THE TRAIL PATHWAY IN CARCINOMAS; REGULATION OF APOPTOSIS AND THERAPEUTIC APPLICATION

By

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ABSTRACT

TRAIL is a member of TNF superfamily and binding of TRAIL to its receptors induces cell death with apparent specificity for carcinoma cells compared with normal epithelium. On this basis, a number of TRAIL-targeted therapies are currently under investigation as anti-cancer agents. Recent studies in our lab have shown that membrane bound forms of both FasL and CD40L are more potent in cell death induction, suggesting that a membrane bound TRAIL could be more effective in cell death induction compared to the wild-type or the soluble counterparts. TRAIL gene therapies have demonstrated that ex vivo infection of cancer cells with a recombinant adenovirus expressing TRAIL (RAdTRAIL) enhances apoptosis and promotes tumour regression. In the present study, we have generated two adenoviral vectors. The first one expressing wild-type TRAIL (RAd wtTRAIL) that subject to cleavage from cell membrane and the second adenoviral vector expressing fusion CD40LTRAIL protein that is resistant to metalloproteinase cleavage (RAd CD40LTRAIL). The direct effects of these viruses were examined on TRAIL receptor positive carcinomas either alone or in combination with different chemotherapeutic drug. The RAd CD40LTRAIL that expressed the membrane bound CD40LTRAIL was found to exhibit more cell death than the RAd wtTRAIL. This cell death was through the activation of caspase 3/7.

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List of abbreviations:

5-FU	5 Fluorouracil
aa	Amino Acid
AFP	α-fetoprotein
B-CLL	B-cell chronic lymphocytic leukaemia
CAR	Coxsackie adenovirus receptor.
c-FLIP	Cellular FLICE inhibitory protein
CHX	Cyclohexaimide
CIS	Cisplatin
CNS	central nervous system
CR2	Conserved region 2
CRD	Cysteine-rich domains
CTL	Cytotoxic T cells
DC	Dendritic cells
DD	Death Domain
DED	Death effector domain
DISC	Death-receptor induced signalling complex.
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signalling-regulated kinase 1 and 2
ESTs	Expressed sequence Tags.
FADD	Fas Associated Death Domain adaptor protein
FCS	Foetal calf serum
FDA	The US Food and Drug Administration
FGF-2	Fibroblast growth factors-2
Flk-1	Fetal liver kinase 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinosito
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factors
IL-2	Interleukin-2
JNK	c-Jun N-terminal kinases
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MMPs	Metalloproteases
MOMP	Mitochondrial outer-membrane Permeabilization.
OPG	Osteoprotegerin

PARP	Poly ADP-ribose polymerase.
PCR	polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet-derived growth factor receptors
PLAD	Pre-ligand binding assembly domain
pRb	Retinoblastoma protein
PSA	Prostate specific antigen
RIP	Receptor-interacting protein
RT-PCR	Real-Time polymerase chain reaction
Tcf	T cell factor
THD	TNF-homology domain
TIL	Tumour-infiltrating lymphocytes
TNF	The tumour necrosis factor
TRADD	TNF receptors associated factor death domain
TRAF	TNF receptors associated factor
TRAIL	TNF related apoptosis-Inducing Ligand
VEGFR	Vascular Endothelial Growth Factor Receptor
Wnt	Wingless integration site
wt	Wild-Type

CHAPTER 1
INTRODUCTION

1.1 : Cancer and its clinical management.

1.1.1 : The hallmarks of cancer.

Cancer is fundamentally a genetic disease developing from mutations in genes encoding for proteins important for normal cell proliferation, differentiation and homeostasis, resulting in defects in the regulatory mechanism governing these processes. Most cancer developed by Deoxyribonucleic acid (DNA) damage that regulates normal cell homeostasis. Tumour suppressor genes and oncogenes are common targeted groups susceptible to genetic lesion. Mutations which occur in tumour suppressor genes results in total loss or down-regulation in functional proteins, whereas mutation that occurs in oncogene results in dominant gain of function in proteins which are required for normal cellular functions (Hahn et al., 1999). Although there are more than 200 different types of human cancers, it has been proposed that normal cells regardless of anatomical location or aetiology require six alterations in cell physiology in order to induce malignant growth. This includes self-sufficiency from growth factor signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, angiogenesis within the tumour mass and tissue invasion/metastasis (Hanahan and Weinberg, 2000). Recently four new hallmarks of cancer have been proposed which including abnormal energy metabolism, evading immune system, genome instability and inflammation (Hanahan and Weinberg, 2011). These "hallmarks of cancer" enable a single cell

to grow into a tumour mass which is independent of its local environmental cues and self-sufficient for nutrient delivery.

Tumour Microenvironment is the cellular environment within the tumours, including carcinoma associated fibroblasts, immune cells, surrounding blood and lymphatic vascular endothelial cells. Recent studies demonstrate a positive correlation between the present of the inflammatory cells and tumour progression (Coussens and Werb, 2002). Hepatic tumours have been associated with chronic inflammation: for example, Hepatitis B and C (Karin et al. 2006). Chronic inflammations create a local microenvironment for tumour progression through the interactions of tumour cells and inflammatory cells. In excessive tumour cell proliferation hypoxia and necrosis stimulate tumour cells and tumour microenvironment cells to release number of growth factor and chemoattractive for monocytes and macrophages (Allavena et al., 2008). In turn, recruited monocytes and macrophages secrete Vascular Endothelial Growth Factor Receptors (VEGF), Fibroblast growth factors (FGF), Hepatocyte growth factors (HGF) and chemokines such as CXC12 and IL-8 within the tumour microenvironment and stimulate tumour cell proliferation, survival and angiogenesis (Lorusso and Ruegg, 2008).

1.1.2 : Cancer treatment.

1.1.2.1 : Current therapeutic regimes.

The three main approaches for cancer treatment are surgical excision, irradiation and chemotherapy, with the preferred choice of regimen depending on the tumour type and stage of disease. Surgical removal and radiotherapy treatment are generally the first line of treatment for localised cancer and can be curative in appropriately selected patients. Chemotherapy may be used palliatively to improve symptoms and prolong survival of cancer patients, or as an adjuvant to surgery or radiotherapy to reduce the risk of recurrent growth. Indeed, combination treatments with chemotherapy and radical surgery or radiotherapy in clinical trials have clearly demonstrate improvement in survival compared to surgery or radiotherapy alone for a number of common cancers, including breast, colorectal, lung, and pancreatic cancers. Combination chemotherapy can effectively afford cure for certain cancers such as germ cell tumours, Hodgkin's disease and Non-Hodgkin's Lymphoma even in the presence of disseminated disease. However, for the most common carcinomas chemotherapy is generally palliative in the setting of advanced disease and benefits must be weighed against potential toxicity of drugs with relatively non-specific mechanisms of action. The most common anti-cancer drugs used currently have been identified more than 50 years ago by *in vitro* cell line screening programmes. These anti-cancer drugs exhibit different mechanisms of

action and side effects. In general anti-cancer drugs function through interfering with the cell cycle and cell division, particularly at the level of DNA synthesis and replication. For example, *Cis*-platin forms cross-linking covalent bonds with DNA and thus impedes DNA replication and enhances apoptosis. 5-fluorouracil is a pyrimidine analogue and thus prevents thymidine synthesis. Doxorubicin and etoposide stabilise the topoisomerase II/DNA complex and thus effectively seize up DNA synthesis (Rang et al., 1995). All these compounds have been extremely useful for cancer treatments, although they have potential severe toxic side effects. However the efficacy of these chemotherapeutic drugs can be significantly reduced due to the genetic alteration resulting in promoting drug resistance. For example, relative unresponsive chemotherapy is associated with tumours bearing mutations in the tumour suppressor protein p53 as a result of function loss (Aas et al., 1996, Hait and Yang, 2006). In addition, acquiring drug resistance after a period of treatment is another challenge as with ovarian carcinoma culminating in disease relapse or progression (Khaider et al., 2012) Given the clinical limitations of conventional chemotherapy, the search for novel therapeutic agents is of paramount importance.

1.1.2.2 : Novel mechanisms of cancer therapy.

Understanding of the mechanisms of tumourigenesis has facilitated the development of selective anti-cancer therapies and reduced the side effect that often exhibited by traditional chemotherapeutic drugs. For example, inhibition of angiogenesis by preventing tumour vascularisation is a recent approach for controlling tumourgenisis where inhibition of the angiogenic growth factor receptors such as Fetal liver kinase 1 (Flk-1/KDR), VEGFR and PDGFR, induced rapid microvasculature cell death and tumour regression (Laird et al., 2002, Burris et al., 2004). Targeting normal endothelial cells would overcome the problem of drug resistance because these cells are genetically stable (Kerbel, 2008). Angiogenic inhibitors drug would suit different tumour types for example Avastin (bevacizumab) (Ferrara et al, 2005). In addition, angiogenesis inhibitor could have side effect such as bleeding, clots, and hypertension (Kristina and William 2010). Furthermore, recent studies show that tumours can develop resistance to angiogenic inhibitors through alternative angiogenesis pathway Such as FGF-2 (Casanovas et al., 2005). A number of recent angiogenic inhibitors used in combination with conventional cytotoxic drugs in clinical trial have now been demonstrated to improve survival (de Bouard et al., 2007, Mackey et al., 2012). Moreover, better understanding of the mechanism of kinases whose dysfunction is critical for the maintenance of the cancer cell phenotype has lead to development of a number of kinase inhibitors. For

example, the anti-cancer agents Imatinib (Gleevec, STI-571), and Lapatinib have received approval from the US Food and Drug Administration (FDA). Imatinib has proved extremely successful for the treatment of Bcr-Abl positive chronic myeloid leukaemia, and c-kit positive Gastrointestinal stromal tumours by specifically inhibiting these hyper-active kinases (O'Dwyer and Druker, 2001). Likewise, the kinase signalling activity, most commonly of VEGFR, in tumour endothelium can be targeted for inhibition of angiogenesis.

Immunotherapy is also a potential strategy for cancer treatment, where better understanding of tumour immune-environment has lead to the development of specific monoclonal antibodies capable of binding key targets. Monoclonal antibodies can trigger specific immune-response to attack cancer cells or to block specific dysfunctional receptors in cancer cells. For example, about 30% of breast carcinomas acquire gene amplification of the epidermal growth factor receptor (EGFR) family member Her-2/neu (ErbB-2) receptor promoting constitutive dimerisation and activation of ErbB family members (Slamon et al., 1987). The development of trastuzumab (Herceptin), an antagonistic monoclonal antibody directed towards Her-2/neu, used in conjunction with chemotherapy has significantly increased the survival rate of patients with Her-2/neu positive breast carcinomas compared to chemotherapy alone (Slamon et al., 2001, Lower et al., 2009).

Furthermore, triggering the Immune system also has demonstrated some clinical activity in those tumours associated with an inflammatory or immune response, such as melanoma, renal cell carcinoma and hepatocellular carcinoma. Most cancers have the potential to be a target for immunotherapeutic manipulation. For example, at the tumour site there is a concurrent recruitment and infiltration of lymphocytes which suggest potential cytotoxic effector cell activation. In addition, tumour-infiltrating lymphocytes (TIL) expanded *ex vivo* in the presence of interleukin-2 (IL-2) display anti-tumour cytolytic activity. However, TIL are frequently only partially activated, proliferate only at very low level and fail to kill tumour cells unless activated by IL-2 *in vitro* suggesting that immunosuppressive mechanisms prevent T cell maturation into useful anti-tumour effectors. The activation of tumour-specific cytotoxic T cells requires three synergistic signals: the presentation of tumour antigen by antigen-presenting cells to specific T-helper cells; the interaction between co-stimulatory factors (e.g. CD40 and its ligand); and the secretion of immunostimulatory cytokines (e.g. IL-2, IL-12) from activated dendritic cells (DCs). Dendritic cells (DC) are the most potent professional antigen-presenting cells that can capture, process and present antigens to naïve T cells, stimulating their proliferation and activation. They provide the optimum co-stimulatory environment, to evoke an immunostimulatory signal and key to this process is ligation of the CD40 receptor on DC by its ligand expressed by T-helper cells.

The direct anti-tumour effects of CD40L ligation have been observed from clinical experiment. Treatment of Burkitt lymphoma and Multiple myeloma with CD40L result in marked decrease in tumour proliferation (Teoh et al, 2000). In addition to the direct anti-proliferative effect of the CD40 ligation in carcinoma, CD40L involve in immune recognition and generation of cytotoxic T lymphocyte cell. The cytotoxic effects are mediated by the interaction of CD40 expressed on the DCs with CD40L expressed on antigen presenting cells (Van Mierlo et al, 2002). CD40-mediated stimulation of DCs and provides help required by $CD8^+$ CTLs, which target tumour cells independently of their CD40 status. Moreover, delivery of CD40L by recombinant adenovirus (RAd-CD40L) to tumour was sufficient to stimulate anti-tumour response depend on $CD8^+$ and CTLs (Eliopoulos and Young et al, 2004).

Furthermore, cytokines and immunotherapy might be a potential strategy as they directly induce tumour cell death. Indeed, the first systemic therapy for cancer, Coley's toxins relied on the production of endogenous TNF for its tumour cytotoxicity, reviewed by (Old, 1985). Large numbers of animal studies have demonstrated cytokines multiple effects within an organism which can potentially contribute to the regression of tumours. In addition cytokines can enhance or initiate a host immune response to the tumour cell by activating cytotoxic T lymphocytes and NK cells (Zamarron and Chen, 2011).

1.2 : The TNF superfamily of receptors and ligands.

1.2.1 : The TNF ligand family.

The tumour necrosis factor (TNF) superfamily of cytokines plays an essential role in immune responses and inflammation by modulating lymphocyte proliferation and apoptosis which is implicated in maintaining homeostasis and self tolerance (Cancro and Smith, 2003). TNF, was first identified in 1975 (Carswell et al., 1975) and consequently with the cloning of human TNF (Pennica et al., 1984), and with the large-scale screening of "expressed sequence tags" (ESTs), it is now known that TNF belongs to a large family of proteins (the TNF superfamily) which mediate cellular effects by activating receptors (the TNF-R superfamily) directly coupled to signalling pathways. To date TNF family ligands include 19 identified members, all of them are Type II transmembrane proteins that have an intracellular N-terminus and an extracellular C-terminus except Lymphotoxin alpha (LTα) and Vascular Endothelial Growth Inhibitor (VEGI).Sequence analysis of TNF ligands indicates that only 25-30% of homology between members exists despite the fact that all members possess the characteristic TNF-homology domain (THD), a conserved sequence of 150 amino acids located at the C-terminal of the extracellular domain. The THD is the biologically active domain of TNF ligands, and consists of a conserved chain of aromatic and hydrophobic residues folding into a β sandwich with "jellyroll" topology. Analysis of

crystals purified from recombinant TNF ligands has confirmed the trimeric formation of recombinant TNF ligands (Pennica et al., 1984, Banner et al., 1993). TNF ligands are biologically active as self assembling, non covalent bound trimers (Peschon et al., 1998), expressed at the cell membrane as membrane attached molecules; however some ligands are subject to extracellular proteolytical cleavage from membrane by the action of specific group of proteases belonging to the family of metalloproteases (MMPs) to produce soluble TNF ligand molecules. Other TNF ligands, such as LTα, are expressed only as soluble molecules but may also be recruited to the cell membrane to form heterotrimeric membrane anchored complexes (Idriss and Naismith, 2000). With the exception of soluble TNF ligands, which appears to mediate a far more potent cytotoxic effect than its membrane bound form (Mohler et al., 1994), most TNF ligands are biologically less active as a soluble cytokine (Schneider et al., 1998).

1.2.2 : The TNF receptor family.

Twenty-nine members of the TNF-receptor family have been identified so far (Table 1.1). The majority of these proteins are Type I transmembrane proteins which are characterized by extracellular N-terminus, intracellular C-terminus, with the exception of the recently identified members BCMA, TAC1, BAFF-R and XEDAR which are Type III transmembrane proteins which lack a signal peptide. Sequence analysis of the TNF receptors has revealed a sequence

homology of 20-25% between TNF receptor members. However, a characteristic cysteine-rich domain (CRD) exists between the TNF receptors. These pseudorepeats are defined by interchain disulphide bonds generated by highly conserved cysteine residues within the receptor chains (Smith et al., 1994). TNF receptors can be categorized into 4 categories based on structural similarities (Table 1.1). The first group, which include Fas, TNF-RI, TRAIL-R1 and TRAIL-R2 belong to the death domain receptors and are defined by a highly homologous region of approximately 80 amino acids within the cytoplasmic domain of the receptor and are termed as Death Domain (DD). Death receptors are the only members of the TNF receptor superfamily that can directly induce cell death. The second group consists of decoy receptors that are incapable of activating signalling pathways but can bind ligand with equal affinity and therefore can possess indirect inhibitory effect on the activation of functional receptors by binding to active ligand. TRAIL-R3 and TRAIL-R4 are membrane attached molecules that lack key domains within the cytoplasmic tail that are responsible for signal transduction. In contrast the TNF receptors DcR3 which binds FasL, and osteoprotegerin (OPG) which binds TRANCE, lack the transmembrane regions and thus function as soluble receptors. The third group of TNF receptors contains the atypical Type III proteins whilst the last group are functional Type I proteins that lack a DD, and includes CD40, CD30, TRANCE and TNF-R2.

Table1. 1: The TNF superfamily of ligands and receptors

Death Domain Receptors	Cognate ligand
TNF-R1	*TNFα/LT-β*
Fas	*FasL*
TRAIL-R1	TRAIL-L
TRAIL-R2	*TRAIL-L*
TRAMP	*TWEAK*
DR6	UN
NGFR	*NGF*
EDAR	*EDA-A2*

Decoy Receptors	Cognate ligand
TRAIL-R3	*TRAIL-L*
TRAIL-R4	*TRAIL-L*
OPG	*TRANCE-L*
DcR3	*FasL*

Other Receptors	Cognate ligand
CD40	*CD40L*
TRANCE	*TRANCE-L*
TNF-R2	*TNFα/LT-β*
LT-βR	*LTα$_1$β$_2$/LIGHT*
CD30	*CD30L*
CD27	*CD70*
4-1BB	*4-1BBL*
OX40	*OX40L*
Fn14	*TWEAK*
HVEM	*LTα/LIGHT*
TROY	UN
GITR	*GITR-L*
RELT	UN

Type III receptors	Cognate ligand
BCMA	*APRIL/BAFF*
TAC1	*APRIL/BAFF*
BAFF-R	*EDA-A1*
XEDAR	*EDA-A2*

UN = unidentified ligand

adapted from (Bodmer et al., 2002).

1.2.3 : TNF receptor-ligand interactions.

The fact that the TNF receptors lack any intrinsic kinase activity highlights the importance of interaction with their ligands for signal transduction, prior to TNF receptor ligand interaction, trimer structures of most TNF ligands and receptors are required. This 3:3 stoichiometry has been confirmed by analysis of the crystal structures of TRAIL bound to TRAIL-R2 (Hymowitz et al., 1999). Membrane attached receptors have been thought to exist as a monomeric structures and that a trimeric ligand binds three units of the receptor, however recent results suggest that some members of the TNF receptors (FAS, TNFRI, TNFRII) preassemble on the cell membrane prior to ligand binding (Chan et al., 2000). The TNF receptor preassembled complex formation on the surface requires the N-terminal domain including parts of the first CRD, termed as pre-ligand binding assembly domain (PLAD), this was confirmed by transplanting the PLAD of the TNFRI onto TNFRII which results in the assembly of heterotrimeric receptor complex. However, not all of the receptors seem to be pre-assembled into trimers prior ligation, as stimulation of cells with TNF ligand significantly enhanced receptor trimer formation (Chan et al., 2000). Therefore, ligation is still critical for receptor trimerisation.

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1.3 : Apoptosis.

1.3.1 : Introduction.

Apoptosis is a programmed cell death which is a morphologically and biochemically different form of cell death from necrosis. Apoptosis is characterised by condensation and fragmentation of DNA, nuclear degradation, cell shrinkage, membrane blebbing, a decrease in mitochondrial membrane potential and the exposure of phoshatidylserine on the outer leaflet of the plasma membrane (Kerr et al., 1972). Apoptosis is a highly controlled process involved in development as in embryo and tissue homeostasis, and to remove unwanted or defective cells from the body without inducing inflammatory responses. Moreover, apoptosis deregulation is involved in various diseases, including cancer, autoimmunity and degenerative disorders. Early genetic studies on the nematode *C. elegans* identified the mechanisms of apoptosis which found that apoptosis is genetically controlled by different initiators, effectors and inhibitors.

1.3.2 : The caspase family of cysteine proteases.

The apoptotic pathway is directly induced and regulated by a group of aspartate-directed cysteine proteases known as caspases. Caspases are highly homologous to the *Caenorhabditis elegans* death gene *ced-3* and are expressed as

inactive zymogens, in order to control their biological activity (Alnemri et al., 1996) reviewed in (Henkart, 1996).

At least 14 mammalian caspases have been identified so far (Fan et al., 2005). Caspases can be classified into activators, effectors and inflammatory inhibitors (Table 1.2), which cleave and activate each other to create amplifiable cascades of proteolysis (Boatright and Salvesen, 2003). Activator caspases including caspase-8, caspase-9, and caspase-10, are synthesized as inactive zymogens, therefore called procaspases which contain motifs that interact with adaptor proteins or undergo self-association, a process which is required for their autocatalytic cleavage into an active form. Activator caspases induce the proteolytic processing of downstream effector caspases or the executioner caspases which include caspase-3, caspase-6 and caspase-7. Effector caspases target several key components critical for cell viability including components of cellular DNA repair machinery such as poly ADP-ribose polymerase (PARP) and DNA-PK (Lazebnik et al., 1994, Casciola-Rosen et al., 1995, Nicholson et al., 1995), structural proteins such as actin, fodrin, lamin and gelsolin, which are important for intact nuclear and cellular morphology (Cryns et al., 1996, Orth et al., 1996, Takahashi et al., 1996, Kothakota et al., 1997, Mashima et al., 1997)

Table 1.2: The Subfamily members of caspase family.

Subfamily	Role	Member
I	Apoptosis activator (Initiator)	Caspase-2 Caspase-8 Caspase-9 Caspase-10
II	Apoptosis executioner (Effector)	Caspase-3 Caspase-6 Caspase-7
III	Inflammatory mediator	Caspase-1 Caspase-4 Caspase-5 Caspase-11 Caspase-12 Caspase-13 Caspase-14

(Fan et al., 2005)

1.3.3 : Pathways initiating apoptosis.

Two distinct pathways can trigger apoptosis mainly through the caspase family proteases. The first pathway involves the activation of caspases via cell surface receptor (death receptor). This pathway requires the recruitment of pro-caspase 8 or 10 into the cytoplasmic domain of the receptor and formation of a complex that results in auto catalytic activation of the pro-caspases to the active caspases. Second route which leads to initiating apoptosis is the more conserved pathway which is activated by either intra or extra stresses for example γ-radiation, chemical and cytotoxic drugs, this pathway leads to DNA damage followed by intracellular changes resulting in mitochondrial membrane potential loss and release of cytochrome c and subsequent activation of caspase-9. Both pathways directly lead to processing and activating downstream effector caspases.

1.3.3.1 : Mechanism and regulation of mitochondrial-mediated apoptosis.

Mitochondrial-mediated apoptosis involves a sequence of process that mainly rely on the release of a number of pro-apoptotic proteins into the cytosol, some of these proteins induce caspase activation (e.g. cytochrome *c*) and others cause caspase-independent cell destruction (e.g. apoptosis-inducing factor and endonuclease G). The release of these proteins is mainly dependent on the integrity of mitochondrial membrane, that could be impaired by intra or extra stresses leading to a mitochondrial outer-membrane permeabilization (MOMP),

a process that results in release of cell-death-inducing mitochondrial molecules and uncoupling of oxidative phosphorylation and production of toxic free radicals as a result of diversion of electrons from the respiratory chain. MOMP induced-cell death is mainly characterised by necrosis (Green and Kroemer, 2004, Spierings et al., 2005), a process whereby cells swell and eventually rupture. Several proteins have been reported to regulate the MOMP process, central to these regulators are members of the Bcl-2 family proteins, a large family that consists of at least of 26 members in humans of both pro-survival and apoptotic–inducing proteins which either induce or inhibit MOMP (Cory et al., 2003, Reed et al., 2004). Bcl-2-like family members which include Bcl-2, Bcl-x_L, Bcl-w, A1 and Mcl-1 are considered to be pro-survival regulators, whereas cell death is controlled by two Bcl-2 groups of proteins, the Bax and BH3-only families.

The Bax-like proteins are cell death inducers. These include Bax, Bak and Bok. Bax and Bak and are thought to directly trigger the cytochrome c release from mitochondria to cytosol, a process which are controlled by both the Bcl-2 family pro-survival and the pro-apoptotic BH3-only proteins (Cory and Adams, 2005). In healthy cells Bax exists in cytosol as an inactive monomer and upon apoptotic stimuli Bax translocates to mitochondria and oligomerises. In contrast, Bak forms an oligomerised mitochondrial membrane structure even in healthy cells and requires additional conformation changes to become pro-

apoptotic. The BH3-only pro-apoptotic family, which consists of Bad, Bid, Noxa and Bim are damage sensors that function by direct inhibition of the Bcl-2 pro-survival proteins via heterodimerising to and locking the survival protein into dysfunctional configuration. This binding is highly selective and varies widely in affinity (Chen et al., 2005, Certo et al., 2006, Kim et al., 2006), where Bim and Bid bind to and block all the pro-survival proteins, Bad is more selective as it binds only to Bcl-2, Bcl-xL and Bcl-w, whilst Noxa binds to Mcl-1 and A1 (Chen et al., 2005). Mitochondrial dysfunctional proteins have been reported during tumourigenesis, in human gastric and colorectal cancers loss of function mutations in Bax and Bak have been detected (Rampino et al., 1997, Kondo et al., 2000). In contrast, Bcl-2 is over-expressed in *cis*-platin-resistant ovarian carcinoma cell lines (Eliopoulos et al., 1995).

The role of anti-apoptotic Bcl-2 proteins in preventing caspase activation has been reported. However, the mechanism is not fully understood. Given that Bcl-2 does not directly interact with Apaf-1 (Moriishi et al., 1999) Bcl-2-like proteins may function by maintaining mitochondrial membrane integrity and thus preventing the release of cytochrome c, a molecule that is required for Caspase 9 activation. One hypothesis suggests that Bax/Bcl-2 heterodimer complex may prevent Bcl-2-like proteins from maintaining pre-existing channels within the mitochondrial membrane in a closed configuration (Narita et al., 1998, Shimizu et al., 1999), such a scenario would lead to subsequent

change in mitochondrial membrane potential, matrix swelling and mitochondrial rupturing enabling the release of cytochrome c (Vander Heiden et al., 1997). This seems unlikely as apoptosis could occur even in the absence of mitochondrial rupture, given that mitochondrial depolarisation occurs downstream of cytochrome c release (Bossy-Wetzel et al., 1998).

1.3.3.2 : Receptor-mediated apoptosis.

Receptor-mediated apoptosis is largely attributed to the death receptors of the TNF receptor superfamily (Table 1.1) via the death domain (DD), which is required for caspase-8 processing and transduction of the apoptotic signals (Peter and Krammer, 2003). Mutations in DD of Fas has been reported to cause lymphoproliferation disorder in mice that is manifested by accumulation of unwanted lymphoid cells as a result of a defect in Fas-mediated apoptosis (Watanabe-Fukunaga et al., 1992). The DD functions by binding to other DD containing-adaptor proteins such as FADD, TRADD and RIP enabling signal transduction and apoptosis. Indeed, ectopic expression of proteins that contain DD alone can initiates cell death (Chinnaiyan et al., 1995). In addition to the DD, FADD importantly possesses a death effector domain (DED) which allow direct recruitment of pro-caspase-8 to the receptor complex (Chinnaiyan et al., 1995). The sequence of events leading to caspase-8 activation is well examined for Fas and TNF-induced apoptosis (Figure 1.1). Ligation of Fas receptor with Fas ligand (FasL) results in higher-order of aggregation of the receptor molecules

and the recruitment of FADD to the Fas cytoplasmic domain via DD-DD interactions (Holler et al., 2003). Upon binding of FADD to the DD of the cytoplasmic tail of Fas, pro-caspases-8 and -10 are then recruited to FADD via the interaction with the FADD death effector domain (DED) to form what is called death-receptor induced signalling complex (DISC) (Sprick et al., 2002). DISC is usually formed within seconds of Fas ligation (Peter and Krammer, 2003). As a result of effective formation of the DISC autocatalytic activity of pro-caspases-8 and -10 is initiated resulting in formation of the corresponding active caspases (Sprick et al., 2002). However, it is still unclear whether caspase-10 can trigger cell death in the absence of caspase-8 (Lavrik et al., 2005), where Fas-mediated cell death requires formation of a single effective complex, TNF-R1 stimulation leads to formation of two signalling complexes. Complex I which consists of the TNFR1, TNF, TRADD, RIP, TRAF-1/2, and probably other molecules which trigger the NF-κB-dependent survival signals. Once this signalling complex is unable to induce survival signals via NF-κB pathway, FADD, pro-caspase-8 and-10 and FLIP$_L$ are recruited to form the so-called traddosome or complex II which results in activation downstream death signalling pathway (Micheau and Tschopp, 2003). The choice between survival and death relies on the efficiency of the complex II formation, caspase-8 activation and the level of FLIP in the cells that blocks the procaspase-8 activation.

1.3.4 : Cross talk between death receptor and mitochondrial-mediated apoptotic pathways.

Previously it was thought that mitochondrial-mediated cell death and receptor-induced cell death were two distinct pathways. However, with caspase-3 being activated in the two pathways suggest that a cross talk between the two pathways is possible. Fas-mediated apoptosis can occur by two distinct biochemical pathways, thus classifying cells as Type I or Type II responsive. In type I cells death receptor are involved, and as a result of recruitment of large quantities of caspase-8 into the DISC, caspase-3 is rapidly activated which precedes the loss of mitochondrial membrane potential. In Type II responsive cells, the level of DISC formation is dramatically reduced and caspase-3 is activated as a result of loss in mitochondrial membrane potential, and a subsequent mitochondria-induced apoptotic signals rather than direct activation of caspase-8. Whilst c-FLIP can inhibit both pathways by interfering with the DISC complex (Scaffidi et al., 1998, Scaffidi et al., 1999), Bcl-2 family members such as Bcl-2 and Bcl-xL can only block type II signalling (Willis et al., 2003). Despite the reduced levels of DISC formation in Type II cells, the small proportion of active caspase-8 produced can induce the cleavage of Bid, cleaved Bid can bind to Bax at the mitochondria and induce a conformational change in Bax enabling the release of cytochrome C (Desagher et al., 1999).

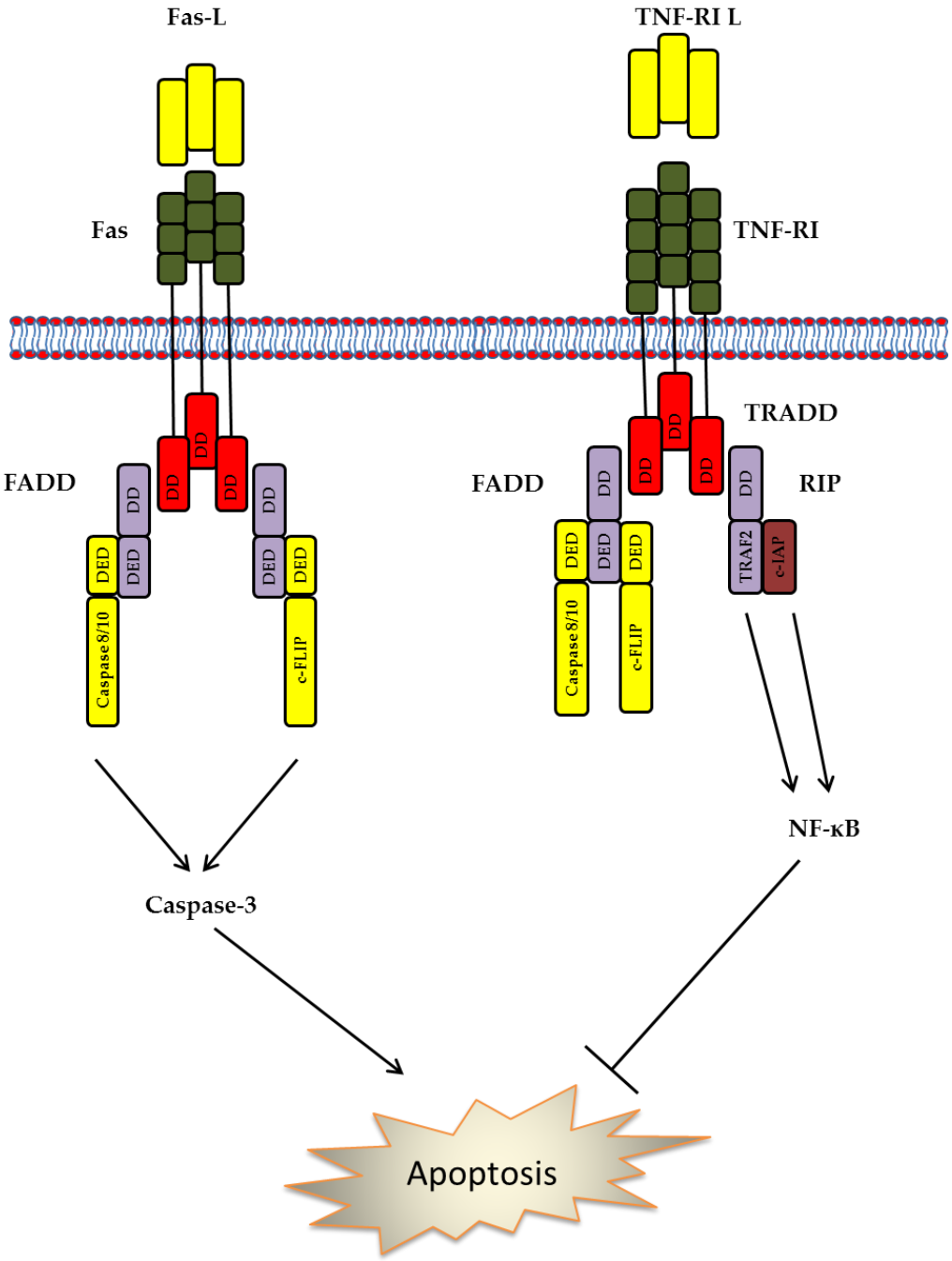

Figure 1.1: Schematic representation of receptor proximal events in death receptor-induced apoptosis.

Trimeric Fas receptor binds FADD enabling direct recruitment and processing of pro-caspase 8. Trimeric TNF-RI directly binds TRADD, but due to the lack of a death effector domain (DED) within TRADD, binds FADD to promotes pro-caspase 8 interactions. TRADD also binds RIP, which via TRAF2 interactions promotes NF-κB signal transduction. C-FLIP inhibits death receptor apoptosis by interfering with the processing of pro-caspase-8. DD; death domain.

1.4 : Structural and functional aspects of TRAILRs-TRAIL interactions.

1.4.1 : TRAIL.

Tumour necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) is also known as Apo2L or (CD253). TRAIL was first reported in 1996 as a novel protein that possessed a sequence homologous to the extracellular domain CD95 ligand (Fas-L) and TNF ligand (Pitti et al., 1996, Wiley et al., 1995). Human TRAIL is a Type II transmembrane protein of 281 amino acids (aa), consisting of a 17 amino acid cytoplasmic domain, a 21 amino acid transmembrane domain, and a 243 amino acid extracellular domain. Recombinant human soluble TRAIL/Apo2L is a 168 amino acid polypeptide spanning the sequence from 114-281 aa of the extracellular domain. It forms the active region of TRAIL that can bind TRAIL receptors and initiate signalling. Like most of the TNF ligand members, TRAIL is naturally expressed at the cell surface as homotrimeric molecules then naturally cleaved into soluble form by the action of specific proteases belong of the metalloproteansaes (MPs). Crystallographic ananlysis of TRAIL has revealed a high similarity with the crystal structure of TNF ligand and CD40L (Wiley et al., 1995).

1.4.2 : The TRAIL receptors.

Five human TRAIL receptors have been reported so far, including TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DR1), TRAIL-R4 (DcR2) and the fifth receptor reported for TRAIL is OPG, a soluble member of the TNF receptor superfamily (Emery et al., 1998). TRAIL-R1 was the first TRAIL receptor cloned by using the death domain sequence of the TNF-R1 to search an EST database (Ashkenazi, 2002). Both TRAIL-R1 and R2 are type I transmembrane proteins, which contain an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The cytoplasmic domain contains the death domain DD, therefore both TRAIL-R1 and R2 are called death receptors. TRAIL-R1 and R2 shares a 58% sequence homology. TRAIL-R1 and R2 are capable of eliciting an apoptotic death response upon binding of TRAIL in a variety of carcinomas and primary tumour cells (Almasan and Ashkenazi, 2003). In addition to the ability TRAIL-R1 and R2 to induce cell death upon ligation with TRAIL, it has been reported that TRAIL-R1 and TRAIL-R2, as well as other members of the TNF receptor family containing DD may also be involved in non-apoptotic functions (Park et al., 2005). TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) lack the complete intracellular DD that is essential for signalling apoptosis and proposed to function as an antagonizing ‘‘decoy’’ receptors for the death domain containing TRAIL receptors (Degli-Esposti et al., 1997, Pan et al., 1997). TRAIL-R3 lacks the the complete intracellular domain and part of transmembrane domain. This

receptor is anchored to the membrane by Glycosylphosphatidylinosito (GPI) residue. Over expression of TRAIL-R3 can inhibit apoptosis in TRAIL sensitive cell (Kimberley & Screaton, 2004). TRAIL-R4 has truncated DD at the cytosolic part. Inhibition of apoptosis was observed with high expression of TRAIL-R4. Studies found overexpression of TRAIL-R4 can activate NF-κB (Degli-Esposti, 1999). The fifth TRAIL receptor is OPG, which is a soluble member of the TNF receptor superfamily (Emery et al., 1998). OPG is involved in regulation of osteoclasts activation in the development and bone remodelling. Like TRAIL-R3 and R4, OPG function as a decoy receptor therefore it can bind to TRAIL and block the TRAIL-R1 and R2 induced apoptosis (Pritzker et al., 2004).

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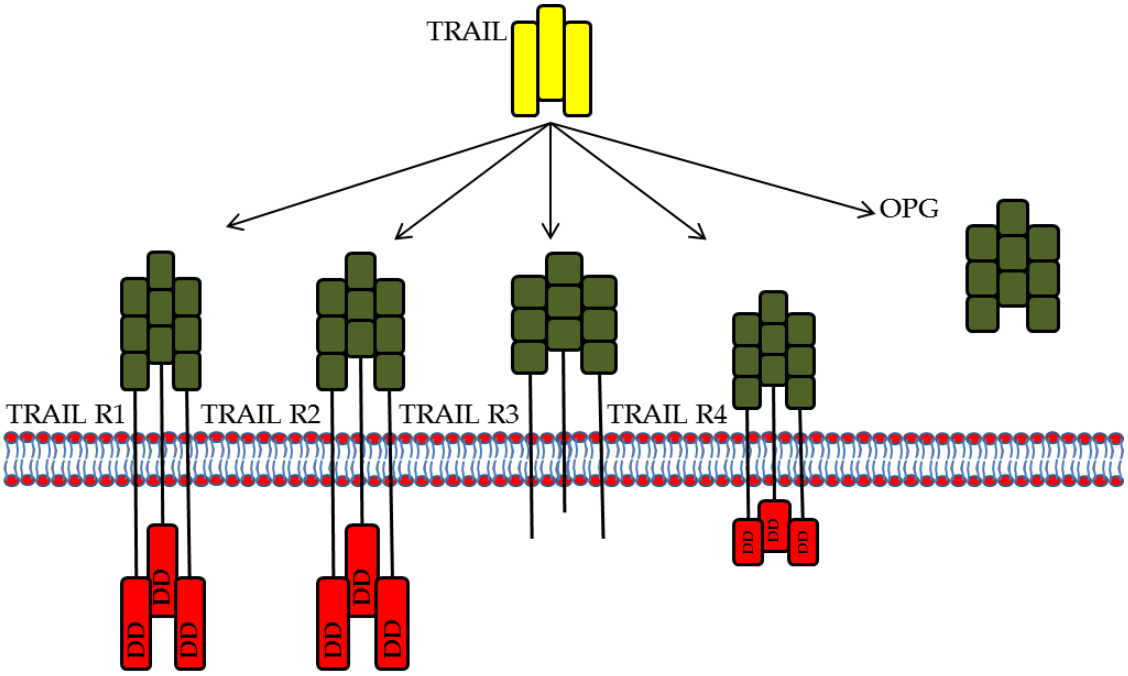


Figure 1.2: TRAIL and its receptors.

TRAIL is a homotrimeric ligand that binds to four closely related and chromosomally linked members of the TNFR superfamily. TRAIL-R1 and TRAIL-R2 which contain a cytoplasmic death domain and signal apoptosis; TRAIL-R3 is linked to the plasma membrane and lacks death domain; TRAIL-R4 has a truncated, non-functional death domain. OPG, a soluble, more distantly related receptor, is capable of binding to Apo2L/TRAIL.

1.4.3 : Functional role of TRAILR-TRAIL interactions in normal cells.

1.4.3.1 : TRAIL expressed in the lymphoid system.

Like other members of TNF ligand involved in immune responses, TRAIL is widely expressed by a variety of immune-regulatory cells, TRAIL expression has been detected in activated and resting B lymphocytes, activated T cells, NK cells, monocytes, macrophages, and dendritic cells (Holoch and Griffith, 2009). The level of TRAIL expression is mainly determined by the proinflammatory cytokine profile, including Interferon (IFN)-γ which stimulate TRAIL expression (Griffith et al., 1999). It has also been reported that IL-2, IL-7, and IL-15 induce the expression of TRAIL in NK cells and that LPS potentiates the cytotoxicity of macrophages and monocytes via TRAIL expression (Johnsen et al., 1999; Kayagaki et al., 1999: Halaas et al., 2000).

The physiological role of TRAIL in the lymphoid system depends on the stimulation of immune cells. However, the precise function is still not fully clear. Recent animal studies found that TRAIL plays an essential role in viral infection as an activator molecule for natural killer cell-mediated cytotoxicity (Cretney et al., 2006). TRAIL also might be implicated in the regulation of haematopoiesis. Expression of TRAIL receptors on the hematopoietic progenitor cells ($CD34^+$ cells) and mature erythroblasts is not detected therefore protected from TRAIL-induced apoptosis, while immature erythroblasts express TRAIL receptors and are sensitive to TRAIL (Sheridan et al., 1997,

Falschlehner et al., 2009). In addition, TRAIL plays an important role in mediating immune surveillance and tumour suppression. Collective studies on mice found that TRAIL can induce rejection or activate the apoptotic process in tumour cells whereas transplantation of TRAIL sensitive tumour into TRAIL-/- mice resulted in increased the size and the growth of the tumour and showed metastases to liver. Other studies similarly show that TRAIL knockout mice are more susceptible to initiate tumour and metastases (Holland, 2011). However, in other studies TRAIL receptor deficient mice showed enhanced tumour metastases without affecting primary tumour growth suggesting that this could be TRAIL specific function or a mouse model specificity (Grosse-Wilde et al., 2008).

1.4.3.2 : TRAIL expressed in normal non-haematopoietic cells.

While the expression of TRAIL on immune-regulatory cells is determined by the inflammatory cytokine profile, TRAIL expression has been detected on several human tissues including spleen, prostate and lung (Wiley et al., 1995, Pitti et al., 1996). Human hepatocytes express TRAIL as well as TRAIL-R1 and R2 which thought to play a critical role in regulation of hepatic cells and inflammation (Zheng et al., 2004). In addition, TRAIL is expressed in the central nervous system (CNS) at high level and plays critical role in induction of cell death after ischemic damage in neurons despite the expression of its four

receptors (Daniels et al., 2005). Death receptors, TRAIL-R1 and R2, are also expressed widely in most of the same tissues as TRAIL. In contrast to TNF and Fas induced cell death, TRAIL is highly selective in cell death-induction and have low toxicity to normal cells (Wiley et al., 1995), This could be attributed to the preferential expression of the decoy receptors, TRAIL-R3 and R4, in normal cells, which might explain the resistance to TRAIL in normal tissues (Pan et al., 1997, Sheridan et al., 1997).

1.4.4 : Functional role of TRAIL in malignancies.

1.4.4.1 : TRAIL expressed on lymphomas.

The effect of TRAIL on lymphomas is highly dependent on the type of the haematological malignancy. In multiple myeloma (MM) cells TRAIL and TRAIL-R1/-R2 are highly expressed compared to normal B cell. It was first reported by (Gazitt et al., 1999) that TRAIL is a potent inducer of apoptosis in MM, then followed by several studies that have indicated that ligation of highly expressed TRAIL and TRAIL-R1/-R2 may activate the autocrine apoptotic mechanism in melanoma cell line (Jourdan et al., 2009). However, studies on acute lymphoblastic leukaemia (ALL) were not fully explained the differential effect of TRAIL on TRAIL receptors which are expressed on ALL cells. Cellular sensitivity to TRAIL may be further regulated at the intercellular level as expression levels of c-FLIP correlates with TRAIL resistance in melanoma and

dendritic cells. In addition, TRAIL sensitivity was paralleled by changes in the c-FLIP expression level when cells were treated with actinomycin D or cycloheximide (Walczak et al., 2000; Griffith et al., 1998). However, the mechanisms of TRAIL sensitivity or resistance are still not fully clear.

1.4.4.2 : TRAIL expression on carcinoma cells.

Since the discovery of TRAIL and its receptors most of the studies have focused on the apoptotic effect of TRAIL on carcinoma cells whilst sparing normal cells. Numerous studies using carcinoma cell lines expressing TRAIL receptors and treated with rsTRAIL have confirmed that TRAIL induces growth inhibition or apoptosis of carcinomas originating from colon, lung, kidney, skin, breast, bladder and pancreas (Ashkenazi et al., 1999, Walczak et al., 1999). Further studies show that TRAIL and its receptors are expressed in normal colonic mucosa but the expression of TRAIL and its receptors was higher in adenomas and carcinomas. In ovarian carcinoma TRAIL mRNA is significantly higher than in normal human ovarian cells. Patients with high TRAIL expression have longer survival (Lancaster et al., 2003.). In addition, in human malignant breast cancer TRAIL expression is higher and can induces cell death and cancer cells are more sensitive to TRAIL than normal cells (Herrnring et al., 2000).

1.5 : Signalling by TRAIL.

1.5.1 : Introduction.

Following TRAIL interaction with TRAIL-R1 and R2 apoptotic adaptors proteins are recruited to the cytoplasmic tail of the TRAIL-R1 and R2, which results in initiating of a cascade of signalling mechanisms followed by apoptosis induction. However, binding of TRAIL to TRAIL-R3 and R4 or osteoprotegerin has a direct apoptotic inhibitory effect because they lack the ability to transduce apoptotic signals.

1.5.2 : Mechanisms of apoptotic signal transduction by TRAIL.

The interaction between TRAIL and its receptors TRAIL R1 andR2 is essential for TRAIL-induced apoptosis in carcinomas. Binding of homotrimeric TRAIL to TRAIL R1/R2 results in clustering and recruitment of the Fas Associated Death Domain adaptor protein (FADD) to TRAIL receptors. The death domain (DD) of FADD is directly interacting with DD of TRAIL-R1/R2. The FADD recruit procaspase-8 or 10 through second domain death-effector domains (DED) which interact with DED of procaspase-8 or 10 via homotypic interaction forming death-inducing signalling complex (DISC) (Kischkel et al., 2001). Recruited procaspase-8 or 10 to the DISC result in their activation and autocatalytic activity that result in activation of effector caspases (3, 6 and 7) and activation of apoptotic cell death (Figure:1.3). At this level cells can be

divided into two deferent type according to the recruitment and sufficient activation of procaspase-8 or 10: type I cells in which DISC activation sufficient to induced cell death, and type II cells, In which DISC formation is not sufficient to trigger cell death through caspase-3 activation and which sequence additional signal amplification to induce cell death through the mitochondrial pathway. This connection between the extrinsic pathway and mitochondrial pathway is regulated through BH3-only protein Bid, which acts as a substrate for caspase-8. Activated caspase-8 at the DISC results in the cleavage of Bid into its truncated form tBid which translocates into the outer-mitochondrial membrane where it activates Bax and Bak. This leads to release of cytochrome *c* and proapoptotic factors from mitochondria and results in the recruitment and activation of caspase-9 which, like the DISC-activated caspases-8 and -10, activates the executioner caspases and induces cell death (Niemoeller and Belka, 2011). Inhibition of TRAIL apoptotic signalling can occur at several levels according to expression of apoptosis inhibitory protein. Studies by (Micheau et al., 2002) found that at the DISC level, the cellular FLICE inhibitory protein (c-FLIP) bind FADD competing with the binding of caspase-8 and -10 resulting in inhibition of caspase activation. Studies found that C-FLIP has two different splice variants, c-FLIPL the long form and c-FLIPS the short form. The c-FLIPS recruited to the DISC site has the ability to inactivate caspase-8 and block

apoptotic signalling. However, the effect of c-FLIPL is not yet clear since it appears to be involved in both the apoptotic and a non-apoptotic signalling.

In addition apoptotic signalling by TRAIL may be inhibited by the X chromosome-linked inhibitor of apoptosis (XIAP) which can bind to caspases-3, -7 and -9 inhibiting their induction of apoptosis that is required in type II cells. A protein called second mitochondrial activator of caspases/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) is released from mitochondria during apoptosis inhibits the activation of XIAP (Maas et al., 2010). Thus, TRAIL has the ability to induce apoptosis in tumour cells, that can be inhibited by several mechanisms at different level endogenously or by modulation of signalling pathway.

1.5.3 : Mechanisms of non-apoptotic signal transduction by TRAIL.

1.5.3.1 : The MAPK pathways.

Mitogen-Activated Protein Kinase (MAPK) signalling cascades regulate a variety of physiological processes, ranging from cell differentiation, movement, proliferation and survival to apoptotic cell death and inflammatory responses. MAPKs can be activated by various stimuli such as growth factors, cytokines and hormones, or in response to environmental stress conditions such as irradiation, DNA damage, osmotic imbalance and heat shock. Higher organisms have three well-characterised subfamilies' of MAPK. The

extracellular signalling-regulated kinase 1 and 2 (ERK1/2) function as regulators of cell growth, survival and differentiation, and inhibition of these kinases can be a target as anticancer treatment. The c-Jun N-terminal kinases (JNK) are essential for transcription and controlling programmed cell death as well as p38 kinases which regulate the expression of different cytokines that are mainly activated by stress and environmental stimuli.

The MAPK pathway is well organized and regulated by phosphorylation of substrate protein which functions as a switch to turn off or on the activity of protein. Thus, MAPKs provide a substrate for MAP Kinase kinase kinases (MAPKKKs) which sequentially phosphorylate and activate MAP kinase kinases (MAPKKs) which result in activation of kinase activity of the ERK, JNK or p38 (Tran et al., 2001).

1.5.3.2 : The JNK MAPK pathway.

JNK pathway is regulated by MAP Kinase Kinase (MKK) family proteins. The MKK4 and MKK7 are members of MKK family involved in the phosphorilation of JNK. Studies indicate that JNK activation is mediated through TRAF2 or RIP which activate MEKK1 that activate MKK4 (Hu et al., 1999). The molecular pathway of JNK activation by TRAIL is not fully understood and it could be activated through a secondary intercellular signalling complex (Varfolomeev et

al., 2005). The involvement of JNK activation induced by TRAIL in cell death is still unclear (Muhlenbeck et al., 1998). However recent studies found that TRAIL induced apoptosis requires the activation of JNK pathway (Corazza et al., 2006) suggesting that it could be a cell type dependent.

1.5.3.3 : The ERK MAPK pathway.

The extracellular signals which trigger the sequential protein kinases leading to activate the ERK pathway are well defined. Signals through activated receptors activate a significant number of well identified proteins such as Ras. The activation of Ras via the Raf Kinase family provoke the phosphorylation and activation of the MAPKKs MEK1 and MEK2. Thus activated MEK1/2 can phosphorylate ERK and induce its kinase activity. Also MEK1/2 can be phosphorylated through a Raf-independent pathway in which Ras binds to and activates the MAPKKK MEKK1(Lange-Carter and Johnson, 1994). Activation of the ERK pathway in general inhibits the effect of TRAIL-induced apoptosis. Studies on Jurkat T cells treated with ERK activators (TPA or OKT3) have found these to inhibit TRAIL-induced apoptosis via a mechanism that involves the inhibition of procaspase-8 cleavage and translocation of Bid (Soderstrom et al., 2002). In addition, JNK and ERK translocate to the nucleus and activate c-Jun and c-Fos, which dimerize to activat the transcripton factor activator protein-1 (AP-1). AP-1 complex composed of the Jun, Fos, ATF, and Maf protein families.

AP-1 is activated by several different agents. Activation occurs both transcriptionally and post-translationally, and signaling occurs predominantly through the mitogen-activated protein kinase cascade (Guseva et al., 2004). This AP-1 regulates the expression of multiple genes essential for cell proliferation, differentiation, and apoptosis (Li W et al., 2007).

1.5.3.4 : The NF-κB pathway.

The NF-κB family is a central regulator of immunity, development, apoptosis and cell proliferation. A variety of signals promote the induction and activation of NF-κB including inflammatory cytokines, bacterial and viral antigens, stresses such as γ-radiation, UV light and oxygen free radicals. NF-κB consists of five family members in mammalian cells; c-Rel (Rel), p65 (RelA), RelB, NF-κB1 (p105 and its processed oroduct p50) and NF-κB2 (p100 and its processed product p52). In unstimulated cells, NF-κB is founds in the cytoplasm as a functionally inactive complex which consists of NF-κB dimers sequestered by IκB inhibitor proteins. The sequence of events of activation and release of NF-κB subunits from IκBα occurs via the activation of the IKK signalosome (IκB Kinase) which phosphorylates serines residues (Ser^{32} and Ser^{36}) on IκBα so that it is recognised and ubiqutinated by beta-transducin repeat containing protein (β-TrCP). This results in protesomal degradation of IκBα and unmasking of nuclear localisation signal on the NF-κB dimer which can enter the nucleus as transcription factors and induce gene transduction. IKK is a large multiprotein

complex with a proximal molecular mass of 700-900 kDa and composed of many hetero-dimer proteins including IKKα (IKK1), IKKβ (IKK2) and IKKγ or termed NEMO. The catalytically active IKKα and IKKβ subunits dimerise and associate with at least two IKKγ subunits. IKKγ functions as a regulatory dimer, but is absolutely essential for IKK function and when a cell deficient in IKKγ it cannot induce IκBα degradation and NF-κB/DNA binding (Yamaoka et al., 1998). IKKα is essential for NF-κB2 processing and the regulation of a specific set of NF-κB responsive genes *via* the formation of p52/RelB heterodimers (Solan et al., 2002). In fact, the formation of specific NF-κB dimers occurs through two pathways. The first pathway, the canonical, involves the IKKα/IκBα pathway resulting in the formation of predominantly p50/p65 dimers, whereas the second non-canonical pathway which requires IKKα-dependent processing of p100 NF-κB2 and the generation of p52/RelB heterodimers. A numbers of studies show that NF-κB can be activated by TRAIL (Jeremias et al., 1998) through ligation to R1,R2 and R4 (Degli-Esposti et al., 1997). Activated NF-κB by TRAIL involves the recruitment of RIP to the cytoplasmic domain of TRAIL-R1 and 2 by TRADD which results in further recruitment of other signalling molecules including TRAF2 that can activate the IKK complex (Devin et al., 2000). In addition, activated NF-κB can upregulate the expression of anti-apoptotic genes like cFLIP that results in inhibition of TRAIL-induced apoptosis (Gonzalvez and Ashkenazi, 2010).

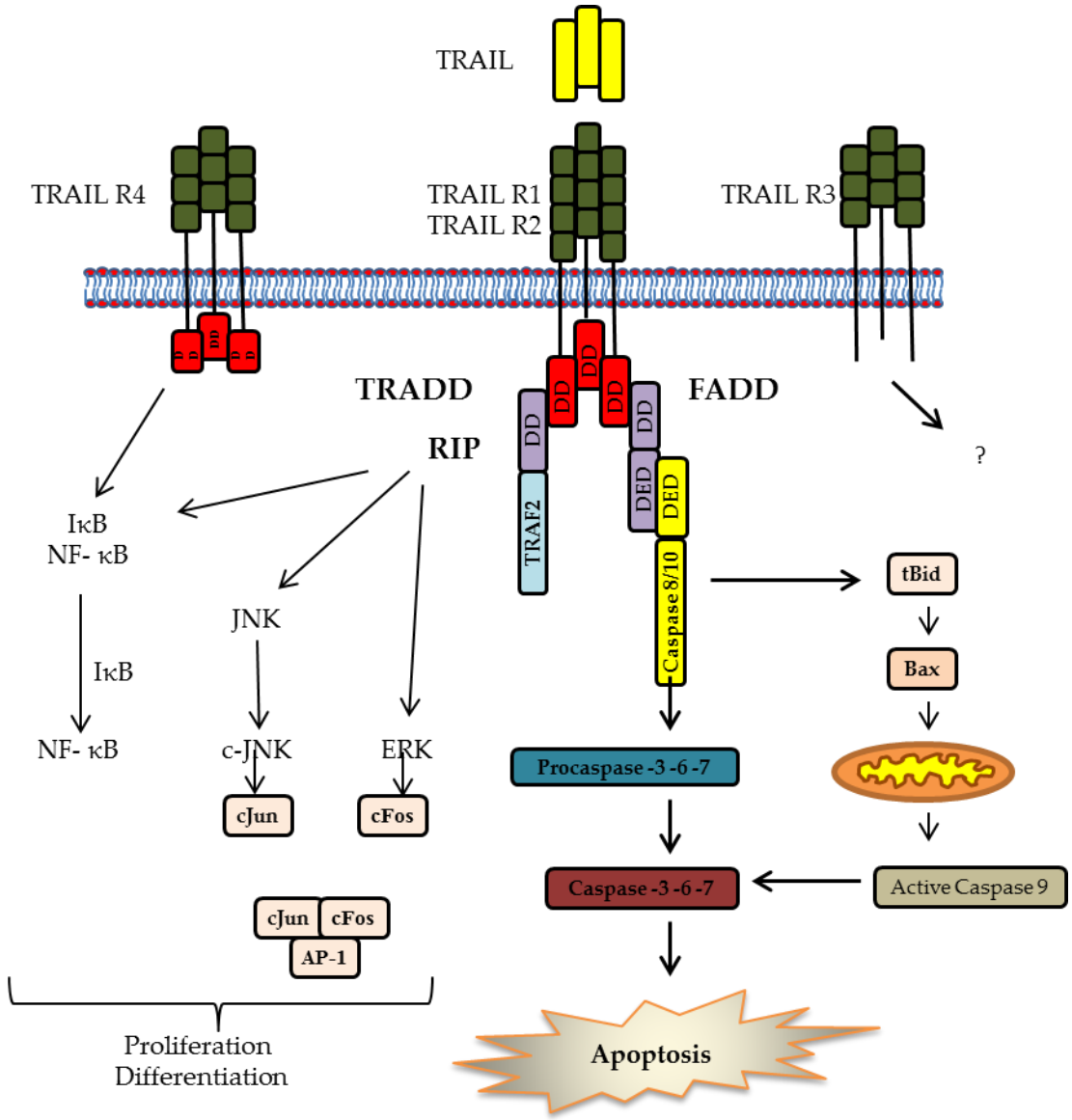


Figure 1.3: Schematic diagram of TRAIL signalling pathway.

TRAIL binding TRAIL R1 and TRAIL R2 and activated death receptors trimerize and recruit Fas-associated death domain (FADD) and recruit the initiator caspase-8/-10. This leads to activate procaspase -3,-6,-7 and subsequently activate caspase-3 and apoptosis. TRAIL can also trigger the intrinsic pathway through caspase-8-mediated cleavage of Bid. Cleaved Bid induces translocation of Bax into the mitochondria leading to release of cytochrome c activate caspase-9. This caspase 9 activates caspase-3 resulting in apoptosis. In addition c-JNK, NF-κB and ERK are activate by TRAIL involved the recruitment of RIP by TRADD.

1.6 : TRAIL-based therapy.

1.6.1 : Introduction.

The outcome from the administration of soluble recombinant TRAIL and monoclonal TRAIL-receptor antibodies in phase I and II clinical trails, and the observation that combination of TRAIL and chemotherapy induces a higher degree of carcinoma cell apoptosis than either agent alone, has highlighted the potential therapeutic role of TRAIL as a potent cancer treatment (Ashkenazi et al., 1999, Ashkenazi et al., 2008). Targeting tumour cells by TRAIL to induce cell death and tumour regression can be achieved by two different strategies. The first strategy involves the use of viral gene therapy delivery systems to introduce the TRAIL gene into the cells. The second strategy involves the direct ligation of TRAIL *via* the systemic administration of recombinant soluble human TRAIL (rshTRAIL) or monoclonal TRAIL-receptor antibodies. Both of these strategies have the potential to induce tumour cytotoxicity directly *via* the up-regulation of apoptotic signalling.

1.6.2 : Recombinant adenovirus TRAIL gene therapy.

1.6.2.1 : Introduction.

Gene therapy is a type of biological therapy for cancer. It was first defined as the deliberate introduction of genetic material into human somatic cells for therapeutic, prophylactic or diagnostic purposes (Gene Therapy Advisory

Committee). In the past 20 years gene therapy has progressed through to clinical trials, and more than 1843 gene therapy clinical trails have now been instigated worldwide (www.abedia.com/wiley/phases.php).

1.6.2.2 : Adenoviral vectors.

Adenovirus is double stranded DNA, non-enveloped icosohedral virus first isolated in 1953 (Rowe et al., 1953). There are at least 52 serotypes that have been identified to cause humans infection. In general, most adenoviral infections cause respiratory illness with mild symptoms and require no treatment (CDC). In general, adenovirus infects host cells by binding to coxsackie adenovirus receptor (CAR) on the cell surface, and then the virus is internalized by interaction with α ν β integrins which act as co receptors (Russell, 2000). Following internalization, virus starts to express its genome through the host nucleus. Adenovirus genome can be divided into two regions according to the expression of gene: early region (E1A, E1B, E2A, E2B, E3, and E4) and late region (L1, L2, L3, L4, and L5). The E1A gene is the first transcriptional activator which activates the transcription of the other late genes. The E1A gene is important in virus replication and cellular transformation. The E1B gene inactivates the p53 protein to prevent apoptosis of host cell. The late gene protein products are essential for virus assembly and structural protein for the virus particles (W. C. Russell, Adenoviruses: update on structure and function, 2009).

1.6.2.3 : Replication deficient adenovirus vectors.

In the first generation of replication-deficient adenoviral vectors E1 and often E3 were deleted and a transgene cassette being inserted into the E1 deleted region (Stratford-Perricaudet et al., 1990, Quantin et al., 1992). Removal of the E1 region from the adenoviral genome renders the virus replication-deficient and eliminates the potential for oncogenicity. Further deletion of the E3 locus increases the capacity for large insertion of transgene cassettes of up to 7.5 kb in size (Jolly, 1994). E1-deleted adenovirus can only propagate in supporting cell lines such as 911 or HEK293 cells due to the removal of removal of the E1A and E1B genes. Because 911 cells contain the nucleotide sequence 79-5789bp of the adenovirus type 5 genome and HEK 293 cells contain the left 11% of the adenovirus type 5 genome they can provide in *trans* the lost E1A and E1B functions (Graham et al., 1977, Fallaux et al., 1996). Regardless of the deletion of the E1 genes in this first generation of adenoviral vectors, a measurable amount of viral gene expression was still detected which was thought to be a result of either transactivation of viral genes by cellular factors similar to E1 gene, or a basal expression of the adenoviral promoters (Yang et al., 1995, Yang and Wilson, 1995).

Next generation adenoviral vectors were rendered 'gutless' by removing all essential viral coding sequences. It has been reported that inserts totalling 35 kb can be inserted into a gutless adenoviral vector (Hardy et al., 1997). These kinds

of adenovirus vectors contain only the viral inverted terminal repeats and the packaging signal and therefore require the presence of a helper virus to enable their replication (Parks et al., 1996). The deletion of viral genes from the vector genome prevents the induction of a host immune response, allowing long term gene expression.

1.6.2.4 : Replication competent adenovirus vectors.

The promising development of conditionally replicating adenoviral vectors for cancer gene therapy creates the possibility of combining the effects of an inserted therapeutic transgene with the cytolytic effects of a replicating adenovirus. One approach to the issue of achieving tumour specific replication is controlling the expression of early region genes using tumour specific promoters. The prostate specific antigen (PSA) promoter is an example of driving expression of E1A specifically in prostate cancer, as the PSA is expressed at all stages of prostate cancers, but not in normal prostatic tissue (Rodriguez et al., 1997). Another example of a tumour-specific promoter is the α-fetoprotein (AFP) to drive E1A expression in hepatocellular carcinoma (Hallenbeck et al., 1999). In addition, using adenovirus with T cell factor (Tcf) binding sites in the early promoters has been found to specifically replicate in colon tumours where the Wingless integration site (Wnt) signalling pathway is

pathologically activated and leads to constitutive transcription from promoters containing (Tcf) binding sites (Fuerer and Iggo, 2002).

Another strategy for the replication of tumour specific adenovirus is the exploitation of tumour specific defects in the control of cell replication such as the pRb and p53 proteins. In normal cells, p53 is induced by activated oncogenes or by DNA damage. The p53 then activates the transcription of downstream effectors, resulting in cell cycle arrest or apoptosis of cells that may have acquired potentially tumourigenic lesions (Levine, 1997, Oren, 2003). However, in the vast majority of human tumours, the p53 tumour suppressor pathway is inactivated, either through direct mutation of p53 or as a result of the loss of upstream regulators such as p14ARF or downstream effectors such as Bax (Sherr and McCormick, 2002). In common with tumour cells, DNA viruses inactivate the p53 pathway, and many encode proteins that bind and degrade p53. Viral proteins that inactivate p53 are therefore believed to play a requisite role in viral replication by preventing p53-mediated cell cycle arrest or apoptosis. In the case of adenovirus, E1B-55K appears to fulfill this role, as together with E4-ORF6 and cellular proteins involved in ubiquitination, it induces the degradation of p53 (Harada et al., 2002, Querido et al., 1997, Querido et al., 2001). An example of this strategy was the development of the oncolytic adenovirus ONYX 015 by Onyx Pharmaceuticals from the adenovirus mutant, dl1520 that was originally described by Baker and Berk in 1987. The generation

of ONYX 015 was based on a mutant deletion of 827 bp from the E1 region, beside a point mutation that generates a stop codon preventing expression of the E1B 55 kDa protein and results in restricting viral replication to tumour cells that lack p53 (Barker and Berk, 1987, Bischoff et al., 1996). Despite the loss of E1B 55KDa expression it has been reported that ONYX 015 can replicate in some tumour cell lines with wild type p53 status (Goodrum and Ornelles, 1998, Hall et al., 1998). However, a recent study has indicated that the loss of E1B 55 kDa mediates late viral RNA export which restricts ONYX 015 replication. RNA export of this type does not exist in normal cells and some tumours, indicating that E1B 55K-mediated late viral RNA export, rather than p53 degradation, restricts ONYX 015 replication in non-malignant cells (O'Shea et al., 2004). ONYX 015 has reached phase I/II clinical trials for head and neck (Khuri et al., 2000, Nemunaitis et al., 2000, Nemunaitis et al., 2001) ,pancreatic (Hecht et al., 2003), ovarian (Vasey et al., 2002), liver (Makower et al., 2003), colorectal (Hamid et al., 2003), lung (Nemunaitis et al., 2001) and oral carcinoma (Ganly and Singh, 2003, Rudin et al., 2003). Moreover, a similar virus to ONYX 015 has now been developed in China named H101 or Oncorine, approved in 2005 by China's State Food and Drug Administration for use in head and neck cancer (Guo and Xin, 2006).

ONYX 411, similar to ONYX 015, was developed based on mutation within the conserved region 2 (CR2) domain of the viral E1A gene region. CR2 is required

for the E1A protein to bind to the retinoblastoma protein (pRb) and inactivate it. This blocks cell proliferation by sequestering and altering the function of E2F transcription factors that control the expression of many genes essential for progression from G1 into S phase (Hatakeyama and Weinberg, 1995). Disrupting the pRb pathway liberates E2Fs and allows cell proliferation, rendering cells insensitive to antigrowth factors. In many human cancers the pRb pathway is interrupted (Hanahan and Weinberg, 2011). Thus an adenovirus with a deletion mutation within the E1A CR2 region can normally replicate in tumour but not in normal cells. Adenovirus ONYX 411 was developed with further tumour specificity by controlling the E1A and E4 expression by replacing their promoters with the E2F1 promoter, highly active in cells with an aberrant pRb pathway. ONYX 411replication was found to be dependent on pRb status of the cells (Johnson et al., 2002).

1.6.3 : Clinical applications of adenoviral delivery of the TRAIL gene.

The first report by Griffith (Griffith et al., 2000) of delivery of TRAIL by a nonreplicative adenoviral vector was in 2000 was followed by further reports that described the use of recombinant adenoviral vectors containing TRAIL cDNA (Armeanu et al., 2003, Zhang et al., 2008). A number of different studies have shown the potent effect of RAd TRAIL compared to the effect of the rshTRAIL on tumour cells. Administration of Ad5-TRAIL into tumour cells

show rapid transcription, translation and rapid expression of TRAIL protein. In addition RAd TRAIL was found to induce apoptosis in TRAIL receptor positive carcinomas and not normal cells. Sustained expression of TRAIL by Ad5-TRAIL in tumour model such as ovarian carcinoma (Seol et al., 2003) pancreatic carcinoma, hepatic carcinoma and lung carcinoma (Hait and Yang, 2006)

has resulted in potent induction of apoptosis compared to rshTRAIL with minimal cytotoxicity to normal cells.

A Phase I clinical trial with RAd TRAIL in patient with prostate cancer have been completed. In this trial a direct injection of adenoviral expressing TRAIL into the prostate tumour has shown tumour regression and cell death without side effects. In addition, sustained TRAIL protein expression was observed after a single intratumoral injection of RAd TRAIL. Such expression of TRAIL would not be achievable with the administration of recombinant TRAIL protein (Griffith et al., 2000). Up to date several methods to enhance RAd TRAIL infection, distribution and expression of TRAIL gene are being investigated.

1.6.4 : Systemic adminstration – based therapy.

1.6.4.1 : Recombinant soluble TRAIL therapy.

Since the first report of TRAIL in 1995, most of the studies have focused on its ability to induce apoptosis in tumour cell *in vitro* and *in vivo*, the target of those studies was to develop TRAIL as an antitumour therapeutic agent. Early studies

found that rsTRAIL induced apoptosis in a broad range of tumour cell lines, while having little to no activity against normal cells. Studies by Walczak *et al* in 1999 to examine the antitumour effect of TRAIL in vivo by intravenous injection of soluble TRAIL demonstrated that there was no toxicity against normal tissues even with high concentrations and for long periods, whereas mice injected with low doses of FasL showed severe toxicity, necrosis and death after short time of treatment. Based on these results, TRAIL was suggested to be used as an anti-tumour therapy without the side effects or toxicity that seen with other TNF family members for example FasL and TNF. Up to date many clinical studies are evaluating the safety of Dulanermin the human recombinant TRAIL. Phase I and II clinical trial of Dulanermin in patients with sold or haematological tumours show the treatment to be well-tolerated. Dulanermin alone or with different drugs such as Rituximab and Cetuximab as well as chemotherapeutic drugs such as Paclitaxel and Carboplatin has been evaluated for the safety and the efficacy on different tumour types. The results from these studies show that the half-life of Dulanermin is short (30-60 min) and may require multiple doses to achieve an effective antitumour response. Dulanermin bind receptor TRAIL-R1 and TRAIL-R2 and because of that it difficult to distinguish which is the specific receptor and whether the decoy receptors are influencing the drug activity. In addition, Dulanermin unlike agonist antibody,

dose not require exogenous Fcγ receptor interaction or cross-linking for its activity (Holland, 2011).

1.6.4.2 : Anti-TRAIL antibodies therapy.

The advantage of mAb over rsTRAIL is the long–life 14-21days (Plummer et al., 2007). A number of mAb against TRAIL receptors have been used inclinical trails. The most investigated mAbs are Mapatumumab which is a fully human agonist IgG monoclonal antibody specific for human TRAIL-R1 and Lexatumamab agonist IgG monoclonal antibody specific for human TRAIL-R2. Mapatumumab is in phase II clinical trials. Studies found that mapatumumab can induce cytotoxic activity in vitro, and has the ability to inhibit growth of human tumour cells in xenograft models (Belyanskaya et al., 2007, Trarbach et al., 2010).

1.6.4.3 : Combination of TRAIL and radiotherapy.

Recombinant soluble TRAIL and TRAIL-receptor agonistic antibodies were shown to exhibit a high selectivity for tumour cells and act synergistically with conventional chemotherapy drugs and radiation. Up to date research has shown that radiotherapy strongly sensitises tumour cells to rsTRAIL and TRAIL-agonistic antibodies. Apoptosis induction in xenograft models was

observed with the combination of radiotherapy and sTRAIL and TRAIL-agonistic antibodies, even in tumour cells with no or weak responses to either treatment alone. However, the efficiency of combined treatment is determined by different factors for example p53-status, the balance between pro- and anti-apoptotic proteins and modulation of TRAIL-receptor signal transduction (Niemoeller and Belka, 2011).

1.6.4.4 : Combination of TRAIL and chemotherapeutic drug strategies.

Treatments of tumour cells with chemotherapy usually results in drug resistance which may be overcome by the combination with TRAIL (Mitsiades et al., 2001). Therefore combination of cytotoxic drugs with rsTRAIL has been tested in preclinical models for synergy and the ability to reduce cancer drug resistance. Results of phase I/II clinical trails of rsTRAIL in combination with chemotherapy drugs are encouraging (Soria et al., 2011).

1.7 : Thesis objectives.

Previous results from our lab and others have demonstrated the efficacy of membrane-bound CD40L and FasL in cell death induction and apoptosis compared to soluble or wild-type counterparts. Given that both CD40L and TRAIL belong to the same family and they share a significant homology (25-30%)(Pitti et al., 1996). In view of the potential therapeutic role of rsTRAIL in carcinoma, the objective of this thesis was to generate a RAd virus expressing wild-type TRAIL and to examine the direct effect of this virus alone or in combination with cytotoxic and novel drugs on TRAIL receptors-positive carcinomas. Furthermore, to examine the direct effect of blocking of the TRAIL cleavage from membrane in RAdwtTRAIL-infected cells on cell viability reduction and apoptosis induction. Moreover, to generate a RAd virus expressing a membrane-bound TRAIL mutant resistant to cleavage from membrane by hybrid fusion between the CD40L N-terminal domain (1-324bp) upstream of the CD40L cleavage site (324-348bp) and the C-terminal domain of TRAIL ligand (340-846bp, containing the TRAIL active site) and we examine the direct effect of this virus alone or in combination with cytotoxic and novel drugs on TRAIL receptors-positive carcinomas. Also, to investigate the possible mechanism responsible for TRAIL-induced cell death caused by the membrane bound-non cleavable TRAIL.

CHAPTER 2
MATERIALS AND METHODS

Unless otherwise stated, all reagents and materials were obtained from Sigma-Aldrich, Poole, UK

2.1 : Molecular Biology techniques.

2.1.1 : PCR amplification for molecular cloning.

The primers and annealing temperatures were optimized according to each primer and product size of each Polymerase Chain Reaction (PCR) reaction. The PCR reaction briefly contains 1µl of forward and reverse primer (50pmol/µl) (synthesised by Alta Biosciences, University of Birmingham, UK), 50ng of DNA template, 5µl of 10x expand high fidelity buffer (Roche), and ddH_2O to complete the total volume up to 49µl, the PCR reaction was then heated to 93°C, Followed by addition of, 1µl of high fidelity proof reading Taq enzyme (3.5U/µl) (Roche) as a hot start reaction. PCR reactions were allowed to cycle in a Gene Amp PCR 2400 system (Perkim-Elmer) according to the conditions described in Table 2.1.

Table 2. 1: Primers and PCR conditions used in molecular cloning.

Name	Oligonucleotide	Tm	PCR Cycle
TRAIL *Sal1* F	5′-A **GTCGAC** ATG-GCT-ATG-ATGG-AGG-TC-3′,	58.1	(93°C5mins (93°C 30 Sec, 58.1°C, 45 Sec, 72°C 2 mins) x 25, 72°C, 5 mins)
TRAIL *Xba1* R	5′ C- **TCTAGA** –TTA-GCC-AAC-TAA-AAA-GGC-CC-3′		

2.1.2 : Agarose gel electrophoresis.

Agarose (Gibco, Paisley UK) gel was prepared by melting the required quantity in TBE buffer (89mM Tris base, 89mM boric acid, 2.5mM EDTA, pH 8.3) ethidium bromide was then added at a concentration of 0.2µg/ml before gel solidification. The solution was subsequently poured into the preassembled tray and all bubbles were removed. The gel solution was then left until solidified and covered with TBE buffer. The DNA samples were mixed with the corresponding volume of 6x loading buffer (100mM Tris HCl, pH 7.5, 20% (v/v) Glycerol, 1mM EDTA, few drops of Bromophenol blue) and loaded into the gel. The gel was run at 100V until the separation was optimal. Gel was visualized under UV light.

2.1.3 : Purification of DNA from agarose.

The desired DNA fragment was visualized by using minimum UV exposure to avoid DNA degradation. The DNA fragment was excised from the gel and DNA was extracted from the agarose gel by Qiagen gel extraction kit (West Sussex, UK) according to the manufacturer's instructions. Following the extraction the DNA was eluted by adding 30µl of pre-warmed sterile water and stored at -20°C.

2.1.4 : Addition of A-overhangs to the PCR products for sub-cloning into TOPO vectors.

The reaction mixture was prepared by mixing 5µl of 10x PCR Buffer, 1µl of dNTPs (2.5mM of each nucleotide) and 0.5µl of Taq polymerase (New England BioLabs), 18.5µl ddH2O was then added to bring the final volume up to 25µl. The reaction mixture was incubated in the Gene Amp PCR 2400 system (Perkim-Elmer) for 20 minutes at 72°C.

2.1.5 : Purification of A-overhanged PCR product.

PCR reaction was mixed with PB buffer supplied within the Gel extraction Qiagen kit (West Sussex, UK) at 1:5 volume. The samples mixture was processed according to the manufacturer's instructions. DNA was then eluted in 30µl of pre-warmed sterile water and stored at -20°C or used immediately in TOPO cloning reactions.

2.1.6 : TOPO-cloning reaction.

Linearized pCR®TOPO2.1 plasmid vectors from (Invitrogen) was ligated with the A-overhanged PCR product. The ligation reaction was prepared according to the manufacturer's instructions. The ligation reaction was incubated overnight at 16°C and was used for transformation or stored at -20°C.

2.1.7 : Vector-insert ligation.

The concentration of the insert and the pre-digested and gel purified plasmid was estimated on an agarose gel. Approximately 1:1 or 1:2 vector : insert DNA were added to a ligation reaction containing 2µl of 10x T4 ligase buffer, 1µl of T4 ligase and sterile water to make up the final volume of 20µl. The reaction mixture was incubated overnight at 16°C. 2µl of the reaction was used to transform 50µl of Top10 F competent cells.

2.1.8 : Preparation of chemically competent cells.

One millilitre of overnight-cultured bacteria in LB medium was used to inoculate 500ml of prewarmed LB broth, bacteria was allowed to grow to ~0.2 OD_{600} (2-3 hours), then chilled on ice for 10 min followed by centrifugation at 5000-6000 rpm at 4ºC for 10 min. Bacterial pellet was resuspended in 250ml of Transformation Buffer-X (50mM $CaCl_2$, 15%(v/v) Glycerol, 10mM MOPS pH 6.6 (with NaOH only), filtered and sterilized). The resuspended cells were incubated on ice in sterile falcon tube for 20 min and centrifuged again for 10 mins (5000-6000 rpm, 4°C). The pellet was resuspended in 2.5ml of transformation buffer-X. Aliquots of 100µl of the cells were prepared into 1.5ml microfuge tubes with fast frozen in liquid nitrogen and stored at -80°C.

2.1.9 : Preparation of electro competent cells.

1ml of the overnight bacterial culture grown in LB medium was inoculated into 500ml of pre-warmed LB broth and grown to ~0.3-0.6 OD_{600} (30-60 minutes). Cells were transferred into 50ml falcon tube and incubated on ice for 30 minutes then pelleted at 5000-6000 rpm at 4°C for 10 minutes. Cells were washed in 250ml ice cold sterile ddH_2O and spun again for 10 minutes at 5000-6000 rpm at 4°C. The washing step was then repeated 3 times using 50ml ice cold sterile ddH_2O. Cells were then washed in 50 ml sterile ice cold 10% glycerol, followed by resuspension in 2.5ml sterile ice cold 10%. 100µl aliquots of the cells were prepared into 1.5ml microfuge tubes, fast frozen in liquid nitrogen and directly stored at -80°C.

2.1.10 : Bacterial transformation.

2.1.10.1 : Heat-shock bacterial transformation of home-made competent cells.

100µl of aliquot competent cells was chilled on ice for 10min to thaw then 2-5µl of DNA was added and the cells were incubated on ice for 30 minutes followed by heat shocked for 5 minutes at 37°C and cooled on ice for 2 minutes. 400µl of recovery SOC medium (L-broth supplemented with 20mM glucose, 2.5mM KCl (pH 7) and 10mM $MgCl_2$) was added to the transformed cells and allowed to recover for 1hr at 37°C in an orbital incubator. After recovery cells were plated on selective LB-agar plates (500ml solutions require 10g LB broth base (Gibco)

and 7.5g selected agar (Gibco), autoclaved and supplemented with an appropriate concentration of the selection antibiotic). Plates were incubated overnight at 37°C.

2.1.10.2 : Bacterial transformation by electroporation.

About 20µl of electrocompetent *E.coli* BJ5183 cells were placed in a 2.0mm pre-cooled cuvette and placed on ice for 5 minutes. DNA was added to cells and incubated for 5 minutes on ice. The mixture was electroporated at 2,500 V, 200 ohms, and 25µF in a Bio-Rad Gene pulser electroporator. Immediately after the pulse, cell mixture was transferred to 500µl of SOC medium and incubated at 37°C with vigorous shaking for 1 hour. Then cells were plated on selective LB-agar plates supplemented with an appropriate concentration of the selection antibiotic and incubated overnight at 37°C.

2.1.11 : Isolation of DNA from mini-prep and maxi-prep cultures.

To extract plasmid DNA, 1.5ml of an overnight bacterial culture was processed with the Qiagen mini-prep kit according to the manufacturer's protocol (Qiagen, West Sussex, UK). To maxi-prep plasmid DNA, 100µl of the mini-prep culture was incubated at 37°C for 16 hours with 100ml of antibiotic-containing LB medium. After the bacteria had propagated, glycerol stocks of the plasmid were made by thoroughly mixing 500µl of crude culture with 500µl of sterile

glycerol and stored at -70°C. Plasmid DNA was extracted using the Qiagen maxi-prep kit according to the manufacturer's instructions. Isolated DNA was re-dissolved with TE buffer (10mM Tris HCl pH 8, 1mM EDTA) and diluted to 1μg/μl stock solution. All plasmids were stored at -20°C.

2.1.12 : Restriction digests.

To digest purified plasmid DNA, 5μl of DNA was mixed with 10U restriction enzyme in total volume of 20μl, or 0.5-1μg of maxi-prep DNA was digested in a total volume of 20μl with 10U restriction enzyme (Roche, Lowes, UK) and the appropriate 10x concentrated buffer (Roche, Lowes, UK). Mixture was incubated at the optimal temperature then analysed by gel electrophoresis.

2.1.13 : Phenol/chloroform extraction of digested DNA.

Phenol/Chloroform mixture was mixed with an equal volume of digested DNA (1:1). The reaction mixture was centrifuged for 10 minutes (13000 rpm, 4°C). Then upper phase was transferred to a new 1.5ml eppendorff tube and thoroughly mixed with an equal volume of chloroform then centrifuged for a further 10 minutes. Again the upper phase was transferred to a new eppendorff tube and 0.1 volume of either 2M NaCl or 3M sodium acetate was added. Isopropanol was added to mixture at an equal volume for DNA precipitation, the mixture was then incubated at -20°C for 2 hours followed by centrifugation

for 10 minutes (12000 rpm, 4°C). The DNA pellet was washed twice with 70% ice cold ethanol and air-dried before dissolving in an appropriate volume of TE buffer.

2.1.14 : Isolation of RNA.

All procedures were performed on ice with ice-cold solutions and RNase free plugged tips. $1x10^6$ cells were trypsinised, washed once with PBS, resuspended with 200µl RNAzol (Biogenesis Ltd, Poole, UK) and 0.1 volume of chloroform. Samples were mixed vigorously by vortex for 15 seconds, incubated on ice for 5 minutes and centrifuged at 13,000rpm for 15 minutes at 4°C. The top aqueous phase was transferred to a pre-chilled eppendorf and an equal volume of iosopropanol (Fisher Scientific, Loughborough, UK) added and vortexed thoroughly. Samples were incubated at –20°C for 1 hour and centrifuged at 13,000rpm for 15 minutes at 4°C. The resultant supernatant was decanted, the RNA pellet washed with 500µl 70% ethanol and re-centrifuged at 13,000rpm for 15 minutes at 4°C. The ethanol was aspirated and the air-dried pellet was re-dissolved in 10-20µl DEPC-treated water and stored at -70°C

2.1.15 : cDNA synthesis.

The cDNA was synthesised by using the RETROscript® RNase reverse transcriptase kit (Ambion Europe) according to the manufacturer's instructions. Briefly, 2µg of RNA was mixed with 2µl of 50µM Oligo (dT) in a total volume of 12µl SDW, and heated to 85°C for 3 minutes. The mixture was quickly chilled on ice and briefly centrifuged then 4µl of dNTP mix (2.5mM each dNTP), 2µl of 10X RT buffer, 1µl of RNase inhibitor (10 units/µl) and 1µl of MMLV-RT (100 units/ml) were added. The reaction was then incubated at 42°C for 1 hour followed by inactivating the reverse transcriptase at 92°C for 10 minutes. cDNA products were stored at -20°C and not repeatedly freeze-thawed.

2.1.16 : PCR amplification of cDNA.

1µl of Real-Time polymerase chain reaction (RT-PCR) cDNA product was incubated in a PCR rea ction tube containing 50pmol of forward and reverse primer (Table 2.2), 5µl x10 buffer (Bioline, London, UK), 200mM dNTP (Roche), 1.5mM MgCl2 and 0.5µl Taq polymerase (Bioline) in a total volume of 50µl. The reaction mixture was done in Perkin Elmer DNA Thermal Cycler as described in Table 2.2.

Table 2. 2: Primers and PCR conditions used for RT-PCR reactions

Name	Oligonucleotide	Tm	PCR Cycle
GAPDHF244	5′CCT CCA AAA TCA AGT GGG GCG 3′	61.8	93°C 4 mins (93°C 30 Sec, 61.5°C, 45 Sec, 72°C 2 min) x 26, 72°C, 5 mins
GAPDHR845	5′ACC ACC AGG TGC TCA GTG TAG 3′		
TR1F	5'-GGAGAGTTGTGTCCACCAG-3'		
TR1R	5'-CGAGTCTGCGTTGCTCAGA-3'		
TR2F	5'-ACGGTAGAGATTGCATCTCC-3'		
TR2R	5'-TGAGCTTCTGTCCACACGC-3'		
TR3F	5'-CCTGTAACCCGTGCACAGA-3'		
TR3R	5'-GGGCTGGTGGTCATTGTC-3'		
TR4F	5'-GCACAGAGGGTGTGGATTAC-3'		
TR4R	5'-GGTGGGCTGCAAGTATCTG-3'		
TR5F	5'-ATGCAAGGAAGGGCGCTAC-3'		
TR5R	5'-CCAATGTGCCGCTGCAC-3'		

2.1.17 : Generation of TRAIL mutants using site-directed mutagenesis.

Deletion mutations within the human TRAIL were generated from pShuttlewtTRAIL vector using the Quick Change site-directed mutagenesis kit (Strategene Europe, Amsterdam, The Netherlands). The forward primers TRAILde105-108F and TRAILde105-115F and the reverse primers TRAILde105-108R and TRAILde109-115R (Table 2.2) were utilised for generating the TRAIL mutants with deletion mutation ^{105}EKQQ^{108} or the ^{105} EKQQHISPLV^{115} respectively. Primers were designed such that the T_m was greater than 72°C. Briefly, PCR reactions contained 125ng of each forward and reverse primers, 50ng of pShuttlewtTRAIL template DNA, 200µM dNTP and 2.5U Pfu polymerase in a total volume of 50µl were heated to 95°C for 30 sec, followed by 16 cycles of 95°C for 30 seconds, 55°C for 1 min and 68°C for 2 min per kb of plasmid length. Finally, the reaction was cooled to 4°C. To get red of the non-mutated parental DNA strand, PCR reactions were treated for 1 hour at 37°C with 10U *Dpn* I enzyme. 1µl of the reaction was used to transform 50µl of XL-1 Blue supercompetent *E. coli* (Strategene Europe, Amsterdam, The Netherlands) and the resultant colonies formed on L-agar plates were further grown for mini-prep analysis.

Table 2.3: Primers and PCR reaction conditions for generating TRAIL mutants.

Name	Oligonucleotide	Tm	PCR Cycle
TRAILde105-108F	5′ GGA AAC CAT TTC TAC AGT TCA AAA TAT TTC TCC CCT AGT G 3′	72°C	93°C 4 mins (93°C 30 Sec, 59.1°C, 45Sec, 72°C 2 min) x 26, 72°C, 5 mins
TRAILde105-108R	5′ CAC TAG GGG AGA AAT ATT TTG AAC TGT AGA AAT GGT TTC C 3′		
TRAILde105-115F	5′ GAG GAA ACC ATT TCT ACA GTT CAA GAA AGA GGT CCT CAG AGA-GTA-GCA-GC-3′		
TRAILde109-115R	5′-CTC-CTT-TGG-TAA-AGA-TGT-CAA-GTT-CTT-TCT-CCA-AGA-GTC-TCT-CAT-CGT-CG 3′		

2.2 : Tissue culture techniques.

2.2.1 : Maintenance of cells lines.

Different carcinoma cell line as shown in (Table 2.3) were maintained in D-MEM supplemented with 10% foetal calf serum (Gibco, Paisley, UK) and 1% penicillin/streptomycin (final concentrations: 100U/ml penicillin, 0.1mg/ml streptomycin). Cells were grown in vented 75cm^2 or 150cm^2 flasks (Iwaki, Japan) at 37°C in 5% CO_2, and routinely passaged when 90% confluent or every 2-3 days. The media was removed, cells washed once with PBS, and diluted trypsin (0.5% w/v trypsin, 0.02% w/v EDTA in a total volume of 20ml PBS) added to cover the cells. Cells were transferred to complete media and pelleted by centrifugation at 1000rpm for 5 minutes. The cell pellet was resuspended in fresh complete media and plated into a 75cm^2 or 150cm^2 flasks.

Table 2.4: Carcinomas cell lines used in this thesis.

Name of cells line	Origin
HepG2	Human hepatic cell carcinoma
Hela	Human cervical carcinoma
OVCAR-3	Human ovarian cell carcinoma
SKOV-3	Human ovarian cell carcinoma
A2780	Human ovarian carcinoma
EJ	Bladder carcinoma
HEK 293	Embryonic kidney

2.2.2 : Freezing and thawing cell stocks.

About $2x10^6$ pelleted cells were resuspended in 1ml of freezing media (complete media supplemented with 10% DMSO (Fisher Scientific, Loughborough, UK)) and transferred to a 1ml vial (Nunc, Nalge Europe Ltd, Hereford, UK). The vials were placed at -80°C in a cooling box for at least 24 hours and then transferred to liquid nitrogen storage. Cells were recovered by thawing vials quickly at 37°C and transferring into 10ml pre-warmed complete media for 1 minute before centrifugation. Cells were resuspended in complete media and transferred to a $75cm^2$ flask and incubated at 37°C in 5% CO_2.

2.2.3 : Transfection of cells with plasmid DNA.

For cell transfection, cells were seeded at $3x10^5$ cells/30mm dish in complete media and the next day transfected overnight using the jetPRIME® Plus kit according to the manufacture's instructions. Briefly, 3-5µg DNA was resuspended into 200µl jetPRIME® buffer, 4µl jetPRIME was then added to the DNA mix and incubated for 10 minutes. The mixture was then added to the cell culture drop by drop and distributed evenly. After 24 hours transfection medium was replaced by complete media and incubated at 37C/5%CO_2.

2.2.4 : Production of recombinant adenovirus.

2.2.4.1 :Generation of recombinant adenoviral plasmids by homologous recombination in *E. coli*.

Typically, 1.0μg of pAdShuttle-CMVTRAIL-CD40L or pAdShuttle-CMV empty vector plasmid were linearized with *Pme*I, purified by phenol/chloroform extraction, and mixed with 0.1μg of pAdEasy-1 in a total volume of 6μl. The DNA mixture was used to transform 20μl of electrocompetent *E. coli* BJ5183 cells by electroporation method. The cell suspension was then plated onto LB-agar medium containing 50μg/ml kanamycin and incubated at 37°C until colonies showed up. The smaller colonies (which usually represented the recombinants) were picked up and grown in 2ml of L-Broth containing 50μg/ml of kanamycin. Clones were first screened by analyzing their supercoiled sizes on agarose gels, comparing them to pAdEasy-1 control. Those clones that had inserts were further examined by *Pac1* restriction endonuclease digestion. Once confirmed, supercoiled plasmid DNA was transformed into Topo 10F chemically competent cells for large-scale DNA amplification.

2.2.4.2 : Production of adenoviruses in mammalian cells.

Approximately 1.5 $x10^6$ of 911 or HEK 293 cells were plated in 25cm^2 flasks 24 hours before transfection, by which time they reached 50–70% confluency. Cells were washed BPS and incubated for 4 hour with jetPRIME as described in

section 2.2.3. 4µg of recombinant adenoviral vector DNA, digested with *Pac*I and phenol/chloroform purified was used for transfection of each 25cm^2 flask. A transfection mix was prepared by adding 4µg of linearized plasmid DNA according to the manufacturer's instructions. Transfected cells were monitored for cytopathic effect and collected 7-10 days after transfection by scraping cells off flasks and pelleting them along with any floating cells in the culture. The supernatant was discarded and the cell pellets were re-suspended in 2ml sterile PBS and viral particles were extracted by vigorous mixing with an equal volume of Arklone P and spinning for 10 minutes at 2000rpm. The viral containing solution was collected and stored at -80°C or used to infect 3–5 x 10^6 293 cells in a 25cm^2 flask. Four days later, viruses were harvested.

2.2.4.3 : Large scale production of the recombinant adenovirus.

For large-scale recombinant adenoviral production, HEK 293 cells were plated out into twelve 150cm^2 flasks (IWAKI). When the cells 80% confluent the complete growth medium was replaced with 5ml 2% FCS D-MEM with 1:100 dilution of the adenoviral extract obtained from 2.2.4.2. After 6 hours cells were supplemented with an additional 12ml of 2% FCS D-MEM medium, zVAD with final concentration 15µM to prevent cell death and incubate at 37°C. 5-6 days post-infection, cells and medium were transferred to a 50ml tube and centrifuged at 2000rpm for 5 minutes at room temperature. The supernatant

was discarded and the cell pellets were re-suspended in 10ml sterile PBS and viral particles were extracted by Arklone P as described in section 2.2.4.2.

2.2.4.4 : Density gradient purification of adenovirus.

In Beckman polycarbonate tube a 2ml of p=1.32 CsCl (32.0g CsCl, 6.8 ml 0.5M Tris pH 7.9 and 61.2ml SDW) was added, followed by 3ml of p =1.45 CsCl (20.5g CsCl, 2.9ml 0.5M Tris pH 7.9 and 25.8ml SDW) drop by drop. Then on top of this, 2ml of 40% glycerol (40g glycerol, 2ml 0.5M Tris pH 7.9 and 0.5ml 0.2M EDTA, 57.5ml SDW) was added, followed by the addition of the crude adenoviral extract slowly so as not to disturb the CsCl gradient. The tubes were topped up with PBS buffer to just below the brim. Tubes were placed in an SW40 rotor in a Beckman Coulter Optima™LE-80K ultracentrifuge and spun at 25000rpm for 2 hours. After the ultra centrifugation two bands could be clearly seen in the tube. The lower band containing the wanted virus was extracted by piercing the tube with a 21-gauge needle allowing the virus to be withdrawn. Viruses were collected and injected into a Slidealyser (3M) 10,000MW dialysis cassettes. Dialysis cassettes were incubated for 4 hours in dialysis buffer (10mM Tris-HCl, pH 8.0, 2mM MgCl, and 5% sucrose) at 4°C with gentle agitation then buffer change and left overnight. Buffer was changed at least four times to ensure all CsCl was removed. The viruses were removed from the Slidealyser and aliquoted into 100µl and stored at -80°C.

2.2.4.5 : Measurement of virus concentration.

Using Adeno-X™ Rapid Titer Kit for viral concentration. HEK 293 cells were cultured in 2% FCS D-MEM at 2.5 X 10^5 cells/ml and plated in 24-well plate. Virus stock was serially diluted with 2% FCS D-MEM as from 10^{-2} to 10^{-7} and 50 µl of viral dilution were add to each well. Cells were incubated at 37°C in 5% CO^2 for 48 hours then assessed according to the manufacture's instructions. The virus concentration was calculated in ifu/ml (Infection Forming Unit).

***2.2.4.6* : Infection of cells with replication deficient adenoviruses.**

Cells were seeded in complete media at 5000-7000 cells per well in a 96-well plate for apoptosis or caspase activation assays, or $3x10^5$ cells/30mm dish for western blotting analysis. Cells were infected in 10% FCS D-MEM for 2 or infected for 24 hours at 37°C with the appropriate MOI of the adenovirus. Culture medium was then replaced by fresh medium and incubated at 37°C.

2.2.4.7 : Chemotherapeutic treatment of cells.

Cells infected with virus or left without infection were cultured in 96 well plate at a density of 5-7 x 10^3 cells/ well for 24 hours .Cells were then treated with different cytotoxic drugs at different concentration [Sorafunib (Sor), Doxorubicin (DOX), 5 Fluorouracil (5FU), Cisplatine (Cis), Pacletoxel (Pac)]. After 48 hours, cell viability was evaluated by WST1 assay.

2.2.5 : Analysis of cell viability by WST-1 assay.

Cells were infected with the adenoviruses (RAd) at an appropriate MOI dose for 2 hours and then plated out in 96-well plate in 10% FCS D-MEM medium f at 5000-7000 cell/100µl/well and incubated at 37°C for either 24, 36 or 48 hours. Cell viability was then assessed by the WST-1 assay reagent (Roche), briefly, the WST-1 reagent was added at 1:10 dilution of the total volume of culture medium. Plates were incubated at 37°C and the OD was then measured by microplate ELISA reader at λ_{450} every 30min up to 3 hours from the time of adding the WST-1 reagent. The number of metabolically active cells is directly correlates to the amount of formazan formed.

2.2.6 : Caspase 3/7 activation measurement assay.

Carcinomas cell lines were grown in complete 10% FCS D-MEM and infected with either RAdMock or RAdwtTRAIL or RAdncCD40LTRAIL or treated with rshTRAIL (100-200ng/ul) described in section 2.2.4.6 or left un-infected as a control. Cells were either treated with the general caspase inhibitor ZVAD at a concentration of 30µM. Cells were cultured in a white-walled tissue culture 96 well plate at density of 5000 cell/100µl/well in triplicate and incubated in a 37°C incubator for 36 hours. The activity of caspases 3/7 was measured using the Caspase-Glo® 3/7 (Promega, UK) assay kit according to the manufacturer's instructions. The caspase reagents were prepared and 100µl/well was added to

cells, mixed for 30 sec in a 96 well plate shaker at room temperature followed by 1 hour incubation at room temperature. Luminescence was then measured using a 96-well plate luminometer. The result of luminescent signal is directly proportional to the amount of caspase activity present in each sample.

2.3 : Protein biochemistry techniques.

2.3.1 : Preparation of protein extracts.

2.3.1.1 : Preparation of protein extracts from mammalian cells.

For protein extraction, cultured cells were washed three times with ice cold PBS. Cells were then lysed *in situ* with a suitable volume of an appropriate concentration of NP-40 lysis buffer. Total cell lysates were collected and incubated on ice for 30 minutes for complete lysis. Lysates were then clarified by centrifugation at 13,000rpm for 15 minutes at 4°C and stored at -80°C.

2.3.1.2 : Determination of protein concentration.

Protein concentration was determined using the Protein Determination assay kit (BioRad Lab Ltd., Hemel Hempstead, UK) by mixing 2.5µl of clarified lysates with 1.2ml SDW and 300µl BioRad reagent (Biorad Lab. Ltd., Hemel Hempstead, UK). Then Absorbance at λ450 was measured by spectrophotometer and protein concentration calculated in reference to the BSA standard curve.

2.3.2 : Immunoblotting techniques.

2.3.2.1 : SDS-PAGE electrophoresis.

Western blotting was performed using a Mini-Protean III tank (BioRad) by the method of Laemnli. Glass plates were cleaned with ethanol and the apparatus assembled according to the manufacturer's instructions. 10ml resolving gel (375mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 30% (w/v) acrylamide/0.8% (w/v) bis mix (National Diagnostics, Geneflow Ltd, Fradley, UK) and TEMED depending on the desired percentage of gel) was poured. Water saturated butanol was layered on top of the resolving gel to remove air bubbles. Gels were allowed to set at room temperature until solidified. Saturated butanol was thoroughly washed out with distilled H_2O. A 4ml stacking gel was prepared (125mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.01% TEMED and 0.67ml 30% (w/v) acrylamide/0.8% (w/v) bis mix) and poured on top of the set resolving gel, a comb inserted and allowed to set at room temperature. Prior to gel loading, the comb was removed and the wells washed extensively with x1 SDS-PAGE running buffer (x10 stock solution; 0.25M Tris-HCl pH 8.3, 1.92M glycine and 1% (w/v) SDS). Samples containing 15-100μg of total protein were denatured by boiling with 3x concentrated gel sample buffer (GSB; 187.5mM Tris-HCl pH 6.8, 6% w/v SDS, 30% glycerol, 0.03% w/v bromophenol blue, 150mM DTT) for 5 minutes. Denatured samples were cooled on ice, pulse centrifuged to remove

condensation and loaded onto the assembled gel. Gels were electrophoresed at 120-150V (constant voltage) for 90-120 minutes.

2.3.2.2 : Western blotting.

Following SDS electrophoresis, transfer of proteins was performed using the BioRad Mini Trans-Blot tank onto nitrocellulose transfer membranes (Pall Gelman laboratory, Portsmouth UK) or methanol soaked 0.45μM PVDF membranes (Millipore, Watford, UK). Tanks containing transfer buffer (50mM Tris-HCl, 190mM glycine, 20% (v/v) methanol) and assembled mini gel holder cassettes were placed on a magnetic stirrer to prevent the build up of heat and electrophoresed for 1 hour at 100V. Membranes were washed in TBS-T (1x TBS; 20mM Tris-HCl pH 7.6, 136mM NaCl, supplemented with 0.1% Tween-20) for 5 minutes and blocked in TBS-T containing 5% low-fat milk or 5% of BSA (phosphrylated proteins) for 1 hour at RT. Blocked membranes were then washed three times for 5 min in TBS-T and incubated overnight at 4°C in the relevant primary antibody (Table 2.4). Membranes were washed three times for 5 minutes each in TBS-T, incubated for 1 hour at RT in the relevant peroxidase conjugated secondary antibody (Table 2.6) diluted in TBS-T, and then washed a further 3 times for 5 minutes each in TBS-T. Proteins were visualised by enhanced chemiluminescence (Amersham).

Table 2.5: Primary antibodies.

Antigen	Dilution	Secondary	Supplier
TRAIL (C-19)	1:500	α- goat	Santa Cruz
β-Actin	1:10000	α-rabbit	Cell Signaling

Table 2.6: Secondary antibodies.

Antigen	Dilution	Supplier
α-Goat HRP	1:4000	Santa Cruz
α-mouse HRP	1:5000	Sigma

2.3.3 : Enzyme-linked immunosorbent assay (ELISA).

Cultured cells in 10 % FCS D-MEM medium were infected with at different MOI of either RAdwtTRAIL, RAdncCD40LTRAIL or RAdMock or left without infection. Then cells plated at 5000-7000 cell/well in 96-well microplates. Twenty four hours later cells were treated with 15μM of MMPI I and II (MPI) or left without treatment for 24 hours. Samples from culture media were collected after 48 hours for assessing the sTRAIL concentration by ELISA assay kit according to manufactures instruction. Briefly, the sTRAIL standard concentrations, in addition to the collected samples, were applied in a total volume of 100μl of the diluent provided within the kit in duplicate to a microwell plate coated with anti-human sTRAIL monoclonal antibody. A 100μl of diluted HRP-conjugate anti-sTRAIL (1:200) monoclonal antibody was added to each well. The plate was covered with a plate cover and incubated for 2 hours at room temperature then carefully washed three times with 300μl wash buffer. 100μl of substrate solution was added to each well and incubated for 10 min at RT on a rotator set at 100rpm. The reaction was stopped by adding 100μl of stop solution into each well. The absorbance of the colour product was measured within 30 minutes at λ450 using an ELISA plate reader.

CHAPTER 3
GENERATION OF REPLICATION-DEFICIENT ADENOVIRUS (RAd) EXPRESSING WILD TYPE-TRAIL

3.1: Introduction.

The utility of TNF ligands in cancer treatment is promising due to their ability to induce cell death and apoptosis. Since the discovery of TNF in 1975 (Carswell et al., 1975), administration of TNF has been found to induce cell death or growth regression of solid human tumours xenotransplanted into SCID mice (Haranaka et al., 1984), the induction of TNF-family ligands for cancer treatment has promised to be an attractive in addition to the classical strategy of treatment cancer with chemotherapy and radiotherapy. Induction of apoptosis in tumour cells through death-domain containing TNF receptor family member is rapid and involves the extrinsic pathway with the cleavage of caspase 8 and activation rather than mitochondrial–mediated pathway. Recent studies have revealed the ability of death receptor ligands of the TNF family to sensitize drug-resistant carcinomas such as ovarian carcinoma (Vignati et al., 2002).

The findings that TRAIL induces apoptosis in tumour but not normal cells, and that combination of TRAIL with other chemotherapeutic drugs or radiation can induces a higher degree of cell death and apoptosis in carcinomas than either agent alone (Chinnaiyan et al., 2000), have highlighted the potential therapeutic role of TRAIL in cancer treatment. To date, several methods have been used to deliver TRAIL into the tumour mass, including direct delivery of TRAIL via systemic administration of recombinant soluble TRAIL (rshTRAIL) or agonistic

monoclonal antibodies (mAbs) (reviewed in Mellier et al., 2010) and these have been tested in clinical trials. Results show that both rshTRAIL and mAbs are promising but required repeated administration and large doses of TRAIL (reviewed in Holoch and Griffith, 2009).

An alternative route of administration involves a gene therapy approach to deliver TRAIL cDNA into the tumour cells. Recent studies of TRAIL gene therapy have demonstrated that ex vivo infection of cancer cells with a recombinant adenovirus expressing TRAIL (RAdTRAIL) enhances apoptosis and promotes tumour regression (Zhang et al., 2008). Direct administration of TRAIL gene by RAdTRAIL into human prostate cancer leads to rapid and sustained expression of functional TRAIL protein resulting in apoptotic cell death in TRAIL positive tumours *in vivo* and *in vitro* (Voelkel-Johnson et al., 2002). In addition, a completed phase I clinical trial of RAdTRAIL in patients with prostate cancer has demonstrated the ability of TRAIL to induce cell death in cancer cells and tumour regression with minimal side effects (reviewed in Griffith et al., 2009). Moreover, modification and improvement of TRAIL gene delivery to increase efficacy are under investigation. For example through enhancement of infection by increasing distribution of adenovirus to infect every tumour cells (Seol et al., 2003, Hait and Yang, 2006). Modification of the mode of ligation may also increase the effect of TRAIL ligation on cell death and apoptosis induction. Indeed previous studies in our laboratory on TNF

ligands particularly FasL and CD40L have confirmed the enhanced effect of membrane-bound forms of these ligands in inducing cell death (Elmetwali et al., 2010, Knox et al., 2003). On this basis, we sought to investigate modification of TRAIL ligation thorough retention of ligand on the cell membrane.

3.2 : Results.

A variety of methods exist for generation of recombinant adenoviruses (Bett et al., 1994, Young et al., 2006). Here in this chapter we have used the AdEasy system (Quantum Biotechnology) using adenovirus vectors and methods as published by Chuan He and co-workers (He et al., 1998), due to the simplicity and the less time consumption compared to other published method.

3.2.1 : Generation of a recombinant adenovirus expressing wild-type TRAIL.

3.2.1.1 : PCR Amplification of full length TRAIL.

To amplify the full-length TRAIL from a pcDNA3.1 vector encoding the full-length TRAIL for cloning into the pShuttle CMV vector, an examination of the endonuclease restriction map of the full length of TRAIL was performed. As a result of this analysis, a couple of restriction sites (*Sal1* and *Xba1*) were chosen for further PCR primers design, these restriction sites do not exist in the TRAIL sequence but are in the multi cloning site (MCS) of the pShuttle CMV vector in an open reading frame downstream of the pShuttle CMV promoter. Together

with a couple of specific forward and reverse primers (Forward primer : TRAIL *Sal1* F, 5'-A **GTCGAC** ATG-GCT-ATG-ATGG-AGG-TC-3', Reverse primer: TRAIL *Xba1* R, 5' C- **TCTAGA** –TTA-GCC-AAC-TAA-AAA-GGC-CC-3') artificially designed with *Sal1* and *Xba1* (bold sequence) restriction sites were utilized to amplify the full-length TRAIL by PCR reaction under the following cycle conditions (93°C 5 mins (93°C 30 Sec, 58.1°C, 45 Sec, 72°C 2 mins) x 25, 72°C, 5 mins), using a proof reading Taq polymerase (Roche) as described in section (2.1.1). As shown in figure 3.1, analysis of the PCR reaction product on 1% gel electrophoresis revealed an amplified fragment at the expected size of full-length TRAIL.

To clone the amplified PCR TRAIL product into the Topo2.1 vector, the amplified PCR product was excised from the gel and gel purified as described in section (2.1.3) then subjected to addition of A-overhangs (section 2.1.4). To clone the A-overhanged PCR product into the Topo2.1 vector, a TA cloning kit (Invitrogen) was utilized as previously described in section (2.1.6). The ligated vector was used to transform the *E.coli* TOPO 10F competent cells, following selection of the transformants on ampicillin-containing LB- agar plates coated with 40µl of each x-Gal (40mg/ml) and IPTG (100mM), positive colonies were selected and miniprepped DNA was digested with *Sal1* and *Xba1* to confirm the cloning of the TRAIL into the Topo2.1 vector. To check whether any mutations

occurred during the cloning process, selected clones were sequenced which have confirmed the absence of any mutations within the TRAIL sequence.

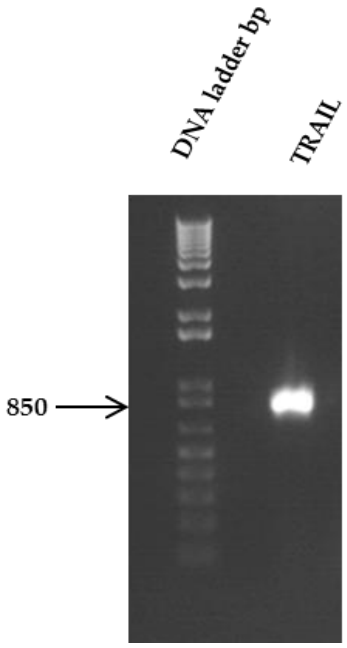


Figure 3.1: Agarose gel electrophoresis of the TRAIL PCR product.

Five microlitres of the purified PCR TRAIL product was run in 1% agarose gel containing 0.2µg/ml EtBr. The amplified fragment runs at an expected size of the TRAIL sequence of 843bp.

3.2.1.2 : Cloning of full-length TRAIL into pShuttle CMV vector.

Following confirmation of the TRAIL sequence cloned into Topo2.1 vector by *Sal1* and *Xba1* restriction digestion and sequence analysis, 2µg of Topo2.1TRAIL construct and 1µg of pShuttle CMV vector were digested with *Sal1* and *Xba1* restriction endonucleases, which resulted in releasing the TRAIL fragment from the Topo2.1 vector and linearizing pShuttle CMV vector. TRAIL fragment and linearized pShuttle CMV vector were excised from 1% agarose gel and gel purified separately from gel by Qiagen gel extraction kit as described in section (2.1.3). Following DNA purification, TRAIL DNA fragment was then ligated into *Sal1* and *Xba1* predigested and purified pShuttle CMV vector, using T4 ligase reaction as described in section (2.1.7). Ligated pShuttle TRAIL vector was used to transform TOPO 10F competent cells, positive clones were selected on kanamycin (50µg/ml) containing LB-agar medium for further analysis with restriction digestion and sequencing. As shown in Figure 3.2, *Sal1* and *Xba1* restriction digestion resulted in releasing a fragment at the expected TRAIL size, furthermore, sequence analysis of the selected pShuttle TRAIL clones revealed the right TRAIL sequence that has been inserted correctly in an open reading frame downstream of the CMV promoter of the pShuttle vector..

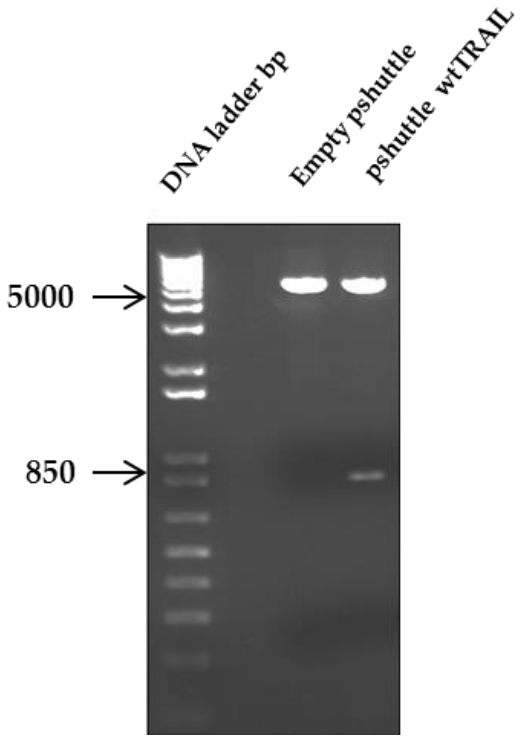


Figure 3.2: Restriction digestion of miniprepped DNA empty pShuttle and pShuttlewt TRAIL constructs.

100 ng of either pShuttle empty vector or pShuttle wtTRAIL construct were digested with *Sal1* and *Xba1* restriction enzymes, which resulted in releasing the wtTRAIL DNA fragment at an expected size of 843bp.

3.2.1.3 : Insertion of TRAIL into the pAdEasy-1 vector by homologues recombination.

The pAdEasy-1 adenoviral plasmid contains all Ad5 sequences except nucleotides 1–3,533 (encompassing the E1 genes) and nucleotides 28,130–30,820 (encompassing E3). The left arm of the pShuttle CMV vector contains the Ad5 nucleotides 34,931-35,935. The right arm contains Ad5 nucleotides 3,534–5,790 which mediate homologous recombination with the PAdEasy-1 vector in *E.coli*, plus inverted terminal repeat (ITR) and packaging signal sequences (nucleotides 1–480 of Ad5) required for viral production in mammalian cells.

To insert the TRAIL sequence into the Adenoviral backbone, the pShuttle wtTRAIL construct was homologously recombined with PAdEasy-1 vector as described by Chuan He and co-workers (He et al., 1998). Typically, 1.0μg of pShuttle wtTRAIL vector was linearized with *PmeI*, purified by phenol/chloroform extraction, and mixed with 0.1μg of pAdEasy-1 in a total volume of 6μl. The DNA mixture was used to transform 20μl of electrocompetent *E. coli* BJ5183 cells by electroporation method. Transformed BJ5183 cells were then recovered in soc medium for 1 hour and plated onto LB-agar medium containing 50μg/ml kanamycin and incubated at 37°C until colonies showed up. The smaller colonies (which usually represented the recombinants) were picked up and grown in 2ml of L-Broth containing 50μg/ml of kanamycin. Clones were first screened by analysing their supercoiled sizes

on agarose gels as shown in figure 3.3A, comparing them to pAdEasy-1 control vector. One of the selected clones was further examined by *Pac1* restriction endonuclease digestion. As shown in Figure 3.3B, *Pac1* restriction digestion results suggest that the homologous recombination has occurred in the desired orientation. This clone was designated as RAdwtTRAIL virus.

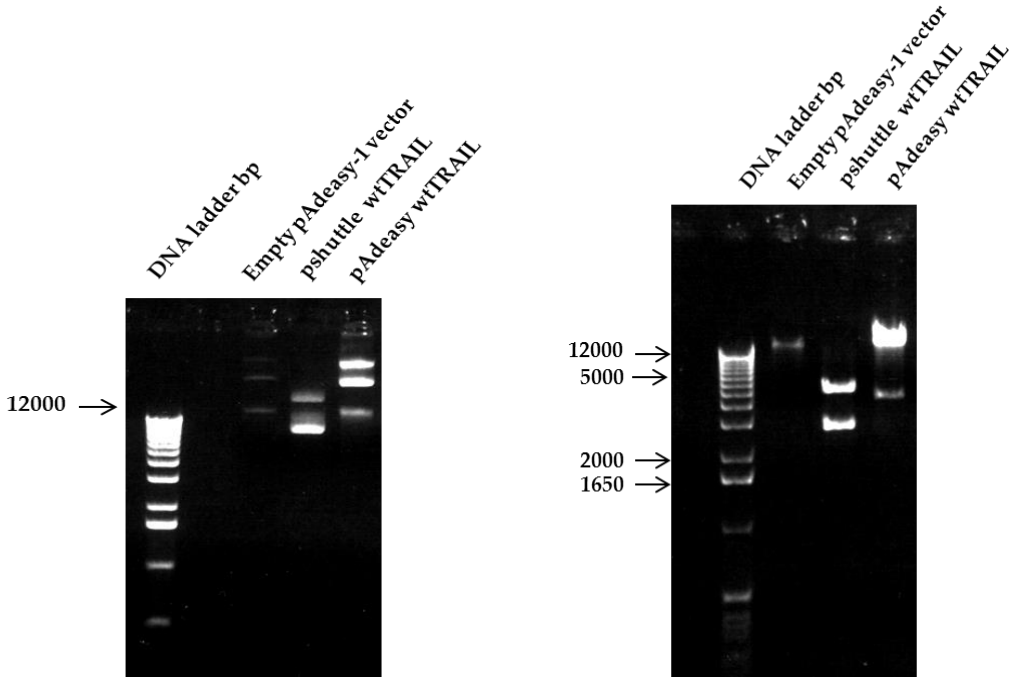


Figure 3.3: Agarose gel electrophoresis of constructed RAdwtTRAIL.

A-500ng of miniprepped DNA of empty pAdeasy-1 vector, pShuttle wtTRAIL, PAdeasy wtTRAIL were run in 1% agarose gel electrophoresis containing 0.2μg/ml EtBr.
B-*Pac1* restriction digestion of empty pAdeasy-1 vector, pShuttle wtTRAIL, PAdeasy wtTRAIL, restriction digestion products were run on 1% agarose gel electrophoresis containing 0.2μg/ml EtBr.

3.2.1.4 : Packaging of RAdwtTRAIL in HEK 293 cells.

In order to generate an infective RAdwtTRAIL, RAdwtTRAIL vector construct was digested with *Pac1* enzyme to expose the lift and the right inverted terminal repeats (ITR), the *Pac1* digested product of RAdwtTRAIL construct was used to transfect HEK293 cells. HEK293 cells are E1 positive and express adenoviral proteins encoded by E1A and E1B genes that are required for replication of E1, E3-deleted adenoviral vectors, thus allowing the packaging and production of the adenovirus. RAdwtTRAIL-transfected HEK293 cells were monitored for 7 days until viral plaques appeared. Figure 3.4, demonstrates the several steps involved in generation of recombinant adenoviruses.

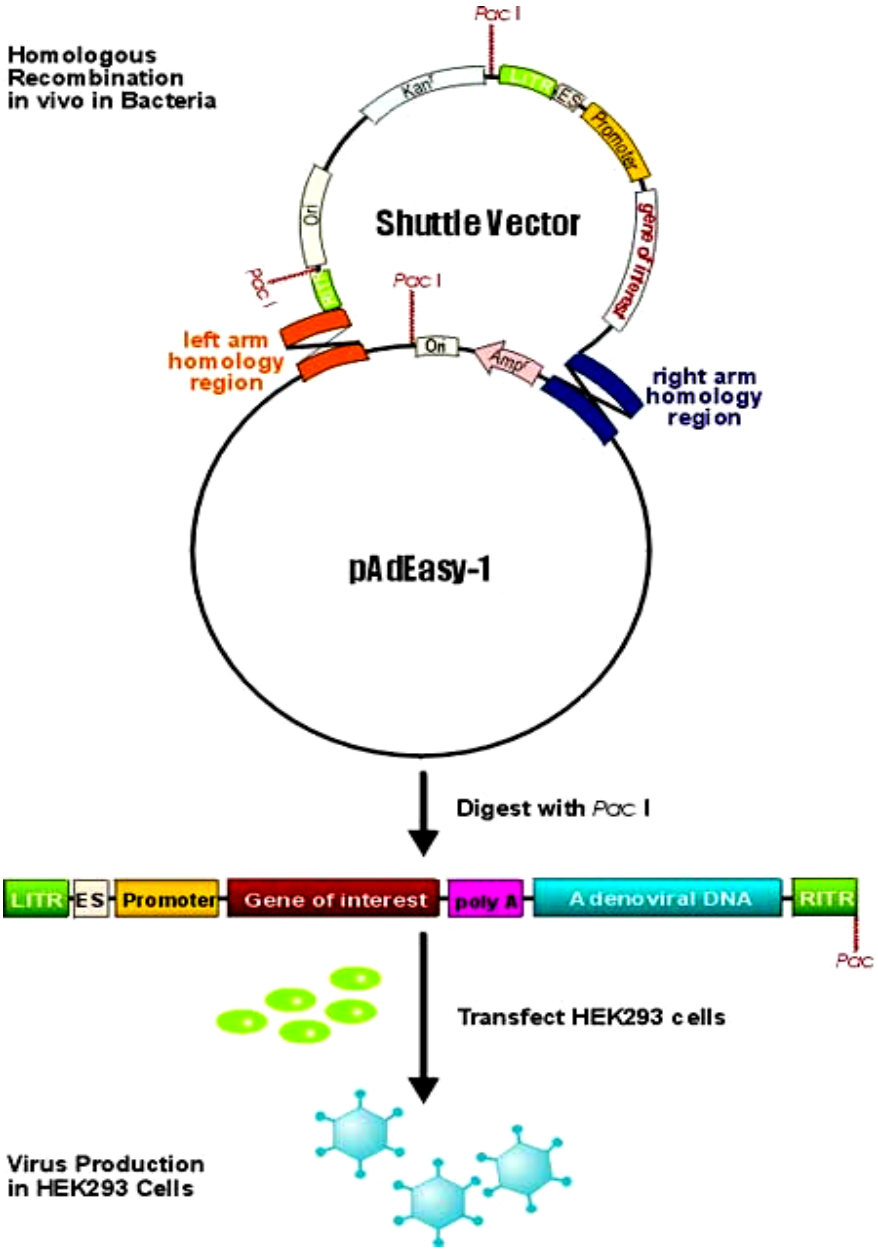


Figure 3.4: Illustrating diagram of the three different stages involved in adenoviral production.

Three different stages are involved in adenovirus production: First stage involves cloning the gene of interest in pShuttle vector, second stage involves integration of the gene of interest with the adenoviral backbone by homologous recombination between the pShuttle construct and the adenoviral backbone vector, final stage involves viral packaging and production in HEK 293 cells.

3.2.2 : Verification of the expression of wtTRAIL from the constructed RAdwtTRAIL virus.

To examine whether the generated RAdwtTRAIL virus expresses the TRAIL protein, HEK 293 cells which are E1-positive and therefore have the ability to support replication-deficient adenoviral vector propagation were infected with either RAdwtTRAIL or RAdMock (been previously generated in our Lab by Dr. Taha Elmitwalli viruses at 1 MOI or left un-infected as a negative control, Given that HEK 293 cells are TRAIL receptor positive cell line, cells were treated with the anti-apoptotic pan-caspase inhibitor z-VAD at a concentration of 20μM to protect cells from an expected TRAIL-induced cell death. Cells were incubated for 48 hours then lysed *in situ* and 100μg of total protein lysates were examined by western blotting analysis for TRAIL protein expression using goat polyclonal antibody raised against TRAIL C-terminus (C-19 Santa cruz biotechnology) region. As demonstrated in figure 4.5, TRAIL expression from RAdwtTRAIL-infected cells but not from RAdMock or uninfected control cells was detected. To ensure that equal amounts of total cell lysates were loaded, β-actin was examined as well using monoclonal mouse anti-β-actin antibody. As seen in figure 3.5, β-actin expression was equal between samples.

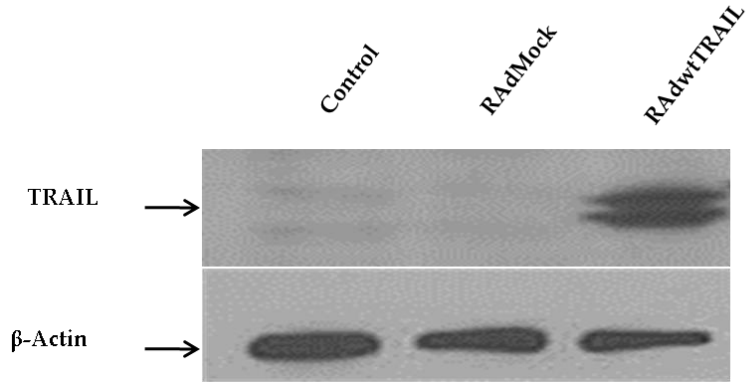


Figure 3.5: Verification of TRAIL expression from the constructed RAdwtTRAIL adenovirus.

HEK293 cells were infected with either RAdwtTRAIL or RAdMock viruses at 1 MOI or left un-infected as a negative control for 48 hours then lysed *in situ*. 100μg of total protein lysates were immunoblotted for TRAIL using goat polyclonal antibody raised against TRAIL C-terminus (C-19 Santa cruz biotechnology) region. β-actin was also examined to ensure equal loading between samples.

3.2.4 : An examination of the TRAIL receptors expression in selected panel of carcinomas.

To test the direct effect of the constructed RAdwtTRAIL adenovirus on TRAIL receptor-positive carcinomas, we examined the expression of TRAIL receptor 1 and 2 at the level of both mRNA and protein in a panel of carcinoma cell lines including the cervical cell line Hela, the hepatic cell line HepG2 and the ovarian cell lines SKOV-3, OVCAR-3 and A2780. Cell lines were grown and mRNA was extracted and subjected to cDNA synthesis as described in section (2.1.12), Following cDNA synthesis, RT-PCR reaction was performed utilizing specific TRAIL-R1 and TRAIL-R2 forward and reverse primers and 1μl of cDNA as a template. As shown in figure 3.6A, adequate levels TRAIL-R1 and TRAIL-R2 were detected in all examined cell lines with the only exception of TRAIL-R1 in the ovarian carcinoma A2780 cell line which was not detected. To examine whether, similar pattern of expression exists at the level of protein, protein samples were collected from these cell lines and examined by western blot analysis using specific antibodies against TRAIL-R1 and TRAIL-R2 proteins. As shown in figure 3.6B, higher levels of TRAIL-R1 and TRAIL-R2 expression were detected in Hela, HepG2 and OVCAR-3 cells, β-actin was also examined to ensure equal loading between samples.

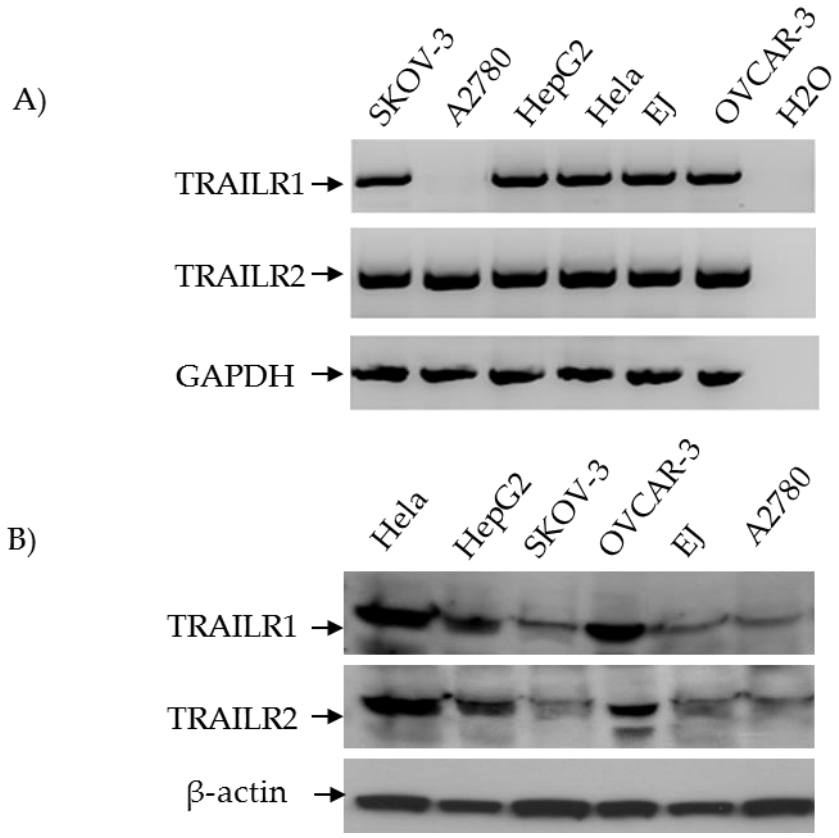


Figure 3.6: Expression of TRAIL-R1 and TRAIL-R2.

A-1μg of total RNA was subjected to cDNA synthesis by reverse transcription method using the retroscript cDNA kit (Invitrogen Cat AM1710). One microliter of total cDNA was then used as a template for amplification of TRAIL-R1 and TRAIL-R2 using specific primers for each receptor, GAPDH was also amplified to ensure equal amount of cDNA input.
B-100μg of total protein lysates were examined for TRAIL-R1 and TRAIL-R2 expression using specific antibodies raised against TRAIL-R1 and TRAIL-R2. β-actin was also examined as a loading control.

3.2.5 An examination of the direct effect of RAdwtTRAIL on TRAIL receptor-positive carcinomas

To examine the effect of wild-type TRAIL on carcinomas cell viability, a panel of TRAILR1 and 2-positive cell lines including the cervical cell line Hela cells, the hepatic cell line HepG2 and the ovarian cell lines OVCAR-3 and A2780 cells which expresses lower levels of TRAILR1 and 2 were infected with either RAdwtTRAIL, RAdMock or left untreated as a negative control at the indicated multiplicities of infection (MOI) for 48 hours. Cell viability was then assessed by WST-1 assay as described in section 2.2.5.1. As shown in figure 3.7, different cell lines exhibited different degrees of sensitivity to the RAdwtTRAIL with the greatest sensitivity observed in Hela, HepG2 and OVCAR-3 cells, where lower sensitivity was noticed with the A2780. RAdwtTRAIL-induced cell viability reduction was enhanced by increasing the MOI, whereas no difference was noticed with similar MOI of RAdMock infection. A2780 cells failed to response to RAdwtTRAIL-infection, which is likely attributed to the level of the TRAIL receptor expression in these cells. To exclude the possibility of reduced adenovirus infectability in A2780 cells, we tested the infectability of A2780 cell using a RAdGFP mock vector. As shown in figure 3.8, 100% infection in A2780 cells was obtained at 150MOI.

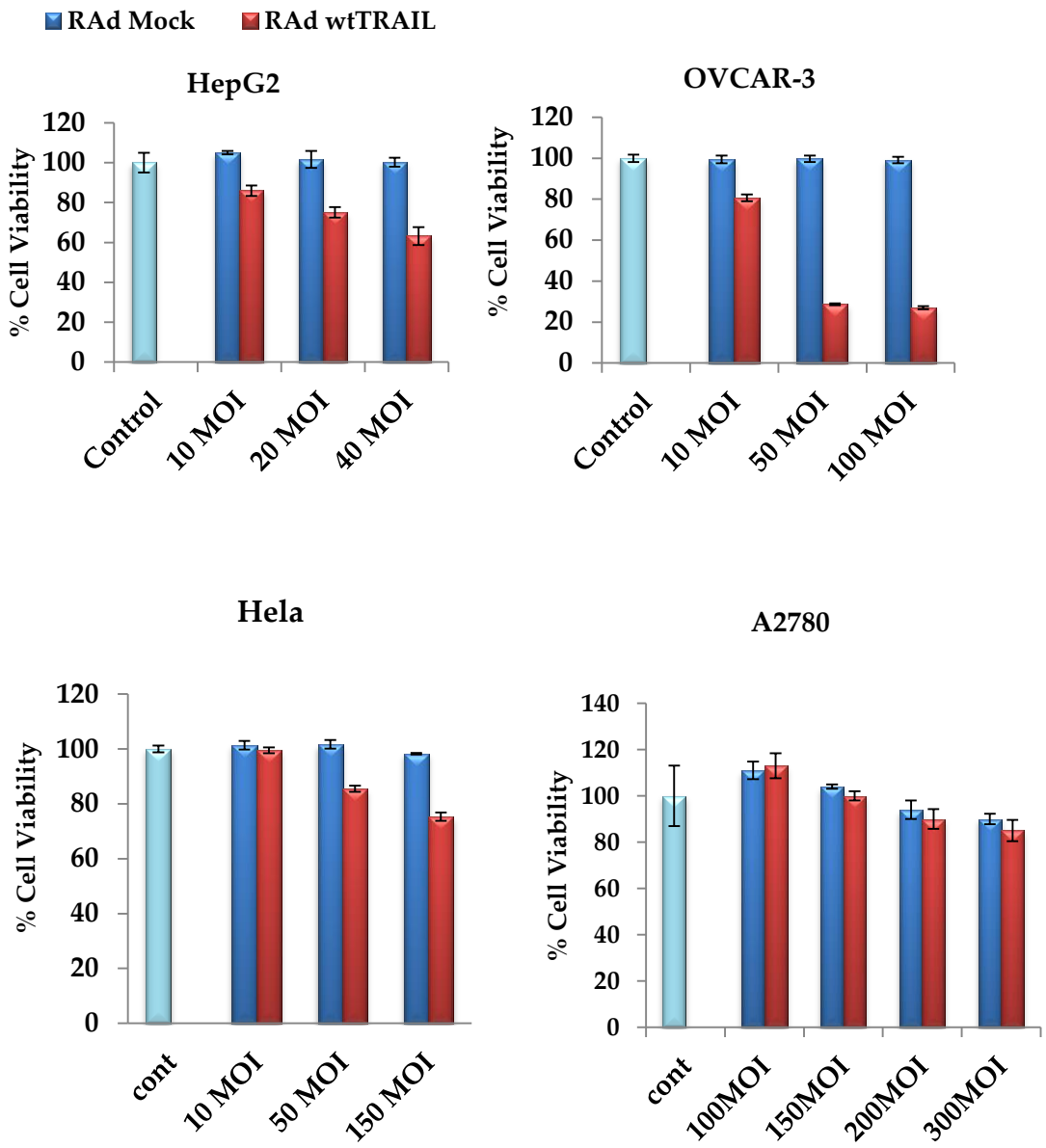
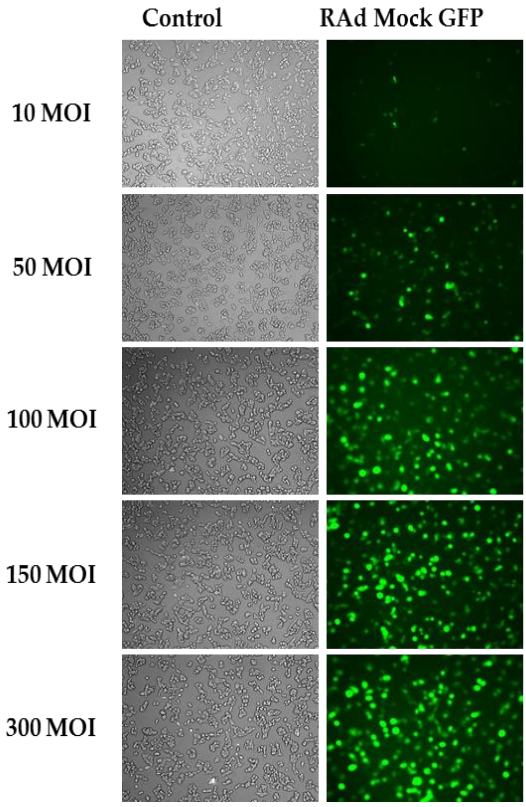


Figure 3.7: Direct effect of RAdwtTRAIL on cell viability.

Hepatocelluler carcinoma HepG2 cell line, ovarian carcinoma cell line OVCAR-3, cervical carcinoma cell line Hela and A2780 were infected with the indicated MOI of RAdwtTRAIL or RAdMock or left without infection for 48 hours. Cell viability was assessed by WST-1ssay. Results represent the mean of triplet samples ±SD.

A)



B)

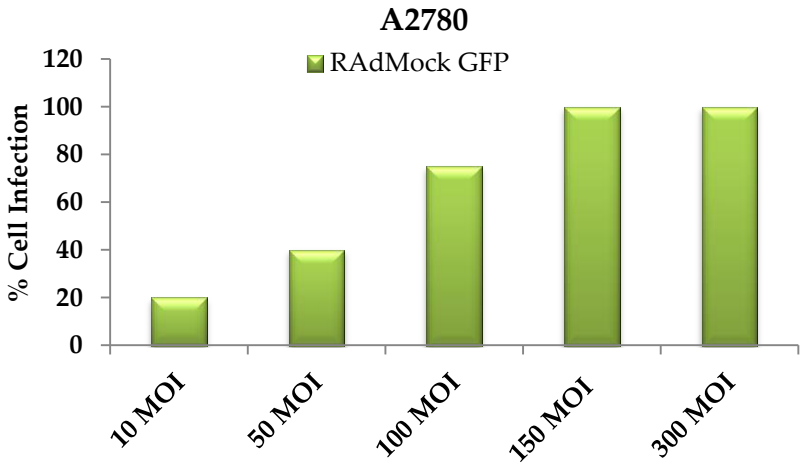


Figure 3.8: A2780 cell line infectability rate by RAdMock GFP.

A) A2780 ovarian carcinoma cell line were infected with RAd Mock GFP at different MOI (10,50,100,150,and 300), GFP expression was visualized under UV microscope.
B) RAd Mock GFP-infected cells were counted and expressed as percentage to uninfected cells.

3.2.6 : An examination of the cleavage of TRAIL from RAdwtTRAIL in RAdwtTRAIL-infected cells.

The majority of TNF ligands are naturally expressed as a membrane bound-structure and upon expression they are naturally cleaved from the membrane by the action of specific group of proteases belonging to the family of metalloproteinases (MMPs). To examine whether TRAIL protein expressed from RAdwtTRAIL is also subject to cleavage from membrane by the action of MMPs, the cervical carcinoma Hela cells were infected with 100MOI of either RAdwtTRAIL or RAdMock or left uninfected as a negative control. Samples were collected from the culture media 24 and 48 hours post-infection for sTRAIL measurement utilising a sTRAIL ELISA assay kit (R&D Biosystems MAB3751). As shown in figure 3.9, a significant quantity of sTRAIL was detected within RAdwtTRAIL-infected cells and the amount of sTRAIL detected increased over time. However, no sTRAIL was detected in culture media collected from either RAdMock-infected cells or uninfected control cells, indicating that wtTRAIL protein is also subject to cleavage from cell membrane.

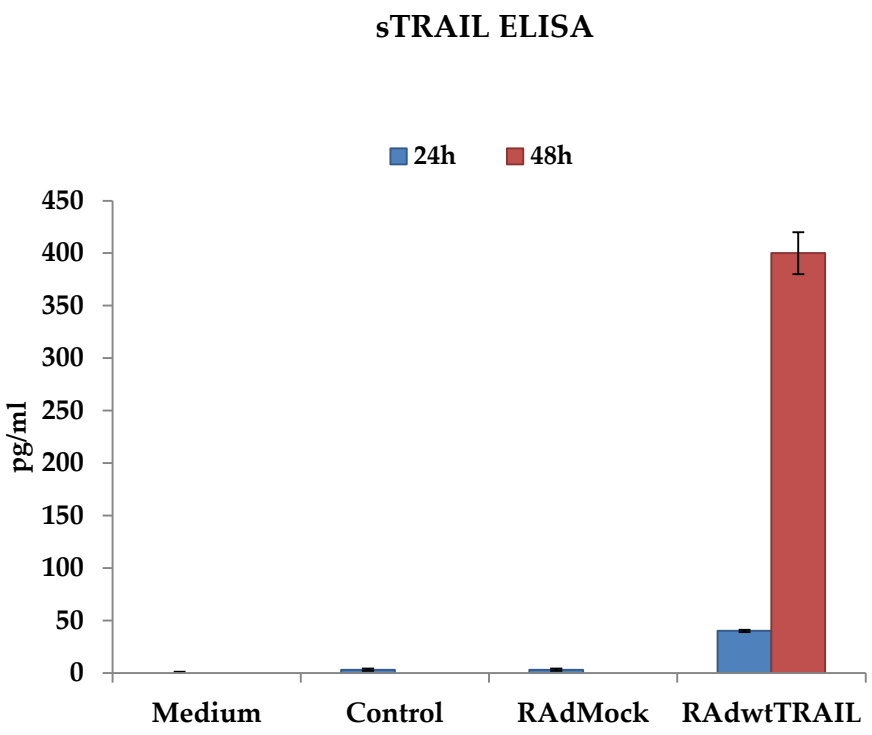


Figure 3.9: TRAIL cleavage from RAdwtTRAIL-infected Hela cells.

Hela cells were infected with 100 MOI of either RAdMock or RAdwtTRAIL or left uninfected as a negative control. Samples were collected from the culture media 24 and 48 hours post-infection for sTRAIL measurement. Results are mean of triplet samples ±SD

3.2.7 : An examination of the direct effect of matrix metalloproteinase inhibitors (MMPIs) on TRAIL cleavage from RAdwtTRAIL adenovirus.

Given that TRAIL expressed from RAdwtTRAIL adenovirus in RAdwtTRAIL-infected Hela cells was also subject to cleavage from the membrane, we next tested whether TRAIL cleavage is mediated by the action of the MMPs family. Thus the human cervical carcinoma Hela cells were infected with 100MOI of either RAd Mock or RAdwtTRAIL or left without infection as a negative control or treated with 100ng/ml of sTRAIL. Cells were either treated with 30μM of MMPI II (Calbiochem, cat 444247), a potent, reversible inhibitor of a broad-range of a number of MMPs at a concentration of 30μM (previously defined as the maximum tolerated non-toxic dose) or left without treatment for a further 48 hours. Samples were collected from the culture media 48 hours post-infection for sTRAIL measurement. Furthermore, culture media were replaced with a fresh medium and cell viability assessed by WST-1 assay reagent. As shown in figure 3.10, MMP inhibitor was capable of blocking the cleavage of soluble TRAIL in RAdwtTRAIL-infected Hela cells at a 30μM concentration. This concentration was well-tolerated by cells, with no cell viability reduction observed in Hela control cells. Interestingly, blocking of cleavage of TRAIL in RAdwtTRAIL-infected cells exhibited a significant cell viability reduction compared to MMPI-untreated cells, indicating that a membrane bound TRAIL

structure is more potent inducer of cell viability reduction compared to wild-type TRAIL which is subject to cleavage from the membrane. Moreover, MMPI treatment by itself does not sensitize cells to sTRAIL treatment or any of the adenoviral proteins, as the MMPI did not induce any cell viability reduction with RAdMock-infected cells or sTRAIL-treated uninfected cells.

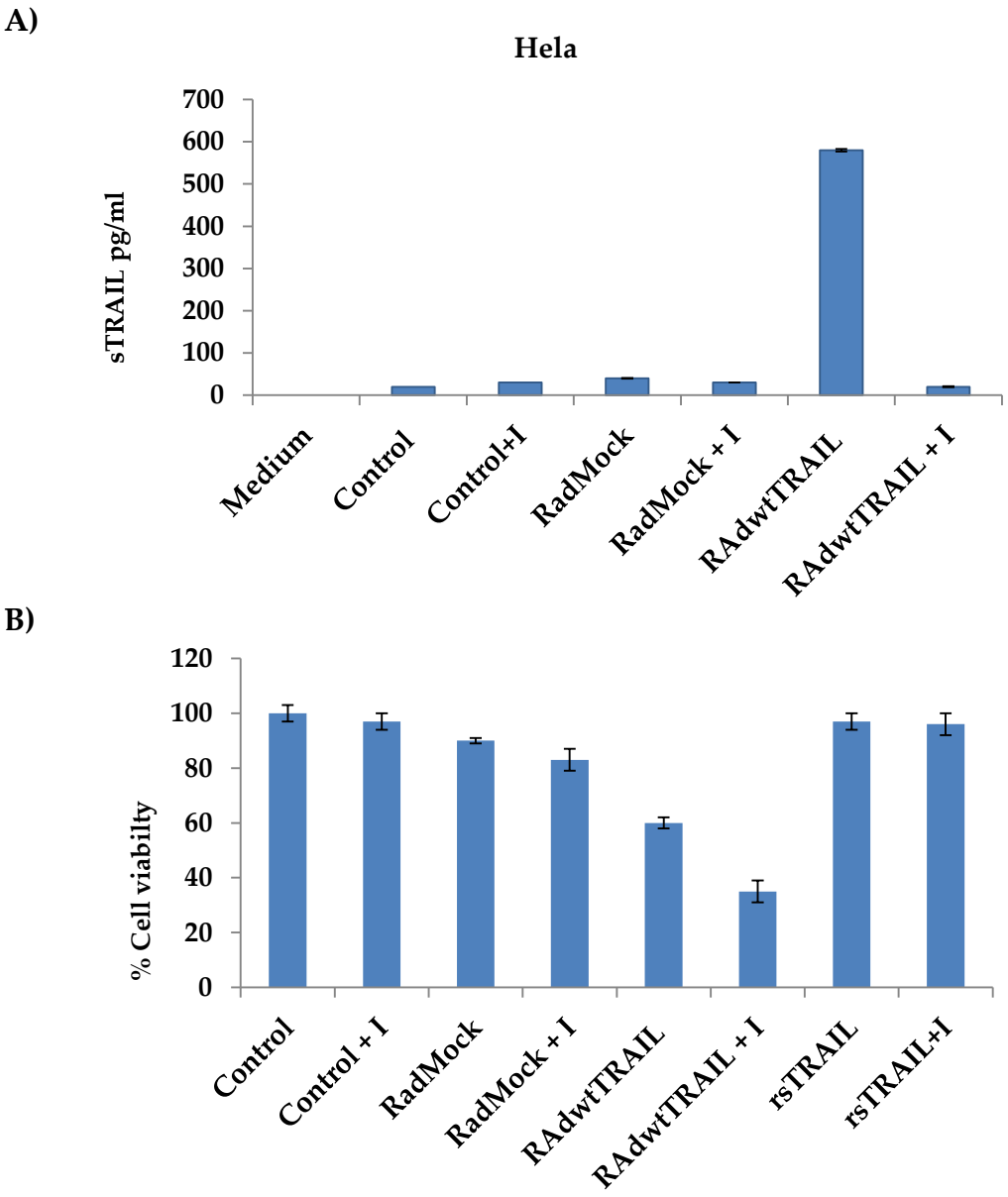


Figure 3.10: Effect of MMPI II on sTRAIL cleavage from RAdwtTRAIL in RAdwtTRAIL-infected Hela cells.

Hela cells infected with 100 MOI of RAdMock, RAdwtTRAIL or left uninfected as a negative control or treated with 100ng/ml of sTRAIL were treated with 30μM of MMPI II or left untreated were grown for 48 hours.
A-Culture media samples were collected from each well except sTRAIL- treated cells for sTRAIL ELISA assessment. Results are mean of triplet samples± SD
B-Cell viability was assessed by WST-1 assay and the results are the mean of triplet samples± SD.

3.2.8 : An attempts to identify the MMPs-targeted cleavage site in wt TRAIL.

The results described in this chapter, have clearly demonstrated that inhibition of sTRAIL cleavage from membrane by the effect of MMPI II enhances the RAdwtTRAIL-induced cell viability reduction in RAdwtTRAIL-infected Hela cells, therefore, highlighting the potential effect of membrane bound TRAIL in inducing cell viability reduction and apoptotic induction in TRAIL receptors-positive carcinomas compared with wt TRAIL.

Previous work from our laboratory has shown that FasL lacking the ^{128}EKQI^{131} cleavage site is more potent in cell death induction. Given that TNF ligands possess a high percentage of sequence homology, a CD40L cleavage site ^{110}SF**EMQK**G^{116} was also identified by sequence alignment between FasL and CD40L (Elmetwali et al., 2010a, 2010b, Knox et al., 2003, Milner et al., 2002). Therefore, a possible alignment between FasL, CD40L and TRAIL may provide some information about the possible TRAIL cleavage site. Therefore, sequence alignment between the published FasL, CD40L, cleavage sites and TRAIL was performed. As shown in figure 3.11, we have identified an ^{105}**EKQQ**^{108} site (bold letters) similar to Fas L cleavage site ^{128}**EKQI**^{131}, so we next aimed to generate a TRAIL mutant that lacks these four amino acids, as well as another TRAIL mutant that lacks the sequence from 105-115aa in case if the EKQQ-deleted TRAIL sequence was still subject to TRAIL cleavage from membrane.

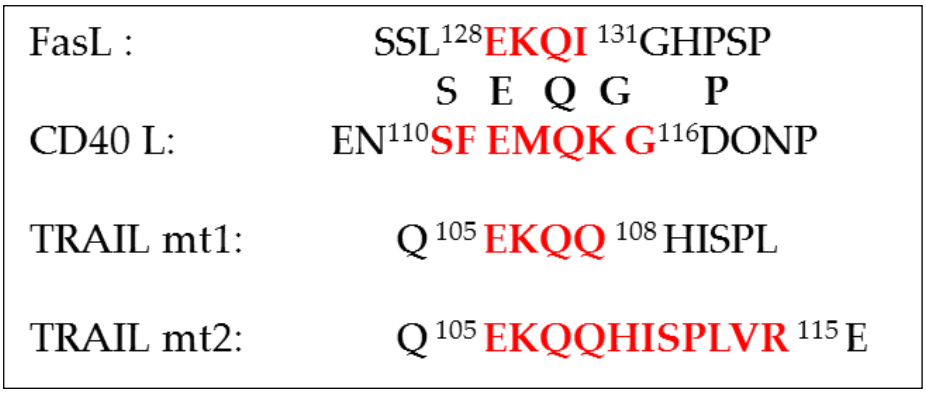


Figure 3. 11: Amino acid alignment of FasL, CD40L and TRAIL.

Sequence alignment identified a possible motif in extracellular domain of TRAIL which is identical to three amino acids in the FasL cleavage site. Deleted sequence in the TRAIL mutant 1 (TRAIL mt1) and mutant 2 (TRAIL mt2) are highlighted in red.

3.2.9 : Generation of pShuttleCMV constructs expressing EKQQ (105-108aa) and EKQQHISPLV (105-115aa)- deleted TRAIL proteins.

To generate two TRAIL mutants (mutant 1; mt1 and mutant 2; mt2) with deletion mutation from 105-108aa and 105-115aa respectively, we utilised the quick change site-directed mutagenesis (SDM) reagent (Stratgene) as described in section (2.1.17). For ^{105}EKQQ^{108} deleted TRAIL sequence, the forward TRAILde105-108F, 5′-GGA AAC CAT TTC TAC AGT TCA AAA TAT TTC TCC CCT AGT G-3′ and the reverse primer TRAILde105-108R, 5′CAC TAG GGG AGA AAT ATT TTG AAC TGT AGA AAT GGT TTC C-3′ were used. For the deletion spanning from ^{105}EKQQHISPLV^{115}, the forward primer TRAILde105-115F, 5′-GAG GAA ACC ATT TCT ACA GTT CAA GAA AGA GGT CCT CAG AGA GTA GCA GC-3′ and the reverse primer TRAILde109-115R, 5′-CTC CTT TGG TAA AGA TGT CAA GTT CTT TCT CCA AGA GTC TCT CAT CGT CG-3′ were used. Obtained PCR reaction was then used to transform XL1-Blue supercompetent cells provided by the SDM producer. The sequence of the TRAIL mutants was then confirmed within the obtained miniprepped DNA clones by restriction digestion with *Sal1* and *Xba1* restriction endonucleases and analysis on low present gel electrophoresis (0.5%). As shown in Figure 3.12, a slight difference was observed with the size of the released TRAIL DNA fragments compared to wild-type TRAIL, as expected from the deletion mutation. To confirm the presence of the deletion mutation, the selected clones

were sequenced and sequence analysis confirmed the presence of the deletion mutations within the majority of the selected clones.

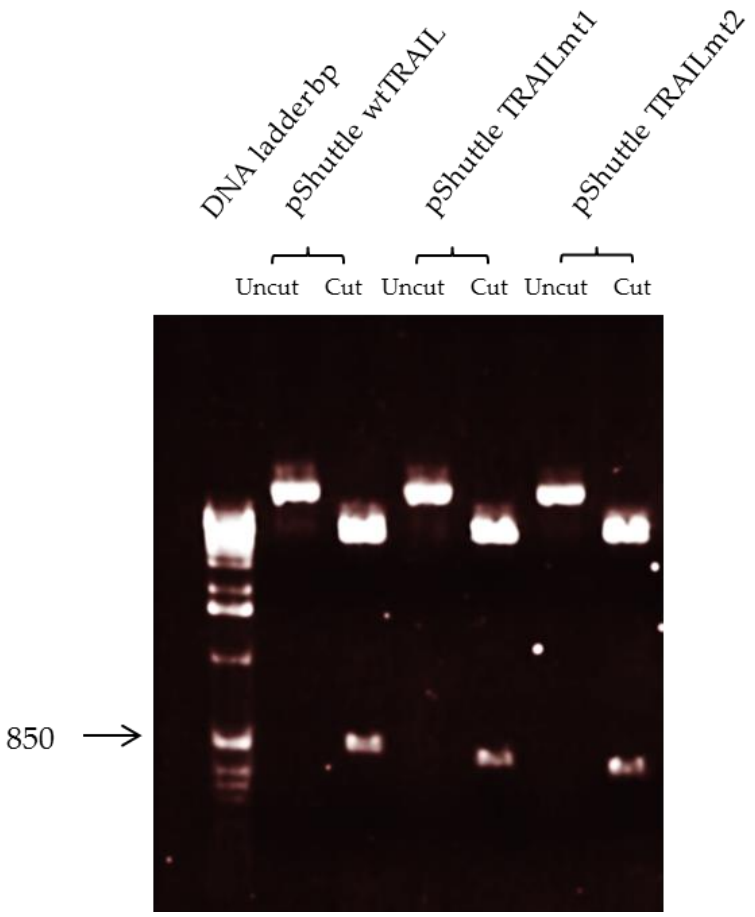


Figure 3.12: *Sal 1* and *Xba1* restriction digestion of the pShuttle -CMV TRAIL constructs.

Half microgram of the selected Miniprepped DNA was digested with *Sal1 and Xba1* restriction endonucleases, the resultant restriction digestion products were resolved on 0.5% agarsoe gel containing 0.2µg/ml ethidium bromide.

3.2.10 : An examination of the cleavage of sTRAIL from the constructed pShuttle TRAIL mutants.

To examine whether the ^{105}EKQQ^{108} or the ^{105} EKQQHISPLV^{115} deletion mutation in the TRAIL mutant 1 and 2 respectively were capable of blocking the cleavage of the TRAIL from the membrane, HEK 293 cells were transfected with 1μg DNA of either pShuttle wtTRAIL or pShuttle TRAILmt1 or pShuttle TRAILmt2 or pShuttle CMV or left untransfected as a negative control. Samples were collected from the culture media 48 hours post-transfection for sTRAIL assessment. As shown in figure 3.13, a significant quantity of sTRAIL was detected within pShuttle wtTRAIL, pShuttle TRAILmt1, and pShuttle TRAILmt2 transfected cells and the quantity of sTRAIL released into the culture medium was similar, indicating that the deletion mutations were not able to block the MMP-mediated cleavage of TRAIL.

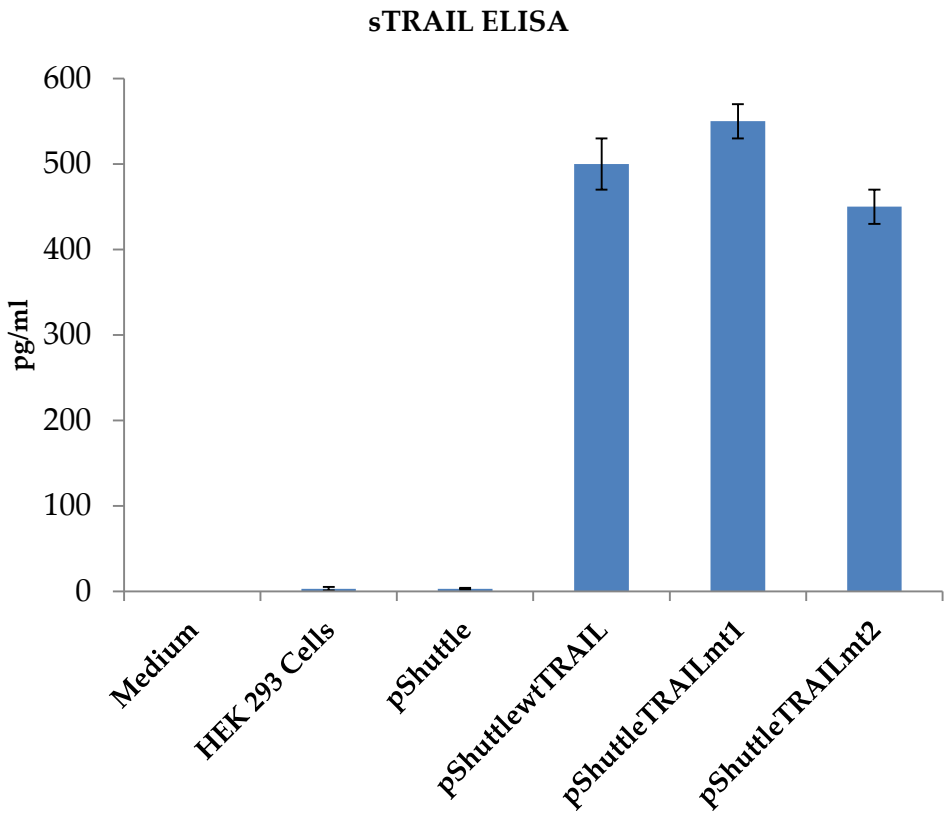


Figure 3.13: An Examination of the TRAIL cleavage from constructed pShuttle CMVTRAIL mutants.

HEK 293 cells were transfected with 1µg DNA pShuttle CMV or pShuttle wtTRAIL or pShuttle TRAILmt1 or pShuttleTRAILmt2 or left non transfected as a negative control. Samples were collected from the culture media 48 hours post-transfection for sTRAIL measurement. Results are mean of triplet samples± SD

3.3: Discussion.

The tumours necrosis factor (TNF) cytokine superfamily plays a crucial role in immune regulation by modulating lymphocyte proliferation and apoptosis (Cancro and Smith, 2003). TNF cytokine superfamily ligands have also been implicated in the induction of tumour cell death induction and apoptosis depending on the mode of ligation. The effect of membrane bound TNF ligand structures compared with wild-type or soluble forms are well characterized in both of CD40 and Fas ligands (Elmetwali et al., 2010, Georgopoulos et al., 2006, Knox et al., 2003, Milner et al., 2002). TNF ligands are naturally expressed as a membrane bound ligands that are subject to cleavage from the membrane by the effect of members of a group of proteases belong to the matrix metalloprotenases (MMPs) family.

To examine whether TRAIL has the same pattern as CD40L and FasL we successfully generated a replication-deficient adenovirus (RAd) expressing wild-type TRAIL (RAdwtTRAIL) protein. The expression of TRAIL protein was confirmed as HEK 293 cells infected with RAdwtTRAIL but not RAdMock or uninfected control cells showed a significant detectable level of TRAIL protein as shown by western blot analysis, confirming the successful generation of RAdwtTRAIL virus. RAdwtTRAIL adenovirus was found to reduce cell viability in TRAIL receptor 1 and 2- positive carcinomas (Hela, OVCAR-3 and HepG2) but not in A2780 cells where the levels of TRAIL-R1 and TRAIL-R2

were confirmed to be very low as shown by western blot analysis have also confirmed that wild-type TRAIL is subject to cleavage from membrane and that such cleavage is mediated by the action of MMPs. Inhibition of MMP activity resulted in enhanced RAdwtTRAIL-induced cell viability reduction and this was mainly attributed to the blocking of the cleavage of the TRAIL from membrane, since RAdMock-infected Hela cells treated with MMPI didn't exhibit any reduction in cell viability compared to MMPI untreated RAdMock-infected cells, thus excluding the possibility of any synergy between the MMPI and the expressed adenoviral proteins. Moreover, MMPI treatment didn't sensitize Hela cells to sTRAIL treatment, suggesting that blocking of the cleavage of TRAIL from membrane by the action of MMPI is the only factor that enhanced the RAdwtTRAIL-induced cell viability reduction in RAdwtTRAIL-infected Hela cells. Given that TNF ligands possess higher sequence homology between each other, it was important to examine whether a similar MMP cleavage site to that exist in FasL and CD40L also exist in TRAIL sequence, sequence alignment between CD40L, FasL and TRAIL has revealed a possible cleavage site in the TRAIL sequence (^{105}EKQQ^{108}), therefore we attempted to generate TRAIL mutants with the identified sequence and with further sequence deletion spanning the ^{105} EKQQHISPLV^{115} motif. However the sequence deletion of ^{105}EKQQ^{108} or the ^{105} EKQQHISPLV^{115} in the TRAIL sequence couldn't block the cleavage of TRAIL from membrane in pShuttle

TRAIL mutant 1 or 2 (mt1 or mt2)-transfected HEK293 cells as sTRAIL was still detected in those cells as confirmed by sTRAIL ELISA assessment. This could be explained by MMPs targeting TRAIL sequences at different or multiple cleavage sites such that more deletion mutations may be needed to block the cleavage from membrane. Since the mutations (^{105}EKQQ^{108} or the ^{105}EKQQHISPLV^{115}) in TRAIL failed to block the cleavage of the TRAIL membrane, other approaches for generating a membrane bound TRAIL were explored.

CHAPTER 4
GENERATION OF REPLICATION-DEFICIENT ADENOVIRUS (RAd) EXPRESSING A MEMBERANE-BOUND CD40LTRAIL FUSION PROTEIN

4.1: Introduction.

Previous results from our lab and others have demonstrated the efficacy of membrane-bound CD40L and FasL in cell death induction and apoptosis compared to soluble or wild-type counterparts. Previous results in chapter 3 have clearly demonstrated the direct effect of MMPI II inhibitor on blocking TRAIL cleavage from membrane in RAdwtTRAIL-infected cells, and that blocking of the cleavage of TRAIL by the MMPI II inhibitor resulted in enhanced RAdwtTRAIL-induced cell viability reduction, suggesting that a membrane-bound TRAIL is more potent in cell death induction than wild-type or soluble TRAIL. Given that both CD40L and TRAIL belong to the same family and they share a significant homology (25-30%) (Pitti et al., 1996), and deletion mutations in the TRAIL sequence identified by sequence alignment between TRAIL sequence and the published FasL and CD40L membrane cleavage sites were unable to block the cleavage of TRAIL from membrane in pShuttle TRAIL mutant 1 and 2 compared to pShuttle TRAIL wt-transfected HEK 293 cells, we thought of generating a hybrid fusion between the CD40L N-terminal domain (1-324bp) upstream of the CD40L cleavage site (324-348bp) and the C-terminal domain of TRAIL ligand (340-846bp, containing the TRAIL active site). This hybrid fusion of CD40LTRAIL is expected to be a membrane bound molecule that resists the MMPs-mediated TRAIL cleavage at the cell surface due to the lack of either CD40L or TRAIL cleavage sites in the CD40LTRAIL hybrid fusion.

Here in this chapter we will describe the construction of CD40LTRAIL hybrid fusion and a replication-deficient adenoviral vector expressing this fusion protein, also we will investigate the direct effect of this virus on cell viability reduction compared to the RAdwtTRAIL virus constructed in chapter 3.

4.2: Results.

4.2.1 : Generation of Adenoviral vector expressing the CD40LTRAIL fusion protein.

4.2.1.1 : Generation of CD40L N-terminal fragment (1-224bp).

To amplify a CD40L fragment (1-324bp) encoding DNA, a pCLneoCD40L construct together with a couple of forward $CD40L_1$Sal1F, 5′-AGTC GAC ATG ATC GAA ACA TAC AAC CA-3′ and reverse primers $CD40L_{324}$R, 5-TTC TTT CTT CGT CTC CTC TTT GTT-3′ were used under the following the PCR condition (93°C 5 mins (93°C 30 Sec, 58.1°C, 45 Sec, 72°C 2 mins) x 25, 72°C, 5 mins). The PCR product was gel purified using a gel extraction purification kit (Quiagen) and the DNA was eluted in 30μL deionized distilled water as described in section 2.1.3. Five microlitres of the purified PCR product was run in 1% gel electrophoresis (Section 2.1.2) as shown in figure 4.1.

Figure 4.1: Analysis of PCR amplified CD40L N-terminal (1-324bp).

Five microlitres of the purified PCR product was run in 1% agarose gel containing 0.2µg/ml EtBr. The amplified fragment runs at an expected size of (324bp). M refer to the DNA ladder, 1 refers to the amplified CD40L fragment (1-324bp).

4.2.1.2 : Generation of the TRAIL C-terminus fragment.

To amplify the C-terminus TRAIL sequence (340-846bp), a pShuttle wtTRAIL construct, was used together with a couple of forward $CD40L_{324}TRAIL_{340}F$, 5'-t ata atg tta aac aaa gag gag acg aag aaa gaa GTG AGA GAA AGA GGT CCT CAG AGA GTA-3 and reverse primer $TRAIL_{846}$ HindIIIR, 5'-A AA GCT TTT AGC CAA CTA AAA AGG CC-3' were used, the $CD40L_{324}TRAIL_{340}F$ primer was designed such that CD40L sequence (290-324bp red color) would be flanked to the amplified TRAIL fragment. The PCR was performed under the following conditions 93°C 5 mins (93°C 30 Sec, 58.4°C, 45 Sec, 72°C 2 mins) x 25, 72°C, 5 mins. The PCR product was gel purified using a gel extraction purification kit (Quiagen) and the DNA was eluted in 30µL deionized distilled water. Five microlitres of the purified PCR product was run in 1% gel electrophoresis as shown in figure 4.2.

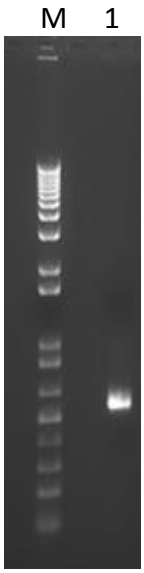


Figure 4.2: Analysis of PCR amplified TRAIL C-terminal (340-846bp).

Five microlitres of the purified PCR product was run in 1% agarose gel containing 0.2µg/ml EtBr. The amplified fragment runs at an expected size of (506bp).

4.2.1.3 : PCR amplification of the CD40LTRAIL fusion.

To combine the above generated CD40L and TRAIL fragments, 100pg of the amplified CD40L and TRAIL fragments were mixed together and used as a template for the following PCR reaction utilizing the forward primer $CD40L_1$Sal1F 5′-AGTC GAC ATG ATC GAA ACA TAC AAC CA-3′ and the reverse primer $TRAIL_{846}$ HindIIIR 5′-A AA GCT TTT AGC CAA CTA AAA AGG CC-3′. As shown in figure 4.3, analysis of the PCR reaction product on 1% gel electrophoresis revealed an amplified fusion of Cd40L-TRAIL at the expected size.

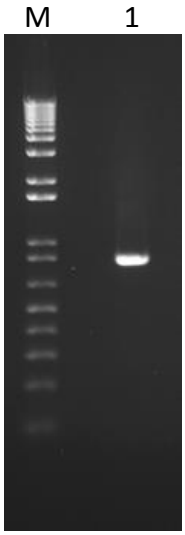


Figure 4.3: Agarose gel electrophoresis of the PCR product of CD40LTRAIL fusion.

Five microlitres of the purified CD40LTRAIL product was run in 1% agarose gel containing 0.2μg/ml EtBr. M refers to the DNA ladder, 1 refers to the amplified CD40LTRAIL fragment which runs at an expected size of the TRAIL of 831bp.

4.2.1.4 : Cloning of CD40LTRAIL into pShuttle CMV vector.

To clone the amplified PCR CD40LTRAIL fusion product into the pShuttle CMV vector, CD40LTRAIL fusion was first cloned into the Topo2.1 vector. The amplified PCR product was excised from the gel and gel purified as described in section 2.1.3 then subjected to addition of A-overhangs (Section 2.1.4). To clone the A-overhanged PCR product into the Topo2.1 vector, a TA cloning kit (Invitrogen) was utilized as previously described in section 2.1.6. Miniprepped DNA was then digested with *SalI* and *HindIII* enzymes to release the CD40LTRAIL fusion. The purified DNA fragment was then ligated into *SalI*/*HindIII* predigested and purified pShuttle CMV vector. The sequence of CD40LTRAIL fusion was then confirmed by sequence analysis and restriction digestions as in figure 4.4.

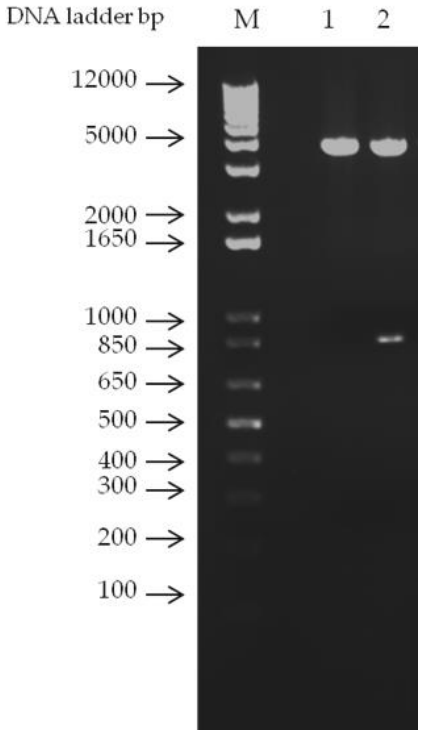


Figure 4.4: Analysis of the pShuttle CMV CD40LTRAIL minipreped DNA.

Predigested pShuttle CMVCD40LTRAIL with *SalI and HindIII m*iniprepped were analysed using 1% agrose gel electrophoresis. M refers to DNA ladder. Lane 1 refers to empty vector, where lane 2 refers to pShuttle-CMVCD40LTRAIL construct.

4.2.2 : Generation of recombinant adenoviral vector expressing the CD40LTRAIL fusion.

Using 1.0μg of *Pme*I, pre-digested and phenol/chloroform purified pShuttle CMVCD40LTRAIL mixed with 0.1μg of pAdEasy-1 in a total volume of 6μl, the DNA mixture was used to transform 20μl of electrocompetent *E. coli* BJ5183 cells by electroporation. Positive clones were first screened by analyzing their supercoiled sizes on agarose gel, comparing them to pAdEasy-1 control. Those clones that had inserts were further examined by *Pac1* restriction endonuclease digestion (Figure 4.5). Once confirmed, supercoiled plasmid DNA was transformed into TOPO 10F chemically competent cells for large-scale DNA amplification. Purified plasmid DNA was digested with *PacI* and used to transfect HEK 293. Transfected HEK 293 cells allowed the packaging and production of adenovirus as previously described in section 3.2.1.4. The packaged adenovirus was designated as RAdCD40LTRAIL virus.

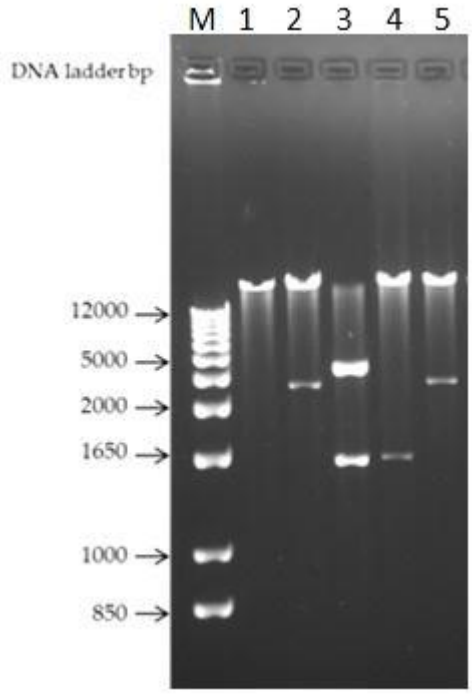


Figure 4.5: Restriction digestion of RAdCD40TRAIL recombinant minipreped DNA.

Miniprepped RAdCD40TRAIL recombinant DNA was digested with *Pac I* and analysed using 1% agarose gel electrophoresis. M refers to DNA ladder. Lane 1 refers to empty pAdEasy vector, Lane 2 and 5 refer to the right clones with successful integration of the pShuttle CMV CD40LTRAIL into the adenoviral pAdEasy vector backbone, and lane 3 refers to pShuttle CMV CD40LTRAIL construct, where lane 4 refers to RAdCD40TRAIL clone where the homologues recombination occurred in wrong orientation.

4.2.3 : Verification of the expression of CD40LTRAIL fusion from RAdCD40LTRAIL virus.

To examine whether the generated RAdCD40LTRAIL virus express the CD40LTRAIL fusion, bladder carcinoma cells EJ were infected with either RAdwtTRAIL or RAdCD40LTRAIL or RAdMock at 100 MOI or left un-infected as a negative control. Cells were incubated for 36 hours then lysed *in situ* and 60μg of total protein lysates were examined by western blotting analysis for TRAIL protein expression. As demonstrated in figure 4.6, TRAIL expression from RAdwtTRAIL and RAdCD40LTRAIL-infected cells but not from RAdMock or uninfected control cells was detected. To ensure that equal amounts of total cell lysates were loaded, β-actin was examined as well using monoclonal mouse anti-β-actin antibody. As seen in figure 4.6, β-actin expression was equal between samples.

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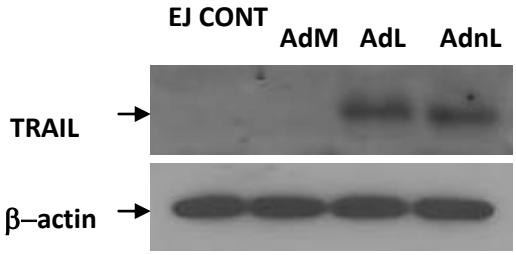

Figure 4.6: Verification the expression of the wild-type TRAIL and CD40L-TRAIL chimera from the generated viruses.

EJ cells infected with 100 MOI of RAd wtTRAIL (AdL) or RAdCD40LTRAIL (AdncL) RAdMock (AdM) or left uninfected as a negative control were grown for 36h, cells were lysed *in situ* and 60μg of total protein lysates were examined for TRAIL expression using specific antibody raised against TRAIL C-terminus domain. β-actin was also examined as a loading control.

4.2.4 : Verification that CD40LTRAIL is not cleaved from cell surface into soluble TRAIL in RAdCD40LTRAIL-infected EJ cells

To examine whether CD40LTRAIL fusion protein expressed from RAdCD40LTRAIL is not cleaved from the cell membrane, bladder carcinoma cells EJ were infected with 100 MOI of either RAdCD40LTRAIL or RAdwtTRAIL or RAdMock or left uninfected as a negative control. Samples were collected from the culture media 24 and 48 hours post-infection for sTRAIL measurement utilising a sTRAIL ELISA assay kit. As shown in figure 4.7, a significant quantity of sTRAIL was detected within infected cells in a time dependant manner. However, no sTRAIL was detected in culture media collected from either RAdMock or the RAdCD40LTRAIL-infected cells indicating that CD40LTRAIL fusion is maintained at the cell surface.

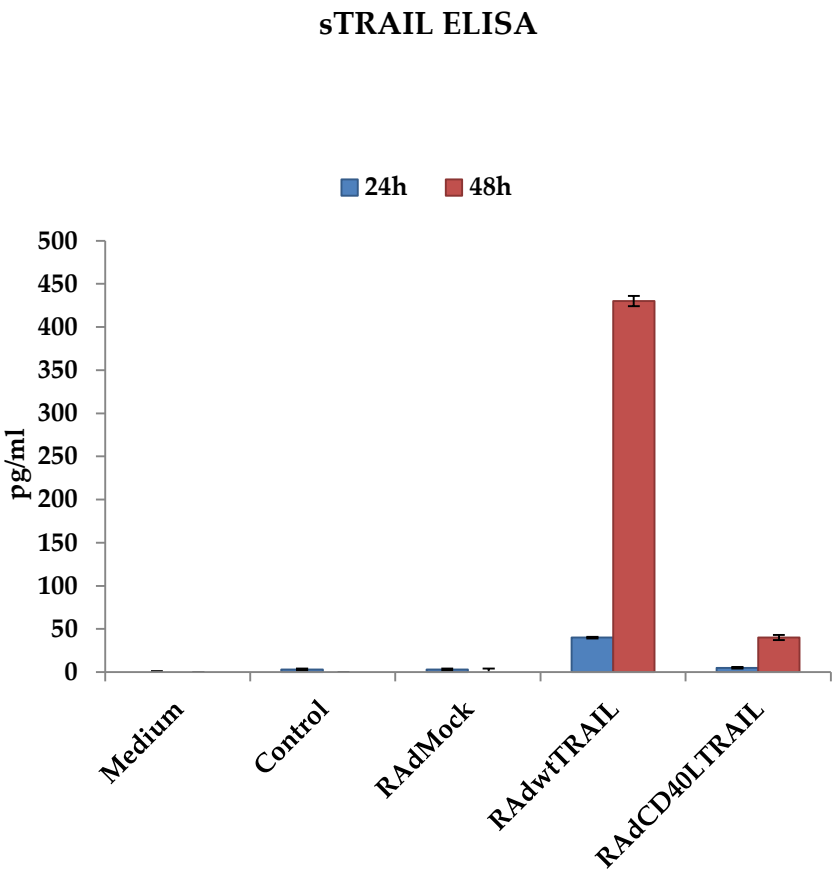

Figure 4.7: Confirmation of the retention of TRAIL expression by RAdCD40LTRAIL at the cell surface.

EJ cells were infected with 100 MOI of either RAdCD40LTRAIL or RAdwtTRAIL or RAdMock or left uninfected as a negative control. Samples were collected from the culture media 24 and 48 hours post-infection for sTRAIL measurement. Results are mean of triplet samples ±SD.

4.3: Discussion.

Results reported in Chapter 3and 4 investigating blocking the cleavage of TRAIL from cell surface in RAdwtTRAIL-infected cells using the MMPI II inhibitor clearly demonstrated the effect of membrane-bound TRAIL in cell death induction and apoptosis in TRAIL receptor 1 and 2 positive cells.

To generate a TRAIL mutant resistant to cleavage from the membrane, several attempts to identify the TRAIL cleavage site based on amino acid sequence alignment with the published FasL and CD40L cleavage site were performed, using the mutagenesis technique we have generated different mutants of TRAIL, however soluble TRAIL (sTRAIL) was always detected in culture media samples collected from cells transfected with these mutants. Therefore, we considered other approaches to generate a membrane-bound TRAIL mutant that can resist the cleavage from the cell surface whilst at the same time maintaining all the biological activity of TRAIL protein. Given that both CD40L and TRAIL belong to the same family and they share a significant homology (25-30%), raised the idea of making a hybrid fusion between CD40L and TRAIL. Therefore, a CD40L N-terminal region lacking the cleavage site (1-324bp) would possibly drive the expression of the active TRAIL ligand fragment (340-846bp, containing the TRAIL active site) at the cell membrane. To test this hypothesis, the CD40LTRAIL fusion was generated by polymerase chain reaction (PCR) technique, and the generated fusion sequence was successfully cloned into the

pShuttle vector in frame with the CMV promoter. Following *Sal1* and *HindIII* restriction and sequencing analysis confirmation of the constructed pShuttle-CMV CD40LTRAIL, we constructed an adenovirus vector that expressed the CD40LTRAIL fusion. The expression of the CD40LTRAIL fusion from the RAdCD40LTRAIL virus was then confirmed by western blotting analysis in bladder carcinoma EJ cells. Furthermore, CD40LTRAIL protein expressed from RAdCD40LTRAIL is maintained at the cell surface and is resistant to the cleavage from membrane by the action of the MMPs since RAdCD40LTRAIL-infected cells failed to release any soluble TRAIL into the culture medium compared with RAd expressing wild-type TRAIL. RAdCD40LTRAIL was also more potent in cell viability reduction than either RAdwtTRAIL or the sTRAIL as RAdCD40LTRAIL exhibited more cell viability reduction compared to other forms of TRAIL.

CHAPTER 5
AN EXAMINATION OF THE DIRECT EFFECT OF RADCD40LTRAIL ALONE OR IN COMBINATION WITH CHEMOTHERAPUITIC DRUGS ON CELL VIABILITY

5.1: Introduction.

Results in chapter 3 and 4 have clearly demonstrated the direct effect of MMPI II inhibitor on blocking TRAIL cleavage from membrane in RAdwtTRAIL-infected cells, and that blocking of the cleavage of TRAIL by the MMPI II inhibitor resulted in enhanced RAdwtTRAIL-induced cell viability reduction, suggesting that a membrane-bound TRAIL is more potent in cell death induction than wild-type or soluble TRAIL. Following successful generation of RAdCD40LTRAIL virus (chapter 4) and the confirmation of retention of the fusion protein at the cell surface, here in this chapter we thought of investigating the direct effect of RAdwtTRAIL and RAdCD40LTRAIL either alone or in combination with cytotoxic drugs on TRAIL receptors positive carcinomas.

5.2 : Results.

5.2.1 : Effect of RAdCD40LTRAIL on TRAIL receptor-positive carcinomas compared to wild-type TRAIL ligands.

To examine the direct effect of membrane-bound TRAIL on carcinomas cell viability, a panel of TRAILR1 and 2-positive carcinomas including Hela, HepG2, A2780, OVCAR-3 and EJ cells were infected with either RAdCD40LTRAIL, RAdwtTRAIL, RAdMock or left uninfected as a negative

control at the indicated of MOI. As shown in figure 5.1, different cell lines exhibited different degrees of sensitivity to the CD40LTRAIL fusion compared to the wild-type TRAIL with the greatest sensitivity to the CD40LTRAIL fusion observed in HepG2, Hela and OVCAR-3 cells, where lower sensitivity was noticed with the bladder carcinoma EJ, and the ovarian carcinoma A2780 cells. Obtained results showed that RAdCD40LTRAIL induced greater cell viability reduction compared to RAdwtTRAIL or RAdMock-infected cells where no cell viability reduction were detected as shown in figure 5.1.

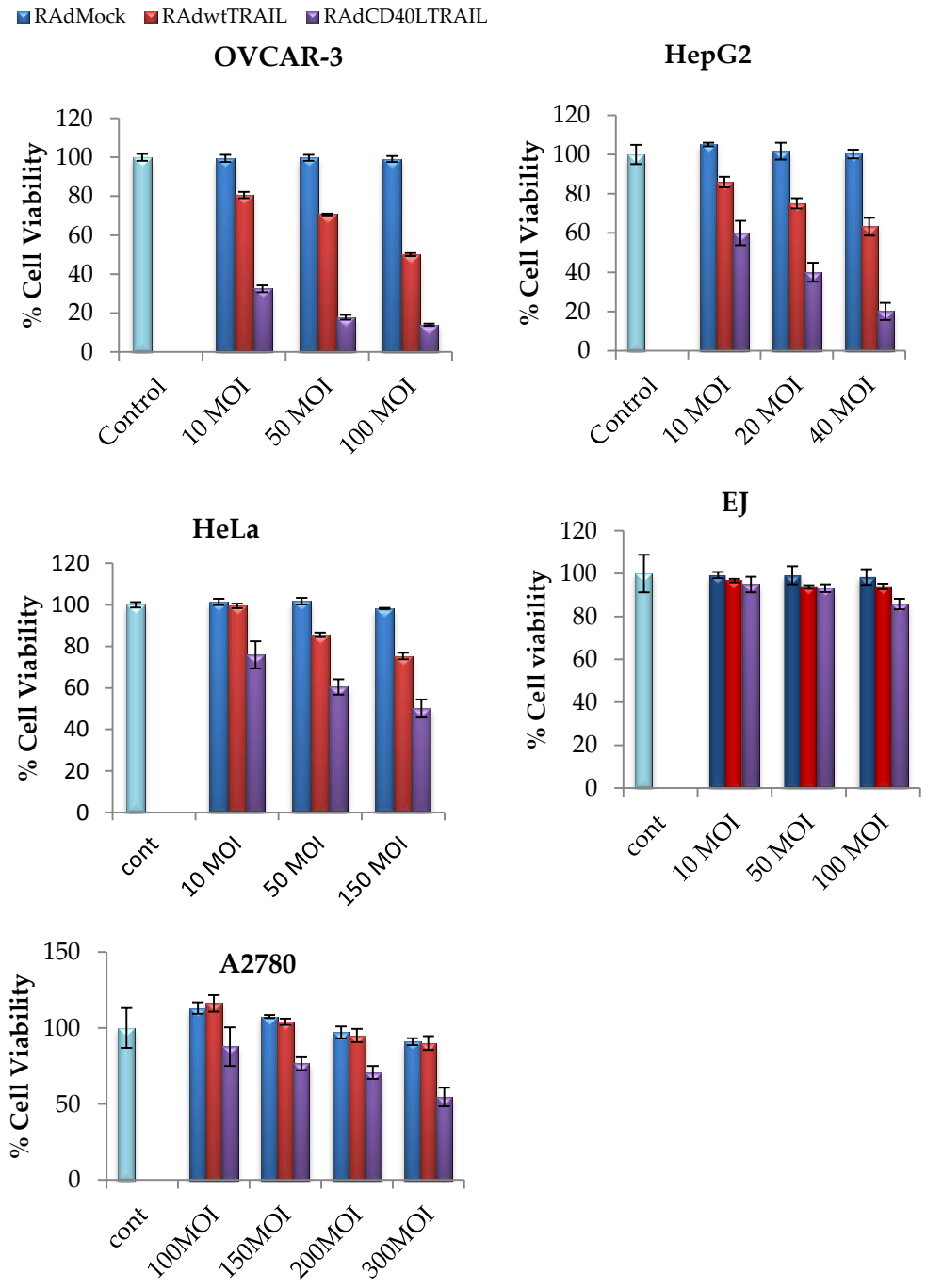

Figure 5.1: Effect of RAdwtTRAIL and RAdCD40LTRAIL on Cell Viability.

Hepatocelluler carcinoma HepG2 cell line, Cervical cell carcinoma Hela, ovarian carcinoma cell lines A2780 and OVCAR-3 and the bladder carcinoma EJ cells were infected with the indicated MOI of RAdwtTRAIL or RAdCD40LTRAIL or RAdMock or left without infection for 48 hours. Cell viability was assessed by WST-1ssay. Results represent the mean of triplet samples ±SD.

5.2.2 : RAdCD40LTRAIL-induced cell viability reduction is attributed to apoptosis induction.

To examine whether RAdCD40LTRAIL-induced cell viability reduction is attributed to apoptosis induction, Hela cells were infected with RAdwtTRAIL or RAdCD40LTRAIL or RAdMock at 100 MOI or let uninfected as negative control or treated with 100ng/ml sTRAIL, cells were either treated with the pan-caspases inhibitor zVAD (Calbiochem) at a concentration of 30μM or left untreated as a negative control and seeded at a density of 6000 cells/100μl/well in 96 well microplate for 28 hours then assessed for caspase 3/7 activity as a marker for apoptotic cell induction, utilizing the Caspase-Glo 3/7 assay reagent (Promega Cat, G8091) according the manufacturer's instructions. As shown in figure 5.2, RAdCD40LTRAIL-infected Hela Cells exhibited higher caspase 3/7 activity compared to RAdwtTRAIL-infected cells, where no activity was detected in either RAdMock or sTRAIL-treated or untreated control cells, indicating that RAdCD40TRAIL-induced cell viability reduction is mainly due to an apoptotic cell induction. Furthermore, the pan-caspase inhibitor zVAD treatment resulted in abolishing the RAdCD40LTRAIL-induced caspase 3/7 activity highlighting the specificity of the used assay.

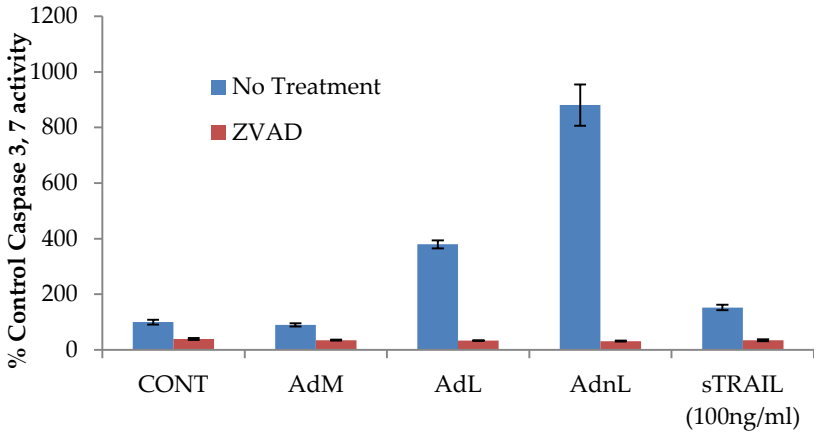

Figure 5.2: Caspase 3/7 activity in RAdCD40LTRAIL-infected Hela cells.

The cervical carcinoma cell line Hela cells were either infected with RAdMock (AdM), RAdwtTRAIL (AdL) or RAdCD40LTRAIL (AdnL) 1t 100 MOI or left uninfected or treated with recombinant soluble TRAIL at 100ng/ml (sTRAIL), were treated with or without the pan-caspase inhibitor zVAD at a concentration of 30μM and seeded at a density of 6000 cells/100μl/well in 96 well microplate for 28 hours. Caspase 3/7 activity was then assessed utilizing the Caspase-Glo 3/7 Assay reagent according to the manufacturer's instructions (Promega, cat G8091). Results represents mean of triplet samples ± SD.

5.2.3 : An *in vitro* examination of synergy between RAd expressing either wild-type TRAIL or CD40LTRAIL fusion proteins.

Results in this chapter and chapter 3 and 4 have demonstrated the direct inhibitory effect of RAd vectors expressing either wild-type TRAIL or CD40LTRAIL fusion proteins on cell viability in TRAIL receptor 1 and 2-positive carcinomas. Given that chemotherapeutic drugs can act in synergy with other members of the TNF family ligands, the possibility of enhancing the TRAIL-mediated growth inhibition by the use of various cytotoxic drugs in combination with RAdwtTRAIL or RAdCD40LTRAIL encouraged us to examine whether RAd-delivered TRAIL would positively interact with these drugs.

5.2.4 : Drug interaction with RAd expressing wtTRAIL or CD40LTRAIL fusion proteins in HepG2 cells

To determine the appropriate concentrations of drugs to be used in examination of the synergy between these drugs and RAd vectors expressing wtTRAIL or CD40LTRAIL fusion, HepG2 cells were cultured with increasing concentrations of different cytotoxic drugs [Cisplatin (CIS), Doxorubicin (DOX), 5 Fluorouracil (5FU), Sorafenib (Sor) and Paclitaxel (Pac); selected on the basis of their differing intracellular targets] and then plated out at a density of 10 $x10^3$ cells/well in 96-well plates. Forty-eight hours later, cell viability was evaluated

by WST-1 assay. A concentration of 10M of CIS, 0.5μM of DOX, 50μM of 5FU, 0.5μM Sor, and 1μM of Pac were sufficient to induce approximately 50-70% cell viability in comparison with the untreated controls.

To examine the interaction between TRAIL-expressing adenoviruses and the cytotoxic drugs at the pre-estimated working concentrations, HepG2 cells were infected with 20 MOI of RAdwtTRAIL, RAdCD40LTRAIL, RAdMock or left without infection as a negative control or treated with sTRAIL at a concentration of 100ng/ml. Cells were then plated out at a density of $10x10^3$ cells/well in 96-well plates. Twenty-four hours post-infection cells were either cultured with the different drugs or left without treatment for a further 24 hours. Cell viability was assessed by WST-1 assay. Results showed that RAd expressing CD40LTRAIL but not RAdwtTRAIL does interact significantly with Sorafenib and to a limited extent with 5FU, and DOX. However, no interaction with Pac was detected (Figure 5.3). However, no positive interaction between sTRAIL and drugs was observed.

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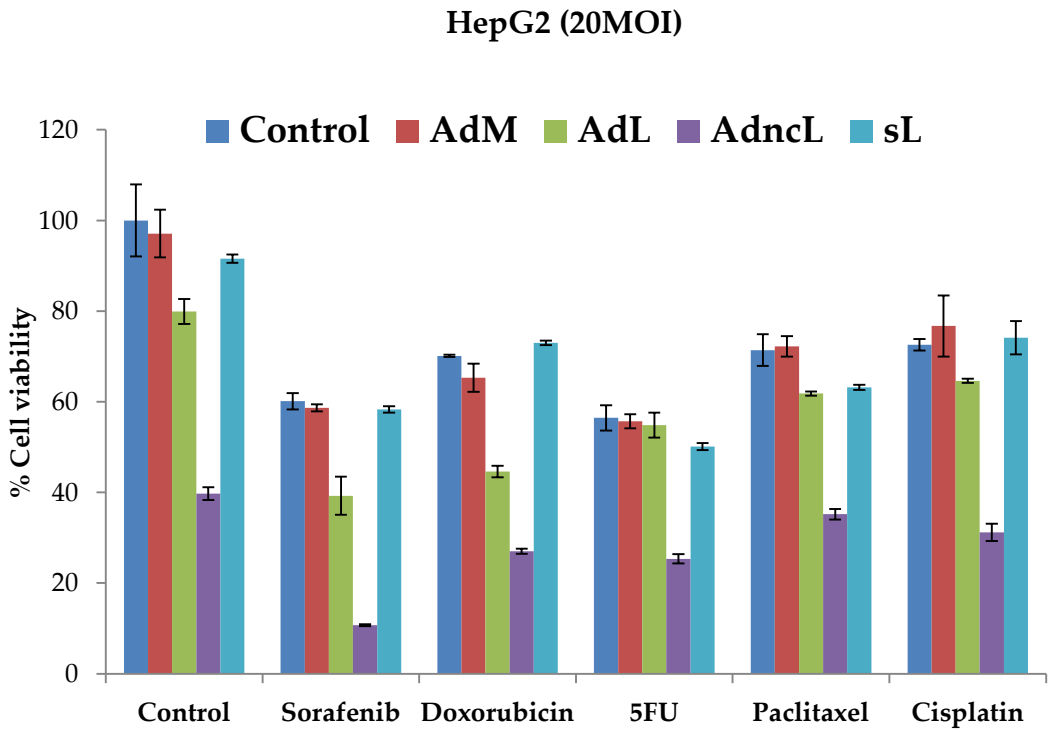

Figure 5.3: Drug interaction with TRAIL in HepG2 cells.

HepG2 cells were infected with 20 MOI of RAdwtTRAIL (AdL), RAdCD40LTRAIL (AdncL), RAdMock (AdM) or left without infection as a negative control or treated with 100ng/ml of sTRAIL for 24 hour then either cultured with 10M of Cisplatin, 0.5µM of Doxrubicin, 50µM of 5FU, 0.5µM Sorafenib, and 1µM of Paclitaxel or left without treatment for another 24 hours. Cell viability assay was then assessed using the WST-1 assay. Results are mean of triplet samples ±SD.

5.2.5 : Drug interaction with RAd expressing wtTRAIL or CD40LTRAIL fusion proteins in OVCAR-3 cells

To determine the appropriate concentrations of drugs to be used to study the interaction with TRAIL-expressing adenoviruses, OVCAR-3 cells were cultured with increasing concentrations of different cytotoxic drugs [Cisplatin (CIS), Doxorubicin (DOX), 5 Fluorouracil (5FU), Sorafenib (Sor) and Paclitaxel (Pac) then plated out at a density of 15 $x10^3$ cells/well in 96-well plates for estimating the dose response. Forty-eight hours later, cell viability was evaluated by WST-1 assay. Results obtained showed that a concentration of 10M of CIS, 0.5μM of DOX, 50μM of 5FU, 0.5μM Sor, and 1μM of Pac induced approximately 60-70% cell viability in comparison with the untreated controls. Therefore, OVCAR-3 cells were infected with 10 MOI of RAdwtTRAIL, RAdCD40LTRAIL, RAdMock or left uninfected as a control or treated with 100ng/ml sTRAIL. Twenty-four hours post-infection cells were either treated with the drugs at the above mentioned concentrations or left without treatment for a further 24 hours. Cell viability was assessed by WST-1 assay. As shown in figure 5.4, RAd expressing wtTRAIL or CD40LTRAIL fusion sensitized OVCAR-3 cells to Sorafenib with greater sensitivity observed in RAdCD40LTRAIL-infected cells, also some positive interaction between RAd expressing either wtTRAIL or CD40lTRAIL were noticed with 5FU, where lower degree of sensitivity were detected as well with sTRAIL-treated cells.

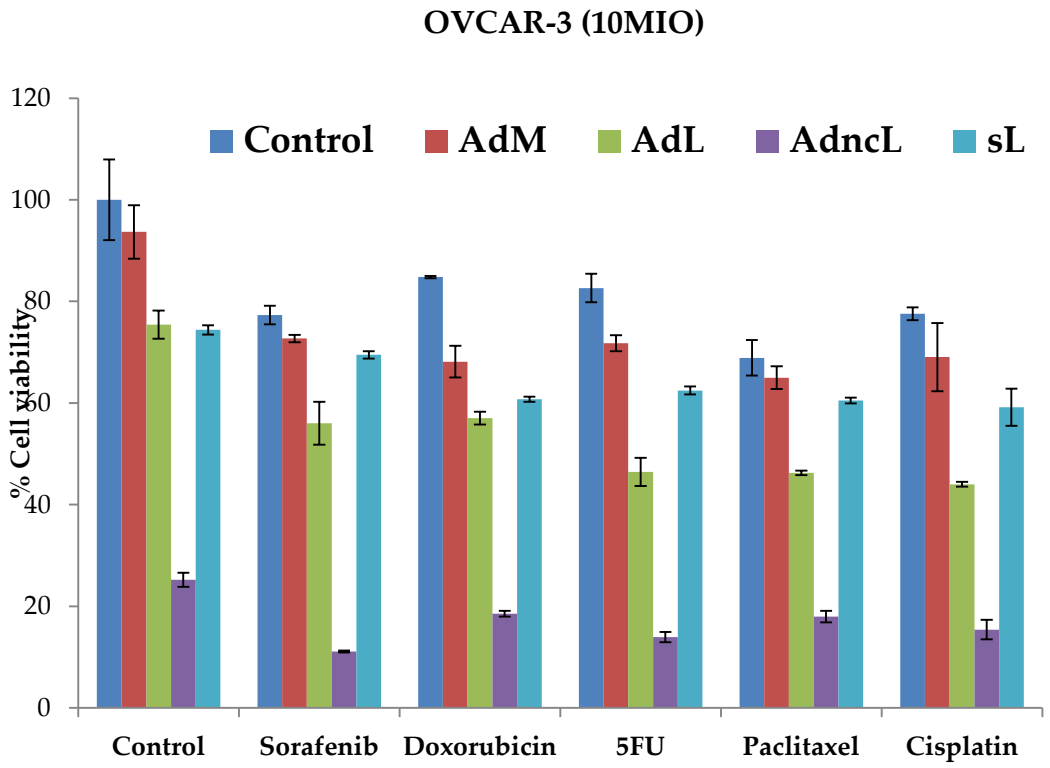

Figure 5.4: Drug interaction with TRAIL in OVCAR-3 cells.

OVCAR-3 cells were infected with 10 MOI of RAdwtTRAIL (AdL), RAdCD40LTRAIL (AdncL), RAdMock (AdM) or left without infection as a negative control or treated with 100ng/ml of sTRAIL for 24 hour then either cultured with 10M of Cisplatin, 0.5µM of Doxrubicin, 50µM of 5FU, 0.5µM Sorafenib, and 1µM of Paclitaxel or left without treatment for another 24 hours. Cell viability assay was then assessed using the WST-1 assay. Results are mean of triplet samples ±SD.

5.3: Discussion.

Following successful generation of RAdCD40LTRAIL virus and confirmation of the retention of the CD40LTRAIL fusion at the cell surface, we tested the

direct effect or RAdCD40LTRAIL on cell viability compared to RAdwtTRAIL or RAdMock viruses. Giving that blocking of the cleavage of TRAIL from cell surface in RAdwtTRAIL-infected cells using the MMPI II inhibitor clearly demonstrated the potential effect of membrane-bound TRAIL in cell death induction and apoptosis in TRAIL receptors positive cells. Indeed RAdCD40LTRAIL was also more potent in cell viability reduction than either RAdwtTRAIL or the sTRAIL as RAdCD40LTRAIL exhibited more cell viability reduction compared to other forms of TRAIL.

RAdwtTRAIL or RAdCD40LTRAIL-induced cell viability reduction could be attributed to apoptotic cell death induction as evidenced with higher caspases 3/7 activity. In line with the observed levels of cell viability reduction induced either by RAdwtTRAIL or RAdCD40LTRAIL, higher levels of caspases 3/7 activity was detected in RAdCD40LTRAIL-infected cells compared to RAdwtTRAIL-infected cells, where no caspases 3/7 activity was detected in uninfected or sTRAIL-treated control cells.

The finding that sorafenib sensitizes TRAIL receptors-positive carcinomas to RAd expressing either wild type TRAIL or non-cleavable CD40LTRAIL fusion than other drugs used in this study might be related to the ability of Sorafenib to down-regulate FLIP protein expression. Sorafenib has been reported to enhance TRAIL-induced cell death via down-regulation of FLIP protein (Liobet et al., 2010), However, there are several other possible explanations. For

example, there may be an additive growth inhibitory effect of Sorafenib due to its ability to induce caspases-independent cell death (Katz et al., 2009). Alternatively, drugs may influence the downstream signalling pathways that mediate TRAIL-induced cell death.

In order to fully elucidate the interactions between TRAIL and conventional chemotherapy further studies are required.

CHAPTER 6
SUMMARY AND FUTURE WORK

6.1: Summary and future work.

The results presented in this thesis describe the generation of replication-deficient adenoviruses expressing either wild-type TRAIL or a membrane bound CD40LTRAIL fusion. The expression of TRAIL proteins have been confirmed by western blotting analysis. We hypothesized that RAd-delivered TRAIL provides more sustained stimulus to the TRAIL receptors by the virtue of the expression at the cell membrane. Furthermore, the ability of RAd wtTRAIL to induce cell death in TRAIL receptor 1 and 2 positive carcinoma was found to be enhanced by inhibition of matrix metalloproteinases (MMP) activity, a further indication of that membrane–bound TRAIL is more potent in inducing cell death than the wtTRAIL and sTRAIL. Therefore an attempt to identify the TRAIL cleavage site was made, given that TRAIL possesses high sequence homology between FasL and CD40L, sequence alignment between CD40L, FasL and TRAIL identified a possible cleavage site in the TRAIL sequence ^{105}EKQQ^{108}. Therefore we generated a couple of TRAIL mutants with the identified sequence and with further sequence deletion spanning the ^{105}EKQQHISPLV^{115} motif. However these deletions failed to block the cleavage of TRAIL from membrane in pShuttle TRAIL mutant 1 or 2 (mt1 or mt2)-transfected HEK293 cells and sTRAIL was still detected in those cells as confirmed by sTRAIL ELISA assessment. This could be as a result of not

targeting the right cleavage site or that MMP cleaves TRAIL at multiple cleavage sites.

The fact that both CD40L and TRAIL belong to the same family and share a significant homology (25-30%) and that we have successfully mutated the CD40L cleavage site stimulated the idea of making a hybrid fusion between CD40L and TRAIL. Therefore, we generated an adenovirus vector that expresses the CD40LTRAIL fusion. The expression of the CD40LTRAIL fusion from the RAdCD40LTRAIL virus was confirmed by western blotting analysis. The results showed that CD40LTRAIL protein expressed from RAdCD40LTRAIL is not susceptible to cleavage from membrane by the action of the MMPs since RAdCD40LTRAIL-infected cells failed to release any soluble TRAIL into the culture medium compared with RAd expressing wild-type TRAIL. Furthermore the RAdCD40LTRAIL was more potent in reducing cell viability than either RAdwtTRAIL or the sTRAIL.

We further observed that RAdwtTRAIL or RAdCD40LTRAIL-induced cell viability reduction is due to apoptosis as evidenced by higher caspases 3/7 activity.

The adenovirus vectors used to deliver TRAIL in these studies have been rendered replication deficient by virtue of deletion of E1 and E3 viral genes. It is increasingly recognised that such vectors provide inefficient gene delivery in patients and that this may be enhanced through the use of selectively

replicating adenoviruses, which are capable of replication in tumour cells but not normal cells in order to amplify transgene expression and, further, to contribute viral oncolysis. Thus, the generation of a replication-competent adenovirus expressing the membrane bound CD40LTRAIL fusion should most likely have significantly greater cytotoxic effects than those obtained with replication-deficient CD40LTRAIL expressing adenovirus through greater CD40LTRAIL expression as well as viral oncolysis. This would also result in membrane bound CD40LTRAIL being expressed within the tumour cell environment enabling an autocrine/paracrine 'bystander' activation of TRAIL receptors. A further advantage of such a vector is that the conditional viral replication provides an additional level of tumour specificity.

Previous studies have shown that phosphorylated JNK and P38 are downstream pathways that contribute to the TRAIL-induced cell death in TRAIL-receptor positive carcinomas, dissecting these pathways may reveal the importance of usage of the specific inhibitors that can block these pathways to enhance RAdCD40LTRAIL-induced cell death, highlighting the importance of such strategy in the treatment of cancer in the future that may allow selection of combination of small molecule inhibitors with sTRAIL or anti TRAIL mAb that can induce same signals as membrane bound TRAIL without problems of gene therapy.

In this thesis, we also report that sorafenib sensitizes TRAIL receptors-positive carcinomas to RAd expressing either wild type TRAIL or non-cleavable CD40LTRAIL fusion to a greater extent than other chemotherapeutics drugs used in this study. This sensitization could be due to the ability of Sorafenib to down-regulate FLIP protein expression (Liobet et al., 2010). In order to fully elucidate the interactions between TRAIL and conventional chemotherapy further studies are required. The time between treatment of chemotherapy and TRAIL are important. Start the treatment with TRAIL then chemotherapy could have more potent effect in cell death. Again, it is important in our future studies to examine the effects of this combination on the expression of death receptor, pro-apoptotic and pro-survival protein expression. The effects of TRAIL and chemotherapy on the ability of stimulate anticancer immune responses and possible anti-angiogenic effect requisite further studies.

The advantage of patients using adenovirus gene therapy is sustained expression of membrane bound TRAIL that are potent in cell death induction.

On the basis of the sound data presented in this thesis, we propose adenovirus delivered CD40LTRAIL fusion to be highly interesting for further study as a novel anti-cancer agent.

The challenges of using adenovirus vectors are highly immunogenic and activate both innate and adaptive immune response; the non-specific immune responses have an inhibitory effect on gene expression. In addition, if the

adenovirus cannot replicate in tumour cells it need inject repeatedly with the same recombinant vectors. However, re-injection of the same vectors may not be effective because induction of host immune response. On the basis of these challenges further studies on recombinant adenovirus virus used for gene therapy are required.

References

AAS, T., BORRESEN, A. L., GEISLER, S., SMITH-SORENSEN, B., JOHNSEN, H., VARHAUG, J. E., AKSLEN, L. A. & LONNING, P. E. 1996. Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nat Med,* 2**,** 811-4.

ALLAVENA, P., SICA, A., SOLINAS, G., PORTA, C. & MANTOVANI, A. 2008. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol Hematol,* 66**,** 1-9.

ALMASAN, A. & ASHKENAZI, A. 2003. Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. *Cytokine Growth Factor Rev,* 14**,** 337-48.

ALNEMRI, E. S., LIVINGSTON, D. J., NICHOLSON, D. W., SALVESEN, G., THORNBERRY, N. A., WONG, W. W. & YUAN, J. 1996. Human ICE/CED-3 protease nomenclature. *Cell,* 87**,** 171.

ARMEANU, S., LAUER, U. M., SMIRNOW, I., SCHENK, M., WEISS, T. S., GREGOR, M. & BITZER, M. 2003. Adenoviral gene transfer of tumor necrosis factor-related apoptosis-inducing ligand overcomes an impaired response of hepatoma cells but causes severe apoptosis in primary human hepatocytes. *Cancer Res,* 63**,** 2369-72.

ASHKENAZI, A. 2002. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer,* 2**,** 420-30.

ASHKENAZI, A., HOLLAND, P. & ECKHARDT, S. G. 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, R. C., FONG, S., LEUNG, S., LAWRENCE, D. A., MARSTERS, S. A., BLACKIE, C., CHANG, L., MCMURTREY, A. E., HEBERT, A., DEFORGE, L., KOUMENIS, I. L., LEWIS, D., HARRIS, L., BUSSIERE, J., KOEPPEN, H., SHAHROKH, Z. & SCHWALL, R. H. 1999. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest,* 104**,** 155-62.

BANNER, D. W., D'ARCY, A., JANES, W., GENTZ, R., SCHOENFELD, H. J., BROGER, C., LOETSCHER, H. & LESSLAUER, W. 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell,* 73**,** 431-45.

BARKER, D. D. & BERK, A. J. 1987. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology,* 156**,** 107-21.

BELYANSKAYA, L. L., MARTI, T. M., HOPKINS-DONALDSON, S., KURTZ, S., FELLEY-BOSCO, E. & STAHEL, R. A. 2007. Human agonistic TRAIL receptor antibodies Mapatumumab and Lexatumumab induce apoptosis in malignant mesothelioma and act synergistically with cisplatin. *Mol Cancer,* 6**,** 66.

BETT, A. J., HADDARA, W., PREVEC, L. & GRAHAM, F. L. 1994. An efficient and flexible system for construction of adenovirus

vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci U S A,* 91**,** 8802-6.

BISCHOFF, J. R., KIRN, D. H., WILLIAMS, A., HEISE, C., HORN, S., MUNA, M., NG, L., NYE, J. A., SAMPSON-JOHANNES, A., FATTAEY, A. & MCCORMICK, F. 1996. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science,* 274**,** 373-6.

BOATRIGHT, K. M. & SALVESEN, G. S. 2003. Mechanisms of caspase activation. *Curr Opin Cell Biol,* 15**,** 725-31.

BODMER, J. L., SCHNEIDER, P. & TSCHOPP, J. 2002. The molecular architecture of the TNF superfamily. *Trends Biochem Sci,* 27**,** 19-26.

BOSSY-WETZEL, E., NEWMEYER, D. D. & GREEN, D. R. 1998. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J,* 17**,** 37-49.

BURRIS, H., 3RD, YARDLEY, D., JONES, S., HOUSTON, G., BROOME, C., THOMPSON, D., GRECO, F. A., WHITE, M. & HAINSWORTH, J. 2004. Phase II trial of trastuzumab followed by weekly paclitaxel/carboplatin as first-line treatment for patients with metastatic breast cancer. *J Clin Oncol,* 22**,** 1621-9.

CANCRO, M. P. & SMITH, S. H. 2003. Peripheral B cell selection and homeostasis. *Immunol Res,* 27**,** 141-8.

CARSWELL, E. A., OLD, L. J., KASSEL, R. L., GREEN, S., FIORE, N. & WILLIAMSON, B. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A,* 72**,** 3666-70.

CASANOVAS O, HICKLIN DJ, BERGERS G, HANAHAN D. 2005.
resistance by evasion of antiangiogenic targeting of VEGF sig naling in late-stage pancreatic islet tumors.*Cancer Cell*. 8(4):299-309

CASCIOLA-ROSEN, L. A., ANHALT, G. J. & ROSEN, A. 1995. DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J Exp Med,* 182**,** 1625-34.

CERTO, M., DEL GAIZO MOORE, V., NISHINO, M., WEI, G., KORSMEYER, S., ARMSTRONG, S. A. & LETAI, A. 2006. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell,* 9**,** 351-65.

CHAN, F. K., CHUN, H. J., ZHENG, L., SIEGEL, R. M., BUI, K. L. & LENARDO, M. J. 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science,* 288**,** 2351-4.

CHEN, L., APGAR, J., HUYNH, L., DICKER, F., GIAGO-MCGAHAN, T., RASSENTI, L., WEISS, A. & KIPPS, T. J. 2005. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. *Blood,* 105**,** 2036-41.

CHINNAIYAN, A. M., O'ROURKE, K., TEWARI, M. & DIXIT, V. M. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell,* 81**,** 505-12.

CHINNAIYAN, A. M., PRASAD, U., SHANKAR, S., HAMSTRA, D. A., SHANAIAH, M., CHENEVERT, T. L., ROSS, B. D. & REHEMTULLA, A. 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**,** 1754-9.

CORAZZA, N., JAKOB, S., SCHAER, C., FRESE, S., KEOGH, A., STROKA, D., KASSAHN, D., TORGLER, R., MUELLER, C., SCHNEIDER, P. & BRUNNER, T. 2006. TRAIL receptor-mediated JNK activation and Bim phosphorylation critically regulate Fas-mediated liver damage and lethality. *J Clin Invest,* 116**,** 2493-9.

CORY, S. & ADAMS, J. M. 2005. Killing cancer cells by flipping the Bcl-2/Bax switch. *Cancer Cell,* 8**,** 5-6.

CORY, S., HUANG, D. C. & ADAMS, J. M. 2003. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene,* 22**,** 8590-607.

COUSSENS, L. M. & WERB, Z. 2002. Inflammation and cancer. *Nature,* 420**,** 860-7.

CRETNEY, E., SHANKER, A., YAGITA, H., SMYTH, M. J. & SAYERS, T. J. 2006. TNF-related apoptosis-inducing ligand as a therapeutic agent in autoimmunity and cancer. *Immunol Cell Biol,* 84**,** 87-98.

CRYNS, V. L., BERGERON, L., ZHU, H., LI, H. & YUAN, J. 1996. Specific cleavage of alpha-fodrin during Fas- and tumor necrosis factor-induced apoptosis is mediated by an interleukin-1beta-converting enzyme/Ced-3 protease distinct from the poly(ADP-ribose) polymerase protease. *J Biol Chem,* 271**,** 31277-82.

DANIELS, R. A., TURLEY, H., KIMBERLEY, F. C., LIU, X. S., MONGKOLSAPAYA, J., CH'EN, P., XU, X. N., JIN, B. Q., PEZZELLA, F. & SCREATON, G. R. 2005. Expression of TRAIL and TRAIL receptors in normal and malignant tissues. *Cell Res,* 15**,** 430-8.

DE BOUARD, S., HERLIN, P., CHRISTENSEN, J. G., LEMOISSON, E., GAUDUCHON, P., RAYMOND, E. & GUILLAMO, J. S. 2007. Antiangiogenic and anti-invasive effects of sunitinib on experimental human glioblastoma. *Neuro Oncol,* 9**,** 412-23.

DEGLI-ESPOSTI, M. A., DOUGALL, W. C., SMOLAK, P. J., WAUGH, J. Y., SMITH, C. A. & GOODWIN, R. G. 1997. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity,* 7**,** 813-20.

DESAGHER, S., OSEN-SAND, A., NICHOLS, A., ESKES, R., MONTESSUIT, S., LAUPER, S., MAUNDRELL, K., ANTONSSON, B. & MARTINOU, J. C. 1999. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol,* 144**,** 891-901.

DEVIN, A., COOK, A., LIN, Y., RODRIGUEZ, Y., KELLIHER, M. & LIU, Z. 2000. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity,* 12**,** 419-29.

ELIOPOULOS, A. G., KERR, D. J., HEROD, J., HODGKINS, L., KRAJEWSKI, S., REED, J. C. & YOUNG, L. S. 1995. The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. *Oncogene,* 11**,** 1217-28.

ELIOPOULOS AG, YOUNG LS. 2004.The role of the CD40 pathway in the pathogenesis and treatment of cancer. *Curr Opin Pharmacol,* 4(4):360-7.

ELMETWALI, T., SEARLE, P. F., MCNEISH, I., YOUNG, L. S. & PALMER, D. H. 2010. CD40 ligand induced cytotoxicity in carcinoma cells is enhanced by inhibition of metalloproteinase cleavage and delivery via a conditionally-replicating adenovirus. *Mol Cancer,* 9**,** 52.

ELMETWALI, T., YOUNG, L. S. & PALMER, D. H. 2010b. CD40 ligand-induced carcinoma cell death: a balance between activation of TNFR-associated factor (TRAF) 3-dependent death signals and suppression of TRAF6-dependent survival signals. *J Immunol,* 184**,** 1111-20.

EMERY, J. G., MCDONNELL, P., BURKE, M. B., DEEN, K. C., LYN, S., SILVERMAN, C., DUL, E., APPELBAUM, E. R., EICHMAN, C., DIPRINZIO, R., DODDS, R. A., JAMES, I. E., ROSENBERG, M., LEE, J. C. & YOUNG, P. R. 1998. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *Journal of Biological Chemistry,* 273**,** 14363-14367.

FALLAUX, F. J., KRANENBURG, O., CRAMER, S. J., HOUWELING, A., VAN ORMONDT, H., HOEBEN, R. C. & VAN DER EB, A. J. 1996. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther,* 7**,** 215-22.

FALSCHLEHNER, C., SCHAEFER, U. & WALCZAK, H. 2009. Following TRAIL's path in the immune system. *Immunology,* 127**,** 145-54.

FAN, T. J., HAN, L. H., CONG, R. S. & LIANG, J. 2005. Caspase family proteases and apoptosis. *Acta Biochim Biophys Sin (Shanghai),* 37**,** 719-27.

FERRARA N, HILLAN KJ, NOVOTNY W. 2005. Bevacizumab (Avastin), ahumanized anti-VEGF monoclonal antibody for cancer therapy.*Biochem Biophys Res Commun* 333:328–335

FUERER, C. & IGGO, R. 2002. Adenoviruses with Tcf binding sites in multiple early promoters show enhanced selectivity for tumour cells with constitutive activation of the wnt signalling pathway. *Gene Ther,* 9**,** 270-81.

GANLY, I. & SINGH, B. 2003. Topical ONYX-015 in the treatment of premalignant oral dysplasia: another role for the cold virus? *J Clin Oncol,* 21**,** 4476-8.

GAZITT, Y., SHAUGHNESSY, P. & MONTGOMERY, W. 1999. Apoptosis-induced by TRAIL AND TNF-alpha in human multiple myeloma cells is not blocked by BCL-2. *Cytokine,* 11**,** 1010-9.

GEORGOPOULOS, N. T., STEELE, L. P., THOMSON, M. J., SELBY, P. J., SOUTHGATE, J. & TREJDOSIEWICZ, L. K. 2006. A novel mechanism of CD40-induced apoptosis of carcinoma cells involving TRAF3 and JNK/AP-1 activation. *Cell Death Differ,* 13**,** 1789-801.

GONZALVEZ, F. & ASHKENAZI, A. 2010. New insights into apoptosis signaling by Apo2L/TRAIL. *Oncogene,* 29**,** 4752-65.

GOODRUM, F. D. & ORNELLES, D. A. 1998. p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection. *J Virol,* 72**,** 9479-90.

GRAHAM, F. L., SMILEY, J., RUSSELL, W. C. & NAIRN, R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol,* 36**,** 59-74.

GREEN, D. R. & KROEMER, G. 2004. The pathophysiology of mitochondrial cell death. *Science,* 305**,** 626-9.

GRIFFITH, T. S., ANDERSON, R. D., DAVIDSON, B. L., WILLIAMS, R. D. & RATLIFF, T. L. 2000. Adenoviral-mediated transfer of the TNF-related apoptosis-inducing ligand/Apo-2 ligand gene induces tumor cell apoptosis. *J Immunol,* 165**,** 2886-94.

GRIFFITH, T. S., STOKES, B., KUCABA, T. A., EAREL, J. K., JR., VANOOSTEN, R. L., BRINCKS, E. L. & NORIAN, L. A. 2009. TRAIL gene therapy: from preclinical development to clinical application. *Curr Gene Ther,* 9**,** 9-19.

GRIFFITH, T. S., WILEY, S. R., KUBIN, M. Z., SEDGER, L. M., MALISZEWSKI, C. R. & FANGER, N. A. 1999. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. *J Exp Med,* 189**,** 1343-54.

GROSSE-WILDE, A., VOLOSHANENKO, O., BAILEY, S. L., LONGTON, G. M., SCHAEFER, U., CSERNOK, A. I., SCHUTZ, G., GREINER, E. F., KEMP, C. J. & WALCZAK, H. 2008. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. *J Clin Invest,* 118**,** 100-10.

GUO, J. & XIN, H. 2006. Chinese gene therapy. Splicing out the West? *Science,* 314**,** 1232-5.

GUSEVA NV, TAGHIYEV AF, STURM MT, ROKHLIN

OW, COHEN MB. 2004. Tumor necrosis factor-related apoptosis inducing ligand-mediated activation of mitochondria associated nuclearfactor kappaB in prostatic carcinoma cell lines. *Mol Cancer Res*.,(10):574-84

HAHN, W. C., COUNTER, C. M., LUNDBERG, A. S., BEIJERSBERGEN, R. L., BROOKS, M. W. & WEINBERG, R. A. 1999. Creation of human tumour cells with defined genetic elements. *Nature,* 400**,** 464-8.

HAIT, W. N. & YANG, J. M. 2006. The individualization of cancer therapy: the unexpected role of p53. *Trans Am Clin Climatol Assoc,* 117**,** 85-101; discussion 101.

HALL, A. R., DIX, B. R., O'CARROLL, S. J. & BRAITHWAITE, A. W. 1998. p53-dependent cell death/apoptosis is required for a productive adenovirus infection. *Nat Med,* 4**,** 1068-72.

HALLENBECK, P. L., CHANG, Y. N., HAY, C., GOLIGHTLY, D., STEWART, D., LIN, J., PHIPPS, S. & CHIANG, Y. L. 1999. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. *Hum Gene Ther,* 10**,** 1721-33.

HAMID, O., VARTERASIAN, M. L., WADLER, S., HECHT, J. R., BENSON, A., 3RD, GALANIS, E., UPRICHARD, M., OMER, C., BYCOTT, P., HACKMAN, R. C. & SHIELDS, A. F. 2003. Phase II trial of intravenous CI-1042 in patients with metastatic colorectal cancer. *J Clin Oncol,* 21**,** 1498-504.

HANAHAN, D. & WEINBERG, R. A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

HARADA, J. N., SHEVCHENKO, A., PALLAS, D. C. & BERK, A. J. 2002. Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. *J Virol,* 76**,** 9194-206.

HARANAKA, K., SATOMI, N. & SAKURAI, A. 1984. Antitumor activity of murine tumor necrosis factor (TNF) against transplanted murine tumors and heterotransplanted human tumors in nude mice. *Int J Cancer,* 34**,** 263-7.

HARDY, S., KITAMURA, M., HARRIS-STANSIL, T., DAI, Y. & PHIPPS, M. L. 1997. Construction of adenovirus vectors through Cre-lox recombination. *J Virol,* 71**,** 1842-9.

HATAKEYAMA, M. & WEINBERG, R. A. 1995. The role of RB in cell cycle control. *Prog Cell Cycle Res,* 1**,** 9-19.

HE, T. C., ZHOU, S., DA COSTA, L. T., YU, J., KINZLER, K. W. & VOGELSTEIN, B. 1998. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A,* 95**,** 2509-14.

HECHT, J. R., BEDFORD, R., ABBRUZZESE, J. L., LAHOTI, S., REID, T. R., SOETIKNO, R. M., KIRN, D. H. & FREEMAN, S. M. 2003. A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. *Clin Cancer Res,* 9**,** 555-61.

HENKART, P. A. 1996. ICE family proteases: mediators of all apoptotic cell death? *Immunity,* 4**,** 195-201.

HERRNRING, C., REIMER, T., JESCHKE, U., MAKOVITZKY, J., KRUGER, K., GERBER, B., KABELITZ, D. & FRIESE, K. 2000. Expression of the apoptosis-inducing ligands FasL and TRAIL in malignant and benign human breast tumors. *Histochem Cell Biol,* 113**,** 189-94.

HOLLAND, P. M. 2011. Targeting Apo2L/TRAIL receptors by soluble Apo2L/TRAIL. *Cancer Lett*.

HOLLER, N., TARDIVEL, A., KOVACSOVICS-BANKOWSKI, M., HERTIG, S., GAIDE, O., MARTINON, F., TINEL, A., DEPERTHES, D., CALDERARA, S., SCHULTHESS, T., ENGEL, J., SCHNEIDER, P. & TSCHOPP, J. 2003. Two adjacent trimeric Fas ligands are required for Fas signaling and formation of a death-inducing signaling complex. *Mol Cell Biol,* 23**,** 1428-40.

HOLOCH, P. A. & GRIFFITH, T. S. 2009. TNF-related apoptosis-inducing ligand (TRAIL): a new path to anti-cancer therapies. *Eur J Pharmacol,* 625**,** 63-72.

HU, W. H., JOHNSON, H. & SHU, H. B. 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.

HYMOWITZ, S. G., CHRISTINGER, H. W., FUH, G., ULTSCH, M., O'CONNELL, M., KELLEY, R. F., ASHKENAZI, A. & DE VOS, A. M. 1999. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol Cell,* 4**,** 563-71.

IDRISS, H. T. & NAISMITH, J. H. 2000. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech,* 50**,** 184-95.

JEREMIAS, I., KUPATT, C., BAUMANN, B., HERR, I., WIRTH, T. & DEBATIN, K. M. 1998. Inhibition of nuclear factor kappaB activation attenuates apoptosis resistance in lymphoid cells. *Blood,* 91**,** 4624-31.

JOHNSON, L., SHEN, A., BOYLE, L., KUNICH, J., PANDEY, K., LEMMON, M., HERMISTON, T., GIEDLIN, M., MCCORMICK, F. & FATTAEY, A. 2002. Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. *Cancer Cell,* 1**,** 325-37.

JOLLY, D. 1994. Viral vector systems for gene therapy. *Cancer Gene Ther,* 1**,** 51-64.

JOURDAN, M., REME, T., GOLDSCHMIDT, H., FIOL, G., PANTESCO, V., DE VOS, J., ROSSI, J. F., HOSE, D. & KLEIN, B. 2009. Gene expression of anti- and pro-apoptotic proteins in malignant and normal plasma cells. *Br J Haematol,* 145**,** 45-58.

KARIN, M., LAWRENCE, T. & NIZET, V. 2006. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell,* 124**,** 823-35.

KATZ, S. I., ZHOU, L., CHAO, G., SMITH, C. D., FERRARA, T., WANG, W., DICKER, D. T. & EL-DEIRY, W. S. 2009. Sorafenib inhibits ERK1/2 and MCL-1(L) phosphorylation levels resulting in caspase-independent cell death in malignant pleural mesothelioma. *Cancer Biol Ther,* 8**,** 2406-16.

KERBEL RS. 2008. Tumor angiogenesis. *N Engl J Med* 358:2039–2049

KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer,* 26**,** 239-57.

KHAIDER, N. G., LANE, D., MATTE, I., RANCOURT, C. & PICHE, A. 2012. Targeted ovarian cancer treatment: the TRAILs of resistance. *Am J Cancer Res,* 2**,** 75-92.

KHURI, F. R., NEMUNAITIS, J., GANLY, I., ARSENEAU, J., TANNOCK, I. F., ROMEL, L., GORE, M., IRONSIDE, J., MACDOUGALL, R. H., HEISE, C., RANDLEV, B., GILLENWATER, A. M., BRUSO, P., KAYE, S. B., HONG, W. K. & KIRN, D. H. 2000. a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med,* 6**,** 879-85.

KIM, H., RAFIUDDIN-SHAH, M., TU, H. C., JEFFERS, J. R., ZAMBETTI, G. P., HSIEH, J. J. & CHENG, E. H. 2006. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol,* 8**,** 1348-58.

KISCHKEL, F. C., LAWRENCE, D. A., TINEL, A., LEBLANC, H., VIRMANI, A., SCHOW, P., GAZDAR, A., BLENIS, J., ARNOTT, D. & ASHKENAZI, A. 2001. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J Biol Chem,* 276**,** 46639-46.

KNOX, P. G., MILNER, A. E., GREEN, N. K., ELIOPOULOS, A. G. & YOUNG, L. S. 2003. Inhibition of metalloproteinase cleavage enhances the cytotoxicity of Fas ligand. *J Immunol,* 170**,** 677-85.

KONDO, S., SHINOMURA, Y., MIYAZAKI, Y., KIYOHARA, T., TSUTSUI, S., KITAMURA, S., NAGASAWA, Y., NAKAHARA, M., KANAYAMA, S. & MATSUZAWA, Y.

2000. Mutations of the bak gene in human gastric and colorectal cancers. *Cancer Res,* 60**,** 4328-30.

KOTHAKOTA, S., AZUMA, T., REINHARD, C., KLIPPEL, A., TANG, J., CHU, K., MCGARRY, T. J., KIRSCHNER, M. W., KOTHS, K., KWIATKOWSKI, D. J. & WILLIAMS, L. T. 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science,* 278**,** 294-8.

LAIRD, A. D., CHRISTENSEN, J. G., LI, G., CARVER, J., SMITH, K., XIN, X., MOSS, K. G., LOUIE, S. G., MENDEL, D. B. & CHERRINGTON, J. M. 2002. SU6668 inhibits Flk-1/KDR and PDGFRbeta in vivo, resulting in rapid apoptosis of tumor vasculature and tumor regression in mice. *FASEB J,* 16**,** 681-90.

LANCASTER, J. M., SAYER, R., BLANCHETTE, C., CALINGAERT, B., WHITAKER, R., SCHILDKRAUT, J., MARKS, J. & BERCHUCK, A. 2003. High expression of tumor necrosis factor-related apoptosis-inducing ligand is associated with favorable ovarian cancer survival. *Clin Cancer Res,* 9**,** 762-6.

LANGE-CARTER, C. A. & JOHNSON, G. L. 1994. Ras-dependent growth factor regulation of MEK kinase in PC12 cells. *Science,* 265**,** 1458-61.

LAVRIK, I. N., GOLKS, A. & KRAMMER, P. H. 2005. Caspases: pharmacological manipulation of cell death. *J Clin Invest,* 115**,** 2665-72.

LAZEBNIK, Y. A., KAUFMANN, S. H., DESNOYERS, S., POIRIER, G. G. & EARNSHAW, W. C. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature,* 371**,** 346-7.

LEVINE, A. J. 1997. p53, the cellular gatekeeper for growth and division. *Cell,* 88**,** 323-31.

LI W, ZHANG X, OLUMI AF. 2007. MG-132 sensitizes TRAIL-resistant prostate cancer cells by activating c-Fos/c-Jun heterodimers and repressing c-FLIP(L). *Cancer Res*. 1;67(5):2247-55.

LLOBET, D., ERITJA, N., YERAMIAN, A., PALLARES, J., SOROLLA, A., DOMINGO, M., SANTACANA, M., GONZALEZ-TALLADA, F. J., MATIAS-GUIU, X. & DOLCET, X. 2010. The multikinase inhibitor Sorafenib induces apoptosis and sensitises endometrial cancer cells to TRAIL by different mechanisms. *Eur J Cancer,* 46**,** 836-50.

LORUSSO, G. & RUEGG, C. 2008. The tumor microenvironment and its contribution to tumor evolution toward metastasis. *Histochem Cell Biol,* 130**,** 1091-103.

LOWER, E. E., GLASS, E., BLAU, R. & HARMAN, S. 2009. HER-2/neu expression in primary and metastatic breast cancer. *Breast Cancer Res Treat,* 113**,** 301-6.

MAAS, C., VERBRUGGE, I., DE VRIES, E., SAVICH, G., VAN DE KOOIJ, L. W., TAIT, S. W. & BORST, J. 2010. Smac/DIABLO release from mitochondria and XIAP inhibition are essential to limit clonogenicity of Type I tumor cells after TRAIL receptor stimulation. *Cell Death Differ,* 17**,** 1613-23.

MACKEY, J. R., KERBEL, R. S., GELMON, K. A., MCLEOD, D. M., CHIA, S. K., RAYSON, D., VERMA, S., COLLINS, L. L., PATERSON, A. H., ROBIDOUX, A. & PRITCHARD, K. I. 2012. Controlling angiogenesis in breast cancer: A systematic review of anti-angiogenic trials. *Cancer Treat Rev*.

MAKOWER, D., ROZENBLIT, A., KAUFMAN, H., EDELMAN, M., LANE, M. E., ZWIEBEL, J., HAYNES, H. & WADLER, S. 2003. Phase II clinical trial of intralesional administration of the oncolytic adenovirus ONYX-015 in patients with hepatobiliary tumors with correlative p53 studies. *Clin Cancer Res,* 9**,** 693-702.

MASHIMA, T., NAITO, M., NOGUCHI, K., MILLER, D. K., NICHOLSON, D. W. & TSURUO, T. 1997. Actin cleavage by CPP-32/apopain during the development of apoptosis. *Oncogene,* 14**,** 1007-12.

MELLIER, G., HUANG, S., SHENOY, K. & PERVAIZ, S. 2010. TRAILing death in cancer. *Mol Aspects Med,* 31**,** 93-112.

MICHEAU, O., THOME, M., SCHNEIDER, P., HOLLER, N., TSCHOPP, J., NICHOLSON, D. W., BRIAND, C. & GRUTTER, M. G. 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.

MICHEAU, O. & TSCHOPP, J. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell,* 114**,** 181-90.

MILNER, A. E., PALMER, D. H., HODGKIN, E. A., ELIOPOULOS, A. G., KNOX, P. G., POOLE, C. J., KERR, D. J. & YOUNG, L. S. 2002. Induction of apoptosis by chemotherapeutic drugs: the role of FADD in activation of caspase-8 and synergy with death receptor ligands in ovarian carcinoma cells. *Cell Death Differ,* 9**,** 287-300.

MITSIADES, C. S., TREON, S. P., MITSIADES, N., SHIMA, Y., RICHARDSON, P., SCHLOSSMAN, R., HIDESHIMA, T. &

ANDERSON, K. C. 2001. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. *Blood,* 98**,** 795-804.

MOHLER, K. M., SLEATH, P. R., FITZNER, J. N., CERRETTI, D. P., ALDERSON, M., KERWAR, S. S., TORRANCE, D. S., OTTEN-EVANS, C., GREENSTREET, T., WEERAWARNA, K. & ET AL. 1994. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature,* 370**,** 218-20.

MORIISHI, K., HUANG, D. C., CORY, S. & ADAMS, J. M. 1999. Bcl-2 family members do not inhibit apoptosis by binding the caspase activator Apaf-1. *Proc Natl Acad Sci U S A,* 96**,** 9683-8.

MUHLENBECK, F., HAAS, E., SCHWENZER, R., SCHUBERT, G., GRELL, M., SMITH, C., SCHEURICH, P. & WAJANT, H. 1998. TRAIL/Apo2L activates c-Jun NH2-terminal kinase (JNK) via caspase-dependent and caspase-independent pathways. *J Biol Chem,* 273**,** 33091-8.

NARITA, M., SHIMIZU, S., ITO, T., CHITTENDEN, T., LUTZ, R. J., MATSUDA, H. & TSUJIMOTO, Y. 1998. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proc Natl Acad Sci U S A,* 95**,** 14681-6.

NEMUNAITIS, J., KHURI, F., GANLY, I., ARSENEAU, J., POSNER, M., VOKES, E., KUHN, J., MCCARTY, T., LANDERS, S., BLACKBURN, A., ROMEL, L., RANDLEV, B., KAYE, S. & KIRN, D. 2001. Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J Clin Oncol,* 19**,** 289-98.

NEMUNAITIS, J., SWISHER, S. G., TIMMONS, T., CONNORS, D., MACK, M., DOERKSEN, L., WEILL, D., WAIT, J., LAWRENCE, D. D., KEMP, B. L., FOSSELLA, F., GLISSON, B. S., HONG, W. K., KHURI, F. R., KURIE, J. M., LEE, J. J., LEE, J. S., NGUYEN, D. M., NESBITT, J. C., PEREZ-SOLER, R., PISTERS, K. M., PUTNAM, J. B., RICHLI, W. R., SHIN, D. M., WALSH, G. L., MERRITT, J. & ROTH, J. 2000. Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J Clin Oncol,* 18**,** 609-22.

NICHOLSON, D. W., ALI, A., THORNBERRY, N. A., VAILLANCOURT, J. P., DING, C. K., GALLANT, M., GAREAU, Y., GRIFFIN, P. R., LABELLE, M., LAZEBNIK, Y. A. & ET AL. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature,* 376**,** 37-43.

NIEMOELLER, O. M. & BELKA, C. 2011. Radiotherapy and TRAIL for cancer therapy. *Cancer Lett*.

O'DWYER, M. E. & DRUKER, B. J. 2001. Chronic myelogenous leukaemia--new therapeutic principles. *J Intern Med,* 250**,** 3-9.

O'SHEA, C. C., JOHNSON, L., BAGUS, B., CHOI, S., NICHOLAS, C., SHEN, A., BOYLE, L., PANDEY, K., SORIA, C., KUNICH, J., SHEN, Y., HABETS, G., GINZINGER, D. & MCCORMICK, F. 2004. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. *Cancer Cell,* 6**,** 611-23.

OLD, L. J. 1985. Tumor necrosis factor (TNF). *Science,* 230**,** 630-2.

OREN, M. 2003. Decision making by p53: life, death and cancer. *Cell Death Differ,* 10**,** 431-42.

ORTH, K., CHINNAIYAN, A. M., GARG, M., FROELICH, C. J. & DIXIT, V. M. 1996. The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J Biol Chem,* 271**,** 16443-6.

PAN, G., O'ROURKE, K., CHINNAIYAN, A. M., GENTZ, R., EBNER, R., NI, J. & DIXIT, V. M. 1997. The receptor for the cytotoxic ligand TRAIL. *Science,* 276**,** 111-3.

PARK, S. M., SCHICKEL, R. & PETER, M. E. 2005. Nonapoptotic functions of FADD-binding death receptors and their signaling molecules. *Curr Opin Cell Biol,* 17**,** 610-6.

PARKS, R. J., CHEN, L., ANTON, M., SANKAR, U., RUDNICKI, M. A. & GRAHAM, F. L. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A,* 93**,** 13565-70.

PENNICA, D., NEDWIN, G. E., HAYFLICK, J. S., SEEBURG, P. H., DERYNCK, R., PALLADINO, M. A., KOHR, W. J., AGGARWAL, B. B. & GOEDDEL, D. V. 1984. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature,* 312**,** 724-9.

PESCHON, J. J., TORRANCE, D. S., STOCKING, K. L., GLACCUM, M. B., OTTEN, C., WILLIS, C. R., CHARRIER, K., MORRISSEY, P. J., WARE, C. B. & MOHLER, K. M. 1998. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J Immunol,* 160**,** 943-52.

PETER, M. E. & KRAMMER, P. H. 2003. The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ,* 10**,** 26-35.

PITTI, R. M., MARSTERS, S. A., RUPPERT, S., DONAHUE, C. J., 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.

PLUMMER, R., ATTARD, G., PACEY, S., LI, L., RAZAK, A., PERRETT, R., BARRETT, M., JUDSON, I., KAYE, S., FOX, N. L., HALPERN, W., COREY, A., CALVERT, H. & DE BONO, J. 2007. Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers. *Clin Cancer Res,* 13**,** 6187-94.

PRITZKER, L. B., SCATENA, M. & GIACHELLI, C. M. 2004. The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival. *Mol Biol Cell,* 15**,** 2834-41.

QUANTIN, B., PERRICAUDET, L. D., TAJBAKHSH, S. & MANDEL, J. L. 1992. Adenovirus as an expression vector in muscle cells in vivo. *Proc Natl Acad Sci U S A,* 89**,** 2581-4.

QUERIDO, E., BLANCHETTE, P., YAN, Q., KAMURA, T., MORRISON, M., BOIVIN, D., KAELIN, W. G., CONAWAY, R. C., CONAWAY, J. W. & BRANTON, P. E. 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev,* 15**,** 3104-17.

QUERIDO, E., MARCELLUS, R. C., LAI, A., CHARBONNEAU, R., TEODORO, J. G., KETNER, G. & BRANTON, P. E. 1997. Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. *J Virol,* 71**,** 3788-98.

RAMPINO, N., YAMAMOTO, H., IONOV, Y., LI, Y., SAWAI, H., REED, J. C. & PERUCHO, M. 1997. Somatic frameshift

mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science,* 275**,** 967-9.

REED, J. C., DOCTOR, K. S. & GODZIK, A. 2004. The domains of apoptosis: a genomics perspective. *Sci STKE,* 2004**,** re9.

RODRIGUEZ, R., SCHUUR, E. R., LIM, H. Y., HENDERSON, G. A., SIMONS, J. W. & HENDERSON, D. R. 1997. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res,* 57**,** 2559-63.

ROWE, W. P., HUEBNER, R. J., GILMORE, L. K., PARROTT, R. H. & WARD, T. G. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med,* 84**,** 570-3.

RUDIN, C. M., COHEN, E. E., PAPADIMITRAKOPOULOU, V. A., SILVERMAN, S., JR., RECANT, W., EL-NAGGAR, A. K., STENSON, K., LIPPMAN, S. M., HONG, W. K. & VOKES, E. E. 2003. An attenuated adenovirus, ONYX-015, as mouthwash therapy for premalignant oral dysplasia. *J Clin Oncol,* 21**,** 4546-52.

RUSSELL, W. C. 2000. Update on adenovirus and its vectors. *J Gen Virol,* 81**,** 2573-604.

SCAFFIDI, C., FULDA, S., SRINIVASAN, A., FRIESEN, C., LI, F., TOMASELLI, K. J., DEBATIN, K. M., KRAMMER, P. H. & PETER, M. E. 1998. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J,* 17**,** 1675-87.

SCAFFIDI, C., SCHMITZ, I., ZHA, J., KORSMEYER, S. J., KRAMMER, P. H. & PETER, M. E. 1999. Differential

modulation of apoptosis sensitivity in CD95 type I and type II cells. *J Biol Chem,* 274**,** 22532-8.

SCHNEIDER, P., HOLLER, N., BODMER, J. L., HAHNE, M., FREI, K., FONTANA, A. & TSCHOPP, J. 1998. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J Exp Med,* 187**,** 1205-13.

SEOL, J. Y., PARK, K. H., HWANG, C. I., PARK, W. Y., YOO, C. G., KIM, Y. W., HAN, S. K., SHIM, Y. S. & LEE, C. T. 2003. Adenovirus-TRAIL can overcome TRAIL resistance and induce a bystander effect. *Cancer Gene Ther,* 10**,** 540-8.

SHERIDAN, J. P., MARSTERS, S. A., PITTI, R. M., GURNEY, A., SKUBATCH, M., BALDWIN, D., RAMAKRISHNAN, L., GRAY, C. L., BAKER, K., WOOD, W. I., GODDARD, A. D., GODOWSKI, P. & ASHKENAZI, A. 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science,* 277**,** 818-21.

SHERR, C. J. & MCCORMICK, F. 2002. The RB and p53 pathways in cancer. *Cancer Cell,* 2**,** 103-12.

SHIMIZU, S., NARITA, M. & TSUJIMOTO, Y. 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature,* 399**,** 483-7.

SLAMON, D. J., CLARK, G. M., WONG, S. G., LEVIN, W. J., ULLRICH, A. & MCGUIRE, W. L. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science,* 235**,** 177-82.

SLAMON, D. J., LEYLAND-JONES, B., SHAK, S., FUCHS, H., PATON, V., BAJAMONDE, A., FLEMING, T., EIERMANN, W., WOLTER, J., PEGRAM, M., BASELGA, J. & NORTON, L.

2001. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med,* 344**,** 783-92.

SMITH, C. A., FARRAH, T. & GOODWIN, R. G. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell,* 76**,** 959-62.

SODERSTROM, T. S., POUKKULA, M., HOLMSTROM, T. H., HEISKANEN, K. M. & ERIKSSON, J. E. 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.

SOLAN, N. J., MIYOSHI, H., CARMONA, E. M., BREN, G. D. & PAYA, C. V. 2002. RelB cellular regulation and transcriptional activity are regulated by p100. *J Biol Chem,* 277**,** 1405-18.

SORIA, J. C., MARK, Z., ZATLOUKAL, P., SZIMA, B., ALBERT, I., JUHASZ, E., PUJOL, J. L., KOZIELSKI, J., BAKER, N., SMETHURST, D., HEI, Y. J., ASHKENAZI, A., STERN, H., AMLER, L., PAN, Y. & BLACKHALL, F. 2011. Randomized phase II study of dulanermin in combination with paclitaxel, carboplatin, and bevacizumab in advanced non-small-cell lung cancer. *J Clin Oncol,* 29**,** 4442-51.

SPIERINGS, D., MCSTAY, G., SALEH, M., BENDER, C., CHIPUK, J., MAURER, U. & GREEN, D. R. 2005. Connected to death: the (unexpurgated) mitochondrial pathway of apoptosis. *Science,* 310**,** 66-7.

SPRICK, M. R., RIESER, E., STAHL, H., GROSSE-WILDE, A., WEIGAND, M. A. & 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**,** 4520-30.

STRATFORD-PERRICAUDET, L. D., LEVRERO, M., CHASSE, J. F., PERRICAUDET, M. & BRIAND, P. 1990. Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector. *Hum Gene Ther,* 1**,** 241-56.

TAKAHASHI, A., ALNEMRI, E. S., LAZEBNIK, Y. A., FERNANDES-ALNEMRI, T., LITWACK, G., MOIR, R. D., GOLDMAN, R. D., POIRIER, G. G., KAUFMANN, S. H. & EARNSHAW, W. C. 1996. Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc Natl Acad Sci U S A,* 93**,** 8395-400.

TEOH G, TAI YT, URASHIMA M, SHIRAHAMA S, MATSUZAKI M, CHAUHAN D, TREON SP, RAJE N, HIDESHIMA T, SHIMA Y, ANDERSON KC. 2000. CD40 activation mediates p53-dependent cell cycle regulation in human multiple myeloma cell lines. *Blood*, 1;95(3):1039-46.

TRAN, S. E., HOLMSTROM, T. H., AHONEN, M., KAHARI, V. M. & ERIKSSON, J. E. 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, C. H., PRZYBOREK, M., SCHULZ, C., SNELLER, V., GALLANT, G. & KANZLER, S. 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**,** 506-12.

VAN MIERLO GJ, DEN BOER AT, MEDEMA JP, VAN DER VOORT EI, FRANSEN MF, OFFRINGA R, MELIEF CJ, TOES RE. 2002. CD40 stimulation leads to effective therapy of CD40(-) tumors through induction of strong systemic cytotoxic T lymphocyte immunity. *Proc Natl Acad Sci*, 16;99(8):5561-6.

VANDER HEIDEN, M. G., CHANDEL, N. S., WILLIAMSON, E. K., SCHUMACKER, P. T. & THOMPSON, C. B. 1997. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell,* 91**,** 627-37.

VARFOLOMEEV, E., MAECKER, H., SHARP, D., LAWRENCE, D., RENZ, M., VUCIC, D. & ASHKENAZI, A. 2005. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. *J Biol Chem,* 280**,** 40599-608.

VASEY, P. A., SHULMAN, L. N., CAMPOS, S., DAVIS, J., GORE, M., JOHNSTON, S., KIRN, D. H., O'NEILL, V., SIDDIQUI, N., SEIDEN, M. V. & KAYE, S. B. 2002. Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. *J Clin Oncol,* 20**,** 1562-9.

VIGNATI, S., CODEGONI, A., POLATO, F. & BROGGINI, M. 2002. Trail activity in human ovarian cancer cells: potentiation of the action of cytotoxic drugs. *Eur J Cancer,* 38**,** 177-83.

VOELKEL-JOHNSON, C., KING, D. L. & NORRIS, J. S. 2002. Resistance of prostate cancer cells to soluble TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) can be overcome

by doxorubicin or adenoviral delivery of full-length TRAIL. *Cancer Gene Ther,* 9**,** 164-72.

WALCZAK, H., MILLER, R. E., ARIAIL, K., GLINIAK, B., GRIFFITH, T. S., KUBIN, M., CHIN, W., JONES, J., WOODWARD, A., LE, T., SMITH, C., SMOLAK, P., GOODWIN, R. G., RAUCH, C. T., SCHUH, J. C. & LYNCH, D. H. 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med,* 5**,** 157-63.

WATANABE-FUKUNAGA, R., BRANNAN, C. I., COPELAND, N. G., JENKINS, N. A. & NAGATA, S. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature,* 356**,** 314-7.

WILEY, S. R., SCHOOLEY, K., SMOLAK, P. J., DIN, W. S., HUANG, C. P., NICHOLL, J. K., SUTHERLAND, G. R., SMITH, T. D., RAUCH, C., SMITH, C. A. & ET AL. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity,* 3**,** 673-82.

WILLIS, S., DAY, C. L., HINDS, M. G. & HUANG, D. C. 2003. The Bcl-2-regulated apoptotic pathway. *J Cell Sci,* 116**,** 4053-6.

YAMAOKA, S., COURTOIS, G., BESSIA, C., WHITESIDE, S. T., WEIL, R., AGOU, F., KIRK, H. E., KAY, R. J. & ISRAEL, A. 1998. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell,* 93**,** 1231-40.

YANG, Y., LI, Q., ERTL, H. C. & WILSON, J. M. 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol,* 69**,** 2004-15.

YANG, Y. & WILSON, J. M. 1995. Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo. *J Immunol,* 155**,** 2564-70.

YOUNG, L. S., SEARLE, P. F., ONION, D. & MAUTNER, V. 2006. Viral gene therapy strategies: from basic science to clinical application. *J Pathol,* 208**,** 299-318.

ZHANG, Y., MA, H., ZHANG, J., LIU, S., LIU, Y. & ZHENG, D.2008. AAV-mediated TRAIL gene expression driven by hTERT promoter suppressed human hepatocellular carcinoma growth in mice. *Life Sci,* 82**,** 1154-61.

ZHENG, S. J., WANG, P., TSABARY, G. & CHEN, Y. H. 2004. Critical roles of TRAIL in hepatic cell death and hepatic inflammation. *J Clin Invest,* 113**,** 58-64.