

University of Groningen

**Trail receptor-targeted therapy : strategies to enhance DR4- and DR5-induced apoptosis**  
van Roosmalen, Ingrid

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*  
2014

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

van Roosmalen, I. (2014). *Trail receptor-targeted therapy : strategies to enhance DR4- and DR5-induced apoptosis*. [Thesis fully internal (DIV), University of Groningen]. [S.n.].

**Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

**Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

TWO DEATH-INDUCING HUMAN  
TRAIL RECEPTORS TO TARGET IN  
CANCER: SIMILAR OR DISTINCT  
REGULATION AND FUNCTION?

Ingrid A.M. van Roosmalen, Wim J. Quax, Frank A.E. Kruyt

*Biochemical Pharmacology 2014; 91(4): 447-456*

2

## ABSTRACT

The emergence during evolution of two tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors, receptor-1/DR4 and -2/DR5, able to induce apoptosis has raised the question whether they differ in function and regulation, which is of key importance for selecting either DR4 or DR5 selective pro-apoptotic agents for cancer treatment. In this review we found practically no information regarding possible differences in DR4 and DR5 function based on structural differences. On the other hand, a panel of different DR4 or DR5 selective pro-apoptotic agonists have been developed that were explored for efficacy in different tumour types in a large number of studies. Leukemic cells appear mainly sensitive for DR4-induced apoptosis, contrasting the situation in other tumour types that show heterogeneity in receptor preference and, in some cases, a slight overall preference for DR5. Both receptors were found to mediate intracellular stress-induced apoptosis, although this is most frequently reported for DR5. Interestingly, DR5 was also found to transmit non-apoptotic signalling in resistant tumour cells and recently nuclear localization and a role in microRNA maturation has been described. DR4 expression is most heavily regulated by promoter methylation, intracellular trafficking and post-translational modifications. DR5 expression is predominantly regulated at the transcriptional level, which may reflect its ability to respond to cellular stressors. It will be important to further increase our understanding of the mechanisms determining TRAIL receptor preference in order to select the appropriate TRAIL receptor selective agonists for therapy, and to develop novel strategies to enhance apoptosis activation in tumours.

## 1. INTRODUCTION

Cancer is one of the leading causes of death in the world. The main treatment strategy of cancer consists of surgical resection in combination with radiation and chemotherapy. More recently, alternative strategies have been developed that directly target molecular mechanisms in tumour cells, such as the activation of apoptosis. Tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is an important representative of biological anti-cancer agents that selectively induce apoptosis in a variety of tumour cells [1]. In humans, TRAIL acts mainly through two receptors, TRAIL-receptor-1 (R1) and -2 (R2), also known as death receptor 4 (DR4) and 5 (DR5), respectively. These receptors reside in the cell membrane and are able to initiate the extrinsic apoptotic or death receptor pathway. This apoptotic route principally does not require the presence of functional p53 implicating a possible broader anti-tumour activity than DNA damaging therapeutics. Additional TRAIL receptors have been identified, Decoy Receptor 1 (DcR1 or TRAIL-R3) and 2 (DcR2 or TRAIL-R4) and soluble receptor osteoprotegerin (OPG). However, these receptors do not transduce death-inducing signals. Intriguingly, humans and chimpanzees have two functional death-inducing TRAIL receptors, whereas rodents have only one receptor [1]. These findings raise the question why during evolution two different death-inducing TRAIL receptors have arisen. A likely explanation is that the receptors differ in signalling ability and functional outcome. In this review we address this possibility by providing an overview of studies that have reported receptor dominance and differences in signalling ability between DR4 and DR5 in cancer cells. The underlying molecular causes that contribute to differential regulation of DR4 and DR5 and DR preference are examined. The implications of receptor preference for the application of therapeutic molecules that selectively target one of the TRAIL receptors are briefly discussed.

## 2. TRAIL SIGNALLING

### 2.1 TRAIL

TRAIL, also known as Apo2 ligand (Apo2L), is a member of the TNF superfamily and displays high sequence homology in the apoptosis-inducing C-terminal region with other TNF family members, such as Fas and TNF. TRAIL is a cytokine that plays a physiological role in the immune system, including anti-tumour immune surveillance [1]. TRAIL is expressed as a type-II transmembrane glycoprotein and can be proteolytically cleaved at the stalk domain to give rise to soluble TRAIL. Crystallographic studies revealed that TRAIL forms a homotrimeric subunit that is stabilized by a zinc ion, which is essential for optimal biological activity [2].

### 2.2 TRAIL-induced apoptosis

TRAIL-induced apoptosis is initiated upon binding of TRAIL to DR4 or DR5, leading to the formation of the death-inducing signalling complex (DISC) consisting of Fas-associated

death domain (FADD) and pro-caspase-8 and/or pro-caspase-10 [3]. The caspases are activated in the DISC by proteolytic cleavage that on their turn cleave the executioner caspases-3 and -7 leading to apoptosis [3]. This so-called extrinsic apoptotic pathway can cross activate the mitochondrial – or intrinsic – apoptotic pathway via caspase-8-dependent cleavage of Bid. Truncated Bid (tBid) interacts with the pro-apoptotic Bcl-2 family members Bax and Bak that form pores in the outer mitochondrial membrane resulting in the release of cytochrome c among other factors. Cytochrome c in the presence of dATP activates caspase-9 in a complex with Apaf-1, called the apoptosome, leading to cleavage and activation of caspase-3. Tumour cells have been categorized into type 1 or type 2 cells, in which TRAIL-induced apoptosis is independent or dependent, respectively, on mitochondrial amplification of the apoptotic signal [3].

### 2.3 TRAIL resistance

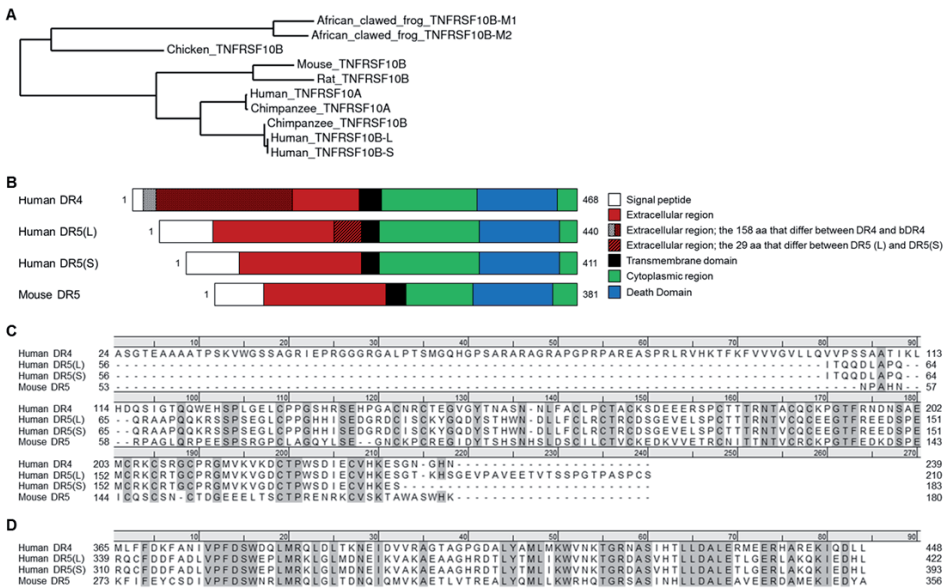
The activation of TRAIL receptors as a powerful anti-cancer strategy has been demonstrated in numerous pre-clinical and clinical studies [3,4]. However, around 50% of tumour cells appear resistant to TRAIL-induced apoptosis [5,6]. Resistance can occur at different levels in the apoptotic pathway. The surface expression levels of DR4 and DR5 can decrease as a result of mutations or methylation of the receptor gene promoters and, on the other hand, high expression levels of DcR1 and DcR2 can lead to inhibition of apoptosis activation [7]. At the DISC, cellular FLICE inhibitory protein (c-FLIP) can bind to FADD and prevent recruitment and cleavage of caspase-8 [7]. Moreover, mutations, epigenetic silencing, decreased stability and ubiquitination of caspase-8 levels can also render cells resistant to TRAIL-induced apoptosis [7]. In type 2 cells, inhibition of mitochondrial apoptosis by anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-XL causes TRAIL resistance. Furthermore, inhibitors of apoptosis proteins (IAPs) that are able to inhibit the functioning of caspases-9, -7 and -3 may hamper apoptosis induced by TRAIL [3].

## 3. DIFFERENCES BETWEEN DR4 AND DR5

### 3.1 Species specific differences

DR4 and DR5 are single-pass type-I membrane proteins and are encoded by two genes located on chromosome 8p [7]. Two splice variants of DR5 have been identified, named long DR5 (DR5(L)) and short DR5(S), that differ in a stretch of 29 amino acids located between the cysteine-rich domains (CRDs) and the transmembrane domain in the extracellular region (figure 1B). Whether the two DR5 isoforms are functionally different is currently unknown [8]. Recently, Picarda *et al.* identified a functional, short isoform of DR4 (bDR4) in Ewing's sarcoma cell lines encoded by an alternative splice variant, which lacks 158 amino acids within the extracellular ligand-binding region (figure 1B). Ectopic overexpression of this alternative transcript sensitized resistant cells to rhTRAIL WT via currently unknown mechanisms [9].

TRAIL and its receptors have been identified in other species. Figure 1A shows a phylogenetic tree depicting the evolutionary relationships among the available sequences of apoptosis-inducing TRAIL receptors identified in various vertebrates. Human and frog (*Xenopus laevis*) DRs are most distinct, whereas human and chimpanzee DRs are closely related [1]. Unlike the name suggests, mouse DR5 (mDR5) is not an orthologue of human DR5 since it is almost equally homologous to human DR4 and DR5. Presently it is unclear why chimpanzees and humans have two apoptosis-inducing receptors, whereas the other vertebrates have only one DR. Apparently there is an evolutionary benefit or need for higher primates to have two DRs. Similar regions and domains can be distinguished in DRs from different species (figure 1B). In figures 1C and D multiple alignments of the amino acid sequences representing the extracellular and death domains (DDs) of DR4,



**Figure 1. Cross species comparison of TRAIL receptors. (A)** Phylogenetic tree of the DRs in various species. The phylogram was generated by making use of the phylogeny.fr website and using the Gblocks program [61]. Protein sequences were obtained from the Protein Knowledgebase (UniProtKB) website. **(B)** Schematic representation of the DRs from human and mouse depicting the different domains. The stretch of 29 amino acids that distinguish DR5(L) from DR5(S), and the 158 amino acids that are truncated in DR4 to give rise to bDR4 are also indicated. **(C)** Sequence alignment of the extracellular domain and **(D)** the Death Domain of the DRs from human and mouse by using the Discovery studio program. The grey blocks indicate fully conserved residues. Gene names and accession numbers of the TNF Receptor Superfamily (TNFRSF) members: human TNFRSF10A (DR4) [O00220], human TNFRSF10B (DR5) [O14763], chimpanzee TNFRSF10A [H2QVW1], chimpanzee TNFRSF10B [K7DCC3], mouse TNFRSF10B (DR5) [Q9QZM4], rat TNFRSF10A [B8YBG7], chicken TNFRSF10B [Q9IAR7], African clawed frog TNFRSF10B-M1 [Q76B99] and TNFRSF10B-M2 [Q76B98].

DR5(L), DR5(S) and mDR5 are displayed. The DDs of these receptors show a higher level of similarity than the extracellular domains. Since DR4 has only 46% and 48% sequence identity with DR5(L) and DR5(S), respectively, this is suggestive of differences in structure, function and regulation.

### 3.2 Functional differences

Thus far only one study by Neumann *et al.* [10] addressed the question if particular subdomains in DR4 and DR5 can be attributed to different TRAIL apoptotic signalling properties. For this, they generated various receptor chimeras containing DR-domains combined with parts from other TNF DR family members. Exchanging the FAS DD with the DDs of DR4 and DR5 resulted in chimeras that were less efficient in transducing apoptosis in comparison to the original Fas DD, thus identifying TRAIL receptor DD as weak apoptosis inducers. Other chimeras in which the transmembrane domain and the adjacent extracellular stalk regions of DR4 and DR5 were fused to portions of FAS or TNFR1 showed strongest caspase-8 and caspase-3 activation by chimeras containing the DR5-derived domain. No differences in ligand binding and internalization kinetics were observed for these chimeras, thus providing no explanation for the differences in TRAIL signalling strength. It was speculated that the presence of a S-palmitoylation site present in the transmembrane domain of DR4, but absent in DR5, may favour the localization of DR4 in lipid rafts, leading to better responsiveness to the ligand. On the other hand a GXXXG motif in DR5, but absent in DR4, may stabilize DR5 homodimerization and subsequent signalling. These notions were not investigated, but were proposed to have a regulatory function in DR signalling [10]. Of note, these human chimeras were tested in immortalized mouse fibroblasts and therefore caution should be taken by generalizing these findings, which may involve species- and cell type-dependent effects. In addition, the authors make use of cross-linked Flag-tagged recombinant human (rh)TRAIL, which has been reported to have different signal-inducing properties compared to non-tagged rhTRAIL [1,7].

## 4. TRAIL RECEPTOR-SELECTIVE AGONISTS

A considerable number of agonistic recombinant TRAIL variants and antibodies have been developed for research and/or therapeutic purposes. A short overview is provided below.

### 4.1 Development of recombinant TRAIL and derived DR-selective variants

Soluble rhTRAIL is a version of TRAIL that comprises the extracellular region of human TRAIL (amino acids 114-281). RhTRAIL, or dulanermin, has been tested in phase I/II clinical trials showing some anti-tumour efficacy while side effects were generally mild [4]. A disadvantage of wild-type (WT) rhTRAIL is its tendency to bind all five TRAIL receptors, including decoy receptors that diminish apoptosis activation. In order to overcome this promiscuous behaviour, several groups have produced DR-specific TRAIL variants in order to improve anti-tumour efficacy by reducing DcR-binding (see also Table 1). The

crystal structure of rhTRAIL WT in complex with the extracellular part of DR5 is presently the only resolved TRAIL/TRAIL receptor structure [11].

The rhTRAIL-DR5 structure was used to generate homology models for DR4, DcR1, and DcR2 and allowed the generation of DR-selective variants [12-15]. Multiple DR4-selective TRAIL variants were generated, named rhTRAIL<sup>D218H</sup> and rhTRAIL<sup>D218Y</sup> [12], 4C7 and 4C9 [13], and rhTRAIL-C3 [14], which displayed lowered affinities to DR5 and DcRs while having increased selectivity and affinity for DR4. Moreover, this translated into higher apoptosis-inducing activity of the DR4-selective variants compared to rhTRAIL WT in tumour cell lines [12-14]. On the other hand, the DR5-selective TRAIL variant D269H/E195R showed enhanced activity in a subset of tumour cell lines compared to rhTRAIL WT [15,16]. Kelley *et al.* applied phage display technology to identify peptides with selectivity for either DR4 or DR5, and subsequently used these to generate Flag-tagged and untagged DR-selective TRAIL variants. Some tumour cells were found to have higher sensitivity for the DR5-selective variant, and cross-linking of Flag-tagged ligand using anti-Flag antibody further enhanced apoptosis. However, TRAIL-resistant hepatocytes were also sensitive for cross-linked rhTRAIL WT or the DR5-selective variant rendering these variants unsuitable for further development [17]. Gasparian *et al.* designed two DR5-selective TRAIL variants, DR5-A and DR5-B, by selecting favourable mutations as identified previously by phage display technology [17] and by *in silico* modelling [15]. DR5-A and DR5-B showed improved selectivity to DR5, decreased affinity to DcR2 and OPG, and high biological activity in several tumour cell lines [18]. Of note and relevant for this review, all studies mentioned above reported that tumour cells showed preferences for either DR4- or DR5-induced apoptosis.

#### 4.2 TRAIL receptor-selective antibodies

Agonistic monoclonal antibodies (mAbs) against DR4 or DR5 provide an alternative pro-apoptotic strategy and different classes have been produced; murine, chimeric, humanized and human mAbs. An advantage of mAbs is their longer half-life (days) compared to rhTRAIL (30-60 minutes) [4]. Consequently, lower doses of mAbs can be applied less frequently when compared to rhTRAIL. An overview of the developed DR4 and DR5 agonistic mAbs and their use in preclinical and clinical studies is given in Table 1.

#### 4.3 Mechanism of increased potency of TRAIL receptor-selective agonists

As mentioned, the developed TRAIL receptor agonists have increased affinity for either DR4 or DR5 and reduced binding to the other TRAIL receptors. This results in faster TRAIL receptor binding when compared to rhTRAIL as was shown by Reis and co-workers using surface plasmon resonance (SPR) assays [19]. More detailed molecular analysis of the TRAIL/TRAIL receptor interactions indicated that trimeric TRAIL (variant) binds first with high affinity to one TRAIL receptor molecule (DR4 or DR5) after which two additional receptor molecules are bound with lower affinity leading to trimeric receptor activation [19]. Furthermore, the formation of heterotrimeric TRAIL receptor complexes consisting



**Table 1.** Agonistic TRAIL variants and TRAIL receptor antibodies. Overview of the developed agonistic rhTRAIL variants and mAbs. TRAIL variants containing His- or Flag-tags are not included because of their toxicity to normal cells [1,7]. Some of the mAbs, such as 2E12 [62], tigatuzumab (CS-1008) [63] and LBY135 [64], require cross-linking for obtaining maximal apoptosis-inducing potential.

Target	Reagent	Description	Clinical status	References	
DR4/5	Dulanermin (rhApo2L/TRAIL, AMG 951)	rhTRAIL WT (residues 114-281)	Phase I/II	[65]	
	2E12	Murine IgG1 mAb	-	[62]	
	4C7	Murine IgG2a mAb	-	[66]	
	4H6	Murine IgG1 mAb	-	[66]	
	DR4-A	Murine IgG mAb	-	[23]	
	M270, M272	Murine IgG1 mAb	-	[67]	
	M271, M273	Murine IgG2a mAb	-	[67]	
	DR4	Mapatumumab (HGS-ETRI)	Human IgG1 mAb	Phase I/II	[68]
		rhTRAIL <sup>G131R/R149I/S159R/N199R/K201H/S215D</sup> (4C7)	rhTRAIL variant (residues 114-281)	-	[13]
		rhTRAIL <sup>G131R/R149I/S159R/S215D</sup> (4C9)	rhTRAIL variant (residues 114-281)	-	[13]
		rhTRAIL <sup>G131R/N199R/K201H</sup> (rhTRAIL-C3)	rhTRAIL variant (residues 114-281)	-	[14]
		rhTRAIL <sup>D218H</sup>	rhTRAIL variant (residues 114-281)	-	[12]
		rhTRAIL <sup>D218Y</sup>	rhTRAIL variant (residues 114-281)	-	[12]
		Conatumumab (AMG 655)	Human IgG1 mAb	Phase I/II	[69]
Drozitumab (Apomab)		Human IgG1 mAb	Phase I/II	[70]	
HGS-TR2J (KMTR2)		Human IgG1 mAb	Phase I	[71]	
LBY135		Chimeric IgG1 mAb	Phase I	[64]	
DR5	LCR211	Murine IgG1 mAb	-	[64]	
	Lexatumumab (HGS-ETR2)	Human IgG1 mAb	Phase I	[72]	
	M411	Murine IgG2b mAb	-	[67]	
	M412, M413	Murine IgG1 mAb	-	[67]	
	rhTRAIL <sup>Y189N/R191K/Q193R/H264R/I266L/D267Q/D269H</sup> (DR5-A)	rhTRAIL variant (residues 114-281)	-	[18]	
	rhTRAIL <sup>Y189N/R191K/Q193R/H264R/I266L/D269H</sup> (DR5-B)	rhTRAIL variant (residues 114-281)	-	[18]	
	rhTRAIL <sup>Y189Q/R191K/Q193R/H264R/I266L/D267Q</sup> (Apo2L-DR5-8)	rhTRAIL variant (residues 96-281)	-	[17]	
	rhTRAIL <sup>D269H/E195R</sup>	rhTRAIL variant (residues 114-281)	-	[15]	
	Tigatuzumab (CS-1008)	Humanized IgG1 mAb	Phase I/II	[63]	
	TRA-8	Murine IgG1 mAb	-	[73]	

of DR4 or DR5 and DcRs were unable to transduce apoptotic signalling and were found to regulate the kinetics of the initiation of TRAIL-induced apoptosis [20]. Interestingly, stepwise computational modelling predicted that high affinity TRAIL agonists would be able to bypass the formation of heteromeric DR/DcR-complexes, which was confirmed experimentally by comparing the efficacy and kinetics of TRAIL-induced apoptosis activation between rhTRAIL WT and the DR5-selective TRAIL D269H/E195R variant [20]. Thus, the high affinity binding of TRAIL receptor-selective variants prevents the formation

of non-active TRAIL-heteromeric receptor complexes, which leads to faster and more potent activation of apoptosis in tumour cells [20].

## 5 DR-SPECIFIC APOPTOSIS ACTIVATION IN TUMOURS

A number of the above mentioned TRAIL receptor agonists have been extensively examined for anti-tumour efficacy in pre-clinical models and a few have also been applied in patients [3,21]. These studies provide a wealth of data for obtaining insight in possible DR4 or DR5 dominance in specific tumour types. Below an overview is provided of reports in which different cancer types were tested for sensitivity towards DR4- and DR5-specific agonists.

### 5.1 Leukemic malignancies signal primarily via DR4

Single agent studies showed that leukemic cells appear to have a preference for DR4-induced apoptosis activation, see Table 2 [12,14,22-24]. The acute T cell leukaemia cell line Jurkat seems an exception to this rule and mainly showed DR5-induced apoptosis that could be explained by a low DR4 and high DR5 membrane cell surface expression [18,24]. Acute myelogenous leukaemia (AML) and chronic myelogenous leukaemia (CML) cell lines treated with DR4-selective variants (rhTRAIL<sup>D218H</sup>, rhTRAIL<sup>D218Y</sup> or TRAIL-C3) were more effectively killed than cells treated with rhTRAIL WT or the DR5-selective D269H/E195R variant [12,14]. In addition, primary AML blasts displayed higher apoptotic levels when treated with TRAIL-C3 compared to rhTRAIL WT and sensitivity for both could be enhanced by blocking NF- $\kappa$ B signalling with the I $\kappa$ B kinase (IKK) inhibitor BMS-345541 [14]. Chronic lymphocytic leukaemia (CLL) cells also appeared mainly DR4-responsive [22-24]. Primary CLL cells and the I-83 CLL-like cell line were sensitive to mapatumumab but resistant to lexatumumab. Combined treatment with the chemotherapeutic fludarabine sensitized both primary and I-83 cells for DR4-mediated apoptosis though I-83 was also sensitized for lexatumumab. Sensitization by co-treatment with fludarabine was explained by the observation that DRs translocated to the lipid rafts [22]. In two other studies, primary CLL cells were found to be resistant for TRAIL receptor-selective agonists DR4-A, LBY135, mapatumumab and lexatumumab and could be sensitized predominantly via DR4 when pre-treated with the histone deacetylase inhibitors (HDACi) depsipeptide, LBH589, or sodium valproate [23,24]. The other haematological tumours, lymphoma and myeloma, showed heterogeneity in receptor preference, similar to most solid tumours (Table 2).

### 5.2 Solid tumours display heterogeneity in TRAIL receptor preference

Table 2 summarizes reported DR4 and/or DR5 sensitivity in different solid tumour types. Breast, hepatocellular, neuroblastoma and ovarian cancer, were in most reports sensitive for DR5-dependent apoptosis in single agent studies [13,14,25-29]. In breast cancer cells, chemotherapeutic agents and the selective protein kinase C inhibitor bisindolylmaleimide (Bis) VIII sensitized cells for rhTRAIL WT, TRA-8 and cross-linked 2E12. Combination of TRA-8 with Bis VIII or chemotherapy agents adriamycin or cisplatin

**Table 2.** TRAIL receptor preference in different tumour types. Overview of reported TRAIL sensitivity in cells representing different tumour types. Included are tumour types for which more than one report was available in literature, and those that used single agent rhTRAIL, agonistic DR4- and DR5-selective variants, that do not contain His- or Flag-tags, and mAbs. \*Indicates the DR that primarily mediated apoptosis activation. DR4 & DR5: indicates studies showing roughly equal sensitivity to DR4- and DR5-induced apoptosis.

Cancer type	Cell line or primary material	DR preference *	Model system	References	
<b>Haematological cancers</b>					
Leukaemia	Acute lymphoblastic leukaemia (ALL)	Jurkat	DR5	<i>In vitro</i>	[18,23,24,72,74]
	Acute myeloid leukaemia (AML)	HL60, ML-1, MOLM-13	DR4	<i>In vitro</i>	[12,14]
	Chronic lymphocytic leukaemia (CLL)	I-83, primary cells	DR4	<i>In vitro</i>	[22,23]
	Chronic myeloid leukaemia (CML)	EM-2	DR4	<i>In vitro</i>	[12,14]
Lymphoma	Anaplastic large cell lymphoma (ALCL)	SU-DHL1	DR5	<i>In vitro</i>	[72]
	Burkitt's lymphoma	BJAB	DR4 & DR5	<i>In vitro</i>	[22]
		Ramos	DR4	<i>In vitro</i>	[23,24,74,75]
	Hodgkin lymphoma	HD-LM-2	DR4	<i>In vitro</i>	[72]
	Lymphoma	primary cells	DR5	<i>In vitro</i>	[72]
	Mantle cell lymphoma (MCL)	Jeko1, SP53	DR4	<i>In vitro</i>	[72]
Non-hodgkin B-cell lymphoma	WSU-FSCCL	DR5	<i>In vitro, in vivo</i>	[76]	
Myeloma	Karpas 620, KMM1, KMS12BM, KMS12PE, KMS18, L363, LP1, MDN, RPMI8226, U266	DR4	<i>In vitro</i>	[77,78]	
		DR5	<i>In vitro</i>	[77,78]	
<b>Solid cancers</b>					
Breast cancer	MDA-MB-231, 2LMP	DR5	<i>In vitro, in vivo</i>	[25,26,28]	
Cervical cancer	HeLa	DR4	<i>In vitro</i>	[49]	
		DR5	<i>In vitro</i>	[18,25]	
Colorectal cancer	HCT15, CL-34, SW480, SW948	DR4	<i>In vitro</i>	[13,49,68,79,80]	
		DR5	<i>In vitro, in vivo</i>	[77,79,81]	
	Colo205	DR4 & DR5	<i>In vitro</i>	[13,82]	
	HCT116	DR4	<i>In vitro</i>	[82]	
		DR5	<i>In vitro</i>	[28,83]	
	RKO	DR4 & DR5	<i>In vitro, in vivo</i>	[79]	
DR4 & DR5		<i>In vitro, in vivo</i>	[28]		
Hepatocellular cancer	Hep3B, HepG2	DR5	<i>In vitro, in vivo</i>	[28]	
	SNU449	DR4 & DR5	<i>In vitro</i>	[28]	

**Table 2.** (Continued)

Cancer type	Cell line or primary material	DR preference *	Model system	References
Lung cancer	H460	DR4	<i>In vitro</i>	[84]
		DR5	<i>In vitro</i>	[28]
	H2122	DR5	<i>In vitro</i>	[84]
Neuroblastoma	LAN-5	DR4	<i>In vitro</i>	[27]
	CHP-212, SH-EP, SK-N-AS	DR5	<i>In vitro</i>	[27]
Ovarian cancer	A2780, UL-3B, UL-3C	DR5	<i>In vitro</i>	[14,25,26]
	primary cells, OVCAR-3	DR4 & DR5	<i>In vitro</i>	[13,29]
Pancreatic cancer	BxPC3, Colo357, DanG, Panc-1, Panc89, Panc98, PancTu1, PaTu8902, primary cells (ULA), T3M4	DR4	<i>In vitro</i>	[85,86]
		DR5	<i>In vitro</i>	[86]
	PT45	DR4 & DR5	<i>In vitro</i>	[85]
Pleural mesothelioma	H28, MSTO-211H	DR4	<i>In vitro</i>	[87]
	H2452, ZL5, ZL34	DR5	<i>In vitro</i>	[87]
	ZL55	DR4 & DR5	<i>In vitro</i>	[87]
Prostate cancer	PC-3	DR5	<i>In vitro</i>	[28]
	DUI145, LNCap	DR4 & DR5	<i>In vitro, in vivo</i>	[28]

increased activation of both the extrinsic and intrinsic apoptotic pathways. Similarly, chemotherapeutics were able to enhance cell death when combined with TRA-8 in astrocytoma, cervix, colon and ovarian cell lines [25]. Moreover, prolonged exposure of DR5-sensitive breast and ovarian cancer cell lines to TRA-8 resulted in resistant cells, and the DEAD-box RNA helicase protein DDX3 was identified to selectively bind to DR5 and block its DD function. TRA-8 resistance was overcome by combined treatment with Bis VIII, adriamycin, cisplatin or taxol that led to caspase-2-dependent cleavage of DDX3 [26]. Furthermore, ovarian cancer cell lines A2780, UL-3B and UL-3C were only or primarily DR5 sensitive [14,25,26], whereas primary ovarian cancer cells and OVCAR-3 cells responded almost equally well to treatment with either DR4 or DR5 agonists [13,29]. In another study, sorafenib, a multi-kinase inhibitor, enhanced TRAIL-induced cell death *in vitro* via both DR4 and DR5 in a panel of solid tumour cell lines, such as breast and liver cancer cells [28]. Combined treatment with lexatumumab and sorafenib was also effective in reducing growth of breast, liver, colon and prostate cancer xenografts in mice. The sensitizing effect of sorafenib was suggested to involve inhibition of the Jak2-Stat3-Mcl1 axis, thus enhancing the intrinsic apoptotic route [28]. In neuroblastoma cells small-molecule IAP inhibitors synergistically enhanced mapatumumab- and lexatumumab-induced apoptosis by triggering the formation of a receptor-activating protein 1 (RIP1)/FADD/caspase-8

complex. In this somewhat uncommon pro-apoptotic complex, RIP1 kinase activity was essential for complex formation since RIP1 inhibition or silencing abolished its formation and protected against TRAIL receptor agonists and IAP inhibitor-induced apoptosis [27]. On the other hand, cells representing cervical cancer, colorectal cancer, lung cancer, pancreatic cancer, pleural mesothelioma and prostate cancer showed no tendency for receptor preference. Of note, the activation of both DR4 and DR5 could be an advantage, since it was shown that combined exposure to DR4- and DR5-selective TRAIL variants in cells sensitive for both receptors was more potent for triggering apoptosis when compared to single agent treatment [13].

## **6 DIFFERENTIAL REGULATION OF DR4 AND DR5 SIGNALLING**

From the above it appears that certain cancer types have a DR preference for inducing apoptosis, whereas other tumour types do not or to a lesser extent. Although, the underlying molecular cause for DR4 or DR5 preference is currently poorly understood, differences in cell surface expression levels, post-translational modifications, DISC formation and downstream signalling have been reported. This is described in more detail below.

### **6.1 TRAIL receptor transcription**

An obvious cause of TRAIL receptor preference is the absence of one of the receptors. Chromosomal deletion of DR4 or DR5 encoding genes in cancer have been sparsely reported, whereas epigenetic silencing of particularly DR4 has been found more frequently as a cause of TRAIL resistance. DNA hypermethylation of the DR4 promoter was found in a proportion of ovarian cancers and the demethylating agent 5-aza-2'-deoxycytidine (5-AZA dC) restored DR4 expression in A2780 cells, which express DR5, and resulted in TRAIL sensitivity [30]. In melanoma cells, 5-AZA dC was also able to restore DR4 expression and sensitivity [31] and similar observations were made in glioma cell lines [32]. MicroRNA (miR)-25-dependent silencing of DR4 was identified as a cause of TRAIL resistance in cholangiocarcinoma cells and inhibition of Hedgehog (Hh) signalling by cyclopamine was able to reduce miR-25 expression and sensitize for apoptosis [33].

Transcription factors also regulate the level of TRAIL receptors. Although initially increased DR5 transcription was linked to stress-induced apoptosis, both DR4 and DR5 appeared to be regulated by stress-induced transcription factors, including p53, NF- $\kappa$ B, CHOP, FOXO3a and AP1 [3,34-36]. However, recent findings again point to DR5 as the dominant receptor in ER stress-induced apoptosis [37]. In this study, unmitigated ER stress resulted in activation of CHOP leading to increased DR5 transcription and induction of ligand-dependent DR5/FADD/caspase-8-mediated apoptosis. Other transcription factors have been reported to selectively regulate only one receptor. For example, cyclopamine-dependent inhibition of Hh-GLI signalling was found to prevent GLI3-dependent repression of the DR4 promoter leading to enhanced DR4 expression in cholangiocarcinoma cells [38].

Furthermore, the transcription factors specificity protein 1 (Sp1) and Yin Yang 1 (YY1) are specific regulators of DR5 transcription [39-48]. The stimulation of pathways that are able to modulate these transcription factors has been used as a strategy to sensitize tumour cells for TRAIL. For example, bile acids and the natural compounds butein and piceatannol were able to activate c-Jun N-terminal kinase (JNK) and the extracellular signal-regulated kinase (ERK) leading subsequently to Sp1 activation, enhanced DR5 levels and sensitization for TRAIL [39-41]. YY1 is a transcriptional repressor of DR5 and is regulated by NF- $\kappa$ B, and inhibition of NF- $\kappa$ B by several chemotherapeutic drugs [44], the Raf-1 kinase inhibitor protein (RKIP) [45], the proteasome inhibitor NPI-0052 [46] and anti-CD20 mAb Rituximab [48] enhanced TRAIL-induced apoptosis in cancer cells. Moreover, immunohistochemical staining of patient-derived lymphoma tissues demonstrated a negative correlation between the expression of DR5 and YY1, thus further indicating the importance of YY1 as a determinant of DR5 expression [48].

### 6.2 Intracellular trafficking

Cell surface expression of DR4 and DR5 is required for their proper functioning and intracellular trafficking mechanisms were found to play an important role for translocation of the receptors to the cell membrane.

Components of the signal recognition particle (SRP) SRP72 and SRP54, a complex that initiates the protein sorting process by targeting secretory and membrane proteins to the ER, were found to specifically regulate DR4 cell surface levels [49]. Down-regulation of SRP72 or SRP54 reduced DR4 levels and conferred significant protection against cell death induction by agonistic mAb DR4-A, but not DR5-A [49]. A splice variant of the adapter protein ArfGAP with RhoGAP domain, Ankyrin repeat and PH domain (ARAP1), a regulator of trafficking, which lacks exon 30 and is named ARAP1- $\Delta$ exon30, was found to bind conserved residues in the DR4 DD, and to a lesser degree to DR5. Depletion of ARAP1 reduced primarily cell surface levels of DR4 and decelerated TRAIL-induced apoptosis in some cell lines [50]. The Golgi-specific Asp-His-His-Cys (DHHC) zinc finger protein (GODZ) that is also involved in membrane expression of proteins was found to increase cell surface localization of DR4 and subsequent TRAIL-induced apoptosis in hepatoma cells [51].

### 6.3 Post-translational modifications

Post-translational modifications of TRAIL receptors play an important role in regulating expression levels and TRAIL sensitivity in tumour cells. S-palmitoylation is a lipid modification of membrane proteins and palmitoylation of DR4 on the cysteine triplet (residues 261-263) positioned in the cytoplasmic portion near the transmembrane domain, which is absent in DR5, resulted in constitutive localization of DR4 in lipid rafts [52]. Lipid rafts are specialized membrane domains enriched in cholesterol and glycosphingolipids, which facilitated homo-oligomerization and subsequent TRAIL-mediated cell death [52].

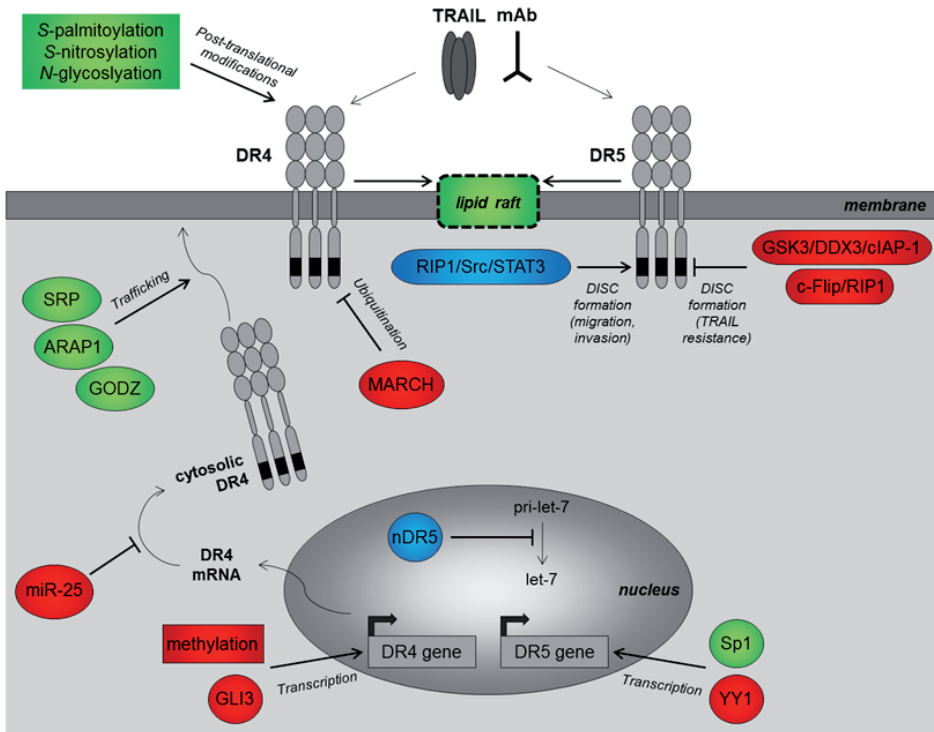
S-nitrosylation of DR4, the covalent coupling of a nitrogen monoxide (NO) group to a reactive cysteine thiol, was demonstrated in cancer cells treated with the nitric oxide-donor nitrosylcobalamin (NO-Cbl), an analogue of vitamin B<sub>12</sub> [53]. NO-Cbl-induced apoptosis in several tumour cell lines, by S-nitrosylation of C336 in the cytoplasmic domain of DR4 and subsequent caspase-8 activation and ectopic expression of a DR4 C336A mutant in tumour cells reduced TRAIL sensitivity [53]. O-glycosylation of both DR4 and DR5 was reported to enhance TRAIL sensitivity in tumour cells [5], whereas DR4 was found to be N-glycosylated [54]. N-glycosylation could be prevented by the induction of ER stress, however, the functional consequences of this modification on TRAIL sensitivity remained unexplored [54].

Furthermore, members of the membrane-associated RING-CH (MARCH) ubiquitin ligases family were found to particularly modify DR4, resulting in altered endosomal trafficking and down-regulation of DR4 cell surface levels [55]. MARCH-1 and -8 ubiquitinated the conserved membrane-proximal lysine 273 and exogenous overexpression of MARCH-1 or -8 reduced TRAIL-induced apoptosis in breast cancer and melanoma cells [55]. MARCH-8 ubiquitination targeted DR4 for lysosomal degradation thus controlling steady-state levels of DR4.

#### **6.4 DISC formation and lipid rafts**

The ability of the TRAIL receptors to recruit and assemble a functional DISC may also vary and result in receptor preference. For example, binding of DDX3 to DR5 was identified as a mechanism responsible for induced resistance to the DR5-selective agonist TRA-8 in breast cancer cells [26]. Interestingly, these TRA-8 resistant cells remained sensitive to 2E12-mediated activation of DR4, suggesting that DDX3 binding was selective for DR5. In another study, DDX3 together in a complex with the glycogen synthase kinase 3 (GSK3) and the cellular inhibitor of apoptosis protein-1 (cIAP1) were found to block DISC formation induced by four major DRs, DR4, DR5, FAS/CD95 and TNF, in breast cancer cells [56]. Inhibition of GSK3 or knockdown of DDX3 released this blockade and sensitized for DR5-induced apoptosis [56]. A more recent study showed that low expression of the DR5/DDX3/cIAP-1 complex correlated with high TRA-8 sensitivity in a panel of breast cancer cell lines, although sensitivity to DR4-induced apoptosis was not examined [57].

As mentioned earlier, the localization of TRAIL receptors to lipid rafts has been associated with their ability to induce apoptosis. Treatment with the chemotherapeutic fludarabine resulted in translocation of DR4 to the lipid rafts together with FADD and caspase-8, but not c-FLIP and RIP, whereas the levels of DR5 in lipid rafts remained unchanged [22]. The localization of the receptors to lipid rafts also appeared to affect their ability to either transmit apoptotic or non-apoptotic signalling. In TRAIL resistant non-small cell lung cancer (NSCLC) cells that expressed higher levels of DR5 compared to DR4, non-apoptotic signalling was mediated by DR5/c-FLIP/RIP1 and knockdown of c-FLIP or RIP1 sensitized for TRAIL [58]. Interestingly, this was associated with redistribution of DR5



**Figure 2. Differential regulation of DR4 and DR5 signalling.** Overview of molecular mechanisms found to differentially regulate DR4 and DR5 expression and functioning. These include regulation at the transcription level, post-translational modifications, intracellular trafficking and localization to the cell membrane and DISC formation. Proteins and modifications that have an activating (green/light grey) or inhibitory (red/dark grey) effect on TRAIL-induced apoptosis, and proteins that induce non-canonical signalling (blue/black) are indicated.

from the nonrafts to the lipid rafts suggesting that DISC assembly determines localization of DR5 in lipid rafts that is associated with apoptosis activation. In another study, TRAIL was able to induce migration and invasion of resistant NSCLC cells, which was mediated by DR5 and not DR4, and involved the RIP1/Src/STAT3 axis [59]. Another difference between DR4 and DR5 was recently discovered by Haselmann and co-workers, who found high levels of DR5 and only low levels of DR4 in the nucleus of different types of tumour cells [60]. Immunoprecipitation studies identified several nuclear proteins present in complex with DR5 and suggested the involvement of DR5 in the regulation of maturation of let-7 miR family members, which are known for their role in development and cancer. Indeed, DR5 was shown to inhibit maturation of let-7 and to promote proliferation. Interestingly, endogenous TRAIL and interactions with membrane localized TRAIL receptors was not required for this DR5-mediated proliferative effect [60].



## 7. CONCLUDING REMARKS

The development and use of DR4- or DR5-selective agents for cancer treatment has brought more attention to the question whether there are functional differences between the two receptors and whether there will be a preferred receptor to target. Here we tried to provide answers to these questions and noted that currently studies are lacking that have aimed to link differences in amino acid sequences and/or domains between DR4 and DR5 in relation to function. Most knowledge on receptor preference has been experimentally obtained by testing DR4- or DR5-selective agents in different tumour types *in vitro*. It appears that of the haematological cancers leukemic cells have a DR4 preference, whereas lymphoma and myeloma demonstrate heterogeneity in receptor preference, which is also mostly seen in solid tumours. However, although somewhat preliminary, breast, hepatocellular, neuroblastoma and ovarian cancer have an overall dominance for apoptosis activation via DR5. Presently, specific biomarkers to determine DR preference are lacking in the clinical setting, which hampers the selection of the optimal TRAIL receptor agonist for treatment. Therefore, in clinical practise it may be best to target both DR4 and DR5 with rhTRAIL WT despite its lower efficacy compared to the receptor selective agonists. Alternatively, DR4- and DR5-selective agents could be combined, although this would require clinical studies to determine the toxicity profile of such combined treatment.

The initial view that DR5 is involved in stress-induced apoptosis has been counteracted by studies showing also such responses to be mediated by DR4. However, more recently additional evidence has been provided that mainly DR5 is involved in ER stress-induced apoptosis and, moreover, in controlling the maturation of miRs in the nucleus. Therefore, the notion that DR5 is primarily more involved in stress-induced signalling appears to gain again more support. Although speculative, the deregulation of oncogenes and tumour suppressor genes in cancer cells leads to significant levels of cellular stress, which may explain increased sensitivity to DR5-targeting strategies. DR5 also appears also to be the preferred receptor for mediating non-apoptotic signals. Furthermore, DR4 preference is predominantly observed in leukaemia and may be indicative of DR4 playing a more dominant role in cells of the immune system.

Several mechanisms have been described to be involved in DR preference, including the regulation of DR levels by promoter methylation or transcription factors, intracellular trafficking of receptors, post-translational modifications and DISC-forming abilities. Overall, one may conclude that DR4 is more heavily regulated than DR5 at the level of promoter methylation, post-translational modification and cellular trafficking processes that determine cell surface expression of DR4. DR5 appears mostly regulated at the level of transcription, perhaps again pointing to sensitivity for signals coming from the microenvironment or intracellular stressors. These notions need further experimental back up and may reveal specific strategies to enhance DR expression levels and sensitize the pathway for TRAIL receptor targeting agents.

## ACKNOWLEDGEMENTS

This research was funded by the Ubbo Emmius Foundation of the University of Groningen and by grant RUG2011-5211 from the Dutch Cancer Society.

## REFERENCES

1. Kelley SK, Ashkenazi A. Targeting death receptors in cancer with Apo2L/TRAIL. *Curr.Opin.Pharmacol.* 2004;4:333-9.
2. Hymowitz SG, O'Connell MP, Ultsch MH, Hurst A, Totpal K, Ashkenazi A, et al. A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. *Biochemistry* 2000;39:633-40.
3. Mahalingam D, Szegezdi E, Keane M, de Jong S, Samali A. TRAIL receptor signalling and modulation: Are we on the right TRAIL? *Cancer Treat.Rev.* 2009;35:280-8.
4. Wiezorek J, Holland P, Graves J. Death receptor agonists as a targeted therapy for cancer. *Clin.Cancer Res.* 2010;16:1701-8.
5. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. *Nat. Med.* 2007;13:1070-7.
6. Stegehuis JH, de Wilt LH, de Vries EG, Groen HJ, de Jong S, Kruyt FA. TRAIL receptor targeting therapies for non-small cell lung cancer: current status and perspectives. *Drug Resist Updat* 2010;13:2-15.
7. Pennarun B, Meijer A, de Vries EG, Kleibeuker JH, Kruyt F, de Jong S. Playing the DISC: turning on TRAIL death receptor-mediated apoptosis in cancer. *Biochim. Biophys.Acta* 2010;1805:123-40.
8. Wang TT, Jeng J. Coordinated regulation of two TRAIL-R2/KILLER/DR5 mRNA isoforms by DNA damaging agents, serum and 17beta-estradiol in human breast cancer cells. *Breast Cancer Res.Treat.* 2000;61:87-96.
9. Picarda G, Surget S, Guiho R, Teletchea S, Berreur M, Tirode F, et al. A functional, new short isoform of death receptor 4 in Ewing's sarcoma cell lines may be involved in TRAIL sensitivity/resistance mechanisms. *Mol. Cancer.Res.* 2012;10:336-46.
10. Neumann S, Bidon T, Branschadel M, Krippner-Heidenreich A, Scheurich P, Doszczak M. The transmembrane domains of TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 co-regulate apoptotic signaling capacity. *PLoS One* 2012;7:e42526.
11. Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF, et al. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol.Cell* 1999;4:563-71.
12. Tur V, van der Sloot AM, Reis CR, Szegezdi E, Cool RH, Samali A, et al. DR4-selective tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) variants obtained by structure-based design. *J.Biol.Chem.* 2008;283:20560-8.
13. Reis CR, van der Sloot AM, Natoni A, Szegezdi E, Setroikromo R, Meijer M, et al. Rapid and efficient cancer cell killing mediated by high-affinity death receptor homotrimerizing TRAIL variants. *Cell.Death Dis.* 2010;1:e83.
14. Szegezdi E, Reis CR, van der Sloot AM, Natoni A, O'Reilly A, Reeve J, et al. Targeting AML through DR4 with a novel variant of rhTRAIL. *J.Cell.Mol.Med.* 2011;15:2216-31.
15. van der Sloot AM, Tur V, Szegezdi E, Mullally MM, Cool RH, Samali A, et al. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. *Proc.Natl. Acad.Sci.U.S.A.* 2006;103:8634-9.
16. Duiker EW, de Vries EG, Mahalingam D, Meersma GJ, Boersma-van Ek W, Hollema H, et al. Enhanced antitumor efficacy of a DR5-specific TRAIL variant over recombinant

- human TRAIL in a bioluminescent ovarian cancer xenograft model. *Clin.Cancer Res.* 2009;15:2048-57.
17. Kelley RF, Totpal K, Lindstrom SH, Mathieu M, Billeci K, Deforge L, et al. Receptor-selective mutants of apoptosis-inducing ligand 2/ tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR)5 than DR4 to apoptosis signaling. *J.Biol.Chem.* 2005;280:2205-12.
  18. Gasparian ME, Chernyak BV, Dolgikh DA, Yagolovich AV, Popova EN, Sycheva AM, et al. Generation of new TRAIL mutants DR5-A and DR5-B with improved selectivity to death receptor 5. *Apoptosis* 2009;14:778-87.
  19. Reis CR, van Assen AH, Quax WJ, Cool RH. Unraveling the binding mechanism of trivalent tumor necrosis factor ligands and their receptors. *Mol.Cell.Proteomics* 2011;10:M110.002808.
  20. Szegezdi E, van der Sloot AM, Mahalingam D, O'Leary L, Cool RH, Munoz IG, et al. Kinetics in signal transduction pathways involving promiscuous oligomerizing receptors can be determined by receptor specificity: apoptosis induction by TRAIL. *Mol.Cell.Proteomics* 2012;11:M111.013730.
  21. den Hollander MW, Gietema JA, de Jong S, Walenkamp AM, Reyners AK, Oldenhuis CN, et al. Translating TRAIL-receptor targeting agents to the clinic. *Cancer Lett.* 2013;332:194-201.
  22. Xiao W, Ishdorj G, Sun J, Johnston JB, Gibson SB. Death receptor 4 is preferentially recruited to lipid rafts in chronic lymphocytic leukemia cells contributing to tumor necrosis related apoptosis inducing ligand-induced synergistic apoptotic responses. *Leuk.Lymphoma* 2011;52:1290-301.
  23. Natoni A, MacFarlane M, Inoue S, Walewska R, Majid A, Knee D, et al. TRAIL signals to apoptosis in chronic lymphocytic leukaemia cells primarily through TRAIL-R1 whereas cross-linked agonistic TRAIL-R2 antibodies facilitate signalling via TRAIL-R2. *Br.J.Haematol.* 2007;139:568-77.
  24. MacFarlane M, Inoue S, Kohlhaas SL, Majid A, Harper N, Kennedy DB, et al. Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. *Cell Death Differ.* 2005;12:773-82.
  25. Ohtsuka T, Buchsbaum D, Oliver P, Makhija S, Kimberly R, Zhou T. Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway. *Oncogene* 2003;22:2034-44.
  26. Li Y, Wang H, Wang Z, Makhija S, Buchsbaum D, LoBuglio A, et al. Inducible resistance of tumor cells to tumor necrosis factor-related apoptosis-inducing ligand receptor 2-mediated apoptosis by generation of a blockade at the death domain function. *Cancer Res.* 2006;66:8520-8.
  27. Abhari BA, Cristofanon S, Kappler R, von Schweinitz D, Humphreys R, Fulda S. RIP1 is required for IAP inhibitor-mediated sensitization for TRAIL-induced apoptosis via a RIP1/FADD/caspase-8 cell death complex. *Oncogene* 2013;32:3263-73.
  28. Abdulghani J, Allen JE, Dicker DT, Liu YY, Goldenberg D, Smith CD, et al. Sorafenib Sensitizes Solid Tumors to Apo2L/TRAIL and Apo2L/TRAIL Receptor Agonist Antibodies by the Jak2-Stat3-Mcl1 Axis. *PLoS One* 2013;8:e75414.
  29. Pasquini L, Petronelli A, Petrucci E, Saulle E, Mariani G, Scambia G, et al. Primary ovarian cancer cells are sensitive to the proapoptotic effects of proteasome inhibitors. *Int.J.Oncol.* 2010;36:707-13.
  30. Horak P, Pils D, Haller G, Pribill I, Roessler M, Tomek S, et al. Contribution of epigenetic silencing of tumor necrosis factor-related apoptosis inducing ligand receptor 1 (DR4) to TRAIL resistance and ovarian cancer. *Mol. Cancer.Res.* 2005;3:335-43.
  31. Bae SI, Cheriya V, Jacobs BS, Reu FJ, Borden EC. Reversal of methylation silencing of Apo2L/TRAIL receptor 1 (DR4) expression overcomes resistance of SK-MEL-3 and SK-MEL-28 melanoma cells to interferons (IFNs) or Apo2L/TRAIL. *Oncogene* 2008;27:490-8.

32. Elias A, Siegelin MD, Steinmuller A, von Deimling A, Lass U, Korn B, et al. Epigenetic silencing of death receptor 4 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in gliomas. *Clin. Cancer Res.* 2009;15:5457-65.
33. Razumilava N, Bronk SF, Smoot RL, Fingas CD, Werneburg NW, Roberts LR, et al. miR-25 targets TNF-related apoptosis inducing ligand (TRAIL) death receptor-4 and promotes apoptosis resistance in cholangiocarcinoma. *Hepatology* 2012;55:465-75.
34. Jin HO, Lee YH, Park JA, Kim JH, Hong SE, Kim HA, et al. Blockage of Stat3 enhances the sensitivity of NSCLC cells to PI3K/mTOR inhibition. *Biochem.Biophys.Res.Commun.* 2014;444:502-8.
35. Shoeb M, Ramana KV, Srivastava SK. Aldose reductase inhibition enhances TRAIL-induced human colon cancer cell apoptosis through AKT/FOXO3a-dependent upregulation of death receptors. *Free Radic.Biol.Med.* 2013;63:280-90.
36. Portanova P, Notaro A, Pellerito O, Sabella S, Giuliano M, Calvaruso G. Notch inhibition restores TRAIL-mediated apoptosis via API-dependent upregulation of DR4 and DR5 TRAIL receptors in MDA-MB-231 breast cancer cells. *Int.J.Oncol.* 2013;43:121-30.
37. Lu M, Lawrence DA, Marsters S, Acosta-Alvear D, Kimmig P, Mendez AS, et al. Cell death. Opposing unfolded-protein-response signals converge on death receptor 5 to control apoptosis. *Science* 2014;345:98-101.
38. Kurita S, Mott JL, Almada LL, Bronk SF, Werneburg NW, Sun SY, et al. GLI3-dependent repression of DR4 mediates hedgehog antagonism of TRAIL-induced apoptosis. *Oncogene* 2010;29:4848-58.
39. Higuchi H, Grambihler A, Canbay A, Bronk SF, Gores GJ. Bile acids up-regulate death receptor 5/TRAIL-receptor 2 expression via a c-Jun N-terminal kinase-dependent pathway involving Sp1. *J.Biol.Chem.* 2004;279:51-60.
40. Moon DO, Kim MO, Choi YH, Kim GY. Butein sensitizes human hepatoma cells to TRAIL-induced apoptosis via extracellular signal-regulated kinase/Sp1-dependent DR5 upregulation and NF-kappaB inactivation. *Mol.Cancer.Ther.* 2010;9:1583-95.
41. Kang CH, Moon DO, Choi YH, Choi IW, Moon SK, Kim WJ, et al. Piceatannol enhances TRAIL-induced apoptosis in human leukemia THP-1 cells through Sp1- and ERK-dependent DR5 up-regulation. *Toxicol.In.Vitro.* 2011;25:605-12.
42. Deng Z, Yan H, Hu J, Zhang S, Peng P, Liu Q, et al. Hepatitis C virus sensitizes host cells to TRAIL-induced apoptosis by up-regulating DR4 and DR5 via a MEK1-dependent pathway. *PLoS One* 2012;7:e37700.
43. Lin T, Chen Y, Ding Z, Luo C, Liu J, Shen J. Novel insights into the synergistic interaction of a thioredoxin reductase inhibitor and TRAIL: the activation of the ASK1-ERK-Sp1 pathway. *PLoS One* 2013;8:e63966.
44. Baritaki S, Huerta-Yeppez S, Sakai T, Spandidos DA, Bonavida B. Chemotherapeutic drugs sensitize cancer cells to TRAIL-mediated apoptosis: up-regulation of DR5 and inhibition of Yin Yang 1. *Mol.Cancer.Ther.* 2007;6:1387-99.
45. Baritaki S, Katsman A, Chatterjee D, Yeung KC, Spandidos DA, Bonavida B. Regulation of tumor cell sensitivity to TRAIL-induced apoptosis by the metastatic suppressor Raf kinase inhibitor protein via Yin Yang 1 inhibition and death receptor 5 up-regulation. *J.Immunol.* 2007;179:5441-53.
46. Baritaki S, Suzuki E, Umezawa K, Spandidos DA, Berenson J, Daniels TR, et al. Inhibition of Yin Yang 1-dependent repressor activity of DR5 transcription and expression by the novel proteasome inhibitor NPI-0052 contributes to its TRAIL-enhanced apoptosis in cancer cells. *J.Immunol.* 2008;180:6199-210.
47. Martinez-Paniagua MA, Baritaki S, Huerta-Yeppez S, Ortiz-Navarrete VF, Gonzalez-Bonilla C, Bonavida B, et al. Mcl-1 and YY1 inhibition and induction of DR5 by the BH3-mimetic Obatoclox (GX15-070) contribute

- in the sensitization of B-NHL cells to TRAIL apoptosis. *Cell.Cycle* 2011;10:2792-805.
48. Vega MI, Baritaki S, Huerta-Yepez S, Martinez-Paniagua MA, Bonavida B. A potential mechanism of rituximab-induced inhibition of tumor growth through its sensitization to tumor necrosis factor-related apoptosis-inducing ligand-expressing host cytotoxic cells. *Leuk.Lymphoma* 2011;52:108-21.
  49. Ren YG, Wagner KW, Knee DA, Aza-Blanc P, Nasoff M, Deveraux QL. Differential regulation of the TRAIL death receptors DR4 and DR5 by the signal recognition particle. *Mol.Biol.Cell* 2004;15:5064-74.
  50. Simova S, Klima M, Cermak L, Sourkova V, Andera L. Arf and Rho GAP adapter protein ARAP1 participates in the mobilization of TRAIL-R1/DR4 to the plasma membrane. *Apoptosis* 2008;13:423-36.
  51. Oh Y, Jeon YJ, Hong GS, Kim I, Woo HN, Jung YK. Regulation in the targeting of TRAIL receptor 1 to cell surface via GODZ for TRAIL sensitivity in tumor cells. *Cell Death Differ.* 2012;19:1196-207.
  52. Rossin A, Derouet M, Abdel-Sater F, Hueber AO. Palmitoylation of the TRAIL receptor DR4 confers an efficient TRAIL-induced cell death signalling. *Biochem.J.* 2009;419:185,92, 2 p following 192.
  53. Tang Z, Bauer JA, Morrison B, Lindner DJ. Nitrosylcobalamin promotes cell death via S nitrosylation of Apo2L/TRAIL receptor DR4. *Mol.Cell.Biol.* 2006;26:5588-94.
  54. Yoshida T, Shiraishi T, Horinaka M, Wakada M, Sakai T. Glycosylation modulates TRAIL-R1/death receptor 4 protein: different regulations of two pro-apoptotic receptors for TRAIL by tunicamycin. *Oncol.Rep.* 2007;18:1239-42.
  55. van de Kooij B, Verbrugge I, de Vries E, Gijzen M, Montserrat V, Maas C, et al. Ubiquitination by the membrane-associated RING-CH-8 (MARCH-8) ligase controls steady-state cell surface expression of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptor 1. *J.Biol.Chem.* 2013;288:6617-28.
  56. Sun M, Song L, Li Y, Zhou T, Jope RS. Identification of an antiapoptotic protein complex at death receptors. *Cell Death Differ.* 2008;15:1887-900.
  57. Oliver PG, LoBuglio AF, Zhou T, Forero A, Kim H, Zinn KR, et al. Effect of anti-DR5 and chemotherapy on basal-like breast cancer. *Breast Cancer Res.Treat.* 2012;133:417-26.
  58. Song JH, Tse MC, Bellail A, Phuphanich S, Khuri F, Kneteman NM, et al. Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and nonapoptotic signals in non small cell lung carcinoma cells. *Cancer Res.* 2007;67:6946-55.
  59. Azijli K, Yuvaraj S, Peppelenbosch MP, Wurdinger T, Dekker H, Joore J, et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3-dependent invasion in resistant non-small cell lung cancer cells. *J.Cell.Sci.* 2012;125:4651-61.
  60. Haselmann V, Kurz A, Bertsch U, Hubner S, Olempska-Muller M, Fritsch J, et al. Nuclear death receptor TRAIL-R2 inhibits maturation of let-7 and promotes proliferation of pancreatic and other tumor cells. *Gastroenterology* 2014;146:278-90.
  61. Dereeper A, Audic S, Claverie JM, Blanc G. BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evol.Biol.* 2010;10:8,2148-10-8.
  62. Ichikawa K, Liu W, Fleck M, Zhang H, Zhao L, Ohtsuka T, et al. TRAIL-R2 (DR5) mediates apoptosis of synovial fibroblasts in rheumatoid arthritis. *J.Immunol.* 2003;171:1061-9.
  63. Yada A, Yazawa M, Ishida S, Yoshida H, Ichikawa K, Kurakata S, et al. A novel humanized anti-human death receptor 5 antibody CS-1008 induces apoptosis in tumor cells without toxicity in hepatocytes. *Ann.Oncol.* 2008;19:1060-7.
  64. Li J, Knee DA, Wang Y, Zhang Q, Johnson JA, Cheng J, et al. LBY135, a novel anti-DR5 agonistic antibody induces tumor cell-specific cytotoxic activity in human colon tumor cell lines and xenografts. *Drug Development Research* 2008;69:69-82.

65. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J.Biol.Chem.* 1996;271:12687-90.
66. Chuntharapai A, Dodge K, Grimmer K, Schroeder K, Marsters SA, Koeppen H, et al. Isotype-dependent inhibition of tumor growth in vivo by monoclonal antibodies to death receptor 4. *J.Immunol.* 2001;166:4891-8.
67. Griffith TS, Rauch CT, Smolak PJ, Waugh JY, Boiani N, Lynch DH, et al. Functional analysis of TRAIL receptors using monoclonal antibodies. *J.Immunol.* 1999;162:2597-605.
68. Pukac L, Kanakaraj P, Humphreys R, Alderson R, Bloom M, Sung C, et al. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types in vitro and in vivo. *Br.J.Cancer* 2005;92:1430-41.
69. Kaplan-Lefko PJ, Graves JD, Zoog SJ, Pan Y, Wall J, Branstetter DG, et al. Conatumumab, a fully human agonist antibody to death receptor 5, induces apoptosis via caspase activation in multiple tumor types. *Cancer Biol.Ther.* 2010;9:618-31.
70. Adams C, Totpal K, Lawrence D, Marsters S, Pitti R, Yee S, et al. Structural and functional analysis of the interaction between the agonistic monoclonal antibody Apomab and the proapoptotic receptor DR5. *Cell Death Differ.* 2008;15:751-61.
71. Motoki K, Mori E, Matsumoto A, Thomas M, Tomura T, Humphreys R, et al. Enhanced apoptosis and tumor regression induced by a direct agonist antibody to tumor necrosis factor-related apoptosis-inducing ligand receptor 2. *Clin.Cancer Res.* 2005;11:3126-35.
72. Georgakis GV, Li Y, Humphreys R, Andreeff M, O'Brien S, Younes M, et al. Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. *Br.J.Haematol.* 2005;130:501-10.
73. Ichikawa K, Liu W, Zhao L, Wang Z, Liu D, Ohtsuka T, et al. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat.Med.* 2001;7:954-60.
74. MacFarlane M, Kohlhaas SL, Sutcliffe MJ, Dyer MJ, Cohen GM. TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. *Cancer Res.* 2005;65:11265-70.
75. Maddipatla S, Hernandez-Ilizaliturri FJ, Knight J, Czuczman MS. Augmented antitumor activity against B-cell lymphoma by a combination of monoclonal antibodies targeting TRAIL-R1 and CD20. *Clin.Cancer Res.* 2007;13:4556-64.
76. Smith MR, Jin F, Joshi I. Bortezomib sensitizes non-Hodgkin's lymphoma cells to apoptosis induced by antibodies to tumor necrosis factor related apoptosis-inducing ligand (TRAIL) receptors TRAIL-R1 and TRAIL-R2. *Clin.Cancer Res.* 2007;13:5528s-34s.
77. Chiron D, Pellat-Deceunynck C, Maillason M, Bataille R, Jego G. Phosphorothioate-modified TLR9 ligands protect cancer cells against TRAIL-induced apoptosis. *J.Immunol.* 2009;183:4371-7.
78. Menoret E, Gomez-Bougie P, Geffroy-Luseau A, Daniels S, Moreau P, Le Gouill S, et al. Mcl-1L cleavage is involved in TRAIL-R1- and TRAIL-R2-mediated apoptosis induced by HGS-ETR1 and HGS-ETR2 human mAbs in myeloma cells. *Blood* 2006;108:1346-52.
79. Marini P, Denzinger S, Schiller D, Kauder S, Welz S, Humphreys R, et al. Combined treatment of colorectal tumours with agonistic TRAIL receptor antibodies HGS-ETR1 and HGS-ETR2 and radiotherapy: enhanced effects in vitro and dose-dependent growth delay in vivo. *Oncogene* 2006;25:5145-54.
80. van Geelen CM, Pennarun B, Le PT, de Vries EG, de Jong S. Modulation of TRAIL resistance in colon carcinoma cells: different contributions of DR4 and DR5. *BMC Cancer* 2011;11:39.
81. Gong J, Yang D, Kohanim S, Humphreys R, Broemeling L, Kurzrock R. Novel in vivo

- imaging shows up-regulation of death receptors by paclitaxel and correlates with enhanced antitumor effects of receptor agonist antibodies. *Mol.Cancer.Ther.* 2006;5:2991-3000.
82. Niyazi M, Marini P, Daniel PT, Humphreys R, Jendrossek V, Belka C. Efficacy of a triple treatment with irradiation, agonistic TRAIL receptor antibodies and EGFR blockade. *Strahlenther.Onkol.* 2009;185:8-18.
83. Zhou L, Wang W, Dicker DT, Humphreys RC, El-Deiry WS. Prediction of proapoptotic anticancer therapeutic response in vivo based on cell death visualization and TRAIL death ligand-receptor interaction. *Cancer. Biol.Ther.* 2011;12:335-48.
84. Luster TA, Carrell JA, McCormick K, Sun D, Humphreys R. Mapatumumab and lexatumumab induce apoptosis in TRAIL-R1 and TRAIL-R2 antibody-resistant NSCLC cell lines when treated in combination with bortezomib. *Mol.Cancer.Ther.* 2009;8:292-302.
85. Lemke J, Noack A, Adam D, Tchikov V, Bertsch U, Roder C, et al. TRAIL signaling is mediated by DR4 in pancreatic tumor cells despite the expression of functional DR5. *J.Mol.Med.(Berl)* 2010;88:729-40.
86. Stadel D, Mohr A, Ref C, MacFarlane M, Zhou S, Humphreys R, et al. TRAIL-induced apoptosis is preferentially mediated via TRAIL receptor 1 in pancreatic carcinoma cells and profoundly enhanced by XIAP inhibitors. *Clin.Cancer Res.* 2010;16:5734-49.
87. Belyanskaya LL, Marti TM, Hopkins-Donaldson S, Kurtz S, Felley-Bosco E, Stahel RA. Human agonistic TRAIL receptor antibodies Mapatumumab and Lexatumumab induce apoptosis in malignant mesothelioma and act synergistically with cisplatin. *Mol.Cancer.* 2007;6:66.





