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Novel molecular mechanisms of cancer cell death regulation

Habilitation thesis

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Abstract

An efficient and selective induction of cancer cell death is a main goal of many therapeutic approaches, and identification of the suitable candidates that are capable to do so, especially by triggering apoptosis, represents a continuous need in oncology. Activation of the apoptotic signalling via TRAIL death receptors (DRs) is an attractive, tumour-specific and biologically relevant approach that can be exploited in the future cancer therapy. However, numerous cancer cells develop the resistance to apoptotic effects of this cytokine and/or activate undesired alternative non-apoptotic signalling resulting in enhancement of their malignant behaviour. The balance between the two types of signalling is crucial in determination of final cancer cell fate and outcome of TRAIL-based therapy, and can be efficiently modulated by different classes of therapeutically relevant compounds.

In the work presented within this thesis, the apoptosis-sensitizing abilities of several classes of conventionally used or recently introduced chemotherapeutic drugs such as cisplatin, LA-12, 5-fluorouracil (5-FU), doxorubicin or etoposide, epigenetic drugs – decitabine or valproic acid (VPA), and dietary compounds like docosahexaenoic acid (DHA) were investigated in order to boost the cytotoxic potential of TRAIL/DR signalling in cancer cells. Several molecular determinants of their stimulating effects on TRAIL/DR apoptotic signalling pathways were newly demonstrated, especially at the level of death-inducing signalling complex (DISC), mitochondria and caspases. In addition, the research included in this thesis also uncovered novel details about the role of kinase-mediated non-apoptotic signalling in regulation of cancer cell sensitivity to the cytotoxic effects of TRAIL, with a particular attention to MAPK/ERK and PI3K/Akt. These results contribute to the better understanding of the complexity of TRAIL-induced signalling and targeting molecular bases of the frequent resistance of tumours to TRAIL-based therapy, which are essential prerequisites for its potentially successful clinical application. In the light of the most recent knowledge on this topic, the theses presents the research findings of the author and her colleagues summarized in 19 publications (18 original articles and 1 review), which demonstrate the proposed efficient strategies for triggering apoptosis in human cancer cells by the above-mentioned therapeutically interesting drugs or their combinations, and the molecular mechanisms involved.

Abstrakt

Účinná a selektivní indukce smrti nádorových buněk je hlavním cílem řady současných terapeutických strategií a identifikace vhodných kandidátů, které mohou zajistit eliminaci těchto buněk zejména prostřednictvím apoptózy, je velkou výzvou v onkologii. Aktivace apoptózy přes receptory smrti (DR) pro TRAIL představuje atraktivní, selektivní a biologicky významný přístup využitelný v budoucí protinádorové terapii. U některých nádorových buněk však dochází k vývoji rezistence k apoptotickým účinkům tohoto cytokinu a/nebo spuštění nežádoucích alternativních "neapoptotických" signálních drah vedoucích spíše k posílení jejich maligního chování. Rovnováha mezi uvedenými dvěma typy signálování je proto naprosto klíčová pro určení finálního osudu nádorové buňky, rozhoduje o efektivitě terapie využívající TRAIL a může být účinně modulována prostřednictvím různých typů terapeuticky zajímavých látek.

Ve výzkumu, který je součástí předkládané habilitační práce, byla studována schopnost různých konvenčně používaných i nových chemoterapeutických látek - cisplatiny, LA-12, 5-fluorouracilu (5-FU), doxorubicinu a etoposidu, epigeneticky působících látek – decitabinu a kyseliny valproové (VPA) a některých složek potravy jako je kyselina dokosahexaenová (DHA) stimulovat cytotoxické signálování spuštěné u nádorových buněk přes DR pro TRAIL. Bylo popsáno několik nových mechanismů posílení apoptózy indukované přes DR, zejména na úrovni signálního komplexu DISC, mitochondrií a kaspáz. V rámci výzkumu obsaženého v této práci byly rovněž představeny nové poznatky o úloze „neapoptotického" signálování zprostředkovaného kinázami v regulaci citlivosti nádorových buněk k cytotoxickým účinkům TRAILu, především s ohledem na zapojení MAPK/ERK a PI3K/Akt. Získané výsledky přispěly k hlubšímu pochopení komplexity signálních drah aktivovaných přes DR pro TRAIL a molekulární podstaty rezistence některých nádorů k terapii založené na působení TRAILu, což je nutným předpokladem jeho potenciálně úspěšné klinické aplikace. V této habilitační práci jsou ve světle recentních poznatků v dané problematice prezentovány výsledky výzkumné práce autorky a jejích kolegů, které jsou součástí 19 přiložených publikací (18 originálních článků a 1 review) popisujících účinné strategie pro spuštění apoptózy nádorových buněk s využitím uvedených látek a jejich kombinací, včetně zapojených molekulárních mechanismů.

1. Introduction – apoptosis

Great numbers of the cells in a human organism die throughout its life, and these death events are often essential for its survival as a whole. So far, a variety of cell death types and subroutines have been discovered and classified, but the main focus has been paid on apoptosis, as this form of cell death seems to be the most frequent, the best understood, and the most significant. Apoptosis (from a Greek word that means "falling off of leaves from tree") represents an active process responsible for elimination of old, damaged, dangerous or unnecessary cells via stimulation of evolutionary conserved and genetically controlled molecular pathways that mediate cellular demise. A general concept of apoptosis was first proposed by Kerr and colleagues (Kerr *et al*, 1972), but the importance and resulting consequences of these findings were underestimated for many years. Subsequently, it has become increasingly evident that apoptosis is implicated in numerous biological processes starting from an early embryonal development to late aging, is crucial for maintenance of homeostasis of a multicellular organism, and often involved in pathogenesis of various human diseases (Favaloro *et al*, 2012; Lockshin & Zakeri, 2007). With its crucial discoveries and their practical applications, research on apoptosis has become one of the hottest fields in biomedical sciences, associated with rapidly increasing numbers of exciting new findings published in journals focused on this topic each year. Importantly, a great proportion of "apoptosis-related papers" is focused on the regulation of this process in cancer cells (Melino & Vaux, 2010).

This theses presents the selection of 19 publications (18 original research articles and 1 review), which summarize the results of our work that contribute with the novel findings to the cell death research field, being especially focused on the investigation of efficient strategies for triggering apoptosis in human cancer cells by selected therapeutically interesting drugs or their combinations, and the molecular mechanisms involved. Within the individual chapters, our results are described in the context of the most recent knowledge on this topic, together with the suggested consequences and future perspectives.

1.1. Apoptosis and cancer

Cancer is a disease characterized by an abnormal accumulation of cells due to an imbalance between their division and death. Evasion of apoptosis has been proposed as an important mechanism implicated in malignant transformation, and one of the hallmarks of cancer (Fig. 1) (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). Furthermore, blocks in apoptosis can contribute to inherent or acquired resistance to a variety of anticancer agents. Thus, apoptosis is implicated in both pathogenesis and treatment of cancer, and involves complex regulation at the different levels inside the cells (Rufini & Melino, 2011; Zhivotovsky & Orrenius, 2006). Targeting apoptotic machinery and specific components of apoptotic pathways is considered as an attractive anticancer strategy, with numerous molecules currently under investigation for their promising therapeutic potential (Fox & MacFarlane, 2016; Pfeffer & Singh, 2018).

1.2. Apoptotic pathways

Apoptosis is manifested by a cellular shrinkage, plasma membrane blebbing, condensation of nuclear chromatin and fragmentation of DNA, followed by separation of the cell fragments into so-called apoptotic bodies, which are phagocytosed and degraded within phagolysosomes, without triggering an inflammatory response (Elmore, 2007; Taylor *et al*, 2008). Generally, the cells undergo apoptosis via two major molecular signalling pathways, the extrinsic (death receptor-mediated) and the intrinsic (mitochondrial) (Fig. 2) (Calvino

Fernandez & Parra Cid, 2010; Fulda & Debatin, 2006). The extrinsic pathway is initiated by extracellular death inducers such as specific ligands of the tumour necrosis factor (TNF) family that bind to their cognate cell surface death receptors (DRs). This results in the recruitment of Fas-associated death domain (FADD) protein, formation of the death-inducing signalling complex (DISC), and activation of initiator caspase-8 and -10 (Dickens *et al*, 2012). This step can be effectively modulated by the cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (cFLIP), which is frequently deregulated in cancer cells (Hughes *et al*, 2016; Shirley & Micheau, 2013). The active caspase-8 can directly cleave effector caspase-3 and -7, and enable further propagation of the cell death (Tummers & Green, 2017).

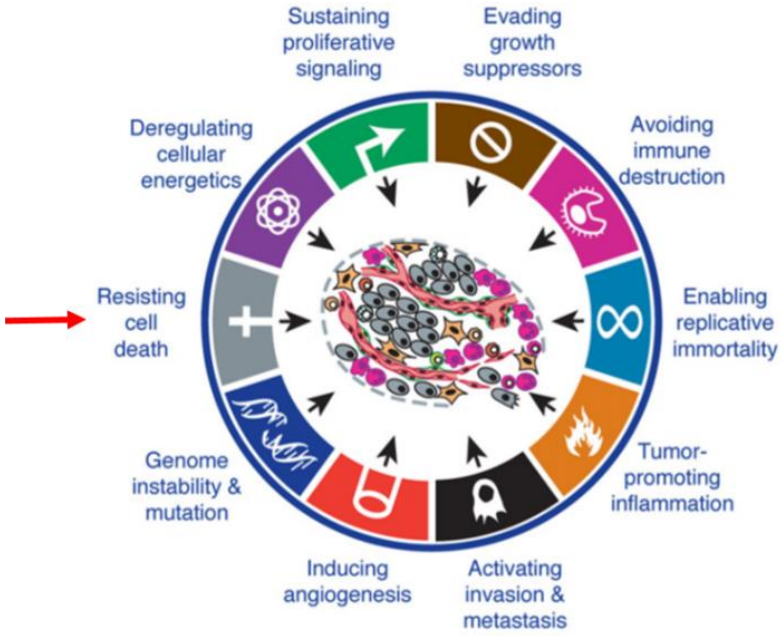

Fig. 1: The hallmarks of cancer (Hanahann & Weinberg, 2011).

The mitochondrial pathway of apoptosis is triggered by intrinsic signals involving growth factor deprivation, DNA damage induced by irradiation or chemicals, oxidative or oncogenic stress (Galluzzi *et al*, 2012). These death signals are sensed and propagated by the Bcl-2 family of proteins, the main regulators of the mitochondrial apoptotic machinery (Adams & Cory, 2018; Czabotar *et al*, 2014; Kale *et al*, 2018), which can be categorized into three main groups: The first group comprises pro-apoptotic BH3-only proteins characterized by the presence of a single BH (Bcl-2 homology) domain in the molecule (Bid, Bim, Puma, Noxa, Bad, Hrk, Bmf, Bik). They monitor numerous cellular processes and transmit the stress signals to the mitochondria, and their cooperation with the multi-domain Bcl-2 family partners at the level of these organelles is critical for triggering of the intrinsic apoptotic pathway (Delbridge & Strasser, 2015). Mimicking their pro-apoptotic action using the small molecule drugs called "BH3 mimetics" offers a promising strategy to promote apoptosis in some resistant tumours (Sarosiek & Letai, 2016). The second group is represented by the pro-apoptotic effectors with three BH domains (Bax, Bak, Bok), capable to form pores in mitochondrial outer membrane (MOM) and thus promote its permeabilization (MOMP) (Chipuk & Green, 2008; Wei *et al*, 2001). The third group consists of anti-apoptotic members with four BH domains (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, A1/Bfl-1), which protect the cells from death by sequestering their pro-apoptotic partners within the Bcl-2 family (Delbridge & Strasser, 2015). Overexpression of these proteins may often be used by cancer cells as a strategy to develop apoptosis resistance (Campbell & Tait, 2018).

The character of mutual interactions and the ratio of pro- to anti-apoptotic Bcl-2 family members represent a "rheostat" that determines the cell susceptibility to apoptosis induced via the intrinsic pathway, which is used to kill cancer cells by most chemotherapeutic and targeted therapies. Importantly, understanding of these interactions enables scientists to assess the cell "priming for death", i.e. how close is the cell to the threshold of apoptosis (Sarosiek *et al*, 2013). Based on these findings, dynamic BH3 profiling, a sophisticated functional approach to study the mitochondrial readiness to undergo apoptosis and to predict the cell chemotherapeutic response was developed (Del Gaizo Moore & Letai, 2013; Letai, 2015; Letai, 2017). By employing specific synthetic BH3 peptides in drug-treated cells, this assay helps to determine the dependency of particular tumour on individual anti-apoptotic Bcl-2 family proteins and its vulnerability to trigger MOMP following the treatment. The MOMP and subsequent release of pro-apoptotic mediators such as cytochrome c from mitochondrial intermembrane space into the cytosol is often considered as the point of irreversible commitment to cell death. Cytochrome *c*, in cooperation with the adaptor protein apoptotic protease activating factor 1 (Apaf1) and in the presence of ATP, participates in the formation of apoptosome, a molecular complex necessary for the activation of caspase-9 (Liu *et al*, 1996; Zou *et al*, 1997).

The intrinsic and extrinsic pathways converge on a "common pathway" resulting in the activation of potent effector caspases (-3, -6, -7), which are involved in disintegration of numerous structurally and functionally important cellular substrates, and apoptosis execution (Inoue *et al*, 2009; Olsson & Zhivotovsky, 2011; Timmer & Salvesen, 2007). The caspases are recognized as crucial players in apoptosis, and determination of their activation and detection of caspase substrate cleavage are considered as useful biochemical markers of apoptosis. The apoptosis detection methods based on evaluation of these parameters both *in vitro* and *in vivo* applications were described in detail by numerous authors and also in our previous work, together with experimental protocols, advantages and disadvantages of these techniques, and background information (Vaculova & Zhivotovsky, 2008) (**Supplement 1**).

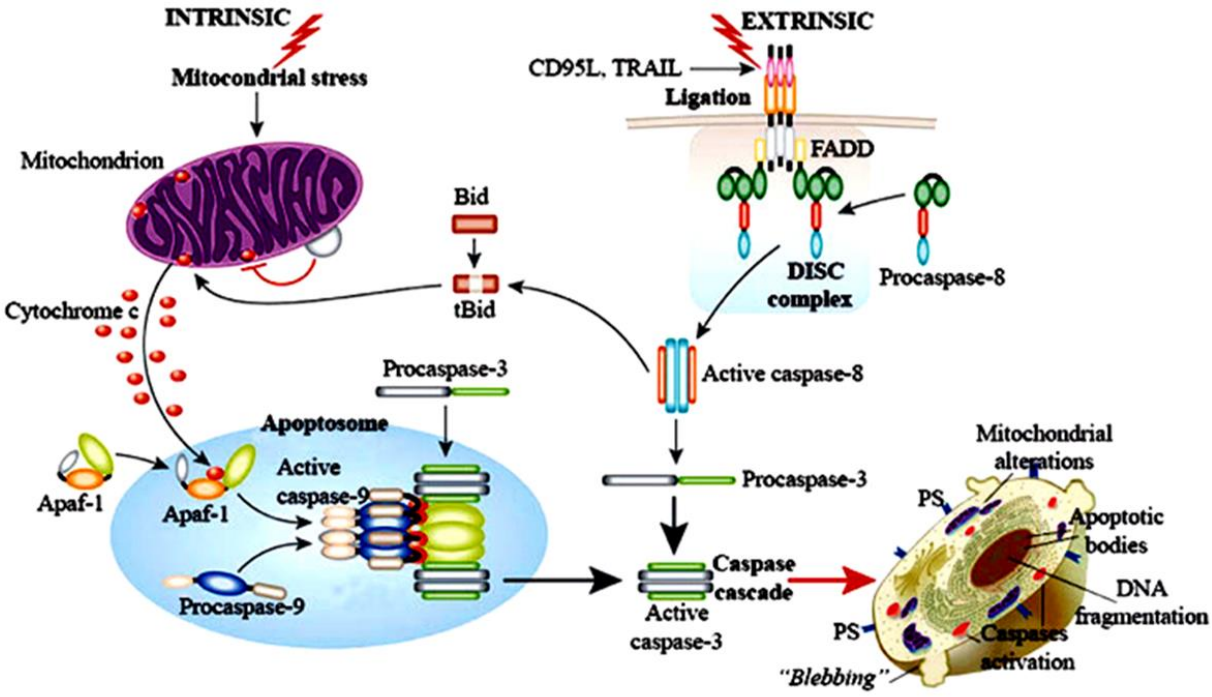

Fig. 2: The extrinsic and intrinsic apoptotic pathways – main characteristics (Calvino-Fernández & Parra Cid, 2010).

Caspase activity can be blocked by endogenous cellular inhibitor of apoptosis (cIAP) proteins (e.g. X-linked inhibitor of apoptosis protein - XIAP, cIAP1/2, survivin) that do so either through direct binding or by targeting caspases for ubiquitin-mediated degradation (Mohamed *et al*, 2017). The cIAPs are often overexpressed in multiple human cancers, being responsible for promoting tumour progression and contributing to treatment failure (Fulda, 2014). The cIAP-mediated inhibition of caspases can be countered by endogenous second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), which is released into the cytoplasm following MOMP (Du *et al*, 2000; Martinez-Ruiz *et al*, 2008; Verhagen *et al*, 2000). Thus, the synthetic small molecule inhibitors mimicking the pro-apoptotic action of this protein, called "Smac mimetics", represent an important class of targeted drugs for cancer treatment (Fulda, 2017).

Importantly, diverse molecular connections exist between intrinsic and extrinsic apoptotic pathways, which can substantially affect the intensity of apoptotic response and a final cell fate. Understanding the complexity of these molecular interactions will enable the design of more efficient therapeutic approaches for particular tumour types. Although apoptosis represents a well-recognized mechanism for elimination of cancer cells exposed to conventional chemotherapy, the research focused on the molecular basis of the selective apoptosis-targeted therapies has been developed more recently. This approach mainly aims to: (i) induce or restore pro-apoptotic pathways by specific death ligands, monoclonal antibodies or fusion proteins (e.g. those targeted to DRs) (Fox & MacFarlane, 2016; Fulda, 2015), or (ii) prevent the action of anti-apoptotic molecules (e.g. cFLIP, cIAP or some Bcl-2 family proteins) that are upregulated in tumours by specific small molecule inhibitors like BH3, Smac mimetics and others (Fulda *et al*, 2010; Gibson & Davids, 2015; Humphreys *et al*, 2018; Timucin *et al*, 2018). Moreover, it has become increasingly evident that the simultaneous application of at least two different approaches will help to maximize the therapeutic outcome, while minimizing the undesired treatment-related toxicities in normal cells.

In our work, a particular attention has been paid to the investigation of the anticancer effects of an interesting cytokine and death ligand with unique ability to selectively induce cancer cell apoptosis, without being toxic to normal tissues, the phenomenon that can be promisingly exploited in cancer therapy. Within the following chapters, the general as well as specific aspects of TNF-related apoptosis-inducing ligand (TRAIL)-induced signalling and novel mechanisms responsible for its modulation are described, and the results of our work related to this topic and their significance are introduced and discussed.

2. TRAIL

2.1. TRAIL – basic characteristics

TRAIL was discovered independently by two groups, based on its sequence homology with TNFα and CD95L (Pitti *et al*, 1996; Wiley *et al*, 1995). The human 20 kb gene coding for TRAIL is located on chromosome 3, and comprises five exons and four introns. The TRAIL is expressed as a type II transmembrane protein consisting of 281 amino acids, which can undergo proteolytic cleavage to release the region of 114-281 amino acids, the soluble ligand. The endogenous TRAIL can be expressed on the membrane of natural killer (NK) cells, macrophages, T-cells and dendritic cells, being involved in immune defence mechanisms by killing virus-infected or cancer cells. The biological activity of TRAIL can be enhanced by its trimerization (Wiley *et al*, 1995).

2.2. TRAIL-induced apoptotic signalling

TRAIL interacts with two DRs, namely DR4 (TRAIL-R1, TNFRSF10A, CD261) (Pan *et al*, 1997a) and DR5 (TRAIL-R2, TNFRSF10B, Killer, CD262) (MacFarlane *et al*, 1997; Screaton *et al*, 1997; Walczak *et al*, 1997), the type I transmembrane proteins with characteristic death domains in their cytoplasmic regions (Fig. 3) (Lemke *et al*, 2014a). Binding of membrane-bound or soluble TRAIL to DR4/5 induces receptor oligomerization and triggers extrinsic apoptotic pathway associated with FADD-dependent formation of DISC and initiator caspase-8/-10 activation. The activity of DISC can also be affected by other proteins recruited to this complex such as Cul3, PP2AC or others (Jin *et al*, 2009; Shirley *et al*, 2011; Xu *et al*, 2013; Xu *et al*, 2014). In so-called type I cells, the amount of caspase-8 activated at the DISC is sufficient for efficient propagation of apoptotic signal and activation of effector caspases, while type II cells rely on the Bid-mediated amplification of apoptotic signal via mitochondrial pathway (Fig. 4) (Ozoren & El-Deiry, 2002; Schneider-Brachert *et al*, 2013). In addition, the TRAIL-induced cytotoxicity may also require the lysosomal permeabilization and the release of non-caspase proteases into the cytoplasm, which is the case in the type L cells (Akazawa *et al*, 2009).

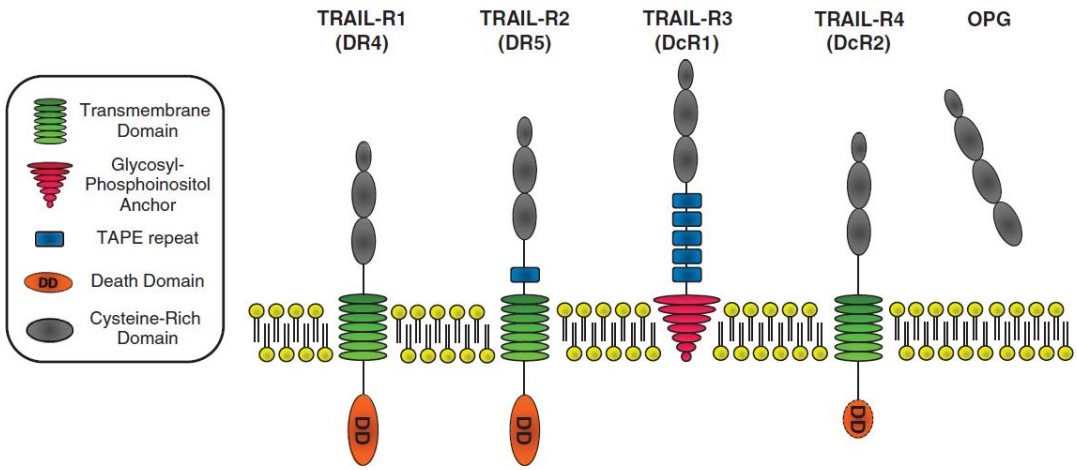

Fig. 3: Structure of TRAIL receptors – schematic picture (Lemke & Walczak, 2014).

The unique ability of TRAIL to act as a selective inducer of cancer cell apoptosis both *in vitro* and *in vivo* was originally demonstrated by two independent groups who reported its cytotoxic effects in various human cancer cell lines and also showed tumour xenograft regression in mice or non-human primates treated with recombinant variants of human TRAIL (Ashkenazi *et al*, 1999; Walczak *et al*, 1999). An additional benefit of TRAIL is that it can initiate cancer cell apoptosis independently of their p53 status (Ravi *et al*, 2004). These discoveries provoked an enormous interest in exploiting TRAIL for cancer therapy and deeper investigation of various preparations of TRAIL. Early on, several alarming publications reported on TRAIL toxicity to normal hepatocytes, an undesired side effect that could disqualify its application in clinic (Jo *et al*, 2000; Lawrence *et al*, 2001). However, later studies clarified that the hepatotoxicity was an artefact caused only by particular tagged forms of TRAIL and could be avoided by the selection of the more safe available variants (Ganten *et al*, 2006).

Based on these findings, various TRAIL-receptor targeting agonists (TRAs) were approved for clinical applications – human recombinant soluble untagged TRAIL (dulanermin/Apo2L.0/AMG-951) and several DR4 (mapatumumab) and DR5 (drozitumumab, lexatumumab, conatumumab, tigatuzumab, LBY-135, DS-8273a) agonistic antibodies (Naoum

et al, 2017). Unfortunately, despite numerous promising preclinical results obtained with dulanermin, clinical trials failed to demonstrate any significant anticancer activity (Dubuisson & Micheau, 2017; von Karstedt *et al*, 2017). These disappointing results are most likely caused by its short plasma half-life and rapid clearance from the circulation, a limited ability to cluster TRAIL DRs, and simultaneous engagement of DcRs. Compared to dulanermin, DR4/5-specific agonistic antibodies are characterized by higher stability, longer half-life, and no interference with TRAIL DcRs. However, similarly as in case of dulanermin, they did not exert sufficient therapeutic activity or improve overall patient survival (von Karstedt *et al*, 2017). The main factors responsible for the clinical failure of these antibodies seem to be their bivalent mode, still insufficient ability to induce higher-order DR clustering, and additional need for more efficient crosslinking. Taken together, although all these "first generation" TRAs are safe and well tolerated in patients, so far they have failed to achieve a significant therapeutic effect in randomized-controlled clinical trials.

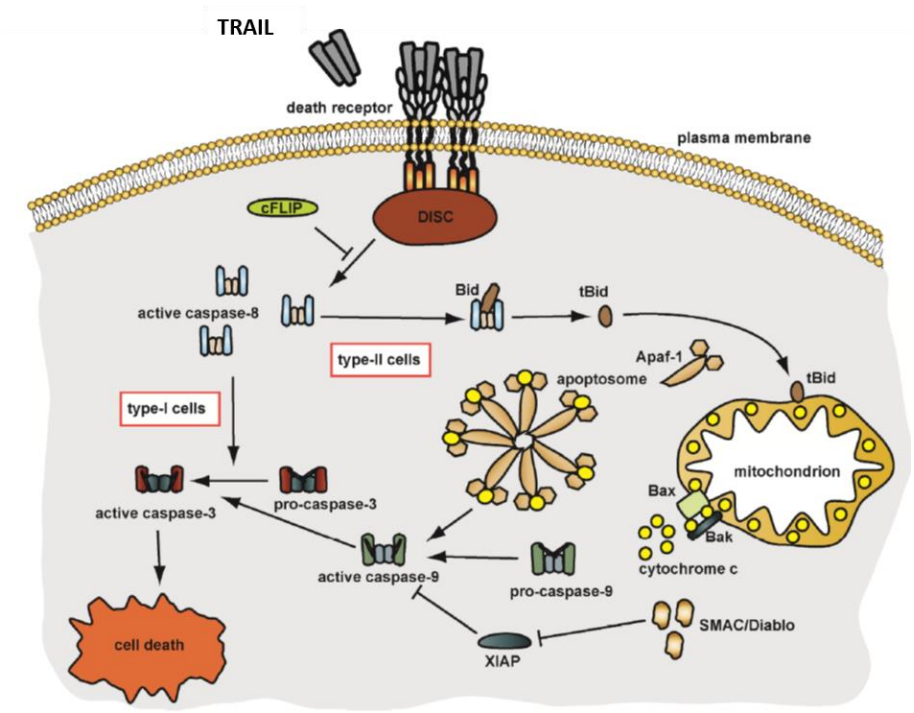

Fig. 4: TRAIL-induced signalling in type I and II cells, basic scheme (Schneider & Brachert, 2013).

In the light of the recent insights on TRAIL signalling, novel "next generation" TRAs with improved agonistic capacities, stability and tumour-specific delivery have been developed. The enhanced stability of TRAIL has been achieved by non-covalent addition of N-terminal tags such as Flag epitope, leucine/isoleucine zipper motifs and tenascin-C (TNC) oligomerization domain, permutation of the ligand (circularly permutated TRAIL) or covalent link to molecules that help to increase its pharmacokinetic and pharmacodynamic properties (Stuckey & Shah, 2013; von Karstedt *et al*, 2017). For an enhancement of tumour delivery, passive or active targeting approaches have been utilized. Passive targeting is often based on enhanced permeation and retention (EPR) effect, and employs TRAIL coupled with nanoparticles, either encapsulated inside or attached to the surface of the nanoparticle, mimicking the physiological membrane-bound protein. Various TRAIL-bearing nanoparticles containing human serum albumin, polyethylene glycol, liposomes or other molecules have been engineered and further examined (de Miguel *et al*, 2016; Guimaraes *et al*, 2018). Active targeting takes advantage of using TRAIL fused with antibody fragments or peptides that

specifically recognize antigens that are highly expressed by cancer cells or cells of tumour stroma, thus enabling to preferentially hit the desired target (de Miguel *et al*, 2016). Next, various types of monoclonal DR4/5-specific antibodies and derivatives functionalized to nanoparticles or linkers that enable to increase their valency, bioavailability and efficacy are being developed (Dubuisson & Micheau, 2017).

Importantly, even the best-designed TRAs may be ineffective in cancer cells that bear serious defects in apoptotic signalling pathways triggered via DR4/5. Thus, in addition to the above-mentioned potential causes of the failure of TRA-based therapy, the intrinsic or acquired resistance can be the crucial factor responsible for the insufficient cytotoxic response observed in TRA-treated tumours. Although TRAIL resistance in cancer cells has been investigated and demonstrated at multiple points in TRAIL apoptotic pathway, as described in the next chapter, the precise molecular mechanisms involved need detailed clarification. It also remains to be understood whether the single factor or the combination of several of them may be responsible for TRAIL resistance in particular cell types and if different cancers develop TRAIL resistance through similar or distinct mechanisms. Deeper understanding of these issues will enable the correct identification of reliable biomarker or biomarker set for precise assessment of tumour response to TRA-based therapy, which is still far from being complete (von Karstedt *et al*, 2017). So far, no sufficient predictive biomarkers have been successfully employed in the clinical trials in order to identify specific group(s) of patients with preferential sensitivity to TRA-based therapy (Ralff & El-Deiry, 2018).

2.2.1. Mechanisms of cell resistance to TRAIL-induced apoptosis

The presence of DR4/5 at the cell surface is an essential but not sufficient precondition for the initiation of apoptotic TRAIL signalling, and the low surface level or unavailability of functional receptors may correlate with decreased sensitivity of cancer cells to TRAIL. It can be caused by specific genetic or epigenetic changes in cancer cells such as gene deletions (Ozoren *et al*, 2000), loss-of-function mutations (Bin *et al*, 2007; McDonald *et al*, 2001; Pai *et al*, 1998) or promoter hypermethylation (Hopkins-Donaldson *et al*, 2003; Horak *et al*, 2005). The surface DR4/5 level can also be modulated by their deficient transport to plasma membrane (Jin *et al*, 2004) or their constitutive endocytosis from the cell surface (Zhang & Zhang, 2008). Although being indispensable for the triggering of TRAIL-induced apoptosis, the presence/amount of DR4/5 at the cell surface is unlikely to serve as an independent biomarker of TRAIL sensitivity that would enable to predict the patient responsiveness to treatment with TRAs. In addition to the changes in the total surface DR4/5 level, their distribution in the plasma membrane within the lipid rafts, membrane micro-domains enriched in cholesterol and sphingolipids, can influence the receptor clustering and the DISC assembly in cancer cells (Marconi *et al*, 2013; Ouyang *et al*, 2011). These events may be affected by posttranslational modifications of DR4 or DR5 such as S-palmitoylation (Rossin *et al*, 2009), N-glycosylation (Dufour *et al*, 2017) or O-glycosylation (Wagner *et al*, 2007). Interestingly, high expression of O-glycosylation enzyme *N*-acetylgalactosaminyltransferase 14 (GALNT14) was suggested as a signature for cancer cell sensitivity to TRAIL, but these findings need further validation (Wagner *et al*, 2007).

In addition to DR4/5, TRAIL can also bind two membrane-bound "decoy" receptors – DcR1 (TRAIL-R3, TNFRSF10C, CD263) (Degli-Esposti *et al*, 1997b; Pan *et al*, 1997b; Sheridan *et al*, 1997) and DcR2 (TRAIL-R4, TNFRSF10D, CD264) (Degli-Esposti *et al*, 1997a; Marsters *et al*, 1997; Pan *et al*, 1998). These receptors are unable to induce apoptosis due to a complete (DcR1) or partial (DcR2) lack of the death domain. However, they can compete with DR4/5 for ligand binding, thus lowering the amount of TRAIL available for initiation of apoptotic signalling. Moreover, DcR2 can inhibit TRAIL-induced apoptosis by forming inactive complexes with DR5 (Merino *et al*, 2006) or stimulating anti-apoptotic

signalling mediated by NF-κB (Degli-Esposti *et al*, 1997a) or PKB/Akt (Lalaoui *et al*, 2011). While the ratio between TRAIL DRs and DcRs was suggested to affect the ability of the cells to trigger TRAIL-induced apoptosis (Sheridan *et al*, 1997), subsequent studies did not confirm the clear correlation between the high/low DcRs level and resistance/sensitivity to TRAIL-induced cytotoxicity (Griffith *et al*, 1999). Thus, it seems that the amount of the surface DcRs level cannot be considered as a sufficient predictor of the cell responsiveness to the cytotoxic effects of TRAIL. Finally, TRAIL can also interact with osteoprotegerin (OPG, TRAIL-R5), a soluble decoy receptor involved in regulation of osteoclastogenesis (Emery *et al*, 1998; Simonet *et al*, 1997). However, as OPG binds TRAIL with lower affinity compared to other TRAIL-Rs, its role in inhibition of TRAIL-induced apoptosis in cancer cells is less apparent.

TRAIL resistance can also be caused by decreased/lost expression or impaired function of the crucial initiator caspase of its signalling, caspase-8 (Crowder & El-Deiry, 2012). These resistance mechanisms include caspase-8 promoter methylation (Grotzer *et al*, 2000; Hopkins-Donaldson *et al*, 2000; Hopkins-Donaldson *et al*, 2003), gene mutations (Kim *et al*, 2003; Soung *et al*, 2005) or a decreased protein stability (Zhang *et al*, 2005) reported in various cancer cell types. Furthermore, several studies highlighted an important role of ubiquitination in both positive (Jin *et al*, 2009) and negative (Xu *et al*, 2017) regulation of caspase-8 activity. Importantly, ubiquitination has also been showed to modulate the function of other TRAIL DISC components such as DRs, FADD or cFLIP, thus tightly controlling the progress of TRAIL signalling (Lafont *et al*, 2018). Therefore, it is likely that "TRAIL's ubiquitin code" may provide a clue for the identification of novel biomarkers of the cell sensitivity to this cytokine, and that modulators of ubiquitination can represent future promising cancer therapeutic targets (Lafont *et al*, 2018).

While the role of caspase-8 in TRAIL-induced apoptosis is well recognized, the involvement of caspase-10 is less clarified and more controversial. Some studies showed that caspase-10 is essential for stimulation of TRAIL signalling (Engels *et al*, 2005; Kischkel *et al*, 2001), while other reports suggested that it is not important (Seol *et al*, 2001; Sprick *et al*, 2002). Recently, caspase-10 has been demonstrated to negatively regulate caspase-8-mediated cell death induced by ligation of CD95 in cancer cells (Horn *et al*, 2017). The further details of the molecular action of caspase-10 in TRAIL-induced signalling remain to be elucidated.

The cFLIP acts as a key determinant of TRAIL sensitivity at the DISC level, and an important modulator of caspase-8/-10 activity (Fulda, 2013a). Aberrant expression of cFLIP has been linked to TRAIL resistance in different types of cancer and is often associated with poor prognosis (MacFarlane *et al*, 2002; Xiao *et al*, 2003; Zhang *et al*, 2004; Zobalova *et al*, 2008). The targeting of this molecule represents a very promising approach in cancer therapy, but is rather complicated by the very complex and dynamic regulation of this molecule at the level of transcription, translation and posttranslational modifications (Shirley et al., 2013). Moreover, the existence of its individual isoforms ($cFLIP_L$, $cFLIP_S$, $cFLIP_R$), their relative representation and mutual interplay make it difficult to predict the TRAIL signalling outcome in particular cancer types, and limit to some extent the usefulness of cFLIP as a biomarker for TRAIL resistance (Dimberg *et al*, 2013).

The type II cancer cells can become resistant to TRAIL-induced apoptosis due to overexpression of anti-apoptotic Bcl-2 family protein such as Mcl-1 (Ricci *et al*, 2007; Schulze-Bergkamen *et al*, 2008; Schulze-Bergkamen *et al*, 2006; Taniai *et al*, 2004), Bcl-X_L (Hinz *et al*, 2000; Schulze-Bergkamen *et al*, 2008) and Bcl-2 (Fulda *et al*, 2002a; Sinicrope *et al*, 2004). On the other hand, loss of expression/function of pro-apoptotic Bcl-2 family members Bax, Bak or both may confer TRAIL resistance, depending on cancer cell type and its genetic background (Kandasamy *et al*, 2003; LeBlanc *et al*, 2002). Complexity of the whole Bcl-2 protein family, and redundancy or multiple interactions among its members make it difficult to predict the final cancer cell response to TRAIL, and the presence/absence of selected single Bcl-2 family

molecule often cannot serve as a sufficient biomarker of the cell response to this cytokine (Dimberg *et al*, 2013). Therefore, functional analyses such as already mentioned dynamic BH3 profiling could help to better assess the readiness of cancer cells to trigger TRAIL-induced mitochondrial apoptotic pathway.

Another efficient mechanism how to develop TRAIL resistance is via upregulation of expression of cIAP family proteins, which has been demonstrated in several cancer cell types (Cummins *et al*, 2004; Lippa *et al*, 2007; Ndozangue-Touriguine *et al*, 2008; Vogler *et al*, 2007). An additional level of regulation of XIAP function following TRAIL treatment is further provided by Smac/DIABLO. The more intensive mitochondrial release of this pro-apoptotic protein was observed in TRAIL-sensitive compared to TRAIL-resistant cancer cells, and the Smac/DIABLO-induced inhibition of XIAP was associated with enhanced caspase-3 activation and TRAIL-induced apoptosis (Zhang *et al*, 2001). Other members of cIAP family may also participate in modulation of cancer cell responsiveness to the apoptotic effects of TRAIL (Finlay *et al*, 2014; Ricci *et al*, 2007).

2.2.2. Sensitization to TRAIL-induced apoptosis – combined therapy

Although the success of TRAs used as a stand-alone cancer therapy is very limited, combined protocols employing TRAs together with other therapeutic approaches are strongly believed to result in better treatment outcome. Numerous preclinical studies demonstrated that the resistance to TRAIL-induced apoptosis can be overcome by co-application of sensitizing agents such as chemotherapeutic drugs, especially platinum-based complexes – cisplatin (Belyanskaya *et al*, 2007; Ding *et al*, 2011; Nagane *et al*, 2000; Shamimi-Noori *et al*, 2008; Tsai *et al*, 2006; Wu & Kakehi, 2009) or oxaliplatin (El Fajoui *et al*, 2011; Galligan *et al*, 2005; Xu *et al*, 2009), pyrimidines – 5-fluorouracil (5-FU) (Galligan *et al*, 2005; Ganten *et al*, 2004; Mizutani *et al*, 2002; Nazim *et al*, 2017; Stagni *et al*, 2010; Wang *et al*, 2015; Yang *et al*, 2017; Zhu *et al*, 2013) or gemcitabine (Hylander *et al*, 2005; Liu *et al*, 2001; Xu *et al*, 2003a; Zhao *et al*, 2011), topoisomerase inhibitors – doxorubicin (El-Zawahry *et al*, 2005; Kang *et al*, 2005; Lacour *et al*, 2003; Wang *et al*, 2010; Xu *et al*, 2003b), etoposide (Baritaki *et al*, 2007; Gibson *et al*, 2000; Liu *et al*, 2001) or camptothecin (Gliniak & Le, 1999; Jayasooriya *et al*, 2014; Singh *et al*, 2003; Yamanaka *et al*, 2000), and taxanes – paclitaxel (Hunter *et al*, 2011; Li *et al*, 2016; Singh *et al*, 2003) or docetaxel (Jaworska & Szliszka, 2017; Yoo *et al*, 2008) in various cancer cell types *in vitro* or *in vivo*.

In addition to "classical" chemotherapeutic drugs and radiation (Chinnaiyan *et al*, 2000; Kim *et al*, 2001; Marini *et al*, 2005; Shankar *et al*, 2004), different classes of drugs such as proteasome inhibitors (de Wilt *et al*, 2013; Koschny *et al*, 2007a; Naumann *et al*, 2011; Saulle *et al*, 2007; Sayers & Murphy, 2006), epigenetic drugs – histone deacetylase (Earel *et al*, 2006; Fulda, 2012; Inoue *et al*, 2004; Kim *et al*, 2004; MacFarlane *et al*, 2005a; Muhlethaler-Mottet *et al*, 2006; Nakata *et al*, 2004; Neuzil *et al*, 2004; Singh *et al*, 2005) or methyl transferase (Braun *et al*, 2015; Hopkins-Donaldson *et al*, 2003; Soncini *et al*, 2013) inhibitors, BH3 (Cristofanon & Fulda, 2012; Huang & Sinicrope, 2008; Song *et al*, 2008; Wang *et al*, 2012) or Smac (Fakler *et al*, 2009; Fulda *et al*, 2002b; Lecis *et al*, 2010; Li *et al*, 2004; Raulf *et al*, 2014) mimetics, various kinase inhibitors (Lemke *et al*, 2014a; Meng *et al*, 2007; Ricci *et al*, 2007; Rosato *et al*, 2007), natural compounds found in food or herbs (Beranova *et al*, 2013; Ganapathy *et al*, 2010; Jacquemin *et al*, 2012; Park *et al*, 2013; Psahoulia *et al*, 2007; Tomasetti *et al*, 2006; Tomasetti *et al*, 2004; Weber *et al*, 2002) and many others have been demonstrated as efficient in elimination of various types of cancer cells when combined with TRAs.

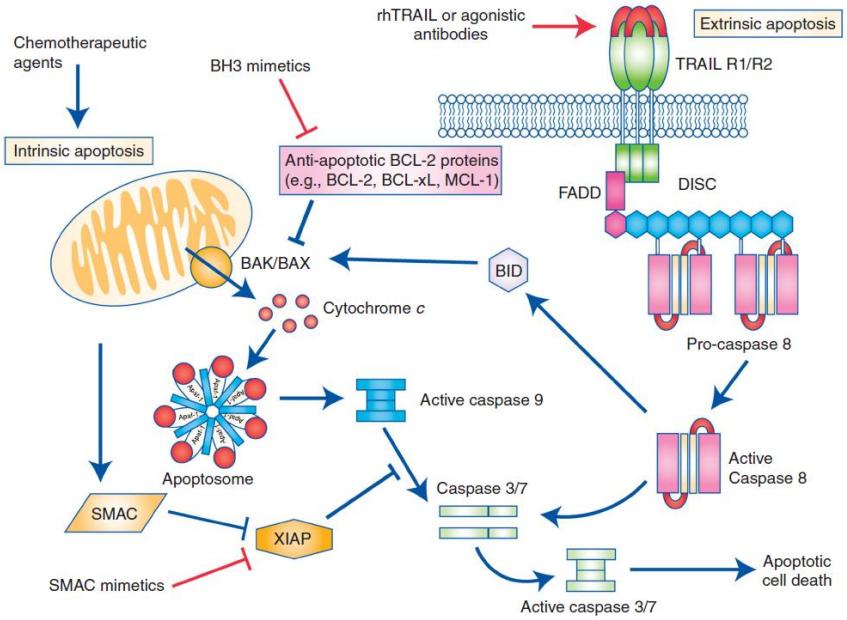

Fig. 5: The extrinsic and intrinsic apoptotic pathways and main points of therapeutic interventions (Fox & MacFarlane, 2016).

Distinct molecular mechanisms have been suggested to be involved in the cooperative cytotoxic action of the combination of TRAs and apoptosis-sensitizing drugs such as upregulation of pro-apoptotic and downregulation of anti-apoptotic proteins, associated with stimulation of DISC formation or enhanced involvement of mitochondria in type II cells (Fig. 5) (Fox & MacFarlane, 2016; Johnstone *et al*, 2008; Lemke *et al*, 2014a; Mahalingam *et al*, 2009; Newsom-Davis *et al*, 2009; Trivedi & Mishra, 2015; von Karstedt *et al*, 2017; Yuan *et al*, 2018). However, in many of them, the detailed knowledge on the molecular crosstalk of signalling pathways simultaneously triggered by these agents, and their complex regulations are far from being understood. Nevertheless, the above-mentioned findings provided a rationale to proceed with investigation of the anticancer effects of the combinations of TRAs and selected promising sensitizers to the already completed or still ongoing phase I/II clinical trials (Dimberg *et al*, 2013; Hellwig & Rehm, 2012; Koschny *et al*, 2007b; Lemke *et al*, 2014a; Naoum *et al*, 2017; Papenfuss *et al*, 2008; Stuckey & Shah, 2013; Yuan *et al*, 2018). Unfortunately, the clinically available drug combinations mostly have not achieved sufficient therapeutic activity, and some of them were discontinued. Therefore, the application of higher affinity TRAs and/or more potent TRA-sensitizing treatments, optimizing their doses and time sequence/duration of the treatment, and considering the relevant biomarkers for preselection of suitable patients may represent the promising approach for maximizing the therapeutic potential of TRAs combined with conventional or novel sensitizers.

Our research has been focused on the investigation of the molecular mechanisms responsible for cancer cell sensitization to TRAIL-induced and DR4/5-mediated apoptosis, which was achieved by application of several classes of therapeutically interesting agents such as conventionally used or novel chemotherapeutic drugs - cisplatin, LA-12, 5-FU, doxorubicin or etoposide, epigenetic drugs – 5´-aza-2´-deoxycytidine (decitabine), valproic acid (VPA) or CI-994, and a dietary polyunsaturated fatty acid with promising anticancer potential – docosahexaenoic acid (DHA). Our work uncovered novel molecular determinants with important role in modulation of TRAIL sensitivity by these drugs in several cancer cell types, especially those of colon, prostate and lung origin, which could be further exploited in future in order to provide potentially valuable targets for design of more successful therapeutic

strategies. The most important results of our work are highlighted and discussed in the following chapters, and more details can be found within the particular Supplements attached.

2.2.2.1. Platinum drug-mediated sensitization to TRAIL-induced apoptosis

Platinum complexes such as cisplatin, carboplatin and oxaliplatin belong to the most commonly used chemotherapeutic drugs in cancer treatment (Apps *et al*, 2015). However, their application is often limited due to serious side effects and the development of cancer cell resistance. These limitations evoke a need to uncover yet unknown mechanisms of regulation of their action and/or search for new analogues with improved anticancer potential. The promising cytotoxic effects of LA-12, a recently introduced platinum(IV) adamantylamin-ligand containing complex (Zak *et al*, 2004) have been demonstrated *in vitro* and *in vivo* by us and others in various cancer cell types (Bouchal *et al*, 2011; Horvath *et al*, 2007; Kvardova *et al*, 2010; Prochazka *et al*, 2007; Roubalova *et al*, 2010; Sova *et al*, 2005; Sova *et al*, 2006). Our group showed that LA-12 induced a favourable cytotoxic potential in several cisplatin-resistant cancer cell lines of a different origin (Horvath *et al*, 2006; Kozubik *et al*, 2005). LA-12 was also able to overcome confluence-dependent resistance observed in colon cancer cells treated with cisplatin or oxaliplatin, which may be a promising advantage in treatment of slowly growing tumours (Svihalkova-Sindlerova *et al*, 2010) (**Supplement 2**). We further showed the ability of LA-12 to overcome the block in the entry to M phase of the cell cycle observed in oxaliplatin-treated colon cancer cells. This was associated with a higher cytotoxicity of LA-12 compared to oxaliplatin, probably due to a lack of time for an efficient repair of cellular damage (Vondalova Blanarova *et al*, 2013) (**Supplement 3**). Importantly, in our recent work, we newly demonstrated that the anticancer properties of LA-12 can be further enhanced by inhibition of checkpoint kinase 1 (Chk1), a serine/threonine-specific protein kinase critical for the cell cycle control and coordination of the cell cycle progression in response to DNA damage (Herudkova *et al*, 2017) (**Supplement 4**). The combined treatment with one of the most specific Chk1 inhibitors, SCH900776 (Guzi *et al*, 2011; Samadder *et al*, 2017), resulted in a significant increase in G1/S phase-related LA-12-induced apoptosis, stimulation of mitotic slippage and senescence in colon cancer cells. We also showed that the presence of functional p53 may facilitate the drug combination-induced cytotoxicity. Furthermore, the cooperative cytotoxic effects were significantly accelerated in the absence of p21 and PTEN, and these proteins were identified as important determinants of the cancer cell response to the combined action of LA-12 and SCH900776. Our work represents the first demonstration of cooperative anticancer effects of LA-12 and SCH900776 at all. Previously, only a few studies investigating combined cytotoxic action of cisplatin and SCH900776 have been reported (Huntoon *et al*, 2013; Montano *et al*, 2012), and in contrast to our observations, they showed no further cancer cell sensitization. Uniquely, our work uncovered so far unknown antitumor potential of SCH900776 also when used in combination with cisplatin, and defined some molecular mechanisms involved. Next, our results also highlighted a remarkable importance of PTEN in modulation of LA-12-induced caspase-dependent apoptosis that was associated with a significant stimulation of mitochondrial apoptotic pathway, and occurred preferentially in G1 phase of the cell cycle (Laukova *et al*, 2015) (**Supplement 5**). The selected crucial aspects of platinum drug-induced cellular signalling, with a particular emphasis on LA-12, were summarized in our review article (Kozubik *et al*, 2008). Taken together, our research uncovered several important determinants of platinum drug-induced cytotoxic response in human cancer cells that can be further exploited in future therapeutic strategies.

Importantly, in addition to exploring its individual potential to trigger cancer cell death on its own, we investigated the ability of LA-12 to stimulate TRAIL-induced apoptosis in human cancer cells, and compared its molecular action with the conventionally used cisplatin. Some studies showed previously that the combined treatment with cisplatin may affect TRAIL-

induced signalling via modulation of different steps in apoptotic pathways like DR4/5, cFLIP, caspases or mitochondria-related events (Ding *et al*, 2011; Nagane *et al*, 2000; Shamimi-Noori *et al*, 2008; Tsai *et al*, 2006; Wu & Kakehi, 2009) in various cancer cell types, but the detailed regulatory mechanisms still remained to be elucidated. Until then, no information regarding the cooperative cytotoxic action of LA-12 and TRAIL could be found in the literature.

Our initial results newly showed that pre-treatment with sub-toxic concentrations of LA-12 enhanced the killing action of TRAIL in human colon and prostate cancer cell lines via stimulation of caspase activity and overall apoptosis (Vondalova Blanarova *et al*, 2011) (**Supplement 6**). The similar cooperative cytotoxic effects were observed using co-treatment with a 20-fold higher dose of cisplatin, suggesting the higher potential to sensitize to TRAIL-induced apoptosis in case of LA-12. Both platinum drugs significantly modulated the initial steps in the TRAIL apoptotic pathway - they increased surface or total DR5 level, stimulated re-localization of DR4 or DR5 into the lipid rafts, and accelerated internalization of TRAIL, the events that may substantially impact on the DISC complex formation and function. In addition, we hypothesized that the more downstream interaction of DR-mediated signalling and mitochondria may be profitable for maximizing of the killing effects of the drug combination, and explored in more detail the possible involvement of these organelles.

Our subsequent results showed that LA-12 primes human colon cancer cells for TRAIL-induced cell death by p53-independent activation of the mitochondrial apoptotic pathway (Jelinkova *et al*, 2014) (**Supplement 7**). The cooperative cytotoxic action of LA-12 and TRAIL was associated with stimulation of activation of Bax and Bak proteins, enhanced MOMP and caspase-9 activation, and an apparent shift of the balance between anti-apoptotic and pro-apoptotic Bcl-2 family proteins in favour of the latter ones. Unlike cisplatin, LA-12 was a potent inducer of ERK-mediated Noxa and Bim_L protein upregulation, and more effectively enhanced TRAIL-induced apoptosis in the absence of Bax. The cooperative pro-apoptotic action of LA-12 and TRAIL was further potentiated by siRNA-mediated silencing of Mcl-1 in both Bax-proficient and -deficient colon cancer cells. We also newly demonstrated that LA-12 triggered ERK-mediated upregulation of c-Myc, and proved that c-Myc silencing inhibited the mitochondria-dependent apoptosis following the treatment with LA-12 and TRAIL. Our results showing similar LA-12-mediated enhancement of TRAIL-induced apoptosis in several colon cancer cell lines derived from different stages of tumour development further underscored more general relevance of these findings.

In addition to colon cancer models, we also demonstrated a notable ability of LA-12 or cisplatin to potentiate TRAIL-induced mitochondrial apoptotic pathway in prostate cancer cells, which was associated with an enhanced Bid cleavage, Bak activation, drop of mitochondrial membrane potential, activation of caspases-8, -9, -10 and -3, and XIAP cleavage (Vondalova Blanarova *et al*, 2017) (**Supplement 8**). Employing RNAi approach, we demonstrated that cisplatin or LA-12 can sensitize cancer cells to TRAIL-induced apoptosis via Bid/Bak-dependent positive mitochondrial feedback amplification loop activation leading to subsequent secondary enhancement of caspase-8 processing. We showed that Bid is a crucial mediator of the drug combination-induced cytotoxicity, and it does not require Bak or caspase-10 for its activation. Although cisplatin/LA-12 and TRAIL combination induced cleavage of caspase-10, we newly proved that it was dispensable for the cooperative apoptotic effects of drugs observed in prostate cancer models (Fig. 6) (Vondalova Blanarova *et al*, 2017). Compared to cisplatin, significantly lower doses of LA-12 were required for similar sensitizing effects on TRAIL-induced prostate cancer cell apoptosis. This is in agreement with our previous work highlighting an outstanding apoptosis-inducing or –sensitizing ability of LA-12 when used in apparently lower doses than some conventional platinum-based drugs (Jelinkova *et al*, 2014; Svihalkova-Sindlerova *et al*, 2010), which might provide favourable advantage especially in resistant tumours. The detailed molecular mechanisms responsible for this phenomenon including the

role of previously reported faster and more efficient cellular uptake of LA-12 compared to cisplatin (Kvardova *et al*, 2010) need further investigation.

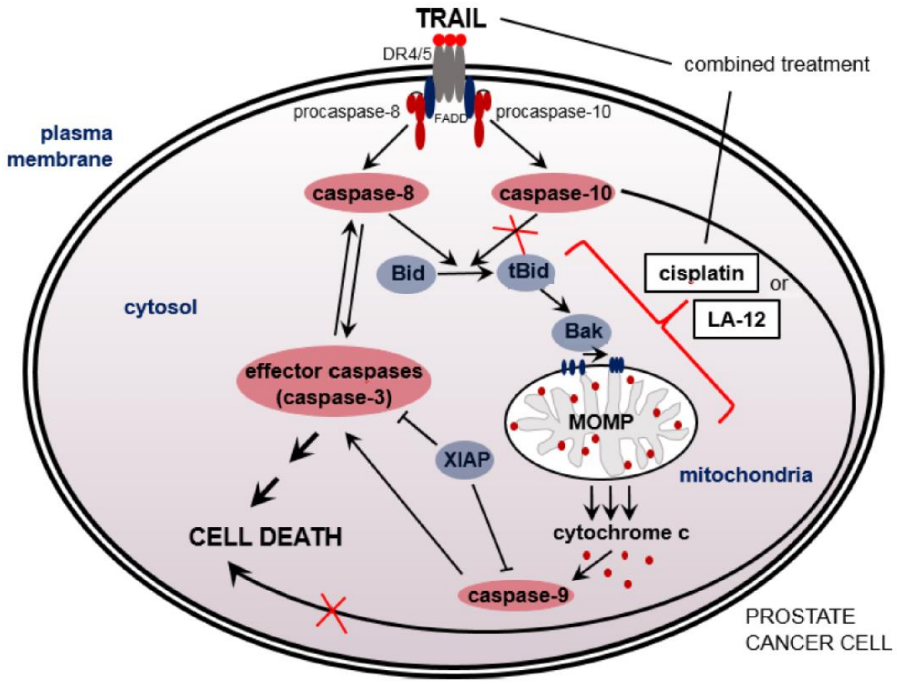

Fig. 6: Cisplatin/LA-12-mediated enhancement of TRAIL-induced apoptosis in prostate cancer cells – main mechanisms (Vondalova-Blanarova *et al*, 2017).

A highly frequent resistance to TRAIL-induced apoptosis caused by severe alterations of crucial components of its signalling pathway was previously reported in a large cohort of patient prostate cancer tissue samples (Anees *et al*, 2011), and our own results also support these findings (Vondalova Blanarova *et al*, 2017) (**Supplement 8**). However, we newly demonstrated the LA-12-mediated enhancement of TRAIL-induced cell death in primary prostate cancer cells isolated from human patient tumour biopsies. In agreement with the results of our experiments employing the prostate cancer cell lines, the drug combination-induced stimulation of cytotoxic response in patient tumour-derived samples was also associated with activation of mitochondrial apoptotic pathway. These results indicate that anticancer strategies employing TRAIL combined with platinum-based drugs may represent an efficient approach for elimination of prostate cancer cells compared to individual action of the drugs. Further molecular mechanisms involved are currently under investigation in our laboratory.

2.2.2.2. 5-fluorouracil-mediated modulation of TRAIL-DR pathways

Another important chemotherapeutic agent implicated in our work aimed to uncover in more detail the mechanisms of regulation of TRAIL DR-mediated signalling was 5-FU, a nucleoside analogue widely used for decades in standard treatment of many cancer types, particularly colorectal. Although initial response rates for 5-FU-based chemotherapy as a first-line treatment for this type of advanced cancer were only 10–15%, further improvement (40-50%) was subsequently achieved by its combination with irinotecan and oxaliplatin (Douillard *et al*, 2000; Giacchetti *et al*, 2000). Nevertheless, as common relapses still occur in a large part of responding patients, new therapeutic strategies are required to improve their survival. The anticancer effects of 5-FU are often based on its ability to block thymidylate synthase (TS) and incorporation of its metabolites into DNA or RNA molecules that finally results in the cell cycle arrest and/or apoptosis of tumour cells (Longley *et al*, 2003). However, frequent chemoresistance is a serious obstacle for successful therapy based on 5-FU. The cell resistance

to the 5-FU effects can be associated with alterations in the drug influx or efflux (Nambaru *et al*, 2011; Oguri *et al*, 2007), high levels of TS (Johnston *et al*, 1995; Peters *et al*, 2002; van Triest *et al*, 1999), defects in the expression or function of DNA mismatch repair genes (Arnold *et al*, 2003; Meyers *et al*, 2004) or overexpression of crucial anti-apoptotic proteins such as cFLIP (Longley *et al*, 2006), Bcl-2, Bcl-x_L or Mcl-1 (Shi *et al*, 2002; Violette *et al*, 2002). The results of these studies suggest that multiple factors may contribute to 5-FU resistance, and precise mechanisms involved need deeper elucidation.

Most chemotherapeutic drugs induce cancer cell apoptosis via activation of mitochondrial pathway, which often requires an involvement of p53. Thus, dysregulation of the function of this protein or related signalling can often result in the cancer insensitivity to the treatment. However, these drugs may also induce stimulation of the extrinsic, DR DISC-mediated apoptotic pathway, which can be both p53- dependent and –independent (Sheikh *et al*, 1998; Takimoto & El-Deiry, 2000), and understanding of the contribution of this signalling to the drug-mediated cytotoxicity and the value of its therapeutic targeting warrants further investigations. Although the status of p53 was proposed as an important player modulating the colon cancer cell response to 5-FU both *in vitro* and *in vivo*, its predictive value is still highly controversial. Some studies reported that the disruption of p53 function rendered some cancer cells resistant to 5-FU-induced apoptosis (Bunz *et al*, 1999; Longley *et al*, 2002), while others including us showed that cancer cells with lost wt p53 or mutant p53 could still trigger the cytotoxic response following treatment with 5-FU, albeit to the lesser extent (Akpinar *et al*, 2015; Backus *et al*, 2003; Garcia *et al*, 2011). Importantly, it has been shown previously that the inducible silencing of DR5 conferred 5-FU resistance of human colon tumours xenografted in immunodeficient mice (Wang & El-Deiry, 2004). These findings strongly suggested that DR5 could be a critical determinant of 5-FU chemosensitivity, but the molecular mechanisms behind still remained to be elucidated, including the possible involvement of p53.

Our work uncovered novel interesting findings about the role of TRAIL/DR5 and associated signalling in 5-FU-mediated cytotoxic action in human colon cancer cells (Akpinar *et al*, 2015) (**Supplement 9**). We showed that 5-FU promoted accumulation of DR5 into the plasma membrane, DISC formation and subsequent caspase activation. The siRNA-mediated downregulation of DR5, overexpression of cFLIP or FADD-DN abrogated 5-FU-induced cell death. In the absence of p53, the 5-FU-mediated DR5-DISC formation was slowed down, but not abrogated. These results indicate that 5-FU can activate DR5-dependent apoptosis regardless of p53 status, however the p53 presence facilitates extrinsic apoptotic signalling. Importantly, we also provided a novel evidence of the mechanism by which tumour cells lacking p53 can be sensitized to 5-FU combinatorial treatment using strategies targeting poly (ADP-ribose) polymerase-1 (PARP-1).

We further continued our research focused on the more detailed molecular mechanisms of regulation of TRAIL/DR5-related signalling in colon cancer cells treated with 5-FU (Akpinar *et al*, 2016) (**Supplement 10**). We demonstrated that disruption of lysosomal function by chloroquine (CQ) suppressed 5-FU-induced apoptosis and delocalized DR5 into cytosol on the lysosomal and autophagosomal membranes. Using chemical inhibitors, RNAi or genetic approaches, we showed that correct DR5 transport rather than autophagy itself is an important factor for 5-FU-induced cytotoxicity. Similarly to CQ, we also showed that brefeldin A (BFA) that blocks ER to Golgi protein transport abrogated 5-FU cytotoxicity, and delocalized DR5. Importantly, neither CQ nor BFA were able to impair TRAIL-induced apoptosis, suggesting a distinct DR5 activation mechanism. We further demonstrated that anticancer response induced by 5-FU is associated with significant alterations of intracellular DR5 signalling that depends on proper cholesterol and lysosomal function. Our results suggested a novel molecular mechanism for DR5 activation induced by 5-FU, important for its anticancer potential.

2.2.2.3. Doxorubicin and etoposide as effective sensitizers to TRAIL-induced apoptosis

In addition to colon and prostate cancer models, our work was also focused on the investigation of TRAIL signalling in small cell lung carcinoma (SCLC), an aggressive tumour entity with a poor prognosis, which represents a great challenge for novel treatment strategies as TRAIL monotherapy is here highly inefficient (Hopkins-Donaldson *et al*, 2003). The failure of SCLC cells to undergo apoptosis in response to this cytokine has been attributed to the significant defects at the level of DISC such as the loss of the expression of DR4, DR5, caspase-10 and caspase-8 (Hopkins-Donaldson *et al*, 2003; Joseph *et al*, 1999; Shivapurkar *et al*, 2002a). Of them, the most prominent characteristics of the majority of SCLC cells is a frequent loss of caspase-8. The same phenomenon was also confirmed in our work, where most SCLC cell lines used in the study were deficient for not only caspase-8, but also caspase-10, thus bearing a complete block in initiator caspase activation via an extrinsic pathway (Vaculova *et al*, 2010) (**Supplement 11**) and therefore resistant to TRAIL-induced apoptosis. Moreover, we also found that even caspase-8 expressing SCLC cells did not respond to the apoptotic effects of TRAIL. Therefore, we investigated whether the cytotoxic effects of TRAIL in SCLC could be restored by co-treatment with selected sensitizers, and used two different approaches separately for caspase-8 expressing or lacking SCLC cells.

Firstly, our results demonstrated that doxorubicin or etoposide, the chemotherapeutic drugs used for treatment of SCLC, could significantly restore the ability of TRAIL to kill caspase-8 expressing SCLC cells. As TRAIL by itself did not induce caspase-8 processing, it was likely that the resistance to TRAIL-induced apoptosis was regulated upstream of this molecule. We demonstrated that doxorubicin mediated sensitization of these cells to the apoptotic effects of TRAIL that was associated with significant increase of surface and total DR5 level, strong caspase-8 activation, a specific cleavage of $cFLIP_L$, and a decrease of $cFLIP_S$ level. These events were further accompanied by an enhanced Bid cleavage, Bax activation, MOMP, cytochrome *c* release, survivin downregulation, and effector caspase activation. Using caspase-8 siRNA or a pan-caspase inhibitor z-VAD-fmk, we showed that the co-treatment with TRAIL and doxorubicin facilitated caspase-8 processing primarily at the DISC level rather than being a secondary result of activation of mitochondrial pathway or effector caspases. The sensitization of SCLC cells expressing caspase-8 to TRAIL-induced apoptosis occurred preferentially at the level DR5 and cFLIP. We proposed that the combined treatment with TRAIL and conventional chemotherapeutic drugs like doxorubicin or etoposide could represent an effective killing strategy for SCLC cells proficient but not deficient for caspase-8.

Our second approach was further aimed at investigation of the potential relevance of TRAIL-based therapy in SCLC cells lacking caspase-8. As the analysis of SCLC tumours revealed that the methylation of CpG islands in the caspase-8 gene was an important factor responsible for the loss of its expression (Shivapurkar *et al*, 2002b), the modulation of epigenetic mechanisms involved seemed to be a valuable target of the therapeutic interventions.

2.2.2.4. Epigenetic drugs in modulation of TRAIL-induced apoptotic signalling

It has been shown previously that co-treatment of SCLC cells with the demethylating agent decitabine and interferon-γ partially restored caspase-8 expression and increased the cell sensitivity to TRAIL-induced apoptosis (Hopkins-Donaldson *et al*, 2003), but activation of other yet uncovered mechanisms seemed to be important for the realization of the full cytotoxic potential of TRAIL. Similarly, the pharmacologically relevant doses of decitabine slightly enhanced the expression of caspase-8 in a panel of SCLC cells in our study, but the amount of caspase-8 was not sufficient to sensitize these cells to TRAIL-induced apoptosis (Kaminskyy *et al*, 2011) (**Supplement 12**). However, we newly demonstrated much more pronounced effects on both caspase-8 re-expression and TRAIL sensitisation when decitabine was combined with HDAC inhibitors valproic acid (VPA) or CI-994. The sensitisation to TRAIL-

induced apoptosis mediated by the two epigenetic drugs also involved activation of mitochondrial pathway and caspase-9, and reduction of the level of anti-apoptotic proteins survivin and cIAP1, indicating that along with the induced level of caspase-8, other factors might contribute to the effect. This could be attributable to the fact that the epigenetics drugs used in the study might simultaneously affect the expression or activation of other molecules with potential impact on apoptotic signalling pathways induced by TRAIL. Nevertheless, we newly demonstrated that the use of the combination of the inhibitors of DNA methyl transferases and HDACs represents an effective treatment option for sensitization of SCLC cells lacking caspase-8 to the killing effects of TRAIL.

2.2.2.5. Docosahexaenoic acid in modulation of TRAIL-induced cell death

Another candidate with potentially interesting sensitizing effects on TRAIL-induced cytotoxicity examined in our work was docosahexaenoic acid (DHA), a polyunsaturated fatty acid of n-6 series known for its ability to exert cytostatic or cytotoxic effects in various cancer cell types, and modulate the expression or activity of molecules relevant for apoptotic signalling (Lee *et al*, 2017; Skender *et al*, 2012). We showed that DHA may affect the cellular lipid composition and apoptotic signalling of some TNF family members in colon cancer cells (Hofmanova *et al*, 2012; Hofmanova *et al*, 2017; Hofmanova *et al*, 2014; Hofmanova *et al*, 2005). We also newly demonstrated that DHA mediated stimulation of TRAIL-induced apoptosis in SW620 human colon cancer cells that was associated with extensive engagement of mitochondrial pathway, manifested by Bid cleavage, Bax/Bak activation, drop of MMP, cytochrome c release, caspase-9 activation and downregulation of XIAP and cIAP1 protein levels (Vaculova *et al*, 2005) (**Supplement 13**), (Skender *et al*, 2014) **(Supplement 14)**. We suggested that DHA can prime mitochondria to make them more prone to TRAIL-induced apoptotic insults. Furthermore, in agreement with previous studies (Jakobsen *et al*, 2008), our results demonstrated that DHA induced an activation of endoplasmic reticulum (ER) stress response. We proposed that ER stress response triggered by DHA could interfere with mitochondrial signalling and contribute to the cancer cell sensitisation to TRAIL-induced killing.

We further demonstrated that DHA-mediated enhancement of TRAIL-induced apoptosis in colon cancer cells was associated with significant changes in the representation of specific sphingolipid molecules, especially ceramides (Skender *et al*, 2014) (**Supplement 14**). They have been established as important regulators of both intrinsic and extrinsic apoptotic pathways and ER stress-induced cell death signalling (de Palma & Perrotta, 2012). It has been shown that specific changes in particular ceramide species can be associated with distinct cellular responses (Hartmann *et al*, 2012). Among them, the increased levels of C16-ceramide have been attributed to the more "pro-apoptotic ceramide phenotype", while the predominance of longer chain ceramides rather supported the cell survival. As an example, this phenomenon was observed in human colon cancer cells isolated from the primary tumour (SW480) and subsequent metastasis (SW620) of the same patient, which differ substantially in their ability to undergo TRAIL-induced apoptosis (Voelkel-Johnson *et al*, 2005). The TRAIL-sensitive SW480 cells contained higher levels of C16-ceramide and less C24-ceramide when compared to TRAIL-resistant SW620 cells. In SW480 but not SW620 cells, TRAIL treatment stimulated further increase in C16-ceramide level, which correlated with apoptosis induction. In addition, the SW620 cells the resistance to TRAIL was overcome by exogenously added ceramide, further confirming that defective ceramide signalling may contribute to TRAIL resistance (Voelkel-Johnson *et al*, 2005). Building on these studies, we newly suggested that DHA-mediated increase in C16:0 in SW620 cells may help to overcome their defects in ceramide signalling, thus facilitating apoptosis after exposure to TRAIL. We also showed that DHA and TRAIL-induced apoptosis was partially blocked by the pre-treatment with fumonisin B1, an

inhibitor of ceramide synthases, which further indicated that the changes in the ceramide signalling may be involved in the stimulation of colon cancer cell apoptosis induced by DHA and TRAIL. Modulation of ceramide synthase 6 levels were also previously shown to affect colon cancer cell sensitivity to TRAIL-induced apoptosis (White-Gilbertson *et al*, 2009). Taken together, our results highlighted the potential of DHA to enhance the TRAIL-induced selective elimination of colon cancer cells via simultaneous targeting of multiple steps in apoptotic pathways.

2.2.2.6. Potential non-selective toxicity of TRAIL-based combination therapy

As specified above, many preclinical studies demonstrated that combination treatments with TRAIL and chemotherapeutic or chemopreventive drugs can overcome the cell death resistance in many cancer cell types. However, requirements of anticancer therapy include not only its high antitumor efficacy, but also minimal toxicity for healthy tissue. Thus, such combinational approaches can be of clinical benefit only in case that they do not simultaneously harm normal cells. This issue has to be particularly considered in the treatment of pediatric cancer patients who have been reported to have a higher risk of developing treatment-associated toxicities. In an interesting recent study, the authors showed that in contrast to adults, the mitochondria in the cells derived from normal somatic tissues such as brain, heart, and kidneys in young children are primed for pro-apoptotic signalling, making them more vulnerable to undergo cell death in response to the action of some chemotherapeutic drugs or ionizing radiation (Sarosiek *et al*, 2017). They showed that apoptosis sensitivity may be dynamically regulated during postnatal development in some healthy tissues, which could substantially affect the occurrence of undesired side effects of the anticancer therapies during the human life.

The relatively limited number of currently available studies focused on potentially undesired toxic effects of TRAIL-based combination therapy in non-cancer cells demonstrated heterogeneous results, depending on the type of the selected TRAs, their sensitizers and the treatment schedules (dose, timing, single/multiple applications). While several combination regimens have been presented as safe for certain types of normal cells, other warn of potentially dangerous toxicities (Finnberg *et al*, 2016; Ganten *et al*, 2006; Koschny *et al*, 2007c; Van Valen *et al*, 2003). The molecular mechanisms involved in the latter scenario remain largely unexplored, as well as strategies how to manipulate them. Generally, it still remains to be answered which sensitizing drugs may open the therapeutic window for more effective but still safe TRAIL-based treatment in particular cancer types.

2.3. TRAIL-induced non-apoptotic signalling

2.3.1. Necroptosis

Numerous recent reports showed that apart from its ability to trigger "classical" caspase-dependent apoptosis, TRAIL can also induce necroptosis, a form of caspase-independent regulated cell death that critically involves activation of receptor-interacting serine/threonine-protein kinase 3 or 1 (RIPK3 or RIPK1) (Galluzzi *et al*, 2018; Grootjans *et al*, 2017; Su *et al*, 2016). If caspase-8 is absent or pharmacologically inhibited, apoptosis is prevented and formation of necrosome, followed by execution of necroptosis may occur alternatively. So far, only a limited number of recent studies elucidated the involvement of specific molecules and related regulatory mechanisms of TRAIL-induced necroptosis, as well as the differences between necroptotic signalling pathways triggered by TRAIL and other TNF family members (Jouan-Lanhouet *et al*, 2012; Sosna *et al*, 2016). It has been shown that TRAIL-induced necroptosis caused death in various cancer cell types, and impaired their clonogenic survival

(Meurette *et al*, 2005; Meurette *et al*, 2007; Voigt *et al*, 2014). Importantly, several chemotherapeutic drugs have been shown to act in cooperation with TRAIL to stimulate cancer cell necroptosis, highlighting a potential of combinatorial therapeutic approaches (Voigt *et al*, 2014). The more precise mechanisms and specific role of necroptosis in TRAIL-induced anticancer action, together with predictive biomarkers for cancer cell sensitivity to necroptosis and also possible off-target effects of necroptosis-based therapy remain to be elucidated. They may uncover yet unknown potential of TRAIL-induced necroptosis as a strategy to overcome apoptosis resistance in tumour therapy, which is so far almost completely unexplored.

2.3.2. Non-cell death signalling

In addition to apoptosis or necroptosis, TRAIL-induced activation of non-cell death gene regulatory signalling pathways resulting in stimulation of proliferation and survival has been demonstrated in various apoptosis-resistant cancer cells or non-transformed normal cells (Belyanskaya *et al*, 2008; Ehrhardt *et al*, 2003; Vilimanovich & Bumbasirevic, 2008). Moreover, activation of DR4/5-mediated pathway can also be involved in enhancement of metastatic properties of cancer cells (Ishimura *et al*, 2006; Trauzold *et al*, 2006), and even affect the crucial processes in the tumour microenvironment such as angiogenesis or secretion of pro-inflammatory and tumour-promoting cytokines (Hartwig *et al*, 2017; Leverkus *et al*, 2003). The results of the above-mentioned studies highlight a dual role of TRAIL signalling not only in elimination of cancer cells, but under some circumstances also in stimulation of their tumorigenic potential. It seems that treatment of certain apoptosis-resistant tumours with TRAs as a single agents may even increase the risk of worsening the disease progress. Therefore, the strategies that will help to overcome apoptosis resistance in cancer cells and simultaneously block the undesired pro-tumorigenic effects of TRAIL are warranted. Selected combined treatments are believed to shift the outcome of the complex TRAIL-induced signalling in favour of cell death, thus reducing both on-target adverse effects and off-target toxicities. For the successful design of such therapeutic strategies, in-depth mechanistic understanding of the cell death and non-cell death pathways, cross-talks between them, and involvement of regulatory proteins that can be shared by these pathways, but may undergo specific modifications that may determine their specific contribution to particular pathway need to be clarified.

In addition to already mentioned DISC (also called TRAIL-R-associated complex I), ligation of DR4/5 can induce formation of a cytosolic complex II, which consists of caspase-8, FADD, RIPK1, TNF receptor-associated factor 2 (TRAF2) and NF-κB essential modulator **(**NEMO), but lacks TRAIL-Rs (Varfolomeev *et al*, 2005). Until recently, TRAIL-induced "non-cytotoxic" gene activating signalling has been considered as solely associated with the activation of cytosolic complex II. However, Lafont et al. showed that this signalling can also be induced via membrane-associated complex I (Lafont *et al*, 2017). Moreover, they newly identified LUBAC (the linear ubiquitin chain assembly complex) as a component of both complex I and II and demonstrated that it is crucial in regulation of the balance between the different outcomes of TRAIL signalling. The authors suggested that TRAIL-induced cell death pathways and "non-cytotoxic" gene activation pathways may not be mediated by spatially distinct signalling complexes, but they are rather finely regulated by ubiquitination events within the complex I and II, which can both act as "DISCs" or "gene activating platforms" (Lafont *et al*, 2018; Lafont *et al*, 2017). Understanding the molecular mechanisms and kinetics of these ubiquitination and de-ubiquitination events as well as the specific involvement of particular kinases will help to understand the "dichotomy" of TRAIL signalling, and identify novel biomarkers and clinical targets. Within the non-apoptotic TRAIL-induced signalling, the involvement of various kinases such as RIPK1, IκB kinase (IKK), mitogen-activated protein kinases (MAPKs) – extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK), phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), Src, transforming

growth factor β-activated kinase 1 (TAK1) or protein kinase C (PKC) have been reported (Fig. 7) (Azijli *et al*, 2013; Kretz *et al*, 2018). However, the preferential activation of specific kinase cascades triggered by TRAIL in particular cell types, their interactions, and the role of tumour microenvironment and other factors in modulation of their activation and function need further clarification.

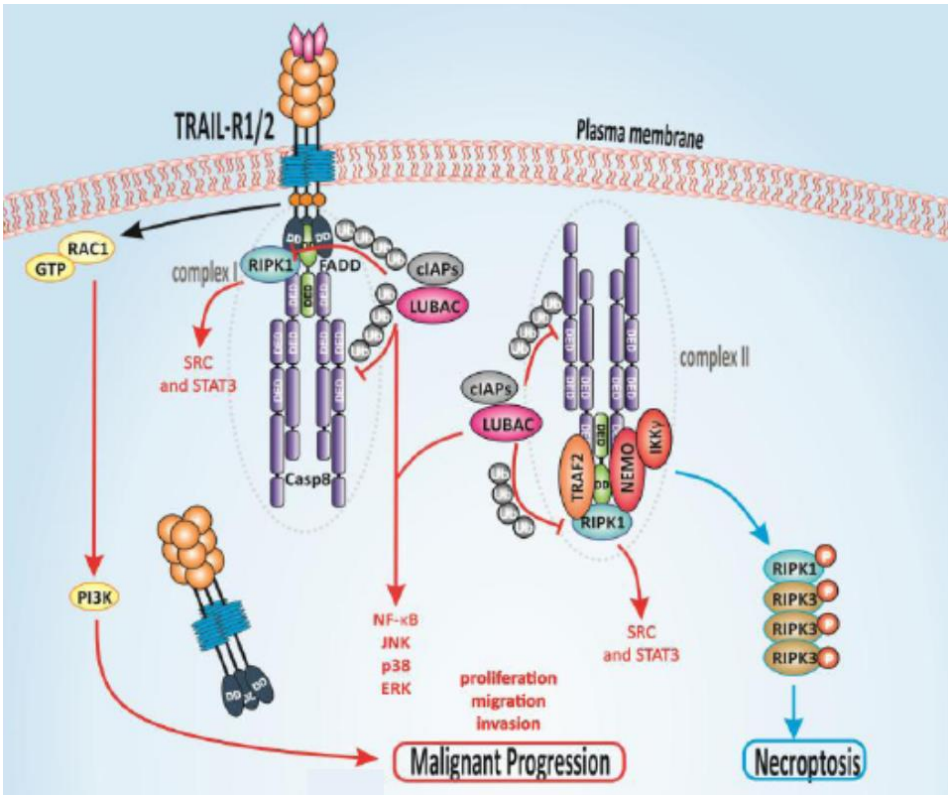

Fig. 7: TRAIL-induced non-apoptotic signalling (Kretz *et al*, 2018).

2.3.2.1. NF-κB pathway

One of the first discovered "non-canonical" kinase signalling pathways triggered by TRAIL was activation of transcription factor NF-κB, which can be mediated via DR4, DR5 or DcR2 (Degli-Esposti *et al*, 1997a; Harper *et al*, 2001; Ravi *et al*, 2001; Shetty *et al*, 2005). It can involve RIPK1-induced activation of IKK, followed by phosphorylation and proteasomal degradation of IκB, nuclear translocation of NF-κB, and transcription of anti-apoptotic genes such as cFLIP, Mcl-1, Bcl-x_L or cIAPs (Azijli *et al*, 2013). Thus, inhibition of NF-κB has been shown as an effective way to enhance TRAIL-induced apoptosis in some cancer cell types (Dalen & Neuzil, 2003; Harper *et al*, 2001; Karacay *et al*, 2004; Roue *et al*, 2007). On the contrary, NF-κB may also mediate the transcriptional upregulation of TRAIL receptors or some "pro-apoptotic" genes, and under some circumstances also support the TRAIL-induced cancer cell apoptosis (Jennewein *et al*, 2011; Jeremias *et al*, 1998). This dual role of NF-κB signalling in modulation of apoptotic response elicited by TRAIL needs further elucidation. Moreover, several studies have shown the importance of NF-κB in TRAIL-induced stimulation of proliferation of some apoptosis-resistant cancer cells (Ehrhardt *et al*, 2003) or promotion of cancer cell migration and invasion both *in vitro* and *in vivo* (Ishimura *et al*, 2006; Trauzold *et al*, 2006). Therefore, without targeted manipulation, TRAIL-induced activation of NF-κB pathway may represent a potential danger especially in some patients with cell death resistant tumours (Malhi & Gores, 2006).

2.3.2.2. ERK pathway

In addition to NF-κB, there is a growing evidence on the role of TRAIL-induced ERK pathway in stimulation of proliferation of cancer or normal cells, or in mediation of cancer cell survival and resistance to apoptosis (Azijli *et al*, 2013; Falschlehner *et al*, 2007). Regarding the first issue, TRAIL induced ERK-mediated stimulation of proliferation of caspase-8-deficient SCLC cells, which was dependent on DR5 and reduced by PD98059, an inhibitor of ERK pathway, was reported (Belyanskaya *et al*, 2008). TRAIL also induced proliferation of human glioma cells mediated via activation of ERK pathway that was dependent on $cFLIP_L$ (Vilimanovich & Bumbasirevic, 2008). TRAIL-induced ERK-mediated enhancement of proliferation was further demonstrated in normal human preadipocytes, vascular endothelial and smooth muscle cells, highlighting the role of TRAIL in modulation of the normal tissue physiology and homeostasis (Funcke *et al*, 2015; Secchiero *et al*, 2003; Secchiero *et al*, 2004).

Second, various studies also showed the importance of ERK pathway in modulation of TRAIL-induced cytotoxicity. In many but not all cases, TRAIL-induced activation of ERK pathway has been associated with the cancer cell protection from apoptosis. TRAIL-induced ERK activation was reported to prevent human melanoma cell apoptosis by inhibition of re-localization of Bax protein to mitochondria and subsequent blockage of mitochondrial release of Smac/DIABLO (Zhang *et al*, 2003). The protective effect of ERK pathway on DR-mediated apoptosis was also demonstrated in HeLa cervical cancer cells, most probably at the level or upstream of caspase-8 (Tran *et al*, 2001). In our work, we showed that TRAIL triggered a strong ERK1/2 phosphorylation accompanied by an increase in Mcl-1 protein level in HT-29 human colon cancer cells. The chemical inhibition of ERK pathway markedly sensitized these cells to apoptosis induction by this cytokine, which was associated with a significant enhancement of caspase-8,-9,-3 activation and their substrate cleavage, drop in MMP, and a loss of Mcl-1 protein (Vaculova *et al*, 2006) (**Supplement 15),** (Hofmanova *et al*, 2008) **(Supplement 16**). In our subsequent study, we further proved that TRAIL-induced activation of ERK was functionally important for early upregulation of Mcl-1 at the level of both mRNA and protein. The siRNA-mediated silencing of Mcl-1 significantly enhanced TRAIL-induced apoptosis and related changes at the level of mitochondria (Vaculova *et al*, 2009) (**Supplement 17**). On the other hand, a pre-treatment with epidermal growth factor (EGF), a well-known activator of ERK pathway, resulted in further increase in TRAIL-induced Mcl-1 protein level and supported the cell resistance to the apoptotic effects of this cytokine. Taken together, our results documented that TRAIL can simultaneously activate both caspase-mediated apoptotic as well as ERK kinase-mediated pro-survival signalling, and described the molecular mechanisms of the cross-talk between them at the level of anti-apoptotic Bcl-2 family member Mcl-1, an important regulator of cancer cell sensitivity to TRAIL (Ricci *et al*, 2007).

2.3.2.3. PI3K/Akt pathway

Similarly as in case of ERK pathway, numerous reports also demonstrated the propensity of TRAIL-induced PI3K/Akt pathway to enhance proliferation or support survival in normal and cancer cells, and inhibition of this pathway often reverted the cancer cell sensitivity to apoptotic effects of TRAIL (Liu *et al*, 2010; Morel *et al*, 2005; Xu *et al*, 2010; Zauli *et al*, 2005). While the elevated Akt activity has been shown to protect many cancer cell types from TRAIL-induced apoptosis (Dida *et al*, 2008; Goncharenko-Khaider *et al*, 2010; Chen *et al*, 2001; Lalaoui *et al*, 2001; Nesterov *et al*, 2001; Puduvalli *et al*, 2005), it was ineffective in others (Efron *et al*, 2006). We showed that LY294002-mediated inhibition of PI3K/Akt pathway significantly enhanced TRAIL-induced apoptosis in colon cancer cells, which was associated with stimulation of caspase activation and involvement of mitochondria (Vaculova *et al*, 2006) (**Supplement 15**). These pro-apoptotic effects induced by the

combination of TRAIL and LY294002 were not mediated via downregulation of Mcl-1 protein as we observed following treatment with TRAIL and ERK inhibitors, suggesting that in these cells ERK or PI3K/Akt employ distinct molecular mechanisms in order to protect from TRAIL-induced apoptosis.

In addition to regulation of the proliferation and survival, PI3K/Akt pathway also plays an important role in modulation of cell invasiveness and migration (Agarwal *et al*, 2013). It has been shown that changes in activity of this pathway may be related to alterations in cell adhesive properties that frequently occur especially in metastatic cancers. In our further work, we showed that compared to adherently cultivated (attached) colon cancer cells, the activation of PI3K/Akt pathway was significantly enhanced in their non-adherently cultivated (detached) counterparts that were resistant to anoikis, a specific variant of apoptosis induced by the loss of integrin-dependent anchorage (Galluzzi *et al*, 2018). Furthermore, we observed that the detachment-induced activation of PI3K/Akt pathway in colon cancer cells was associated with an increase in their resistance to TRAIL-induced apoptosis, and inhibition of this pathway resulted in re-activation of apoptotic response to this cytokine (Koci *et al*, 2011) (**Supplement 18**). In contrast, another study showed a decrease in Akt phosphorylation and an enhancement of TRAIL-induced apoptosis in detached ovarian cancer cells (Lane *et al*, 2008). An additional increase in TRAIL-induced killing of these cells was further observed under the inhibition of Akt pathway by LY294002. The results obtained by us and others suggest that despite the differences in regulation of basal PI3K/Akt activity in different cell/tumour types, targeted inhibition of this pathway may serve as a useful approach to prime numerous resistant cancer cells to TRAIL-induced apoptosis.

2.4. TRAIL-induced apoptotic versus non-apoptotic signalling

TRAIL-induced apoptotic signalling belongs to the best-characterized apoptotic pathways and harbours a great potential for exploitation in cancer therapy. However, despite its name ("apoptosis-inducing ligand"), the induction of apoptosis is only one of the biological effects that can be triggered by this cytokine (Fulda, 2013b). Although so far less well understood, it is unequivocally clear that so-called non-canonical TRAIL-induced signalling represents a potential risk of TRAIL-based anticancer therapies. As already mentioned, the engagement of non-canonical signalling not only hampers TRAIL-induced apoptosis in cancer cells, but also encourages the development of the malignant phenotype by enhancing their proliferation, survival, migration and metastasis (Fig. 8) (Bertsch *et al*, 2014). However, it remains to be better understood if or under which conditions TRAIL induces such signalling events preferentially/exclusively in apoptosis-resistant cancer cells or how relevant it is in tumours that are responsive to TRAIL-induced killing. It has been shown that TRAIL can activate both canonical and non-canonical signalling pathways in clonal cancer cell populations within the heterogeneous tumour, where some of the cell subpopulations undergo apoptosis, while other survive and develop acquired resistance to TRAIL-induced killing. This phenomenon called a fractional survival may contribute to failure of TRAIL-based therapy and enable further tumour propagation (Flusberg *et al*, 2013; Shlyakhtina *et al*, 2017).

2.4.1. Pleiotropic function of DR4/5 during carcinogenesis

Different types of deregulations at the DR4/5 level expression and function, and changes in sensitivity/resistance to TRAIL effects may frequently occur during carcinogenesis in various tumours. A high frequency of the deficiency of DR4/5 on the surface plasma membrane has been demonstrated in various cancer cell types (Gallmeier *et al*, 2013; Hopkins-Donaldson *et al*, 2003; Horak *et al*, 2005; Jin *et al*, 2004; Ozoren *et al*, 2000; Zhang & Zhang, 2008). Although the complete loss of both surface DR4 and DR5 in cancer cells is not so frequent, the

absence of one of them may be sufficient to induce TRAIL resistance. In some of these cases, combination therapies that either facilitate TRAIL-induced apoptotic signalling via the remaining receptor or help to re-establish the expression of the missing receptor can restore the cancer cell sensitivity to the killing effects of TRAIL (Pennarun *et al*, 2010). However, the increase in surface DR level mediated by the sensitizing drugs used in combination with TRAs may under some circumstances serve as a double-edge sword as in addition to potentiation of apoptotic effects, it can also stimulate the non-canonical signalling in cancer cells. Therefore, combination of TRAIL DR agonists with cell death sensitizers and at the same time compounds hampering the stimulation of undesired pro-tumorigenic events (e.g. specific kinase inhibitors) is required to ensure the efficient execution of cancer-selective death while countering the manifestation of tumour-promoting features (Shlyakhtina *et al*, 2017).

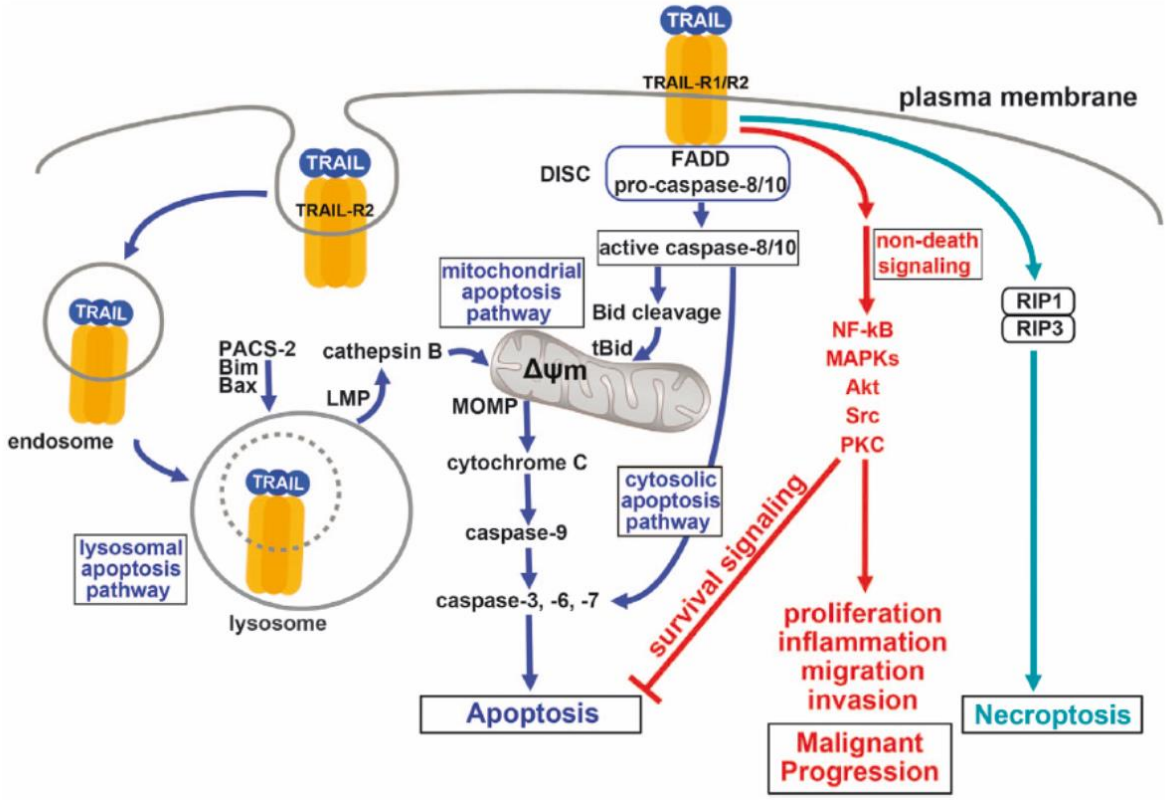

Fig. 8: Pleiotropic functions of plasma membrane-localized TRAIL DRs (Bertsch *et al*, 2014).

On the contrary, there are also studies showing an increase in DR4/5 expression during carcinogenesis, e.g. in cervical, pancreatic or colorectal cancer cell lines or patient-derived colorectal tumours (Jalving *et al*, 2014; Koornstra *et al*, 2003; Ozawa *et al*, 2001; Ryu *et al*, 2000). In addition, a high DR4/5 expression was associated with worse overall patient survival in some cancers (Dong *et al*, 2008; Ganten *et al*, 2009; McCarthy *et al*, 2005; van Geelen *et al*, 2006). These findings suggest that the elevation of DR4/5 expression during cancer development may provide tumours with growth advantages rather than support their pro-apoptotic phenotype. In such tumours, the activation of DR4/5 signalling predominantly contributes to cancer promotion, and its targeted inhibition may be beneficial in cancer treatment strategies.

Thus, depending on the tumour type or the cellular context, TRAIL DRs have been shown stimulate pleiotropic signalling resulting either in suppression of tumour initiation and metastasis (Cretney *et al*, 2002; Grosse-Wilde *et al*, 2008) or promotion of cancer cell migration

and invasion (Ishimura *et al*, 2006; Trauzold *et al*, 2006; von Karstedt *et al*, 2015). Interestingly, the oncogenic K-Ras was proposed as a critical factor in switching the pro-apoptotic function of TRAIL DRs into pro-metastatic ones in human and mouse colorectal cancer cells, suggesting the potential adverse effects of treatment with DR4/5 agonists in patients with K-Ras mutated tumours (Hoogwater *et al*, 2010; von Karstedt *et al*, 2015). The results of these studies showed that TRAIL DR signalling is much more pleiotropic than initially thought, and further clarification of its molecular complexity and crucial determinants is urgently needed in order to make a right selection of patients who may benefit from TRAIL treatment.

2.4.2. DR4/5-specific signalling in cancer

Another important aspect that has to be considered is the phenomenon of differences in a relative contribution of surface DR4 or DR5 to apoptotic and non-apoptotic signalling in various cancer cell types. For example, primary cells derived from patients with chronic lymphocytic leukemia, mantle cell lymphoma or human pancreatic ductal adenocarcinoma cells trigger apoptosis almost exclusively through DR4 but not DR5 (Lemke *et al*, 2010; MacFarlane *et al*, 2005). In contrast, cancer cell lines derived from colon and breast tumours signal primarily through DR5 although they express both DRs at the surface (Kelley *et al*, 2005). Although there are reports showing preferential induction of apoptosis via either DR4 or DR5 in different cell types and conditions, DR5 is generally considered as more potent apoptotic mediator compared with DR4 (Kelley *et al*, 2005; Truneh *et al*, 2000). In agreement with these findings, results of our work also showed that the chemotherapy-mediated sensitization of SCLC or colon cancer cells to TRAIL-induced apoptosis occurred preferentially at the level of DR5 (Vaculova *et al*, 2010) (**Supplement 11)**, (Vondalova Blanarova *et al*, 2011) **(Supplement 6**). Similarly, ABT-737- or homoharringtonine-mediated enhancement of TRAIL-induced apoptosis also proceeded predominantly via the DR5 receptor in colon cancer cells (Nahacka *et al*, 2018).

Compared to apoptosis pathways, fewer studies explored the relative contribution of DR4/5 to TRAIL-induced non-apoptotic kinase-mediated signalling. Activation of NF–κB following TRAIL stimulation was exclusively driven by DR4 and not inhibited by DR5 blocking antibody in pancreatic ductal adenocarcinoma cells (Lemke *et al*, 2010). In contrast, TRAIL-induced non-canonical kinase-mediated migratory and invasive features in apoptosis resistant NSCLC cells were predominantly mediated by DR5 (Azijli *et al*, 2012). Another group also reported a differential contribution of DR4/5 to JNK-mediated signalling in HeLa cells treated with TRAIL or agonistic DR-selective monoclonal antibodies (Muhlenbeck *et al*, 2000). An interesting recent study provided a first systematic insight into complex DR4/5-specific signalling in colorectal and pancreatic cell lines. The authors showed that DR5 plays a major role in TRAIL-induced non-canonical kinase signalling, but not in the induction of necroptosis (Nahacka *et al*, 2018). It is evident that the efficacy of the combined TRAIL-based therapy can be substantially affected by the appropriate selection of particular DR4/5-specific recombinant TRAIL ligand variants or monoclonal antibodies and/or the co-treatment with sensitizing agents primarily influencing the regulation of DR4- or DR5-specific signalling.

2.4.3. DR4/5 compartmentalization in TRAIL signalling

Until recently, mostly the total or surface level of DR4/5 was investigated as a prognostic marker for cancer development and therapy. However, there is an increasing number of publications showing that DR4/5 are abundantly present in other previously unexplored subcellular localizations, especially in cancer cells. Besides plasma membrane and the membranes of secretory or endocytic/lysosomal compartments, TRAIL DRs can also be found in autophagosomes, soluble within the cytoplasm or in the nucleus (Bertsch *et al*, 2014; Twomey *et al*, 2015). In these locations, DR4/5 seem to exert specific yet uncharacterized

functions rather than only contribute to apoptotic or non-apoptotic signalling pathways initiated within the plasma membrane (Bertsch *et al*, 2014). The recent discoveries clearly showed that subcellular localization of DR5 and the related regulatory events represent important factors that determine the final output of DR-mediated signalling, and elucidation of these mechanisms will substantially change the understanding of TRAIL biology. Importantly, the presence of DR4/5 in specific subcellular compartments could be used as a predictive biomarker to identify the patients that are most likely to respond to TRA-based therapy (Mert & Sanlioglu, 2017).

One of the events that can significantly contribute to the modulation of the DR4/5 intracellular trafficking and localization is endocytosis (Repnik *et al*, 2013). The internalized receptors can be transported through early to late endosomes and lysosomes, which may be followed by receptor degradation. On the other hand, internalized receptors can be delivered back to the plasma membrane during the process of their recycling. The functional relevance of the dynamic endosomal DR compartmentalization is still incompletely understood, especially with regard to its involvement in modulation of related apoptotic and non-apoptotic cellular signalling. In contrast to TNF – TNF-R1 or Fas – FasL, where the endocytosis has been shown as a positive factor in enhancing their pro-apoptotic signalling, the role of endocytosis in apoptosis triggered via TRAIL – DR4/5 is much more controversial and the cell type-specific (Schneider-Brachert *et al*, 2013; Twomey *et al*, 2015). Blockage of TRAIL – DR4/5 internalization did not inhibit the formation of the DISC, caspase-8 activation and further propagation of apoptosis signal in BJAB Burkitt lymphoma B cells (Kohlhaas *et al*, 2007). On the contrary, inhibition of endocytosis restored cell surface DR4/5 expression and sensitized breast cancer cells to TRAIL-induced apoptosis (Zhang & Zhang, 2008). DR5 internalization was also essentially required for rapid lysosomal permeabilization, cathepsin B release and apoptosis execution in hepatocellular and cholangiocarcinoma cells (Akazawa *et al*, 2009). Besides apoptosis, even less is known about the possible impact of DR4/5 endocytosis on the outcome of TRAIL-induced non-apoptotic intracellular signalling.

In our work, we investigated in detail the role of endocytosis and endosomal trafficking in modulation of TRAIL-induced apoptotic and non-apoptotic signalling in human colon cancer cells (Horova *et al*, 2013) (**Supplement 19**). We showed that TRAIL-induced apoptosis can be significantly reduced upon blocking endosomal acidification by the vacuolar ATPase (V-ATPase) inhibitors bafilomycin A1 or concanamycin A. In the cells pre-treated with these inhibitors, the TRAIL-induced processing of caspase-8 and the aggregation and trafficking of the TRAIL receptor complexes were temporarily attenuated. These effects were not related to the possible suppression of lysosomal membrane permeabilization or activity of acidic sphingomyelinase. Importantly, the V-ATPase inhibitors did not affect the TRAIL-induced NF-κB or ERK activation observed in these cells, suggesting that their function was restricted mainly to the pro-apoptotic arm of TRAIL signalling. Our data demonstrated that endosomal acidification represents an important regulatory node in the proximal part of TRAIL-induced pro-apoptotic signalling, while it did not seem to substantially affect the TRAIL-induced non-apoptotic events. In addition to the continuous need to delineate the detailed role of endocytosis in regulation of DR4/5-mediated signalling, there are also numerous open questions on whether and how specific DR signalling reciprocally regulates the endocytic machinery. Recently, it has been shown that TRAIL-induced early stimulation of caspase-8 results in activation of dynamin 1 (Dyn1), Dyn1-dependent endocytosis of DR4/5, and suppression of TRAIL-induced apoptosis in some cancer cells (Reis *et al*, 2017). Thus, the deregulations at the level of DR4/5 endocytosis may represent an interesting adaptation of cancer cells to modulate their survival and tumorigenic potential, and a potential therapeutic target to combat this disease.

Furthermore, as mentioned above, a nuclear expression of DR5 in some cancer cell types was recently recognized as a novel phenomenon significantly affecting the outcome of TRAIL signalling (Kriegl *et al*, 2012; Leithner *et al*, 2009). The DR5 molecule contains two nuclear

localization signals and possess the ability to undergo β1-importin-mediated nuclear translocation (Kojima *et al*, 2011). The origin of nuclear DR5 as well as the mechanisms responsible for shuttling between different compartments remain to be elucidated in more detail. Within the nucleus, DR5 has been demonstrated to interact with the microprocessor complex and its accessory proteins, which results in impaired maturation of let-7 miRNA, a negative regulator of several proliferation-driving molecules including K-Ras mRNA (Haselmann *et al*, 2014; Kretz *et al*, 2018). Thus, nuclear DR5 can enhance cancer cell proliferation and contribute to malignancy, even in a TRAIL-independent manner (Mert & Sanlioglu, 2017). These findings revealed additional crucial aspects of TRAIL DR-mediated signalling and stimulated the search for yet unknown nuclear functions of not only DR5, but also DR4 or DcR1/2, which may open new therapeutic opportunities based on targeting these receptor-dependent nuclear events.

Thus, based on the results of numerous publications, the preferential induction of apoptosis in cancer versus normal cells has been recognized as a strong selective advantage of TRAIL-based therapy for a long time. Recently, an increasing knowledge on the ability of TRAIL to stimulate non-cell death pathways in cancer cells revealed the need to block these pathways in order to maximize the therapeutic benefit of the TRAs therapy. However, as similar pro-survival pathways are also activated by TRAIL in normal cells, it remains to be explored whether the application of this combined treatments could simultaneously enhance undesired toxic side effects on normal cells, reducing the therapeutic window of TRAIL-based therapy. The limited evidence obtained so far showed that combined treatments that block the TRAIL-induced non-cell death pathways may preferentially sensitize cancer but not most normal cells to TRAIL-induced cytotoxicity (Fulda, 2013b). However, further investigations will reveal whether this phenomenon may also be observed repeatedly using other yet unexplored normal cell types, and finally also in the clinical settings.

3. Summary and future perspectives

An efficient and selective induction of cancer cell death is a main aim of many therapeutic approaches, and identification of the suitable candidates that are capable to do so represents a continuous need in oncology. Within this work, we investigated the TRAIL signalling and its fascinating biology from the different points of view, exploring its outstanding ability to act as a unique cancer-selective killer and, on the other hand, as a dangerous weapon that could be used by some cancer cells to support their malignant behaviour. To distinguish between these potent contradictory properties of this enormously interesting cytokine is an essential precondition of its successful therapeutic application and a great challenge for many scientists around the world including us. Our work contributed to the better understanding of the "two faces" of TRAIL signalling in cancer cells, with a particular focus on the mechanisms of resistance to TRAIL-induced apoptosis observed in numerous cancer cell types. We put our efforts into elucidation of molecular mechanisms and strategies how to enhance the anticancer potential of TRAIL and at the same time to shed more light on the "dark side" of its signalling that should be prevented in order to eliminate the undesired side effects of TRAIL-based therapy. In order to boost the cytotoxic potential of TRAIL in cancer cells, we investigated the apoptosis-sensitizing abilities of several classes of potentially interesting therapeutic agents - conventionally used or recently introduced chemotherapeutic drugs such as cisplatin, LA-12, 5-FU, doxorubicin or etoposide, epigenetic drugs – decitabine or VPA, and dietary polyunsaturated fatty acid DHA. We newly described several molecular determinants of their cooperative cytotoxic action with TRAIL at the different levels of apoptotic signalling pathways, including DRs, DISC, mitochondria and caspases in several cancer cell types. In addition, our work also uncovered novel details about the role of kinase-mediated non-cell death signalling in regulation of cancer cell sensitivity to the apoptotic effect of TRAIL, with a particular attention to MAPK/ERK and PI3K/Akt. Our results identified possible cross-talks between TRAIL-induced apoptotic and kinase signalling in cancer cells, and their impact especially at the cell death-related events at the level of mitochondria.

Due to the complexity of TRAIL/TRAIL-R system, further intensive research is required to fully understand the dichotomy of its signalling, individual regulatory pathways, cross-talks between them, molecular switches and factors that may affect it, and the biological consequences. Many of these issues that deserve our attention were already mentioned within this theses, and represent important milestones for the future research efforts. Unfortunately, the ability of some cancer cells to escape from TRAIL-induced killing action still remains a serious limitation that prevents its successful therapeutic application. The deeper insights into the underlying mechanisms of TRAIL resistance in cancer and normal cells and expanded knowledge about the new ways of sensitization to TRAIL-induced apoptosis will enable to understand which conditions need to be fulfilled to efficiently exploit the anticancer potential of TRAIL. These findings will also be crucial for determination of predictive biomarkers for individualized selection of patients eligible for tailored DR4/5-targeted therapy. Finally, combining TRAIL receptor agonists with current or recently developed agents that will sensitize the cancer cells to apoptosis and simultaneously inhibit the kinase signalling promoting cancer cell survival seems to be the best way how to provide a greater clinical benefit of this extraordinary "apoptotic" molecule. The future research will show if we are able to design such efficient anticancer strategies and prove their clinical relevance, and confirm that we are on the "right trail".

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(Alena Hyrslova Vaculova is an author or co-author of the citations marked in bold.)

5. Supplement list

The Supplement part contains a selection of 18 original research articles and 1 review (out of 39 author´s publications, according to PubMed, October 2018), which are directly related to the topic of the theses. These publications provide a detailed overview of the author´s contribution to the research focused on the regulation of the cancer cell death, especially molecular mechanisms of apoptosis and the novel possibilities of its modulation by selected therapeutically interesting agents. Alena Hyrslova Vaculova is the first or corresponding author of 11 out of the 19 publications, and substantially contributed also to the others as a co-author.

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6. Supplements 1 - 19