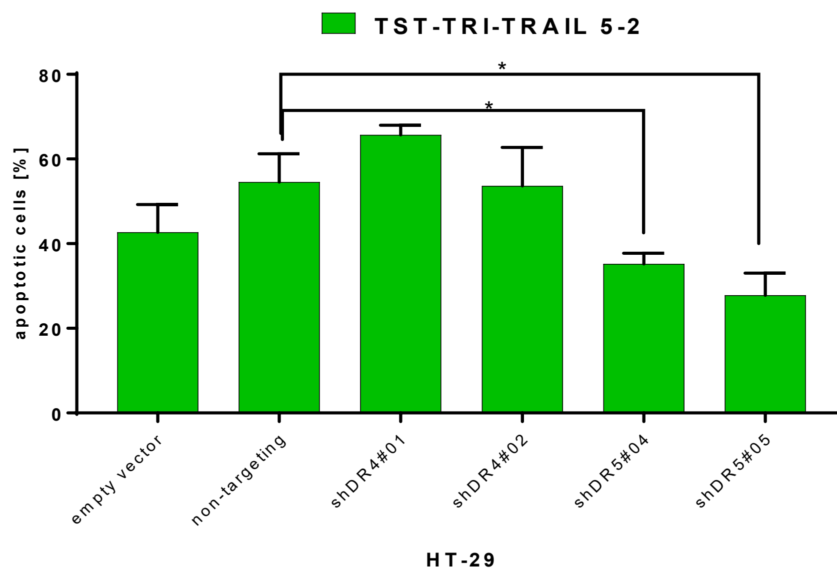
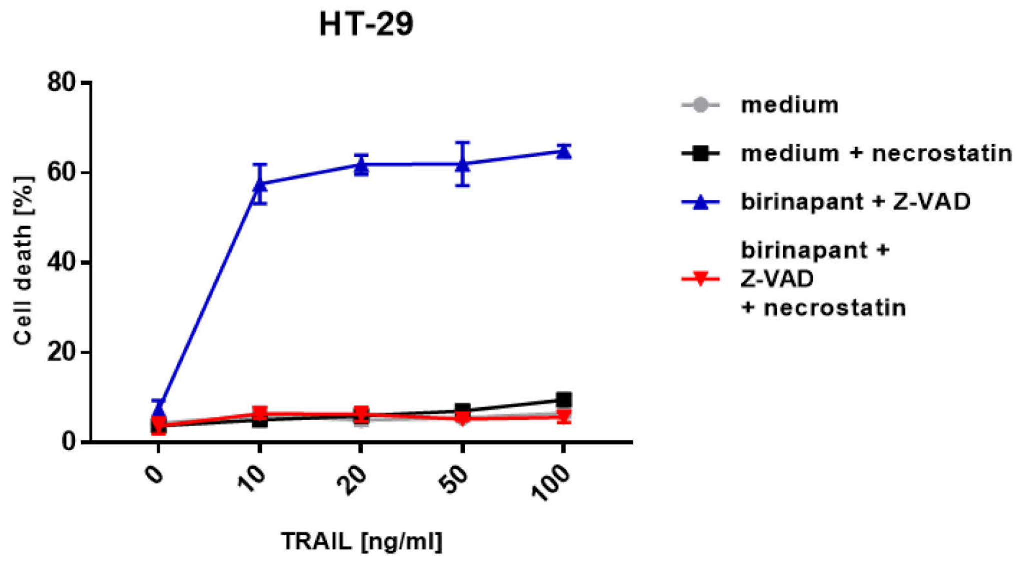


Figure 4.11: DR5 but not DR4 downregulation attenuates TST-TRAIL WT induced apoptosis of HT-29 cells. For the enhanced induction of apoptosis (A-D), HT-29 were pre-treated with 100 nM HHT for 1 h and then treated with 100 ng/ml TRAIL WT (A) or TRAIL receptor specific variants (B-D) for 3 h. TRAIL-induced apoptosis was quantified using annexin V-FITC staining and flow cytometry. Data in Fig. 4.11 A-D (three biological replicates) were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant.

4.1.6 Both DR4 and DR5 receptor-specific ligands efficiently induce necroptosis in HT-29 cells

Besides inducing apoptosis, activated death receptors can, upon inhibition of apoptosis, trigger a regulated necrotic cell death - necroptosis. We examined the efficacy of TRAIL receptor-specific ligands in the induction of necroptotic signalling. Efficient induction of death receptors (including TRAIL receptors) triggered necroptosis requires blocking caspase activity e.g. with pan-caspase inhibitor z-VAD, and in addition cytosolic availability of RIPK1, an essential component of the necrosome - necroptosis-activating intracellular complex. RIPK1 is in cells often ubiquitinated by cIAP1/2 (especially in activated death receptors DISCs) and thus unavailable for the necrosome formation. Therefore, using IAP inhibitor/SMAC mimetic such as birinapant, which suppresses cIAP1/2 expression and thus increases the cytosolic pools of RIPK1, is recommended for efficient induction of DR-mediated necroptosis. Necroptotic character of the cell death was confirmed using known inhibitors of necroptosis - RIPK1 inhibitor necrostatin-1 and MLKL assembly inhibitor necrosulfonamide. Initially we titrated TST-TRAIL WT and assessed an effective concentration of the ligand to induce necroptosis of HT-29 cells (pre-treated with zVAD and birinapant). The titration uncovered that TST-TRAIL WT efficiently induced necroptosis of about 60% of HT-29 cells even at the low concentration of 10 ng/ml, which just slightly increased at saturating TST-TRAIL WT concentration 100 ng/ml to about 65-67% of dead, PI-positive cells. Necroptotic mode of this cell death was confirmed by its efficient suppression with either RIPK1 inhibitor necrostatin-1 (Fig. 4.11 A-B) or the MLKL inhibitor necrosulfonamide (Fig. 4.11 B). Necroptotic character of HT-29 cell death was also supported by confirming RIP3 kinase-mediated phosphorylation of the necroptosis effector MLKL protein and by the absence of caspase-3-processed PARP (Fig. 4.11 D). Interestingly and in a contrast to apoptotic signalling, we did not notice any significant differences between DR4- and DR5-specific ligands in inducing necroptotic cell death at high, saturating concentrations of the ligands (100 ng/ml) (Fig. 4.11 B). However, at low concentration (10 ng/ml and less) of the TST-TRAIL ligands, DR5-selective ones (notably DR5.2) were significantly more effective than TST-TRAIL WT of the TST-TRAIL DR4.2 ligand (Fig. 4.11 C).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60
40
20
0
Cell death [%]
0
10
20
50
100
TRAIL [ng/ml]
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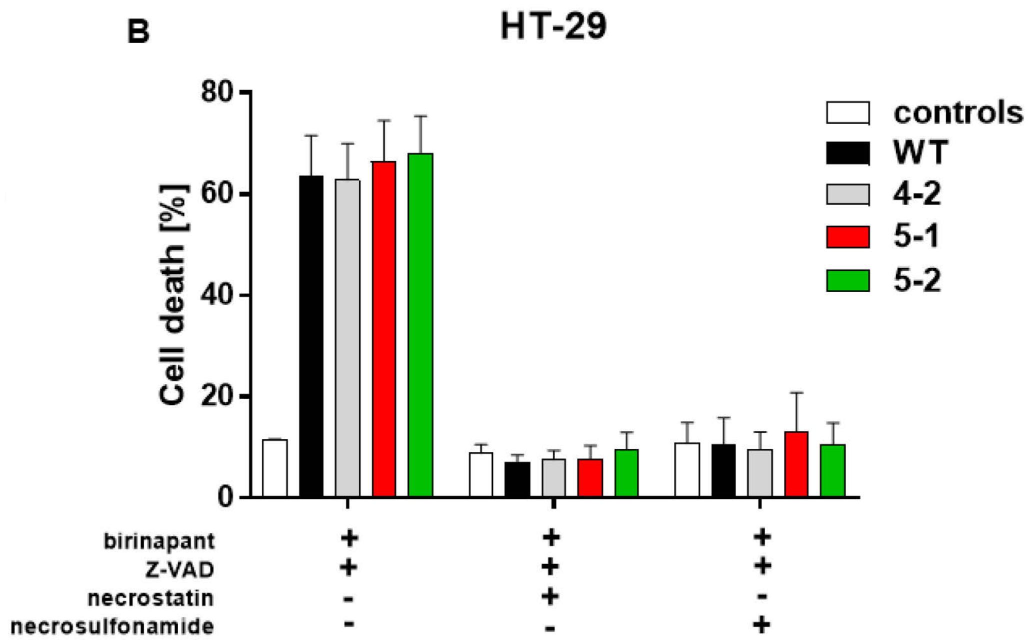
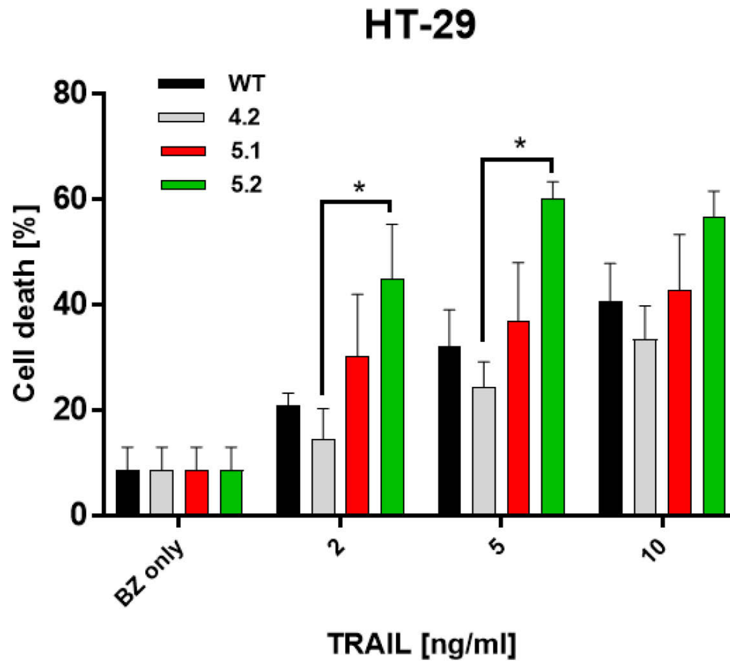
medium + necrostatin
birinapant + Z-VAD
birinapant +
Z-VAD
+ necrostatin


B
HT-29
80
60
40
20
0
Cell death [%]
controls
WT
4-2
5-1
5-2
birinapant
Z-VAD
necrostatin
necrosulfonamide
+ + - -
+ + + -
+ + - +

C



D

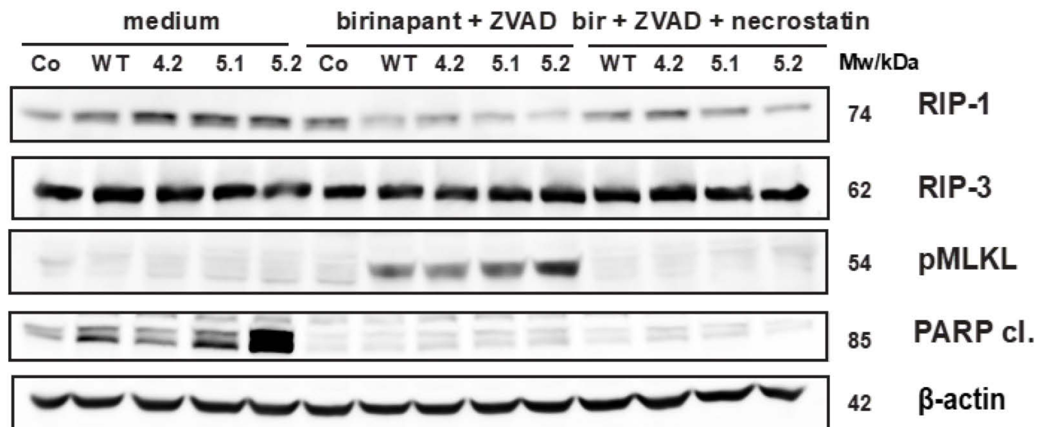


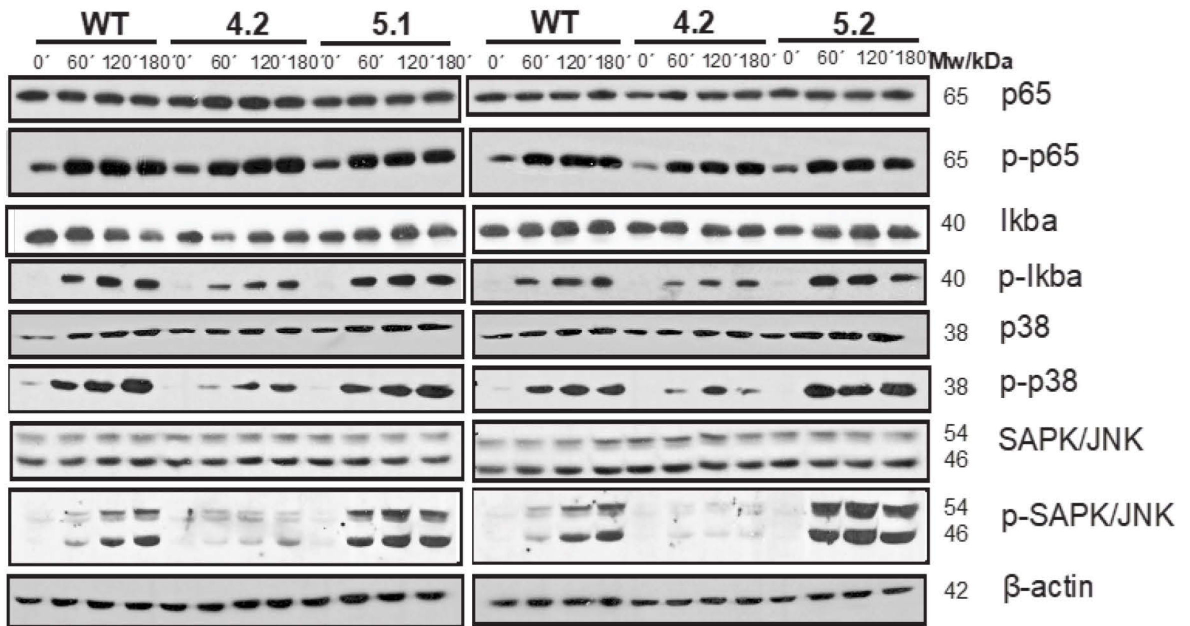
Figure 4.12: Both DR4 and DR5 receptor-specific TRAIL ligands efficiently induce necroptosis in HT-29 cells. HT-29 cells were pre-treated with combinations of 10 nM birinapant plus 50 µM z-VAD +/- 50 µM necrostatin-1 for 1 h and then treated with increasing concentration of TST-TRAIL WT for 8 hrs (A). HT-29 were pre-treated with the combinations of 10 nM birinapant, 50 µM z-VAD +/- 50 µM necrostatin-1 or 20 µM necrosulfonamide for 1 h, and then treated with 100 ng/ml of TST-TRAIL WT, 4.2, 5.1 and 5.2 for 8 hrs (B). HT-29 cells were pre-incubated with 10 nM birinapant and 50 µM z-VAD for 1 h and then treated with increasing concentrations of TST-TRAIL ligands for 6 hrs (C). Cell death was in all cases

quantified by propidium iodide staining and flow cytometry. HT-29 cells were pre-treated with the combination of 10 nM birinapant, 50 μM z-VAD and 50 μM necrostatin-1 for 1 h, and then TRAIL ligands were added at the concentration of 100 ng/ml for 3hrs (D). Samples were analysed by Western blotting. Data in Fig. 4.11 A-C were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The presented data are representative of at least three biological replicates.

4.1.7 DR5 receptor-specific TRAIL ligands efficiently induce NF-κB, p38 and JNK signalling under both apoptotic and necroptotic conditions

Along the induction of cell death, ligands from the TNF family can upon binding to their cognate receptors activate a number of non-apoptotic signalling pathways such as the NF-κB, MAP and stress kinases that might drive cell proliferation, migration and even grant cell survival. Similarly, as for the apoptotic and necroptotic signalling, we were interested whether and how TRAIL receptor-specific ligands might also affect these auxilliary signallings in HT-29 or PANC-1 cells under either pro-apoptotic or pro-necroptotic conditions. We examined an effect of receptor-specific TRAIL variants on the activation of NF-κB, MAP kinase p38 and stress kinase JNK in these cells. In general, under pro-apoptotic conditions, DR4-specific TRAIL variant was less effective in the efficacy of activation of these signalling pathways both in HT-29 and PANC-1 cells (Fig. 4.13 A-B). Notably, activation/phosphorylation of JNK kinases and IκB phosphorylation was very inefficient in TST-TRAIL DR4.2-treated HT-29 cells and apparently less efficient also in PANC-1 cells. Under necroptotic conditions, these differences among receptor-specific TRAIL variants were blunted in TST-TRAIL-treated HT-29 cells (Fig. 4.13 C), possibly reflecting similar efficacy of wild-type and receptor-specific variants in triggering necroptosis of HT-29 cells.

A



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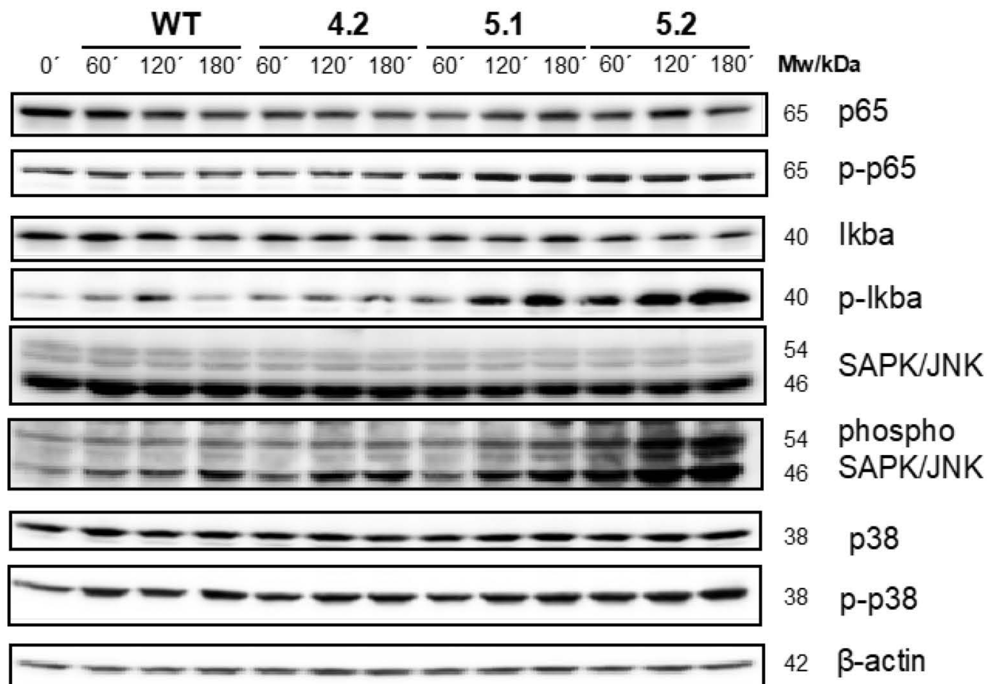


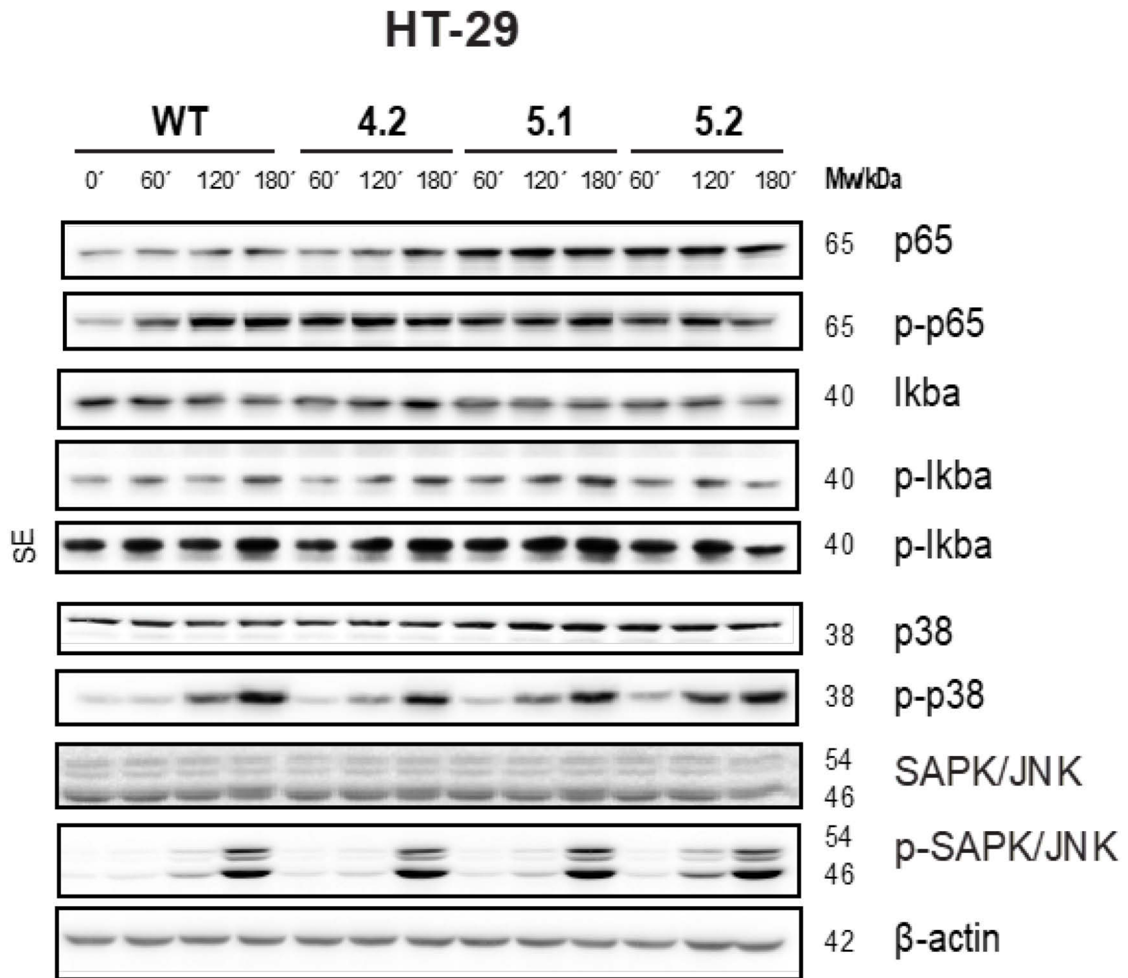
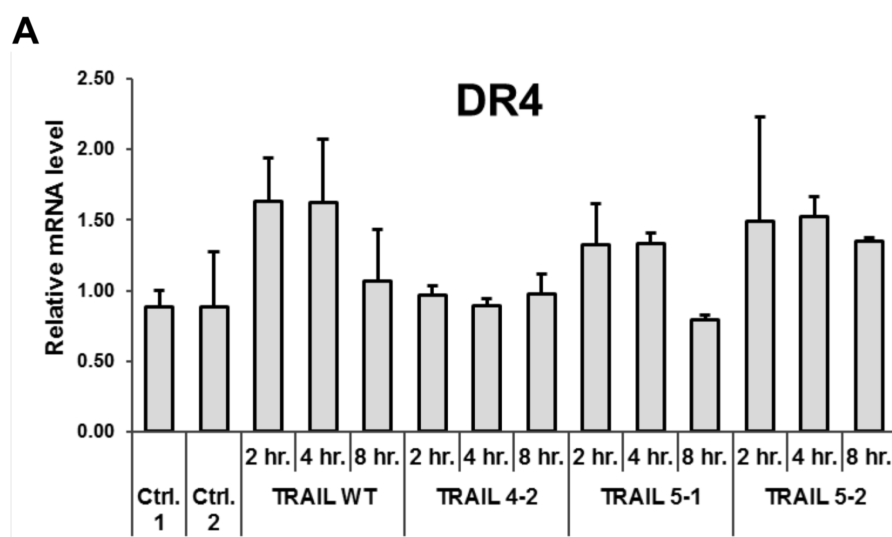


Figure 4.13: DR5 receptor-specific TRAIL ligands effectively induce NFκB, p38 and JNK signalling under apoptotic and necroptotic conditions.

HT-29 cells (A) and PANC-1 (B) were treated with 100 ng/ml of TST-TRAIL WT, 4.2, 5.1 and 5.2 for 60-180 min (A, B) or were pre-treated with both 10 nM birinapant and 50 µM z-VAD for 1 h and then treated with 100 ng/ml of TST-TRAIL ligand variants for 60-180 min (C). Cell lysates were analysed by Western blotting. SE stands for the stronger exposition. The presented data are representative of at least three biological replicates.

4.1.8 TRAIL ligands upregulate mRNA expression of several cell death-related genes including DR4 and DR5 receptors

To further explore the TRAIL signalling induced by different receptor specific TRAIL ligands and being puzzled by our finding that shRNA-mediated downregulation of DR4 receptor slightly enhanced cell surface expression of DR5 receptor and vice versa (see Fig. 4.10) we examined an effect of TRAIL ligand variants on the mRNA expression of receptors DR4 and DR5 and some other cell death-related genes (cIAP1/2). Interestingly, we found that treatment of HT-29 cells with TST-TRAIL WT and DR5 specific TRAIL ligands in HT-29 cells for different time periods led to increased mRNA expression of DR4 receptor (Fig. 4.14 A). Similarly, TST-TRAIL WT and DR5 selective ligands also enhanced mRNA expression of DR5 (Fig. 4.14 B). The increase of DR4 and DR5 mRNA expression was traceable after 2 and 4 hours and declined after 8 hours of incubation of HT-29 with TRAIL ligands. Likely we observed increased levels of both cIAPs mRNA using all TRAIL ligands but mostly in case of TST-TRAIL DR5.01 and DR5.02.



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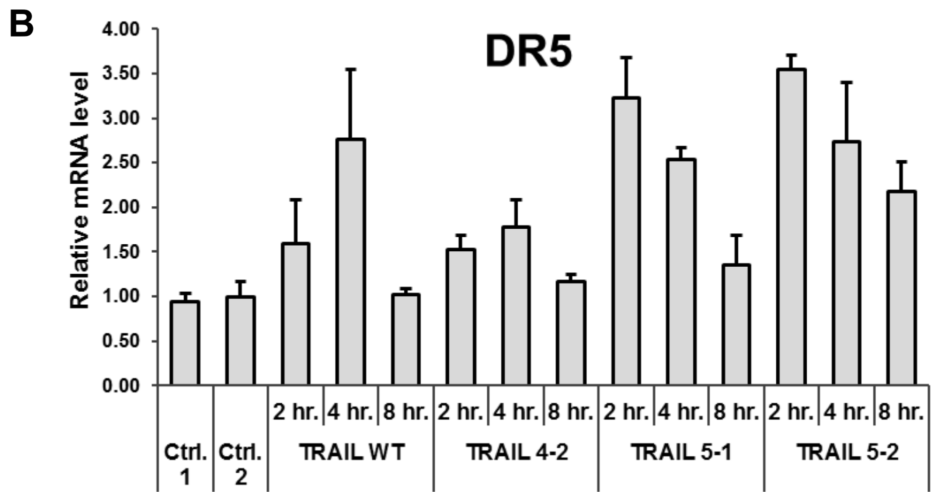

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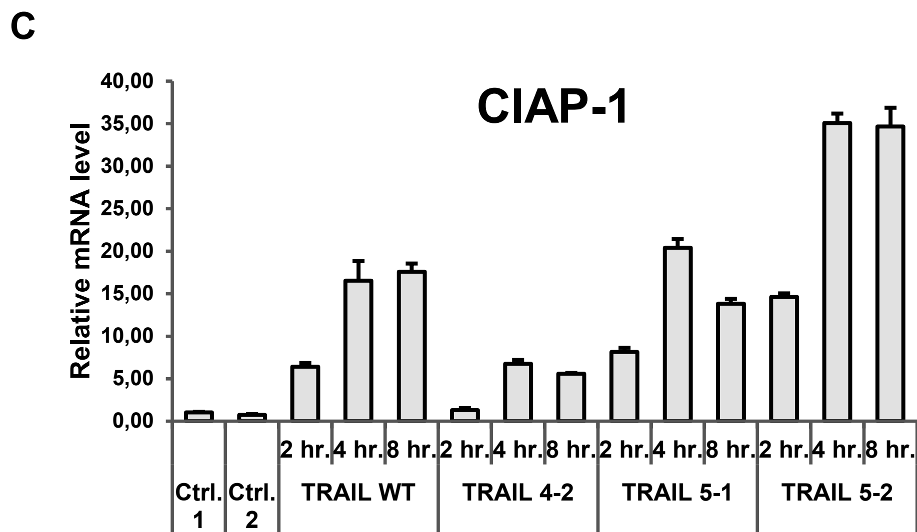

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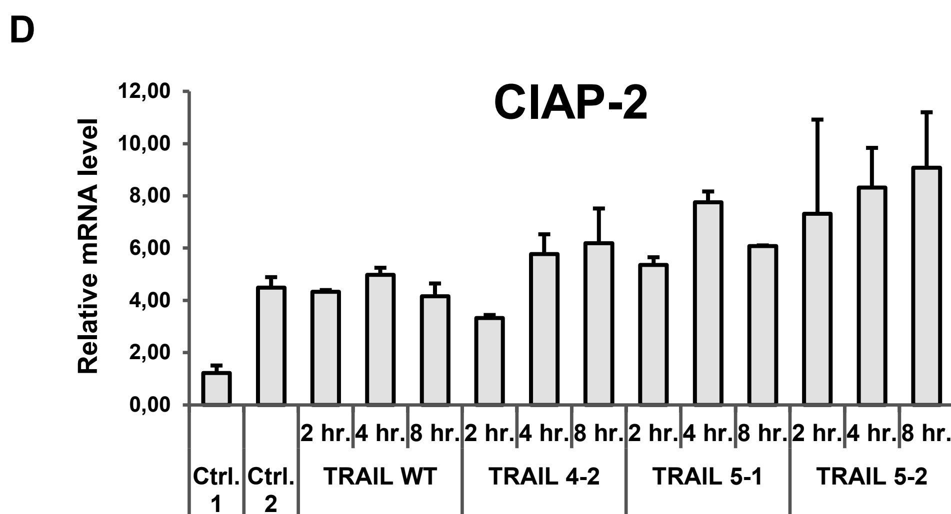


Figure 4.14: TRAIL ligands induce up-regulation of DR4, DR5 and cIAP1/2 mRNAs expression in HT-29 cells. HT-29 cells were treated with 100ng/ml TST-TRAIL ligands WT, 4.2, 5.1, 5.2 for 2, 4 and 8 hrs and mRNA for DR4 (A), DR5 (B), cIAP1 (C), cIAP2 (D) were quantified by RT-qPCR. Samples were normalized to 4 house-keeping genes GAPDH, β-actin, RPL37A and SDH. Ctrl.1 and ctrl.2 are non-treated cells. Data were analysed by GraphPad Prism 6 software and the presented data are representative of at least two biological replicates.

4.2 Human embryonic and induced pluripotent stem cells express TRAIL receptors and can be sensitized to TRAIL-induced apoptosis

Summary

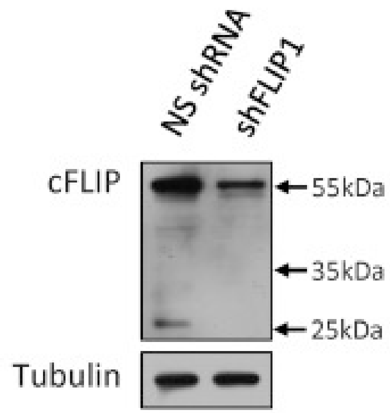
Inducing of apoptotic signalling in somatic cells is broadly characterised but not much is known about regulated cell death occuring in human stem cells. In colaboration with prof. Ales Hampl laboratory we tried to describe several main inducers of apoptosis from TNFR receptor family, which are well known triggers of cell death in somatic cells. We were interested whether essential components of these pathways are expressed and able to activate cell death in human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC). Even though both cell types expressed TNFR1, DR4/5 receptors and proteins inevitable for efficient induction and progression of extrinsic apoptotic signalling , they were not responsive to TRAIL induced apoptosis. Using inhibitor of translation homoharringtonine (HHT) resulted in sensitizig of hESC and hiPSC cell lines to TRAIL induced apoptosis. Further experiments revealed that HHT treatment caused decrease of anti-apoptotic proteins cFLIP and Mcl-1, which in combination with TRAIL ligand enhanced processing of initiator caspase-8 and downstream effector caspase-3. Downregulation of cFLIP protein by shRNA proved the important regulatory function of this protein in TRAIL-induced apoptosis in hESC and hiESc. We provided the first evidence that, irrespective of their origin, human pluripotent stem cells express canonical components of the extrinsic apoptotic system and on stress can activate death receptor-mediated apoptosis.

Declaration of honour

Author contributed to this project by preparing cell line with downregulated cFLIP. Initially were prepared lentiviral particles containing either non-targeting (in this work shown as non-silencing NS) control shRNA and two shRNA against cFLIP subcloned into pLKO plasmid containing puromycine resistance. These lentiviral particles were purified as described in Materials and Methods and subsequently used for transduction of hESC cell line. Transduced, puromycin-resistant cells were selected and used in further experiments as showed in Fig. 4.15-4.16. Experiments in Fig. 4.15-4.16 were done in prof. Ales Hampl laboratory, Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno, Czech Republic by Vladimir Vinarsky and his colleagues.

4.2.1 cFLIP represents a safeguard protecting human pluripotent stem cells against TRAIL-induced apoptosis

In our previous work from Beranova et al., we showed that homoharringtonine (HHT) is a potent sensitizer for TRAIL induced apoptosis in several colorectal cancer cell lines. Sensitizing effect of homoharringtonine is mainly mediated through downregulation of cFLIP and Mcl-1 anti-apoptotic proteins. In this work Vinarsky et al. showed that human hESC and hiPSC were resistant to TRAIL-induced apoptosis and could be sensitized by using HHT. To address a role of cFLIP and Mcl-1 in resistance of hESC to TRAIL-induced apoptosis were prepared recombinant lentiviruses expressing shRNA specific to each of these proteins, one non-targeting control (non-silencing NS), two different shRNA against cFLIP (shFLIP1/2) and three shRNA for Mcl-1. Selected cells were tested for downregulation of either of the protein as showed for cFLIP in Fig. 4.15 A (only shFLIP1 is shown), downregulation of Mcl-1 was not successful (data not shown). Photo in Fig. 4.15 B displays the light microscopy of non-treated and TRAIL-treated NS and shFLIP1 transduced cell lines. While non-treated or NS transduced TRAIL treated cells had no morphological features of apoptosis, cells with downregulated cFLIP and treated with 200 ng/ml of TRAIL WT ligand showed rounded and detached morphology after 6 hours of treatment. This observation was also confirmed by flow cytometry with antibody specific for PARP cleavage where was revealed significant increase in apoptotic cell death in shFLIP1 cell line (Fig. 4.15 C). Consistently with previous results apical caspases-8 and -10 and effector caspase-3 manifested pronounced cleavage in case of shFLIP1 (Fig. 4.15 D). It was published that cFLIP could contribute to not only apoptosis but also could prevent cells from necroptotic cell death (He et al., 2013). The exclude the possibility of contribution of necroptosis to the pool of observed dead cells, were cells exposed to pan-caspase inhibitor Z-VAD-FMK and caspase-8 inhibitor Z-IETD-FMK, respectively, cells were also treated with inhibitor of RIPK1 necrostatin-1, which is supposed to inhibit necroptotic signalling pathway. In these conditions, no changes in proceeding cell death were determined either by specific antibody against cleaved PARP1 (Fig. 4.16 A) or by DNA content release by subG1 method (Fig. 4.16 B) in cell lines with NS shRNA or shRNA against cFLIP when using necrostatin-1. On the contrary both caspase inhibitors were able to inhibit either cell death measured by flow cytometry or detected by cleavage of caspase-8 and -3 on Western blot (Fig. 4.16 A-C).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NS shRNA
shFLIP1
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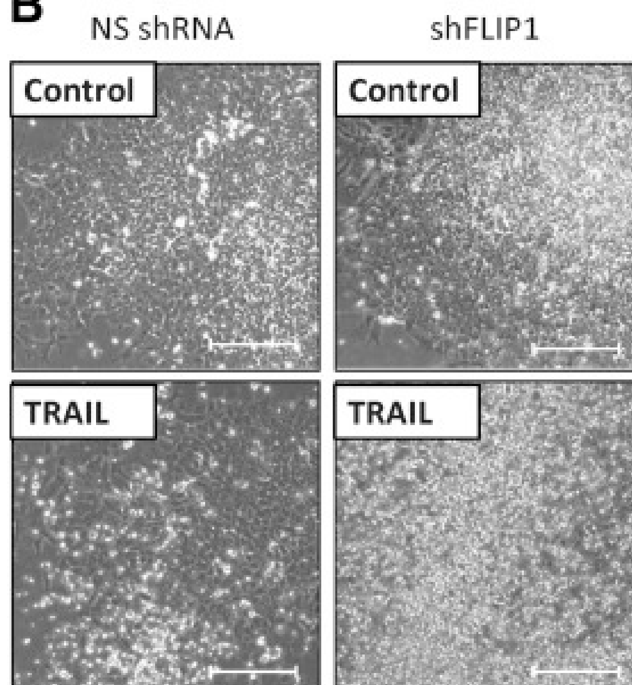
35kDa
25kDa
Tubulin


B
NS shRNA
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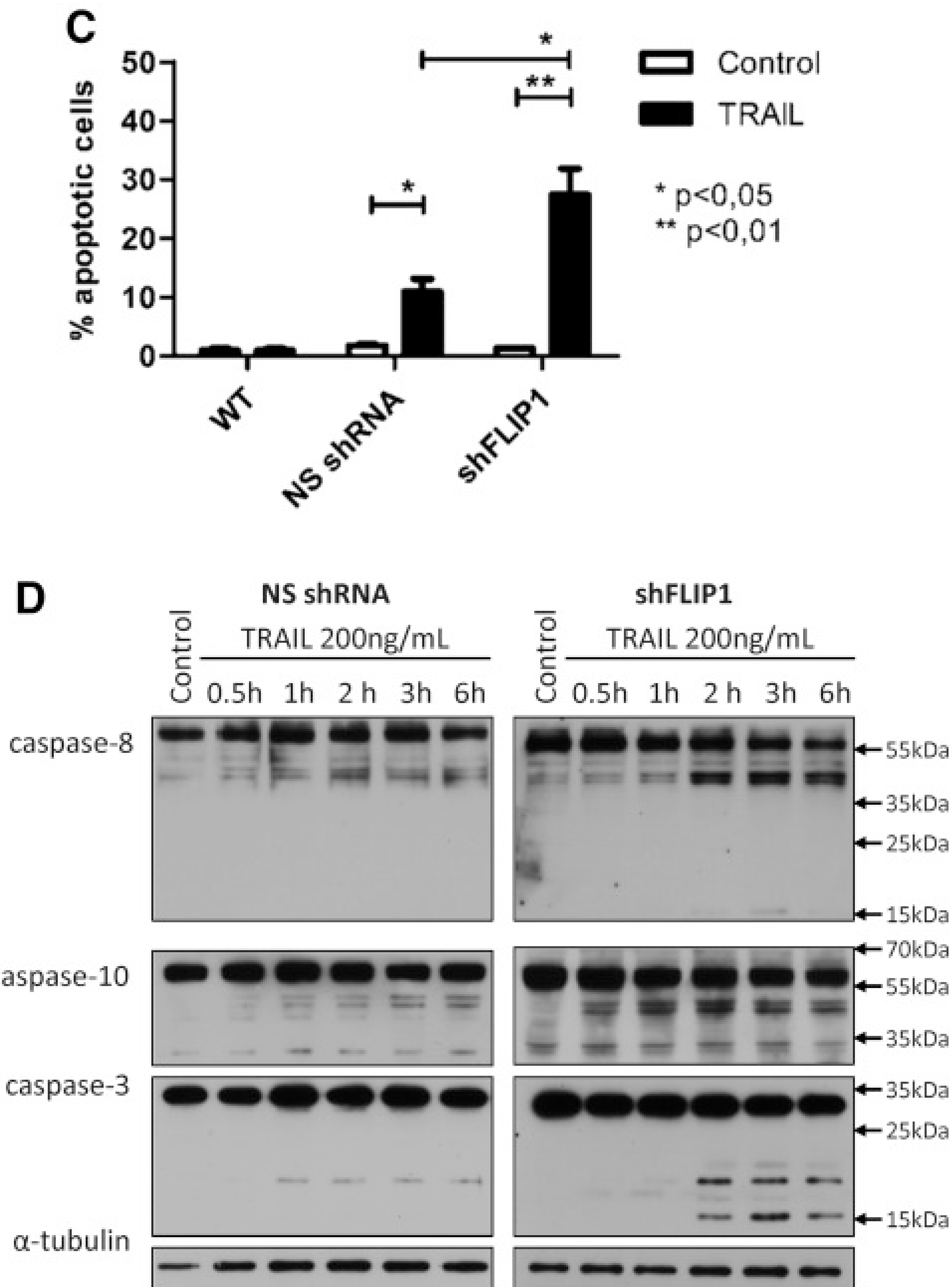


Figure 4.15: Knock-down of cFLIP sensitizes hESC to TRAIL-induced apoptosis. hESC line CCTL14 was transduced using lentiviruses expressing non-silencing shRNA (NS) or shRNA targeting cFLIP mRNA (shFLIP1). Western blot showing a decrease in cFLIP levels in shFLIP1-transduced cells comparing to NS cells (A). Nontransduced (WT), NS shRNA-transduced and shFLIP1-transduced cells were left untreated (control) or treated with 200 ng/mL TRAIL for 6 h. Induction of apoptosis was determined by light microscopy showing morphology of colonies and cell detachment (B) or by staining with antibody specific to PARP cleavage and analysed by flow cytometry (C). Western blot analysis of the activation of the

extrinsic apoptotic pathway in NS shRNA and shFLIP1-transduced cells after 30 min and 1, 2, 3, and 6 h of treatment with 200 ng/mL TRAIL (D) . Data in C were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The presented data are representative of three biological replicates.

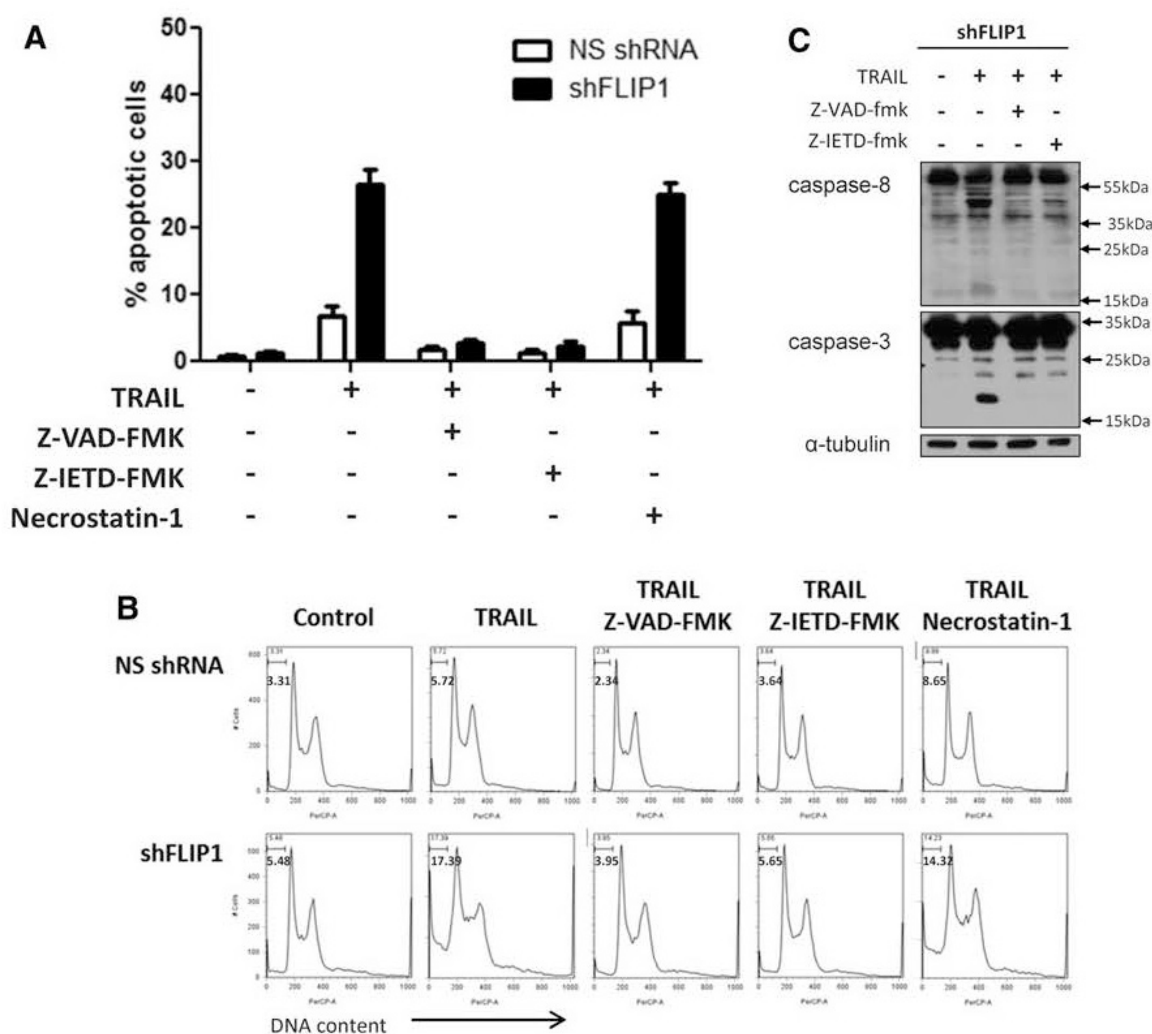


Figure 4.16 Caspase inhibitors suppressed TRAIL-induced apoptosis of hESCa with downregulated expression of cFLIP. Non-silencing (NS) shRNA and shFLIP1 cells were untreated (control), treated with 200 ng/mL TRAIL, or co-treated with 200 ng/mL TRAIL and 20 mM Z-VAD-FMK, Z-IETD-FMK or necrostatin-1. Induction of apoptosis was determined

by staining with an antibody specific to cleaved PARP (A) or determined by DNA content realease (B) and analysed by flow cytometry. Western blot analysis of activation of the extrinsic apoptotic pathway in cells with down-regulated expression of cFLIP after 3 h of treatment (C). Data in A-B were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The presented data are representative of three biological replicates.

4.3 Streptomycetes metabolites of the Manumycin family can via enhanced production of reactive oxygen species sensitize colon cancer cells to TRAIL-induced apoptosis (not published)

Summary

One of the main limitation of a use of TRAIL-induced apoptosis in cancer therapy is an acquired resistance of tumour tissue to its induction. Currently a number of chemical enhancers or sensitizers of TRAIL-induced apoptosis is being proposed including those targeting known anti-apoptotic proteins such as cFLIP and Mcl-1 targeting HHT or Bcl-2 proteins antagonists ABT-199 and ABT737 (Beranova et al. 2013, Konopleva et al., 2006). In colaboration with Dr. Petr Bartunek´s Laboratory of cell differentiation (Institute of Molecular Genetics, AV CR) we, in high throughput screenings for senziters of TRAIL induced apoptosis, identified Manumycin A as a compound strongly enhancing TRAIL-induced apoptosis of human colorectal carcinoma cell lines. In follow-up experiments we in addition to Manumycin A analysed effect of its relatives Manumycin B and Asukamycin, all of them known as inhibitors of farnesyltransferases (proteins responsible for post-translational modulation and maturation of Ras proto-oncogene). In our work we aimed to explore a mechanism of Manumycin A-mediated senzitization of colorectal carcinoma cell lines to TRAIL induced apoptosis.

Declaration of honour

Author participated in this project by measuring apoptosis in RKO and SW-620 cell lines and by measuring production of mitochondrial ROS after cell treatment with Manumycin A, Manumycin B and Asukamycin in Fig. 4.17 B and 4.19 respectively. Western blots and detection of caspase activation in Fig. 4.18 was performed by Martin Klíma.

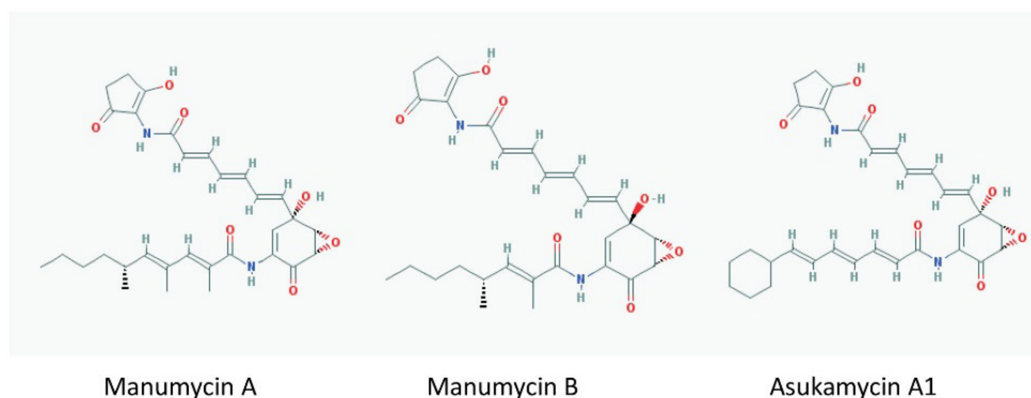
4.3.1 Manumycin A but not related Manumycin B or Asukamycin strongly enhances TRAIL- and BH3 analogs-induced apoptosis of resistant human colorectal cancer cells.

High throughput screening analysis of Manumycins-mediated enhancement of TRAIL-induced apoptosis of colorectal carcinoma cells lines showed that Manumycin A was superior to to its analogs Manumycin B and Asukamycin (Fig. 4.17 A) in their sensitizing to TRAIL-induced apoptosis (Fig.4.17 B). Both colorectal carcinoma cell lines SW-620 and RKO are resistant to TRAIL- and BH3 analogues-induced apoptosis however upon their pre-incubation

with Manumycin A (and to some extent also with Manumycin B) became largely sensitized to apoptosis induced by either of these agents. Interestingly another analogue Asukamycin was largely ineffective in enhancing apoptotic signalling (Fig. 4.17 B).

Enhancing effect of Manumycin A on TRAIL-induced apoptosis of RKO and SW-620 cells was also confirmed by Western blotting of cell lysates from treated colorectal cancer cells. As one can see on Fig. 4.18 pre-treatment with Manumycin A strongly enhanced self-processing and cleavage of caspases - 3 and -9 as well as some of their targets (caspase-8-cleaved Bid and caspase-3-cleaved XIAP) and thus further confirmed sensitizing effect of Manumycin A on TRAIL-induced apoptosis.

A



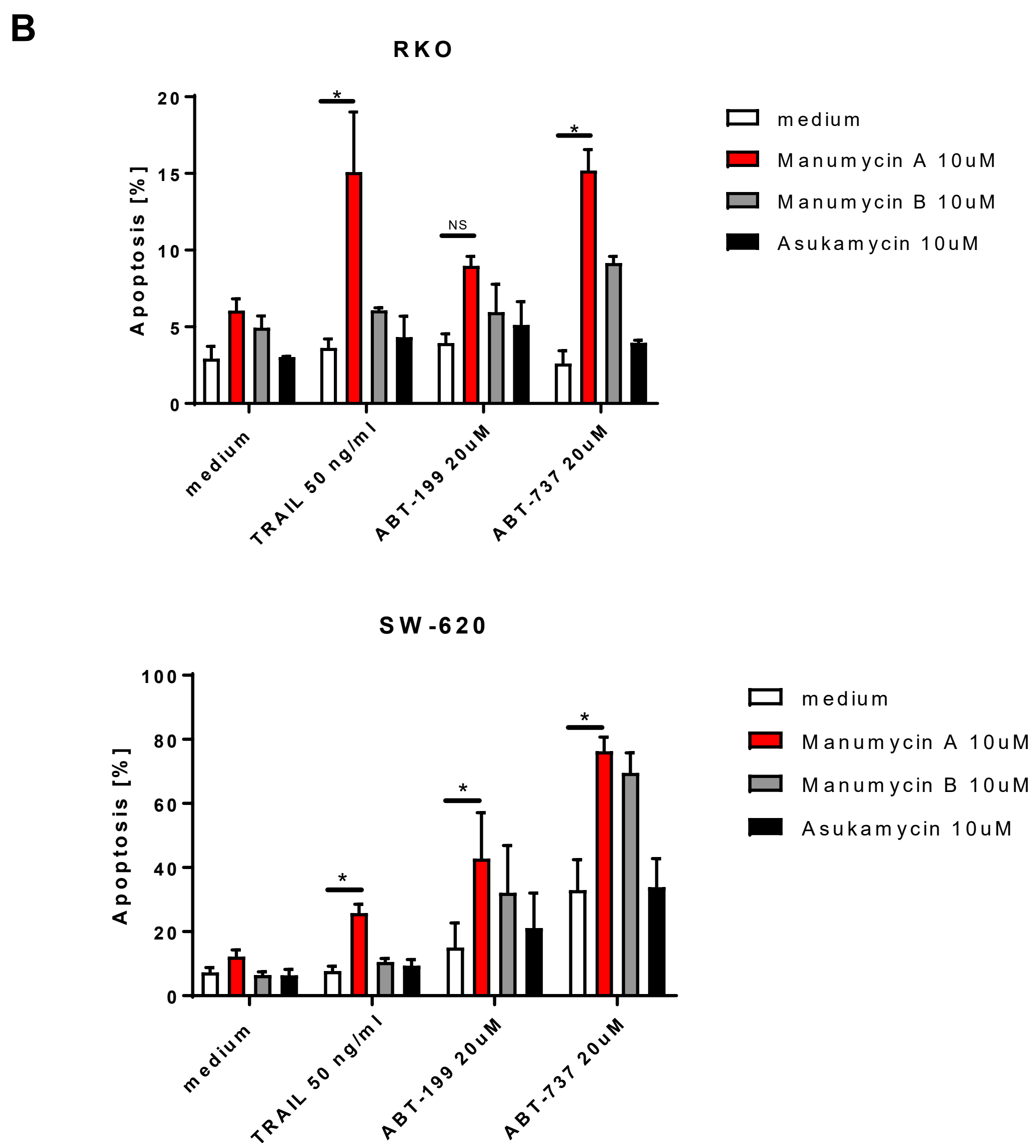


Figure 4.17: Manumycin A to lesser extent Manumycin B but not Asukamycin enhance TRAIL- or BH3 analogues-induced apoptosis of resistant human colorectal cancer cells. The structure of Manumycin A, Manumycin B and Asukamycin (A). For the quantification of apoptosis SW-620 and RKO colorectal cancer cells were treated with inhibitors 20 µM ABT-199/737, 50 ng/ml His-TRAIL ligand WT or combination with 10 µM of Manumycin A, Manumycin B and Asukamycin for 3 hrs. Cells were evaluated for apoptosis using annexin V-FITC and analysed by flow cytometry (B). Data in B were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The presented data are representative of two biological replicates for RKO cell line and three biological replicates for SW-620.

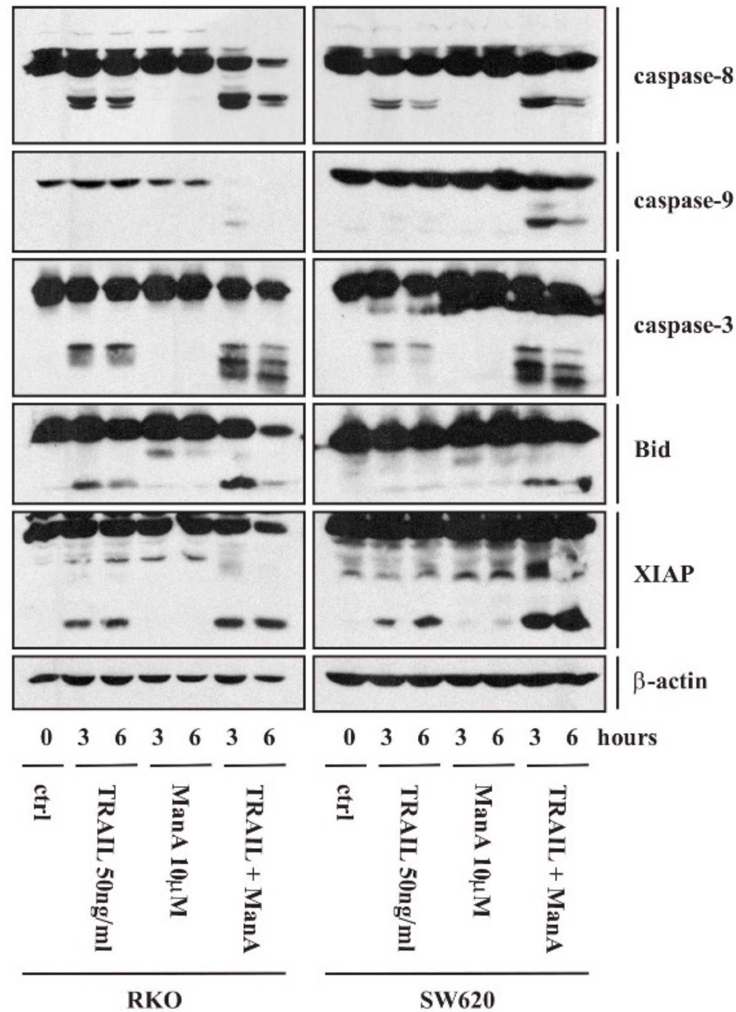


Figure 4.18: Manumycin A enhances TRAIL-triggered activation and activity of caspases. RKO or SW-620 cells were incubated with Manumycin A, TRAIL and their combination for 3 and 6 hrs and their lysates were analysed by Western blotting using antibodies given on the right. The Western blot is a representative of at least three biological replicates.

4.3.2 Manumycin A triggers mitochondrial ROS production

From previous results we observed the strong cleavage of apical caspase-9 in Manumycin A-pre-treated cells, which might indicate involvement of mitochondria induced apoptotic signalling such as increased ROS production. In order to test this hypothesis, three colorectal carcinoma cell lines HT-29, RKO and SW-620 were analysed for production of mitochondria induced ROS by using MitoSOX™ Red Mitochondrial Superoxide Indicator. Importantly, all three cell lines showed significantly increased production of ROS after Manumycin A

treatment, to notably lesser extent after Manumycin B treatment but no enhanced ROS production in Asukamycin-treated cells (Fig. 4.19).

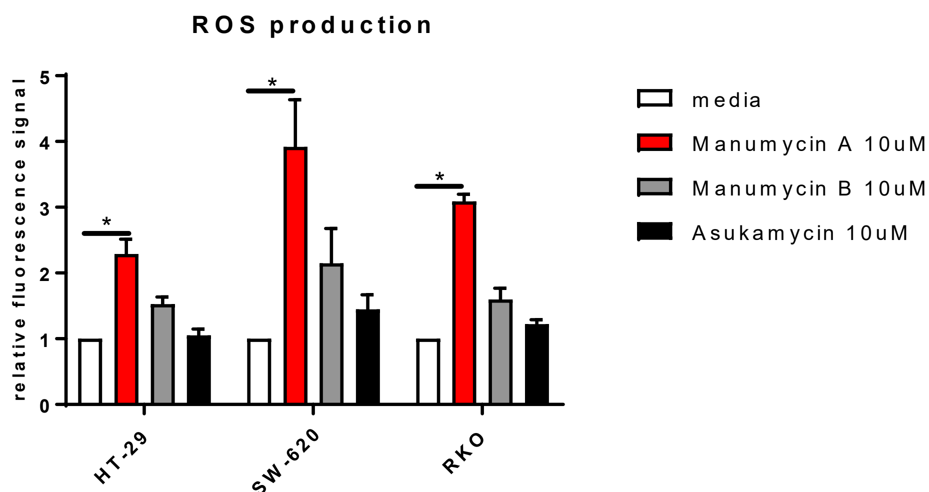


Figure 4.19: Manumycin A triggers significant elevation of mitochondria produced ROS in human colorectal cancer cells. HT-29, SW-620 and RKO colorectal cancer cells were treated with 10 µM of Manumycin A, Manumycin B and Asukamycin for 3 hrs and then ROS production was measured using MitoSOX™ Red Mitochondrial Superoxide Indicator and fluorescence signal in PE channel was detected by flow cytometry. Relative fluorescence signal was determined as mean fluorescence signal relative to control (media). Data were analysed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The presented data are representative of two biological replicates.

5 DISCUSSION

5.1 TRAIL induces apoptosis but not necroptosis in colorectal and pancreatic cancer cells preferentially via the TRAIL-R2/DR5 receptor

Dysregulation of apoptosis belongs among fundamental features of cancer progression (Hanahan and Weinberg, 2011). Therefore discovery and clinical use of cancer cell death promoting or enhancing drugs is one of the essential goals of cell death-related research. Identification of TRAIL ligand as a selective inducer of apoptosis in cancer cells led to the development of TRAIL receptor agonists (Yuan et al., 2018). In addition to its selectivity towards cancer cells, the use of TRAIL agonists in tumour therapy might take an advantage from being supported by other drugs with apoptosis-sensitizing effect. Importantly, apoptosis induced by these agonists is triggered even in human tumours with mutated p53 tumour suppressor. Two kinds of TRAIL agonists have been developed and tested in clinical studies: recombinant proteins and agonistic antibodies. Dulanermin, a zinc-coordinated, homotrimeric recombinant protein consisting of 114-281 amino acids of the endogenous polypeptide and recently other recombinant IgG fusion ligand ABBV-621 entered clinical trials. Apart from recombinant TRAIL ligands a number of agonistic antibodies targeting DR4 or DR5 (conatumumab, lexatumumab, mapatumumab etc.) were or are being either alone or in combination with other anti-cancer drugs evaluated in phase I-II clinical trials. Albeit all TRAIL agonists used in clinical trials were well tolerated, no statistically significant anticancer activity was achieved. Moreover many cancer cell lines were found to be resistant to TRAIL-induced apoptosis. Though unsuccessful, the outcome from the trials might lead to the formulation of better and possibly patient-tailored therapeutic protocols, which include the use of novel sensitizers of TRAIL-induced apoptosis in resistant cancer cells and the discovery and preparation of modified TRAIL-based agents (e.g. HERA-TRAIL/ABBV-621).

In the fundamental part of this study we focused on the receptor-selective analysis of TRAIL-induced signalling in human cells. The promiscuity of TRAIL ligand for binding of five different receptors, three of them non-apoptotic, and formation of hetero-trimeric non-apoptotic complexes of DR4/DR5 with decoy receptors Dcr1/DcR2 encouraged the concept that death receptor specific selectivity could be the possible way to find more potent inducers of TRAIL triggered apoptosis. Differential contribution of either of the TRAIL receptor was

already proposed in several reports in cancer cells of various origin but no detail study dealing with all TRAIL receptors specific signalling outcomes was published so far (Kelley et al., 2005; MacFarlane et al., 2005; Lemke et al., 2010; Lemke et al., 2014). Thus our systemic approach using modified/enhanced DR4- and DR5-selective ligands might be in addition to enhanced systemic knowledge of their signalling of a potential applied outreach in tailoring cancer therapy in TRAIL receptor-selective way.

5.1.1 Preparation of TRAIL receptor-specific ligands

Our novel/upgraded soluble recombinant ligands are composed of the extracellular region of human TRAIL with amino acids 95-281 containing, in addition to the mutated extracellular part of TRAIL, trimerisation/stabilisation (TRI) motif and TwinStrep tag at their N-termini. Moreover, trimerisation of DR receptors is required for effective induction of apoptosis and thus agonistic antibodies are rather weak inducers of apoptosis due to their bivalent nature allowing only dimerization of TRAIL receptors. To enhance purification and stability of the recombinant TRAIL a number of approaches introducing different tags (His, Flag,) and trimerising domains (leucine zipper (LZ) or isoleucine zipper (iz)) were developed. Though some of these tags extended the stability of recombinant proteins, they were also accompanied by increased hepatotoxicity *in vivo* (Yuan et al., 2018). In our modified recombinant TRAIL ligands, T4 phage trimerisation/stabilisation (TRI) motif enhances stability and trimeric state of the ligands and the Twin Strep tag allowed (in addition to its use for the purification of *E.coli*-expressed recombinant ligands) the most effective approach for the purification of TRAIL DISC complexes (Papanikolopoulou et al., 2005). A selective and computer design-based point mutations of sequence of human TRAIL ligand led to the preparation of novel highly active and DR4- or DR5-specific variants of the ligand. These mutations were published to be selective for each cognate receptor and efficiently induced apoptosis in cancer cells (Gasparian et al., 2009; Reis et al., 2010; van der Sloot et al., 2006).

5.1.2 TRAIL-induced apoptosis

Efficacy and selectivity testing of our modified TRAIL receptor-specific ligands was carried out using hematopoietic cell lines Ramos and Jurkat, which were previously shown to transduce apoptotic signalling in TRAIL receptor-specific manner (Jang et al., 2003; Sung et al., 2009). The modified DR4- and DR5- targeted TRAIL variants were, as expected from already published data, highly selective, inducing apoptosis more efficiently than TST-TRAIL

WT via DR5-mediated signalling in Jurkat T cells or DR4-mediated signalling in Ramos B cells.

For detailed analysis of DR4- vs. DR5-specific signalling were chosen as model cell lines, colorectal HT-29 and pancreatic PANC-1 cells. We confirmed the published data that these cells are resistant to TRAIL WT-induced apoptosis and thus for its enhancement we used two types of enhancing agents: a/ inhibitor of translation homoharringtonine (HHT), which downregulates expression of short-lived anti-apoptotic protein cFLIP and Mcl-1 or b/ Bcl-2/Bcl-XL inhibitor ABT-737. The detail analysis of TRAIL receptor specific ligands signalling in colorectal HT-29 cells revealed that DR5-specific ligands are more effective than DR4-targeted ligands or even wt TRAIL in the activation of caspases and following initiation of apoptosis in HT-29 cells. Interestingly, early apoptotic process of DISC formation proceeded similarly for the TST-TRAIL WT and all TRAIL receptors-specific variants in HT-29 cells, suggesting enhanced downstream processing of caspase-8 in cells treated with DR5-selective ligand. This phenomenon could for example happen due to different turn-over of DISC proteins. In support of this option, two articles published by McDonald and col. and Jin and col. suggest that caspase-8 ubiquitination influences the processing of available caspase-8 cellular pool (McDonald et al., 2004; Jin et al., 2009).

Majority of so-far published data point to DR5 receptor as a dominant receptor signalling in TRAIL-induced apoptosis in colorectal cancer cells (Kelley et al., 2005; Mohr et al., 2015). However, recently published data by Dufour and col. obtained using DR4 or DR5 gene editing in HCT-116 and SW-480 colorectal cancer cells, show a bit contradictory outcome (Dufour et al., 2017). Cells with inactivated expression of DR4 become upon induction of ER stress resistant to His TRAIL or DR5 receptor specific antibody lexatumumab, pointing to DR4 as a main apoptotic receptor in ER induced apoptosis. Similarly Glab and col. published that DR5 and even caspase-8 are dispensable for ER induced apoptosis (Glab et al., 2017). However, their data became later questioned by Lam and his colleagues who used the same cell lines but were not able to reproduce the same results and confirmed the dominant role of DR5 in ER-induced apoptosis (Glab et al., 2017; Lam et al., 2018). Additionally, other recent report documents that DR5 receptor is the one, which responds to stress triggered by unfolded protein response (Lu et al., 2014). Our data obtained using modified TRAIL receptor-specific mutants support major impact of DR5-triggered apoptotic signalling in colorectal HT-29 cells. In addition to apoptosis, DR5-selective ligands are also superior to DR4-selective ligand in the activation of auxiliary signalling (stress kinases and NFkB). Especially strong DR5.2-mediated

activation of the long isoforms of JNK1 kinase might enhance DR5-triggered apoptotic signalling in colorectal cancer cell lines (Mahalingam et al., 2009). Our results are additionally supported by shRNA-mediated knockdown of either DR4 or DR5 expression in HT-29 cells, which in contrast to gene editing-mediated inactivation of these receptors, do not entirely eliminate the expression of either of these receptors, and thus allow for their possible cross-talk. In addition, we found that engagement of DR5 receptor led to an increase in the transcription of DR5 gene, which could lead to increased level of DR5 receptor and subsequent more efficient DR5 triggered apoptosis. Moreover, several reports document that ubiquitin mediated degradation preferentially targets DR4 receptor, which also likely contributes to the enhanced DR5-mediated apoptotic signalling (Liu et al., 2007; Song et al., 2010; van de Kooij et al., 2013). However, DR4/DR5 requirement is likely cell/cancer specific and in some cells could be DR4 required to support DR5-mediated signalling and thus its deletion could severely compromise DR5-triggered apoptosis. From the reasons mentioned above and according to our and recently published data we believe that DR5 receptor is a dominant inducer of apoptosis in colorectal cancer cells. An interesting work from posttranslational modification of TRAIL receptors might be also behind different response of cancer cells to DR5- or DR4-specific signalling. Colorectal cancer cells which are not responsive to DR5, but are responsive to DR4 (DLD-1 and HCT-116) have decreased levels of fucosyltransferase 3 (FUT3) and -6 (FUT6), enzymes responsible for the fucosylation of DR5 receptor in colon cancer cells. Ectopic expression of these enzymes restored the dominant character of DR5-mediated apoptosis in both DLD-1 and HCT-116 cell lines (Zhang et al., 2019). Importantly fucosylation of DR5 has impact on ligand-independent receptor association resulting in better DISC formation. Basement for the use of DR5 specific ligands in treatment of colorectal cancer favours also the study pointing to the mRNA levels of different apoptosis involved genes: *DR4*, *DR5*, *c-IAP1/2*, *XIAP* and *BIRC5*/survivin genes in 100 colorectal cancer tissues from tumours from colorectal cancer patients. Analyses showed up-regulation of *DR5*, *XIAP* and *BIRC5*/survivin while *c-IAP1* and *c-IAP2* were downregulated (Devetzi et al., 2016). CIAP1/2 is required for NFkB activation and likely negatively regulates necroptosis via ubiquitinilation of RIP1 kinase. Notably, among genes that we found upregulated in DR5-selective ligands treated cells were in addition to DR5 itself also cIAP1 and cIAP2. Interestingly, up-regulation of DR5 was observed independently on *KRAS* mutation status, while recent data showed involvement of DR5 in editing of tumour microenvironment and supporting the cancer progression in TRAIL apoptosis resistant *KRAS* mutated cancer cell lines (Karstedt et al., 2015; Karstedt et al., 2017).

To broaden our study we included into our analysis of DR4/5-selective signalling pancreatic cancer cells. PANC-1 cell line showed similarly as HT-29 cells preferential signalling via DR5 receptor. Both DR5-specific ligands and, notably, more efficient DR5.2 ligand were, despite lower cell surface expression of DR5 comparing to HT-29 cells, significantly more efficient in triggering apoptosis in PANC-1 cells. Notably, DR5.2 ligand triggered already significant apoptosis in PANC-1 cells even in the absence of a sensitizer/enhancer. Although the initial steps in TRAIL-induced apoptosis involving assembly of DISC and DISC mediated activation of caspase-8 proceeded similarly for the TST-TRAIL WT and all TRAIL receptors-specific variants in HT-29 cells, DR5-specific ligands are then superior in the amplification of apoptotic signalling. In contrast to our data, several reports mainly employing TRAIL receptor agonistic antibodies claim that pancreatic PANC-1 cancer cells preferentially use DR4 receptor for the induction of apoptosis (Lemke et al., 2010; Stadel et al., 2010). These apparent controversies could have several reasons likely including enhanced activity and stability of our TRAIL receptors-specific ligands. Enhanced efficacy of our ligands could be related to the trimerisation motif that allows forming of receptor trimers comparing to monoclonal antibodies. It was also published that post-translational modifications could have impact on DR signalling. For example positive impact on signalling was showed for O-glycosylation of DR5 or N-glycosylation of DR4 thus our ligands might interact differently than other similar recombinant TRAIL receptor specific ligands with differently glycosylated cognate receptors in cancer cell specific manner (Wagner et al., 2007; Dufour et al., 2017). Very attractive reason for more effective kinetics in DR5 apoptotic signalling could be the result of more efficient chain formation of caspase-8 in DR5-selective DISC complexes with rapid kinetics of caspase-8 processing (Dickens et al., 2012; Schleich et al., 2013). Additionally, screening of pancreatic and colorectal cancer cells for their response to TRAIL receptor-specific variants revealed the preference of DR5 receptor for PANC-1 cells (Mohr et al., 2015).

Other support of DR5 preference for apoptotic signalling in pancreatic cancer comes from the report documenting that pancreatic cancer stem cells from patient xenografts respond to DR5 agonistic antibody drozitumab (Eng et al., 2016). Treatment of immunocompromised SCID mice bearing patient-derived pancreatic tumour xenografts (PDX) with drozitumab alone inhibited growth of pancreatic cancer xenografts as well as inhibited development of tumours in mice implanted with pancreatic cancer stem cells (CSCs). Importantly drozitumab treatment was efficient even in the PDX with previously characterized (resistant) responses to Apo2L/TRAIL treatment. Additionally authors proved that nearly all pancreatic CSCs express DR5 and are responsive to drozitumab while only 25% of tumour mass express DR5.

Interestingly, though the majority of the bulk tumour did not express DR receptor, the continuous administration with drozitumab led to increase of DR5 expression and tumour regression. Moreover, the in vitro study with PANC-1 cell line showed that mRNA of DR5 receptor increases with the time of drozitumab treatment, which is in an agreement with our data documenting increased DR5 mRNA expression in HT-29 cells treated with DR5-selective ligand. To sum up authors of this paper showed that targeting DR5 alone is sufficient to eliminate the population of pancreatic CSCs derived from patients as well as inhibit the further tumour growth of PDX in mice. The results from this study and importance of DR5 receptor in cancer stem cells are also strongly supported by *in vitro* experimental model mimicking breast cancer microenvironment of blood stream with circulating cancer cells which were found out to be resistant to TRAIL-induced apoptosis due to autophagy of DR5 receptor (Twomey et al., 2019). Interestingly autophagy of not only DR5 but other death receptors including TNFR1, FasR and DR4 occurred but DR5 especially was targeted for lysosomal degradation.

5.1.3 TRAIL-induced necroptosis

TRAIL can similarly as TNFR1 receptor induce other mode of regulated cell death, RIPK1/RIPK3 dependent necroptosis (Meurette et al., 2005; Jouan Lanhouet et al., 2012). One of the crucial players deciding whether the cell commits apoptosis or necroptosis is caspase-8. Caspase-8 mediated cleavage of RIPK1 and RIPK3 kinases, the core components of necrosome complex, blocks necroptosis and drives cells to apoptotic mode of cell death (Sosna et al., 2016). Other key factors influencing necroptotic cell death are IAP proteins. These anti-apoptotic E3 ubiquitin ligases target RIPK1 to proteosomal degradation (Sosna et al., 2016). Thus only combined treatment of HT-29 cells with pan-caspase inhibitor zVAD and IAP antagonist/Smac mimetic birinapant efficiently induced dose-dependent necroptosis in TRAIL WT-treated HT-29 cells. The efficient induction of necroptosis was proved by several approaches. At first pre-treatment of HT-29 cells with specific inhibitor of RIPK1, necrostatin-1, and inhibitor of MLKL protein, necrosulfonamide, efficiently suppressed proceeding of cell death. Using pan-caspase inhibitor z-VAD prevented the activation and activity of caspase-8, displayed by decreased cleavage of the downstream apoptotic target of caspase-3, PARP protein. Moreover induction of necroptosis in HT-29 cells was also confirmed by phosphorylation of MLKL protein, which is together with RIPK1 and RIPK3 essential component of necrosome complex in death receptor-induced necroptosis (Voigt et al., 2014). However, in contrast to apoptosis, all DR4- and DR5-specific ligands were, at saturating

concentrations, equally effective in triggering RIP1- and MLKL-dependent necroptosis in HT-29 cells. Notably, in spite of equal activation of necroptosis at saturating concentration of TST-TRAIL ligands, we observed higher efficacy of the DR5.2 ligand at its lower concentration, likely reflecting its higher binding affinity for the DR5 receptor (Gasparian et al., 2009).

5.1.4 NFκB, p38 and JNK kinase signalling

TRAIL-induced activation of DR4 or DR5 receptors in both permissive and resistant cancer cells leads in addition to cell death signalling also to the activation of non-canonical/auxiliary signalling pathways often enhancing cell survival or proliferation. Activation of these signalling pathways especially in cancer cells resistant to TRAIL-induced apoptosis would be counterproductive and taking in account recent studies indicating metastasis-promoting activity of TRAIL, suppressing these auxiliary signalling would be essential for any consideration of TRAIL-based therapy in cancer treatment (Karstedt et al., 2015; Hartwig et al., 2017). Activated TRAIL receptors DR4/5 depending on circumstances and cellular origin/background can activate following signalling: RIPK1, IκB/NFκB, MAPK p38, MAP3K TAK1, JNK1/2, ERK1/2, PKC, PI3K/Akt or Src (Karstedt et al., 2017). In our study we mainly focused on the activation of NFκB pathway, p38 and JNK kinases. TRAIL-mediated activation of NFκB signalling can attenuate TRAIL-induced apoptosis via transactivation of anti-apoptotic proteins as cFLIP or Bcl-XL and in some cases could promote tumour cell migration and invasion without influencing the proliferation (Karstedt et al., 2015; Hartwig et al., 2017). Treatment of colorectal HT-29 as well as pancreatic PANC-1 cell lines with TRAIL ligands led to the efficient induction of NFκB signalling pathway, reflected mainly in the phosphorylation its inhibitor IκBa. In an agreement with apoptotic signalling, DR5-selective ligands were also most effective inducers of NFkB signalling. Interestingly, NFκB can in addition to its anti-apoptotic transcriptome also mediate up-regulation of death receptors DR4 and DR5. We were interested whether dominant character of DR5 receptor could be reflected also in the transcription level of both receptors DR4 and DR5. Using WT ligand and ligands specific to DR4 and DR5 we observed that WT TRAIL induced higher expression of DR5 than DR4 receptor. DR5 ligands, in consistence with previous data, induced increase of DR5 receptor expression and interestingly also 2-times folder increase in the expression of DR4 receptor. Surprisingly ligand specific to DR4 receptor did not induce higher expression of its own cognate receptor but induced 2-times fold enhancement of DR5 expression. The enhancement of DR5 mRNA expression thus also reflects increased efficacy of DR5-selective ligands in the

activation of NFkB signalling suggesting that this signalling is in our cells likely responsible for the increased expression of DR5.

Similarly the activation of other non-canonical pathways, members of stress kinase subfamily of MAP kinases, p38 and JNK, was significantly stronger in cells treated with DR5-specific ligands. p38 was shown to have anti-apoptotic as well as pro-apoptotic effect in TRAIL induced signalling. For example in prostate cancer cells led phosphorylation of p38 by induction of TRAIL signalling to up-regulation of anti-apoptotic protein Mcl-1 while in HeLa cell line was activating of p38 kinase substantial for proceeding of cell death (Azijli et al., 2013). JNK kinases can have either opposite effects on cell survival and depending on circumstances and cellular context might promote survival or enhance pro-apoptotic signalling. JNK kinase can synergistically contribute to TRAIL-induced apoptosis by phosphorylating Bim protein and thus facilitating mitochondrial apoptosis. Additionally JNKs activated by TRAIL receptors can phosphorylate the key component of autophagic pathway, Beclin-1, resulting in autophagy induced cell death in HCT116 cells (Azijli et al., 2013). We report that JNK kinase is predominantly induced by DR5 receptor in HT-29 and PANC-1 and this activation could have contributed to higher levels of measured cell death in apoptotic conditions in both cell lines. Therefore, very low activation of JNKs upon DR4.2 treatment of HT-29 cells could be an additional reason for poor performance of DR4.2 ligand in inducing apoptosis of these cells.

We were also interested, whether the necroptotic conditions would influence non-death auxiliary signalling induced by TRAIL ligands. Interestingly, upon necroptotic conditions TRAIL receptor-specific ligands enhanced activation these signalling pathways to a similar extent blunting differences between DR4- and DR5-triggered signalling. One of the reasons could be related to the endocytosis and endosomal acidification in a process of pro-death signalling from TRAIL receptors (Horova et al., 2013). Other reason could be that in apoptotic signalling, dynamin 1-mediated endocytosis likely attenuates TRAIL-mediated apoptosis, but it apparently does not affect TRAIL-triggered necroptosis (Zhang et al., 2008; Sosna et al., 2016; Reis et al., 2017).

In conclusion, we showed, in correlation with the published data, that combination of several sensitizing compounds as homoharringtonine or ABT-737 efficiently induced apoptosis in TRAIL resistant colon cancer HT-29 and pancreatic cancer PANC-1 cells. We also showed efficient induction of necroptosis in HT-29 cells. Using a number of approaches, we proved that TRAIL receptor-specific pro-apoptotic signalling in colorectal and in pancreatic cancer cells does largely rely on DR5 death receptor and this dependence is blunted in TRAIL-

triggered necroptosis. Importantly, we showed that in resistant HT-29 and PANC-1 cells were efficiently induced several kinase pathways which were predominantly relying signalling from activated DR5 receptor.

5.2 Human embryonic and induced pluripotent stem cells express TRAIL receptors and can be sensitized to TRAIL-induced apoptosis

The potent capability of stem cells to reconstitute the damaged and aged tissues drove attention to the stem cell research and their application in regenerative medicine. Moreover cancer patients undergoing radiation and chemotherapy are exposed to many harmful agents causing depletion of tissue stem cells pool. Secondary processes, such as oxidative stress and inflammation that accompany the radiation therapy not only regulate regeneration of normal tissue but could have impact on cell survival of any engrafted or transplanted stem cells. It was shown that human embryonic and induced pluripotent stem cells were sensitive to several stress factors inducing DNA damage and undergo apoptosis even at low exposure. This apoptosis is driven through the activation of intrinsic mitochondrial pathway, but not much is known about the extrinsic, death receptors-mediated one. Our study with Vladimir Vinarsky and col. from Masaryk University in Brno revealed the first insight into expression profile and functional state of major death domain-containing receptors TNFR1, Fas/CD95 and especially TRAIL receptors DR4 and DR5 in hESC and hiPSC. We showed that expression of Fas receptor and TNFR1 was rather low or none comparing to TRAIL receptors DR4 and DR5. Therefore we focused in further experiments on characterizing of TRAIL-induced signalling in stem cells. We found that despite moderate protein expression and cell membrane localization of both pro-apoptotic TRAIL receptors were both hESCs and hiPSCs resistant to TRAIL-induced apoptosis. hESCs treatment with inhibitor of translation homoharringtonine suppressed expression of two anti-apoptotic proteins Mcl-1 and cFLIP and led to their sensitization to TRAIL-induced apoptosis. This data are in agreement with the previous results from our lab, where we showed that using HHT efficiently led to lower protein level of Mcl-1 and cFLIP in colorectal carcinoma cell lines (Beranova et al., 2013). Experiments presented in this Thesis concerned the involvement of cFLIP in resistance to TRAIL-induced apoptosis in hESC and hiPSC. cFLIP protein is coded by *CFLAR* gene located on human chromosome 2q33-34 adjacent to genes coding caspase-8 and -10. In contrast to caspase-8/10, cFLIP, which is highly similar to pro-caspase-8, does not possess a catalytically active domain. Three isoforms: FLIP-

L, FLIP-S and FLIP-R act as inhibitors of caspase-8 activation by hetero-dimerizing with the molecules of pro-caspase-8 at DISC, only FLIP-L was shown to have an ability to activate caspase-8, although this activation has rather limited capacity to cleave the substrates. The proposed anti-apoptotic function of cFLIP and its important regulatory function in cell survival of hESC and hiPSC was confirmed by shRNA-mediated knock-down of cFLIP expression, which led to increased sensitization of these cells to TRAIL-induced apoptosis. The protecting role of cFLIP in human pluripotent stem cells was also suggested in hematopoietic stem cells showing that stem cell factors caused increase of cFLIP levels and inhibited FasL-mediated apoptosis of human erythroid progenitor cells (Chung et al., 2003). In addition, suppression of cFLIP expression in breast cancer stem cells resulted in selective TRAIL-mediated cell death of these cells (Piggott et al., 2015). Interestingly, cFLIP can in addition to apoptosis play some role in necroptosis, specific type of cell death proceeding under caspase compromised conditions and depending on the activity of RIPK1 kinase. Not much is known about necroptosis in stem cells, but few papers refer to necroptotic signalling to be responsible for the bone marrow failure in patients (Roderick et al., 2014). To exclude the possibility of contribution of necroptosis to the pool of dying cells, were cell lines with downregulated expression of cFLIP tested for the involvement of RIPK1 kinase. Our data indicate that cell death observed in hESC and hiPSC mediated by TRAIL ligand was caspase dependent and incubation of cells with inhibitor of RIPK1 necrostatin-1 did not modulate their survival in any way. To conclude, we showed that cFLIP protein plays an important regulatory signalling node in human stem cells hESC and hiPSC and contributes to their defence against potent death inducing factors.

5.3 Streptomycetes metabolites of the Manumycin family differently sensitize colon cancer cells to TRAIL-induced apoptosis via enhanced production of reactive oxygen species (not published).

Manumycin A (Man A), Manumycin B (Man B) and Asukamycin (Asuc) are all Streptomyces secondary metabolites from the manumycin family. These compounds are known as inhibitors of farnesyl transferase (FTase), enzyme responsible for the post-translational modification of Ras protein family (Tuladhar et al., 2018). Blocking Ras farnesylation leads to retaining of Ras in the cytosol where this normally membrane associated protein cannot target its downstream interaction partners. *Ras* belongs to the most frequent mutated oncogenes in

human cancers and is involved in regulation of a number of important cellular processes. In our screening for the potent sensitizers of TRAIL-induced apoptosis in colorectal cancer cell lines, we revealed Man A as the only one capable to efficiently increase level of apoptosis comparing to Man B and Asuc. Man A was shown to have anti-tumour capability in several types of cancers (Cho et al., 2015; Zhang et al., 2016). This capability of Man A was mostly understood as the attribution of FTase to impair the activity of Ras, but recent studies and our results show that Man A can stimulate signalling pathways independently of the inhibitory function of FTase and the other possible regulatory mechanisms are involved (Cho et al., 2015; Zhang et al., 2016; Tuladhar et al., 2018). As we documented, Man A strongly enhanced apoptotic signalling in colorectal carcinoma RKO and SW-620 cells, triggered either by TRAIL ligand or by inhibitors of Bcl-2 and Bcl-Xl proteins, while the other metabolites, Man B and Asuc, were less effective or ineffective in this enhancement. These results point to participation of Man A in regulation of extrinsic as well as intrinsic apoptotic pathway. The extrinsic apoptotic pathway is in most cell lines insufficient to induce apoptosis by itself and the signal triggered from death receptors is amplified by engagement of mitochondria by caspase-8-cleaved tBid. We showed that Man A in combination with TRAIL ligand strongly enhances processing of tBid and subsequently downstream mitochondria activated caspases, thus supporting the role of Man A in extrinsic mediated cell death, underlined by stronger cleavage of caspase-8. The possible mechanism along the regulatory node at the DISC level was proposed from Juang Jae Cho and his colleagues, who published that ManA induced increase of DR4 and DR5 expression in dose dependent manner in oral squamosus cell carcinoma cells (Cho, Chae et al. 2015). They suggested that increase of TRAIL receptors is mediated through CHOP protein, which was shown to be involved in ER stress induced apoptosis (Yamaguchi and Wang 2004). Other regulatory node of the apoptotic pathway presents mitochondria mediated or intrinsic signalling regulated mainly by family of Bcl-2 proteins. Increased cell death induced by incubation of Man A with inhibitor of Bcl-2 anti-apoptotic protein (ABT-199) or Bcl-2/Bcl-Xl (ABT-737) revealed involvement of Man A in regulation of mitochondria induced apoptosis independently on ligand induced extrinsic apoptosis. To analyse the mechanism behind the regulation of intrinsic apoptotic signalling by Man A we subjected HT-29, RKO and SW-620 cells for the mitochondrial ROS production. Indeed, we found out that ManA but not Man B or Asuc largely enhanced production of mitochondrial ROS. Mitochondrial ROS were already proposed as very effective triggers of intrinsic apoptotic signalling and potent sensitizers of cancer cells to other pro-apoptotic agents such as TRAIL. For example vitamin E succinate and its mitochondria-targeted analogue mitoVES inhibit electron transfer in mitochondrial complex II, which leads

to increased ROS production and potent sensitization of resistant mesotheliomal cells to TRAIL-induced apoptosis (Tomassetti et al., 2004). Thus similarly enhanced ROS production triggered by ManA might be behind its strong sensitizing effect to TRAIL and BH3 analogues triggered apoptosis (Zhang et al., 2016). However, the presumed mitochondrial target of ManA and mode of its mitochondrial uncoupling resulting in boost of ROS production is still unknown.

6 CONCLUSIONS

1. I prepared several novel TRAIL receptor specific ligands, which can efficiently and selectively induce signalling through their cognate receptors DR4 or DR5.
2. I showed that designing new recombinant TRAIL receptor agonists, specifically targeting DR4 or DR5, can lead to more effective induction of apoptosis in cancer cells comparing to wild type ligand. Thus receptor specific ligands could be used for a patient tailored therapy due to their ability of specific targeting the cognate TRAIL receptors dominantly signalling in different types of cancer.
3. I also proved that DR4 and DR5 receptor are not equally regulated during apoptosis in colorectal HT-29 and pancreatic PANC-1 cells and the resulting cell response not only in apoptosis, but also in other auxiliary pathways could be affected by different events downstream from the receptor level.
4. From my results is obvious that apoptosis and necroptosis could be induced by the same DR4 and DR5 receptors but the signalling from these two receptors is differently regulated during apoptotic and necroptotic conditions.
5. In second part I showed that human embryonic and induced pluripotent stem cells express all components of TRAIL-induced apoptotic signalling and apoptosis in these cells is regulated by anti-apoptotic protein cFLIP.
6. Finally in the last part, I showed that Manumycin-A is a potent sensitizer of TRAIL-induced apoptosis in resistant colorectal cancer cell lines and this sensitizing effect might be at least partially achieved by increased production of mitochondrial ROS.

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8 LIST OF PUBLICATIONS IN WHICH AUTHOR PARTICIPATED

1. **Human embryonic and induced pluripotent stem cells express TRAIL receptors and can be sensitized to TRAIL-induced apoptosis.**

   Vinarsky V, Krivanek J, Rankel L, **Nahacka Z**, Barta T, Jaros J, Andera L, Hampl A. **Stem Cells Dev. 2013 Nov 15**;22(22):2964-74. doi: 10.1089/scd.2013.0057. Epub 2013 Aug 2.

2. **Manipulating Wnt signalling at different subcellular levels affects the fate of neonatal neural stem/progenitor cells.**

   Kriska J, Honsa P, Dzamba D, Butenko O, Kolenicova D, Janeckova L, **Nahacka Z**, Andera L, Kozmik Z, Taketo MM, Korinek V, Anderova M**. Brain Res. 2016 Nov 15**;1651:73-87. doi: 10.1016/j.brainres.2016.09.026. Epub 2016 Sep 19.

3. **TRAIL induces apoptosis but not necroptosis in colorectal and pancreatic cancer cells preferentially via the TRAIL-R2/DR5 receptor.**

   Nahacka Z, Svadlenka J, Peterka M, Ksandrova M, Benesova S, Neuzil J, Andera L. **Biochim Biophys Acta Mol Cell Res. 2018 Mar**;1865(3):522-531. doi: 10.1016/j.bbamcr.2017.12.006. Epub 2017 Dec 24.

4. **Induction, regulation and roles of neural adhesion molecule L1CAM in cellular senescence.**

   Mrazkova B, Dzijak R, Imrichova T, Kyjacova L, Barath P, Dzubak P, Holub D, Hajduch M, **Nahacka Z**, Andera L, Holicek P, Vasicova P, Sapega O, Bartek J, Hodny Z. **Aging (Albany NY). 2018 Mar 28**;10(3):434-462. doi: 10.18632/aging.101404.

5. **<u>Reactivation of Dihydroorotate Dehydrogenase-Driven Pyrimidine Biosynthesis Restores Tumor Growth of Respiration-Deficient Cancer Cells.</u>**

Bajzikova M, Kovarova J, Coelho AR, Boukalova S, Oh S, Rohlenova K, Svec D, Hubackova S, Endaya B, Judasova K, Bezawork-Geleta A, Kluckova K, Chatre L, Zobalova R, Novakova A, Vanova K, Ezrova Z, Maghzal GJ, Magalhaes Novais S, Olsinova M, Krobova L, An YJ, Davidova E, **Nahacka Z**, Sobol M, Cunha-Oliveira T, Sandoval-Acuña C, Strnad H, Zhang T, Huynh T, Serafim TL, Hozak P, Sardao VA, Koopman WJH, Ricchetti M, Oliveira PJ, Kolar F, Kubista M, Truksa J, Dvorakova-Hortova K, Pacak K, Gurlich R, Stocker R, Zhou Y, Berridge MV, Park S, Dong L, Rohlena J, Neuzil J. **Cell Metab. 2018 Nov 9**. pii: S1550-4131(18)30646-6. doi: 10.1016/j.cmet.2018.10.014.