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Review

Exploring death receptor pathways as selective targets in cancer therapy

Maria Russo [1], Annalisa Mupo [1], Carmela Spagnuolo, Gian Luigi Russo *

Institute of Food Sciences, National Research Council, 83100 Avellino, Italy

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ABSTRACT

A recent and innovative strategy in cancer therapy is the activation of apoptosis in tumour cells specifically expressing death receptors (DR) belonging to the tumour necrosis factor (TNF) receptor superfamily and including several members known since the early '90. Among these, those largely studied for clinical purpose are TNF, CD95, and TRAIL receptors. Promising results are expecting from ongoing phases I/II clinical trials proving the therapeutic efficacy of DR agonistic antibodies and/or recombinant proteins alone or in association to classic and novel chemotherapeutic drugs. However, two key issues need extensive studies, before clinical and safe applications of DRs as effective anticancer drugs can be accepted: i. DR-based cancer therapy must be selective and effective against a broad range of cancers and reduce excessive systemic toxicity toward normal cells and tumour resistance after recurrent treatments; ii. an improved knowledge of mechanisms of alternative signalling triggered by DR ligands and leading to cell survival and apoptotic resistance. Activation of survival pathways regulated by key factors, such as NF-κB, JNK, p38, ERK and PI_3K are the focus of several studies revealing the dark side of DR signalling. The present review focuses on new insights in the signalling and clinical application of TNF, CD95 and TRAIL receptors.

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1. Introduction

Since its first appearance in the Literature [1], the term "apoptosis" has been used to describe a mode of cell death, morphologically distinct from "necrosis". In recent years, apoptosis became more commonly known as "programmed cell death", to indicate a genetically programmed cell suicide which play a central homeostatic role during development [2,3]. Apoptosis of pre-malignant or malignant cells represents a protective mechanism against tumour formation and development, since it removes from the body genetically damaged cells induced to proliferate under uncontrolled mitogenic stimuli. Apoptosis can be triggered by two major mechanisms: the intrinsic pathway involving mitochondrial dysfunction, and an extrinsic pathway associated with stimulation of death receptors (DRs) located on the cell membrane (Fig. 1). These DRs belong to the tumour necrosis factor (TNF) receptor (TNF-R) superfamily and include well-known members listed in Table 1. DR-induced apoptosis is an innovative way to selectively kill cancer cells compared to modern anticancer drugs (protein kinase inhibitors or monoclonal antibody agonists for growth

* Corresponding author at: Istituto Scienze dell'Alimentazione, Via Roma 64, 83100 Avellino, Italy. Tel.: +39 0825 299331; fax: +39 0825 781585.
E-mail address: glrusso@isa.cnr.it (G.L. Russo).
[1] These two authors equally contributed to the preparation of the present work.

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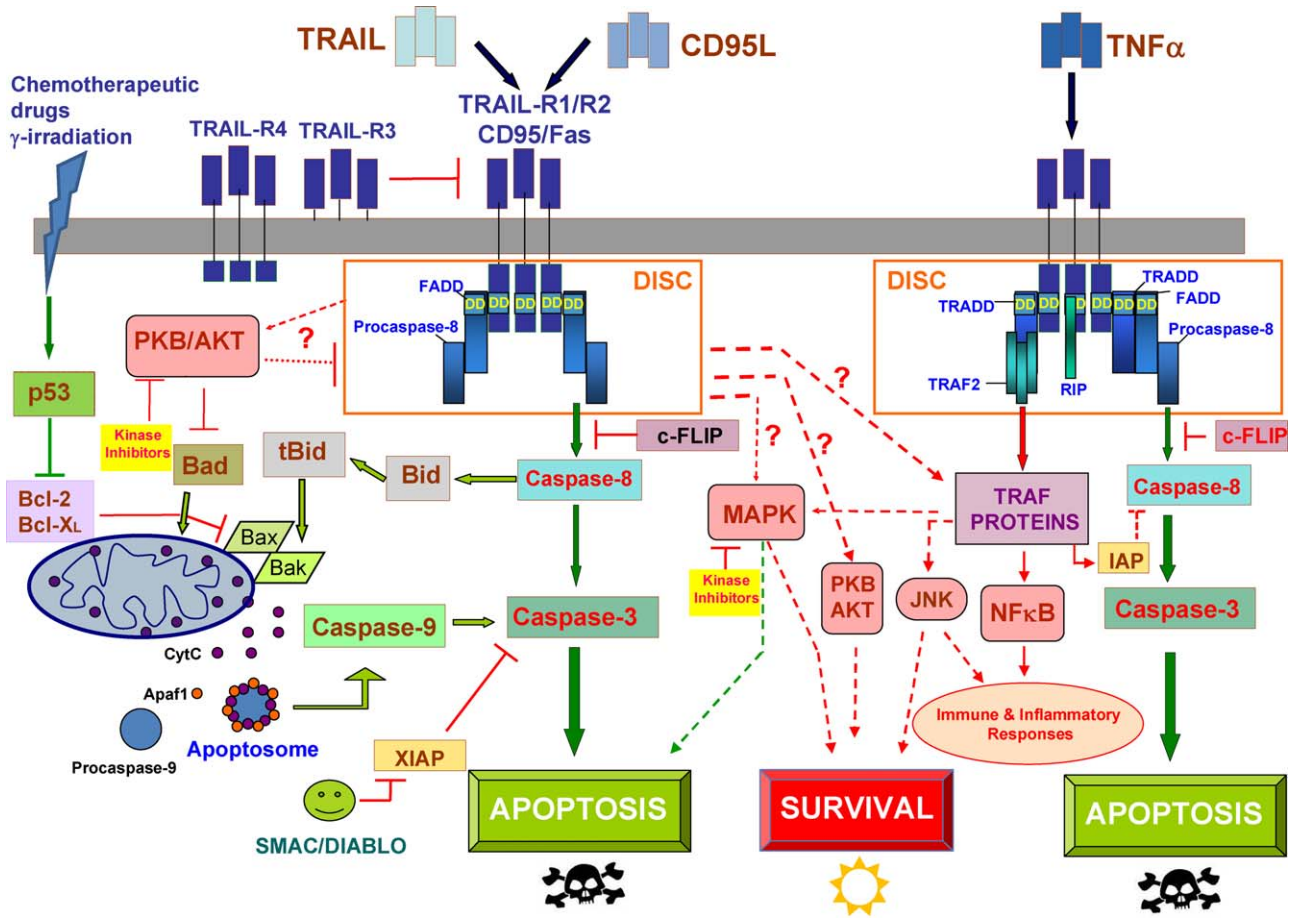

Fig. 1. Principal pathways activated by death receptors containing death domains (see text for details).

Table 1
Death receptors containing a death domain, decoy receptors and their relative ligands.

Death Receptors		Ligand
Common name	Alternative names	
Death receptors containing a DD		
TNF-R1	p55 CD120a	TNF-alpha
CD95	Fas APO-1	CD95L
TRAIL-R1	DR4 TNFRSF10A	TRAIL
TRAIL-R2	DR5 APO-2 KILLER TRICK2 TNFRSF10B	TRAIL
TRAMP	APO-3 DR3 WSL-1 LARD	TL1A
DR6	TR-7	?
Decoy Receptors		
TRAIL-R3	DcR1 TRID TNFRSF10C	TRAIL
TRAIL-R4	DcR2 TNFRSF10D	TRAIL

receptors), since DR-based cancer therapy could be selective and effective against a broad range of cancers. In addition, DR engagement, using recombinant death ligands, or agonistic antibodies, activates the extrinsic apoptosis pathway, while, generally, chemotherapy or radiotherapy trigger the mitochondrial/intrinsic pathway (Fig. 1). Therefore, the conventional therapeutic approach could be complemented and implemented by DR-induced apoptosis, when DRs are expressed and functional on tumour cells. This double strategy may reduce excessive systemic toxicity toward normal cells and tumour resistance after recurrent treatments.

Excellent reviews have been recently published on different therapeutic approaches to specifically target tumours by activating DR pathways [4,5]. Here, we will discuss new insights in the signalling and clinical application of TNF, CD95 and TRAIL receptors.

2. Death receptor signalling

The TNF-R superfamily includes several members, which can be divided into three major groups based on the structure of their cytoplasmic region and the signalling generated by interaction with downstream ligands. The first group includes the six receptors listed in Table 1 possessing a death domain (DD) which, upon interaction and trimerization with their specific ligands, recruits intracellular DD containing adaptors, such as FADD (FAS-associated death domain) and TRADD (tumour necrosis factor receptor type 1-associated death domain) leading to the activation of the caspase cascade resulting in apoptosis (Fig. 1). The second

group of TNF-Rs includes about 19 members characterized by the presence of a TRAF (tumour necrosis factor receptor associated factor)-interacting motif (TIM) in their cytoplasmic tails. In this case, the association between TIM-containing TNF-R with TRAF members triggers survival pathways regulated by key factors, such as NF-κB, JNK (c-Jun N-terminal kinase), p38, ERK and PI_3K (phosphatidylinositol-3 kinase). Finally, a third group of TNF-Rs includes the so-called decoy receptors (TRAIL-R3, TRAIL-R4, and osteoprotegerin [OPG]) characterized by the absence of intracellular domains which make them unable to activate downstream pathways of cell death or cell survival. However, these decoy receptors possess important functions since they may compete with the other two groups of TNF-Rs for the same ligands resulting in an attenuation of the signalling.

CD95, TRAIL-R1/R2 and TNF-R1 are type I transmembrane protein characterized by two to five copies of cysteine-rich extracellular repeats and an intracellular DD [6] which transmits a death signal to the cell [7,8]. CD95 exists as a homotrimer and is activated through binding to trimers of its ligand, CD95L [9]. Similarly, active TRAIL-R ligand (TRAIL) is a homotrimer which binds one of two cell surface receptors: TRAIL-R1 and TRAIL-R2 (with two splice variants). Both receptors share a sequence homology of 58%. There are five cellular receptors showing binding affinity for TRAIL (Table 1). Cytoplasmic domain of TRAIL-R1/R2 shares significant homology to DD of different DRs, such as CD95 and TNF-R1. Binding of CD95L and TRAIL to their functional receptors triggers the assembly of the DISC (death-inducing signalling complex), consisting in the ability of their respective DDs to recruit FADD which leads to the activation of initiating caspases (-8 and -10) [10,11] at the DISC (Fig. 1). This multimolecular complex in turns activates downstream effector caspases, such as caspase-3, -6 and -7 acting on a wide range of substrates and leading to apoptosis. The DISC is a pivotal trigger of apoptosis [12]. Until now, the dominant model for DISC formation proposed that trimeric CD95L cross-linked units of preformed trimeric CD95 embedded in local membrane rafts, leading to higher order arrangements [13]. While illustrating the overall process, this initial model did not provide a mechanism for the actual clustering of CD95 DDs within the cell and, most importantly, the relationship between CD95 clustering and FADD binding. Very recently, a model has been proposed to explain this clustering based on crystal structure: only when a sufficient number of CD95 molecules are in close proximity, as it is expected on CD95L binding, the open forms of CD95, exposing the stem helix of DD, can become stabilized. Subsequently, CD95 molecules connect each other through functional bridges, setting the stage for a chain reaction. These bridges link the DD in the trimeric units establishing an interaction via the globular portion of the open forms of CD95, directly resulting in rapid and processive clustering on the inside of the cell membrane. At this stage, FADD is able to bind the open trimeric CD95, key for DISC signalling, and additionally increasing stabilization of the CD95–CD95 bridges, thereby further fostering DISC formation and clustering [14].

DR-dependent apoptosis could be amplified at mitochondrial level depending on cell type (I or II) [15]. The intrinsic pathway is activated by various stimuli, including DNA damage, growth factor withdrawal or cytokine deprivation [16]. These signals affect the function of Bcl-2 family members [17] characterized by specific regions of homology termed Bcl-2 homology (BH1, BH2, BH3, and BH4) domains, which are critical to the function of these proteins, including their impact on cell survival and their ability to interact with other family members and regulatory proteins. The Bcl-2 family can be divided into two classes: those suppressing cell death (e.g., Bcl-2, Bcl-X_L and Mcl-1), and others promoting apoptosis (e.g., Bak and Bax). Oligomerization of proteins such as Bak (Bcl-2 antagonist killer) and Bax (Bcl-2-associated X protein) causes permeabilization of the outer mitochondrial membrane, release of cytochrome C and activation of initiator caspase 9 in the apoptosome (Fig. 1). Effector caspase-9 then activates caspase-3, caspase-6 and caspase-7, which ultimately results in cell death [17,18]. The identification of the BH3 domain as a potent mediator of cell death led to the characterization of an additional family of pro-apoptotic proteins called "BH-3 only protein" (Bad, Bik, Bid, Bim) for they share identity with Bcl-2 only within this BH3 domain. In type I cells, Bcl-2 over-expression blocks the mitochondrial changes associated with cell death, but cells undergo apoptosis upon DR activation. In fact, type I cells have massive caspase-8 activation to the DISC, sufficient to activate executive caspases. In type II cells, extrinsic pathway activated by DR is ineffective to form, at the DISC level, enough caspase-8 to activate effector caspases. However, through homotypic aggregation at DISC, caspase-8 is stabilized in an active form, the so-called "induced conformation model", A re-evaluation of the "induced proximity dimerization model" theorized for caspase-9 activation [19], and released into the cytosol, where it cleaves its target proteins, most notably the pro-apoptotic BH3-only protein Bid (BH3-interacting-domain death agonist). Truncated Bid (tBid), with its BH3 domain exposed, translocates to the mitochondria surface interacting with Bax/Bak and facilitating their pro-apoptotic activity (Fig. 1). In this cell type, caspase-8 cleaves and activates the BH3 pro-apoptotic protein Bid, connecting mitochondrial to DR pathway [18]. In this scenario, oncogenic mutations affecting molecules involved in intrinsic, mitochondrial pathway might cause resistance in type II cells, while mutation in DR pathway could confer resistance to DR-dependent apoptosis especially in type I cells.

Until recently, there were no studies describing substantial differences between TRAIL and CD95 signalling at DISC level. In the past, molecules such as TRADD and DAP3 (Death Associated Protein 3) have been indicated as adaptors in FADD recruitment after TRAIL binding. However, current studies suggest that TRAIL-R1/R2 directly bind to FADD in DISC formation and, perhaps, in activating survival pathways after DR engagement (Fig. 1). The C-terminal tails of TRAIL-R1/R2, outside the DD, positively regulate FADD binding, activating caspase cascade and finally apoptosis. In contrast, the corresponding C terminal region in the CD95 receptor shows the opposite effect, inhibiting binding to the receptor DD [20]. In addition, the same authors demonstrated that some agonistic antibodies display an absolute requirement for the C-terminal tail for FADD binding and signalling while other therapeutic antibodies (such as, lexatumumab/HGS-ETR2; see below and Table 2) can induce apoptosis in the absence of this mechanism, for example in cancers with mutations or deletion in the C-terminal domain of TRAIL receptors. This observation may have fundamental consequences in DR-based cancer therapy, because different agonists could activate opposite signalling mechanisms triggering the same receptor due to subtly different conformational changes in the intracellular receptor domain interacting with FADD.

Similarly to CD95 and TRAIL-R1/R2, also TNF-R1, differently from TNF-R2, possesses a DD which interacts with its ligand, TNF-alpha, but induces apoptosis only when protein synthesis is inhibited. In most circumstances, TNF-R1 interacts with TRADD (TNFR associated DD) leading to the activation of transcription factors, such as NF-κB and JNK promoting cell survival, differentiation and transcription of inflammatory genes [21,22] (Fig. 1). Briefly, the interaction between TNF-R1 and TRADD respective DDs generates a stand-by station for further signalling molecules. DD of TRADD can interact with [22], or be substituted by DD of FADD allowing the complex to dissociate from the membrane and initiate the caspase-8 activation pathway [23]. Alternatively, TRADD can recruit TRAF-2, TRAF-1 and RIP (receptor-interacting protein) forming a so-called complex I [24]. TRAF-2 is able to act on

Table 2
Ongoing clinical trials targeting TRAIL receptors.

Trial Lead Organizations	Treatment		Phase		Type of cancer
	TRAIL	Other Drugs			
Amgen	rApo2L/TRAIL	Rituximab	Ib II	Completed Active	CD20+ NHL[1]
Amgen	AMG 951[a]	Chemotherapy Bevacizumab	II	Closed	untreated NSCLC[2]
Amgen	AMG 655[b]	Panitumumab	I II	Closed	metastatic colorectal cancer
Amgen	AMG 655[b]	Doxorubicin C	I II	Closed	soft tissue sarcoma
Amgen	AMG 655[b]	Paclitaxel Carboplatin	I II	Closed	advanced NSCLC[2]
Amgen	AMG 655[b]	mFOLFOX6 Bevacizumab	I II	Closed	metastatic colorectal cancer
Amgen	AMG 655[b]	Bortezomib Vorinostat	Ib	Active	Lymphomas
Amgen	AMG 655[b]	Gemcitabine AMG 479[c]	I II	Active	metastatic pancreatic cancer
Amgen	AMG 655[b]	AMG 479[c]	I II	Active	refractory solid tumours
Daiichi Sankyo	CS1008[d]		I	Completed	solid malignancies lymphomas
Daiichi Sankyo	CS1008		II	Closed	untreated and unresectable pancreatic cancer
Daiichi Sankyo Inc.	CS1008	Paclitaxel Carboplatin	II	Active	advanced or metastatic ovarian cancer
Daiichi Sankyo Inc.	CS1008	Paclitaxel Carboplatin	II	Active	metastatic or unresectable NSCLC[2]
Daiichi Sankyo, Inc.	CS-1008	Irinotecan	II	Active	metastatic colorectal carcinoma
Daiichi Sankyo Inc.	CS1008	Sorafenib	II	Approved not active	advanced liver cancer
Human Genome Sciences	TRM-1[e]		II	Completed	NSCLC[2] NHL[1]
Human Genome Sciences	Mapatumumab[f]	Bortezomib	II	Closed	relapsed or refractory multiple myeloma
Human Genome Sciences	Mapatumumab[f]	Paclitaxel Carboplatin	II	Closed	NSCLC[2]
Human Genome Sciences	Mapatumumab[f]	Sorafenib	I II	Active	advanced hepatocellular carcinoma
Human Genome Sciences	Lexatumumab[g]	Chemotherapy	I	Active	advanced solid tumours
Genentech Inc.	PRO95780[h]	Rituximab	II	Closed	NHL[1]
Genentech Inc.	rApo2L/TRAIL	FOLFOX Bevacizumab	I	Active	untreated metastatic colorectal cancer
Genentech Inc.	rApo2L/TRAIL	Camptosar(R) Erbitux(R) Chemotherapy FOLFIRI	I	Active	previously treated metastatic colorectal cancer
NCI	Lexatumumab[g]	Interferon-γ	I	Active	pediatric patients with relapsed or refractory solid tumours or lymphoma
NCI	Conatumumab[i]	Gemcitabine Radiotherapy Capecitabine	I II	Active	advanced pancreatic cancer

[1]Non-Hodgkin's Lymphoma.
[2]Non-Small Cell Lung Cancer.
[a] Recombinant human Apo2L/TRAIL.
[b] AMG 655 is a fully human monoclonal agonist antibody that binds human TRAIL receptor 2 (TR-2/DR5).
[c] AMG 479 is a fully human monoclonal antibody that targets type 1 insulin-like growth factor receptor (IGF-1R).
[d] Humanized agonistic monoclonal antibody directed against human tumour necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2).
[e] TRAIL-R1 monoclonal antibody.
[f] A fully human agonistic monoclonal antibody to TRAIL-R1 (also known as ETR-1).
[g] An agonistic human monoclonal antibody against TRAIL-R2 (also known as ETR-2).
[h] DR5-agonist antibody (PRO95780) is a fully human monoclonal antibody which is a DR5-targeted.
[i] Fully human monoclonal agonist antibody directed against the extracellular domain of human TRAIL-R2.

different pathways: i. it can recruit cIAP-1 (cellular inhibitor of apoptosis-1) and cIAP-2 into the signalling complex, inhibiting caspase-8 processing [22]; ii. it can activate NF-κB via its translocation into the nucleus and subsequent activation of inflammatory responsive genes [25]; iii. TRAF-2 can also trigger JNK activation via the MAP-kinase cascade leading to the transcription of TNF-responsive genes [25].

Similarly to TNF-R1, pro-inflammatory and anti-apoptotic activities have been recently described also for CD95 and TRAIL receptors, although the mechanisms for the proliferative signalling have not been fully understood [26,27]. Two hypotheses are under investigation: for some DRs, DISC components might mediate the non-apoptotic pathways, whereas other DRs might use specialized anti-apoptotic members such as TRADD, RIP and TRAF. As an example, CD95 can activate the canonical NF-κB pathway, but the existence of a specific factor(s) linking CD95 receptor to IkB kinase (IKK) is still missing [28]. In addition, T-cells from transgenic mice expressing a dominant-negative mutant of FADD and from mice lacking FADD show defects in re-entering the cell cycle, suggesting that FADD is an important determinant in T-cell development, activation and proliferation [29]. It seems that serine-194 phosphorylation by casein kinase Iα is essential for the non-apoptotic function of FADD [30]. In the case of TRAIL-R1/R2, RIP has been detected in TRAIL DISC and mediates TRAIL-induced IKK [26]. Similarly to TNF-mediated activation of NF-κB, TRAF2 also seems to be involved in TRAIL-induced NF-κB via TRADD [26]. Among the non-apoptotic NF-κB target gene, cFLIP (cellular FADD-like interleukin-1beta-converting enzyme-inhibitory protein) probably has the most prominent role by interfering with the activation of caspase-8 [31]. cFLIP is also a target of the anti-apoptotic PKB/Akt and MAPK pathways. However, its central role as molecular switch between cell death and cell survival in the same cell has not been confirmed [27]. According to some authors, a DISC complex, DR-independent and including FADD, might be formed in the cell and regulate proliferation and cell cycle progression [26]. Activation of MAP kinases by CD95 is not fully understood and apparently does not require an active DD [32]. More controversial is the TRAIL-R1/R2-induced activation of PKB/Akt and MAP kinases [26]. The three key kinases of MAPK pathways, i.e., ERK, p38-MAPK and JNK have been often associated to the anti-apoptotic function of TRAIL receptors. Paradoxically, pro-apoptotic effects connect TRAIL and MAPK pathways in different cell models [33,34]. The existence of a DD-alternative DISC complex, mentioned above, has also been suggested to explain the TRAIL-dependent activation of MAPK pathways. FADD, caspase-8, RIP and TRAF2 might form a cytoplasmic complex upon TRAIL stimulation leading to p38 and JNK activation [35]. In HUVECs (human umbilical vein endothelial cells) and synovial fibroblasts, TRAIL has a direct effect on endothelial cells survival and proliferation stimulating the phosphorylation and activation of PKB/Akt kinase depending on PI_3K (phosphatidylinositol-3 kinase) activation. This event occurs without activation of NF-κB [36].

Following a different hypothesis, DR signalling toward cell death or cell survival could be regulated by membrane localization. As an example, if TRAIL-R1/R2 are localized into lipid rafts, they stimulate apoptosis mediated by DISC formation and caspase-8 activation. However when TRAIL-R1/R2 are not associated to lipid rafts they induced not apoptotic signalling such as NF-κB and ERK1/2 activation [37]. Similarly, CD95 and TNF-alpha could be regulated by their membrane domain localization such as cholesterol and sphingolipids-rich lipid rafts in the plasma membrane [13].

3. Cancer resistance targeting DRs

The balance between cell death and cell survive is tightly controlled at several levels. In cancer cells, an enhanced anti-apoptotic response might generate resistance to DR-mediated apoptosis. This is particularly true for TRAIL-R1/R2, considering their potential clinical applications (see below). An interesting characteristic in the family of DR is that normal cells are TRAIL resistant, but the molecular basis for TRAIL tumour selectivity is still unclear. Defects in either of different molecules involved in TRAIL signalling can lead to TRAIL resistance. In fact, over expression of cFLIP correlates with TRAIL resistance in several types of cancer, especially in type I cells [38]. cFLIP protein has two DEDs (death effector domains) that facilitate binding to the DED of FADD, thereby inhibiting the activation of caspase-8. Over expression of Bcl-2, Bcl-X_L, or Mcl-1, loss of Bax or Bak function, increased expression of IAPs and reduced release of Smac/Diablo (second mitochondria-derived activator of caspases) from the mitochondria to the cytosol are all events resulting in TRAIL resistance in type II cancer cells [39]. In the case of IAP family, XIAP inhibits the autocatalytic activation of caspase-3, preventing a further transmission of the death signal and consequently apoptosis [5]. The adaptor protein FADD and caspase-8 are essential for the assembly of the death-inducing signalling complex, and most tumours mutations map the intracellular domain of TRAIL-R2 that binds FADD [40]. Mechanism of resistance has also been linked to the presence of decoy receptors (Table 1). They do not have a functional (DcR1) or have a truncated (DcR2) DD and are ineffective to induce apoptosis after TRAIL binding. Their physiological role is unclear. In experimental models, decoy receptors over-expression could sequester TRAIL decreasing the functional binding to TRAIL-R1/R2 and attenuating the apoptotic signalling, but experiments under physiological conditions are still missing. TRAIL-R3/R4 mRNA are preferentially expressed in normal cells, but subsequent studies, using specific monoclonal antibodies, demonstrated that TRAIL sensitivity was not correlated with the relative expression of functional TRAIL receptors or decoy receptors [10]. They may have important functional effects in human cancers, even if their impact on cell signalling is poorly understood. It is now accepted that the simple expression of a death or decoy receptors is not an essential feature for apoptosis sensitivity. In fact, as many chemotherapeutic drugs, TRAIL is not universally active against tumour cell lines, especially primary tumour cells, even expressing DRs on their surface. Dysfunctions of the TRAIL-R1/R2 due to oncogenic mutations have been found in different tumours and in different cancer patients (breast, lung, head and neck cancer and non-Hodgkin lymphoma) [41,42]. A microarray-based expression profiling on 100 human cell lines indicates that post-translational modification such O-glycosylation is essential for TRAIL-R1/R2 full functionality [43]. Protein glycosylation could enhance ligand-mediated receptor clustering. The glycosylation status of TRAIL R1/R2 could be a marker for TRAIL sensitivity. Others suggested that c-Myc expression and alternative TRAIL signalling are important factors in determining whether a cell is sensitive to TRAIL-induced apoptosis [44]. In this study, it was found that Myc sensitized cells with defective ($Bax^{-/-}$) intrinsic signalling to TRAIL. TRAIL itself induced expression of anti-apoptotic Mcl-1 and cIAP2 through activation of MAPKs and NF-κB. Both Myc and the drug multi-kinase inhibitor sorafenib, with different mechanisms, blocked NF-κB. Combining sorafenib with TRAIL, in vivo, the authors showed a dramatic effect in TRAIL-resistant tumour xenografts, suggesting that the combination of TRAIL treatment with kinase inhibitors holds promise for a successful combination therapy in DR resistant tumours [44].

4. Cancer therapy targeting DRs

4.1. TNF-alpha

The limited use of TNF-alpha in clinical oncology has been due to the powerful and toxic systemic side effects of this cytokine,

which many research groups are trying to bypass targeting TNF-alpha specifically to tumours. In fact, TNF-alpha is not only able to initiate cellular apoptosis at higher doses, as discussed above, but a further advantage of TNF-alpha treatment in cancer therapy is related to its anti-vascular activity which can be used clinically to destroy tumour vasculature also improving permeability to cytostatic drugs [5,45,46]. From the recent literature emerges that targeting TNF-R1 with appropriate strategies, avoiding systemic administration of TNF-alpha and improving the local delivery into the cancer site, may prove to be highly beneficial for tumour resection. This approach showed its efficacy since 1995 with the administration of high-dose TNF-alpha by a technique called isolated limb perfusion on unresectable tumours such as soft-tissue sarcomas [47]. As suggested [5], the isolated limb perfusion approach can be potentiated if coupled to chemical conjugation of TNF-alpha to polyethylene glycol, a technique (PEGylation) which prolongs plasma half-life and promotes accumulation of target factor in tumours due to enhanced permeability and retention effect. Using this strategy, a lysine-deficient mutant of TNF-alpha (mTNF-K90R) generated by phage display technique and mono-PEGylated at the NH_2 terminus, showed a higher in vitro bioactivity and longer plasma half-life than native TNF-alpha. Regarding its in vivo effectiveness and safety, the antitumour therapeutic window of the PEGylated mTNF-K90R was 60-fold wider than that of the native TNF-alpha [48].

A different approach to deliver locally high doses of TNF-alpha has been the generation of fusion proteins where TNF-alpha was linked to antibodies or natural ligands targeting surface proteins on cancer cells. The efficacy of the initial formulations of conjugated TNF-alpha to anti-melanoma antibody was limited by the size, poor distribution and low update of the drug [49]. More recently, an improvement has been reached by the generation of a fusion protein between GX-1 peptide, which binds selectively to human gastric cancer vasculature and a recombinant mutant of human TNF-alpha. In nude mice, the fusion protein concentrates the TNF-alpha in human gastric cancer cells delaying their growth and causing less systemic toxicity than TNF-alpha alone [50]. In this study, the anti-angiogenic effect of TNF-alpha was coupled to its pro-apoptotic activity. Finally, the most promising approach in the TNF-based fusion protein therapy is probably represented by the so-called TNF-alpha pro-drugs. Here, a homotrimeric molecule comprised of an N-terminal single-chain antibody variable fragment targeting FAP (fibroblast activation protein, a membrane protein present in virtually all solid tumours and limited expressed in normal cells), a trimerization domain (derived from tenascin), TNF-alpha and a C-terminal TNF-R1 fragment which protects TNF-alpha from activation. The insertion of a protease-sensitive linker (MMP-2 or uPA) between TNF-alpha and TNF-R1 module allows activation of pro-drug by endogenous proteases expressed on the surface of tumour stroma [51]. The pro-drug exerts minimal TNF-alpha activity, but can be activated in vitro several thousand-fold by proteolytic digest, showing the principal feasibility of this approach [51]. The same approach was proved for other DR ligands, such as CD95L with encouraging results [52]. However, this strategy requires additional confirmatory data from animal model before hypothesizing clinical applications. From this point of view, TNFerade, a new gene therapy drug (GenVec, Gaithersburg, MD, USA) deserves interest. It employs a replication-deficient adeno-vector carrying the gene for human TNF-alpha, regulated by inserting radio- and chemo-inducible elements from the Egr-1 promoter. Transduction of tumour cells with TNFerade and then treatment with radiation or chemotherapy is associated with spatial and temporal control of TNF-alpha secretion and enhanced antitumour activity. Preclinical toxicity studies indicated that TNFerade is safe and well tolerated in mice [53]. Currently, it is under evaluation in several advanced phase I, II and III clinical trials for patients with sarcomas, melanomas and cancers of the pancreas, esophagus, rectum and head and neck.[2] Since the ongoing phase III trial for pancreatic cancer appears successful, TNFerade might likely become the first gene therapy approved for cancer in the United States [54].

4.2. CD95/Fas

In the early 1990s, the demonstration that systemic administration of agonistic CD95 antibodies and of recombinant CD95L led to severe hepatotoxicity and subsequent death of the treated mice strongly reduced the wave of enthusiasm on the use of CD95 agonists as cancer therapeutics [5]. To bypass the problem of toxicity, several strategies have been attempted. One possibility is to use tissue-specific CD95 agonistic antibodies to induce apoptosis. As an example, the hamster anti-mouse CD95 antibody RK8, and the mice anti-human CD95 antibody HFE7A showed limited cytotoxicity against hepatocytes [5]. In this context, the most promising anti-CD95 antibody, which shows tissue-specific activity, is R-125224. This reagent selectively induced apoptosis in type I activated lymphocytes, but not in type II cells [55]. A second approach is based on targeted gene therapy able to induce a tissue-specific effect and bypass toxicity, increasing safety of CD95 treatment. Inducible tetracycline adenoviral vectors under tissue-specific promoters [56] and tamoxifen inducible constructs [57] have been developed. Adenoviral constructs expressing CD95L have been shown to be much more potent in the induction of apoptosis than some anti-CD95 antibodies and tumour regression of mouse epithelial carcinoma and colon carcinoma following the application of a CD95L-containing adenoviral vector was proved [58,59]. A third approach is based on the idea to create cell surface-targeted CD95L fusion proteins to induce a "fratricide massacre" of tumour cells only in cancer sites [60]. An example is the trimeric fusion protein, sc40-FasL, consisting of the extracellular domain of CD95L fused to a single-chain antibody that specifically recognizes the tumour stroma marker FAP. The antibody fragment recognizing FAP allows for tumour-specific immobilization of CD95L and, upon binding, converts the inactive protein into a protein with membrane-bound-CD95L-like activity. In line with this, sc40-FasL solely induced apoptosis in FAP-expressing cells in vitro and intravenous injection into mice did not result in systemic toxicity. As FAP is highly expressed in the majority of epithelial cancers including colon cancer, the rationale of FAP-fusion to CD95 seems to be promising [5,60]. A very recent study, used a CD95L recombinant protein, pET-22b(+)/FasL, in combination with the well-known cytotoxic chemotherapeutic reagent adriamycin (ADM) to induce apoptosis in vitro and in vivo model of hepatocellular carcinoma. The apoptotic levels of cells treated with CD95L-ADM were significantly higher than those treated with CD95L or ADM alone, and the CD95L-ADM combination resulted in more than additive effect on tumour growth delay in this model. The results suggested that combined treatment of CD95L and other chemotherapeutic agents may be a new approach to improve the efficacy of chemotherapy for hepatocellular carcinoma [61]. Recently, micro (mi)RNAs, a class of non-coding RNAs of 18–24 nucleotides that post-transcriptionally regulate protein expression, has been proposed as regulators of DR signalling and possible targets for cancer therapy. The known roles of miRNAs have expanded from their initially identified functions in development to various biological activities including proliferation, cell death, and differentiation [62]. The deregulation of various miRNAs is associated with many diseases including cancer. Recently, thirty-four miRNAs were reported to cause a change in caspase activity. Using the miRBase

[2] www.cancer.gov/clinicaltrials.

Target database[3] a subset of these 34 miRNAs was selected and several interesting candidates were found. Among them, miR-144, miR-182 and miR-155 target caspase-3. miR-145 and miR-216 had TRAIL-R1 and TRAIL-R2 as predicted targets, respectively. miR-182 and miR-96 were predicted to target FADD. Finally, miR-7 and let-7c targeted Bad and CD95L, respectively [63].

However, despite the huge scientific information and the interesting findings described so far, none of these molecules has been tested in clinics. An exception is represented by ABT-510 (Abbott Laboratories). ABT-510 is a nonapeptide analogue of an antiangiogenic sequence from thrombospondin-1, and a single substitution confers 1000-fold greater anti-angiogenic activity. ABT-510 competes with thrombospondin-1 for binding to endothelial cells, induces CD95L expression in endothelial cells, inhibits VEGF and basic fibroblast growth factor, stimulates migration of human microvascular endothelial cell [64]. Blocking angiogenesis is a promising approach in cancer therapy. Thrombospondin-1 and ABT-510 act by inducing endothelial cell apoptosis in some cases via CD36 cell surface receptor. Pro-apoptotic signal elicited by thrombospondin-1 generates CD95L [65]. However, expression of CD95 receptor on vascular endothelial cell is independent from thrombospondin-1; thus, accessible CD95 limits the rate of apoptosis and antiangiogenesis due to thrombospondin-1 and consequently determines, at least in part, the efficacy of thrombospondin-1-based cancer treatments. To improve the efficacy of ABT-510, the molecule is often combined in treatments with classical chemotherapeutic agents. Multiple chemotherapy agents, such as cyclophosphamide, cisplatin, and docetaxel, induced endothelial CD95 in vitro and in vivo at low doses that failed to kill endothelial cells. An antiangiogenic cocktail combining ABT-510 with cytoxan or cisplatin increased in vivo endothelial cell apoptosis and angiosuppression. Moreover, in a mouse model, ABT-510 and cytoxan, applied together at low doses, acted in synergy to delay tumour, to stabilize the growth of established tumours and to cause a long-term progression delay of PC-3 prostate carcinoma. These antitumour effects were accompanied by major decreases in microvascular density and concomitant increases of the vascular CD95, CD95L and apoptosis [66]. ABT-510 is currently under evaluation in phase II clinical trials, and actually 9 studies are assessing safety and effectiveness of the peptide on different type of tumours.[4]

Finally, two-centre, open label, uncontrolled, dose-finding phase I study, should determine the safety and tolerability of APO010, a recombinant form of CD95L, administered by intravenous bolus injection once per week in patients with solid tumours, for whom therapy of proven efficacy does not exist or is no longer effective. This study is still enrolling participants[3] and its main aim is to identify the recommended dose for future clinical trials. APO010 is a humanized, recombinant mega-CD95L showing anticancer activity in vitro and in animal models carrying a human xenograft of a variety of cancers, including multiple myeloma, non-small cell lung cancer (NSCLC) and ovarian cancer. Its activity is cell cycle independent; it does not cross-react with known multi-drug resistance (MDR) mechanism and appears to be synergistic with a variety of commonly used anticancer drugs. Hence, APO010 may an attractive candidate for combination anticancer therapy and may be an effective drug in overcoming MDR. Based on preclinical studies of APO010 may cause liver toxicity and a drop in platelets, that recover within 5 days.

4.3. TRAIL

The first steps before developing effective TRAIL based cancer therapy is to identify molecular signalling triggered by TRAIL in physiological and pathological settings, eventually linked to tumours resistance. Although soluble recombinant TRAIL (rTRAIL) as well as TRAIL-R1/R2 agonistic antibodies are not free from toxicity, both reagents elicit little cytotoxicity when administered systematically compared to CD95 receptor agonists; therefore, TRAIL preclinical studies are more encouraging for the significant lower hepatotoxicity [67,68]. The effect of TRAIL on tumour cell lines and responsiveness of primary tumour cells have been extensively reviewed [5,69]. From these in vitro and ex vivo studies emerged that not all tumours display simultaneously TRAIL-R1/R2 or have both functional receptors. For example, breast and colon cancer express both TRAIL-R1/R2 receptors but only TRAIL-R2 is functional as apoptotic inducer [70]. On the contrary, primary cells from CLL (chronic lymphocytic leukaemia) selectively activate TRAIL-R1 dependent apoptosis [71]. Despite these difficulties, clinical applications of TRAIL-R agonists represents a frontline for many pharmaceutical companies which are involved in clinical trials where different formulation of rTRAIL or agonistic anti-TRAIL-R1/R2 monoclonal antibodies are employed in single form or in combinatorial treatments with a variety of chemotherapeutic agents or ionizing radiation to sensitize cancer cells to TRAIL-mediated apoptosis. Excellent reviews on preclinical studies targeting TRAIL pathways have been recently published [4,67,68]. In general, soluble forms of rTRAIL suppress the growth of human tumour xenografts implanted in mice without a relevant systemic toxicity [67,68]. Similarly, an enormous number of studies have been performed on combinatorial therapy including rTRAIL or anti-TRAIL-R1/R2 in association with conventional chemotherapeutic drugs, inhibitors of NF-κB, kinase, histone deacetylase inhibitors, immune-based therapies. Table 1 in Ref. [4] reports a list of these studies highlighting the supposed mechanism(s) of action. Unfortunately, only in few cases, the efficacy of the combination treatments has been proved in vivo and a general consensus on how chemotherapy and radiotherapy may synergize with TRAIL therapy is far to be reached. Accordingly to [4], three issues must be considered before suggesting a combinatory therapy including TRAIL: i. efficacy in vivo compared to monotherapy; ii. the existence of a strong molecular rationale suggesting a combination treatment; iii. knowledge of the detailed molecular mechanism(s) by which an anticancer agent enhances TRAIL effects.

Initial data on clinical trials testing the efficacy of rTRAIL and/or anti-TRAIL-R1/2 on cancer patients have been published since 2004 (Table 2 in [4] and references therein). Table 2 of the present review reports an updated list of currently active clinical trials deducted from National Cancer Institute web site.[5] The first company to start and complete phase II clinical trials with TRAIL-R1 specific mAbs was Human Genome Sciences (mapatumumab/HGS-ETR1) in subjects with relapsed or refractory non-Hodgkin's Lymphoma (NHL), colorectal cancer and NSCLC [72]. The results of this study confirmed that clinical response or stable disease with TRAIL-R1 monoclonal antibody was more evident in NHL patients (14/17) than in solid tumours (30%). In advanced solid tumours and multiple myeloma, mapatumumab/HGS-ETR1 is currently used in combination with different chemotherapeutic drugs (gemcitabine, cisplatin, paclitaxel, carboplatin and bortezomib). Human Genome Sciences (lexatumumab/HGS-ETR2), Genetech (PRO95780), and Daiichi Sankyo (CS1008) are also studying human antibodies specific for TRAIL-R2. These studies are in phase I as single and chemotherapy combined-agent in advanced solid tumours. It should note that mapatumumab and lexatumumab treated patients developed several adverse effects including anaemia, fatigue and dehydration, even if some adverse effects could be due to the associated chemotherapeutic drugs. Amgen is studying a

[3] http://microrna.sanger.ac.uk/cgibin/targets/v3/download.pl.
[4] www.cancer.gov/clinicaltrials.
[5] www.cancer.gov/clinicaltrials.

fully human TRAIL-R2 monoclonal antibody (AMG655) in combination with AMG479 (anti IGF-receptor mAb) in advanced, refractory solid tumours (NSCLC, colorectal, pancreatic, ovarian cancer and sarcoma). AMG655 is in phase Ib study also in lymphoma patients in combination with bortezomib and vorinostat (Table 2).

The major pharmaceutical companies involved in testing anti-TRAIL-R1/R2 antibodies are also designing clinical trials to prove the efficacy of human rTRAILs. Previous studies demonstrated that apoptosis of normal cells induced by rTRAIL is dependent on the method used for production of the recombinant soluble protein [73]. Amgen and Genentech have ongoing phase Ib and II clinical trials to test safety and tolerability in patient with advanced solid and hematological tumours (Table 2).

5. Conclusion and perspectives

More than one century ago, Paul Ehrlich introduced the concept of "magic bullet", a compound which selectively targets disease-causing microorganisms, seeking out and destroying them, avoiding other organisms and having no harmful effects on the bodies of patients [74]. Hence, the "magic bullet" represents, still now, the goal in molecular oncology: it should eradicate malignant cells without killing normal cells. However, cancer is a complex and heterogeneous disease, and until recent years, cancer therapy consisted in killing as many transformed cells as possible using larger dose of cytotoxic drugs, keeping the patient alive as long as possible. Targeted therapy, for some solid tumours and leukaemias is now a reality, but the emerging resistance to cytotoxic drugs as well as to selective drugs is an actual and still unsolved problem. In the next future "combined anticancer therapy" could consider DR derived drugs and specific protein kinase inhibitors and/or selective growth receptor agonists to realize a modern version of Ehrilch's "magic bullet" and optimize cancer therapy.

From this review emerges that promising results are expecting from the large number of clinical trials actually ongoing on TRAIL-R agonists, both antibodies and recombinant proteins. Expectations also come from drugs triggering CD95 and TNF-R1, although, in these cases, the clinical applicability is still far to become a reality. For now, the best results are obtaining from tumours which are primarily sensitive to DR activation or might acquire sensitivity by means of combinatory therapy with canonical anticancer drugs [4]. These combinatorial approaches allow the contemporary activation of multiple apoptotic targets acting on both extrinsic and intrinsic cell death pathways. In the case of TRAIL, the use of a cocktail of monoclonal antibodies against different TRAIL receptors could increase tumour-specific activity, but also increase toxicity. In fact, they have a significant higher half-life (14–21 days) respect to recombinant ligands (about 30 min in non-human primates). However, until now, there are no studies comparing simultaneously tumour efficacy and toxic side effects of recombinant ligands versus monoclonal antibodies. The idea that a cocktail of a few monoclonal antibodies are substantially more potent than individual antibodies has stimulated the initiation of clinical trials with promising results. In oncology, combination of two mAbs targeting the HER2/neu receptor in breast cancer cells resulted in synergistic cell death. Similarly, combination of anti-CD20 and CD22 in patients with recurrent or refractory non-Hodgkin's lymphoma demonstrated promising antilymphoma activity (reviewed in [75]). To our knowledge, no data are available on similar approaches using cocktails of monoclonal antibodies raised against DRs. In addition, this therapeutic approach must face practical issues related to high costs of production and treatment. When alternative and innovative strategies will allow development and manufacturing of recombinant monoclonal antibodies without substantially increasing the cost, the efficacy of combination therapy may even reduce the economic cost of therapy by lowering doses of antibodies to be administered to patients [75].

A key point underlined by several authors on the clinical applicability of DR agonists regards the limited knowledge, at cellular and molecular level, of the mechanisms producing tumour resistance. As we described above, a black hole in DR signalling is the simultaneous existence, after DR engagement, of classical apoptotic and alternative pathways leading to cell proliferation or apoptosis resistance. As demonstrated for TNF-alpha, CD95 and TRAIL, their activation can induce not only the classical caspase cascade, but also a variety of non-apoptotic pathways leading to cell survival and involving activation of NF-κB, PKB/AKT and MAPKs. Decision to one pathway versus the other may not simple depends on the concentration of DR ligand applied, but also on the kinetic and stoichiometry of multiple protein interacting DISC level. Other key elements are the specific physiological state of the cell and the role of death ligands in interacting with the immune system in both normal and cancer cells.

Certainly, the alternative pathways triggered by DRs and leading to NF-κB and kinases activation are still confusing and their functional importance requires stronger evidence, deriving from both in vitro and in vivo studies. A better understanding of the Janus-like behavior of DRs to progress from one condition, cell death, to another, cell survival, will clarify many of the fundamental mechanisms leading to tumour resistance.

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