Ollscoil na Gaillimhe
University of Galway

Provided by the author(s) and University of Galway in accordance with publisher policies. Please cite the published version when available.

Title	Decoy Receptors block tumour cell eradication by TRAIL - Molecular engineering of TRAIL to evade decoy receptors
Author(s)	O' Leary, Lynda
Publication Date	2012-06-29
Item record	http://hdl.handle.net/10379/3150

Downloaded 2024-03-13T08:38:51Z

Some rights reserved. For more information, please see the item record link above.

Decoy receptors block tumour cell eradication by TRAIL

Molecular engineering of TRAIL to evade decoy receptors

A thesis submitted to the National University of Ireland in fulfillment of the requirement for the degree of

Doctor of Philosophy

By

Lynda O' Leary

Discipline of Biochemistry, School of Natural Sciences, National University of Ireland, Galway

Thesis supervisors: Prof. Afshin Samali
Dr Eva Szegezdi

March 2012

Table of Contents

Acknowledgements

I would like to sincerely thank my supervisors Dr. Eva Szegezdi and Prof. Afshin Samali. I am extremely grateful for all their help, support, endless knowledge and the opportunities they have provided me with.

I also would like to thank Dr. Adrienne Gorman for helpful discussions and the many collaborators involved in this project especially Carlos R. Reis, Robbert H. Cool and Wim J. Quax of the Groningen lab, my second home and Almer van der Sloot of CRG in Barcelona, your help throughout has been invaluable.

I need to thank all the members of my lab, Mairead, Paul, Tamas, Anna, Aoife and Marianne as well as the members of the ER stress lab, Donna, Karen, Shane, Neysan, Sveta, Lisa, Chris, Trish, Dani, Reka, Laura, Richard and those that have already left. Thanks for all your support, all the coffees and all the laughs.

A big thank you to my family especially my mum Dairine, for never-ending support and encouragement. Finally to my lovely Jeroen, I cannot thank you enough for your encouragement and support, for keeping me sane and keeping me smiling.

Thank you.

Abstract

Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) is a cytokine expressed by immune cells which selectively eradicates a wide range of cancer cells while leaving normal healthy cells unharmed. TRAIL binds to 4 different membrane-bound receptor molecules. However, only two of these receptors, DR4 and DR5, can launch the death machinery into action leading to cancer cell death. The two remaining receptors, decoy receptor-1 (DcR1) and DcR2 can also bind TRAIL but are unable to induce apoptosis. These decoy receptors can attenuate TRAIL-induced apoptosis by competing with DR4 and DR5 for TRAIL binding or directly binding and inhibiting the death inducing receptors. Here we show that decoy receptors either expressed by the tumour cells or on the surrounding normal cells can greatly reduce the efficacy of wild type TRAIL. While TRAIL shows promise as a potential anti-cancer agent, its promiscuous receptor binding capacity limits its utilisation as a therapeutic. To address this limitation we engineered a TRAIL mutant (TRAIL-45) using computational rational design that retained the ability to bind to DR4 and DR5 with high affinity, but not to the DcRs. We found that mutating a threonine to a leucine at position 261 (T261L) could reduce binding to the DcRs without significantly reducing binding to DR4 and DR5. Furthermore, combination of T261L with G160E where glutamic acid takes the place of glycine at position 160 resulted in enhanced favourable characteristics. The mutants were capable of activating both DR4 and DR5 and proved to be potent inducers of cell death compared to WT TRAIL. Unlike native TRAIL the efficacy of the mutants was not altered in the presence of non-transformed cells overexpressing decoy receptors indicating that they can successfully evade these receptors. In this thesis it is clearly illustrated the necessity for these decoy receptor insensitive TRAIL mutants and their potential power in the clinic.

Abbreviations

AIF	Apoptosis inducing factor
AML	Acute myeloid leukemia
Apaf-1	Apoptosis protease-activating factor 1
ASM	Acid sphingomyelinase
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2 associated protein X
Bcl-2	B-cell lymphoma 2
Bcl-x_L	B-cell lymphoma 2- extra large
BID	BH3-interacting domain death agonist
BOK	Bcl-2-related ovarian killer
CAD	Caspase activated DNase
CAF	Cancer associated fibroblast
CD95	Cluster of differentiation 95
CNS	Central nervous system
CRD	Cysteine rich domain
dATP	Deoxyadenosine triphosphate
DcR1	Decoy receptor 1
DcR2	Decoy receptor 2
DD	Death domain
DED	Death effector domain
DNA	Deoxyribonucleic acid

DR4	Death receptor 4
DR5	Death receptor 5
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EM-DR	Environmental mediated-drug resistance
EST	Expressed sequence tag
FADD	Fas-associated protein with death domain
FAP	Fibroblast-activating protein
FLIP	FLICE inhibitory protein
FSP	Fibroblast-specific protein
GPI	Glycosyl phosphatidylinositol
HGF	Human growth factor
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
IFN	Interferon
IL	Interleukin
MCF7	Breast cancer cell line with acronym Michigan Cancer Foundation - 7
MMP9	Matrix metallopeptidase 9
MOMP	Mitochondrial outer membrane permeabilization
MPC	Mesenchymal progenitor cells
MS	Multiple Sclerosis

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHEK	Normal human epidermal keratinocytes
OPG	Osteoprotegerin
PARP	Poly (ADP-ribose) polymerase
PCD	Programmed cell death
PCR	Polymerase chain reaction
PLAD	Pre-ligand assembly domain
RIP	Receptor interacting protein
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
Smac/DIABLO	Second mitochondria-derived activator of caspases (Smac)/direct IAP binding protein with low pI
STAT3	Signal transducer and activator of transcription 3
TME	Tumour microenvironment
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis inducing ligand
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

Chapter 1:Introduction

1.1 Cancer

Cancer is a term given to a group of more than 100 diseases in which once normal cells of the body have developed a number of acquired capabilities and evasion strategies. Cancer can stem from a single cell which has developed a mutation and can be caused by a multitude of factors including radiation, inherited genetic flaws, infectious agents and environmental factors and lifestyle choices. The features which separate cancer cells from normal cells are a self-sufficiency in growth factors, a limitless potential for replication, evasion of growth inhibitory factors, the ability to induce angiogenesis, ability to invade and metastasize to other parts of the body and evasion of cell death (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). These characteristics allow damaged and mutated cells to grow unrestrained when they would normally be removed by programmed cell death or apoptosis.

The evidence of the complexity of cancers continues to grow with each new development in the field and now it is not only the cancer cells themselves that have come under intense scrutiny but also the environment in which they evolve and thrive and how it contributes to its survival. This "tumour microenvironment" (TME) consists of cells, soluble factors, signalling molecules and signals from the extracellular matrix that can promote and enable tumour growth, invasion and metastasis. The normal cells comprising the TME have been found to be active contributors in the evolution and progression of cancer and a critical factor that may influence tumour sensitivity to therapy. A number of cell types have been identified and implicated including, endothelial cells, cancer associated fibroblasts, immune inflammatory cells. Endothelial cells play a prominent role due to there involvement in the formation of the tumour-associated vasculature and lymphatic vessels (tammela and Alitalo, 2010). Receptors present on the surface of endothelial cells have been found to activate a wide array of signaling pathways via recently discovered ligands crucial for tumour development and angiogenesis (Ahmed and Bicknell, 2009; Dejana et al.,2009). Fibroblasts are found in abundance in the microenvironment of many solid tumours and evidence has shown them to contribute to tumour growth, invasion and metastasis doing so through release of growth factors such as HGF and EGF in addition to various chemokines also shown to influence tumour behaviour (Kalluri and Zeisberg, 2006; Shimoda et al., 2010; Pietras and Ostman, 2010). Infiltrating cells of the immune system can also modulate tumour behaviour with both tumour-

antagonizing and tumour-promoting cells present, tumour-associated macrophages being a prime example of the latter. These macrophages are known to produce many tumour-promoting factors such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) (Lewis et al., 2000; O'Sullivan et al., 1993) as well as promoting angiogenesis, invasion and metastasis through secretion of VEGF and matrix metallopeptidase 9 (MMP9) (Lewis et al., 2006; Manotvani et al., 2006). In addition to this they have also been demonstated to down regulate anti-angiogenic factors such as interleukin-12 (IL-12) (Sica et al., 2000). Marini and colleagues demonstrated that bone-marrow derived mesenchymal progenitor cells (MPCs) were recruited into primary tumours where they then differentiated into cancer-associated fibroblasts (CAFs). These CAFs expressed fibroblast-activation protein (FAP) and fibroblast-specific protein (FSP) and were found to enhance growth of the tumour and enable immune evasion (Spaeth *et al.*, 2009). Yves DeClerck reported that MPC expression of IL-6 was enhanced when in the presence of tumour cells leading to activation of STAT3 resulting in upregulation in the expression of survivin, Mcl-1 and Bcl-x_L, increasing the resistance of these tumour cells to cytotoxic therapies (Ara and DeClerck, 2010). CAFs were also shown to facilitate tumour cell migration by remodeling the ECM causing track formation which allows cancer invasion (Gaggioli *et al.*, 2007; Wyckoff *et al.*, 2007)., This remodeling by CAFs was found to be dependent on Rho-ROCK signaling (Gaggioli *et al.*, 2007). It is becoming increasingly evident that the TME must be taken into account in the design of therapeutic strategies. Currently efforts are focused on targeting tumour cells in combination with the microenvironment by either blocking the pathways which mediate the recruitment and activation of stromal cells in the TME or by targeting pathways with allow the TME to modulate the sensitivity of a tumour to therapy.

While research in the last couple of decades has made tremendous progress at elucidating the mechanisms underpinning the initiation and progression of cancer, the National Cancer Registry of Ireland reports 50% more cancer cases per year than for the recorded period in the mid '90s. While this can also be attributed to raised awareness, improved and increased screening and detection methods, this also highlights the importance of novel therapeutics. Conventional cancer treatment includes surgery, chemotherapy and radiotherapy, the latter two of which cause severe side-effects for cancer patients and are not specifically targeted to cancer cells. Both forms of these adjuvant therapies, along with the cancer cells, also target fast dividing,

normal cells such as intestinal endothelium cells, bone marrow cells and hair follicles. With the identification of the so-called "hallmarks of cancer" there has been an increase in mechanism-based targeted therapeutics focusing on the molecular culprits responsible for these aberrant characteristics. Some such therapies include epidermal growth factor receptor (EGFR) inhibitors, vascular endothelial growth factor (VEGF) inhibitors, telomerase inhibitors and a plethora of potential therapeutics charged with the task of circumventing the cancer cell's evasion of apoptosis.

A cancer cell's ability to evade death is one characteristic which scientists have sought to reverse making them once again sensitive to apoptosis. One potential therapeutic which has been gathering a lot of attention is TNF-related apoptosis-inducing ligand (TRAIL) due to its ability to selectively induce cell death in a wide range of cancer cells while leaving normal cells of the body unharmed (Pitti *et al.*, 1996; Wiley *et al.*, 1995).

1.2 Programmed Cell Death

Programmed cell death (PCD) is a rigorously controlled process critical during the development of multicellular organisms and homeostasis of their tissues. The idea that cell death was not accidental but instead a highly orchestrated series of events leading to the self-destruction of a cell was introduced by Lockshin in 1964 (Lockshin and Williams, 1964). Since then its importance in the role of development as well as disease has been greatly explored. It is this process that is responsible for the elimination of damaged, infected or superfluous cells from the body that may otherwise prove harmful. Aberrant cell death can lead to many human diseases including cancer, autoimmune-, neurodegenerative- and immunodeficiency diseases. Understanding and deciphering the regulation of cell death pathways is crucial for the development of novel therapeutics. Several types of PCD are known and can be classified according to their morphological appearance and also enzymological criteria.

1.3 Apoptotic cell death

The term apoptosis was first used by Kerr, Wyllie and Currie in 1972 to describe a morphologically distinct form of PCD (Kerr *et al.*, 1972). Cells undergoing cell death by apoptosis display quite specific features including cell membrane blebbing, cell

shrinkage, chromatin condensation, nucleosomal fragmentation, and the ultimate breaking up of the cell and packaging into a number of membrane-bound vesicles known as apoptotic bodies (Wyllie *et al.*, 1980). Biochemically, these features are the result of the activation of a subset of proteolytic enzymes known as caspases (**c**ysteinyl **asp**artate specific prote**ases)** which cleave a multitude of protein substrates and are responsible for the majority of the events which eventually culminate in the death of the cell.

Apoptosis can be triggered by a variety of stimuli from outside or inside the cell activating the extrinsic or intrinsic pathways respectively. The extrinsic pathway can be stimulated by activation of cell surface death receptors by their associated ligands such as FasL, TNF and TRAIL while the intrinsic pathway is initiated when the cell has suffered some insult, for example by DNA damage as a result of failed DNA repair mechanisms, treatment with cytotoxic drugs or irradiation or insufficient survival signals, which triggers cytochrome *c* release from mitochondria.

Both intrinsic and extrinsic pathways cause the activation of caspases, the central components in the apoptotic machinery. It is also through these cysteine proteases that these two pathways can converge.

1.4 Necrotic –like cell death

In contrast to apoptosis, necrosis is a response to severe trauma to the cell for instance exposure to high concentrations of detergents, oxidants and extreme pathological insult. However recent observations indicate that necrosis can be programmed and is known as necrotic-like cell death. Programmed necrosis was long thought to be a tissue culture phenomenon. In a study by Chan and colleagues (Chan *et al.*, 2003) they observed that virally infected cells undergo this form of cell death and that viruses produce proteins that can modulate the necrotic program. Necrotic death of these virally infected cells and the consequent inflammation caused by this demonstrated the potential importance of programmed necrosis. However it has since been found to be induced by death receptors and the mechanisms responsible delineated. This pathway was found to be dependent on the kinase activity of the receptor-interacting protein

(RIP) and the formation of ROS (Holler *et al.*, 2000; Matsumura *et al.*, 2000; Vercammen *et al.*, 1998)

1.5 Autophagic cell death

Before the discovery of caspases and their pinnacle role in programmed cell death most cell death was believed to be lysosomal based. The term "autophagic cell death" was later used as the relationship between lysosomes, autophagic vacuoles and autophagosomes was further explored. Morphologically this form of cell death can be defined by the absence of chromatin condensation but most notably by the presence of massive autophagic vacuolization of the cytoplasm. Autophagy is a well-known normal physiological process which is responsible for the routine degradation of the cells constituents and considered a process promoting survival activated during nutrient depravation and other stresses. To date many papers have presented data suggesting that tumour cell autophagy induced by anti-cancer treatment inhibits tumour cell killing. However, autophagy is also a cell death mechanism thought to be used when apoptosis is blocked or disabled. At present there are on-going clinical trials in patients both using autophagy inhibitors or inducers in combination with other cancer treatments highlighting the importance of disentangling this process's function and the implications in relation to cancer.

1.6 Other forms of cell death

1.6.1 Anoikis

Induction of anoikis occurs when anchorage dependent cells lose attachment to the surrounding extracellular matrix (ECM), or adhere to an inappropriate type of ECM, the latter of which is more common *in vivo*. (Frisch and Francis, 1994; Meredith *et al.*, 1993) The importance of anoikis *in vivo* can readily be seen during tumour metastasis, a process which requires the cell to survive and proliferate in potentially inappropriate environments. When the normal function of anoikis is altered, tumour metastasis was found to be enhanced (Douma *et al.*, 2004).

1.6.2 Mitotic Catastrophe

Cell death in mitosis, also known as mitotic catastrophe, is caused by the cell's inability to complete mitosis. It is distinguished by the formation of large cells with multiple micronuclei and decondensed chromatin. While there are several studies which link apoptosis and mitotic catastrophe, their relationship still remains unclear. Several studies have shown that mitotic cell death involves activation of caspases, cytochrome c release, chromatin condensation and DNA degradation (Castedo *et al.*, 2004a; Castedo *et al.*, 2004b; Jordan *et al.*, 1996; Merrit *et al.*, 1997). In contrast, other studies have reported and concluded that death in mitosis is an apoptosis-independent event that may be followed independently by apoptosis (Lock and Stribinskiene, 1996; Roninson *et al.*, 2001).

1.6.3 Paraptosis

Paraptosis is a term used to describe a form of cell death with morphologically and biochemically distinct features from apoptosis. The characteristics of this type of cell death include cytoplasmic vacuolization and mitochondrial swelling and this is induced for example by insulin-like growth factor receptor 1. The signalling pathway was found to be independent of caspase activation as caspase inhibition had no effect on paraptosis induction. Similarly overexpression of the anti-apoptotic Bcl-2 proteins has no effect indicating that this pathway employs novel players to mediate cell death (Sperandio *et al.*, 2000).

1.7 TRAIL

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) also known as Apo2 ligand (Apo2L) (Pitti *et al.*, 1996; Wiley *et al.*, 1995) is a member of the TNF superfamily of ligands which possess the ability to induce apoptosis in cancer cells. Since its discovery in 1995, TNF-related apoptosis-inducing ligand (TRAIL) is continuing to ignite interest and is being described as potentially one of the most promising natural immune molecules for cancer treatment.

This family is composed of 19 members which exert their ability via 29 receptor members (reviewed in (Aggarwal *et al.*, 2012; Tansey and Szymkowski, 2009)). TNF-α was the first family member identified to have potential as an anti-cancer therapeutic. However this potent cytotoxic activity towards cancer cells was accompanied with a strong inflammatory response limiting its usage. TRAIL was originally identified based on its sequence homology to TNF-α and Fas ligand but unlike these, TRAIL selectively induces apoptosis in cancer cells while having no effect on healthy untransformed cells (Pitti *et al.*, 1996; Sadarangani *et al.*, 2007; Wiley *et al.*, 1995). TRAIL is expressed on the cell surface of interferon (IFN)-stimulated monocytes, natural killer (NK) cells, dendritic cells, fibroblasts and T-cells and also interleukin-2 (IL-2) stimulated NK cells (Almasan and Ashkenazi, 2003; Bouralexis *et al.*, 2005; Falschlehner *et al.*, 2009; Takeda *et al.*, 2001). TRAIL is a type II transmembrane protein; however, its extracellular domain can be proteolytically cleaved from the cell surface by cysteine proteases generating a soluble form of the ligand (Wajant *et al.*, 2001). Each TRAIL subunit consists of two antiparallel β -pleated sheets that form a β-sandwich and interacts with the adjacent subunits in a head-to-tail fashion forming a bell-shaped homotrimer (Cha *et al.*, 1999; Hymowitz *et al.*, 1999; Mongkolsapaya *et al.*, 1999). Each TRAIL monomer contains one cysteine at position 230. The side chains of each Cys-230 form a unique zinc-binding site buried at the core of the trimer (Hymowitz *et al.*, 2000). It has been shown that this zinc ion is crucial for structure and stability of the ligand and hence for biological activity (Bodmer *et al.*, 2000b; Hymowitz *et al.*, 2000).

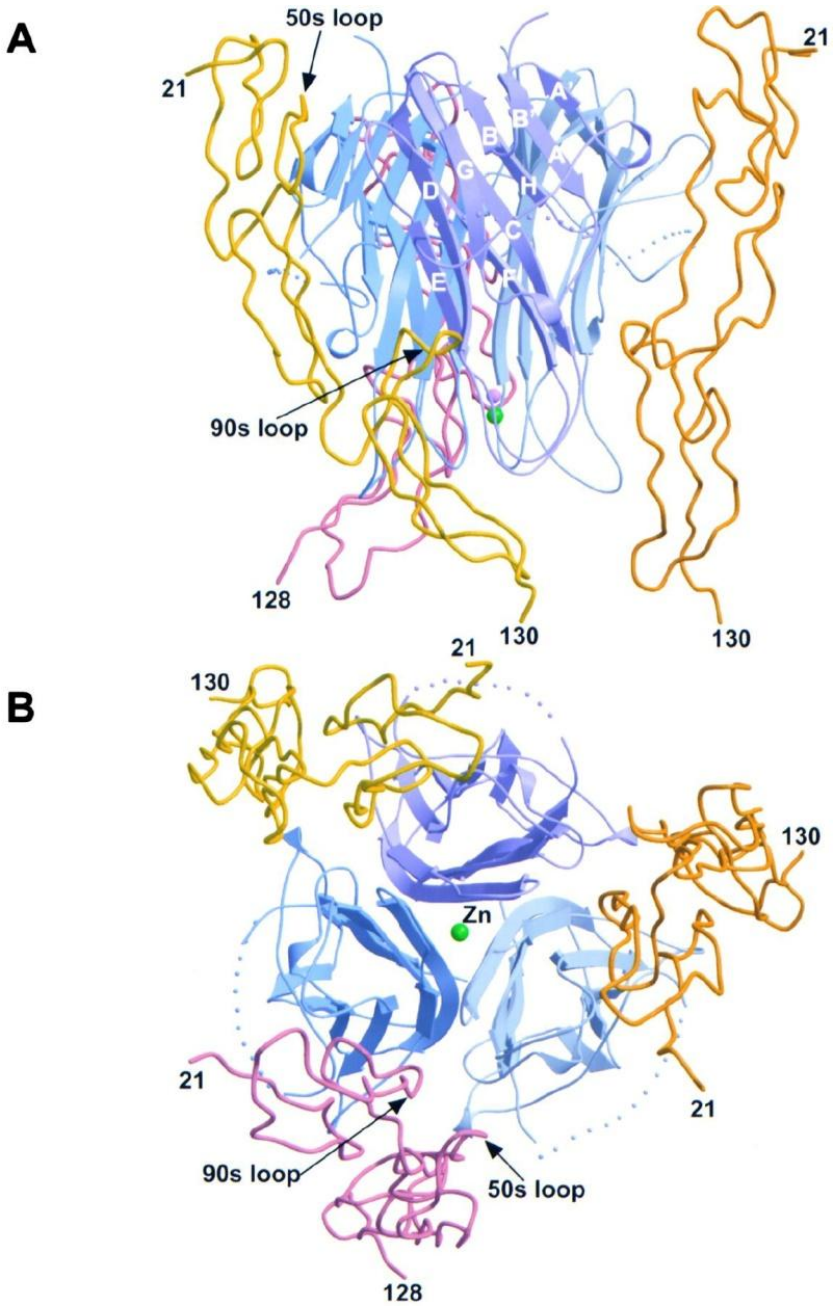


Figure 1.1. *Crystal structure of the TRAIL homotrimer (Hymowitz et al., 1999).* (A) Ribbon structure of TRAIL trimer in complex with its receptor DR5. (B) Top view. The bound zinc atom is coloured green, and the bound chloride ion is pink

Crystal structure studies, have found that like other members of the TNF family of ligands, TRAIL forms a homotrimer which binds one copy of the receptor in each of the identical clefts between the TRAIL subunits.

1.8 TRAIL Receptors

Death receptors are a subgroup in the TNF receptor superfamily. They share a homologous region consisting of around 80 amino acids in their cytoplasmic tail, this region is known as the "death domain" (DD) (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). It is this region which allows transmission of the death-inducing signal. Early studies with TRAIL established that this ligand induced apoptosis independent of Fas or TNFR1 which sparked the search to identify its receptors. Following this, two receptors which could bind TRAIL were discovered in quick succession. Firstly death receptor 4 (DR4, TRAIL-R1, TNFRSF10A) (Pan *et al.*, 1997b) was discovered by searching expressed sequence tag (EST) DNA databases for ESTs with homology to the death domain of TNFR1. DR5 (TRAIL-R2, TNFRSF10B, TRICK2, KILLER) was discovered by several groups simultaneously by different approaches, Sheridan and colleagues employed the same approach as was used to identify DR4 (Sheridan *et al.*, 1997). Screaton's group identified DR5 based on ESTs that showed homology to the cysteine-rich domains (CRDs) found in the extracellular domains (ECDs) of TNFR family members as well as ESTs showing homology to death domains (Screaton *et al.*, 1997). Numerous other groups utilised the newly available DR4 sequence in their bid to also uncover new receptors resulting in their discovery of DR5 (Chaudhary *et al.*, 1997; MacFarlane *et al.*, 1997; Pan *et al.*, 1997a; Schneider *et al.*, 1997a), while others discovered DR5 by ligand-based affinity purification (Walczak *et al.*, 1997) and during a search for p53 down-stream targets involved in apoptosis (Wu *et al.*, 1997). Subsequently, three more TRAIL receptors were identified, decoy receptor 1 (DcR1, TRAIL-R3, TNFRSF10C, TRID, LIT) (Degli-Esposti *et al.*, 1997b; Mongkolsapaya *et al.*, 1998; Pan *et al.*, 1997a; Schneider *et al.*, 1997a; Sheridan *et al.*, 1997), DcR2 (TRAIL-R4, TNFRSF10D, TRUNDD) (Degli-Esposti *et al.*, 1997a; Marsters *et al.*, 1997; Pan *et al.*, 1998) and osteoprotegerin (OPG) (Emery *et al.*, 1998; Simonet *et al.*, 1997).

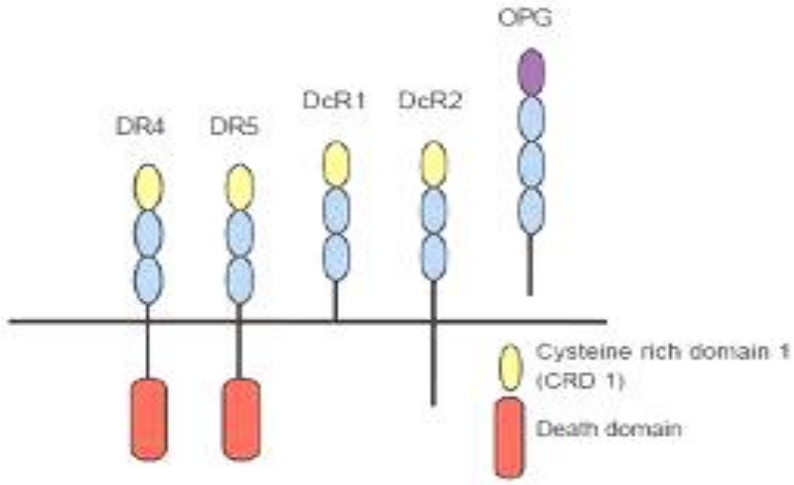


Figure 1.2. *Structure of TRAIL Receptors (Kimberley and Screaton, 2004).* The extracellular cysteine-rich domains are in yellow, for the first partial CRD1 and the other full CRDs necessary for ligand binding are in blue.

DR4 and DR5 both contain three cysteine rich domains (CRDs) in their extracellular region the first of which is a highly conserved incomplete CRD, with complete CRDs 2 and 3 being important for ligand binding (Cha *et al.*, 1999; Hymowitz *et al.*, 1999; Mongkolsapaya *et al.*, 1999). The crystal structure of TRAIL incomplex with DR5 show that two loops on DR5, the 50s loop (residues 51-65) in CRD2 and the 90s loop (residues 91-104) in CRD3 are crucial in facilitating most of the interactions with TRAIL (Cha *et al.*, 2004; Cha *et al.*, 2000; Hymowitz *et al.*, 1999). The function of this first NH_2-terminal CRD in other TNFR family members such as Fas, TNFR-1 and TNFR-2 was identified to be the pre-assembly of homotypic receptor complexes independent of ligand stimulation (Chan *et al.*, 2000; Papoff *et al.*, 1999; Siegel *et al.*, 2000). While this CRD is truncated in TRAIL receptors its function appears to be the same with this so called pre-ligand assembly domain (PLAD) interestingly mediating both homotypic and heterotypic associations between the TRAIL receptors (Chan, 2007; Clancy *et al.*, 2005; Lee *et al.*, 2005). These heterotypic preassociations are suggested to have a regulatory function in TRAIL-induced apoptosis which will be further discussed later (Clancy *et al.*, 2005).. It is thought that these pre-assembled receptors may be able to bind their ligands with higher affinity as it was found that deleting this domain resulted in diminished ligand binding (Clancy *et al.*, 2005). It has also been reported that the ligand binding causes a conformation change in the pre-

assembled receptor complex, supposedly replacing it with a more stable ligand-receptor complex mediating apoptotic signaling (Chan *et al.*, 2001).

DR4 and DR5 also contain a death domain (DD) in the cytoplasmic region which is characteristic of death-inducing receptor members of the TNF superfamily. This ~80 amino acid domain is essential in transmitting the apoptotic signal (Ashkenazi and Dixit, 1998; Nagata, 1997). The extracellular domain of these death receptors displays a high degree of homology, with 58% identity and 70% similarity (Chaudhary *et al.*, 1997), however a clear distinction in the roles of DR4 and DR5 has yet to be established. It is not known why cells will preferentially express one receptor over another, nor is it clear why if both receptors are present on the cell, TRAIL may favour to signal via one.

DcR1 is glycosyl phosphatidylinositol (GPI)-linked to the membrane and contains two complete CRDs but unlike the death-inducing receptors contains no cytoplasmic region. While it can still bind TRAIL there is no death domain to transmit the signal and therefore it cannot induce the apoptotic machinery. DcR2 has a type I transmembrane topology resembling that of DR4 and DR5 and similarly contains two CRDs, but like DcR1 it cannot induce apoptosis in this case it is due to the presence of a truncated non-functional death domain (Ashkenazi and Dixit, 1998; Ashkenazi and Dixit, 1999). Along with the four described membrane-bound receptors, TRAIL also binds OPG, a secreted member of the TNF receptor family (Emery *et al.*, 1998; LeBlanc and Ashkenazi, 2003). Like DcR1 and DcR2 it is also dubbed a decoy receptor due to its capacity to bind TRAIL, coupled with its failure to induce apoptosis. At physiological conditions OPG has the lowest affinity for TRAIL of all its receptors (Emery *et al.*, 1998; Shipman and Croucher, 2003; Vitovski *et al.*, 2007). Despite this there is growing evidence that OPG may act as a survival factor for different tumour types, this will be discussed later. OPG also has a role in inhibiting bone resorption, facilitated by its binding to the receptor activator of NF-κB (RANK) ligand (Simonet *et al.*, 1997; Vitovski *et al.*, 2007). While TRAIL expression as mentioned earlier is somewhat specialised, TRAIL receptors are expressed on the surface of most cell types (Daniels *et al.*, 2005).

1.9 Physiological Role of TRAIL

TRAIL is primarily known for its potential as a cancer therapeutic, with the volume of research in this area exploding upon discovery of this attribute, so it is not surprising that information regarding its physiological function is greatly limited in comparison and also came considerably later with first significant results published in 2002. However, there are several important studies which have begun to elucidate the importance of TRAIL in the immune system. It was expected that this is where it would exert its main function as TRAIL is expressed by various immune cells. TRAIL has been suggested to be involved in infectious disease, and the development of autoimmune diseases. TRAIL was also found to be instrumental in immune surveillance against tumour development and metastasis.
In an initial investigation using $TRAIL^{-/-}$ mice, it was found that they were both viable and fertile and no developmental defects were observed eliminating any role for TRAIL in embryonic development (Cretney *et al.*, 2002; Sedger *et al.*, 2002).

TRAIL has been found to be involved in innate immunity. TRAIL, along with FasL and perforin is responsible for the cytotoxic effect of NK cells (Falschlehner *et al.*, 2009; Kayagaki *et al.*, 1999). It has been shown that foetal and neonatal nice have mostly immature NK cells which are TRAIL positive in the liver and spleen while adult mice had only a sub-population of immature NK cells in the liver which express TRAIL. It was suggested by Takeda and colleagues that $TRAIL^{-}$ NK cells develop from $TRAIL^{+}$ NK precursor cells. Their data indicated that TRAIL expression was a hallmark of immature cytotoxic NK cells (Takeda *et al.*, 2005).

TRAIL- and TRAIL-R-deficient mice did not spontaneously develop autoimmune diseases; however, many studies found that these mice were more prone to disease development as well as showing accelerated progression of disease when autoimmunity was induced (Cretney *et al.*, 2005; Hilliard *et al.*, 2001; Lamhamedi-Cherradi *et al.*, 2003). TRAIL deficiency also increases the susceptibility of mice to autoimmune arthritis and diabetes (Lamhamedi-Cherradi *et al.*, 2003). Studies were carried out to investigate TRAILs involvement in multiple sclerosis (MS) a disease which is characterised by infiltration of immune cells into the central nervour system (CNS) causing the destruction of the myelin surrounding axons. These results contradict the previous findings that TRAIL is involved in preventing the progression

of disease as it was found that upon blocking TRAIL directly in the CNS by injecting TRAIL-R2-Fc intracisternally into mice in which experimental autoimmune encephalomyelitis (EAE) had been induced. In this case inhibiting TRAIL caused prevention of the disease (Aktas *et al.*, 2005). It is therefore proposed that TRAIL may have opposing roles in MS in that it plays a part in the damage inflicted on the CNS during neuroinflammation and the death of oligodendrocytes and neurons

A major physiological function of TRAIL is immune surveillance against tumour development and metastasis. The fact that TRAIL could play a role in tumourigenesis first came to light when it was observed that TRAIL could cause a reduction of tumour growth in tumour xenografts in SCID mice (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999). In order to deduce the physiological role of TRAIL in tumour development, Sedger and colleagues generated TRAIL gene-targeted mice. As previously stated these TRAIL-deficient mice develop normally. $TRAIL^{-/-}$ mice do not spontaneously develop tumours but when transplanted with a B cell lymphoma line they are notably more susceptible to death from overpowering tumuor burden. A considerable increase in metastasis to the liver was also observed(Sedger *et al.*, 2002). In a similar study using TRAIL-deficient mice or mice treated with a TRAIL-neutralizing antibody, it was observed that mice had increased incidence of both induced and spontaneous tumour development combined with increased metastasis (Cretney *et al.*, 2002; Grosse-Wilde *et al.*, 2008; Seki *et al.*, 2003; Takeda *et al.*, 2001; Takeda *et al.*, 2002).

However there are several studies which oppose the above results and instead metastases promotion is in fact observed. It was found that in TRAIL-resistant cells, metastasis was enhanced following treatment with TRAIL. This resistance was found to be attributed to high expression levels of FLIP, XIAP, anti-apoptotic members of the Bcl-2 family or insufficient expression of pro-apoptotic members of this same family (Hinz *et al.*, 2000; LeBlanc and Ashkenazi, 2003; shi *et al.*, 2003; Walczak *et al.*, 2000). In the instance of human pancreatic ductal adenocarcinoma that is known to express Bcl-x_L at exceptionally high levels, TRAIL was found to increase the number and volume of metastases in a xenograft model using SCID mice (Trauzold *et al.*, 2006).

Studies by Secchiero and colleagues have found TRAIL to have a role in the biology of the vascular system (Secchiero *et al.*, 2003; Secchiero *et al.*, 2004). TRAIL was found to promote survival in human umbilical vein endothelial cells (HUVEC) which

was mediated by Akt, while also increasing proliferation in an ERK dependent manner (Secchiero *et al.*, 2003). They also found TRAIL to have an influence over the survival, proliferation and migration of vascular smooth muscle cells (VSMCs) which was facilitated by activation of the ERK pathway (Secchiero *et al.*, 2004). TRAIL's ability to stimulate migration was also demonstrated in multipotent stromal cells (MSC), again ERK was found to be the mediator (Secchiero *et al.*, 2008)

1.10 TRAIL-induced apoptotic signalling pathway

Upon TRAIL binding to its cognate receptors, the apoptotic machinery is initiated and the cell death program of the cell commenced. There are two well-characterised apoptotic signalling pathways; the intrinsic pathway and the extrinsic pathway of which the latter is mediated by TRAIL.

1.11 Extrinsic pathway

Similar to FasL, TRAIL initiates the apoptotic program upon binding to its death receptors expressed on the surface of the cell. Binding of TRAIL leads to trimerization of the receptors, inducing the recruitment of specific cytoplasmic proteins to the intracellular death domain (DD) of the receptor, which form the death-inducing signalling complex (DISC). Fas-associated death domain (FADD) an adaptor protein is one of the proteins recruited where it then interacts via its DD with the DD of the receptors. FADD via its other functional domain, the death effector domain (DED) can recruit and bind the inactive form of the initiator caspase, pro-caspase-8 (Bodmer *et al.*, 1997; Bodmer *et al.*, 2000a; Kischkel *et al.*, 2000; Sprick *et al.*, 2000). In this complex, pro-caspase-8 is auto-catalytically activated, a crucial event in order for the transmission of the apoptotic signal, it is then released as active caspase-8 where it is available to cleave and activate the executioner caspases-3, -6 and -7 and thus initiate the caspase cascade, the proteolytic machinery responsible for dismantling the dying cell (Bodmer *et al.*, 2000a; Sprick *et al.*, 2000). While Caspase 10 was also found to be recruited to and activated at the DISC, it was shown not to be required for induction of apoptosis (Sprick *et al.*, 2002). Caspases are pinnacle to the death machinery and their activation leads to the cleavage of an array of cellular substrates which ultimately result in the demise and dismantling of the cell. A variety of cytoskeletal proteins have

been established as caspase substrates and include gelsolin, fodrin, nuclear lamins A and B, keratin 18 and β catenin (Caulín *et al.*, 1997; Kothakota *et al.*, 1997; Martin *et al.*, 1995; Neamati *et al.*, 1995; Takahashi *et al.*, 1996). Cleavage of these proteins may lead to the characteristic reorganisation of the cell body that occurs following caspase activation. Also cleaved during apoptosis is poly(ADP-ribose) polymerase (PARP) nuclear protein that normally functions in DNA damage detection. This cleavage effectively neutralizes the ability of PARP to aid in DNA repair, and instead contributes to a cell's commitment to undergo apoptosis (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). ICAD (inhibitor of caspase-activated DNase) is another important caspase substrate. Cleavage of this substrate results in caspase-activated DNase (CAD) activation and the subsequent degradation of DNA (Enari *et al.*, 1998; Liu *et al.*, 1997).

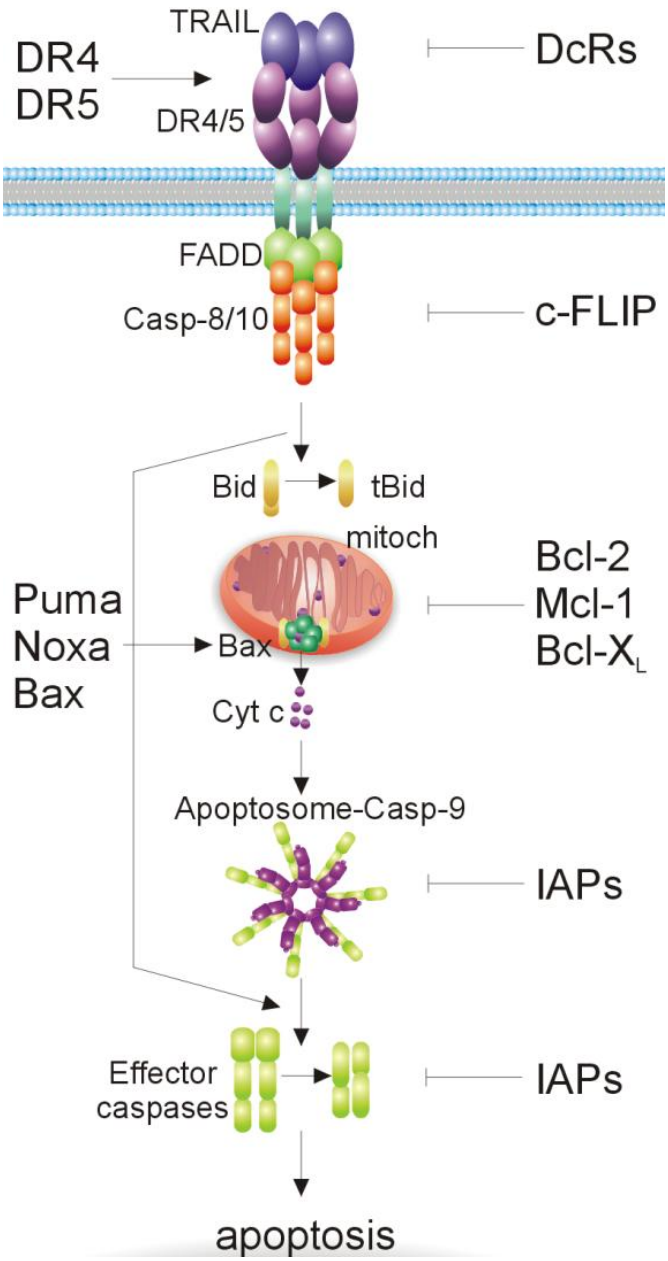


Figure 1.3. *TRAIL-induced apoptotic pathway (Mahalingam et al., 2009).* Upon TRAIL binding, the death receptors DR4 and/or DR5 trimerize allowing the recruitment of FADD and pro-caspase-8, forming the DISC. The DISC mediates pro-caspase-8 autoactivation and release and where it is then able to cleave and activate executioner caspases, pro-caspase-3, -6 and -7, culminating in cell death.

1.12 Intrinsic Pathway

As mentioned previously, the intrinsic pathway is induced by numerous stimuli such as hypoxia, reactive oxygen species (ROS), ultra-violet (UV) or gamma radiation, growth factor deprivation along with kinase inhibitors such as staurosporine and several other cytotoxic compounds (Decaudin *et al.*, 1998; Green and Kroemer, 2004). This pathway is controlled by the Bcl-2 family of proteins and when activated these multidomain pro-apoptotic proteins lead to mitochondrial outer membrane permeabilization (MOMP) (Green and Kroemer, 2004). Disruption of the outer mitochondrial membrane results in the release of pro-apoptotic proteins into the cytosol, they are then available to activate the executioner caspases and ensure cell death.

The receptor and the mitochondrial pathways had originally thought to be two independent pathways. However, it is now clear that these two pathways may converge and this is mediated by a member of the Bcl-2 protein family, the "BH3 domain only" protein Bid (Li *et al.*, 1998; Luo *et al.*, 1998) cleavage of Bid by activated caspase-8 results in truncated Bid (tBid) (Werner *et al.*, 2002; Yamada *et al.*, 1999).

Activation of Bcl-2-associated X protein (BAX) and Bcl-2 antagonist or killer (BAK) by tBid is essential for MOMP, and allows the release of crucial pro-apoptotic proteins such as cytochrome *c*, second mitochondria-derived activator of caspases (Smac)/direct IAP binding protein with low pI (DIABLO) (Smac/DIABLO), Omi/HtrA2, apoptosis inducing factor (AIF) and endonuclease G (Daugas *et al.*, 2000; Du *et al.*, 2000; Kluck *et al.*, 1997; Li *et al.*, 2001; Suzuki *et al.*, 2001; Verhagen *et al.*, 2000). These proteins are normally found between the inner and outer membrane of the mitochondria and once released they can fulfill their duties in executing cell death via activation of caspases (Saelens *et al.*, 2004) in the case of cytomchrome *c*, Smac/DIABLO and Omi/HtrA2 or by induction of DNA degradation as is the case with AIF and endonuclease G.

Release of cytochrome c induces the formation of a multi-protein complex, similar to the DISC in function, which is the platform for caspase activation in the intrinsic apoptotic pathway. This complex is composed of cytochrome *c*, apoptosis protease-activating factor 1 (Apaf-1), dATP and pro-caspase-9 and is known as the apoptosome (Cain *et al.*, 2000; Jiang and Wang, 2000; Kim *et al.*, 2005a; Li *et al.*, 1997; Saleh *et*

al., 1999; Yu *et al.*, 2005; Zou *et al.*, 1999). In the apoptosome, pro-caspase-9, the apical caspase of the caspase cascade in the intrinsic apoptotic pathway becomes activated and serves to activate the executioner caspases, pro-caspases -3, -6 and -7 which are instrumental in completing apoptosis (Li *et al.*, 1997). Activation of the intrinsic apoptotic pathway downstream of death receptor activation is believed to amplify the apoptotic signal ensuring that the cell commits to death (Hinz *et al.*, 2000; Sun *et al.*, 2001).

Based on the requirement of this intrinsic pathway amplification loop for TRAIL-induced apoptosis, type I and type II cells are distinguished. In so-called type I cells, binding of TRAIL can trigger the formation of large number of DISC complexes and in turn can activate sufficiently large amounts of caspase-8 to activate the caspase cascade and commit the cell to apoptosis. In type II cells however, only low levels of caspase-8 get activated upon exposure to TRAIL, possibly due to inefficient DISC formation, which is not sufficient to directly activate the caspase cascade. In such cells, caspase-8-mediated cleavage of Bid and consequent cytochrome *c* release can amplify the pro-death signal and commit the cell to apoptosis (Li *et al.*, 1998; Luo *et al.*, 1998; Scaffidi *et al.*, 1998). It has been shown that this tendency towards one pathway or another can be dependent on cell surface receptor expression and that by up-regulating one of the death receptors the phenotype of the cell can be switched from type II to type I (LeBlanc *et al.*, 2002; Meng *et al.*, 2011; Wang and El-Deiry, 2003).

1.13 Mechanisms of TRAIL resistance

Despite the potential and early promising results, studies have shown that around half of cancers are resistant to TRAIL, including highly malignant tumours such as pancreatic cancer, neuroblastoma and malignant melanoma. Resistance to TRAIL can be mediated by numerous factors at various levels in the apoptosis signalling pathway. While many different mechanisms of resistance have been delineated, there is no one general mode of resistance that all tumour cells share.

1.13.1 Resistance at the receptor level

Binding of TRAIL to its cognate receptors DR4 and DR5 initiates the TRAIL-induced signalling pathway; it is responsible for formation of the DISC, the platform for activation of caspases. Therefore these receptors can be the first point of resistance. Death receptors must be expressed on the cell surface in sufficient amounts and must be functional in order to sucessfully commence signalling. Studies have shown that a lack of expression of DR4 in ovarian cancer cells correlated with resistance to TRAIL-induced apoptosis, this lack of expression was caused by epigenetic silencing (Horak *et al.*, 2005). Similarly, regardless of expression of the receptor it is useless unless it is expressed on the cell surface. Resistance of colon cancer cells can be attributed to deficient transport of the DR4 to the cell surface (Jin *et al.*, 2004). In several cancers such as head and neck squamous cell carcinoma, breast cancer cells and non-Hodgkin's lymphoma, mutations in either DR4 or DR5 have been observed which result in a loss-of-function and consequent resistance to TRAIL-mediated cell death (Bin *et al.*, 2007; Fisher *et al.*, 2001; Lee *et al.*, 1999; Lee *et al.*, 2001; Pai *et al.*, 1998; Shin *et al.*, 2001). In addition to surface expression and mutation it has been shown than post-translational modification of the death receptors can play a role in TRAIL-resistance. In a study by Wagner and colleagues a link between death receptor O-glycosylation and TRAIL signalling was clearly demonstrated. They showed that O-glycosylation can promote ligand-stimulated clustering of DR4 and DR5, which is crucial for recruitment of pro-caspase-8 and formation of the DISC. mRNA expression of GALNT14, a peptidyl O-glycosyltransferase, was found to correlate with TRAIL sensitivity, where high expression of GALNT14 was indicative of responsiveness to TRAIL. This was found to be true for non-small cell lung carcinoma, melanoma cell lines and pancreatic carcinoma and has been proposed as a potential biomarker for TRAIL responsiveness (Wagner *et al.*, 2007).

Lipid rafts are plasma membrane microdomains enriched in cholesterol and glycosphingolipids. They have been suggested to be important in the clustering or aggregating of certain surface receptors and this is essential for death receptor-mediated cell death (Cremesti *et al.*, 2002; Delmas *et al.*, 2004; Hueber *et al.*, 2002; Lim *et al.*, 2011; Muppidi and Siegel, 2004). Sphingomyelin is found in the outer leaflet of the plasma membrane where it is hydrolyzed by acid sphingomyelinase

(ASM), thus releasing ceramide and forming ceramide membrane platforms. These microdomains or ceramide membrane platforms allow clustering of the death receptors already present at the surface of the cell. This type of receptor clustering is known to occur in the case of other receptor members of TNF family receptors like TNF and CD95. Aggregation of death receptors at the cell surface is also thought to enhance the sensitivity of tumor cells to ligand-induced apoptosis. DcR1 is the only TRAIL receptor to be found readily in lipid rafts while the remaining TRAIL receptors, DR4, DR5 and DcR2 are present in non-lipid raft-containing membranes (Merino *et al.*, 2006). TRAIL-mediated DISC formation occurring in lipid rafts has only been observed in a few instances (Min *et al.*, 2009; Song *et al.*, 2007).. Several studies have found that certain drugs can induce redistribution of the death receptors to the lipid rafts and thus sensitize to TRAIL-induced apoptosis. Evidence supporting this was provided by Psahoulia and colleagues; they showed that quercetin sensitizes colon adenocarcinoma cells to TRAIL-induced apoptosis mediated by redistribution of death receptors in lipid rafts without any change in the expression or levels of the death receptors (Psahoulia *et al.*, 2007). Similarly the redistribution of death receptors to lipid rafts and the subsequent enhancement of TRAIL-induced apoptosis has been shown following the administration of various therapeutics (Delmas *et al.*, 2004; Gajate and Mollinedo, 2005; Maldonado-Celis *et al.*, 2009; Xu *et al.*, 2009). In addition, it was shown that S-palmitoylation can target death receptors (Chakrabandhu *et al.*, 2008; Rossin *et al.*, 2009) to the lipid rafts and has been shown to be necessary for efficient oligomerization (Rossin *et al.*, 2009).

1.13.2 The role of the decoy receptors in TRAIL-mediated apoptosis

Promiscuity of this ligand exceeds that of any other TNF family member and it is this characteristic that may hinder its progress as a cancer therapeutic despite its great potential. With TRAIL able to bind to the non-apoptosis-inducing decoy receptors it can reduce the amount of ligand available to bind to the death-inducing receptors, thereby having reduced efficacy. This logic was the basis for the widely accepted idea that decoy receptors were the means by which normal cells could protect themselves from the cytotoxic activity of TRAIL. By mopping up the TRAIL there would be insufficient levels to bind to the death receptors and induce cell death, thus protecting the cell. It was published that these receptors were only present on normal cells at high

levels and therefore must be the reason these cells were protected (Degli-Esposti *et al.*, 1997a; Pan *et al.*, 1997a; Sheridan *et al.*, 1997). While since it has emerged that this is not the case and inhibition of these receptors will not sensitize non-transformed cells of the body (Kim *et al.*, 2000). While decoy receptors are present on normal cells, evading them will not cause the normal cells to succumb to TRAIL-mediated apoptosis. As discussed previously many anti-apoptotic molecules have since been identified as being able to inhibit TRAIL-mediated apoptosis at various points in the program, however it has not yet been elucidated what exactly protects normal cells from TRAIL.

While this theory of how normal cells are protected proved to be false, it has been found that many cancer cell lines protect themselves in this manner. Numerous studies show that expression of decoy receptors on cancer cells can be responsible for the reduction of the efficacy of TRAIL, or even the cell's resistance to TRAIL in certain cancer cell lines (Bernard *et al.*, 2001; Chamuleau *et al.*, 2011; Koksal *et al.*, 2008; Riccioni *et al.*, 2005; Sanlioglu *et al.*, 2005; Sanlioglu *et al.*, 2007). This resistance can occur by two methods, the first involved the mopping up of TRAIL by the decoy receptors so that it does not reach its target of the death receptors and the second is by forming a heteromeric complex between the decoy receptors and the death-inducing receptors and thereby inhibiting functional formation of the DISC.

DcRs are expressed on the surface of many cancer cells and when overexpressed they can reduce or completely block cell death induced by TRAIL. BTK-143 osteogenic sarcoma cells shows sensitivity to TRAIL-induced apoptosis in early passage however, increasing passage was accompanied with increasing resistance and this was found to be a result of acquired DcR2 expression. The sensitivity could be restored upon chemotherapy probably due to upregulation of the death receptors (Bouralexis *et al.*, 2003). Resistance of acute myeloid leukemic (AML) cells to TRAIL has also been largely attributed to decoy receptor expression (Riccioni *et al.*, 2005). Sanlioglu and colleagues found that DcR2 expression is responsible for resistance to TRAIL in both breast cancer cell line MCF7, lung cancer and prostate carcinoma cells where it was also correlated with poor prognosis (Aydin *et al.*, 2007; Koksal *et al.*, 2008; Sanlioglu *et al.*, 2005; Sanlioglu *et al.*, 2007). DcR2 does not dominate this resistance to TRAIL-induced apoptosis however and high DcR1 expression levels have also been implicated in several studies linking TRAIL resistance and poor outcome (Bernard *et al.*, 2001;

Chamuleau *et al.*, 2011). In a study by Merino and colleagues they found that DcR1, which localizes in sphingolipid- and cholesterol-enriched membranes, structures also known as lipid rafts or raft microdomains, inhibits TRAIL-induced cell by sequestering TRAIL in these lipid rafts (Merino *et al.*, 2006). This same group has also explored decoy mediated resistance by means of forming heterocomplexes with the death receptors which result in a dysfunctional DISC and failure to transmit the death-inducing signal. Overexpressing the decoy receptors was as efficient at inhibiting apoptosis as the pan caspase inhibitor zVAD-fmk, in the case of DcR1 as previously mentioned this was as a result of sequestration of TRAIL, however, in the case of DcR2 this was a result of incorporation of DcR2 into the DR5-DISC. DcR2 allowed DR5-mediated DISC formation but prevented initiator caspase activation within the DISC (Merino *et al.*, 2006). They also show that chemotherapy can circumvent these inhibitory events (Morizot *et al.*, 2011). In this instance they report the DcR2 interaction with DR5 at the DISC is mediated by TRAIL (Merino *et al.*, 2006). This is in conflict with previous reports that DcR2 and DR5 interaction at the DISC occurs through the pre-ligand-assembly domain (PLAD) which is a ligand independent event (Clancy *et al.*, 2005). Since then, there have also been more reports regarding decoy-mediated inhibition of TRAIL-induced apoptosis in a ligand-independent manner. It is suggested that expression of DcR2 can trigger the AKT pathway independent of TRAIL leading to cell survival and proliferation and this was found to be the case in cervical carcinoma HeLa cells (Lalaoui *et al.*, 2011). It is also important to note that DcR2 can be upregulated by radiation-induced genotoxic stress as was the case in colon carcinoma cells (Sreekumar *et al.*, 2001). Ionizing radiation has been previously shown to up-regulate DR5 (Chinnaiyan *et al.*, 2000) making TRAIL an attractive choice to combine with this form of therapy. However, it is crucial to be aware of the parallel upregulation of this inhibitor of apoptosis which may cancel out any benefit of increased expression of DR5.

Decoy receptors are also expressed by a range of normal cell types in the human body, including the peripheral blood lymphocytes, spleen, kidneys, liver, pancreas etc. (Daniels *et al.*, 2005; LeBlanc and Ashkenazi, 2003; Marsters *et al.*, 1997; Pan *et al.*, 1997a; Pan *et al.*, 1998; Sheridan *et al.*, 1997). While there is evidence that their expression on cancer cells can reduce and even inhibit TRAIL-mediated cell death, there is a significant lack of knowledge regarding what effect decoy expression on normal cells will have on the TRAIL sensitivity of a tumour.

There is a growing body of evidence highlighting the cross-talk between tumor cells and the surrounding stroma (Perez *et al.*, 2008) and its participation in the processes of tumor cell survival, growth, adhesion, invasion, metastasis, and therapy resistance. Decoy receptors expressed on the cell surface of normal cells, while they do not protect normal cells from TRAIL it is possible that their presence may result in environmental mediated-drug resistance (EM-DR).

Bone marrow stroma has been found to confer resistance to TRAIL in multiple myeloma. They found this resistance could be attributed to soluble factors released by the stroma which increased FLIP expression levels in the supported tumour cells (Perez *et al.*, 2008).

Another study by Anees and colleagues looked for a correlation between TRAIL and TRAIL receptor expression and recurrence-free survival in prostate cancer. When investigating the protein expression of TRAIL receptors using a tissue microarray they found that 26.6% of transformed cells expressed the death receptors (combined) compared to 4.5% of non-malignant stromal cells. They discovered that DcR1 was expressed in 64.3% of tumour cells and in 35.7% of benign tissue. The trend was similar for DcR2 where 70.3% of the cancer cells expressed this receptor and 29.6% of normal cells. Such high levels of decoy receptors being expressed on both transformed and non-transformed cells warrants concern as to how this may effect TRAIL efficacy however it was never investigated whether this additionally mediated resistance to TRAIL (Anees *et al.*, 2011).

1.13.3 Resistance at the DISC level

In many cases resistance to TRAIL-induced apoptosis has been found to be a result of high levels of cellular FLICE inhibitory protein (c-FLIP) (Tschopp *et al.*, 1998). c-FLIP has a short and a long isoform, as a result of two different splice variants. The short form, c-$FLIP_S$, consists only of two DEDs and the long form, c-$FLIP_L$ (also called I-FLICE, for inhibitor of FLICE) consists of two DEDs and a caspase-like domain, and closely resembles caspase-8, with the important difference that it contains an inactive enzymatic site. Following stimulation with TRAIL, both forms of c-FLIP may be recruited to the DISC (Scaffidi *et al.*, 1999). These two isoforms differ in that if c-$FLIP_S$ is incorporated into the DISC it can competitively inhibit pro-caspase-8

recruitment, while c-FLIP$_L$ allows pro-caspase-8 to be recruited to the DISC and it can even be partially cleaved from the 55 and 57 kDa proforms to 41 kDa and 43 kDa polypeptides. However, c-FLIP$_L$ prevents any further proteolytic processing of caspase-8 to generate the active 18 kDa (p18) and 10 kDa (p10) subunits, the mechanism of which is unclear (Krueger *et al.*, 2001). All forms of c-FLIP are generally accepted as having anti-apoptotic functions however, c-FLIP$_L$ when transiently overexpressed was found to have both anti- and pro-apoptotic effects (Goltsev *et al.*, 1997; Han *et al.*, 1997; Inohara *et al.*, 1997; Shu *et al.*, 1997). c-FLIP$_L$ has also been found to potentially promote caspase-8 activity through heterodimerization of c-FLIP$_L$ and procaspase-8 within the DISC. This is thought to mimic procaspase-8 dimerization, leading to its activation in the absence of procaspase-8 cleavage (Micheau *et al.*, 2002). While active caspase-8 remains sequestered within the DISC as it is not fully processed and therefore not released, it is still capable of cleaving a number of substrates including c-FLIP, RIP and also procaspase-3 (Hughes *et al.*, 2009; Micheau *et al.*, 2002; Yu *et al.*, 2009). Cleavage of c-FLIP$_L$ by procaspase-8 was found to further enhance proteolytic activity of the unprocessed caspase-8 (Yu *et al.*, 2009). These findings indicate a dual role for c-FLIP in the regulation of TRAIL-signalling

Caspase-8/c-FLIP ratio is thought to determine TRAIL sensitivity, evidence of this can be seen in cancers where high levels of c-FLIP are expressed, there include, lung and breast cancer, colon carcinoma, malignant melanoma, hepatocellular carcinoma, B-cell chronic lymphocytic leukemia and glioblastoma (Dolcet *et al.*, 2005; Geserick *et al.*, 2008; Guseva *et al.*, 2008; Panner *et al.*, 2005; Wang *et al.*, 2008). Additionally it has been demonstrated that low caspase-8 expression is a determinant of TRAIL-resistance in small cell lung carcinoma, leukemia, some neuroblastoma types and the colon cancer cell line, DLD1. Similar to the death receptors, caspase-8 can also be silenced epigenetically by methylation or by mutation, both resulting in low caspase-8 protein expression (Hopkins-Donaldson *et al.*, 2003; Zhang *et al.*, 2005).

1.13.4 Resistance at the mitochondrial level

1.13.4.1 Bcl-2 family

While it is not always necessary, TRAIL-mediated apoptosis may require the participation of the mitochondrial, intrinsic apoptotic pathway in order to amplify the signal (type II cells) to ensure that the ultimate goal of cell death is achieved. With the involvement of this pathway also comes a wide array of inhibitory proteins along with it.

Members of the B-cell lymphoma-2 (Bcl-2) family of proteins are major regulators of apoptosis and these regulatory effects are mainly centered on the mitochondria. Every member of the Bcl-2 family of proteins contains at least one of the four conserved _α-helical motifs known as Bcl-2 homology (BH1–4) domains (Oltvai *et al.*, 1993). This family is composed of anti- and pro-apoptotic members, and ultimately it is their balance within the cell that determines cell fate.

Antiapoptotic Bcl-2 proteins are characterized by four BH domains (BH1–4) and include Bcl-2-related gene A1 (A1), Bcl-2, Bcl-2-related gene, long isoform (Bcl-x_L), Bcl-w, and myeloid cell leukemia 1 (Mcl-1) while the proapoptotic Bcl-2 proteins are further divided into two subgroups – the multidomain proteins containing BH domains 1-3 (Bak, Bax and Bok) and the BH3-only proteins (Bcl-2 antagonist of cell death (Bad), Bcl-2-interacting domain death agonist (Bid) and Bcl-2-interacting mediator of cell death (Bim) Bik, Bmf, Hrk, Puma and Noxa) which as the name suggested contain only the BH3 domain.

The antiapoptotic members can inhibit the function of the proapoptotic members through direct interactions (Chaudhary *et al.*, 2001; Hacki *et al.*, 2000; Morishima *et al.*, 2004; Murakami *et al.*, 2007; Wei *et al.*, 1998; Zhang *et al.*, 2001). Bcl-x_L and Mcl-1, were shown to target Bak,while Bcl-2 could not and all of the antiapoptotic members can interact with Bax (reviewed by (Chipuk *et al.*, 2010)).

Anti-apoptotic proteins Bax and Bak are central to regulating this pathway as guardians of the mitochondria (Wei *et al.*, 2001). Following an apoptotic stimulus, Bax translocates to the mitochondrial outer membrane (MOM) where both Bax and Bak have been proposed to homo-oligomerize into pores, allowing the release of cytochrome *c*, into the cytosol, a step which is critical to apoptosome formation and

execution of cell death (Antonsson *et al.*, 2000; Antonsson *et al.*, 2001; Nechushtan *et al.*, 2001; Wei *et al.*, 2001).

Based on the binding between different Bcl-2 members, two models of Bax and Bak activation exist, the *direct* and the *indirect* model (O' Neill *et al.*, 2006). In the *direct* model, BH3-only proteins such as Bid, Bim, and Puma can bind directly to Bax and Bak. In the *indirect* model, Bax and Bak are activated following their displacement from prosurvival proteins which is mediated by BH3-only proteins (Chipuk and Green, 2008; Dewson and Kluck, 2009; Leber *et al.*, 2010).

Bax and Bak are crucial for inducing the intrinsic apoptotic pathway with deletion of both proteins renders the cells completely resistance to cell death. Of note, in either $Bax^{-/-}$ or $Bak^{-/-}$ mouse embryonic fibroblasts TRAIL was capable of inducing cytochrome *c* release and apoptosis but a double Bax/Bak knockout was completely resistant (Kandasamy *et al.*, 2003).

Bcl-x_L and Bcl-2, anti-apoptotic proteins, have been found to protect against TRAIL-mediated apoptosis in some tumour cell types when overexpressed. Bcl-x_L expression was correlated with TRAIL sensitivity in some pancreatic adenocarcinoma cell lines (Hinz *et al.*, 2000), while Bcl-2 was found to confer resistance to TRAIL in neuroblastoma, glioblastoma and breast cancer cell lines (Fulda *et al.*, 2002).

1.13.4.2 IAPs

Members of this family of inhibitors of apoptosis (IAP) are defined by the presence of a variable number of Baculoviral IAP repeat (BIR) motifs. These BIR domains bind directly to and inhibit the proteolytic activity of caspases, the critical executioners of apoptosis (Eckelman *et al.*, 2006). In addition to the characteristic BIR domains, some IAPs also contain a RING domain (**R**eally **I**nteresting **N**ew **G**ene) (Weissman, 2001). RING domains can confer ubiquitin protein ligase (E3) activity and, together with ubiquitin activating enzyme (E1) and a ubiquitin conjugating enzyme (E2) can catalyze the transfer of ubiquitin to proteins and target them for proteosomal degradation (Lorick *et al.*, 1999). The most extensively characterised of these IAPs are X-linked IAP (XIAP), c-IAP1 and c-IAP2. While all IAPs are thought to have the potential to bind to caspases only XIAP has found to be a direct inhibitor, with caspase-9, -3 and -7

the targets (Eckelman *et al.*, 2006; Srinivasula *et al.*, 2001). XIAP binds to these caspases via their IAP-binding motif (IBM) resulting in the blocking of their substrate binding site (Eckelman *et al.*, 2006). c-IAP1 and c-IAP2 are more commonly known to be involved in receptor-mediated signaling events involving their association with TNF receptor associated factor (TRAF) 1 and 2, further upstream of the mitochondria (Rothe *et al.*, 1995). An abundance of these proteins can confer protection against cell death while conversely downregulation or inhibition of these IAPs can restore the balance in favour of cell death (Gill *et al.*, 2009; Lee *et al.*, 2006a; Mori *et al.*, 2007; Shrader *et al.*, 2007; Symanowski *et al.*, 2009) and this can be mediated by other BIR-interacting proteins which also contain an IAP binding motif such as Smac/DIABLO and Omi/Htr2 (Yang *et al.*, 2003). TRAIL stimulation can induce Smac/DIABLOs release from the mitochondria so it can then promote polyubiquitination of both c-IAP1 an c-IAP2 leading to their degradation (Vaux and Silke, 2005; Yang and Du, 2004). Some reports have found that Smac/DIABLO can also cause the degradation of XIAP but this remains somewhat controversial (Vaux and Silke, 2005). More commonly XIAP's inhibitory effects are neutralised by the binding of Smac/DIABLO and Omi/Htr2 via their IAP-binding motif, allowing the release of the captured caspase (Du *et al.*, 2000; Srinivasula *et al.*, 2001; Suzuki *et al.*, 2001) It is also possible that cancer cells may acquire resistance to apoptosis by downregulating molecules that block the inhibitory action of XIAP. This is the case in 70% of patients with childhood acute lymphoblastic leukemia (ALL) where expression of the mitochondrial protein ARTS (apoptosis-related protein in the TGF-β signalling pathway) is lost (Gottfried *et al.*, 2004). It was also found that by reintroducing ARTS into resistant leukemic cell lines which had lost ARTS sensitized these cells to chemotherapeutic drugs (Elhasid *et al.*, 2004).

1.13.5 Influence of the tumour microenvironment

Many therapeutics which display promising cytotoxicity *in vitro* fail to exert their same potential *in vivo*. While there are various factors which could reduce delivery and/or efficacy of cancer therapeutics *in vivo*, one which has become recognised as a critical factor influencing sensitivity of tumour cells to therapies is the tumour microenvironment. TRAIL is one such therapy which shows varying results in *in vivo*

studies, where resistance is proving an obstacle. However this resistance may not only stem from the tumour cells themselves but may also be mediated by the tumour microenvironment. This possibility is neatly demonstated by Kim and colleagues whereby mesothelioma cell lines grown *in vitro* as a monolayer were sensitive to TRAIL in combination with cyclohexamide. They then tested the sensitivity of patient mesothelioma fragments grown as spheroids *in vitro*. These fragment spheroids allow the heterogeneity of the original tumour to be preserved as they contained not only tumour cells but also non-transformed cells commonly found in the tumour and tumour extracellular matrix. Upon treatment with TRAIL plus cyclohexamide the spheroids proved resistant indicating that sensitivity to therapies can be influenced by the tumour microenvironment (Kim *et al.*, 2005b). It has previously been demonstrated that the TME can protect a variety of carcinoma cells from TRAIL-mediated apoptosis through the production of various soluble factors and chemokines such as IL-8, CD40 and matrix metalloproteinases (Abdollahi *et al.*, 2003; Abdollahi *et al.*, 2005; Ahonen *et al.*, 2003; Travert *et al.*, 2008). In the case of follicular lymphoma B cells, CD40 signaling was found to have a protective effect against TRAIL-induced apoptosis with the activation of NFκB leading to the up-regulation of the anti-apoptotic proteins c-FLIP and Bcl-$x_{L.}$ Inhibiton of NFκB was capable of reversing the protective effect (Travert *et al.*, 2008).

Bone Marrow stromal cells have been implicated numberous times for their role in aiding cancer cell survival and confering TRAIL resistance. Perez and colleagues reported that bone marrow stroma is responsible for the TRAIL resistance seen in myeloma cell lines. Their release of soluble factors lead to the up-regulation of the anti-apoptotic protein c-FLIP and sensitivity could be restored with cyclohexamide treatment (Perez *et al.*, 2008). OPG is known to be expressed by bone marrow stromal cells and osteoblasts and reports have shown it to have a role in the protection of myeloma, breast and prostate cancer cells (Neville-Webbe *et al.*, 2004; Nyambo *et al.*, 2004; Shipman and Croucher, 2003) against TRAIL-induced apoptosis. While myeloma cells down regulate OPG, it was shown that the concentration is still sufficient to confer protection (Shipman and Croucher, 2003). Agonisitc antibodies for TRAIL's death receptors were found to overcome this particular OPG-mediated resistance (Locklin *et al.*, 2007). Contrary to these findings, another group reported that *in vivo* TRAIL can retain its cytotoxic effect even in the presence of OPG at supraphysiological concentrations. While they also showed that OPG over-expression

in vitro could reduce TRAIL-induced apoptosis, upon evaluating TRAIL's efficacy in the bone microenvironment in a xenogenic tumour model they show TRAIL treatment resulted in high levels of growth inhibition. However in this study the OPG was over-expressed by the cancer cells and not by stromal cells and they reported that treatment with TRAIL led to a decrease in levels of OPG due to death of the cancer cells; the outcome may have varied had the OPG been expressed constantly by nearby stromal cells (Zinonos *et al.*, 2011).

The differences in the responsiveness of a cancer cell to therapeutics *in vitro* versus *in vivo* can be polar opposites. It is crucial to identify the factors involved in aiding and protecting cancer cells so that a combined targeted approache may be investigated.

1.14 TRAIL-induced pro-survival signalling

While TRAIL is more famous for its role in cancer cell death, it has been described that TRAIL has somewhat of a Jekyll and Hyde complex in that it can also induce pro-survival pathway leading to cell proliferation. It achieves this through activation of NF-κB, Akt and MAPK pathways.

1.14.1 NF-κB

NF-κB is a transcription factor which holds a central role in innate and adaptive immunity, development, apoptosis and cell proliferation. It has five component subunits cRel, cRelA/p65, cRelB, NF-κB1/p50 and NF-κB2/p52 which form homo- or heterodimeric complexes, they control transcription by binding NF-κB consensus sequences in the promoter regions of target genes (Bernard *et al.*, 2001; Harper *et al.*, 2001; Karin *et al.*, 2002). TRAIL and NF-κB have a somewhat controversial relationship with some groups reporting NF-κB protects cells from TRAIL mediated cell death (Ehrhardt *et al.*, 2003; Kim *et al.*, 2002; Plantivaux *et al.*, 2009) while other report conflicting results, where NF-κB has a pro-apoptotic role (Jennewein *et al.*, 2012; Karl *et al.*, 2009; Radhakrishnan and Kamalakaran, 2006; Shetty *et al.*, 2002). These inconsistencies are said to be due to the varying function and amounts of the subunits (Chen *et al.*, 2003). Evidence of this is clear in the overexpression of the RelA

subunit, which results in the inhibition of caspase-8, DR4 and DR5 expression while enhancing c-IAP1 and c-IAP2 following TRAIL treatment. Overexpression of cRel has the opposite effect, enhancing DR4 and DR5 while inhibiting c-IAP1 and c-IAP2 (Chen *et al.*, 2003).

NF-κB activation has been shown to be stimulated by TRAIL upon its binding to DR4, DR5 and DcR2 (Degli-Esposti *et al.*, 1997a; Harper *et al.*, 2001; Hu *et al.*, 1999; Kim *et al.*, 2011; MacFarlane, 2003). It is believed that activation of NF-κB by TRAIL occurs in a similar manner to its activation by TNF. In the case of TNF the receptor-interacting protein (RIP) is recruited to the ligand-receptor complex via its death domain. RIP has been found in the TRAIL DISC (Harper *et al.*, 2001) where it mediates TRAIL-induced NF-κB inducing kinase (NIK) activation in turn leading to the phosphorylation and activation of IκB kinase (IKKα/β) (Chaudhary *et al.*, 2000; Ehrhardt *et al.*, 2003). This results in the phosphorylation and degradation of IκB allowing NFκB to translocate to the nucleus and induce transcription of target genes. However there is some debate regarding the recruitment of TRADD and RIP to the DISC, the mechanism by which this occurs and if it occurs under normal conditions. TRADD and RIP have been found to interact with TRAIL when FADD was overexpressed (Chaudhary *et al.*, 1997; Lin *et al.*, 2000b; Schneider *et al.*, 1997b). Studies undertaken to unravel the components of the DISC have found differing results, some reported that neither TRADD nor RIP were present (Kischkel *et al.*, 2000; Sprick *et al.*, 2000) others have shown RIP to be part of this complex (Lin *et al.*, 2000b) as well as being found associated with FADD upon TRAIL stimulation in secondary complexes which also contain TRADD and caspase-10 (Jin and El-Deiry, 2006; Varfolomeev *et al.*, 2005). Like TRAIL in this case, RIP also plays a dual role; following stimulation by TRAIL, RIP can be cleaved by caspase-8 and this cleavage resulted in cell death (Lin *et al.*, 1999). In contrast a mutant form of RIP which is non-cleavable activates NF-κB and renders the cells resistant to apoptosis (Lin *et al.*, 2000b)..

1.14.2 AKT

PKB/Akt protein kinase is another important signalling protein found to be involved in TRAIL-induced apoptosis. This PI3-kinase activated protein kinase is known to play a major role in regulation of cellular functions including nutrient metabolism, cell growth, apoptosis and survival. There have been many reports in recent years

elucidating the important function of PKB/Akt signalling pathways in cell survival in several cancers. This has been attributed to its ability to increase the expression of anti-apoptotic proteins such as c-FLIP, XIAP and Bcl-2 (Panner *et al.*, 2005; Shrader *et al.*, 2007; Wang *et al.*, 2008) or inactivate pro-apoptotic members such as Bad. This Bcl-2 family member was one of the first Akt phosphorylation targets to be identified (Datta *et al.*, 1997). Bad has the ability to bind to and block the activity of anti-apoptotic members Bcl-2 and Bcl-x_L. Phosphorylation of Bad by Akt prohibits this interaction and keeps Bad localised in the cytosol (Sakamaki *et al.*, 2011). Similarly Akt is also responsible for the phosphorylation of the proapoptotic protein Bax preventing its translocation to the mitochondria (Gardai *et al.*, 2004). In addition to this it can also inhibit pro-caspase-9 activation (Cardone *et al.*, 1998) and also prevent the cleavage of the nuclear factor Acinus by caspase-3, an important factor responsible for chromatin condensation (Hu *et al.*, 2005). Many studies using inhibitors that directly or indirectly target the Akt pathway, such as arsenic trioxide (Szegezdi *et al.*, 2006), complestatin (Kim *et al.*, 2004), EGFR inhibitor (Shrader *et al.*, 2007), sulphoraphane (Kim *et al.*, 2008), and amiloride (Kim and Lee, 2005) have shown to enhance TRAIL-induced apoptosis.

1.14.3 MAPK

The MAP kinases (MAPKs) are a family of proteins involved in different cellular processes, including inflammation, cell proliferation and differentiation as well as apoptosis. There are three main subfamilies, p38-MAPKs, ERKs and JNKs. It was originally thought that ERK was important for cell survival (Zhuang and Schnellmann, 2006) while p38-MAPKS and JNK were believed to be involved in apoptosis. However, it has since been shown that all three kinases are activated upon TRAIL stimulation, in addition to this there are numerous conflicting and controversial reports that describe each of these kinases to be suppressors or enhancers of TRAIL-mediated apoptosis (Jin *et al.*, 2007; Lee *et al.*, 2006b; Mucha *et al.*, 2009; Secchiero *et al.*, 2003; Shenoy *et al.*, 2009; Soderstrom *et al.*, 2002; Tran *et al.*, 2001; Zou *et al.*, 2004). In agreement with the idea that ERKs have an antiapoptotic effect, it was found that TRAIL induced the prompt activation of ERK1/2 in a subset of melanoma cells, inhibition of this event sensitized the cells to TRAIL-induced apoptosis (Zhang *et al.*,

2003) this data was also confirmed by another group who independently demonstrated the same result (Lee *et al.*, 2006b).

There is also controversy surrounding p38 MAPK and JNK and their role in TRAIL-mediated cell death. It has been suggested that TRAIL incorporated into a secondary signalling complex distinct from the DISC (Varfolomeev *et al.*, 2005).

This theory is supported by other findings in which FADD, TRAF2, RIP, caspase-8 and NEMO were found to be recruited to this signalling complex (Jin and El-Deiry, 2006). While p38 activation is dependent on RIP and also TRAF2, JNKs stimulation is independent which would suggest distinct signalling pathways. However, as with much of this research these findings are in dispute, with a study using RIP- and TRAF2-deficient fibroblasts showing them to be involved in JNK activation (Lin *et al.*, 2000a)

1.15 The potential of TRAIL as a cancer therapeutic

A cancer therapeutic which has the ability to effectively target cancer cells but leave non-transformed cells unharmed is the goal of cancer therapy. TNF-related apoptosis inducing ligand (TRAIL) appears to fulfil this criterion. Pre-clinical studies demonstrated the potential of soluble recombinant TRAIL as well as agonistic antibodies targeting either DR4 or DR5 where they selectively induced apoptosis in a variety of cell lines. This led to the progression of TRAIL-based targeted therapies to clinical trials either as single agents or in conjunction with a range of conventional cancer therapeutics whose effects have been shown to be enhanced upon combination (Jin *et al.*, 2008; Luster *et al.*, 2009; Marini *et al.*, 2009).

Recombinant human (rh) TRAIL (Dulanermin) is a soluble protein based on the native ligand that activates the full repertoire of TRAIL receptors just as the wild type would. In several mouse xenograft models of human cancer rhTRAIL has demonstrated significant antitumor activity and very little toxicity (Ashkenazi *et al.*, 1999; Kelley and Ashkenazi, 2004; Lawrence *et al.*, 2001). Preclinical studies have found that rhTRAIL can cause significant growth reduction and cell death in a wide variety of malignant tumours (Ashkenazi *et al.*, 1999; Kelley and Ashkenazi, 2004; Walczak *et al.*, 1999). A number of phase 1 clinical studies with rhTRAIL as a monotherapy have been carried out in patients with advanced solid tumours to assess its safety and anti-tumoural effects. Findings showed rhTRAIL to be safe and well tolerated at the doses

tested and no hepatotoxicity was observed (Herbst *et al.*, 2010a; Herbst *et al.*, 2006; Ling *et al.*, 2006; Pan *et al.*, 2007). Results showed achievement of stable disease, with two patients with chondrosarcoma showing a partial response.

However promising, rhTRAIL can still bind to the ligand-sequestering decoy receptors, which can limit its therapeutic potential. Many groups decided to address this problem and to date there are a number of agonistic antibodies and rhTRAIL mutants which have been generated to be specific for either death receptor DR4 or DR5. Some agonistic antibodies currently in clinical trials include mapatumumab, an anti-DR4 antibody from Human Genome Sciences (HGS) and several anti-DR5 antibodies, lexatumumab (HGS), conatumumab (Amgen), LBY135 (Novartis), tigatuzumab (Daiichi-Sankyo) and drozitumab (Genentech).

Phase 1 studies involving the anti-DR4 antibody mapatumumab resulted in stable disease in 19 out of the 49 patients with advanced solid tumours in the first study (Tolcher *et al.*, 2007) and 12 out of 41 in the second (Hotte *et al.*, 2008).
Phase 2 trials with mapatumumab in patients with relapsed NHL, colorectal cancer and NSCLC have been completed. Results showed a more pronounced effect of the therapy in patients with NHL with 2 complete responses and 1 partial response observed and a further 11 patients achieving stable disease from a total cohort of 40 patients (Younes *et al.*, 2010). In patients with colorectal cancer and NSCLC the best outcome was stable disease in 12 out of 38 and 9 out of 32 patients respectively (Greco *et al.*, 2008; Trarbach *et al.*, 2010). The agonistic DR5 antibody lexatumumab has also been involved in two phase 1 trials in patients with advanced solid tumours. The first study saw 9 patients out of 31 achieve stable disease and one patient experienced possible dose-related toxicity at a dose of 10 mg/kg (Wakelee *et al.*, 2010). In the second study, 12 out of 35 patients sustained stable disease while 3 patients from this study also exhibited dose-limiting toxicity at the highest dose tested which was 20 mg/kg (Plummer *et al.*, 2007). DR5 agonistic antibodies drozitumab, tigatuzumab, conatumumab and LBY135 have also been the subjects of phase 1 studies. Doses of drozitumab up to 20 mg/kg were investigated and no maximum dose reached. From 41 patients, 20 achieved stable disease (Camidge *et al.*, 2010). In the case of tigatuzumab, for 7 of the 17 patients in the study the outcome was stable disease with 1 patient experiencing prolonged stabilisation for more than 2 years (Forero-Torres *et al.*, 2010).

A phase 1 study involving conatumumab observed one partial response in a patient with NSCLC in a study of 37 patients treated with doses up to 20 mg/kg every 2 weeks, 15 patients achieved stable disease one of which had a 24% reduction in tumour size (Herbst *et al.*, 2010b). Another phase 1 study with conatumumab reported 9 out of 18 patients with advanced solid tumours attained stable disease (Doi *et al.*, 2011). LBY135 was tested in 32 patients with advanced solid tumours, one sarcoma patient experienced a minor response while two other patients (NSCLC and prostate cancer) showed decreases in tumour markers of 50% and 40% respectively (Sharma *et al.*, 2008)

Protein- based therapeutics have significant advantages making them valuable in the clinic. They are usually highly specific, have low cross-reactivity, fewer off-target effects and better patient tolerance (Leader *et al.*, 2008). Computational design is at the forefront in the design of these engineered protein therapeutics. Its use has led to many successes in improving antibody affinity (Lippow *et al.*, 2007), increasing the stability of potential therapeutic proteins (Zakrzewska *et al.*, 2005), reducing immunogenicity (Vivona *et al.*, 2008) and in targeting protein-protein interactions (Kortemme *et al.*, 2004; Steed *et al.*, 2003; Szegezdi *et al.*, 2011; van der Sloot *et al.*, 2006). In the case of TRAIL, the specificity of interaction is altered for therapeutic effect. Using the computational design algorithm FoldX, we have previously described the generation of rhTRAIL mutants which can successfully discriminate between the two death receptors and induce cell death selectively in cancer cells which signal either through DR4 or DR5. Among the first TRAIL mutants generated was the DR5 specific mutant D269H/E195R which displayed increased biological activity compared to WT rhTRAIL in a range of cancer cell lines which mediate apoptosis through the DR5 receptor (van der Sloot *et al.*, 2006). The efficacy of this mutant was evaluated in combination with cisplatin in a bioluminescent human A2780 intraperitoneal ovarian cancer xenograft model. Cisplatin was found to strongly upregulate DR5 and both WT rhTRAIL and the DR5-specific mutant induced higher levels of apoptosis compared to cisplatin alone, with the mutant displaying superior cytotoxity over WT rhTRAIL (Duiker *et al.*, 2009). The same approach was also used to generate DR4-specific mutants such as 4C7 and 4C9 (Reis *et al.*, 2010). Both of these mutants induced apoptosis in a variety of cell lines including human colon adenocarcinoma, Burkitt's lymphoma as well as the WT rhTRAIL resistant cell lines, PANC-1 and MCF-7, a

pancreatic carcinoma and human breast adenocarcinoma respectively. Another mutant specifically inducing apoptosis via DR4 is rhTRAIL-C3. AML cell lines were particularly sensitive to this mutant, which had showed higher apoptosis unduction compared to WT rhTRAIL. However, while affinity to DR4 was increases so too was the mutant's affinity towards DcR1; DR5 and DcR2 binding remained unchanged from the WT (Szegezdi *et al.*, 2011). Several other TRAIL mutants were successfully engineered to signal via DR4, while specific, proved to be less biologically active on DR4-responsive cell lines than WT rhTRAIL (Tur *et al.*, 2008). Several other groups have also generated receptor specific TRAIL mutants with varying success (Gasparian *et al.*, 2009; Kelley *et al.*, 2005; MacFarlane *et al.*, 2005b). These receptor-specific variants can be a valuable therapeutic where it is determined which TRAIL receptor is expressed and active such is the case for mantle cell lymphoma and chronic lymphocytic leukemia which signal primarily through DR4 (MacFarlane *et al.*, 2005a; MacFarlane *et al.*, 2005b). However, in most cancer types it is not known through which receptor TRAIL would induce apoptosis.

While some of these receptor-targeted therapies show some promise in clinical trials such as mapatumumab and lexatumumab, to date there is no TRAIL mutant or agonistic antibody which is capable of binding to both death-inducing TRAIL receptors while evading the decoy receptors.

1.16 Combination of TRAIL with other cancer therapeutics.

TRAIL shows potential in pre-clinical studies when combined with targeted therapeutics, such as histone deacetylase inhibitors, or proteasome inhibitors, as well as classical chemotherapeutics in selected tumour cell types. The mechanism of sensitisation to TRAIL-induced apoptosis has been revealed for many of these studies and been found to involve reducing the expression of c-FLIP, XIAP or changing the ratio of Bcl-2 protein expression in favour of the pro-apoptotic members. Combining TRAIL with these other types of therapies allows the dual activation of both the intrinsic and extrinsic apoptotic pathway which has been shown to amplify their affects. Clinical trials have been carried out to evaluate the safety and efficacy of both recombinant TRAIL (dulanermin) and DR agonistic antibodies in combination with various other therapeutics. In a phase 1b study investigating the combination of

dulanermin and rituximab in a group of seven low-grade NHL patients, two complete responses and one partial response was observed. (Yee *et al.*, 2007). Following this a randomized phase 2 trial was carried out to assess the safety and efficacy of dulanermin plus rituximab compared to rituximab alone in patients with relapsed follicular NHL. This study found that the addition of dulanermin did not improve the objective response rate in these patients (Belada *et al.*, 2010). Another phase 1b trial saw 24 patients with advanced NSCLC undergo treatment with dulanermin in combination with paclitaxel, carboplatin and bevacizumab. This study yielded one complete response and 13 partial responses (Soria *et al.*, 2010). A randomized phase 2 study further examining this combination showed it to be well tolerated however the addition of dulanermin to paclitaxel and carboplatin or to paclitaxel, carboplatin and bevacizumab did not improve response rate or result in a better progression free survival (Soria *et al.*, 2011). While safe, the efficacy of these combinations remains to be seen. TRAIL receptor agonists in combination with other chemotherapies have also entered clinical trials with varying results. Mapatumumab has been combined with gemcitabine and cisplatin and also paclitaxel and carboplatin in phase 1 studies. In both trials, the best outcome was partial response and was observed in 12 out of 49 patients and 5 out of 27 patients respectively (Leong *et al.*, 2009; Mom *et al.*, 2009). A phase 2 study was carried out combining mapatumumab with carboplatin and paclitaxel as a first-line therapy in patients with advanced NSCLC. In this case mapatumumab was not found to have any beneficial effect and did not improve response rate or progression-free survival (Von Pawel *et al.*, 2010). However, very few, if any of these combinations can circumvent the problem of the decoy receptors. In view of the findings here, as well as the recent study of the Micheau group that shows that DcR2 together with c-FLIP is the major determinant of TRAIL resistance (Morizot *et al.*, 2011), a TRAIL mutant that is not recognised by the decoy receptors, but still retains full DR4/DR5-agonistic activity holds the greatest potential of all the TRAIL-based therapeutics in succeeding in the clinic.

1.17 Aims and objectives

TNF-related apoptosis-inducing ligand (TRAIL) is a promising potential cancer therapeutic due to its ability to selectively induce apoptosis in a wide range of cancer cell lines while leaving non-transformed cells unharmed. However, TRAIL is a promiscuous ligand and binds to five receptors, only two of which can initiate the cell death pathway culminating in cell death. The other three ligands, dubbed 'decoy receptors' are expressed on most non-transformed cells of the body as well as many cancer cells, with their primary function to inhibit TRAIL-mediated apoptosis. This promiscuity limits TRAILs potential in the treatment of cancer. By evasion of the decoy receptors we can circumvent their antagonistic effects, and efficiently and potently induce tumour cell death.

This thesis aims to demonstrate the inhibitory capabilities of the decoy receptors, be it present on cancer cells or on non-transformed cells in the surrounding stroma. Using molecular engineering and rational design techniques, TRAIL mutants which can evade the decoy receptors will attempt to overcome this problem and highlight the need for such mutants in the clinic.

Chapter 2: Materials and Methods

2.1 Computational Design of TRAIL variants

2.1.1 Modelling of TRAIL-Receptor Complexes.

At present only the crystal structure of TRAIL in complex with the DR5 receptor is known. The template selected for this study was 1D4V, the structure at 2.2 Å resolution and of monomeric human TRAIL in complex with the ectodomain of DR5 (TRAILR2) receptor. The homotrimer was generated using the protein quaternary structure server from the EBI (http://pqs.ebi.ac.uk), having the symmetry coordinates in the PDB file. From the sequence alignment of the different TRAIL receptors it is observed that the receptor cysteine-rich domains (CRDs) involved in the interaction with TRAIL (CRD2 and CRD3) are highly conserved, with the exception of the soluble receptor OPG. Indeed, when compared to DR5, the sequence identity of any other membrane-attached TRAIL receptor is higher than 50% in each case, and there are neither insertions nor deletions in the sequence (with the exception of a glycine deletion in the middle of the CRD3 in DcR1). In addition, all of the cysteines involved in the formation of internal disulfide bridges are conserved and share the same sequence position. Thus, it was possible to build homology models of all TRAIL receptors except for OPG. The homology model of TRAIL-DR4 was built using the protein design capabilities of FoldX. The DR5 amino acid residues were mutated into the corresponding DR4 amino acids, and subsequently, all amino acid side chain interactions were optimized in order to accommodate TRAIL and receptor residues to their new interface.

2.1.2 Computational Design of the Mutants.

A detailed description of the empirical force field FoldX (version 3.0) is available elsewhere (at http://foldx.crg.es). Briefly, this force field calculates the free energy of unfolding (ΔG) of a target protein or protein complex combining the physical description of the interactions with empirical data obtained from experiments on proteins. Force field components (polar and hydrophobic solvation energies, van der Waals interactions, van der Waals clashes, H-bond energies, electrostatics in the complex and its effects on the *k*on and backbone and side chain entropies) are calculated evaluating the properties of the structure, such as its atomic contact map, the accessibility of its atoms and residues, the backbone dihedral angles, the H-bond

network, and the electrostatic network of the protein. Water molecules making two or more H-bonds with the protein are also taken into account.

FoldX is able to perform amino acid mutations and simultaneously accommodate the new residues and its surrounding amino acids. FoldX first mutates the selected position to alanine and annotates the side chain energies of the neighbour residues. Then it mutates this alanine to the selected amino acid and recalculates the side chain energies of the same neighbouring residues. Those that exhibit an energy difference are then mutated to themselves to see if another rotamer will be more favourable. This procedure was also used to reconstruct the binding interface of TRAIL in complex with the modeled DR4 receptor: In order to repair residues with bad torsion angles, residues having bad van der Waals clashes, or to build up the putative interactions between TRAIL and the modelled receptor, the most optimal amino acid conformation was chosen using rotamer substitution. The crystal structure of TRAIL in complex with the DR5 receptor was also refined in this way.

The modeling and computational design of the TRAIL mutants was carried out by our collaborators in the Centre for Genomic Regulation (CRG) in Barcelona, Spain.

2.2 Generation of Decoy insensitive TRAIL variants

2.2.1 Cloning and PCR

Sequence of TRAIL 114-281:

```
      catggtgagag  aaagaggtcc  tcagagagta  gcagctcaca  taactgggac  cagaggaaga  agcaacacat
114     M  V  R     E  R  G     P  Q  R  V     A  A  H     I  T  G     T  R  G  R     S  N  T
      tgtcttctcc  aaactccaag  aatgaaaagg  ctctgggccg  caaaataaac  tcctgggaat  catcaaggag
137   L  S  S     P  N  S  K     N  E  K     A  L  G     R  K  I  N     S  W  E     S  S  R
      tgggcattca  ttcctgagca  acttgcactt  gaggaatggt  gaactggtca  tccatgaaaa  agggttttac
160   S  G  H  S     F  L  A     N  L  H     L  R  N  G     E  L  V     I  H  E     K  G  F  Y
      tacatctatt  cccaaacata  ctttcgattt  caggaggaaa  taaaagaaaa  cacaaagaac  gacaaacaaa
183     Y  I  Y     S  Q  T     Y  F  R  F     Q  E  E     I  K  E     N  T  K  N     D  K  Q
      tggtccaata  tatttacaaa  tacacaagtt  atcctgaccc  tatattgttg  atgaaaagtg  ccagaaatag
206   M  V  Q     Y  I  Y  K     Y  T  S     Y  P  D     P  I  L  L     M  K  S     A  R  N
      ttgttggtct  aaagatgcag  aatatggact  ctattccatc  tatcaagggg  gaatatttga  gcttaaggaa
229   S  C  W  S     K  D  A     E  Y  G     L  Y  S  I     Y  Q  G     G  I  F     E  L  K  E
      aatgacagaa  tttttgtttc  tgtaacaaat  gagcacttga  tagacatgga  ccatgaagcc  agtttttttg
252     N  D  R     I  F  V     S  V  T  N     E  H  L     I  D  M     D  H  E  A     S  F  F
      gggccttttt  agttggctaa
275   G  A  F     L  V  G  -
```

Before designing the mutagenic primers it was necessary to consult the codon usage table for E. coli (Maloy *et al.*, 1999)

Table 2.1 Codon usage table for *E.coli*. The % represents the average frequency this codon appears per 100 codons. The ratio represents the abundance of the codon relative to all the codons for that individual amino acid.

CODON USAGE IN *E. COLI* GENES[1]

	Codon	Amino acid[2]	%[3]	Ratio[4]	Codon	Amino acid	%	Ratio	Codon	Amino acid	%	Ratio	Codon	Amino acid	%	Ratio	
U	UUU	Phe (F)	1.9	0.51	UCU	Ser (S)	1.1	0.19	UAU	Tyr (Y)	1.6	0.53	UGU	Cys (C)	0.4	0.43	U
	UUC	Phe (F)	1.8	0.49	UCC	Ser (S)	1.0	0.17	UAC	Tyr (Y)	1.4	0.47	UGC	Cys (C)	0.6	0.57	C
	UUA	Leu (L)	1.0	0.11	UCA	Ser (S)	0.7	0.12	UAA	STOP	0.2	0.62	UGA	STOP	0.1	0.30	A
	UUG	Leu (L)	1.1	0.11	UCG	Ser (S)	0.8	0.13	UAG	STOP	0.03	0.09	UGG	Trp (W)	1.4	1.00	G
C	CUU	Leu (L)	1.0	0.10	CCU	Pro (P)	0.7	0.16	CAU	His (H)	1.2	0.52	CGU	Arg (R)	2.4	0.42	U
	CUC	Leu (L)	0.9	0.10	CCC	Pro (P)	0.4	0.10	CAC	His (H)	1.1	0.48	CGC	Arg (R)	2.2	0.37	C
	CUA	Leu (L)	0.3	0.03	CCA	Pro (P)	0.8	0.20	CAA	Gln (Q)	1.3	0.31	CGA	Arg (R)	0.3	0.05	A
	CUG	Leu (L)	5.2	0.55	CCG	Pro (P)	2.4	0.55	CAG	Gln (Q)	2.9	0.69	CGG	Arg (R)	0.5	0.08	G
A	AUU	Ile (I)	2.7	0.47	ACU	Thr (T)	1.2	0.21	AAU	Asn (N)	1.6	0.39	AGU	Ser (S)	0.7	0.13	U
	AUC	Ile (I)	2.7	0.46	ACC	Thr (T)	2.4	0.43	AAC	Asn (N)	2.6	0.61	AGC	Ser (S)	1.5	0.27	C
	AUA	Ile (I)	0.4	0.07	ACA	Thr (T)	0.1	0.30	AAA	Lys (K)	3.8	0.76	AGA	Arg (R)	0.2	0.04	A
	AUG	Met (M)	2.6	1.00	ACG	Thr (T)	1.3	0.23	AAG	Lys (K)	1.2	0.24	AGG	Arg (R)	0.2	0.03	G
G	GUU	Val (V)	2.0	0.29	GCU	Ala (A)	1.8	0.19	GAU	Asp (D)	3.3	0.59	GGU	Gly (G)	2.8	0.38	U
	GUC	Val (V)	1.4	0.20	GCC	Ala (A)	2.3	0.25	GAC	Asp (D)	2.3	0.41	GGC	Gly (G)	3.0	0.40	C
	GUA	Val (V)	1.2	0.17	GCA	Ala (A)	2.1	0.22	GAA	Glu (E)	4.4	0.70	GGA	Gly (G)	0.7	0.09	A
	GUG	Val (V)	2.4	0.34	GCG	Ala (A)	3.2	0.34	GAG	Glu (E)	1.9	0.30	GGG	Gly (G)	0.9	0.13	G
		U				C				A				G			

TRAIL (114-281) Primers:

NCOI_For	AAAAAAAAAAAAACCATGGTGAGAGAAAGAGG
BAMHI_Rev	AAAAAAAAAAAAAGGATCCCTATTAGCCAAC

Mutagenic Primers:

E271F_For	CATGGACCATTTTGCCAGTTTTTTTG
E271F_Rev	CAAAAAAACTGGCAAAATGGTCCATG
H270D_For	GACATGGACGATGAAGCCAGT
H270D_Rev	ACTGGCTTCATCGTCCATGTC
H270E_For	GACATGGACGAAGAAGCCAGT
H270E_Rev	ACTGGCTTCTTCGTCCATGTC
D267Y_For	AGCACTTGATATATATGGACCATG
D267F_Rev	AGCACTTGATATTTATGGACCATG
T261H_For	GTTTCTGTACATAATGAGCACTTGAT

T261H_Rev	ATCAAGTGCTCATTATGTACAGAAAC
T261L_For	GTTTCTGTACTGAATGAGCACTTG
T261L_Rev	CAAGTGCTCATTCAGTACAGAAAC
I220M_For	CCTGACCCTATGTTGTTGATG
T214D_For	TACAAATACGATAGTTATCCTGACCCT
T214D_Rev	AGGGTCAGGATAACTATCGTATTTGTA
T214I_For	TACAAATACATTAGTTATCCTGACCCT
T214I_Rev	AGGGTCAGGATAACTAATGTATTTGTA
T200H_For	AAAGAAAACCATAAGAACGACAAAC
N199M_For	GAAATAAAAGAAATGACAAAGAACGAC
K197D_For	AGGAGGAAATAGATGAAAACACAAAG
K197E_For	AGGAGGAAATAGAAGAAAACACAAAG
Q193M_Rev	CTTTTATTTCCTCCATAAATCGAAAGTA
Q193N_Rev	CTTTTATTTCCTCATTAAATCGAAAGTA
H161F_Rev	GCTCAGGAATGAAAACCCACTCCT
H161M_Rev	GCTCAGGAATGACATCCCACTCCT
H161I_Rev	GCTCAGGAATGAAATCCCACTCCT
H161D_Rev	GCTCAGGAATGAATCCCCACTCCT
H161E_Rev	GCTCAGGAATGATTCCCCACTCCT
G160D_Rev	GAATGAATGATCACTCCTTGATGA
G160E_Rev	GAATGAATGTTCACTCCTTGATGA
S159D_Rev	TGAATGCCCATCCCTTGATGATTC
S159E_Rev	TGAATGCCCTTCCCTTGATGATTC
L147K_Rev	ATTTTGCGGCCTTTAGCCTTTTCAT
L147P_Rev	ATTTTGCGGCCAGGAGCCTTTTCAT

cDNA corresponding to human soluble TRAIL (aa 114-281) was cloned into pET15b (Novagen, Cat. No. 69661-3) using the restriction sites NcoI and BamHI. The identified mutations were introduced by polymerase chain reaction (PCR) using a modified megaprimer method. This method involved the use of three oligonucleotide primers and two rounds of PCR. One nucleotide is mutagenic; the remaining two are forward and reverse primers that lie upstream and downstream of the binding site for the mutagenic oligonucleotide. The mutagenic primer and the nearer of the external primers were used in the first PCR to generate and amplify the mutated fragment of DNA. The resulting amplified fragment, known as the megaprimer, was used in the second PCR in conjunction with the remaining external primer to amplify the target region of the template DNA. The resulting product is the 515bp fragment of TRAIL containing the desired mutation. The polymerase used was phusion polymerase from Finnzymes. The presence of the desired mutations was confirmed by DNA sequencing (Macrogen).

2.2.2 Expression and purification of TRAIL variants

The TRAIL variants constructed were transformed into *Escherichia Coli* BL21 (DE3). Starter cultures (50 ml) of rhTRAIL WT and mutants were grown in Luria-Bertani (LB) medium (per liter: 10 g Trypton, 5 g Yeast extract, 5 g NaCl, pH ~7) + 100 µg/ml Ampicillin overnight at 37ºC and 250 rpm. 10 ml was pelleted and resuspended in 1 ml of fresh LB. 1 litre 2XLB (per liter: 16 g Trypton, 10 g Yeast extract, 5 g NaCl), 100 µg/ml Amp was inoculated with 1 ml starter culture, incubated at 37ºC 250 rpm until OD_{600} reached 0.5. Expression was induced with 1 mM IPTG and 100 µM $ZnSO_4$ was added to promote trimerisation. Cultures were grown for 5 hours at 28ºC at 250 rpm or alternatively 20ºC O/N. Cells were harvested by centrifugation and the isolated pellet was resuspended in 3 volumes of extraction buffer (buffer A) (20 mM NaPi, 200 mM NaCl, 10% glycerol, 7 mM β-mercapto-ethanol, pH 8), cells were disrupted by sonication (3x 90 s, 50% duty cycle, 50-60 % output) and extracts were clarified by centrifugation for 60mins at 40000g at 4ºC. For purification the AKTA explorer automated purification system was utilized. The supernatant was loaded on a nickel-charged Hisprep FF 16/10 column supplied by GE healthcare. Unbound sample was washed out with buffer A followed by 3 column volumes (CV) of 90% buffer A, 10% buffer B (20 mM NaPi, 200 mM NaCl, 10 % (v/v) glycerol, 100 mM imidazole, 7 mM

β-mercapto-ethanol, pH 8). The TRAIL containing fraction was eluted with 100% buffer B. A desalting step was carried out and the buffer exchanged for buffer C (20 mM NaPi, 100 mM NaCl, 10 % (v/v) glycerol, 3.5 mM DTT, 20 μM $ZnSO_4$, pH 7.6). The TRAIL variants were further purified by cation-exchange chromatography on a HI-Trap SP HP column (GE Healthcare). The desalted fraction was loaded onto the cation exchange column and the column washed with buffer C. It was then washed with 3 CV of 97% buffer C and 2.5% buffer D (20 mM NaPi, 1 M NaCl, 10 % (v/v) glycerol, 3.5 mM DTT, 20 μM $ZnSO_4$, pH 7.6). TRAIL containing fraction was then eluted with 70% buffer C, 30% buffer D. Finally analytical gel filtration was employed using a Hiload Superdex 75 16/60 (GE Healthcare) confirming the presence of the trimeric TRAIL proteins. TRAIL was eluted in buffer E (20 mM NaPi, 150 mM NaCl, 10 % (v/v) glycerol, 3.5 mM DTT, 20 μM $ZnSO_4$, pH 7.4). Purity was confirmed using SDS-PAGE gel stained with coomassie brilliant blue. Purified protein was aliquoted, flash frozen in liquid nitrogen and stored at -80ºC

The cloning, expression and purification of TRAIL mutants was carried out by me at the University of Groningen, The Netherlands with the assistance of our collaborators there.

2.3 Cell Culture, treatments and cell death detection

Colon carcinoma Colo205 cells, acute myeloid leukaemia cells ML-1 and acute T-cell leukaemia Jurkat cells were maintained in RPMI1640 medium, supplemented with 10% FBS, 2 mM glutamine, 1 mM pyruvate, 50 U/ml penicillin and 50 ug/ml streptomycin. ML-1 cells were a kind gift from Dr. Heinz-Peter Nasheuer, Colo205 cells and Jurkat cells were obtained from ATCC. Human umbilical vein endothelial cells (HUVEC) and normal human epidermal keratinocytes (NHEK) were procured from PromoCell, Germany, and cultured in the recommended ready-to-use medium from the suppliers. Human dermal fibroblasts (hFb) were a kind gift Dr. Linda Howard of REMEDI, National University of Ireland, Galway. They were maintained in low glucose DMEM, supplemented with 10% FBA and 50 U/ml penicillin and 50 ug/ml streptomycin. All cells were maintained in a humidified incubator at 37°C and 5% CO_2 environment

2.3.1 Externalization of phosphatidylserine (Annexin V assay)

Cells were seeded 24 hours prior to treatment in 24 well plates (0.5 ml/well) at appropriate density. Wild type TRAIL and TRAIL variants were added at concentrations ranging from 2.5-50 ng/ml and incubated for 24 hours. Cells were transferred into eppendorf tubes and collected by spinning at 5000 x g for 5 mins. Cell pellets were resuspended in 50 µl of annexin v incubation buffer (10 mM HEPES/NaOH. pH7.5, containing 1 µl of home-made Annexin V-FITC. The cells were then incubated in the dark for 15 mins. 300 µl of Annexin V incubation buffer was added and the samples were analysed immediately using the FACS CantoII flow cytometer (BD Sciences).

2.3.2 Cell viability assay MTT

Cells were seeded 24 hours prior to treatment in a 96 well plate (0.1 ml/well) at the appropriate density. Wild type TRAIL and TRAIL variants were added at concentrations ranging from 5-500 ng/ml and the plate incubated for 24 hours or to treatment in a 96 well plate (0.1 ml cell culture/well) at the appropriate density. Wild type TRAIL (WT TRAIL) and TRAIL variants were added at concentrations ranging from 5-500 ng/ml and the plate incubated for 24 hours. 10 µl of MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma dissolved in Hanks balanced salt solution, 5 mg/ml) was added and incubated for a period of 3 hours exactly at 37°C. After 3 hours the reaction was stopped with the addition of 100 µl of stop mix (20% SDS in 40% dimethyl formamide). The blank was generated using three wells of the plate where no MTT was added. When stopping the MTT reaction, stop mix was added to these wells followed by the addition of 10 µl of MTT. The plate was placed on an orbital shaker to allow the formazan precipitate to dissolve. The formed colour intensity was measured at 550 nm using the Wallac plate reader. Cell viability was then calculated using the untreated samples as 100% and expressing the viability of the treated samples relative to the control.

2.4 Neutralization of Decoy Receptors

Cells were seeded 24 hours prior to treatment in 24 well plates (0.5ml/well) at appropriate density. Neutralizing antibodies for DcR1 and DcR2 (R & D systems) were added individually or together at a concentration of 2 µg/ml1 hour prior to treatment with WT TRAIL and TRAIL variants at a dosage corresponding to the ED50 of the ligand. Induction of apoptosis was measured 3 hours after treatment using annexin V staining and flow cytometry. An increase in apoptosis in response to decoy receptor neutralization is represented as fold increase compared to the level of apoptosis induced by the ligands in the absence of the neutralizing antibodies.

2.5 Rational combination of single mutants

Single mutants were combined and generated by Integrated DNA technologies (IDT Coralville, IOWA). Mutants were expressed and purified as described in section 2.2.2. This was carried out by our collaborators in the Department of Pharmaceutical biology, University of Groningen, The Netherlands.

2.6 TRAIL Receptor Immunocytochemistry

Cells were collected following a gentle trypsinization and then incubated for 10 min at 37^{o}C to allow membrane recovery. The cells were pelleted by centrifugation at 5,000 rpm followed by two washes with 1% BSA in PBS and incubated with mouse monoclonal antibodies against all four membrane bound TRAIL receptors (Alexis) for 45 min on ice. The cells were again washed twice and then incubated with anti-mouse IgG-FITC (Sigma) for 45 min on ice and in the dark. As negative controls, isotype control antibodies were used (mouse IgG1). After staining, cells were either resuspended in PBS and measured immediately or they were fixed in 1% formaldehyde in PBS and receptor expression was measured within 24 hours on a FACSCantoII flow cytometer (BD Biosciences). Analysis was performed using WinMDI 12.9 software.

2.7 Receptor binding by ELISA

Nunc maxisorb 96-well plates were coated with Fc-receptor (R & D systems) at a concentration range of 5-320 ng/well. Coating was carried out in 0.1 M sodium bicarbonate buffer (pH 8.6) for 2 hours at4^{o}C, control wells contained coating buffer without Fc-receptor. All wells were subsequently blocked with 2% BSA/0.1 M sodium bicarbonate buffer for 1 hour. Following the blocking wells were washed three times with Tris-buffered saline/0.5% Tween 20 (TBST) (pH7.5), followed by 2 washes with PBS. WT TRAIL and TRAIL45 variants in PBS (pH 7.4) were then added to the wells at a concentration of 25 ng/well and incubated at room temperature for 30 mins. After 6 washes with TBST, anti-TRAIL antibody (R & D systems) was added using a 1:400 dilution, the plates were then incubated for 1 hour at room temperature. The washing step was repeated before the addition of a 1:4,000 dilution of horse radish peroxidase-conjugated anti-goat IgG (R & D systems). The wash step was repeated and 100 µl of 1-Step Ultra TMB (Thermo Scientific Pierce) was added to initiate the reaction and the plate was incubated in the dark until the colour change was observed. The reaction was terminated by addition of 100 µl of 2 M sulfuric acid and the absorbance was subsequently measured at 450 nm on a microplate reader. Binding of WT TRAIL to the highest concentration of Fc-receptor was taken as 100% binding, and binding at lower concentrations and the binding of the TRAIL45 variants was calculated relative to this value.

2.8 Receptor Binding by Surface Plasmon Resonance:

Initial binding experiments were performed with a SPR-based biosensor Biacore 3000 (GE Healthcare, Munich, Germany). Research grade CM5 sensor chips, N-hydroxysuccimide, N-ethyl-N_-(3-diethylaminopropyl) carbodiimide, ethanolamine-HCl and standard buffers, e.g. HBS-N and HBS-EP were purchased from the manufacturer. All the buffers were filtered and degassed. Immobilization of Protein A on the sensor surface of a Biacore (CM5) sensor chip was performed following a standard amine coupling procedure according to the manufacturer's instructions. Receptor chimeras were captured at a level of 200–500 response units (RU). Reference surfaces consisted of activated CM dextran, subsequently blocked with ethanolamine. 100 ul aliquots of WT rhTRAIL or variants were injected in 3-fold at concentrations

ranging from 0.5 to 500 nM at 50 ul/ml and at 37^{o}C using HBS-N supplemented with 0.005% surfactant P20 (Biacore, Uppsala, Sweden) as running and sample buffer. Binding of ligands to the receptors was monitored in real time. Pre-steady state data were obtained by recording the responses 30 sec. after the end of injections for all concentrations. The response data as a function of TRAIL concentration were fitted by using a four-parameter equation to give apparent affinity constants. Between injections, the receptor/sensor surface was regenerated using 10 mM glycine.HCl pH 2.0 and a contact time of 25 sec.

To obtain an overview of rate constants for trimer:monomer complex between rhTRAIL WT and mutants for all the receptors, experiments were conducted using a C1 sensor chip as previously described (Reis *et al.*, 2011). This was carried out by collaborators at the University of Groningen, The Netherlands.

2.9 Generation of decoy receptor overexpressing cell lines

2.9.1 Lentivirus production

Full length cDNA clones for both DcR1 (IRCMp5012H1033D) and DcR2 (IRAUp969C08104D) were purchased from Imagene. A digest was performed using EcoRI and XhoI to cut both cDNAs from their respective vectors. They were then ligated into the lentiviral vector pCDH-RFP using the restriction sites EcoRI and XhoI (Vector map for pCDH-RFP can be found at Systems Biosciences, cat # CD512A-1). Lentivirus for DcR1 or DcR2 was generated by co-transfecting the pCDH-DcR1-RFP or pCDH-DcR2-RFP lentiviral vector with a 2nd generation lentivirus packaging system (Addgene, pMD2.G Cat#12259, psPAX2 Cat#12260, pRSV-Rev Cat#12253) using JET PEI transfection reagent (Polyplus Transfection, Cat#101-01N) into HEK293T cells, virus supernatant was harvested at 24 and 48 hour and filtered via a 0.45 µm nalgene filter. The virus was stored in cryotubes at -80ºC.

2.9.2 Transduction of human primary fibroblast cells

Human dermal fibroblasts (hFb) were seeded at 100,000 cells/well in a 6 well plate. 24h post seeding the cells were at ~ 30% confluency. The virus was then defrosted and incubated with 5µg polybrene per ml of virus at 37°C for 5 mins. After the 5 min

incubation the media was removed from the hFb's in the 6 well plates and replaced with 2ml of the virus/polybrene mixture. The 6 well plates were then centrifuged at 1500rpm for 90mins at 37°C. Following centrifugation the plates were placed in 37°C incubator overnight. The next day the media containing virus was removed and fresh culture media was applied. Transduction efficency was < 90% which was monitored by florescent microscopy and quantified by FACS analysis using RFP fluorescent tag as a marker for transduced cells.

2.10 Co-culture of primary human dermal fibroblasts with Colo205 cells

Primary human dermal fibroblasts (hFb) containing empty vector pCDH and hFb cells overexpressing DcR1 and DcR2 were trypsinized and labelled with 5 µM CellTracker red CMTPX (molecular Probes) in serum free media for 45 min, shaking intermitantly. The stained cells were then mixed with the Colo205 cells at a 1:1 ratio and seeded in 24 well plates. Colo205 cells were also seeded without hFb cells. 24 hours after seeding the cells were treated with either WT TRAIL or the TRAIL45 variants at a concentrate range of 2.5-50 ng/ml for 3 hours after which they were harvested and cell death determined by annexin V staining (previously described).

2. 11 3D minitumour generation

2.11.1 Methocellulose solution preparation

To prepare the methocellulose solution, 0.6 g of methylcellulose (Sigma) was added to a 50 ml tube, keeping everything sterile. 25 ml of EGM-2 medium (Promocell, Germany) was heated to 60°C and following this added to the methylcellulose powder and rotated for 20 min or until completely dissolved. 25 ml of EBM-2 medium at RT was added and the solution rotated for a further 2 hour at 4 °C. Finally the solution was centrifuged at 5,000 x g for 2 h at RT.

2.11.2 Spheroid production

hFb cells and HUVEC cells were trypsinized and counted and the required number of cells incubated with the red cell tracker CMTPX or CM-DiI (Invitrogen) at a concentration of 5µM for 45 min at 37ºC shaking every 5-10 min. Following staining, 7.5 x 10^4 HUVEC and 3.75 x 10^4 hFb were spun down along with 7.5 x 10^4 MDA-MB-231 added to 15 ml of EGM-2 medium containing 20% of methocel solution. 150 µl of the 20% methocel solution containing the different cells was added to each well of a 96 U-shaped well suspension plate (Greiner BioOne, UK). The plate was incubated for 24 h at 37ºC to allow for spheroid formation.

2.11.3 Harvesting Spheroids

A 1.5 mg/ml collagen type-I solution was prepared by diluting rat tail collagen type-I (BD Biosciences, UK) into an appropriate volume of EGM-2 medium, and neutralizing the by addition of a 1 M NaOH solution. Solution was filter sterilised. 40 µl of the collagen-I solution was added to the bottom of 35mm dishes with 14 mm glass slide bottom for microscopy (MatTek Corporation) previously warmed to 37ºC. These were then incubated at 37ºC for 45 min to allow for the collagen gel to set. Spheroids were harvested gently from the 96 well plate into a 15 ml tube using a 10 ml pipette and centrifuged at 300 x g for 5 min. The supernatant was carefully removed and the freshly prepared 1.5 mg/ml collagen type-I solution was added (50 µl X number of dishes to be seeded) and the spheroid pellet was carefully resuspended. 50µl of this spheroid suspension in collagen was then added on top of each collagen gel in the dish, before being placed in the incubator at 37ºC for ~2 hour to allow the second collagen gel to set. 2 ml of EGM-2 medium is then added to each well and incubate for 24 hour. Cells were then treated with WT TRAIL or TRAIL45-a at a concentration of 250 ng/ml for 24 hour. 1-2 hours before analysis 1µg/ml of Hoechst33342 and 1µM of nucview or SYTOX green nucleic acid dye (Invitrogen S7020) to the media and incubate at 37ºC.

2.12 Statistical analysis

Statistical analysis was carried out using the statisitical software Minitab 15. Significant differences between treatments were determined using a paired student t-test and $p<0.05$ was deemed significant. All error bars represent standard error of mean (SEM).

Chapter 3: Results

3.1 The Design and Generation of Decoy-insensitive TRAIL mutants

In order to study the role of the decoy receptors in TRAIL-induced apoptosis, and the potency of a TRAIL-based therapeutic capable of avoiding neutralisation by the decoy receptors *in vivo*, a decoy receptor-insensitive TRAIL mutant has been generated using a computational design algorithm, called FoldX (Schymkowitz *et al.* 2005, van der Sloot *et al*, 2006, Tur *et al*, 2008 and Reis *et al*, 2010). To date, crystal structures for only TRAIL in complex with DR5 exist (Hymowitz *et al.*, 1999, Mongkolsapaya *et al.*, 1999 & Cha *et al.*, 2000). Since sequence alignment of the different membrane-bound TRAIL receptors shows that the ligand-binding cysteine rich domains (CRD2 and CRD3) are highly conserved between receptors with more than 50% homology to the DR5 CRDs (Chaudhary *et al.*, 1997) and there are no significant insertions or deletions (Strausberg *et al.*, 2002; van der Sloot *et al.*, 2006), it was possible to create homology models for the complex of TRAIL with DR4, DcR1 and DcR2 (Reis *et al.*, 2010; van der Sloot *et al.*, 2006). The accuracy of the homology models and the FoldX predictive computational model has been demonstrated previously in our laboratory by the successful generation of previously described receptor-specific TRAIL mutants (van der Sloot *et al*, 2006, Tur *et al*, 2008 and Reis *et al*, 2010).

To predict mutations in WT TRAIL that could diminish binding to the decoy receptors and/or enhance binding to DR4 and DR5, the amino acid residues of TRAIL present on the ligand-receptor interface (i.e. TRAIL-DR4/DR5/DcR1/DcR2) were identified. Of these residues, the ones which were conserved among the four receptors and those known to be necessary for DR4/DR5 binding have been identified and excluded from the consequent mutational analysis. FoldX was then used to introduce all the possible single amino acid substitutions at these positions and to calculate how the amino acid replacement changes the affinity of the ligand for the four TRAIL receptors. From this, mutations that increased the energy of interaction (i.e. reduced the binding affinity) between the complex of the ligand (TRAIL mutant) and DcR1/DcR2 (Figure 3.1) as well as the ones that decreased the energy of interaction between the ligand and DR4/DR5 have been identified (Figure 3.2). This work was carried out by our collaborators in the Centre for Genomic Regulation (CRG) in Barcelona, Spain.

The change in the energy of interaction was expressed by calculating the difference in the change in free energy ($\Delta\Delta G_i$) between the ligand-receptor complexes of WT TRAIL vs. the TRAIL mutants. For example, if the affinity of a mutant for DcR1 was

lower than that of WT TRAIL, the following equation for ΔΔGi gave a positive value: $\Delta\Delta Gi=\Delta Gi_{\text{WT TRAIL-DcR1}}- \Delta Gi_{\text{TRAIL mutant}}$.

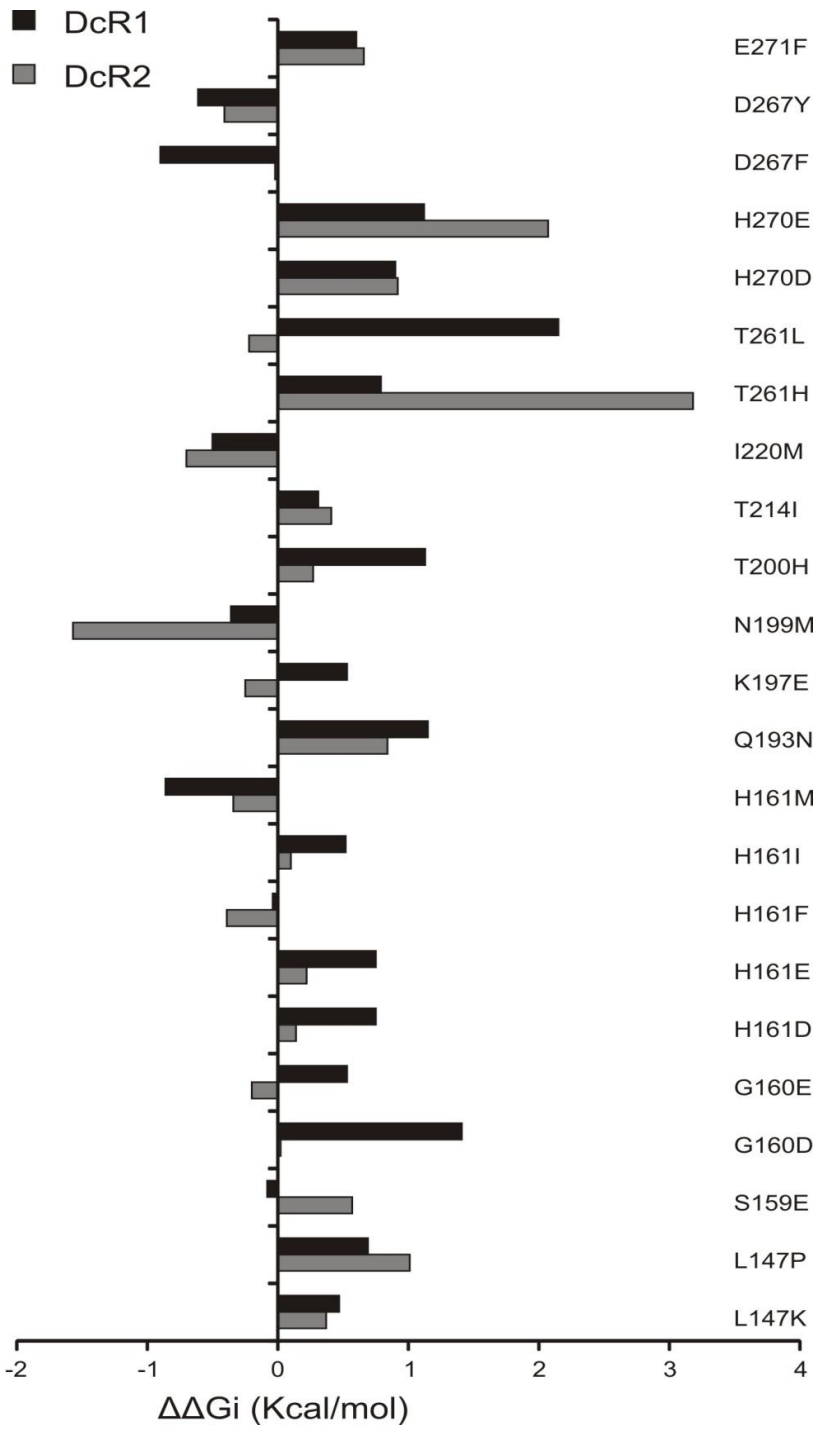


Figure 3.1. *ΔΔGi values of single mutations reducing binding to decoy receptors predicted by FoldX.* Depicted is the predicted difference in binding energy ($\Delta\Delta G_i$) of the 24 designed decoy-insensitive TRAIL mutants to the decoy receptors when compared to WT TRAIL. A negative $\Delta\Delta G_i$ indicates an enhanced binding to the receptor and a positive $\Delta\Delta G_i$ indicates a diminished binding capacity. This data was generated by our collaborators at CRG.

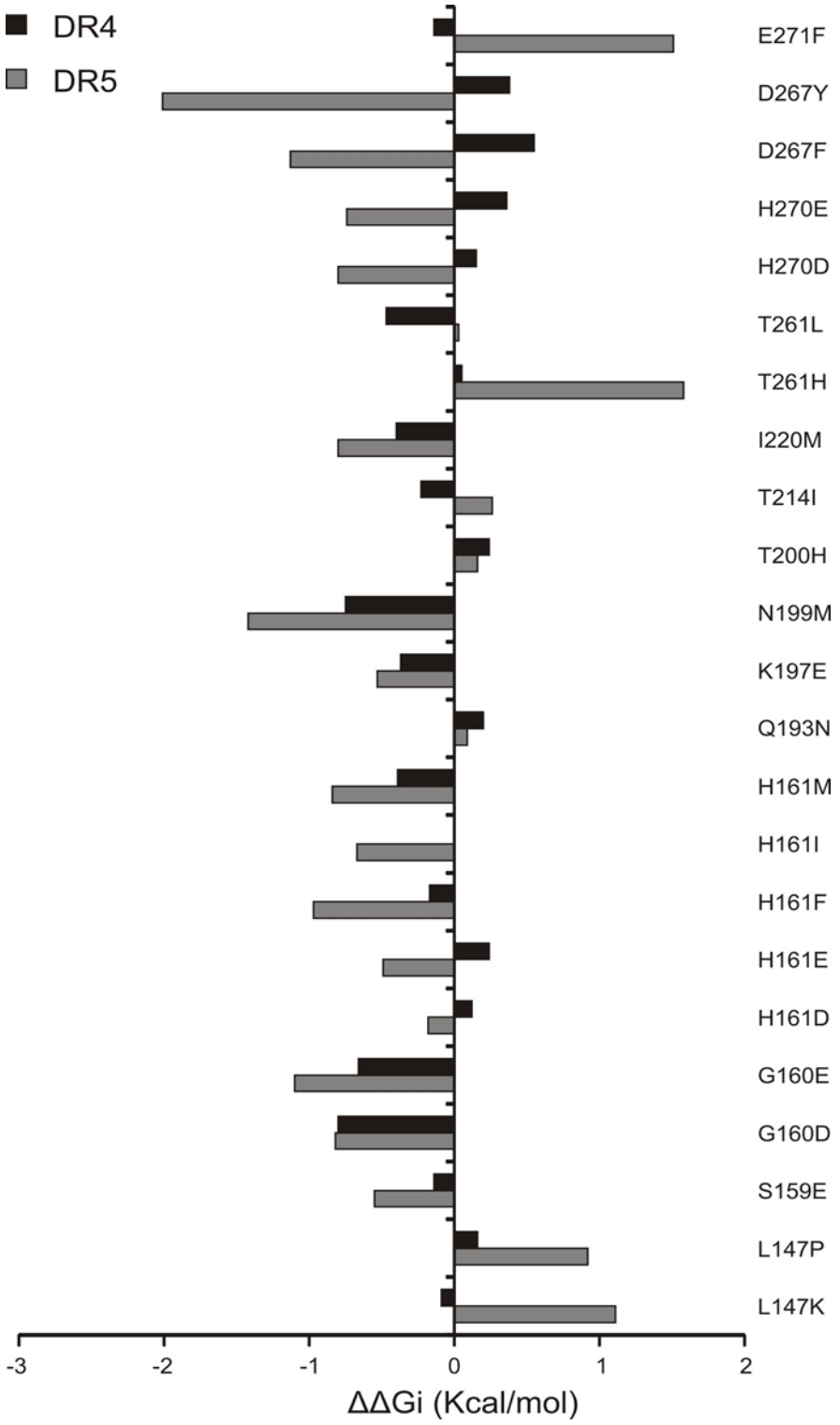


Figure 3.2. *ΔΔGi values of decoy-insensitive TRAIL mutations predicted by FoldX*. Depicted is the predicted difference in binding energy ($\Delta\Delta G_i$) of the 24 designed decoy-insensitive TRAIL mutants to the death-inducing receptors when compared to WT TRAIL. A negative $\Delta\Delta G_i$ indicates an enhanced binding to the receptor and a positive $\Delta\Delta G_i$ indicates a diminished binding capacity. This data was generated by our collaborators at CRG.

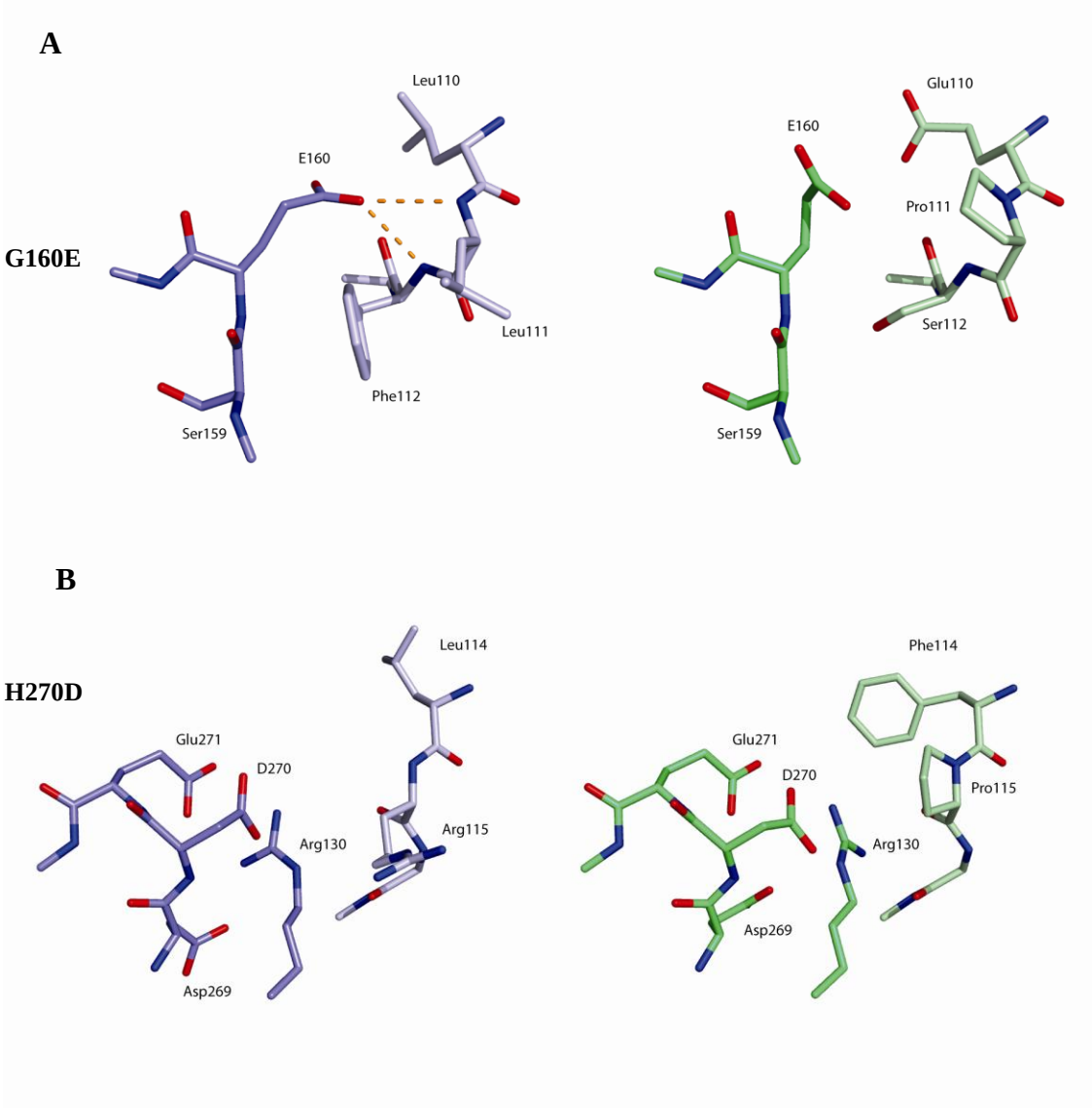


***Figure 3.3**. Structural impression of mutants G160E (A) and H270D (B) in complex with DR5 (left) or DcR1 (right.) according to FoldX prediction.*

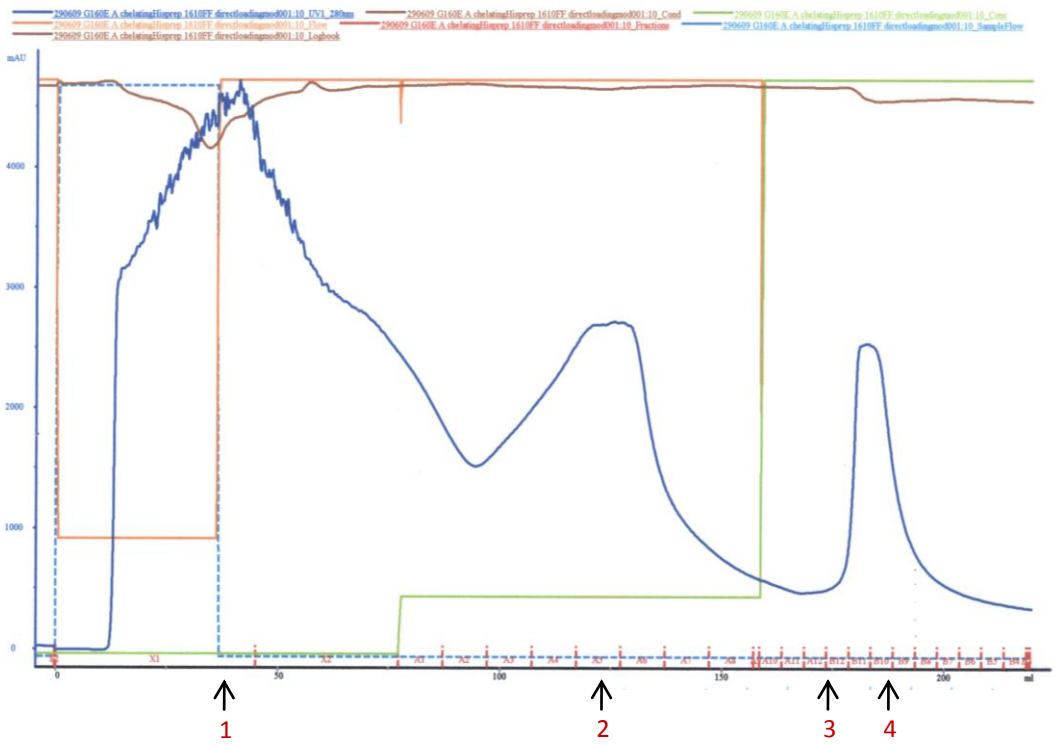
Figure 3.3(A) shows TRAIL mutant G160E in complex with DR5 (left) and in complex with DcR1 (right). The side chain OE2 of Glu160 is able to form a weak hydrogen bond with the backbone amides of Leu 111 and Leu 112 of DR5, in DR4 Glu110 causes electrostatic repulsion with Glu160 and no hydrogen bond can be formed with the N of Pro111. This results in an improved binding of TRAIL mutant G160E towards DR5, while it negatively affects binding towards DcR1. In DR4 and DcR2 residue 110 is, like in DR5, leucine, therefore the G160E mutation does not cause electrostatic repulsion in this binding pocket. Like DcR1, residue 111 is a Pro and hence is unable to form a hydrogen bond with OE2 (or OE1) of Glu160 of G160E. (B) depicts TRAIL mutant H270D in complex with DR5 (left) and in complex with DcR1 (right). Amino acid position 115 of DR4 and DcR1 is Pro while the structural

equivalent position in DR5 and DcR2 is Arg and Leu, respectively. One of the carbons of the His270 packs tightly against Pro115 of DR4/DcR1 and less tightly against Arg115 and Leu115 of DR5 and DcR2, respectively. Upon substitution with Asp this favorable interaction is removed and the unfavorable solvation of Pro115 adds an additional energy penalty.

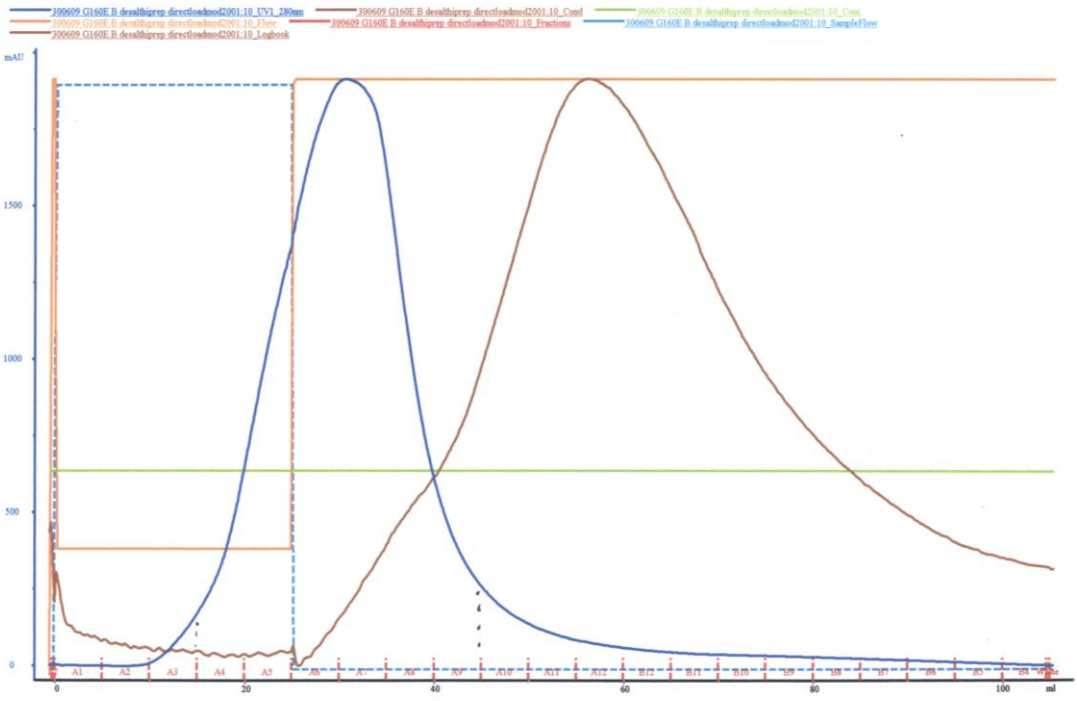
Although the prediction of the interaction energy of the T261L single mutant in complex with DcR1 correlates with experimental receptor binding data, upon examination of the FoldX structure output files it was revealed that the magnitude of the predicted interaction energy was most likely caused by an artifact which caused conformational distortions in adjacent amino acids. The cause is currently being investigated (personal communication A.M. van der Sloot).

The predicted amino acid replacements were generated by site-directed mutagenesis and confirmed by DNA sequencing. Following this the rhTRAIL mutant proteins were expressed in *E. coli* and purified sucessfully with a protein yield of ~1.8-7.3 mg/l (Table 3.1).

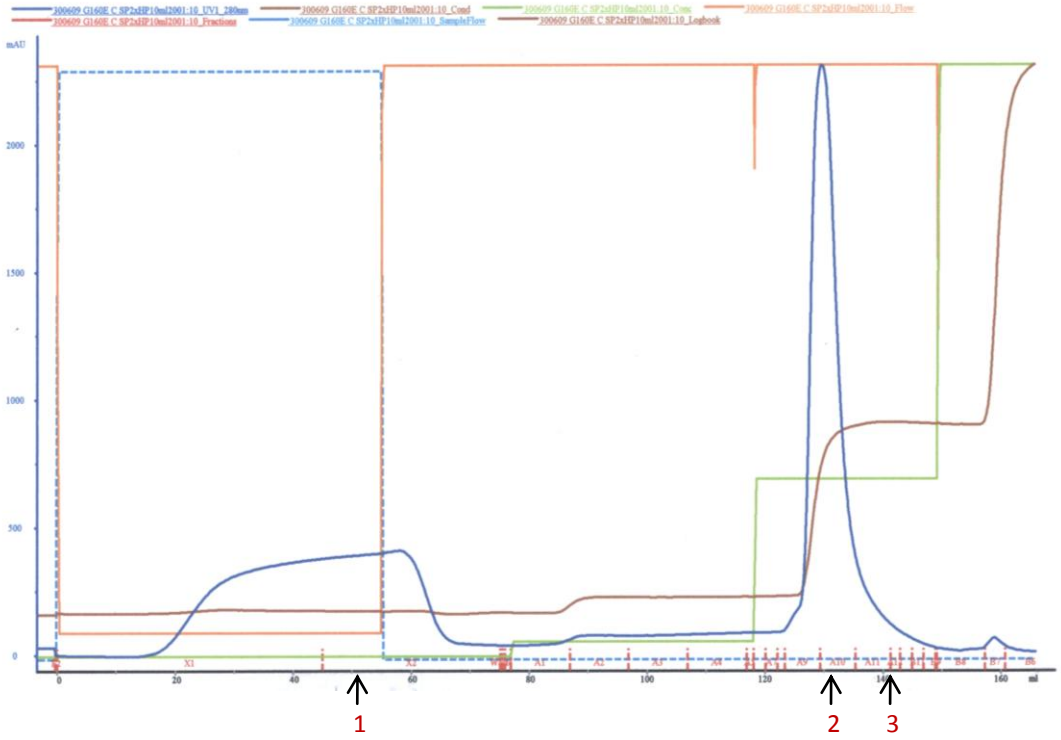
A


290609 G160E A chelatingHisprep 1610FF directloadingmod001:10_UV1_280nm
290609 G160E A chelatingHisprep 1610FF directloadingmod001:10_Cond
290609 G160E A chelatingHisprep 1610FF directloadingmod001:10_Conc
290609 G160E A chelatingHisprep 1610FF directloadingmod001:10_Flow
290609 G160E A chelatingHisprep 1610FF directloadingmod001:10_Fractions
290609 G160E A chelatingHisprep 1610FF directloadingmod001:10_SampleFlow
290609 G160E A chelatingHisprep 1610FF directloadingmod001:10_Logbook
mAU
4000
3000
2000
1000
0
0
50
100
150
200
ml
1
2
3
4

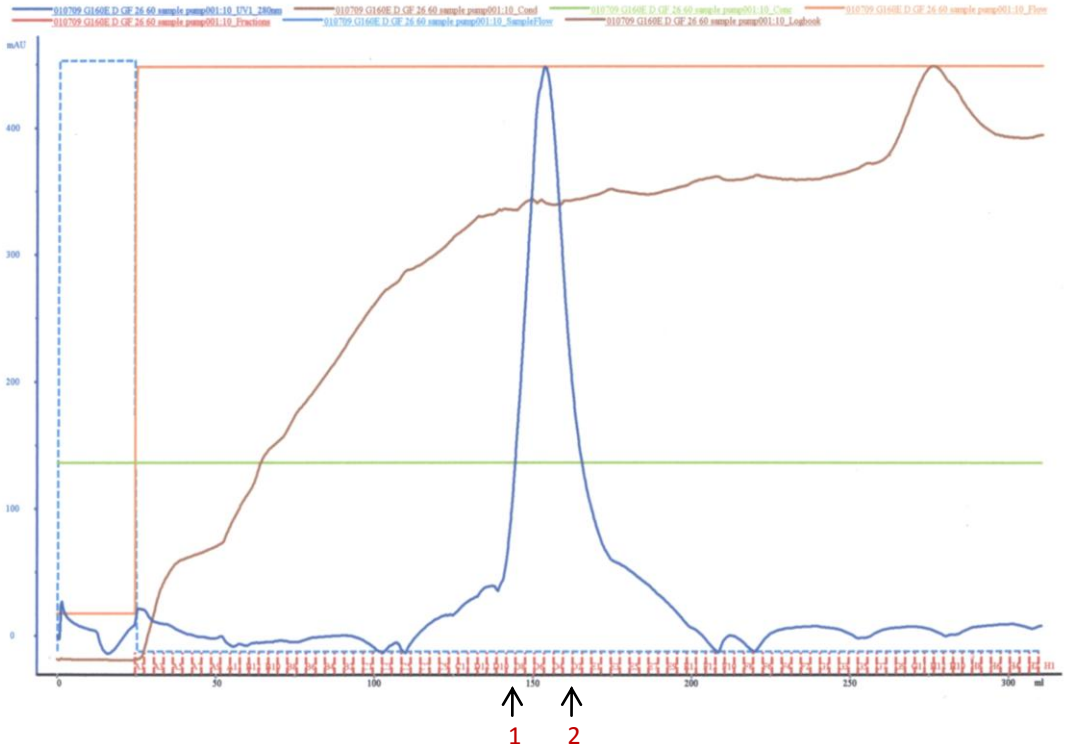
B


300609 G160E B desalthisprep directloadmod2001:10_UV1_280nm
300609 G160E B desalthisprep directloadmod2001:10_Cond
300609 G160E B desalthisprep directloadmod2001:10_Conc
300609 G160E B desalthisprep directloadmod2001:10_Flow
300609 G160E B desalthisprep directloadmod2001:10_Fractions
300609 G160E B desalthisprep directloadmod2001:10_SampleFlow
300609 G160E B desalthisprep directloadmod2001:10_Logbook
mAU
1500
1000
500
0
0
20
40
60
80
100
ml

C


mAU
2000
1500
1000
500
0
0
20
40
60
80
100
120
140
160
ml
1
2
3

D


mAU
400
300
200
100
0
0
50
100
150
200
250
300
ml
1
2

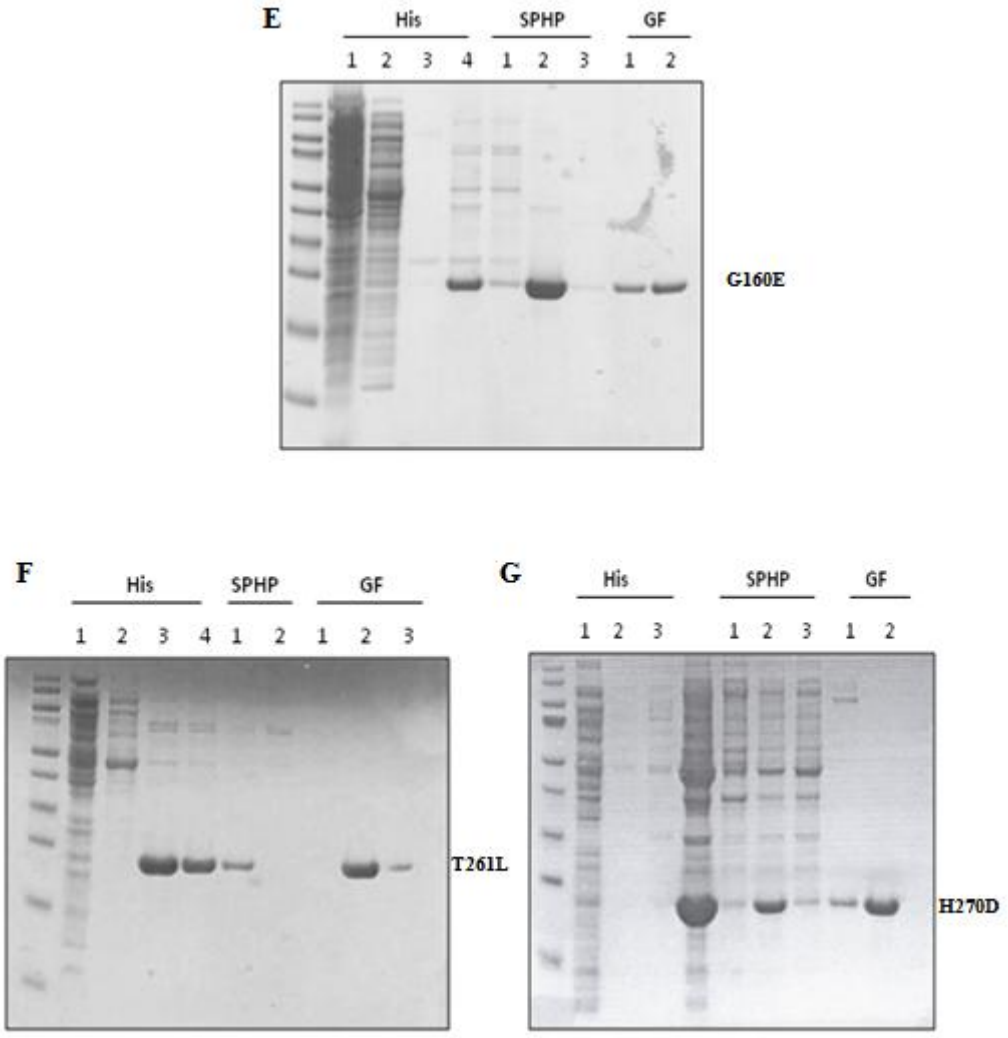


Figure 3.4. *Example of chromatograms for the TRAIL mutant G160E* for the various steps of purification, His-trap (A), Desalting (B), Cation exchange (C) and Gel filtration (D). The various fraction and resulting purity for this mutant is seen in part (E), along with two other examples T261L (F) and H270D (G) which together are representative of the purity obtained for all mutants.

For previous TRAIL mutants Far-UV CD wavelength spectra was carried to ensure that all mutants were folded correctly and displayed the characteristics of a β-sheet containing protein comparable to WT TRAIL. Gel filtration and dynamic light scattering measurements were used to show that all mutant protein solutions contained the protein in its trimeric oligomerization state. Following this purification procedure endotoxin levels were found to be < 5 EU/ml although further steps to remove contaminants was taken prior to animal studies (Duiker *et al.*, 2009; van der Sloot *et al.*, 2004; van der Sloot *et al.*, 2006). In the case of these mutants endotoxin analysis and removal will be carried out prior to any *in viv*o studies undertaken. Gel filtration profiles for subsequent TRAIL mutants were compared to the WT profile for confirmation of the oligomerization state of the protein (Szegezdi *et al.*, 2011). All

mutations introduced were on the outer surface of the protein and not at the core to ensure no interference in folding or trimerization. No deviation from the expected gel filtration profile was seen.

Table 3.1 Protein yields for TRAIL mutants obtained form 1 Litre of bacterial culture.

Mutant TRAIL protein yield mg/liter culture			
L147K	~ 4	N199M	~ 3.6
L147P	~ 3.5	T200H	~ 6.3
S159E	~ 4.7	T214I	~ 2.5
G160D	~ 3.1	I220M	~ 4.2
G160E	~ 4.2	T261H	~ 1.8
H161D	~ 5.4	T261L	~ 2.7
H161E	~ 3.9	D267F	~ 3.9
H161F	~ 2.6	D267Y	~ 3.6
H161I	~ 1.8	H270D	~ 7.3
H161M	~ 3	H270E	~ 3.7
Q193N	~ 5.7	E271F	~ 4
K197E	~ 3.5		

3.2 Decoy-insensitive TRAIL mutants retain their cytotoxic potential

The ability of the TRAIL mutants to reduce cell viability in the TRAIL-sensitive colon carcinoma cell line, Colo205 was measured as a first screen and used as an indication of retained binding to DR4 and/or DR5. This cell line is known to express all four membrane-bound TRAIL receptors on their cell surface and are highly sensitive to TRAIL-induced apoptosis (van der Sloot *et al.*, 2006). Induction of cell death was quantified by measuring cell viability with MTT assay after 24 h treatment (Figure 3.5 and Figure 3.6). From this, the dose inducing 50% cell death (ED_{50}) was determined (Table 3.2).

According to their ED_{50} values, the TRAIL mutants were divided in three groups (Table 3.2). Mutants displaying a minimum of two-fold lower ED_{50} than WT TRAIL (two-fold higher activity than WT TRAIL, i.e. $ED_{50\text{-mutant}}/ED_{50\text{-WT TRAIL}} \leq 0.5$) were designated to group 1 (Figure 3.5A-D). Group 1 (mutants with the highest pro-apoptotic activity) consisted of the mutants T261L, where a hydrophilic threonine residue at position 261 was replaced with a hydrophobic leucine residue, T200H where at position 200 a threonine was substituted with the positively charged histidine,

H270D (histidine to aspartate mutation), and lastly G160E (glutamate replacing the glycine at position 160). The second group of mutants reduced Colo205 cell viability to a comparable level as WT TRAIL with an ED_{50} ratio between 0.5-2 and these were: L147K, G160D, H161F, H161I, H161M Q193N, K197E and H270E (Figure 3.6A) . The third group of mutants displayed greatly reduced cytotoxicity with an ED_{50} ratio of ≥ 2. These mutants were: L147P, S159E, H161E, I220M, T261H and D267Y (Figure 3.6B). Lastly, a number of mutants failed to induce any cell death and were deemed inactive, these mutants were: H161D, N199M, T214I, D267F and E271F (data not shown). The first group of TRAIL mutants were further studied to investigate whether they possess their predicted characteristics along with the four best preforming members of group 2 which were L147K, G160D, Q193N and K197E.

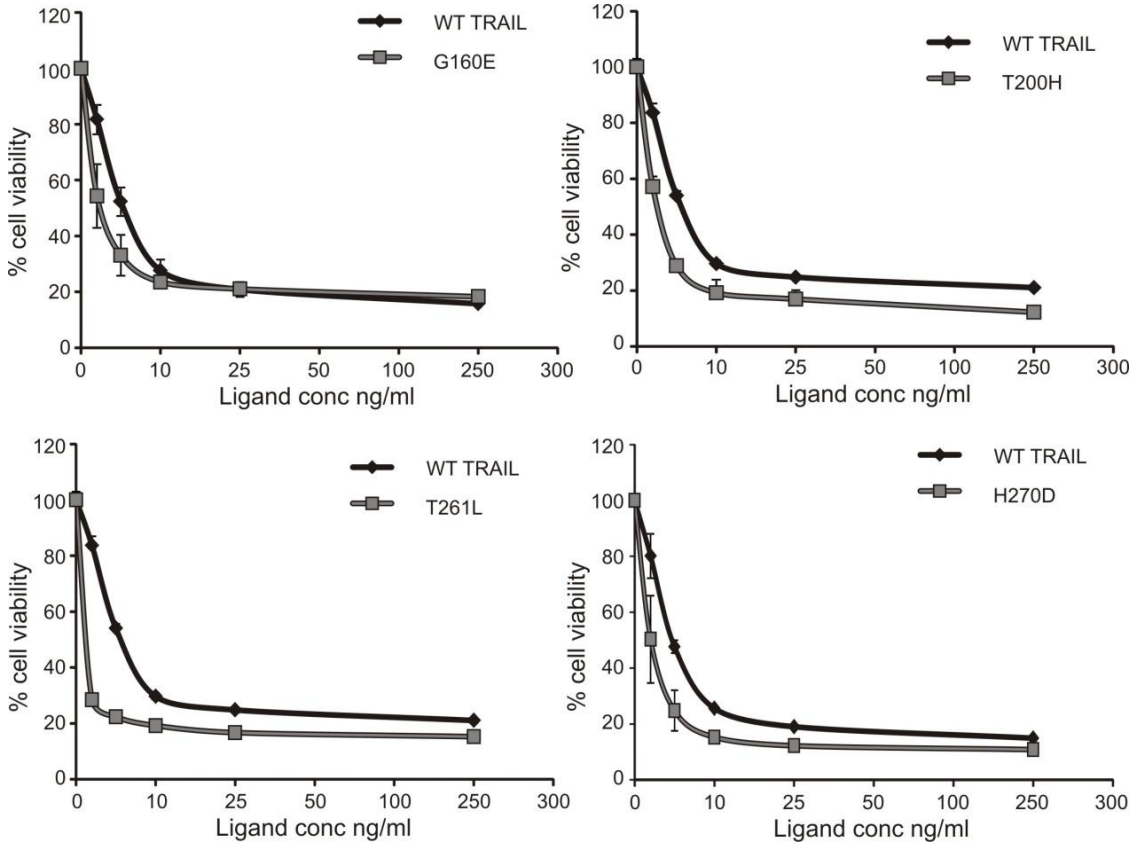


Figure 3.5. *Decoy-insensitive TRAIL mutants can reduce cell viability in vitro.* Group 1 TRAIL mutants, G160E (A), T200H (B), T261L (C) and H270D (D) have the ability to strongly reduce cell viability with T261L being the most potent. Colo205 cells were treated with concentrations from 5-250 ng/ml of WT TRAIL or TRAIL mutants for 24 h after which cell viability was determined using MTT assay. The graphs show average cell viability compared to untreated control ± SEM from three independent experiments.

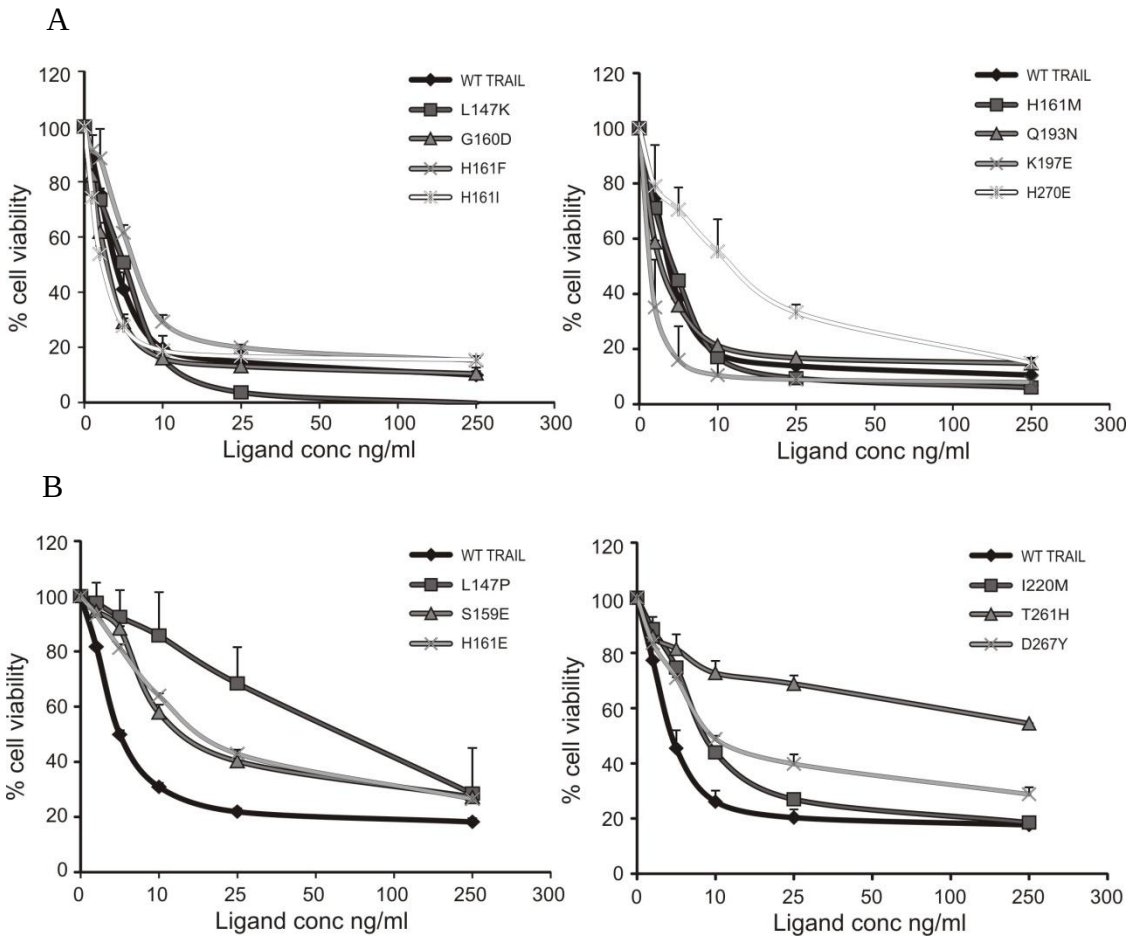


Figure 3.6*. Decoy-insensitive TRAIL mutants can reduce cell viability in vitro.* (A) Group 2 TRAIL mutants can reduce cell viability to a level similar to WT TRAIL, while (B) group 3 mutants are less potent than WT TRAIL with all mutants in this group unable to reduce viability to a level similar to that of its native counterpart. Colo205 cells were treated with concentrations from 5-250 ng/ml of WT TRAIL or TRAIL mutants for 24 h after which cell viability was determined using MTT assay. The graphs show average cell viability compared to untreated control ± SEM from three independent experiments.

Table 3.2. ED_{50} ratios of decoy-insensitive TRAIL single mutants to WT TRAIL.

G160E	0.3036	**L147K**	0.7141	**L147P**	5.7266
T200H	0.1433	**G160D**	0.6135	**S159E**	3.1679
T261L	0.0005	**H161F**	1.3673	**H161E**	3.2720
H270D	0.2501	**H161I**	0.8471	**I220M**	2.2071
		H161M	0.7476	**T261H**	9.8430
		Q193N	0.5218	**D267Y**	2.7663
		K197E	0.8235		
		H270E	1.8344		

We further investigated the apoptosis-inducing potency of the most promising group of TRAIL mutants by measuring phosphatidyl serine exposure by the dying cells (Figure 3.7). Colo205 cells were treated with a dosage of 2.5-50 ng/ml of the mutants or WT TRAIL for 3 h after which induction of cell death was quantified with annexin V-FITC staining and flow cytometry as described in Materials and Methods.

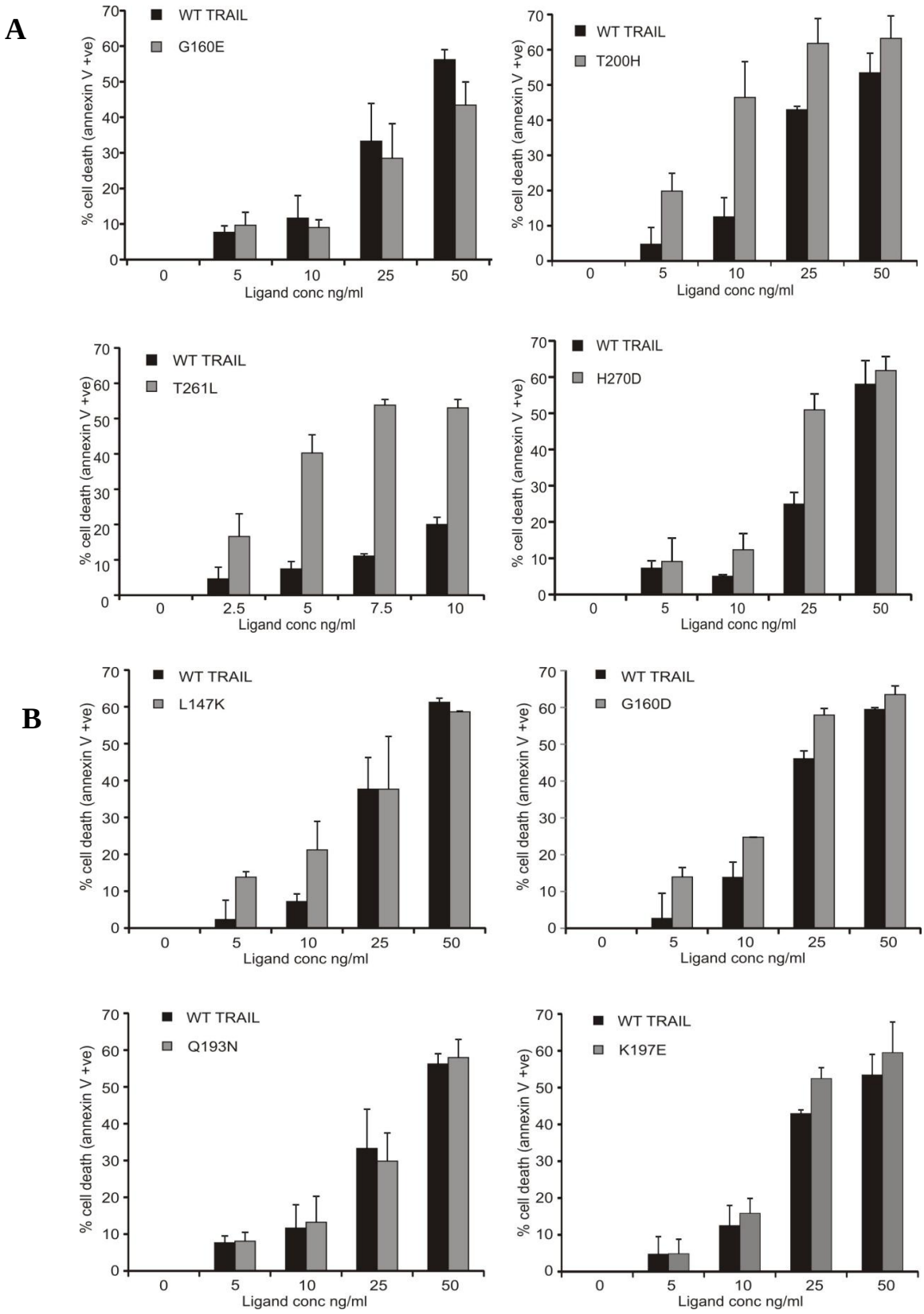


Figure 3.7. *Mutants T200H, T261L, H270D show stronger death-inducing ability than WT TRAIL.* Biological activity of WT TRAIL, group 1 TRAIL mutants (A) and the four best performing members of group 2 TRAIL mutants (B) was tested in Colo205 cells. Percentage cell death was measured as percentage annexin V positivity following a 3 hour incubation period. The graphs show average percentage of cell death ±SEM from 3 independent experiments.

Mutants of the first group T200H, T261L and H270D showed an enhanced apoptosis-inducing ability compared to WT TRAIL verifying the results of the viability assay (Figure 3.7A). T261L showed a 5.4- fold higher pro-apoptotic activity than WT TRAIL at a concentration of only 5 ng/ml. These results identified 3 mutants with superior death-inducing ability compared to that of WT TRAIL and thus the consequent characterisation focused on these TRAIL mutants. Although G160E showed a cytotoxic ability comparable to WT TRAIL it was also included in further experiments due to the fact that the FoldX analysis predicted a very robust effect of this mutation on decoy receptor binding. Group 2 TRAIL mutants Q193N and K197E showed apoptosis-inducing ability similar to WT TRAIL, while L147K and G160D showed a slight increase in apoptosis compared to WT TRAIL (Figure 3.7B).

3.3 TRAIL mutants can evade decoy receptor-mediated inhibition

To investigate whether the generated TRAIL mutants can overcome decoy receptor-mediated inhibition of DR4/DR5 activation, the apoptosis-potentiating effect of DcR1 and DcR2 inhibition using neutralising antibodies was measured in Colo205 cells. Neutralisation of DcR1 and DcR2 enhanced induction of apoptosis by WT TRAIL indicating that TRAIL binding to the decoy receptor limits its efficacy (Figure 3.8A). From the group 1 mutants, T200H also showed an increase in induction of apoptosis upon decoy receptor neutralisation, indicating that this mutant can also still bind to both decoy receptors. On the other hand, the remaining group 1 TRAIL mutants G160E, T261L and H270D did not show increased pro-apoptotic activity after neutralisation of either DcR1 or DcR2, indicating that none of these mutants bound to either decoy receptors to a level that affected their efficacy (Figure 3.8A). All members of group 2 mutants showed an increase in cell death following decoy receptor neutralization indicating that all members of this group can still bind to these receptors (Figure 3.8B). These results coupled with the mutants' apoptotic potency lead to elimination of group 2 mutants from further exploration.

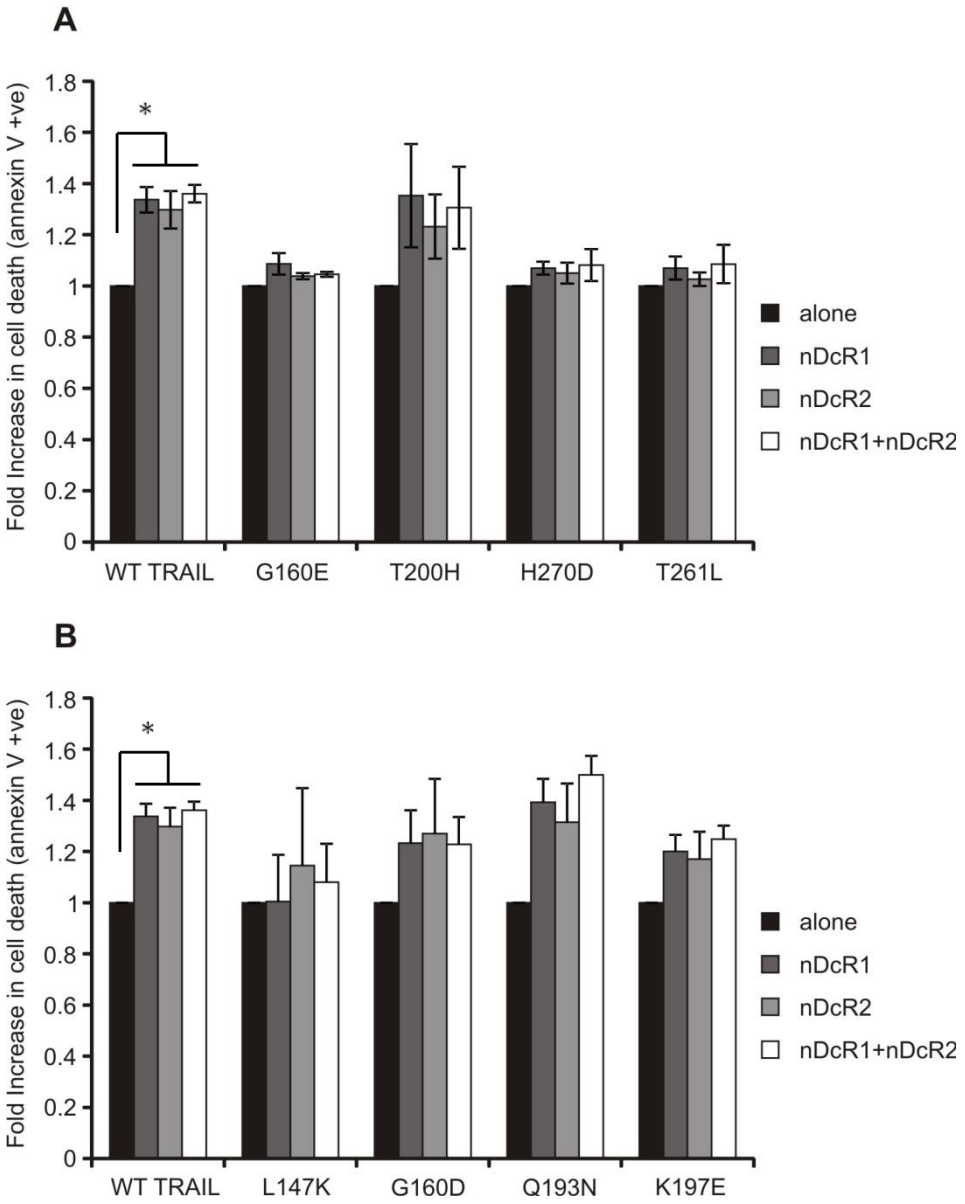


Figure 3.8. *TRAIL mutants G160E, T261L and H270D appear to have reduced binding to the decoy receptors.* Colo205 cells were pre-treated with 2 µg/ml of neutralizing anti-DcR1 and/or anti-DcR2 antibodies for one hour prior to treatment with an ED_{50} dose of WT rhTRAIL or (A) group 1 mutants or (B) group 2 TRAIL mutants. Induction of apoptosis was measured 3 hours after treatment using annexin V staining. An increase in apoptosis in response to decoy receptor neutralization was calculated as fold increase compared to the level of apoptosis induced by the ligands in the absence of the neutralizing antibodies. The graph shows the average of 3 independent experiments ±SEM.

3.4 TRAIL mutants are capable of activating both DR4 and DR5 and have limited or no binding to the decoy receptors

We have identified mutants which can potently induce apoptosis in the colon carcinoma cells, Colo205, however the generated mutant must be able to bind to and activate both DR4 and DR5 equally. To test the ability of the TRAIL mutants to activate both DR4 and DR5 to at least a comparable level as WT TRAIL, cell lines in which TRAIL has been shown to signal specifically through either of these death receptors were treated with the mutants belonging to group 1. ML-1 cells express all four TRAIL receptors on their cell surface (van der Sloot *et al.*, 2006) but only induce apoptosis via DR4 and were chosen as a model of DR4-responsive cells (Figure 3.9A-D) and Jurkat cells only expression DR5 on their cell surface were used as DR5-sensitive cells (Figure 3.10A-D) (Reis *et al.*, 2010; van der Sloot *et al.*, 2006).

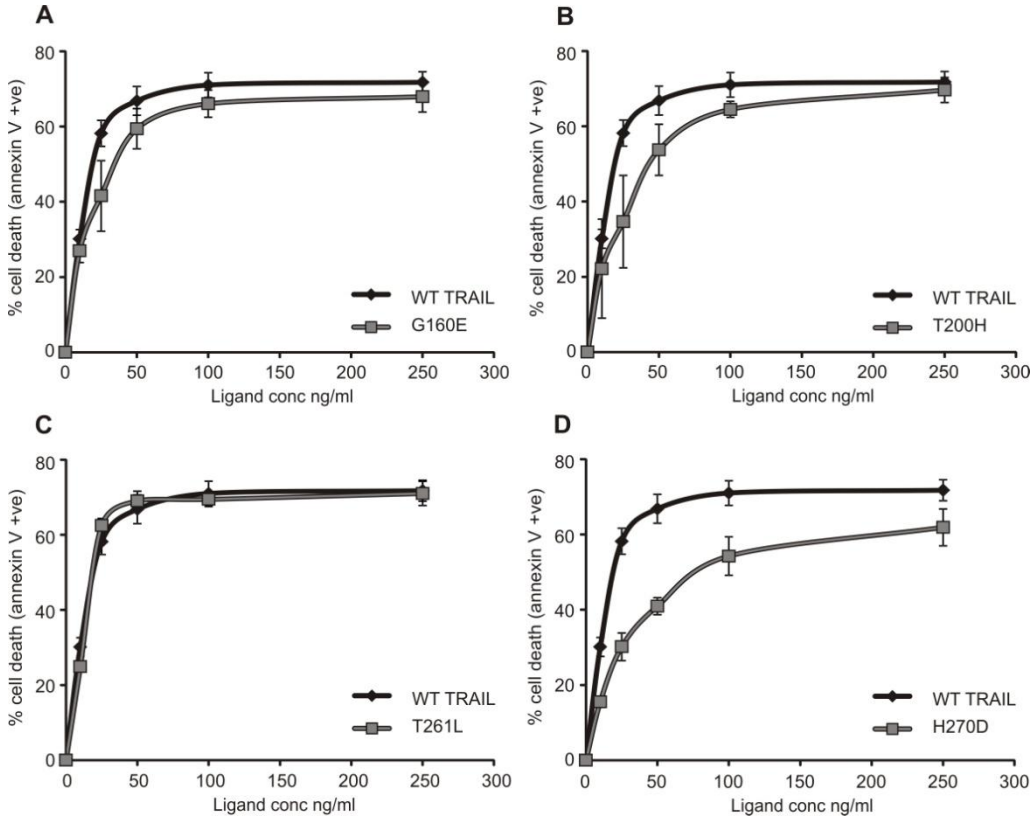


Figure 3.9. *Group 1 TRAIL mutants activate DR4 to a comparable level as WT TRAIL.* ML-1 cells which signal via DR4 were treated with the indicated doses of WT TRAIL or group 1 TRAIL mutants for 24 h. Cell death was quantified by annexin V staining and flow cytometry. The graph shows the average of 3 independent experiments ±SEM.

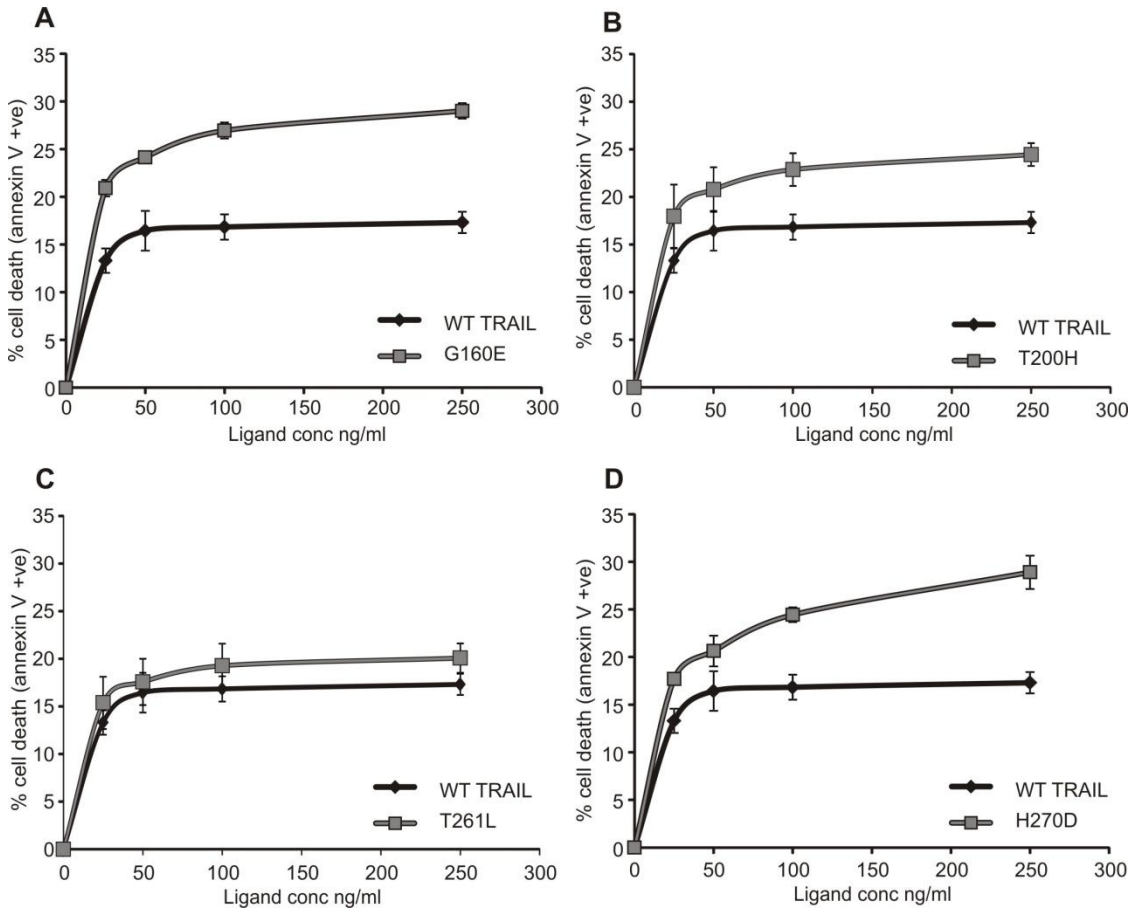


Figure 3.10. *Group 1 TRAIL mutants activate DR5 to at least the same level as WT TRAIL.* Jurkat cells which signal via DR5 were treated with the indicated doses of WT TRAIL or group 1 TRAIL mutants for 24 h. Cell death was quantified by annexin V staining and flow cytometry. The graph shows the average of 3 independent experiments ±SEM.

All four TRAIL mutants were capable of inducing apoptosis in both cell lines to a similar or higher extent than WT TRAIL, indicating that they are capable of binding to and activating both DR4 and DR5.

3.5 Rational combination of single mutants

Upon testing, four single amino acid mutants displayed favourable characteristics with some showing potent induction of apoptosis in cancer cells as was the case for mutant T261L, and some also showing reduced binding to the decoy receptors such as G160E. To attempt to further reduce binding to the decoy receptors, combinations of these single mutations were generated. Based on the computationally determined binding affinities as well as the experimental data, we selected the mutant T261L to be the core

mutation for all combinations due to its potent biological activity. This mutant could bind to both death receptors inducing levels of apoptosis superior to that of WT TRAIL while completely evading the decoy receptors, making it a potentially valuable mutant. While G160E proved to have apoptotic activity quite similar to WT TRAIL the value of this mutant lay in its reduction in binding to the decoy receptors. This was proposed to be the most promising combination while other combinations involving the other mutants of group 1 were also constructed with T261L at their core. They were as follows:

TRAIL45-a:T261L/G160E
TRAIL45-b:T261L/H270D
TRAIL45-c:T261L/G160E/H270D
TRAIL45-d:T261L/G160E/H270D/T200H

The mutants were generated by site directed mutagenesis, expressed and purified in the same manner as the single mutants.

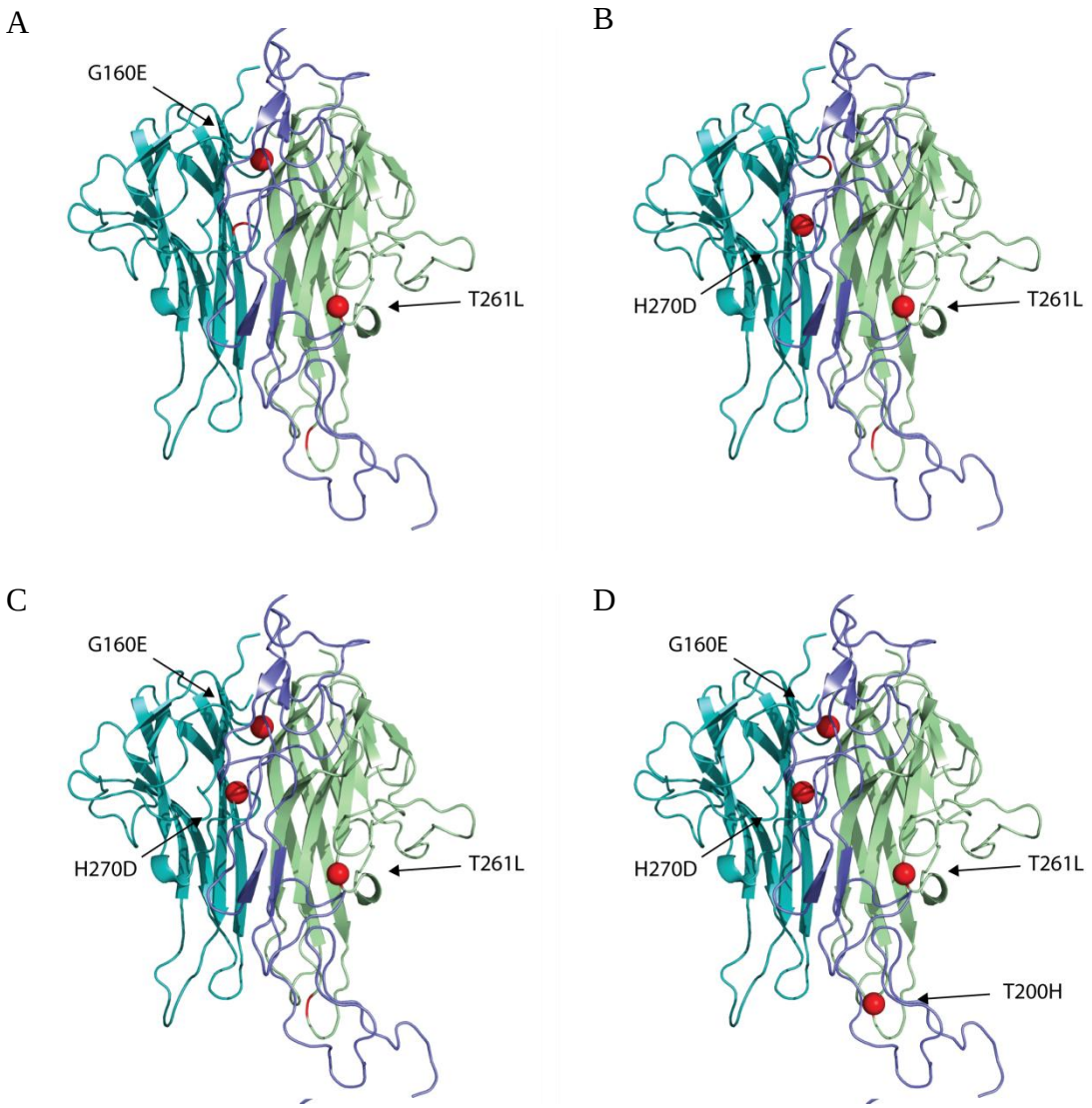


Figure 3.11 *Structural depiction of predicted mutations* TRAIL45-a (A), TRAIL45-b (b) TRAIL45-c (C) and TRAIL45-d (D), in complex with DR5.

3.6 TRAIL45 mutants retain their cytotoxic potential

In order to first investigate whether the new combination mutants retained their death-inducing potential, Colo205 cells were treated with the TRAIL45-a-d mutants. TRAIL45-c and TRAIL45-d showed activity similar to that of WT TRAIL while TRAIL45-a and TRAIL45-b displayed a significant increase in apoptotic activity, with an $ED_{50\text{-TRAIL45}}$:$ED_{50\text{-WT TRAIL}}$ ratio of 0.2 and 0.26 respectively (Figure 3.12).

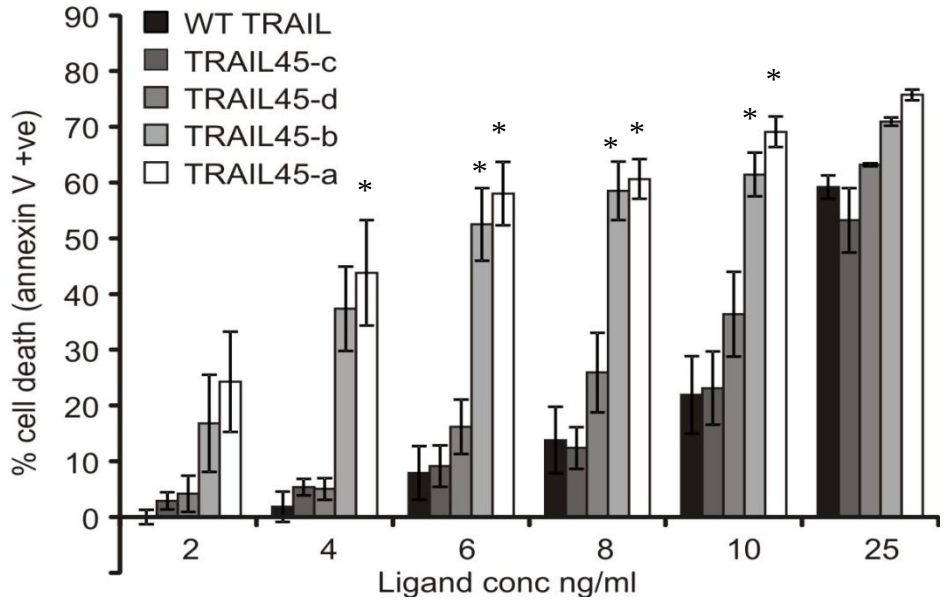


Figure 3.12. *TRAIL45-a and TRAIL45-b show strong apoptosis-inducing ability while mutants TRAIL45-c and –d show biological activity similar to WT TRAIL.* Biological activity of WT TRAIL and TRAIL45 mutants was tested in Colo205 cells at concentrations from 2-25 ng/ml. Induction of cell death was quantified by measuring percentage annexin V positivity following a 3 hr incubation period. The graph shows average percentage of annexin V positivity ± SEM from three independent experiments. $P < 0.05$

3.7 TRAIL45 mutants show no increase in binding affinity for the death receptors

Receptor binding of the combination TRAIL mutants to all four membrane bound TRAIL receptors was assessed in real time by Surface Plasmon Resonance (SPR). Apparent dissociation constants (K_D) were calculated based on pre-steady state response values.

Table 3.3: K_D ratios calculated relative to rhTRAIL WT as measured by Surface Plasmon Resonance as determined using a pre-steady state approach.

Abbreviation	**Mutations**	**K_D ratio (mutant/WT)**			
		DR4-Fc	DR5-Fc	DcR1-Fc	DcR2-Fc
TRAIL45-a	T261L; G160E	1.3 +/- 0.2	1.5 +/- 0.1	5.0 +/- 1.2	10.7 +/- 4.5
TRAIL45-b	T261L; H270D	1.9 +/- 0.8	1.7 +/- 0.5	4.7 +/- 0.3	8.5 +/- 2.8
TRAIL45-c	T261; G160E; H270D	6.5 +/- 0.3	7.4 +/- 2.0	13.3 +/- 4.6	35.4 +/- 13.4
TRAIL45-d	T261L; G160E; H270D; T200H	8.1 +/- 2.6	12.7 +/- 1.2	15.4 +/- 5.2	33.5 +/- 1.6

Both TRAIL45-a and TRAIL45-b variants show a nearly equivalent binding to DR4-Fc when compared to rhTRAIL WT. From these mutants, the largest reduction is observed by mutant TRAIL45-a binding to DcR2-Fc (~10-fold). A reduction in binding to DcR1-Fc was also observed, ranging from 4 to 5 fold decreased affinity for TRAIL45-b and TRAIL45-a, respectively. TRAIL 45-c and TRAIL45-d show the largest reduction in binding to DcR1-Fc and DcR2-Fc, with up to 35-fold lowered affinity as compared to rhTRAIL WT, as seen for TRAIL45-c binding to DcR2-Fc. However, this is also accompanied by a less pronounced, but significant reduction in death receptor affinity (between 6.5 and 12.7-fold lowered affinity to both DR4-Fc and DR5-Fc). The data in Table 3.3 was generated by our collaborators in the University of Groningen, The Netherlands.

To further investigate and confirm the binding capacity of the TRAIL mutants to the four TRAIL receptors, ELISA assays were carried out. Recombinant DR4, DR5, DcR1 or DcR2 was coated in increasing concentration to maxisorb plate wells and incubated with 25 ng/well WT TRAIL or TRAIL45-a-d. Unbound ligands were washed off and the amount of receptor-bound ligand was determined as described in Materials and Methods. TRAIL45-a and TRAIL45-b showed binding to DR4 similar to WT TRAIL, while TRAIL45-c and –d showed significant reduction in DR4 binding. All mutants showed a small reduction in binding to DR5 (Figure 3.13A and 3.13B).

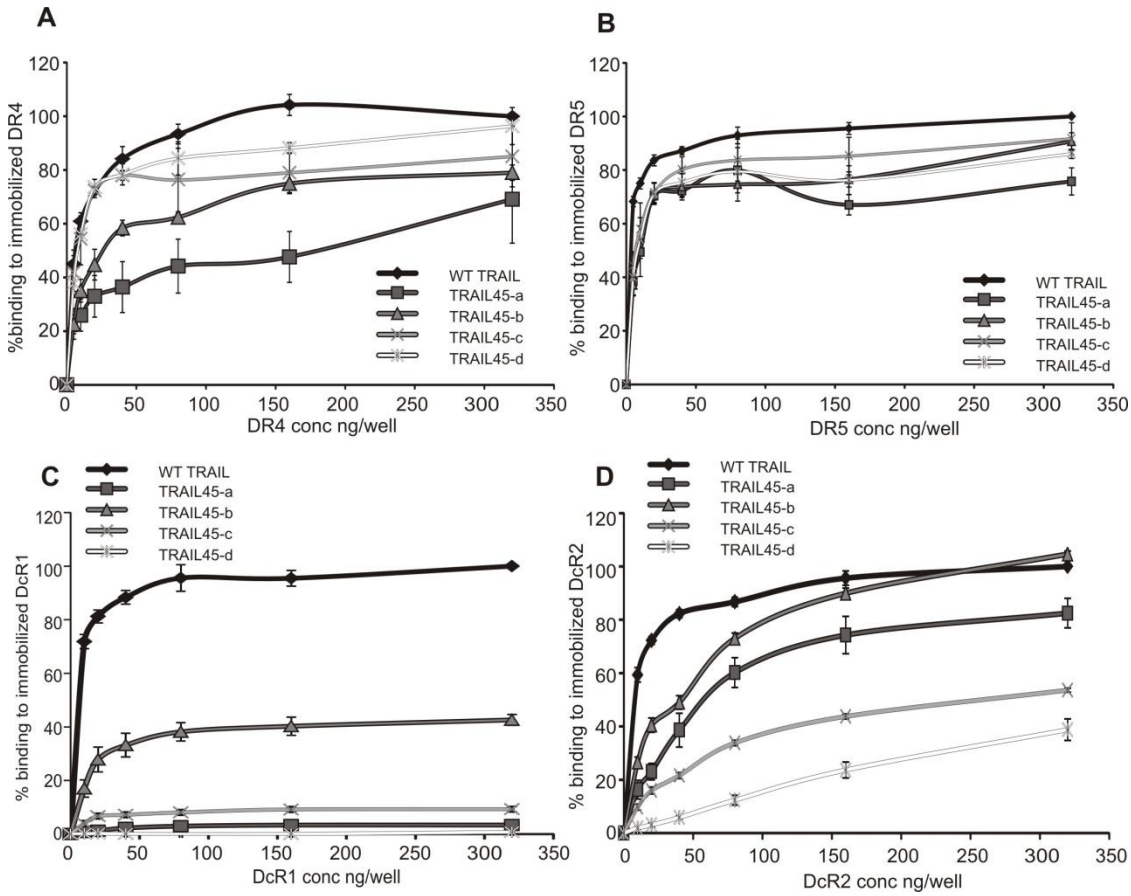


Fig 3.13. *Binding affinity of TRAIL45 mutants to DR4 and DR5 is comparable or reduced than WT TRAIL while decoy receptor binding is greatly reduced for all TRAIL45 mutants.* Binding of WT TRAIL and TRAIL mutants to immobilised DR4 (A), DR5 (B), DcR1 (C) and DcR2 (D) was tested by incubating 25 ng/well of WT TRAIL, or TRAIL45 mutants with increasing amount of Receptor-Fc fusion proteins coated to the wells for 30 minutes. Unbound ligands were removed by a series of washes before detecting the amount of ligand bound. Ligand binding was calculated by taking the value of the well with the 320 ng per well coated receptor as 100% and the value of the well with no receptor coated as blank. The graph shows the average of three independent experiments ± SEM.

The ELISA assays carried out to test binding of the combination mutants to DcR1 and DcR2 revealed that all four mutants had a substantial reduction in binding to the decoy receptors compared to WT TRAIL (Figure 3.13C and 3.13D) as was also observed by SPR (Table 3.3). In the case of TRAIL45-a at the lowest concentration of DcR1 receptor immobilized, a 100-fold decrease in binding to the receptor was evident compared to WT TRAIL. As the amount of decoy receptor bound to the well surface increased, this difference in binding was reduced to a 30-fold decrease compared to WT TRAIL. A substantial decrease could also be seen in binding to DcR2, however it

was not as striking as for DcR1; a 3.6 fold decrease was observed at the lowest concentration of receptor bound, which was reduced to a 1.2-fold decrease at the highest concentration of the receptor coated. TRAIL45-b showed a 5 fold reduction in binding to the immobilized DcR1, again as the receptor concentration increased the fold reduction decreased to 2.3. When DcR2 was immobilized to the plate we observed a 3-fold decrease in binding compared to WT TRAIL and as the receptor concentration reached the highest concentration of 320 ng/well, the difference in binding between the TRAIL45-b and WT TRAIL disappeared. The TRAIL45-c mutant displayed a decrease in binding to DcR1 up to 18 - 10 fold lower than that of WT TRAIL across the concentration range of bound receptor tested. Binding to DcR2 showed a 7 - 2 fold change in comparison to WT TRAIL from the lowest to the highest concentration of bound receptor, respectively. TRAIL45-d showed almost no binding to DcR1 by ELISA and also showed the biggest reduction of 31-fold to DcR2 (Figure 3.13 C and D).

While the decoy receptor binding properties of TRAIL45-d appear to be the best, this mutant displayed a significant reduction of DR4-binding and correlating reduction of pro-apoptotic potential. TRAIL45-a clearly stood out as the most promising mutant showing enhanced cytotoxic potential and a greatly reduced decoy receptor binding. This reduction in binding appears to be a direct result of the combination of the two single mutations, T261L and G160E, as the single mutants also have a reduced binding to the decoys as examined by ELISA (Figure 3.14). It appears from these studies that the effect of the two single mutations on decoy receptor binding had an additive effect, as the combination mutant showed a much bigger reduction in decoy receptor binding capacity.

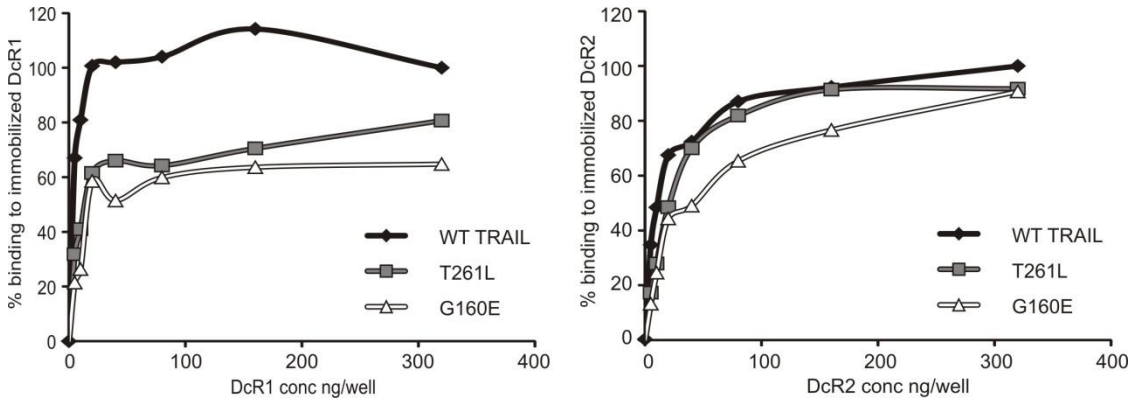


Figure 3.14. *T261L and G160E both have reduced binding to the decoy receptors.* Binding of WT TRAIL and TRAIL mutants T261L and G160E to immobilised DcR1 and DcR2 was tested by incubating 25 ng/well of WT TRAIL, or TRAIL mutants with increasing amount of receptor-Fc fusion proteins coated to the wells for 30 minutes. Unbound ligands were removed by a series of washes before detecting the amount of ligand bound. Ligand binding was calculated by taking the value of the well with the 320 ng per well coated receptor as 100% and the value of the well with no receptor coated as blank.

Based on these findings, the increase in cytotoxic potency cannot be attributed to an increase in death receptor affinity, but likely to be due to the reduced binding to the decoy receptors.

3.8 TRAIL45 mutants can activate both DR4 and DR5

To investigate the ability of the combination mutants to activate DR4 and DR5, the same approach as with the single mutants was taken (Figure 3.15 and Figure 3.16). The results showed that all the mutants could bind to and activate both DR4 and DR5 (in ML-1 and Jurkat cells respectively) with TRAIL45-a being the most potent inducer of apoptosis, closely followed by TRAIL45-b. Both of these mutants could potently induce apoptosis in ML-1 cells to a level far surpassing the abilities of WT TRAIL (Figure 3.15A and B) while TRAIL45-a also showed superior induction of cell death in Jurkat cells compared to WT TRAIL (Figure 3.16A). TRAIL45-c and –d were also capable of activating both DR4 and DR5, but with an activity similar to or lower than that of WT TRAIL (Figure 3.15 and Figure 3.16, C and D).

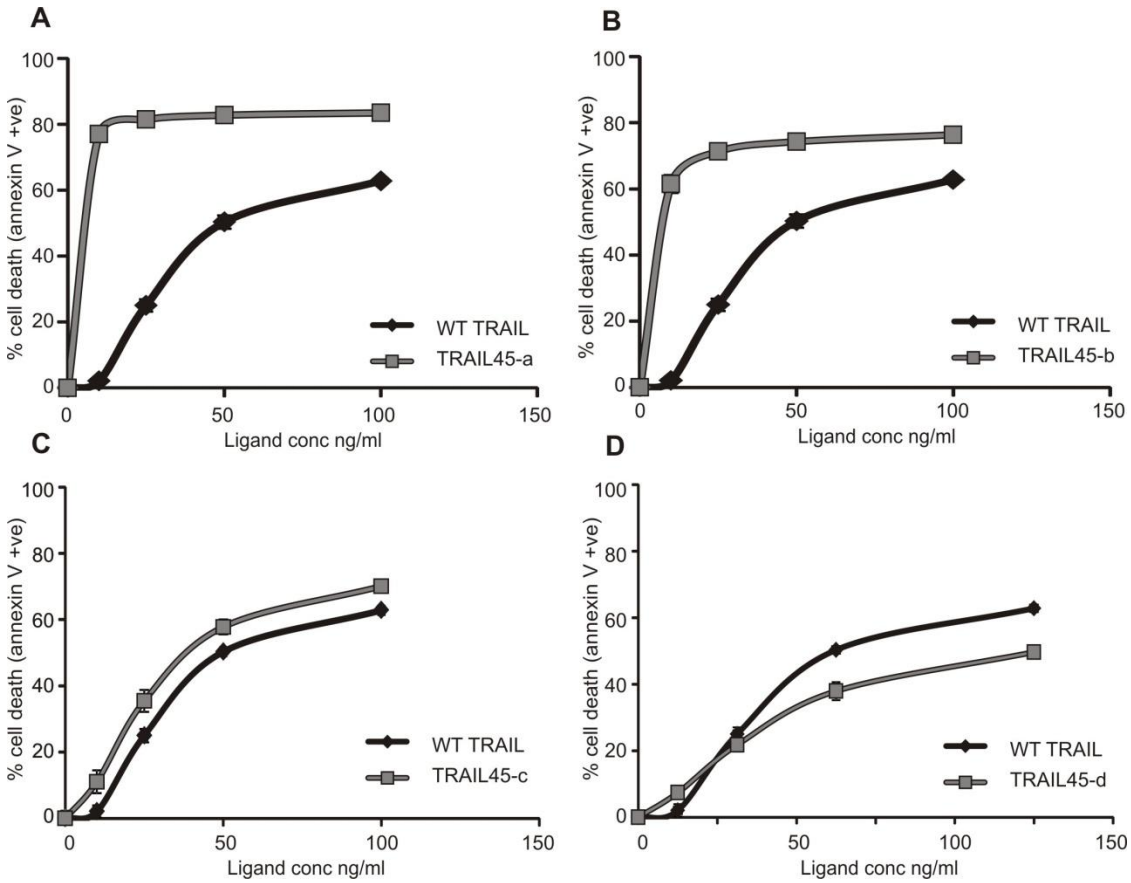


Figure 3.15. *TRAIL45-a and -b mutants retain DR4-agonistic activity comparable to that of WT TRAIL.* Induction of cell death via DR4 in ML-1 cells by TRAIL45 mutants. Cells were treated with the indicated doses of WT TRAIL or TRAIL45 mutants for 24 h. Induction of cell death was quantified as percentage of annexin V positive cells determined by flow cytometry. The graphs show average cell death ± SEM from three independent experiments.

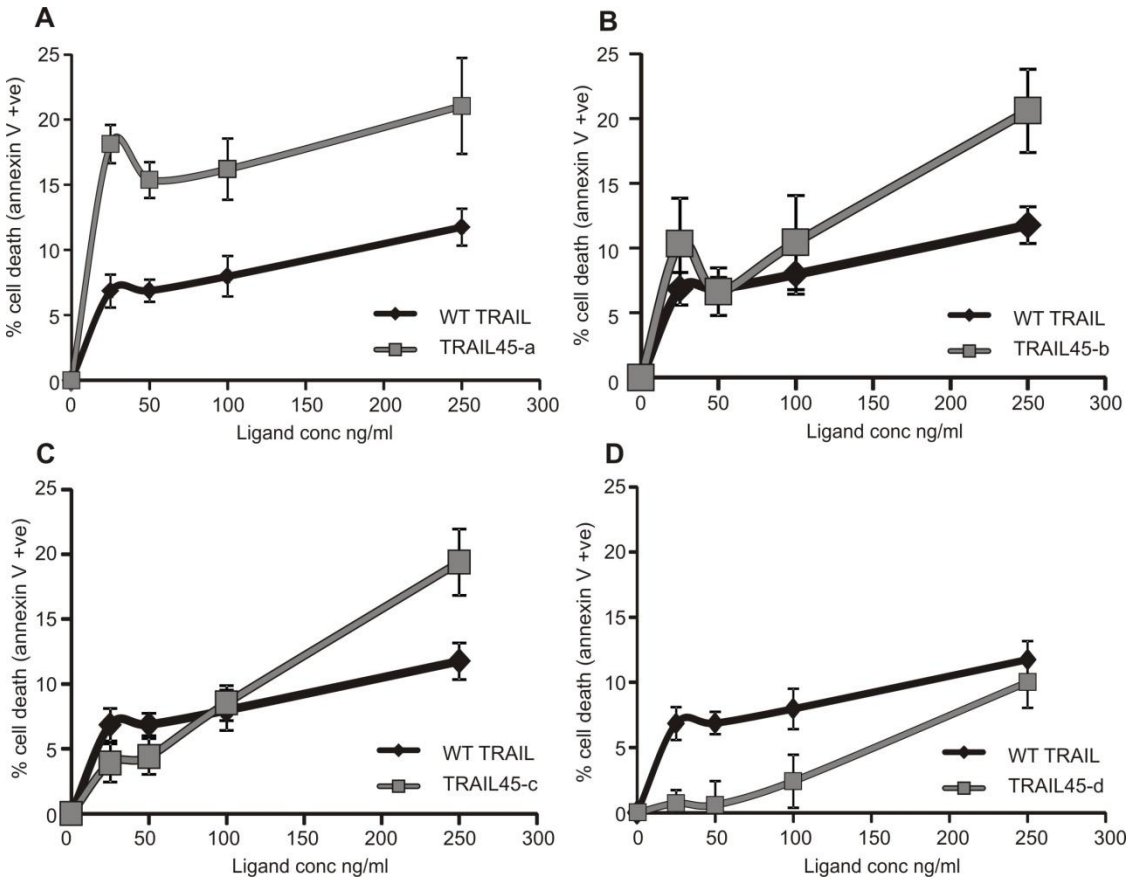


Figure 3.16. *TRAIL45-a and -b mutants retain DR5-agonistic activity comparable to that of WT TRAIL.* Induction of cell death via DR5 was investigated in Jurkat cells with annexin V staining. Cells were treated with the indicated doses of WT TRAIL or TRAIL45 mutants for 24 h. Induction of cell death was quantified as percentage of annexin V positive cells determined by flow cytometry. The graphs show average cell death ± SEM from three independent experiments.

3.9 TRAIL45 mutants can escape the antagonistic effects of decoy receptors

Finally, the ability of the TRAIL45 mutants to avoid DcR-mediated inhibition was studied by inhibiting DcR1 and DcR2 expressed on the surface of Colo205 cells using neutralizing antibodies. While neutralization of DcR1 and DcR2 enhanced TRAIL-mediated apoptosis as expected, it had no effect on the pro-apoptotic potency of TRAIL45-a, -c and -d indicating that these mutants do not bind to the decoy receptors (Figure 3.17). These results also mirror the results gained with the ELISA assays

(Figure 3.13C and 3.13D) where TRAIL45-b showed the least reduction in binding to the decoy receptors.

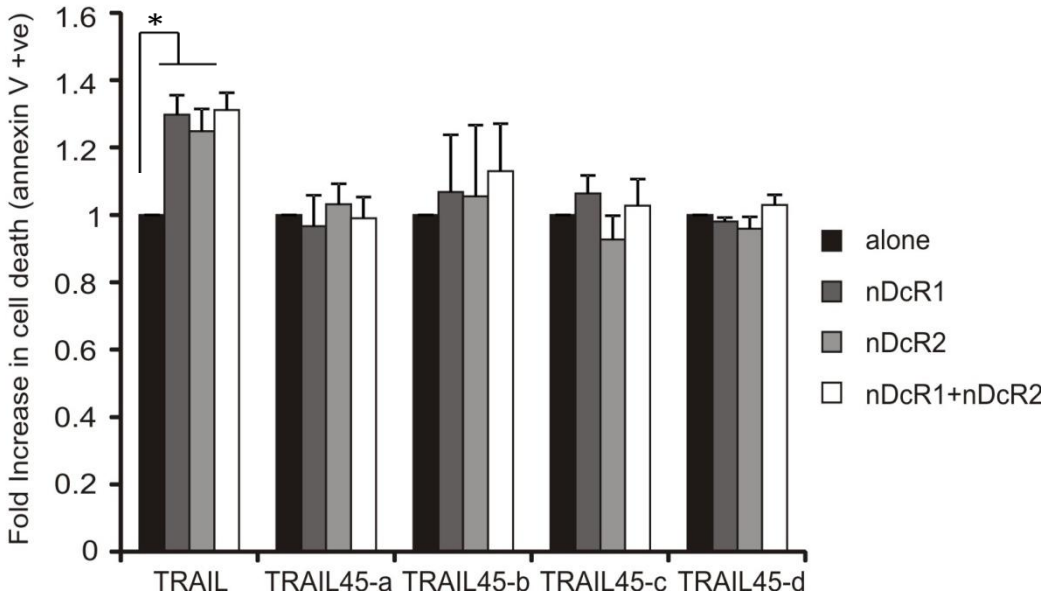


Figure 3.17. *The effect of decoy receptor neutralisation on the apoptosis-inducing activity of TRAIL45 mutants.* Colo205 cells were treated with 2 µg/ml of DcR1 and/or DcR2 receptor-neutralizing antibodies for one hour prior to treatment with the ED_{50} dose of WT TRAIL or TRAIL45 mutants. Induction of apoptosis was measured 3 hours after treatment using annexin V staining and flow cytometry. An increase in apoptosis in response to decoy receptor neutralization is represented as fold increase compared to the level of apoptosis induced by the ligands in the absence of the neutralizing antibodies.

3.10 Decoy receptors greatly reduce the efficacy of WT TRAIL

We showed that the decoy receptors expressed at endogenous levels in tumour cells, such as Colo205 cells can significantly reduce TRAIL-induced apoptosis. Previously in the lab we have screened an array of 25 tumour cell lines for the expression of the four membrane-bound TRAIL receptors on their surface and we found that 75% of the partially resistant cell lines have very high DcR1 or DcR1+DcR2 expression (data not shown).

To study whether the high DcR1/2 expression contributes to the reduced TRAIL sensitivity in these cells, representative cell types, namely MDA-MB-231, DLD1, HL-60 and HCT116 cells were treated with WT TRAIL and decoy-insensitive TRAIL

mutants, T261L and TRAIL45-a. All cell types showed increased sensitivity towards the TRAIL mutants compared to WT TRAIL, indicating that high DcR expression in the tumour cells correlates with reduced TRAIL sensitivity (Figure 3.18A-E). The TRAIL resistant cell line T47D, also expressing high levels of DcRs was also treated with WT TRAIL and the same decoy-insensitive TRAIL mutants; however, these cells did not show increased sensitivity towards TRAIL45-a indicating that alternative, or additional mechanisms of resistance exist in these cells (Figure 3.18F).

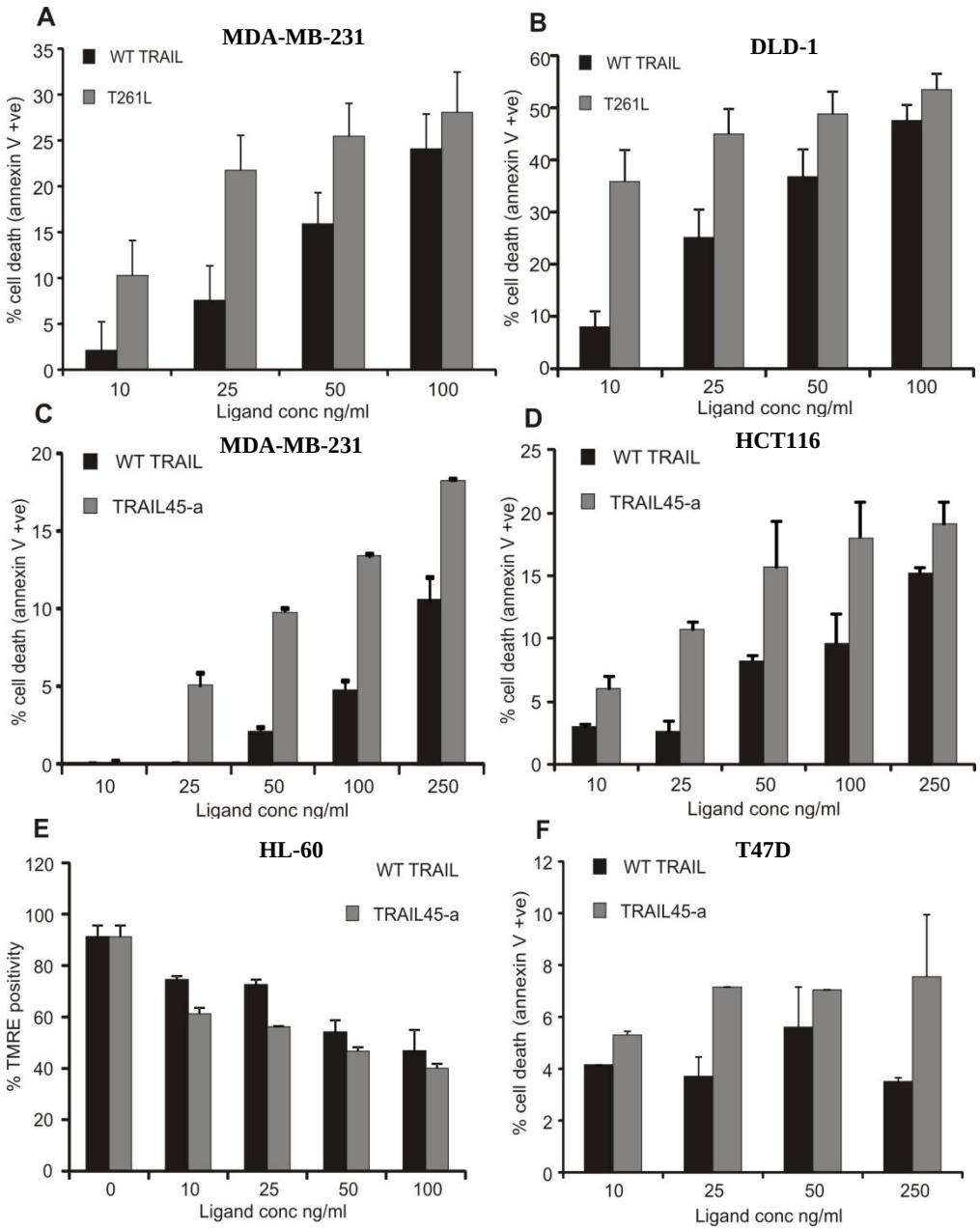


Figure 3.18. *Decoy-insensitive TRAIL mutants can potently induce apoptosis in cell lines with high decoy receptor expression.* Cell lines partially resistant to WT TRAIL with high DcR expression, MDA-MB-231 cells (A and C), DLD-1 cells (B), HCT116 cells (D) HL-60 cells (E) and (F) the TRAIL resistant T47D cells were treated with either WT TRAIL, T261L or TRAIL45-a at the indicated doses for 24 h and induction of cell death was measured by annexin V or in the case of HL-60, mitochondrial membrane potential was examined with TMRE staining. Induction of cell death was quantified as percentage of annexin V positive or TMRE negative (HL-60) cells determined by flow cytometry. The graphs show average cell death ± SEM from two or three independent experiments.

To further model the potency of DcRs to block TRAIL-induced apoptosis in tumour cells, Jurkat cells overexpressing either empty vector (EV), DcR1 or DcR2 have been utilised (Morizot *et al.*, 2011). While the pro-apoptotic effect of WT TRAIL dropped by approximately 50% in both the DcR1- and DcR2-overexpressing Jurkat cells, all

four TRAIL45 mutants retained their full efficacy, while only TRAIL45-b showed a slight reduction in cell death in the DcR2 overexpressing Jurkat cells (Figure 3.19), which is in keeping with previous findings that this mutant still retains some binding to the decoy receptors. This provides evidence that DcRs present on the same membrane as DR4 and/or DR5 exert a significant inhibitory effect; however these DcR-insensitive TRAIL mutants escape DcR-mediated regulation and maintain high efficacy.

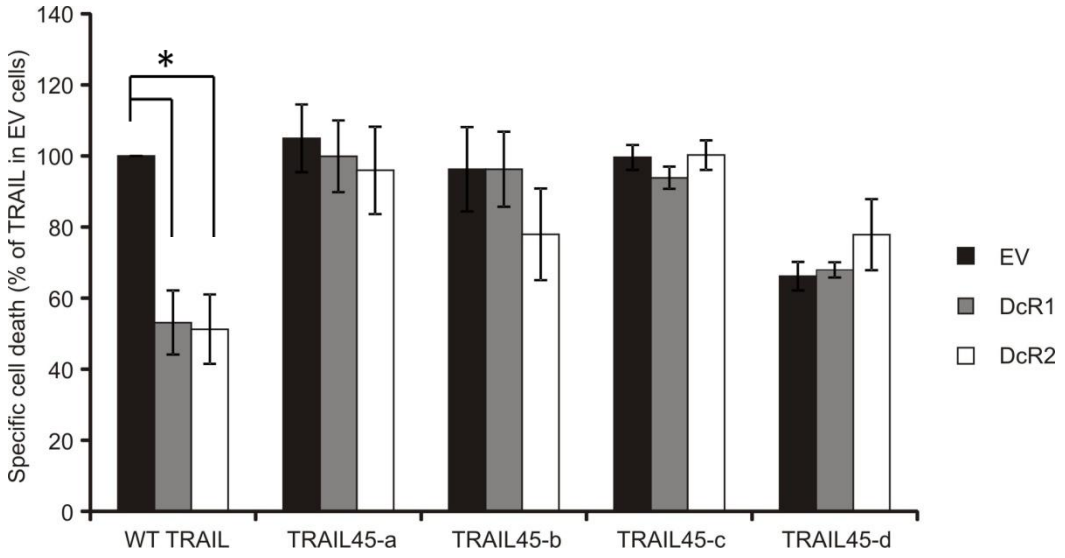


Figure 3.19. *Expression of decoy receptors by cancer cells can greatly reduce the efficacy of WT TRAIL, but not of the TRAIL45 mutants.* Jurkat cells expressing either an empty vector (EV), DcR1 or DcR2 were treated with either WT TRAIL or TRAIL45 mutants at the doses indicated for 24h and cell death measured by annexin V and flow cytometry. The graph depicts percentage of cell death relative to WT TRAIL treated EV sample ± SEM from three independent experiments.

3.11 DcR-insensitive TRAIL mutants are not toxic to human non-transformed cells

In order to determine whether loss of binding to the decoy receptors and the evident increase in potency of these mutants would result in toxicity in non-transformed cells, primary human dermal fibroblasts were treated with WT TRAIL or the TRAIL45 mutants at concentrations ranging from 25-250 ng/ml. No significant induction of cell death was observed for any of the DcR-insensitive TRAIL mutants (Figure 3.20). In

addition to hFB, primary human keratinocytes, known to be moderately sensitive to WT TRAIL have also been tested. Human primary keratinocytes did not show increased sensitivity towards the DcR-insensitive TRAIL mutants either, confirming that the decoy receptors expressed on the surface of non-transformed cells are not the sole factors maintaining their TRAIL resistance and also alluding to the safety of these TRAIL mutants *in vivo.*

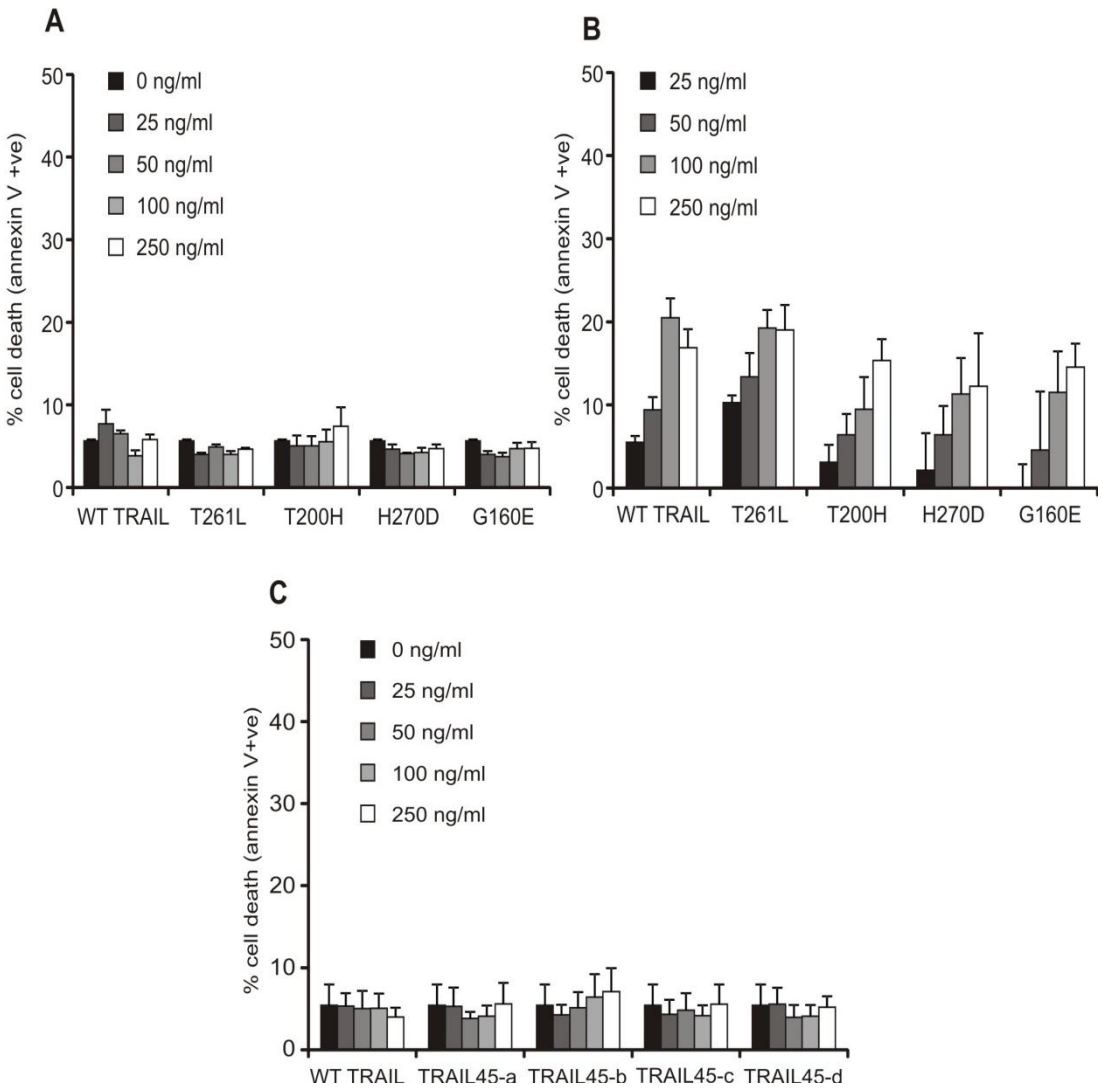


Figure 3.20. *Decoy-insensitive TRAIL mutants do not exert any cytotoxic effects in non-transformed cells.* Apoptosis-inducing activity of WT TRAIL and decoy-insensitive TRAIL mutants in primary, non-transformed human fibroblasts (hFb) (A and C) and in primary human keratinocytes (B). hFB and keratinocytes were treated with the stated doses of WT TRAIL or TRAIL mutants for 24 h. Induction of cell death was quantified as percentage of annexin V positive cells determined by flow cytometry. The graphs show average percentage of cell death ± SEM from three independent experiments.

The effect of DcR expression on TRAIL efficacy *in vivo*, especially DcR expression by normal somatic cells in the body or in the stroma surrounding the tumour has not been examined in the literature largely due to the lack of suitable animal models. Mouse models are suitable to study the function of DR4 and DR5, as the mouse death-inducing TRAIL receptor, mTRAIL-R, is homologous to human DR4/DR5 (Wu *et al.*, 1999). On the contrary, while similarly to humans, two TRAIL decoy receptors exist in mice, they are barely homologous to the human DcRs. To demonstrate that the DcRs expressed by non-transformed cells surrounding tumour cells significantly reduce the cytotoxic potency of WT TRAIL, we have developed a co-culture system. This system aims to model the environment that the ligands may encounter *in vivo*, in the microenvironment of the tumour itself. In this model, primary human dermal fibroblasts (hFb) expressing empty vector (EV), or modest levels of DcR1 or DcR2 together with the red fluorescent protein (RFP, to enable separation of the co-cultured cell types during analysis), were cultured in direct contact with Colo205 cells in a 1:1 cell ratio. The expression level of the decoy receptors on the hFB cells is shown in Figure3.21. The direct co-culture of cells was then treated with WT TRAIL or the TRAIL45 mutants and induction of cell death in the tumour cells was measured by annexin V staining (Figure 3.22).

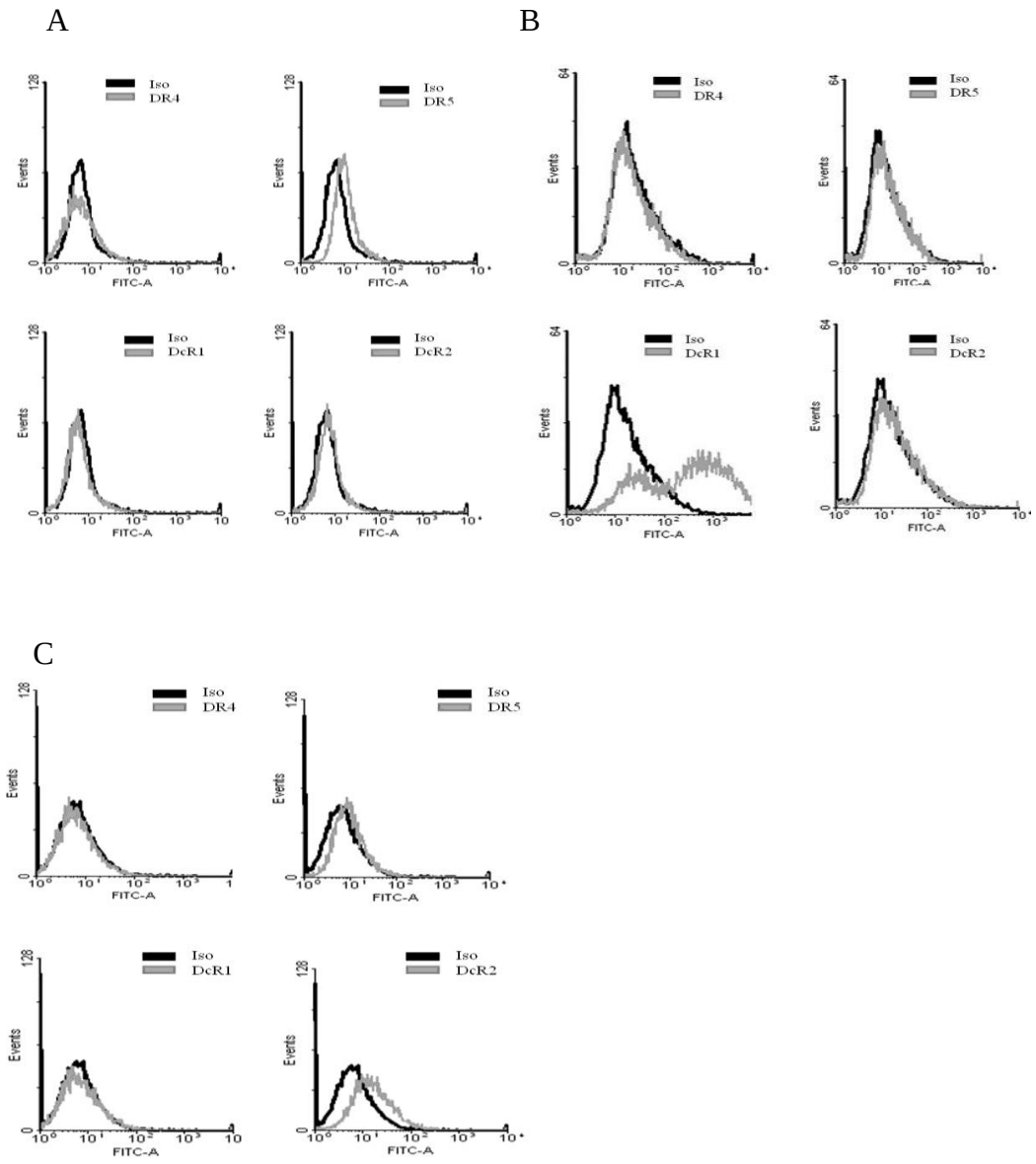


Figure 3.21. *Expression of TRAIL receptors on human primary dermal fibroblasts (hFb) overexpressing DcR1 or DcR2.* Cell surface expression of TRAIL receptors on hFb transduced with a lentivirus overexpressing (A) red fluorescent protein (RFP, empty vector, EV), (B) DcR1 or (C) DcR2. Histograms depicts TRAIL receptor expression on the cell surface of each transduced hFb cell line analysed by flow cytometry. The histograms are representative of 3 independent experiments.

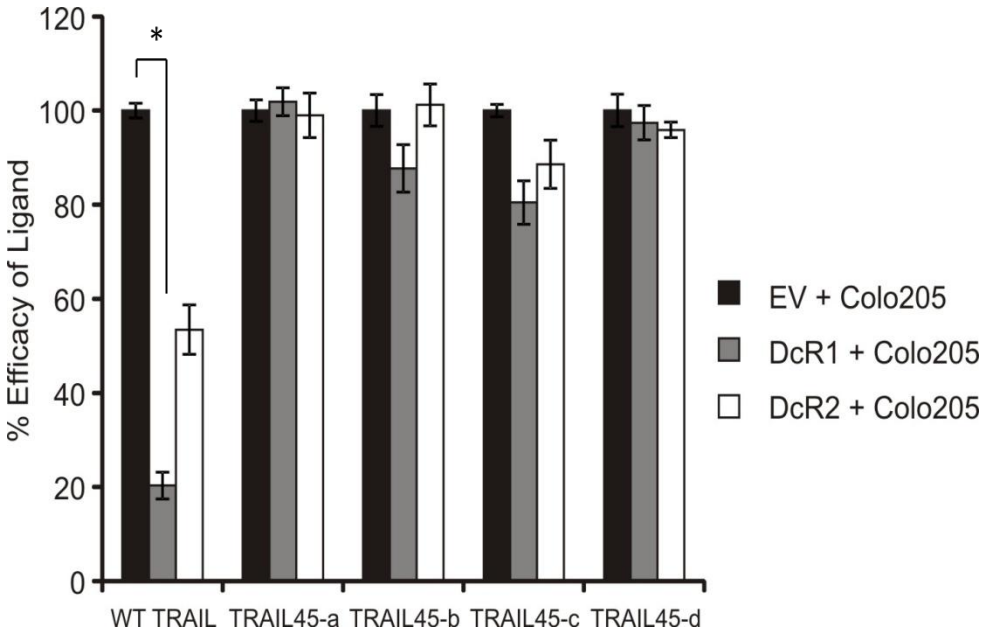


Figure 3.22. *Expression of decoy receptors by neighbouring non-transformed cells can greatly reduce the efficacy of WT TRAIL, but not of the TRAIL45 mutants.* Colo205 cells were cultured together with hFb expressing an empty vector (EV), DcR1 or DcR2 along with red fluorescent protein (RFP) to allow the non-transformed cells to be distinguished from the colon carcinoma cells. The cells were treated with WT TRAIL or TRAIL45 mutants at a concentration of 25 ng/ml for a period of 3 h and induction of cell death was measured in the RFP negative Colo205 cells by annexin V staining and flow cytometry. The graph shows the efficacy of the ligand relative to that of treated Colo205 cells co-cultured with hFb expressing EV ± SEM from three independent experiments.

Expression of DcR1 reduced the efficacy of WT TRAIL by approximately 5-fold whereas an increase in DcR2 expression caused a 2-fold reduction in efficacy of WT TRAIL (Figure 3.22). On the other hand, TRAIL45-a and –d were completely insensitive to the antagonistic effects of the decoy receptors on the non-transformed cells. While TRAIL45-c did not show any inhibition mediated by the presence of endogenously expressed decoy receptors on the Colo205 cells (Figure 3.17), when DcR1 and DcR2 were expressed on the hFb cells surrounding the tumour cells, they could slightly reduce the efficacy of TRAIL45-c. This was also the case for TRAIL45-b which also showed minimal binding to the decoy receptor in previous results (Figure 3.17 and 3.19), in this instance the efficacy of TRAIL45-b seemed to be slightly reduced by DcR1 expression on the hFb cells. However, this reduction was very small compared to the effect of the decoy receptors on the activity of WT TRAIL.

To further demonstrate the potential of the decoy receptors in blocking the *in vivo* efficacy of WT TRAIL, we used an *in vitro* three dimensional (3D) tumour model (shared with us by Prof Gillian Murphy, Cancer Research UK, Cambridge Research Institute, and modified by us). We generated tumour mini-spheroids by which we aimed to better mimic the cellular composition and structure of tumours. The minitumours are comprised of endothelial cells (HUVEC), stromal cells (hFB) and MDA-MB-231 breast cancer cells embedded in a methylcellulose matrix.

Each minitumour consisted of approximately 1000 cells and had a spherical shape of 30-50 μm in diameter. To trace the tumour cells in the spheroids, they were labelled with a long-term cell tracker, CM-DiI which is a membrane-incorporating, red-fluorescent dye. The structure of the tumour spheroids seen with light- and with fluorescent microscopy is shown in Figure 3.23. To detect apoptotic cells, 2 fluorescent labelling methods have been tested. The first dye was the cell permeable, fluorescent caspase-substrate, NucView (green fluorescence). Of note, generation of minitumours with Colo205 cells has also been attempted, but probably due to the semiadherent nature of these cells, they tended to grow outside of the spheres.

NucView penetrates into the nucleus and emits green fluorescence upon cleavage by caspases and thus, it is well suited for *in situ* imaging. The second method tested was the classical Annexin V labelling of dying cells. This method however has some disadvantages. Annexin V has limited penetrability deep in the tumour spheres, and thus, for detection of cell death with Annexin V, the tumour spheres had to be dismantled by tryptic digestion. Secondly, it only stains the cell membrane and thus the disintegrating apoptotic cells may be difficult to be visualised.

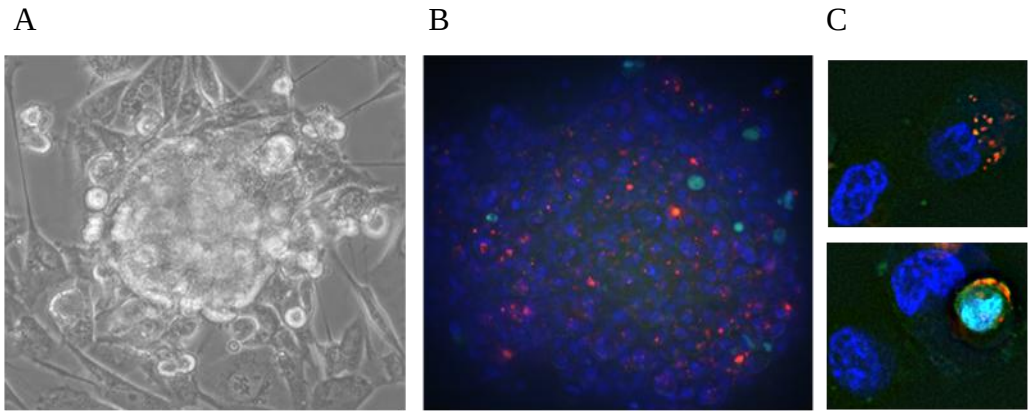


Figure 3.23*. Optimisation of the tumour-sphere model.* Minitumours consisted of endothelial cells (HUVEC), breast cancer cells (MDA-MB-231) and stromal cells (human primary fibroblasts). Structure of the tumour spheroids as seen with light (A). (C) Visualisation of NucView positivity in apoptotic tumour cells. The MDA-MB-231 tumour cells in the spheres were stained with a red cell-tracker (CM-DiI) and all cells were stained with 1µg/ml of Hoechst33342. The spheres were treated with WT TRAIL as in part B and the apoptotic cells labelled with NucView. The image shows an example of a live tumour cell (top image, blue nucleus with red-stained internal membrane elements) and an apoptotic tumour cell (bottom image), with the green NucView signal in the fragmenting nucleus. Images are representative of two independent experiments and were generated on an Optigrid structured illumination microscope with Volocity 3D/4D image capture software, and 40x magnification. Z-stacks were taken to image the spheroid in its entirety. Image processing involved deconvolution of stacked image using the Volocity software.

In order to determine whether NucView staining reliably and quantitatively detects cell death, tumour spheres were treated with the chemotherapeutic drug, cytarabine (AraC) or WT TRAIL for 24 h after which NucView (1µM) was added to the tumour spheres. MDA-MB-231 cells have been reported to be highly resistant to AraC (Daly *et al.*, 1990; Ma *et al.*, 2011) and thus this treatment was used as a negative control. At the end of the treatment the tumour spheres were separated into individual cells by tryptic digestion and the percentage of NucView positive cells was determined by flow cytometry. In parallel samples, the percentage of apoptotic cells was determined using Annexin V staining (Figure 3.24).

The percentage of NucView positive cells was found to be comparable to the percentage of Annexin V positive cells in the same treatment groups, confirming that NucView is a specific and suitable dye to visualise apoptotic cells in the tumour spheres (Figure 3.24). Fluorescent microscopy was also carried out to confirm that the NucView positivity appears in the nuclei of dying tumour cells. To this end, the

NucView staining has been combined with Hoechst33342 labelling of the nuclei on WT TRAIL-treated tumour spheres. The gained microscopic images confirmed that the green fluorescence of the NucView was preferentially present in the nuclei of the MDA-MB-231 cells (identified by the presence of the red fluorescence of CM-DiI cell tracker) and it was only visible in cells that also displayed nuclear fragmentation (Figure 3.23C).

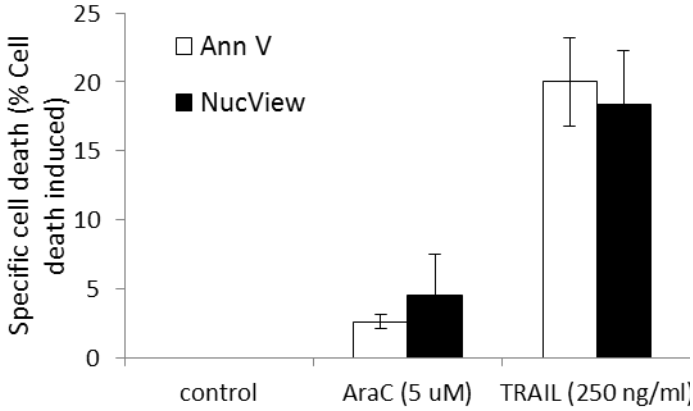
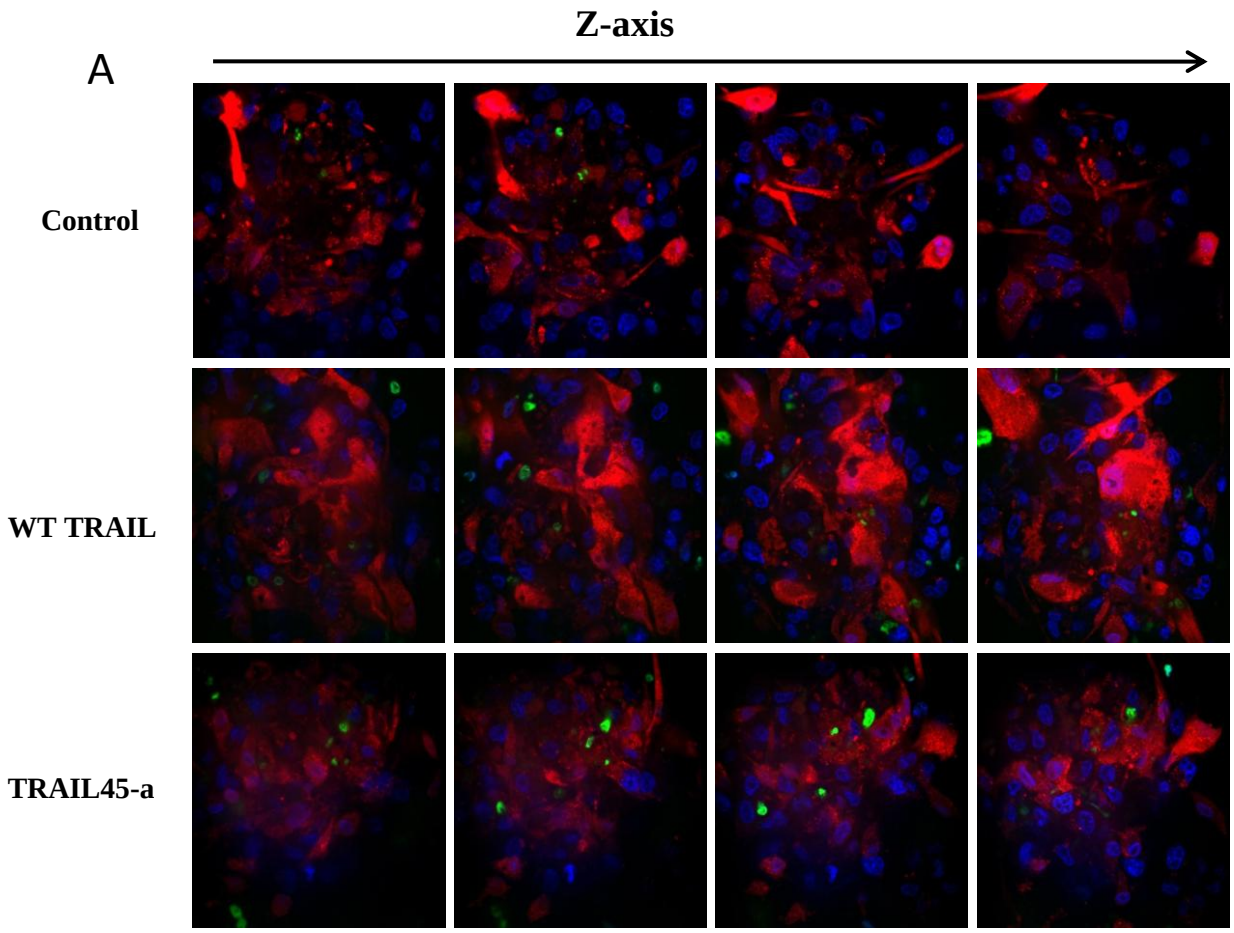
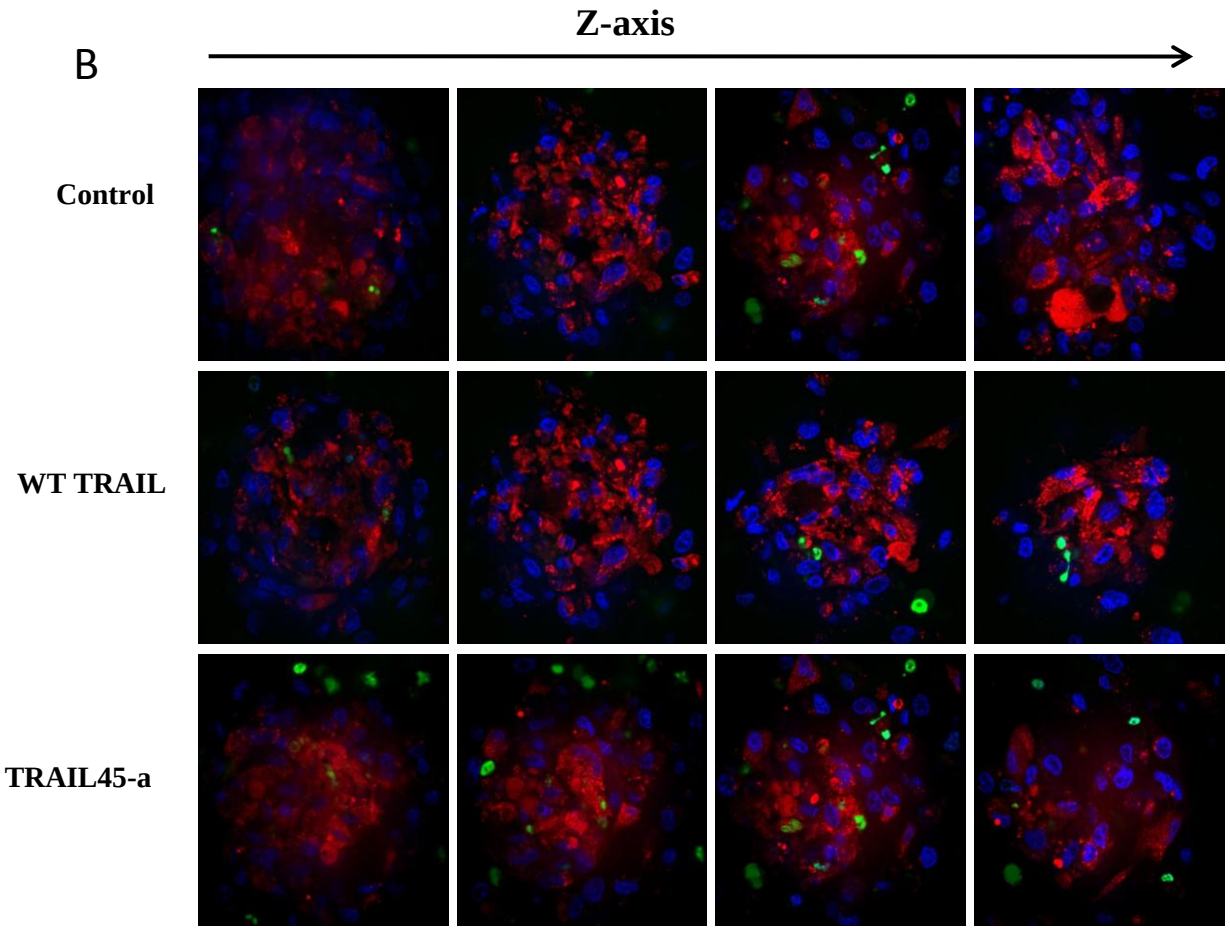


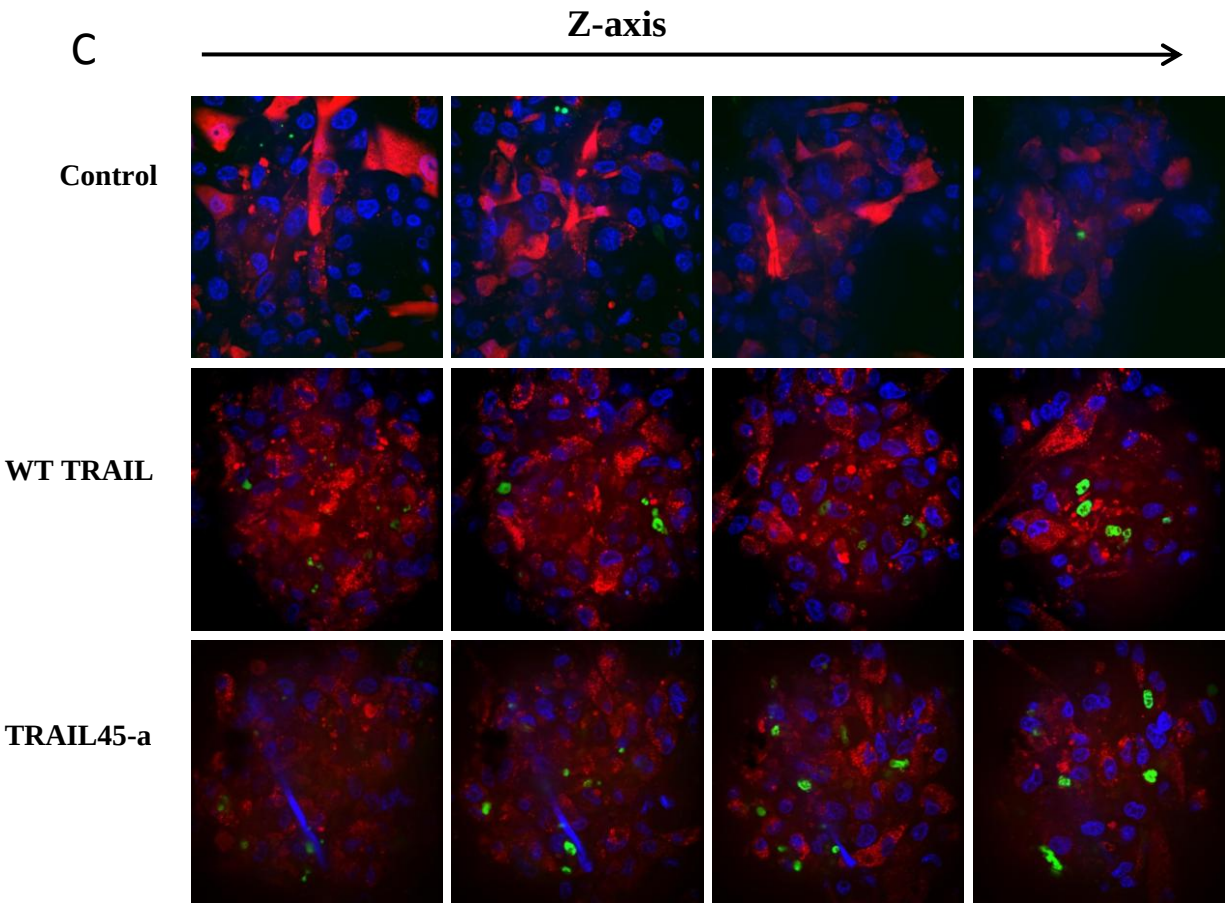
Figure 3.24 *Determination of apoptosis-induction using NucView versus Ann V*. Tumour spheres were treated with 5 μM Cytarabine (AraC) or WT TRAIL (250 ng/ml) for 24 h after which the cells of the spheres were either stained with the fluorescent caspase-3 substrate, NucView (1 μM for 1 h) or left untreated. The cells of the spheres were separated and the samples that have not been labelled with NucView were stained with Annexin V-FITC. The percentage of apoptotic cells indicated by NucView or Annexin V was determined by flow cytometry.

However when changing to the more powerful Andor Revolution Spinning Disk Confocal system we experienced problems with overlapping of the TRITC (red) signal and the FITC (green) signal leading us to substitute NucView for SYTOX green nucleic acid dye (Invitrogen) which readily penetrates membrane compromised cells giving a very strong fluorescence. As we intended to use the decoy receptor expressing fibroblast cells which also expressed RFP we dual stained with a red cell tracker to ensure all fibroblasts would be identifiable. The cell tracker CM-DiI was changed for CMTPX a red cell tracker (Invitrogen) with a more diffuse staining allowing easy identification of non-transformed cells in the spheroid.

To analyse the importance of decoy receptor-insensitivity for the efficacy of TRAIL in this minitumour model, we substituted parental fibroblasts for fibroblasts expressing DcR1 and DcR2 in the minitumours. We could show that WT TRAIL as well as TRAIL45-a could penetrate into the minitumour and kill the breast cancer cells. Cell death induced by WT TRAIL was markedly reduced when hFB in the minitumour expressed DcR1 and DcR2, with DcR1 expression causing the most significant reduction (Figure 3.25 D). In addition to this HUVEC cells also express DcR1 (Chen and Easton, 2008) which also may have contributed to the strong reduction in efficacy of WT TRAIL. On the other hand, expression of the decoy receptors did not reduce the efficacy of TRAIL45-a; and efficient killing of the tumour cells could only be achieved with the DcR-insensitive TRAIL variant. These results mirror those obtained in the monlayer co-culture experiments of colo205 cells mixed with the decoy receptor-expressing fibroblasts, indicating that these TRAIL mutants may have potential *in vivo*.




B
Z-axis
Control
WT TRAIL
TRAIL45-a


C
Z-axis
Control
WT TRAIL
TRAIL45-a

D

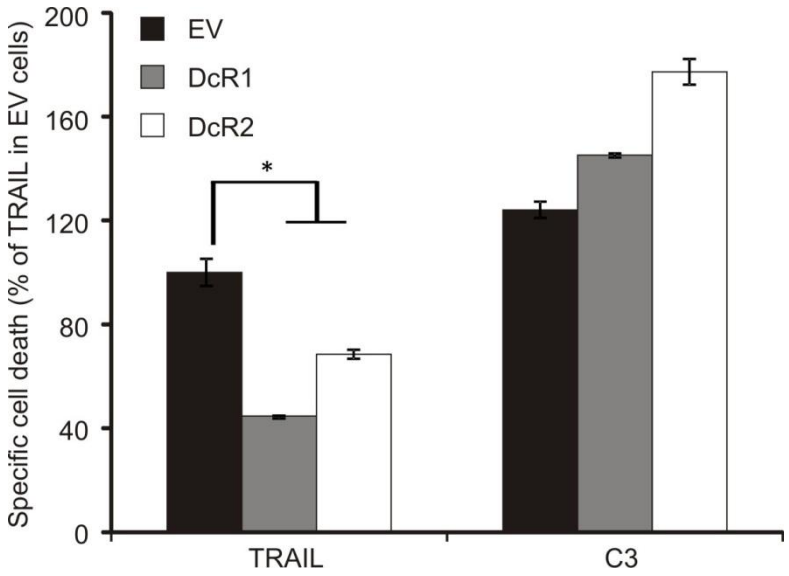


Figure 3.25. *TRAIL45-a retains its cytotoxic efficacy in 3D minitumours expressing EV, DcR1 and DcR2.* Minitumours expressing either EV (A), DcR1 (B) or DcR2 (C) were treated with 250 ng/ml of either WT TRAIL or TRAIL45-a for 24 h, after which 1µM of SYTOX green was added for 1 h to visualise cells with compromised plasma membranes, all cells were also stained with 1µg/ml of Hoechst33342. Images are representative of four independent experiments and were generated on the Andor Revolution Spinning Disk Confocal system at 40x magnification. Z-stacks were taken to image the spheroid in its entirety, with an image captured every 0.2µm. Image analysis (D) was carried out using ImageJ software and calculating % area occupied by a particular population, cell death was then expressed as percentage of cell death relative to WT TRAIL treated EV sample ± SEM from four independent experiments. * = $P<0.05$, ** = $P<0.005$

Chapter 4:Discussion

4.1. Discussion

In this study we have highlighted the potential value of a death-receptor specific TRAIL mutant with the ability to evade the antagonistic effects of decoy receptors. We sought not only to demonstrate that these receptors can cause a real reduction in the efficacy of TRAIL when expressed on cancer cells but also to prove that the expression of these receptors on normal cells of the body can reduce the efficacy of this ligand.

Table 4.1 Summary of findings for both single and combination mutants identified compared to WT TRAIL.

	Apoptosis-inducing ability Colo205	**Activity in DR4-responsive ML-1 cells**	**Activity in DR5-responsive Jurkat cells**	**Binding to DcR1**	**Binding to DcR2**
G160E	<	<	>	<	<
T200H	>	<	>	=	=
T261L	>	=	>	<	<
H270D	>	<	>	<	<
TRAIL45-a	>	>	>	<	<
TRAIL45-b	>	>	>	<	<
TRAIL45-c	>	>	<	<	<
TRAIL45-d	>	=/<	<	<	<

Through the use of computational design we have identified four point mutations in WT TRAIL that results in reduced decoy receptor binding while retaining the dual agonistic activity towards DR4 and DR5. These mutations are: G160E, T200H, T261L and H270D. Rational combination of these single mutations was found to enhance these favourable characteristics; with the combination of the mutations T261L and G160E resulting in the best characteristics combining potency with evasion of the decoy receptor binding. The double mutant T261L/G160E (named: TRAIL45-a) showed diminished binding to DcR1 and an 8-fold reduction in DcR2-binding (Figure 3.13), and enhanced pro-apoptotic activity compared to WT TRAIL through either DR4 or DR5 (Figure 3.12). In the DR4-responsive cell line ML-1 the single mutants T261L and G160E only induced apoptosis to a level lower than or equal to WT TRAIL

(Figure 3.9), however on combining these two mutations in one mutant we saw a dramatic increase in apoptosis in this cell line compared to WT TRAIL and both single mutants (Figure 3.15). The same was true for combining T261L and H270D (Figures 3.9 and 3.15). We observed that the mutations in all four TRAIL45 mutants did not increase, but rather slightly reduced the affinity toward DR4 and DR5 (Figure 3.13), highlighting that the increased pro-apoptotic potential must be due to the abolished interaction between the TRAIL mutants and DcR1/DcR2. Blocking the decoy receptors' ability to sequester TRAIL or to complex with the death-inducing receptors upon exposure to the ligand and thus form non-responsive receptor oligomeres resulted in increased efficacy of WT TRAIL, reiterating the findings of other groups that decoy receptors regulate the function of the death-inducing receptors in tumour cells. The generated decoy-insensitive mutants, TRAIL45-a, -c and -d on the other hand could escape the inhibitory effect of the decoy receptors as their neutralization did not affect the efficacy of these mutants (Figure 3.17). Ectopic expression of the decoy receptors has been demonstrated to vastly reduce TRAIL-induced apoptosis or even diminish the effect of WT TRAIL completely. Utilising Jurkat cells overexpressing either DcR1 or DcR2, we could confirm these antagonizing effects of the decoy receptors by showing that in both DcR1- and DcR2-overexpressing Jurkat cells, the pro-apoptotic potency of WT TRAIL was reduced by approximately 50%. Again, the pro-apoptotic capacity of the TRAIL45 mutants was unaffected and they retained full efficacy with the exception of TRAIL45-b which showed a slight reduction in efficacy in the case of the DcR1-overexpressing Jurkat cells (Figure 3.19). To address the potential effect of DcRs expressed by non-transformed cells in the environment of the tumour cells, we have developed two models; either a simple, direct co-culture of tumour cells (Colo205) with non-transformed fibroblasts expressing a moderate level of DcR1 or DcR2 (Figure 3.22), and a tumour spheroid model consisting of tumour cells (MDA-MB-231), DcR1/2-expressing human primary fibroblasts and endothelial cells (HUVEC) in a methylcellulose matrix (Figure 3.23 and Figure 3.25). Both models showed that WT TRAIL can lose between 50-80% of its efficacy when the tumour-surrounding fibroblasts express at least one of the decoy receptors. This substantial drop in efficacy was achieved by a moderate overexpression of the decoy receptors and only one of them at a time. Given the fact that the tumour:normal tissue ratio can be much smaller *in vivo* than in our models, it is safe to assume that *in vivo* the DcRs have the ability to sequester a large amount of administered TRAIL and block its effect

greatly. This finding was corroborated by our experiment where we tested the TRAIL45 mutants. These mutants continued to efficiently induce apoptosis despite decoy receptor expression with only a slight reduction in efficacy observed for TRAIL45-b and –c, while TRAIL45-a was capable of fully retaining its pro-apoptotic activity in both models (Figure 3.22 and Figure 3.25), underscoring that a recombinant TRAIL mutant, with the ability to potently target both death-inducing TRAIL receptors combined with the ability to evade the decoy receptors has the capacity to be a powerful treatment of cancer.

A cancer therapeutic which has the ability to effectively target cancer cells but leave non-transformed, cells unharmed is the goal of cancer therapy. TRAIL appears to fulfil this criterion. Since the discovery of this cytokine and subsequently of its receptors, there has been steady progress in illuminating the pathway through which it can selectively kill a wide range of cancer cell lines and the identification of a wide array of pro- and anti- apoptotic proteins which can aid or hinder this process respectively. While TRAIL is currently in phase Ib/II clinical trials and progressing (Ashkenazi *et al.*, 1999; Soria *et al.*, 2011; Soria *et al.*, 2010; Yee *et al.*, 2009; Yee *et al.*, 2007), resistance is a significant problem with 50-60% of tumour cell lines resistant to TRAIL-induced apoptosis.

A major point of regulation of TRAIL-mediated apoptosis is at the level of the receptors (Clancy *et al.*, 2005; Ganten *et al.*, 2009; Merino *et al.*, 2006). Firstly, cellular stresses regulate the expression of TRAIL receptors and thus TRAIL sensitivity; this is one of the mechanisms exploited by some chemotherapeutic drugs in order to re-activate apoptosis and sensitize the tumour cells to TRAIL. Secondly, a number of regulatory proteins bind to the protein complex formed on the intracellular side of the receptor upon ligand binding, such as c-FLIP, RIP (receptor-interacting protein), DAPK (death-associated protein kinase) and these various adaptor proteins can play a part in determining the fate of the cell. Finally, the promiscuity of TRAIL plays a direct role in its regulation with the functionality of DR4 and DR5 being controlled by the two TRAIL decoy receptors.

In an effort to specifically target this promiscuous ligand to its death inducing receptors, several agonistic antibodies specific to DR4 or DR5 have been developed as an anti-cancer therapeutics (reviewed in (Mahalingam *et al.*, 2009)). While TRAIL can bind to both death receptors in most cases it will preferentially signal through only one

in a given cell line, the mechanism regulating this is unknown. For example, in mantle cell lymphoma cells it has been shown that only the DR4 receptor is active (MacFarlane *et al.*, 2005a), similarly, previous studies from our laboratory found that in acute myeloid leukemia cells, despite the often high expression of DR5, WT TRAIL induces apoptosis preferentially via DR4 (Szegezdi *et al.*, 2011). As a possible mechanism of DR5 inhibition, Riccioni and colleagues have shown that in primary AML blasts, high DR5 expression tightly correlated with high DcR2 expression. Results from our laboratory confirmed these findings, which indicate that DcR2 may play a significant role in regulating the activity of DR5 (Riccioni *et al.*, 2005; Szegezdi *et al.*, 2011). A range of various tumour cell types shows activity of both DR4 and DR5, but a preference towards one receptor, i.e. activation of one receptor triggers higher percentage of apoptosis than the other, can be seen. For example, the pancreatic cancer cell lines, Colo357 and BxPC are more sensitive to DR4 activation than DR5, while HepG2 hepatocellular carcinoma cells and Colo205 colon carcinoma cells are more sensitive to DR5 activation (unpublished data from our laboratory). Finally, there are examples of cells that only respond to DR5, but not to DR4 activation, such as A2780 ovarian carcinoma cells, or Jurkat T-cell leukemia cells (Reis *et al.*, 2010; Reis *et al.*, 2009). In the latter group, the selective DR5-responsiveness can be explained by the very low or undetectable expression of DR4 on the cell surface. On the other hand, there is no similar, universal explanation for the selective responsiveness to DR4. Most of these cell types express reasonable or even high levels of DR5 and currently very little is understood what the differential events of the DR4- versus DR5-mediated apoptotic signal transduction pathways are. DR4 is either not expressed on the cell surface, or at a very low level.

Further to this, there is no diagnostic method to predict which receptors are expressed and/or active in a given tumour in a patient. In the absence of this information, agonisitic antibodies have only marginal advantage, if any, over WT TRAIL. Taking this into account, a recombinant TRAIL ligand which retains its affinity towards both death receptors in contrast to just one, combined with an ability to evade the inhibitory effects of the decoy receptors would be of extreme clinical benefit.

While some cell lines are inherently resistant to TRAIL, some cells which appeared to be previously sensitive can acquire resistance. It has been shown that resistance can be induced by repeated exposure to TRAIL where sub-cytotoxic concentrations of for

example DR5 agonistic antibody has been used (Li *et al*., 2006). Interestingly these cancer cells although acquiring resistance to DR5 mediated apoptosis were still sensitive to DR4 mediated cell death, the mechanism of which is unknown. These results indicate that acquired resistance may also be a concern resulting from the prolonged presence of an agonistic antibody. As the antibody concentration in the body gradually declines between two administrations, it can reach levels insufficient to induce apoptosis, this may then be sufficient to confer resistance. This example illustrates the need not only for a recombinant TRAIL, but also one that can target both death-inducing receptors simultaneously for fast and efficient tumour cell killing, eliminating the chance for resistance to occur. Pairing this with the added characteristic of evasion of the decoy receptors suggests a powerful tool in the treatment of cancer.

The predictive computational design algorithm FoldX used by us to design the mutants in this study has also been successfully used in the study of various proteins (Kempkens *et al.*, 2006; Kiel *et al.*, 2005; van der Sloot *et al.*, 2004) and we too have used it previously in the design of TRAIL mutants selective for either DR4 or DR5 with great success (Reis *et al.*, 2010; Tur *et al.*, 2008; van der Sloot *et al.*, 2006). These TRAIL mutants can very effectively distinguish between DR4 and DR5. The DR5-selective TRAIL mutant, D269H/E195R also have significantly reduced affinity towards DcR1, but only slightly reduced affinity towards DcR2 (van der Sloot *et al.*, 2006), while the DR4-selective TRAIL variant, 4C9 retained the high binding affinity of WT TRAIL towards both decoy receptors (Reis *et al.*, 2010). The combination of these two individual receptor-specific mutants was tested to investigate the effect of targeting both receptors simultaneously. The results saw increased apoptosis levels compared to each mutant tested alone indicating that dual targeting is useful in cancer cell lines which may signal through both receptors or in cells where the expression profile is unknown (Reis *et al.*, 2010). While this type of combination can be of use it does not overcome the antagonistic effects of the decoy receptors as binding of these mutants still occurs and reduces their potential efficacy. The successful design and generation of these recombinant TRAIL mutants and lack of a mutant which could bind to both death receptors while escaping sequestration by the decoy receptors inspired us to adopt the same approach for the generation of the mutants reported in this thesis.

Several other receptor selective mutants also exist and were achieved using other methods. A phage display technique was employed by Kelley and colleagues, resulting in a DR5-selective TRAIL mutant. This TRAIL mutant contained 6 amino acid substitutions in an attempt to elucidate the importance of each mutation they found they could not eliminate any without seeing a loss in either selectivity or biological activity. In addition to this, the decoy receptor-binding ability of their mutant is not reported, and the phage display technique used to identify this TRAIL mutant did not employ any step that aimed to select a TRAIL mutant with reduced decoy receptor binding (Kelley *et al.*, 2005). Using a computational approach, while not all mutations followed the prediction, we were successful in identifying several single mutations of importance. As currently only TRAIL in complex with DR5 has been crystalized, solving of the crystal structures for TRAIL in complex with the remaining receptors will allow for improvement of such structure based design and increased precision and accuracy of predictions. Using FoldX allows a vast amount of possibilities to be screened and outcomes predicted for the introduction of amino acid substitutions compared to what would be possible if these substitutions would be investigated by the labourious chore of simple inspection. In the case of our most promising mutant TRAIL45-a only two mutations were required to produce a mutant with superior biological activity and reduced decoy receptor binding. The benefit of which will be a reduced risk of an immunogenic response *in vivo*. Taken together this highlights the benefits and value of computational design. Also, DR4-selective TRAIL mutants have also been generated by the Cohen laboratory (MacFarlane *et al.*, 2005b) but again these mutants have up to 6 amino acid substitutions and also these mutants were not designed to have reduced affinity towards the decoy receptors, nor they have been studied for such properties, only to distinguish between DR4 and DR5.

Decoy receptors have been shown to be highly expressed by a number of tumour types, such as acute myeloid leukemia, colorectal cancer or prostate cancer *in vivo* (Anees *et al.*, 2011; Chamuleau *et al.*, 2011; Ganten *et al.*, 2009; Koksal *et al.*, 2008; Riccioni *et al.*, 2005) and their expression has been correlated with poor prognosis. Further, ectopic overexpression of the decoy receptors in tumour cells has been shown to be capable of reducing or even in some cases, fully blocking TRAIL-induced apoptosis (Degli-Esposti *et al.*, 1997a; Morizot *et al.*, 2011; Pan *et al.*, 1997a; Pan *et al.*, 1998; Sheridan *et al.*, 1997). The mechanism of this inhibition has been demonstrated to be two-fold; the decoy receptors can mediate their effect by either sequestration of

TRAIL from the death-inducing TRAIL receptors, or formation of DcR-DR heteromeric receptor complexes which are non-functional (Bouralexis *et al.*, 2003; Clancy *et al.*, 2005; Davidovich *et al.*, 2004; Merino *et al.*, 2006; Morizot *et al.*, 2011; Riccioni *et al.*, 2005; Sheridan *et al.*, 1997). The formation of these heteromeric complexes mediated by the PLAD domain have been reported to be independent of ligand binding, indicating that targeting the death receptors will not overcome this mode of inhibition (Chan *et al.*, 2001; Clancy *et al.*, 2005) however it is also reported that while the receptors may be held in this complex, ligand stimulation provokes a conformational change in favour of a more stable complex. This could infact allow for the re-organisation of the receptors particularly if no stable heteromeric complex can be formed which would be the true in the case of a decoy-insensitive TRAIL mutant. Over-expression of the death receptors leads to spontaneous induction of the apoptotic pathway independent of ligand stimulation (Kischkel *et al.*, 2000; Sheridan *et al.*, 1997; Wu *et al.*, 1997); While the discussed experiments were carried out in decoy receptor over-expressing cell lines and could be argued physiologically irrelevant, we have demonstrated the ability of the decoy receptors to reduce TRAIL's efficacy with the use of neutralising antibodies in Colo205 cells which normally express the decoy receptors on their cell surface (Figure 3.8 and Figure 3.17).

Decoy receptors are also expressed by a range of normal cell types in the human body, including the peripheral blood lymphocytes, spleen, kidneys, liver, pancreas etc. (Daniels *et al.*, 2005; LeBlanc and Ashkenazi, 2003; Marsters *et al.*, 1997; Pan *et al.*, 1997a; Pan *et al.*, 1998; Sheridan *et al.*, 1997). While DcRs can inhibit TRAIL-induced apoptosis in cancer cell lines, their expression on non-transformed cells is not required for their protection (Kim *et al.*, 2000) and thus, inhibition of these receptors will not sensitise non-transformed cells to TRAIL (Kim *et al.*, 2000; van Dijk *et al.*, MS in preparation). DcRs expressed by non-transformed cells throughout the body as well as in the tumour stroma may be a major factor in determining the *in vivo* efficacy of TRAIL. With the realisation that the tumour microenvironment has significant influence over the survival and progression of a tumour as well as responsiveness to therapy, investigation into how it influences TRAIL is also ongoing. Despite TRAIL's significant potency *in vitro* this is not as evident in clinical trials and it may be in part due to the influences of the tumour microenvironment. Reports show TRAIL effected by many factors originating from the microenvironment such as CD40, IL-8 and OPG and probably many more yet to be elucidated (Abdollahi *et al.*, 2003; Abdollahi *et al.*,

2005; Travert *et al.*, 2008). While there have been studies investigating the protection of cancer cells from TRAIL-induced apoptosis mediated by the surrounding stroma (Perez *et al*., 2008) and even TRAIL and TRAIL receptor expression in the stroma (Anees *et al*., 2011), whether the decoy receptors are mediating any protective effects is being overlooked. This lack of elucidation may in part be due to the fact, that there are no or very few appropriate experimental models that could address this question at a mechanistic level. Mouse models are suitable to study the function of DR4 and DR5, as the mouse death-inducing TRAIL receptor mTRAIL-R is homologous to human DR4/DR5 (Wu *et al.*, 1999). On the contrary, while just as in humans, two TRAIL decoy receptors exist in mice, they are barely homologous to their human counterparts (Schneider *et al.*, 2003). Thus, their role in regulating TRAIL apoptosis-signalling is unlikely to be comparable between the two species.

WT TRAIL shows potential in pre-clinical studies when combined with targeted therapeutics, such as histone deacetylase inhibitors, or proteasome inhibitors, as well as classical chemotherapeutics in selected tumour cell types. The mechanism of sensitisation to TRAIL-induced apoptosis has been revealed for many of these studies and been found to involve reducing the expression of c-FLIP, XIAP or changing the ratio of Bcl-2 protein expression in favour of the pro-apoptotic family members. However, very few, if any of these combinations can circumvent the regulatory effect of the decoy receptors, the first line of defence. In view of the findings here, as well as the recent study of the Micheau group that shows that DcR2 together with c-FLIP is the major determinant of TRAIL resistance (Morizot *et al.*, 2011), a TRAIL mutant that is not recognised by the decoy receptors has the greatest promise in becoming a cancer-therapeutic targeting DR4/DR5.

While there are many agonistic antibodies against the death receptors (mainly DR5) available and in various clinical trials, the amount of recombinant TRAIL mutants in trials is significantly less despite obvious advantages. While agonistic antibodies are hailed for their pharmacokinetic properties, i.e. their greatly extended half-life compared to a recombinant TRAIL, this so-called advantage could also lead to unwanted affects with prolonged exposure to antibody-mediated apoptosis thought to also increase the risk of toxic side-effects. The mechanism by which death receptor agonistic antibodies mediate their affects is still not fully elucidated and their ability to penetrate deep in the mass of a solid tumour is questionable. Due to its significantly

smaller size, the ability of a recombinant TRAIL to penetrate a tumour may be greater than that of an agonistic antibody. Though on a small scale, we have also clearly demonstrated the penetrability of the TRAIL45-a mutant in this work.

To summarise, we have successfully generated several recombinant TRAIL mutants which can induce apoptosis via both DR4 and DR5 and which can successfully evade the antagonistic effects of the decoy receptors. While there are many receptor-selective TRAIL variants in existence, to be truly receptor selective they must be decoy-receptor insensitive in order to retain their efficacy and reach their full potential. Such TRAIL mutants have been described in this thesis and their potential power in the clinic illustrated.

4.2. Future Perspectives

In this thesis, it has been shown that TRAIL45 mutants which can avoid the antagonistic decoy receptors have superior death-inducing ability over WT TRAIL in a range of cancer cell lines. Decoy insensitive TRAIL mutants displayed higher cytotoxic potential compared to WT TRAIL in cell lines previously characterised as partially-resistant to TRAIL due to decoy receptor expression (Figure 3.18). In this panel of partially-resistant cell lines 75% showed high decoy receptor expression, this correlation can be further examined utilising the TRAIL45 mutants on a larger panel of cell lines. While our 3D minituomour model attempts to mimic the composition of a real tumour, it can not impersonate the real thing. Tumour slices cultured *in vitro* are often used to evaluate different therapies allowing the tumour to maintain some of its *in vivo* characteristics. This would be an interesting model to investigate how the TRAIL45 mutants behave in a near-*in vivo* model. In this way we could also investigate using neutralising antibodies how much influence the decoy receptors expressed on both the tumour cells and the stromal cells have over WT TRAIL efficacy and explore our mutant's ability to evade their antagonising effects.

At the moment there is only one version of recombinant human TRAIL (rhTRAIL) being evaluated in clinical trials (Genentech, San Francisco, CA, USA), it retains binding to all five TRAIL receptors. Phase Ib/II trials investigating pharmacokinetic properties and safety of this recombinant TRAIL were carried out in patients suffering from low-grade non-Hodgkin lymphoma. The results showed that rhTRAIL is active against this tumour and well tolerated with no dose-limiting toxicities or severe adverse effects. A high percentage of patients receiving this monotherapy achieved partial response or stable disease (Ashkenazi *et al.*, 2008). If such results are observed for this recombinant version, then we could assume interesting results if the TRAIL45 mutants should enter into *in vivo* pre-clinical studies and this should indeed be explored. Upon promising results in mouse tumor xenograft models, the pharmacokinetic properties (absorption, best route of administration, blood half-life time, tissue distribution, etc.) of TRAIL45-a could be determined.

Various conventional chemotherapies have been found to upregulate one or both of the TRAIL death-inducing receptors, making recombinant TRAIL or agonistic antibodies for DR4 or DR5 attractive agents for combination therapy. *In vitro* studies determined which of the two TRAIL death receptors is induced in response to various

chemotherapeutics, but how it correlates with primary tumour samples and how consistent it is across various tumour types is not known. It is likely that this question cannot be answered with the necessary confidence, due to the high variability of primary tumours and their fast evolution/alteration during their progression. Thus, a recombinant TRAIL with the ability to efficiently activate both death-inducing receptors may be a better route for testing and establishing combination therapies with chemotherapeutics. In addition to this it was reported that as well as the up-regulation of DR5 observed with irradiation, DcR2 levels were also increased (Sreekumar *et al.*, 2001), this study does not refer to the effect on DcR2 expression on the normal surrounding tissue, which may also undergo the same effects. This strengthens the argument that a TRAIL mutant which can evade the decoys receptors may be of particular value in this kind of combination treatment.

It is evident from results presented in this thesis that the role the decoy receptors in TRAIL-resistance and their expression on non-transformed cells as well as the tumour cells themselves can be important in mediating this resistance. Many groups have found that decoy receptor expression on transformed cells can reduce and in some cases even inhibit TRAIL-induced apoptosis completely. However, the focus should not be limited to expression of the decoy receptors on the surface of the cancer cells but also but on the tissues surrounding the tumour. Resistance mediated by decoy receptor expression in the stroma has previously been overlooked and investigation would highlight the importance of these mutants in a clinical setting.

References

Abdollahi T, Robertson NM, Abdollahi A, Litwack G (2003). Identification of interleukin 8 as an inhibitor of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in the ovarian carcinoma cell line OVCAR3. *Cancer Res* **63:** 4521-4526.

Abdollahi T, Robertson NM, Abdollahi A, Litwack G (2005). Inhibition of TRAIL-induced apoptosis by IL-8 is mediated by the p38-MAPK pathway in OVCAR3 cells. *Apoptosis* **10:** 1383-93.

Aggarwal BB, Gupta SC, Kim JH (2012). Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. *Blood* **119:** 651-65.

Ahonen M, Poukkula M, Baker AH, Kashiwagi M, Nagase H, Eriksson JE *et al* (2003). *Oncogene* **22:** 2121-2134.

Aktas O, Smorodchenko A, Brocke S, Infante-Duarte C, Schulze Topphoff U, Vogt J *et al* (2005). Neuronal damage in autoimmune neuroinflammation mediated by the death ligand TRAIL. *Neuron* **46:** 421-32.

Almasan A, Ashkenazi A (2003). Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. *Cytokine Growth Factor Rev* **14:** 337-48.

Anees M, Horak P, El-Gazzar A, Susani M, Heinze G, Perco P *et al* (2011). Recurrence-free survival in prostate cancer is related to increased stromal TRAIL expression. *Cancer* **117:** 1172-82.

Antonsson B, Montessuit S, Lauper S, Eskes R, Martinou JC (2000). Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. *Biochem J* **345:** 271-8.

Antonsson B, Montessuit S, Sanchez B, Martinou JC (2001). Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem* **276:** 11615-23.

Ara T, DeClerck YA (2010). Interleukin-6 in Bone Metastasis and Cancer Progression. *Eur J Cancer* **46:** 1223-1231.

Ashkenazi A, Dixit VM (1998). Death receptors: signaling and modulation. *Science* **281:** 1305-8.

Ashkenazi A, Dixit VM (1999). Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* **11:** 255-60.

Ashkenazi A, Holland P, Eckhardt SG (2008). Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). *J Clin Oncol* **26:** 3621-30.

Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA *et al* (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* **104:** 155-62.

Aydin C, Sanlioglu AD, Karacay B, Ozbilim G, Dertsiz L, Ozbudak O *et al* (2007). Decoy receptor-2 small interfering RNA (siRNA) strategy employing three different siRNA constructs in combination defeats adenovirus-transferred tumor necrosis factor-related apoptosis-inducing ligand resistance in lung cancer cells. *Hum Gene Ther* **18:** 39-50.

Belada D, Mayer J, Czuczman MS, W. FI, Durbin-Johnson B, Bray GL (2010). Phase II study of dulanermin plus rituximab in patients with relapsed follicular non-Hodgkin's lymphoma (NHL). *J Clin Oncol* **28:** (Suppl abstr 8104).

Bernard D, Quatannens B, Vandenbunder B, Abbadie C (2001). Rel/NF-kappaB transcription factors protect against tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by up-regulating the TRAIL decoy receptor DcR1. *J Biol Chem* **276:** 27322-8.

Bin L, Thorburn J, Thomas LR, Clark PE, Humphreys R, A. T (2007). Tumor-derived Mutations in the TRAIL Receptor DR5 Inhibit
TRAIL Signaling through the DR4 Receptor by Competing for
Ligand Binding
J Biol Chem **282:** 28189-28194.

Bodmer JL, Burns K, Schneider P, Hofmann K, Steiner V, Thome M *et al* (1997). TRAMP, a novel apoptosis-mediating receptor with sequence homology to tumor necrosis factor receptor 1 and Fas(Apo-1/CD95). *Immunity* **6:** 79-88.

Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P *et al* (2000a). TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* **2:** 241-3.

Bodmer JL, Meier P, Tschopp J, Schneider P (2000b). Cysteine 230 is essential for the structure and activity of the cytotoxic ligand TRAIL. *J Biol Chem* **275:** 20632-7.

Bouralexis S, Findlay DM, Atkins GJ, Labrinidis A, Hay S, Evdokiou A (2003). Progressive resistance of BTK-143 osteosarcoma cells to Apo2L/TRAIL-induced apoptosis is mediated by acquisition of DcR2/TRAIL-R4 expression: resensitisation with chemotherapy. *Br J Cancer* **89:** 206-14.

Bouralexis S, Findlay DM, Evdokiou A (2005). Death to the bad guys: targeting cancer via Apo2L/TRAIL. *Apoptosis* **10:** 35-51.

Cain K, Bratton SB, Langlais C, Walker G, Brown DG, Sun XM *et al* (2000). Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4-MDa apoptosome complexes. *J Biol Chem* **275:** 6067-70.

Camidge DR, Herbst RS, Gordon MS, Eckhardt G, Kurzrock R, Durbin B *et al* (2010). A phase I safety and pharmacokinetic study of the death receptor 5 agonistic antibody PRO95780 in patients with advanced malignancies. *Clin Cancer Res* **16:** 1256-63.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E *et al* (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science* **282:** 1318-21.

Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G (2004a). Cell death by mitotic catastrophe: a molecular definition. *Oncogene* **23:** 2825-37.

Castedo M, Perfettini JL, Roumier T, Valent A, Raslova H, Yakushijin K *et al* (2004b). Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene* **23:** 4362-70.

Caulín C, Salvesen GS, Oshima RG (1997). Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *J Cell Biol* **138:** 1379-94.

Cha SS, Kim MS, Choi YH, Sung BJ, Shin NK, Shin HC *et al* (1999). 2.8 A resolution crystal structure of human TRAIL, a cytokine with selective antitumor activity. *Immunity* **11:** 253-61.

Cha SS, Song YL, Oh BH (2004). Specificity of Molecular Recognition Learned from the Crystal Structures of TRAIL and the TRAIL:sDR5 Complex. *Vitam Horm* **67:** 1-17.

Cha SS, Sung BJ, Kim YA, Song YL, Kim HJ, Kim S *et al* (2000). Crystal structure of TRAIL-DR5 complex identifies a critical role of the unique frame insertion in conferring recognition specificity. *J Biol Chem* **275:** 31171-7.

Chakrabandhu K, Huault S, Garmy N, Fantini J, Stebe E, Mailfert S *et al* (2008). The extracellular glycosphingolipid-binding motif of Fas defines its internalization route, mode and outcome of signals upon activation by ligand. *Cell Death Differ* **15:** 1824-37.

Chamuleau ME, Ossenkoppele GJ, van Dreunen L, Jirka SM, Zevenbergen A, Schuurhuis GJ *et al* (2011). High TRAIL-R3 expression on leukemic blasts is associated with poor outcome and induces apoptosis-resistance which can be overcome by targeting TRAIL-R2. *Leuk Res* **35:** 741-9.

Chan FK (2007). Three is Better Than One: Pre-Ligand Receptor Assembly in the Regulation of TNF Receptor Signaling. *Cytokine* **37:** 101-107.

Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ (2000). A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* **288:** 2351-4.

Chan FK, Shisler J, Bixby JG, Felices M, Zheng L, Appel M *et al* (2003). A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. *J Biol Chem* **278:** 51613-21.

Chan FK, Siegel RM, Zacharias DA, Swofford R, Holmes KL, Tsien RY *et al* (2001). Fluorescence resonance energy transfer analysis of cell surface receptor interactions

and signaling using spectral variants of the green fluorescent protein. *Cytometry* **44:** 361-8.

Chaudhary KS, Abel PD, Stamp GW, Lalani E (2001). Differential expression of cell death regulators in response to thapsigargin and adriamycin in Bcl-2 transfected DU145 prostatic cancer cells. *J Pathol* **193:** 522-9.

Chaudhary PM, Eby M, Jasmin A, Bookwalter A, Murray J, Hood L (1997). Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. *Immunity* **7:** 821-30.

Chaudhary PM, Eby M, Jasmin A, Kumar A, Liu L, Hood L (2000). Activation of NF-kappaB pathway of caspase-8 and its homologs. *Oncogene* **19:** 4451-4460.

Chen PL, Easton A (2008). Apoptotic Phenotype Alters the Capacity of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand to Induce Human Vascular Endothelial Activation. *J Vasc Res* **45:** 111-122.

Chen X, Kandasamy K, Srivastava RK (2003). Differential roles of RelA (p65) and c-Rel subunits of nuclear factor kappa B in tumor necrosis factor-related apoptosis-inducing ligand signaling. *Cancer Res* **63:** 1059-66.

Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL *et al* (2000). Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proc Natl Acad Sci U S A* **97:** 1754-1759.

Chipuk JE, Green DR (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* **18:** 157-64.

Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR (2010). The BCL-2 family reunion. *Mol Cell* **37:** 299-310.

Clancy L, Mruk K, Archer K, Woelfel M, Mongkolsapaya J, Screaton G *et al* (2005). Preligand assembly domain-mediated ligand-independent association between TRAIL receptor 4 (TR4) and TR2 regulates TRAIL-induced apoptosis. *Proc Natl Acad Sci U S A* **102:** 18099-104.

Cremesti AE, Goni FM, Kolesnick R (2002). Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? *FEBS Lett* **531:** 47-53.

Cretney E, McQualter JL, Kayagaki N, Yagita H, Bernard CC, Grewal IS *et al* (2005). TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L suppresses experimental autoimmune encephalomyelitis in mice. *Immunol Cell Biol* **83:** 511-9.

Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, Smyth MJ (2002). Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol* **168:** 1356-61.

Daly PF, Zugmaier G, Sandler D, Carpen M, Myers CE, Cohen JS (1990). Regulation of the cytidine phospholipid pathways in human cancer cells and effects of 1-beta-D-arabinofuranosylcytosine: a noninvasive 31P nuclear magnetic resonance study. *Cancer Res* **50:** 552-7.

Daniels RA, Turley H, Kimberley FC, Liu XS, Mongkolsapaya J, Ch'En P *et al* (2005). Expression of TRAIL and TRAIL receptors in normal and malignant tissues. *Cell Res* **15:** 430-8.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y *et al* (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91:** 231-41.

Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N *et al* (2000). Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J* **14:** 729-39.

Davidovich IA, Levenson AS, Levenson Chernokhvostov W (2004). Overexpression of DcR1 and survivin in genetically modified cells with pleiotropic drug resistance. *Cancer Lett* **211:** 189-97.

Decaudin D, Marzo I, Brenner C, Kroemer G (1998). Mitochondria in chemotherapy-induced apoptosis: a prospective novel target of cancer therapy (review). *Int J Oncol* **12:** 141-52.

Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG (1997a). The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* **7:** 813-20.

Degli-Esposti MA, Smolak PJ, Walczak H, Waugh J, Huang CP, DuBose RF *et al* (1997b). Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med* **186:** 1165-70.

Delmas D, Rébé C, Micheau O, Athias A, Gambert P, Grazide S *et al* (2004). Redistribution of CD95, DR4 and DR5 in rafts accounts for the synergistic toxicity of resveratrol and death receptor ligands in colon carcinoma cells. *Oncogene* **23**.

Dewson G, Kluck RM (2009). Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. *J Cell Sci* **122:** 2801-8.

Doi T, Murakami H, Ohtsu A, Fuse N, Yoshino T, Yamamoto N *et al* (2011). Phase 1 study of conatumumab, a pro-apoptotic death receptor 5 agonist antibody, in Japanese patients with advanced solid tumors. *Cancer Chemother Pharmacol* **68:** 733-41.

Dolcet X, Llobet D, Pallares J, Rue M, Comella JX, Matias-Guiu X (2005). FLIP is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis. *Lab Invest* **85:** 885-95.

Douma S, van Laar T, Zevenhoven J, Meuwissen R, van Garderen E, Peeper DS (2004). Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB. *Nature* **430:** 1034-1039.

Du C, Fang M, Li Y, Li L, Wang X (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102:** 33-42.

Duiker EW, de Vries EG, Mahalingam D, Meersma GJ, Boersma-van Ek W, Hollema H *et al* (2009). Enhanced antitumor efficacy of a DR5-specific TRAIL variant over recombinant human TRAIL in a bioluminescent ovarian cancer xenograft model. *Clin Cancer Res* **15:** 2048-57.

Eckelman BP, Salvesen GS, Scott FL (2006). Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO J* **7:** 988-94.

Ehrhardt H, Fulda S, Schmid I, Hiscott J, Debatin KM, Jeremias I (2003). TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB. *Oncogene* **22:** 3842-52.

Elhasid R, Sahar D, Merling A, Zivony Y, Rotem A, Ben-Arush M *et al* (2004). Mitochondrial pro-apoptotic ARTS protein is lost in the majority of acute lymphoblastic leukemia patients. *Oncogene* **23:** 5468-75.

Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C *et al* (1998). Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem* **273:** 14363-7.

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

Falschlehner C, Schaefer U, Walczak H (2009). Following TRAIL's path in the immune system. *Immunology* **127:** 145-154.

Fisher MJ, Virmani AK, Wu L, Aplenc R, Harper JC, Powell SM *et al* (2001). Nucleotide substitution in the ectodomain of trail receptor DR4 is associated with lung cancer and head and neck cancer. *Clin Cancer Res* **7:** 1688-97.

Forero-Torres A, Shah J, Wood T, Posey J, Carlisle R, Copigneaux C *et al* (2010). Phase I trial of weekly tigatuzumab, an agonistic humanized monoclonal antibody targeting death receptor 5 (DR5). *Cancer Biother Radiopharm* **25:** 13-19.

Frisch SM, Francis H (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* **124:** 619-626.

Fulda S, Meyer E, Debatin KM (2002). Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. *Oncogene* **21:** 2283-94.

Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K *et al* (2007). Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol* **9:** 1392-1400.

Gajate C, Mollinedo F (2005). Cytoskeleton-mediated death receptor and ligand concentration in lipid rafts forms apoptosis-promoting clusters in cancer chemotherapy. *J Biol Chem* **280:** 11641-7.

Ganten TM, Sykora J, Koschny R, Batke E, Aulmann S, Mansmann U *et al* (2009). Prognostic significance of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor expression in patients with breast cancer. *J Mol Med* **87:** 995-1007.

Gardai SJ, Hildeman DA, Frankel SK, Whitlock BB, Frasch SC, Borregaard N *et al* (2004). Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. *J Biol Chem* **279:** 21085-95.

Gasparian ME, Chernyak BV, Dolgikh DA, Yagolovich AV, Popova EN, Sycheva AM *et al* (2009). Generation of new TRAIL mutants DR5-A and DR5-B with improved selectivity to death receptor 5. *Apoptosis* **14:** 778-787.

Geserick P, Drewniok C, Hupe M, Haas TL, Diessenbacher P, Sprick MR *et al* (2008). Suppression of cFLIP is sufficient to sensitize human melanoma cells to TRAIL- and CD95L-mediated apoptosis. *Oncogene* **27:** 3211-20.

Gill C, Dowling C, O' Neill AJ, Watson RW (2009). Effects of cIAP-1, cIAP-2 and XIAP triple knockdown on prostate cancer cell susceptibility to apoptosis, cell survival and proliferation. *Mol Cancer* **8**.

Goltsev YV, Kovalenko AV, Arnold E, Varfolomeev EE, Brodianskii VM, Wallach D (1997). CASH, a novel caspase homologue with death effector domains. *J Biol Chem* **272:** 19641-4.

Gottfried Y, Rotem A, Lotan R, Steller H, Larisch S (2004). The mitochondrial ARTS protein promotes apoptosis through targeting XIAP. *EMBO J* **23:** 1627-35.

Greco FA, Bonomi P, Crawford J, Kelly K, Oh Y, Halpern W *et al* (2008). Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. *Lung Cancer* **61:** 82-90.

Green DR, Kroemer G (2004). The pathophysiology of mitochondrial cell death. *Science* **305:** 626-9.

Grosse-Wilde A, Voloshanenko O, Bailey SL, Longton GM, Schaefer U, Csernok AI *et al* (2008). TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. *J Clin Invest* **118:** 100-10.

Guseva NV, Rokhlin OW, Taghiyev AF, Cohen MB (2008). Unique resistance of breast carcinoma cell line T47D to TRAIL but not anti-Fas is linked to p43cFLIP(L). *Breast Cancer Rec Treat* **107:** 349-57.

Hacki J, Egger L, Monney L, Conus S, Rossé T, Fellay I *et al* (2000). Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene* **19:** 2286-95.

Han DK, Chaudhary PM, Wright ME, Friedman C, Trask BJ, Riedel RT *et al* (1997). MRIT, a novel death-effector domain-containing protein, interacts with caspases and BclXL and initiates cell death. *Proc Natl Acad Sci U S A* **94:** 11333-8.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. *Cell* **100:** 57-70.

Hanahan D, Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell* **144:** 646-74.

Harper N, Farrow SN, Kaptein A, Cohen GM, MacFarlane M (2001). Modulation of tumor necrosis factor apoptosis-inducing ligand- induced NF-kappa B activation by inhibition of apical caspases. *J Biol Chem* **276:** 34743-52.

Herbst RS, Eckhardt G, Kurzrock R, Ebbinghaus S, O' Dwyer P, Gordon MS *et al* (2010a). Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic receptor agonist, in patients with advanced cancer. *J Clin Oncol* **28:** 2839-46.

Herbst RS, Kurzrock R, Hong DS, Valdivieso M, Hsu CP, Goyal L *et al* (2010b). A first-in-human study of conatumumab in adult patients with advanced solid tumors. *Clin Cancer Res* **16:** 5883-91.

Herbst RS, Mendolson DS, Ebbinghaus S, Gordon MS, O' Dwyer P, Lieberman G *et al* (2006). A phase I safety and pharmacokinetic (PK) study of recombinant Apo2L/TRAIL, an apoptosis-inducing protein in patients with advanced cancer. *J Clin Oncol* **24:** 3013.

Hilliard B, Wilmen A, Seidel C, Liu TS, Goke R, Chen Y (2001). Roles of TNF-related apoptosis-inducing ligand in experimental autoimmune encephalomyelitis. *J Immunol* **166:** 1314-9.

Hinz S, Trauzold A, Boenicke L, Sandberg C, Beckmann S, Bayer E *et al* (2000). Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis. *Oncogene* **19:** 5477-86.

Holler N, Zaru R, Micheau O, Thorne M, Attinger A, Valitutti S *et al* (2000). Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol* **1:** 489-95.

Hopkins-Donaldson S, Ziegler A, Kurtz S, Bigosch C, Kandioler D, Ludwig C *et al* (2003). Silencing of death receptor and caspase-8 expression in small cell lung carcinoma cell lines and tumors by DNA methylation. *Cell Death Differ* **10:** 356-64.

Horak P, Pils D, Haller G, Pribill I, Roessler M, Tomek S *et al* (2005). Contribution of epigenetic silencing of tumor necrosis factor-related apoptosis inducing ligand receptor 1 (DR4) to TRAIL resistance and ovarian cancer. *Mol Cancer Res* **3:** 335-43.

Hotte SJ, Hirte HW, Chen EX, Siu LL, Le LH, Corey A *et al* (2008). A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. *Clin Cancer Res* **14:** 3450-5.

Hu WH, Johnson H, Shu HB (1999). Tumor necrosis factor-related apoptosis-inducing ligand receptors signal NF-kappaB and JNK activation and apoptosis through distinct pathways. *J Biol Chem* **274:** 30603-10.

Hu Y, Yao J, Liu Z, Liu X, Fu H, Ye K (2005). Akt phosphorylates acinus and inhibits its proteolytic cleavage, preventing chromatin condensation. *EMBO J* **24:** 3543-54.

Hueber AO, Bernard AM, Herincs Z, Couzinet A, He HT (2002). An essential role for membrane rafts in the initiation of Fas/CD95-triggered cell death in mouse thymocytes. *EMBO Rep* **3:** 190-6.

Hughes MA, Harper N, Butterworth M, Cain K, Cohen GM, MacFarlane M (2009). Reconstitution of the death-inducing signaling complex reveals a substrate switch that determines CD95-mediated death or survival. *Mol Cell* **35:** 265-79.

Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF *et al* (1999). Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol Cell* **4:** 563-71.

Hymowitz SG, O'Connell MP, Ultsch MH, Hurst A, Totpal K, Ashkenazi A *et al* (2000). A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. *Biochemistry* **39:** 633-40.

Inohara N, Koseki T, Hu Y, Chen S, Núnez G (1997). CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proc Natl Acad Sci U S A* **94:** 10717-22.

Itoh N, Nagata S (1993). A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J Biol Chem* **268:** 10932-7.

Jennewein C, Karl S, Baumann B, Micheau O, Debatin KM, Fulda S (2012). Identification of a novel pro-apoptotic role of NF-κB in the regulation of TRAIL- and CD95-mediated apoptosis of glioblastoma cells. *Oncogene* **31:** 1468-1474.

Jiang X, Wang X (2000). Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J Biol Chem* **275:** 31199-203.

Jin CY, Moon DO, Lee JD, Heo MS, Choi YH, Lee CM *et al* (2007). Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis through downregulation of ERK and Akt in lung adenocarcinoma A549 cells. *Carcinogenesis* **28:** 1058-66.

Jin H, Yang R, Ross J, Fong S, Carano K, Totpal K *et al* (2008). Cooperation of the agonist DR5 antibody apomab with chemotherapy to inhibit orthotopic lung tumor growth and improve survival. *Clin Cancer Res* **14:** 7733-7740.

Jin Z, El-Deiry WS (2006). Distinct signaling pathways in TRAIL- versus tumor necrosis factor-induced apoptosis. *Mol Cell Biol* **26:** 8136-48.

Jin Z, McDonald ER, 3rd, Dicker DT, El-Deiry WS (2004). Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. *J Biol Chem* **279:** 35829-39.

Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L (1996). Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res* **56:** 816-25.

Kandasamy K, Srinivasula SM, Alnemri ES, Thompson CB, Korsmeyer SJ, Bryant JL *et al* (2003). Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c and Smac/DIABLO release. *Cancer Res* **63:** 1712-21.

Karin M, Cao Y, Greten FR, Li ZW (2002). NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* **2:** 301-310.

Karl S, Pritschow Y, Volcic M, Hacker S, Baumann B, Wiesmuller L *et al* (2009). Identification of a novel pro-apopotic function of NF-kappaB in the DNA damage response. *J Cell Mol Med* **13:** 4239-56.

Kayagaki N, Yamaguchi N, Nakayama H, Eto H, Okumura K, Yagita H (1999). Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: A novel mechanism for the antitumor effects of type I IFNs. *J Exp Med* **189:** 1451-60.

Kelley RF, Totpal K, Lindstrom SH, Mathieu M, Billeci K, Deforge L *et al* (2005). 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* **280:** 2205-12.

Kelley SK, Ashkenazi A (2004). Targeting death receptors in cancer with Apo2L/TRAIL. *Curr Opin Pharmacol* **4:** 333-9.

Kempkens O, Médina E, Fernandez-Ballester G, Ozuyaman S, Le Bivic A, Serrano L *et al* (2006). Computer modelling in combination with in vitro studies reveals similar binding affinities of Drosophila Crumbs for the PDZ domains of Stardust and DmPar-6. *Eur J Cell Biol* **85:** 53-67.

Kerr JF, Wyllie AH, Currie AR (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26:** 239-57.

Kiel C, Wohlgemuth S, Rousseau F, Schymkowitz J, Ferkinghoff-Borg J, Wittinghofer F *et al* (2005). Recognizing and defining true Ras binding domains II: in silico prediction based on homology modelling and energy calculations. *J Mol Biol* **348:** 759-75.

Kim EC, Yun BS, Ryoo IJ, Min JK, Won MH, Lee KS *et al* (2004). Complestatin prevents apoptotic cell death: inhibition of a mitochondrial caspase pathway through AKT/PKB activation. *Biochem Biophys Res Commun* **313:** 193-204.

Kim HE, Du F, Fang M, Wang X (2005a). Formation of apoptosome is initiated by cytochrome c-induced dATP hydrolysis and subsequent nucleotide exchange on Apaf-1. *Proc Natl Acad Sci U S A* **102:** 17545-50.

Kim JY, Lee JY, Kim DG, Koo GB, Yu JW, Kim YS (2011). TRADD is critical for resistance to TRAIL-induced cell death through NF-kB activation. *FEBS Lett* **585:** 2144-2150.

Kim K, Fisher MJ, Xu SQ, el-Deiry WS (2000). Molecular determinants of response to TRAIL in killing of normal and cancer cells. *Clin Cancer Res* **6:** 335-46.

Kim K, Wilson SM, Abayasiriwardana KS, Collins R, Fjellbirkeland L, Xu Z *et al* (2005b). A novel in vitro model of human mesothelioma for studying tumor biology and apoptotic resistance. *Am J Respir Cell Mol Biol* **33:** 541-548.

Kim KM, Lee YJ (2005). Amiloride augments TRAIL-induced apoptotic death by inhibiting phosphorylation of kinases and phosphatases associated with the P13K-Akt pathway. *Oncogene* **24:** 355-66.

Kim YH, Lee DH, Jeong JH, Guo ZS, Lee YJ (2008). Quercetin augments TRAIL-induced apoptotic death: involvement of the ERK signal transduction pathway. *Biochem Pharmacol* **75:** 1946-58.

Kim YS, Schwabe RF, Qian T, Lemasters JJ, Brenner DA (2002). TRAIL-mediated apoptosis requires NF-kappaB inhibition and the mitochondrial permeability transition in human hepatoma cells. *Hepatology* **36:** 1498-508.

Kimberley FC, Screaton G (2004). Following a TRAIL: Update on a ligand and its five receptors. *Cell res* **14:** 359-372.

Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A (2000). Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* **12:** 611-20.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275:** 1132-6.

Koksal IT, Sanlioglu AD, Karacay B, Griffith TS, Sanlioglu S (2008). Tumor necrosis factor-related apoptosis inducing ligand-R4 decoy receptor expression is correlated with high Gleason scores, prostate-specific antigen recurrence, and decreased survival in patients with prostate carcinoma. *Urol Oncol* **26:** 158-65.

Kortemme T, Joachimiak LA, Bullock AN, Schuler AD, Stoddard BL, Baker D (2004). Computational redesign of protein-protein interaction specificity. *Nat Struct Mol Biol* **11:** 371-9.

Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K *et al* (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278**.

Krueger A, Baumann S, Krammer PH, Kirchhoff S (2001). FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol Cell Biol* **21:** 8247-54.

Lalaoui N, Morlé A, Mérino D, Jacquemin G, Iessi E, Morizot A *et al* (2011). TRAIL-R4 promotes tumor growth and resistance to apoptosis in cervical carcinoma HeLa cells through AKT. *PLoS One* **6:** e19679.

Lamhamedi-Cherradi SE, Zheng S, Maguschak KA, Peschon J, Chen YH (2003). Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL-/- mice. *Nat Immunol* **4:** 255-60.

Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B *et al* (2001). Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* **7:** 383-5.

Leader B, Baca QJ, Golan DE (2008). Protein therapeutics: a summary and pharmacological classification. *Nat Rev Drug Discov* **7:** 21-39.

Leber B, Lin J, Andrews D (2010). Still embedded together binding to membranes regulates Bcl-2 protein interactions. *Oncogene* **29:** 5221-30.

LeBlanc HN, Ashkenazi A (2003). Apo2L/TRAIL and its death and decoy receptors. *cell Death Differ* **10:** 66-75.

LeBlanc HN, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P *et al* (2002). Tumor-cell resistance to death receptor--induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nat Med* **8:** 274-81.

Lee HW, Lee SH, Lee HW, Ryu YW, Kwon M, H. , LKim YS (2005). Homomeric and heteromeric interactions of the extracellular domains of death receptors and death decoy receptors. *Biochem Biophys Res Commun* **330:** 1205-12.

Lee SH, Shin MS, Kim HS, Lee H, K., Park WS, Kim SY *et al* (1999). Alterations of the DR5/TRAIL receptor 2 gene in non-small cell lung cancers. *Cancer Res* **59:** 5683-6.

Lee SH, Shin MS, Kim HS, Lee HK, Park WS, Kim SY *et al* (2001). Somatic mutations of TRAIL-receptor 1 and TRAIL-receptor 2 genes in non-Hodgkin's lymphoma. *Oncogene* **20:** 399-403.

Lee TJ, Jung EM, Lee JT, Kim S, Park JW, Choi KS *et al* (2006a). Mithramycin A sensitizes cancer cells to TRAIL-mediated apoptosis by down-regulation of XIAP gene promoter through Sp1 sites. *Mol Cancer Ther* **5:** 2737-46.

Lee TJ, Lee JT, Park JW, Kwon TK (2006b). Acquired TRAIL resistance in human breast cancer cells are caused by the sustained cFLIP(L) and XIAP protein levels and ERK activation. *Biochem Biophys Res Commun* **351:** 1024-30.

Leong S, Cohen RB, Gustafson DL, Langer CJ, Camidge DR, Padavic K *et al* (2009). Mapatumumab, an antibody targeting TRAIL-R1, in combination with paclitaxel and carboplatin in patients with advanced solid malignancies: results of a phase I and pharmacokinetic study. *J Clin Oncol* **27:** 4413-21.

Li H, Zhu H, Xu CJ, Yuan J (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94:** 491-501.

Li LY, Luo X, Wang X (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412:** 95-9.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES *et al* (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91:** 479-89.

Lim SC, Duong HQ, Choi JE, Lee TB, Kang JH, Oh SH *et al* (2011). Lipid raft-dependent death receptor 5 (DR5) expression and activation are critical for ursodeoxycholic acid-induced apoptosis in gastric cancer cells. *Carcinogenesis* **32:** 723-731.

Lin Y, Devin A, Cook A, Keane MM, Kelliher M, Lipkowitz S *et al* (2000a). The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. *Mol Cell Biol* **20:** 6638-45.

Lin Y, Devin A, Cook A, Keane MM, Kelliher M, Lipkowitz S *et al* (2000b). The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. *Mol Cell Biol* **20:** 6638-45.

Lin Y, Devin A, Rodriguez Y, Liu ZG (1999). Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev* **13:** 2514-26.

Ling J, Herbst RS, Mendelson DS, Eckhardt G, O'Dwyer P, Ebbinghaus S *et al* (2006). Apo2L?TRAIL pharmacokinetics in a phase 1a trial in advanced cancer and lymphoma *J Clin Oncol* **24:** 3047.

Lippow SM, Wittrup KD, Tidor B (2007). Computational design of antibody-affinity improvement beyond in vivo maturation. *Nat Biotechnol* **25:** 1171-6.

Liu X, Zou H, Slaughter C, Wang X (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **89:** 175-84.

Lock RB, Stribinskiene L (1996). Dual modes of death induced by etoposide in human epithelial tumor cells allow Bcl-2 to inhibit apoptosis without affecting clonogenic survival. *Cancer Res* **56:** 4006-12.

Locklin RM, Croucher PI, Russell RGG, Edwards CM (2007). Agonists of TRAIL death receptors induce myeloma cell apoptosis that is not prevented by cells of the bone marrow microenvironment. *Leukemia* **21:** 805-812.

Lockshin RA, Williams CM (1964). Programmed cell death. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. *J Insect Physiol* **10:** 643-649.

Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci U S A* **96:** 11364-9.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors.
. *Cell* **94:** 481-90.

Luster TA, Carrell J, McCormick K, Sun D, Humphreys R (2009). 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* **8:** 292-302.

Ma S, Zhao L, Zhu Z, Liu Q, Xu H, Johansson M *et al* (2011). The multisubstrate deoxyribonucleoside kinase of Drosophila melanogaster as a therapeutic suicide gene of breast cancer cells. *J Gene Med* **13:** 305-11.

MacFarlane M (2003). TRAIL-induced signalling and apoptosis. *Toxicol Lett* **139:** 89-97.

MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM, Alnemri ES (1997). Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J Biol Chem* **272:** 25417-20.

MacFarlane M, Inoue S, Kohlhaas SL, Majid A, Harper N, Kennedy DBJ *et al* (2005a). Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. *Cell Death Differ* **12**
773-782.

MacFarlane M, Kohlhaas SL, Sutcliffe MJ, Dyer MJ, Cohen GM (2005b). TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. *Cancer Res* **65:** 11265-70.

Mahalingam D, Szegezdi E, Keane M, de Jong S, Samali A (2009). TRAIL receptor signalling and modulation: Are we on the right TRAIL? *Cancer Treat Rev* **35:** 280-8.

Maldonado-Celis ME, Bousserouel S, Grossé F, Lobstein A, Raul F (2009). Apple procyanidins activate apoptotic signaling pathway in human colon adenocarcinoma cells by a lipid-raft independent mechanism. *Biochem Biophys Res Commun* **388:** 372-6.

Maloy S, Stewart V, Taylor R (1999). Genetic analysis of pathogenic bacteria. *Cold Spring Harbour Laboratory Press*.

Marini P, Junginger D, Stickl S, Budach W, Niyazi M, Belka C (2009). Combined treatment with lexatumumab and irradiation leads to strongly increased long term tumour control under normoxic and hypoxic conditions. *Radiat Oncol* **4:** 49.

Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D *et al* (1997). A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr Biol* **7:** 1003-6.

Martin SJ, O' Brien GA, Nishioka WK, McGahon AJ, Mahboubi A, Saido TC *et al* (1995). Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. *J Biol Chem* **270:** 6425-8.

Matsumura H, Shimizu Y, Ohsawa Y, Kawahara A, Uchiyama Y, Nagata S (2000). Necrotic death pathway in Fas receptor signaling. *J Cell Biol* **151:** 1247-56.

Meng XW, Peterson KL, Dai H, Schneider P, Lee S, Zhang J *et al* (2011). High Cell Surface Death Receptor Expression Determines Type I Versus Type II Signaling. *J Biol Chem* **286:** 35823-35833.

Meredith JE, Fazeli B, Schwartz MA (1993). The extracellular matrix as a cell survival factor. *Mol Biol Cell* **4:** 953-61.

Merino D, Lalaoui N, Morizot A, Schneider P, Solary E, Micheau O (2006). Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2. *mol cell biol* **26:** 7046-55.

Merrit AJ, Allen TD, Potten CS, Hickman JA (1997). Apoptosis in small intestinal epithelial from p53-null mice: evidence for a delayed, p53-independent G2/M-associated cell death after gamma-irradiation. *Oncogene* **14:** 2759-66.

Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW *et al* (2002). The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem* **277:** 45162-71.

Min Y, Shi J, Zhang Y, Liu Y, Zheng D (2009). Death receptor 5-recruited raft components contributes to the sensitivity of Jurkat leukemia cell lines to TRAIL-induced cell death. *IUBMB Life* **61:** 261-7.

Mom CH, Verweij J, Oldenhuis CN, Gietema JA, Fox NL, Miceli R *et al* (2009). Mapatumumab, a fully human agonistic monoclonal antibody that targets TRAIL-R1, in combination with gemcitabine and cisplatin: a phase I study. *Clin Cancer Res* **15:** 6744.

Mongkolsapaya J, Cowper AE, Xu XN, Morris G, McMichael AJ, Bell JI *et al* (1998). Lymphocyte inhibitor of TRAIL (TNF-related apoptosis-inducing ligand): a new receptor protecting lymphocytes from the death ligand TRAIL. *J Immunol* **160:** 3-6.

Mongkolsapaya J, Grimes JM, Chen N, Xu XN, Stuart DI, Jones EY *et al* (1999). Structure of the TRAIL-DR5 complex reveals mechanisms conferring specificity in apoptotic initiation. *Nat Struct Biol* **6:** 1048-53.

Mori T, Doi R, Kida A, Nagai K, Kami K, Ito D *et al* (2007). Effect of the XIAP inhibitor Embelin on TRAIL-induced apoptosis of pancreatic cancer cells. *J Surg Res* **142**.

Morishima N, Nakanishi K, Tsuchiya K, Shibata T, Seiwa E (2004). Translocation of Bim to the endoplasmic reticulum (ER) mediates ER stress signaling for activation of caspase-12 during ER stress-induced apoptosis. *J Biol Chem* **279:** 50375-81.

Morizot A, Merino D, Lalaoui N, Jacquemin G, Granci V, Iessi E *et al* (2011). Chemotherapy overcomes TRAIL-R4-mediated TRAIL resistance at the DISC level. *Cell Death Differ* **18:** 700-11.

Mucha SR, Rizzani A, Gerbes AL, Camaj P, Thasler WE, Bruns CJ *et al* (2009). JNK inhibition sensitises hepatocellular carcinoma cells but not normal hepatocytes to the TNF-related apoptosis-inducing ligand. *Gut* **58:** 688-98.

Muppidi JR, Siegel RM (2004). Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death. *Nat Immunol* **5:** 182-9.

Murakami Y, Aizu-Yokota E, Sonoda Y, Ohta S, Kasahara T (2007). Suppression of endoplasmic reticulum stress-induced caspase activation and cell death by the overexpression of Bcl-xL or Bcl-2. *J Biochem* **141:** 401-10.

Nagata S (1997). Apoptosis by death factor. *Cell* **88:** 355-65.

Neamati N, Fernandez A, Wright S, Kiefer J, McConkey DJ (1995). Degradation of lamin B1 precedes oligonucleosomal DNA fragmentation in apoptotic thymocytes and isolated thymocyte nuclei. *J Immunol* **154:** 3788-95.

Nechushtan A, Smith CL, Lamensdorf I, Yoon SH, Youle RJ (2001). Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. *J Cell Biol* **153:** 1265-76.

Neville-Webbe HL, Cross NA, Eaton CL, Nyambo R, Evans CA, Coleman RE *et al* (2004). Osteoprotegerin (OPG) produced by bone marrow stromal cells protects breast cancer cells from TRAIL-induced apoptosis. *Breast Cancer Rec Treat* **86:** 269-79.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M *et al* (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376:** 37-43.

Nyambo R, Cross N, Lippit J, Holen I, Bryden G, Hamdy FC *et al* (2004). Human bone marrow stromal cells protect prostate cancer cells from TRAIL-induced apoptosis. *J Bone Miner Res* **19:** 1712-21.

O' Neill JW, Manion MK, Maguire B, Hockenbery DM (2006). BCL-XL dimerization by three-dimensional domain swapping. *J Mol Biol* **356:** 367-81.

Oltvai ZN, Milliman CL, Korsmeyer SJ (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74:** 609-19.

Pai SI, Wu GS, Ozoren N, Wu L, Jen J, Sidransky D *et al* (1998). Rare loss-of-function mutation of a death receptor gene in head and neck cancer. *Cancer Res* **58:** 3513-8.

Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM (1997a). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277:** 815-8.

Pan G, Ni J, Yu G, Wei YF, Dixit VM (1998). TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Lett* **424:** 41-5.

Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J *et al* (1997b). The receptor for the cytotoxic ligand TRAIL. *Science* **276:** 111-3.

Pan Y, Xu R, Peach ML, Huang CP, Branstetter DG, Durbin B *et al* (2007). Application of phamacodynamic assays in a phase 1a trial of APO2L/TRAIL in patients with advanced tumors.
. *J Clin Oncol* **25:** 3535.

Panner A, James CD, Berger MS, Pieper RO (2005). mTOR controls FLIPS translation and TRAIL sensitivity in glioblastoma multiforme cells. *Mol Cell Biol* **25:** 8809-23.

Papoff G, Hausler P, Eramo A, Pagano MG, Di Leve G, Signore A *et al* (1999). Identification and characterization of a ligand-independent oligomerization domain in the extracellular region of the CD95 death receptor. *J Biol Chem* **274:** 38241-50.

Perez LE, Parquet N, Shain K, Nimmanapalli R, Alsina M, Anasetti C *et al* (2008). Bone marrow stroma confers resistance to Apo2 ligand/TRAIL in multiple myeloma in part by regulating c-FLIP. *J Immunol* **180:** 1545-55.

Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A (1996). Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* **271:** 12687-90.

Plantivaux A, Szegezdi E, Samali A, Egan L (2009). Is there a role for nuclear factor kappaB in tumor necrosis factor-related apoptosis-inducing ligand resistance? *Ann N Y Acad Sci* **1171:** 38-49.

Plummer R, Attard G, Pacey S, Li L, Razak A, Perrett R *et al* (2007). Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers. *Clin Cancer Res* **13:** 6187-94.

Psahoulia FH, Drosopoulos KG, Doubravska L, Andera L, Pintzas A (2007). Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts *Mol Cancer Ther* **6:** 2591-9.

Radhakrishnan SK, Kamalakaran S (2006). Pro-apoptotic role of NF-kappa B: implications for cancer therapy. *Biochim Biophys Acta* **1766:** 53-62.

Reis CR, van Assen AH, Quax WJ, Cool RH (2011). Unraveling the binding mechanism of trivalent tumor necrosis factor ligands and their receptors. *mol cell proteomics* **10**.

Reis CR, Van der Sloot AM, Natoni A, Szegezdi E, Setroikromo R, Meijer M *et al* (2010). Rapid and efficient cancer cell killing mediated by high-affinity death receptor homotrimerizing TRAIL variants. *cell Death Dis* **21**.

Reis CR, van der sloot AM, Szegezdi E, Natoni A, Tur V, Cool RH *et al* (2009). Enhancement of antitumor properties of rhTRAIL by affinity increase toward its death receptors. *Biochemistry* **48:** 2180-91.

Riccioni R, Pasquini L, Mariani G, Saulle E, Rossini A, Diverio D *et al* (2005). TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL. *haematologica* **90:** 612-24.

Roninson IB, Broude EV, Chang BD (2001). If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat* **4:** 303-13.

Rossin A, Derouet M, Abdel-Sater F, Hueber AO (2009). Palmitoylation of the TRAIL receptor DR4 confers an efficient TRAIL-induced cell death signalling. *Biochem J* **419:** 185-92.

Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV (1995). The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* **83:** 1243-52.

Sadarangani A, Kato S, Espinoza N, Lange S, Llados C, Espinosa M *et al* (2007). TRAIL mediates apoptosis in cancerous but not normal primary cultured cells of the human reproductive tract. *Apoptosis* **12:** 73-85.

Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P (2004). Toxic proteins released from mitochondria in cell death. *Oncogene* **23:** 2861-74.

Sakamaki JI, Daitoku H, Ueno K, Hagiwara A, Yamagata K, Fukamizu A (2011). Arginine methylation of BCL-2 antagonist of cell death (BAD) counteracts its phosphorylation and inactivation by Akt. *Proc Natl Acad Sci U S A* **108:** 6085-6090.

Saleh A, Srinivasula SM, Acharya S, Fishel R, Alnemri ES (1999). Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J Biol Chem* **274:** 17941-5.

Sanlioglu AD, Dirice E, Aydin C, Erin N, Koksoy S, Sanlioglu S (2005). Surface TRAIL decoy receptor-4 expression is correlated with TRAIL resistance in MCF7 breast cancer cells. *BMC Cancer* **5:** 54.

Sanlioglu AD, Karacay B, Koksal IT, Griffith TS, Sanlioglu S (2007). DcR2 (TRAIL-R4) siRNA and adenovirus delivery of TRAIL (Ad5hTRAIL) break down in vitro tumorigenic potential of prostate carcinoma cells. *Cancer Gene Ther* **14:** 976-84.

Scaffidi C, Fulda S, Srinivasula SM, Friesen C, Li F, Tomaselli KJ *et al* (1998). Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* **17:** 1675-87.

Scaffidi C, Schmitz I, Krammer PH, Peter ME (1999). The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem* **274:** 1541-8.

Schneider P, Bodmer JL, Thome M, Hofmann K, Holler N, Tschopp J (1997a). Characterization of two receptors for TRAIL. *FEBS Lett* **416:** 329-34.

Schneider P, Olson D, Tardivel A, Browning B, Lugovskoy A, Gong D *et al* (2003). Identification of a New Murine Tumor Necrosis Factor Receptor Locus That Contains Two Novel Murine Receptors for Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL). *J Biol Chem* **278:** 5444-5454.

Schneider P, Thome M, Burns K, Bodmer JL, Hofmann K, Kataoka T *et al* (1997b). TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. *Immunity* **7:** 831-6.

Screaton GR, Mongkolsapaya J, Xu XN, Cowper AE, McMichael AJ, Bell JI (1997). TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. *Curr Biol* **7:** 693-6.

Secchiero P, Gonelli A, Carnevale E, Milani D, Pandolfi A, Zella D *et al* (2003). TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways. *Circulation* **107:** 2250-6.

Secchiero P, Melloni E, Corallini F, Beltrami AP, Alviano F, Milani D *et al* (2008). Tumor necrosis factor-related apoptosis-inducing ligand promotes migration of human bone marrow multipotent stromal cells. *Stem Cells* **26:** 2955-63.

Secchiero P, Zerbinati C, Rimondi E, Corallini F, Milani D, Grill V *et al* (2004). TRAIL promotes the survival, migration and proliferation of vascular smooth muscle cells. *Cell Mol Life Sci* **61:** 1965-74.

Sedger LM, Glaccum MB, Schuh JC, Kanaly ST, Williamson E, Kayagaki N *et al* (2002). Characterization of the in vivo function of TNF-alpha-related apoptosis-inducing ligand, TRAIL/Apo2L, using TRAIL/Apo2L gene-deficient mice. *Eur J Immunol* **32:** 2246-54.

Seki N, Hayakawa Y, Brooks AD, Wine J, Willtrout RH, Yagita H *et al* (2003). Tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis is an important endogenous mechanism for resistance to liver metastases in murine renal cancer. *Cancer Res* **63:** 207-13.

Sharma S, de Vries EG, Infante JR, Oldenhuis C, Chiang L, Bilic S *et al* (2008). Phase I trial of LBY135, a monoclonal antibody agonist to DR5, alone and in combination with capecitabine in advanced solid tumors. *J Clin Oncol* **26**.

Shenoy K, Wu Y, Parvaiz S (2009). LY303511 enhances TRAIL sensitivity of SHEP-1 neuroblastoma cells via hydrogen peroxide-mediated mitogen-activated protein kinase activation and up-regulation of death receptors. *Cancer Res* **69:** 1941-50.

Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D *et al* (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* **277:** 818-21.

Shetty S, Gladden JB, Henson ES, Hu X, Villanueva J, Haney N *et al* (2002). Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) up-regulates death receptor 5 (DR5) mediated by NFkappaB activation in epithelial derived cell lines. *Apoptosis* **7:** 413-20.

shi J, Zheng D, Man K, Fan ST, Xu R (2003). TRAIL: a potential agent for cancer therapy. *Curr Mol Med* **3:** 727-36.

Shin MS, Kim HS, Lee SH, Park WS, Kim SY, Park JY *et al* (2001). Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers. *Cancer Res* **61:** 4942-6.

Shipman CM, Croucher PI (2003). Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. *Cancer Res* **63:** 912-6.

Shrader M, Pino MS, Lashinger L, Bar-Eli M, Adam L, Dinney CP *et al* (2007). Gefitinib reverses TRAIL resistance in human bladder cancer cell lines via inhibition of AKT-mediated X-linked inhibitor of apoptosis protein expression. *Cancer Res* **67:** 1430-5.

Shu HB, Halpin DR, Goeddel DV (1997). Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity* **6:** 751-63.

Siegel RM, Frederiksen JK, Zacharias DA, Chan FK, Johnson M, Lynch D *et al* (2000). Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. *Science* **288:** 2354-7.

Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R *et al* (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89:** 309-19.

Soderstrom TS, Poukkula M, Holmstrom TH, Heiskanen KM, Eriksson JE (2002). Mitogen-activated protein kinase/extracellular signal-regulated kinase signaling in activated T cells abrogates TRAIL-induced apoptosis upstream of the mitochondrial amplification loop and caspase-8. *J Immunol* **169:** 2851-60.

Song JH, Tse MC, Bellail A, Phuphanich S, Khuri FR, Kneteman NM *et al* (2007). 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* **67:** 6946-55.

Soria JC, Mark Z, Zatloukal P, Szima B, Albert I, Juhasz E *et al* (2011). Randomized Phase II Study of Dulanermin in Combination With Paclitaxel, Carboplatin, and Bevacizumab in Advanced Non-Small-Cell Lung Cancer. *J Clin Oncol* **[Epub ahead of print]**.

Soria JC, Smit E, Khayat D, Besse B, Yang X, Hsu CP *et al* (2010). Phase 1b study of dulanermin (recombinant human Apo2L/TRAIL) in combination with paclitaxel, carboplatin, and bevacizumab in patients with advanced non-squamous non-small-cell lung cancer. *J Clin Oncol* **28:** 1527-33.

Spaeth EL, Dembinski JL, Sasser AK, Watson K, Klopp A, Hall B *et al* (2009). Mesenchymal Stem Cell Transition to Tumor-Associated Fibroblasts Contributes to Fibrovascular Network Expansion and Tumor Progression. *PLoS ONE* **4:** e4992.

Sperandio S, de Belle I, Bredesen DE (2000). An alternative, nonapoptotic form of programmed cell death. *Proc Natl Acad Sci U S A* **97:** 14376-14381.

Sprick MR, Rieser E, Stahl H, Grosse-Wilde A, Weigand MA, Walczak H (2002). Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *EMBO J* **21:** 4520-4530.

Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J *et al* (2000). FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity* **12:** 599-609.

Sreekumar A, Nyati MK, Varambally S, Barrette TR, Ghosh D, Lawrence TS *et al* (2001). Profiling of Cancer Cells Using Protein Microarrays
Discovery of Novel Radiation-regulated Proteins. *Cancer Res* **61**.

Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J *et al* (2001). A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* **410:** 112-6.

Steed PM, Tansey MG, Zalevsky J, Zhukovsky EA, Desjarlais JR, Szymkowski DE *et al* (2003). Inactivation of TNF signaling by rationally designed dominant-negative TNF variants. *Science* **301:** 1895-8.

Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS *et al* (2002). Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc Natl Acad Sci U S A* **99:** 16899-903.

Sun SY, Yue P, Zhou JY, Wang Y, Choi Kim HR, Lotan R *et al* (2001). Overexpression of BCL2 blocks TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in human lung cancer cells. *Biochem Biophys Res Commun* **280:** 788-97.

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* **8:** 613-21.

Symanowski J, Vogelzang N, Zawel L, Atadja P, Pass H, Sharma S (2009). A histone deacetylase inhibitor LBH589 downregulates XIAP in mesothelioma cell lines which is likely responsible for increased apoptosis with TRAIL. *J Thorac Oncol* **4:** 149-60.

Szegezdi E, Cahill S, Meyer M, O'Dwyer M, Samali A (2006). TRAIL sensitisation by arsenic trioxide is caspase-8 dependent and involves modulation of death receptor components and Akt. *Br J Cancer* **94:** 398-406.

Szegezdi E, Reis CR, van der Sloot AM, Natoni A, O' Reilly A, Reeve J *et al* (2011). Targeting AML through DR4 with a novel variant of rhTRAIL. *J Cell Mol Med* **15:** 2216-31.

Takahashi A, Musy PY, Martins LM, Poirier GG, Moyer RW, Earnshaw WC (1996). CrmA/SPI-2 inhibition of an endogenous ICE-related protease responsible for lamin A cleavage and apoptotic nuclear fragmentation. *J Biol Chem* **271:** 32487-90.

Takeda K, Cretney E, Hayakawa Y, Ota T, Akiba H, Oqasawara K *et al* (2005). TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. *Blood* **105:** 2082-9.

Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S *et al* (2001). Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med* **7:** 94-100.

Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Kayagaki N, Yagita H *et al* (2002). Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. *J Exp Med* **195:** 161-9.

Tansey MG, Szymkowski DE (2009). The TNF superfamily in 2009: new pathways, new indications, and new drugs. *Drug Discov Today* **14:** 1082-8.

Tartaglia LA, Ayres TM, Wong GH, Goeddel DV (1993). A novel domain within the 55 kd TNF receptor signals cell death. *Cell* **74:** 845-53.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR *et al* (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81:** 801-9.

Tolcher AW, Mita M, Meropol NJ (2007). Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *J Clin Oncol* **25:** 1390-1395.

Tran SE, Holmstrom TH, Ahonen M, Kahari VM, Eriksson JE (2001). MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. *J Biol Chem* **276:** 16484-90.

Trarbach T, Moehler M, Heinemann V, Kohne CH, Przyborek M, Schulz C *et al* (2010). Phase II trial of mapatumumab, a fully human agonistic monoclonal antibody that targets and activates the tumour necrosis factor apoptosis-inducing ligand receptor-1 (TRAIL-R1), in patients with refractory colorectal cancer. *Br J Cancer* **102**.

Trauzold A, Siegmund D, Schniewind B, Sipos B, Egberts J, Zorenkov D *et al* (2006). TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. *Oncogene* **25:** 7434-7439.

Travert M, Ame-Thomas P, Pangault C, Morizot A, Micheau O, Semana G *et al* (2008). CD40 Ligand Protects from TRAIL-Induced Apoptosis in Follicular Lymphomas through NF-κB Activation and Up-Regulation of c-FLIP and Bcl-xL. *J Immunol* **181:** 1001-1011.

Tschopp J, Irmler M, Thorne M (1998). Inhibition of fas death signals by FLIPs. *Curr Opin Immunol* **10:** 552-8.

Tur V, van der Sloot AM, Reis CR, Szegezdi E, Cool RH, Samali A *et al* (2008). DR4-selective tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) variants obtained by structure-based design. *J Biol Chem* **283:** 20560-8.

van der Sloot AM, Mullally MM, Fernandez-Ballester G, Serrano L, Quax WJ (2004). Stabilization of TRAIL, an all-beta-sheet multimeric protein, using computational redesign. *Protein Eng Des Sel* **17:** 673-80.

van der Sloot AM, Tur V, Szegezdi E, Mullally MM, Cool RH, Samali A *et al* (2006). Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. *Proc Natl Acad Sci U S A* **103:** 8634-9.

Varfolomeev E, Maecker H, Sharp D, Lawrence D, Renz M, Vucic D *et al* (2005). Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. *J Biol Chem* **280:** 40599-608.

Vaux DL, Silke J (2005). IAPs, RINGs and ubiquitylation. *Nat Rev Mol Cell Biol* **6:** 287-97.

Vercammen D, Brouckaert G, Denecker G, van de Craen M, Declercq W, Fiers W *et al* (1998). Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J Exp Med* **188:** 919-30.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE *et al* (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102:** 43-53.

Vitovski S, Phillips JS, Sayers J, Croucher PI (2007). Investigating the interaction between osteoprotegerin and receptor activator of NF-kappaB or tumor necrosis factor-related apoptosis-inducing ligand: evidence for a pivotal role for osteoprotegerin in regulating two distinct pathways. *J Biol Chem* **282:** 31601-9.

Vivona S, Gardy JL, Ramachandran S, Brinkman FS, Raghava GP, Flower DR *et al* (2008). Computer-aided biotechnology: from immuno-informatics to reverse vaccinology. *Trends Biotechnol* **26:** 190-200.

Von Pawel J, Harvey JH, Spigel DR, Dediu M, Reck M, Cebataru CL *et al* (2010). A randomized phase II trial of mapatumumab, a TRAIL-R1 agonist monoclonal antibody, in combination with carboplatin and paclitaxel in patients with advanced NSCLC. *J Clin Oncol* **28:** (suppl abstr LBA7501).

Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K *et al* (2007). Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. *Nat Med* **13:** 1070-7.

Wajant H, Moosmayer D, Wuest T, Bartke T, Gerlach E, Schonherr U *et al* (2001). Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. *Oncogene* **20:** 4101-6.

Wakelee HA, Patnaik A, Sikic BI, Mita M, Fox NL, Ullrich SJ *et al* (2010). Phase I and pharmacokinetic study of lexatumumab (HGS-ETR2) given every 2 weeks in patients with advanced solid tumors. *Ann Oncol* **21:** 376-81.

Walczak H, Bouchon A, Stahl H, Krammer PH (2000). Tumor necrosis factor-related apoptosis-inducing ligand retains its apoptosis-inducing capacity on Bcl-2- or Bcl-xL-overexpressing chemotherapy-resistant tumor cells. *Cancer Res* **60:** 3051-7.

Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N *et al* (1997). TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *Embo J* **16:** 5386-97.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M *et al* (1999). Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* **5:** 157-63.

Wang S, El-Deiry WS (2003). Requirement of p53 targets in chemosensitization of colonic carcinoma to death ligand therapy. *Proc Natl Acad Sci U S A* **100:** 15095-100.

Wang X, Chen W, Zeng W, Bai L, Tesfaigzi Y, Belinsky SA *et al* (2008). Akt-mediated eminent expression of c-FLIP and Mcl-1 confers acquired resistance to TRAIL-induced cytotoxicity to lung cancer cells. *Mol Cancer Ther* **7:** 1156-63.

Wei H, Wei W, Bredesen DE, Perry DC (1998). Bcl-2 protects against apoptosis in neuronal cell line caused by thapsigargin-induced depletion of intracellular calcium stores. *J Neurochem* **70:** 2305-14.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ *et al* (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**.

Weissman AM (2001). Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* **2:** 169-78.

Werner AB, de Vries E, Tait SW, Bontjer I, Borst J (2002). TRAIL receptor and CD95 signal to mitochondria via FADD, caspase-8/10, Bid, and Bax but differentially regulate events downstream from truncated Bid. *J Biol Chem* **277:** 40760-7.

Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK *et al* (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* **3:** 673-82.

Wu GS, Burns TF, McDonald ER, 3rd, Jiang W, Meng R, Krantz ID *et al* (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* **17:** 141-3.

Wu GS, Burns TF, Zhan Y, Alnemri ES, El-Deiry WS (1999). Molecular Cloning and Functional Analysis of the Mouse Homologue of the KILLER/DR5 Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Death Receptor *cancer Res* **59:** 2770-5.

Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER *et al* (2007). Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res* **67:** 2649-56.

Wyllie AH, Kerr JF, Currie AR (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* **68:** 251-306.

Xu L, Qu X, Zhang Y, Hu X, Yang X, Hou K *et al* (2009). Oxaliplatin enhances TRAIL-induced apoptosis in gastric cancer cells by CBL-regulated death receptor redistribution in lipid rafts. *FEBS Lett* **583:** 943-8.

Yamada H, Tada-Oikawa S, Uchida A, Kawanishi S (1999). TRAIL causes cleavage of bid by caspase-8 and loss of mitochondrial membrane potential resulting in apoptosis in BJAB cells. *Biochem Biophys Res Commun* **265:** 130-3.

Yang QH, Church-Hajduk R, Ren J, Newton ML, Du C (2003). Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. *Genes Dev* **17:** 1487-96.

Yang QH, Du C (2004). Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and livin in HeLa cells. *J Biol Chem* **279:** 16963-70.

Yee L, Burris HA, Kozloff M, Wainberg Z, Pao M, Skettino S *et al* (2009). Phase Ib study of recombinant human Apo2L/TRAIL plus irinotecan and cetuximab or FOLFIRI in metastatic colorectal cancer (mCRC) patients (pts): Preliminary results. *J Clin Oncol* **27:** 4129.

Yee L, Fanale K, Dimick S, Calvert C, Robins J, Ing J *et al* (2007). A phase IB safety and pharmacokinetic (PK) study of recombinant human Apo2L/TRAIL in combination with rituximab in patients with low-grade non-Hodgkin lymphoma. *J Clin Oncol* **25:** 8078.

Younes A, Vose JM, Zelenetz AD, Smith MR, Burris HA, Ansell SM *et al* (2010). A Phase 1b/2 trial of mapatumumab in patients with relapsed/refractory non-Hodgkin's lymphoma. *Br J Cancer* **103:** 1783-7.

Yu JW, Jeffrey PD, Shi Y (2009). Mechanism of procaspase-8 activation by c-FLIPL. *Proc Natl Acad Sci U S A* **106:** 8169-74.

Yu X, Acehan D, Ménétret JF, Booth CR, Ludtke SJ, Riedl SJ *et al* (2005). *Structure* **13:** 1725-35.

Zakrzewska M, Krowarsch D, Wiedlocha A, Olsnes S, J. O (2005). Highly stable mutants of human fibroblast growth factor-1 exhibit prolonged biological action. *J Mol Biol* **352:** 860-75.

Zhang L, Zhu H, Teraishi F, Davis JJ, Guo W, Fan Z *et al* (2005). Accelerated degradation of caspase-8 protein correlates with TRAIL resistance in a DLD1 human colon cancer cell line. *Neoplasia* **7:** 594-602.

Zhang QH, Sheng HP, Loh TT (2001). bcl-2 protects HL-60 cells from apoptosis by stabilizing their intracellular calcium pools. *Life Sci* **68:** 2873-83.

Zhang XD, Borrow JM, Zhang XY, Nguyen T, Hersey P (2003). Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. *Oncogene* **22:** 2869-81.

Zhuang S, Schnellmann RG (2006). A death-promoting role for extracellular signal-regulated kinase. *J Pharmacol Exp Ther* **319:** 991-7.

Zinonos I, Labrinidis A, Lee MH, Liapis V, Hay S, Ponomarev V *et al* (2011). Anticancer Efficacy of Apo2L/TRAIL Is Retained in the
Presence of High and Biologically Active Concentrations
of Osteoprotegerin In Vivo
J Bone Miner Res **26**.

Zou H, Li Y, Liu X, Wang X (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* **274:** 11549-56.

Zou W, Liu X, Yue P, Zhou Z, Sporn MB, Lotan R *et al* (2004). c-Jun NH2-terminal kinase-mediated up-regulation of death receptor 5 contributes to induction of apoptosis by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1, 9-dien-28-oate in human lung cancer cells. *Cancer Res* **64:** 7570-8.