University of Alberta

c-FLIP and Resistance to TRAIL-Induced Apoptosis in Pancreatic Cancer

by

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Abstract

Currently, pancreatic cancer is one of the most lethal of malignant tumors due to its aggressive local behavior, rapid progress and its resistance to conventional radiation therapy and chemotherapy. No effective therapy currently exists to treat pancreatic cancers. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis in cancer cells but does not affect normal cells supporting its role as an anti-cancer agent. However, many human pancreatic cancer cells are resistant to TRAIL. In this study we investigate the molecular basis of TRAIL resistance in pancreatic cancers. We found that the death-inducing signaling complex (DISC) is the critical regulator of TRAIL-induced apoptosis signalling, and modulation of the DISC by c-FLIP is a critical upstream event in this process. Anti-cancer agents such as cisplatin, camptothecin and celecoxib can inhibit c-FLIP-mediated cell survival signalling pathways thus overcoming TRAIL-resistance in pancreatic cancers. These findings may lead to more effective therapy for pancreatic cancer in the clinic.

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List of Abbreviations

The following abbreviations and definitions have been used throughout this thesis.

ADC ....5-Aza-2′-deoxycytidine

Act D ....actinomycin D

Apaf1 ....apoptotic protease-activating factor 1

Bak ....Bcl-2 antagonist/killer

Bax ....Bcl-2 associated x protein

Bcl-2............................B-cell lymphoma 2

Bcl-X_L ....long form of Bcl-X

Bcl-X_S ....short form of Bcl-X

Bid..............................Bcl-2 inhibitory BH3 domain-containing protein

bp...............................base pair

caspase ....cysteinyl aspartic acid-protease

cDNA ....complementary DNA

CDDP..........................cisplatin

c-$FLIP_L$.......................cellular FADD-like, IL-1β-converting enzyme inhibitory protein long form

c-$FLIP_S$.......................cellular FADD-like, IL-1β-converting enzyme inhibitory protein short form

CPT ....camptothecin

C-terminal ....carboxy terminal

Cys ....cysteine (amino acid)

Cyto c ....cytochrome *c*

DR...............................death receptor

DcR ....decoy receptor

DD...............................death domain

DED ....death effector domain

DFF ....DNA fragmentation factor

DISC ....death inducing signalling complex

DOX............................doxorubicin

ECL ....enhanced chemiluminescence
EDTA ....ethylenediaminetetraacetic acid di-sodium salt
ERK1/2 ....extracellular signal-regulated kinase
FACScan ....fluorescent activated cell sorting scan
FADD ....Fas-associated death domain
FBS ....fetal bovine serum
5-FU ....5- fluorouracil
Gly ....glycine (amino acid)
HDAC ....inhibitor of histone deacetylase
HRP ....horseradish peroxidase
HUVECs ....human umbilical vein endothelial cells
IAP ....inhibitor of apoptosis proteins
IgG ....immunoglobulin G
IFN-γ ....interferon gamma
kD ....kilodalton
LOH ....loss of heterozygosity
mAbs ....monoclonal antibodies
mRNA ....messenger ribonucleic acid
NF-κB ....nuclear factor kappa B
NK cells ....natural killer cells
N-terminal ....amino-terminal
OPG ....osteoprotegerin
p-Akt ....phosphorylated Akt
PARP ....poly(ADP-ribose)polymerase
PBLs ....peripheral blood lymphocytes
PBS ....phosphate buffered saline
PI ....propidium iodide
PI3K ....phosphoinositide 3-kinase
PKB ....protein kinase B otherwise known as Akt
PKC ....protein kinase C
PMSF ....phenylmethylsulfonyl fluoride

p value........................probability (of incorrectly rejecting the null hypothesis)

RNAi..........................RNA interference

RIP.............................receptor interacting protein

RISC...........................RNA-induced silencing complex

SD..............................standard deviation of the mean

SDS............................sodium dodecyl sulphate

siRNA........................small interfering RNA

SEM...........................standard error of the mean

Ser..............................serine (amino acid)

Smac/DIABLO..........second mitochondria-derived activator of caspase/direct inhibitor of apoptosis binding protein [IAP] with low pI

tBid............................truncated Bid

TBST..........................Tris buffered saline with Tween-20

Thr.............................threonine (amino acid)

TNFα.........................tumor necrosis factor alpha

TNFR.........................TNF receptor

TRADD......................TNFR1 associated death domain

TRAIL........................TNF-related apoptosis-inducing ligand

TRAIL-R....................TRAIL receptor

Tris-HCl.....................tris[hydroxymethyl]-amino methane hydrochloride

TSA............................Trichostatin A

XIAP..........................X-linked inhibitor of apoptosis proteins

CHAPTER 1

INTRODUCTION

1.1 Background

Pancreatic cancer, accounting for more than 30,000 deaths annually in the United States [1] and 3,300 deaths annually in Canada (Canadian Cancer Statistics, National Cancer Institute of Canada, 2004), is one of the most lethal cancers because there is no curative treatment currently available. The high mortality rate in patients with pancreatic cancer results from the difficulty in early diagnosis and its resistance to conventional therapy [2]. After diagnosis, the average survival time is between 4–5 months [1]. Although the mainstay for therapy is cytotoxic agents, this approach has been unsuccessful in pancreatic cancers for two reasons: ①current chemotherapeutic agents are not selective and target both cancer and normal cells causing a broad range of unwanted side effects at therapeutic doses; ②clinical investigations show that fluorouracil based radiotherapy or nucleoside analogue gemcitabine [3] based chemotherapy do not prolong survival rates [4, 5] suggesting primary or *de novo* resistance of tumor cells to therapy. Therefore, understanding the molecular mechanism

of drug resistance is an important goal which may lead to more effective therapy for pancreatic cancer.

Recently, several studies have shown that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is capable of inducing apoptosis in some pancreatic cancer cells [6-9], but others are resistant to TRAIL-induced apoptosis [7-9]. The molecular mechanism of this resistance to TRAIL-induced apoptosis is largely unknown.

1.2 Apoptosis

1.2.1 Apoptosis definition and morphology

Apoptosis is a form of programmed cell death that allows multicellular organisms to remove old or damaged cells in a process that is distinct from necrosis [10, 11]. Necrosis, a form of pathological or accidental cell death, is instigated upon acute injury to a cell. Necrosis results in irreversible swelling of a cell's cytoplasm and organelles followed by the eventual lysing and release of its contents to its surroundings [12]. The release of cellular components affects neighbouring cells and can potentially cause a damaging inflammatory response. The process of necrosis typically occurs when cells are exposed to traumatic conditions such as hypoxia, ischemia, hyperthermia, complement attack from the innate immune system, metabolic toxins as well as direct cell trauma [13].

Unlike necrosis, apoptosis is morphologically characterized by chromatin condensation and fragmentation, cell shrinkage and membrane blebbing [11, 14, 15] (Figure 1.1). Changes in the plasma membrane composition during apoptosis provide signals for phagocytes to absorb the apoptotic cell without provoking a generalized

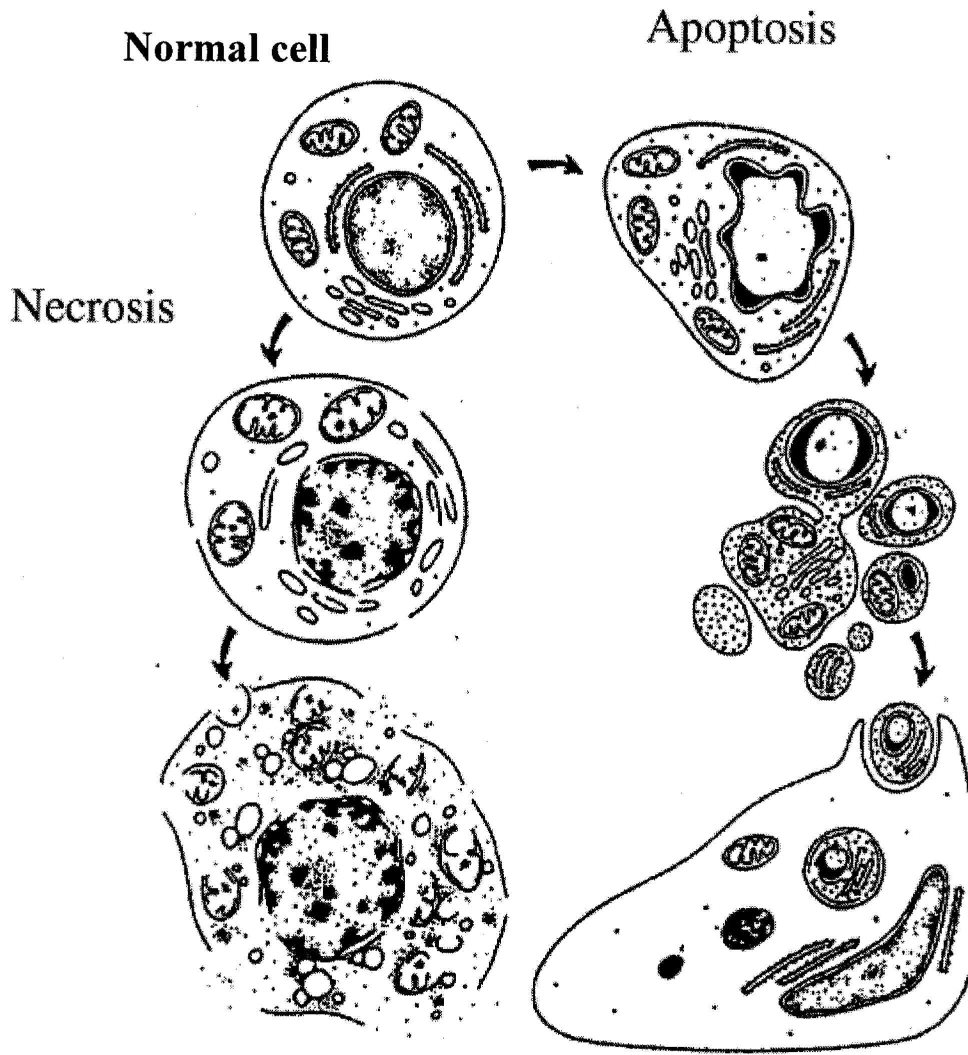


Figure 1.1 Morphology of necrosis and apoptosis. Necrotic cells and their organelles are characteristically swollen. There is early membrane damage with eventual loss of plasma membrane integrity and leakage of cytosol into extracellular space. Despite early clumping, the nuclear chromatin undergoes lysis (karyolysis). In contrast, apoptotic cells are shrunken and develop blebs containing dense cytoplasm. Membrane integrity is not lost until late, after cell death. Nuclear chromatin undergoes striking condensation and fragmentation. The cytoplasm becomes divided to form apoptotic bodies containing organelles and/or nuclear debris. Terminally, apoptotic cells and fragments are engulfed by phagocytes or surrounding cells. (Reproduced from *Robbins Pathologic Basis of Disease*, 6th ed., Cotran RS, Kumar V, Collins T, eds., Saunders, 1999, p. 18.)

inflammatory response [14, 15]. This process not only prevents cellular contents from being released into the surrounding tissue and initiating an inflammatory reaction, but also allows for recycling of the organic components of the dying cell [13].

Apoptosis is an important physiological event that is required for normal cell development as well as controlling pathological processes [11, 13]. Because of its importance, apoptosis is a tightly regulated process, hence any abrogation of the normal apoptotic regulation can lead to a number of diseases [12]. For example, accumulation of uncontrolled cells because of insufficient apoptosis can lead to cancer, and cell loss due to excess apoptosis results in stroke, neurodegeneration and heart failure [12]. In multicellular organisms, apoptosis is a highly regulated and programmed form of cell suicide that is involved in embryogenesis, tissue homeostasis and in the development of various disorders, such as autoimmunity, cancer and neurodegenerative disease [12, 16].

1.2.2 Caspases and apoptosis

The execution of apoptosis is carried out by a set of intracellular proteins called caspases, which are responsible for inducing the morphological and biochemical changes that are associated with apoptosis [17, 18]. The caspases are cysteine proteases which constitutively exist in almost all cell types. These proteases contain a conserved QACXG (where X is R, Q or G) pentapeptide which includes the active-site cysteine [17]. They are normally present in an inactive proenzyme (zymogen) form. The pro-caspases (32–56 kDa) contain four domains: an N-terminal prodomain, a large subunit (17–21 kDa), a small subunit (10–13 kDa) and a short linker region between the large and small subunits

[19]. Caspase activation involves proteolytic processing of the proenzyme at specific aspartate residues (Asp-X) between the domains, resulting in removal of the prodomain as well as the linker region and formation of a heterodimer containing one large and one small subunit [20]. The active caspase is a tetramer composed of two such heterodimers (Figure1.2).

Since their discovery in Caenorhabditis elegans (*C. elegans*), fourteen caspases have been identified in mammalian cells [17, 21]. They can be subdivided into two groups, initiator and effector caspases, based on their structure and function [17, 21] (Figure1.2). 'Initiator' caspases or caspases -2, -8, -9, or -10, have long prodomains, which allow them to interact with protein 'adaptor' molecules [17, 21]. In the event of apoptotic stimuli, recruitment of these caspases through clustering of adaptor molecules results in their close proximity to each other. This leads to their autocleavage and activation [22]. When activated, these 'initiator' caspases can go on, either directly or indirectly, to activate caspases with shorter prodomains usually less than 20 amino acids, termed 'effector' caspases that include caspases -3 and -6 [17, 21, 23]. Once these 'effector' caspases are activated, they can go on to cleave substrates such as structural and regulatory proteins leading eventually to apoptosis. This caspase cascade is selective since caspases recognize a specific protein motif in their substrates, including other caspases, resulting in cleavage after aspartate residues within the specific protein motif [21]. Substrates cleaved by 'effector' caspases include poly (ADP-ribose) polymerase (PARP), DNA fragmentation factor (DFF) as well as lamins, actin, cytokeratins and Bcl-2 and Bcl-X_L. Cleavage of these substrates results in the morphological and biochemical changes associated with apoptosis [21]

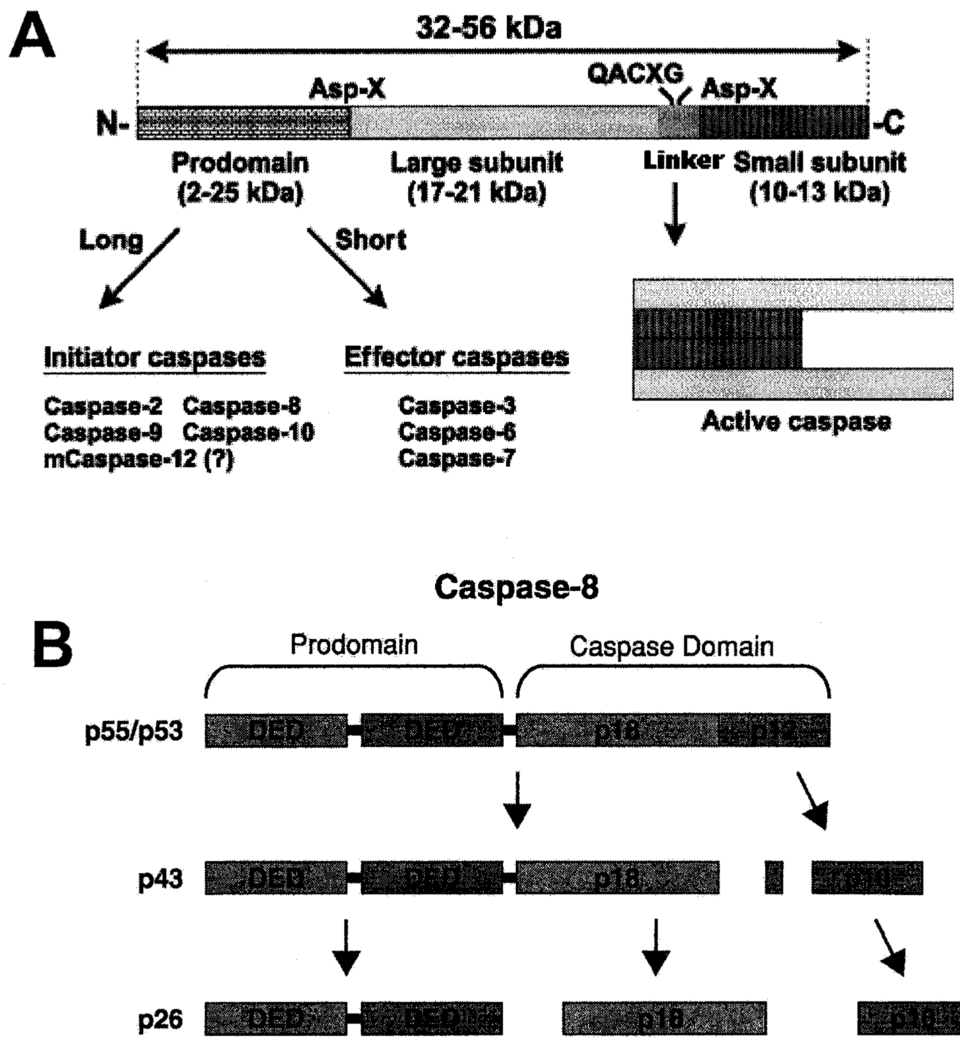


Figure 1.2 A. The structural and functional organization of caspases. The pro-caspases (32–56 kDa) contain four domains: an N-terminal prodomain, a large subunit (17–21 kDa), a small subunit (10–13 kDa) and a short linker region between the large and small subunits. A conserved QACXG (where X is R, Q or G) pentapeptide includes the active-site cysteine. There are specific aspartate residues (Asp-X) between the domains (Adapted from *Morphological and Biochemical Aspects of Apoptosis, Oncosis and Necrosis* Anat.Histol.Embryol. 31, 214–223 (2002) S. Van Cruchten and W.V an den Broeck) **B. Structure of Caspase-8 and cleavage mechanism.** Cleavage sites and the size of cleavage products are indicated. (Reproduced from Vitamines and Hormones, Volume 67, 192 (2004))

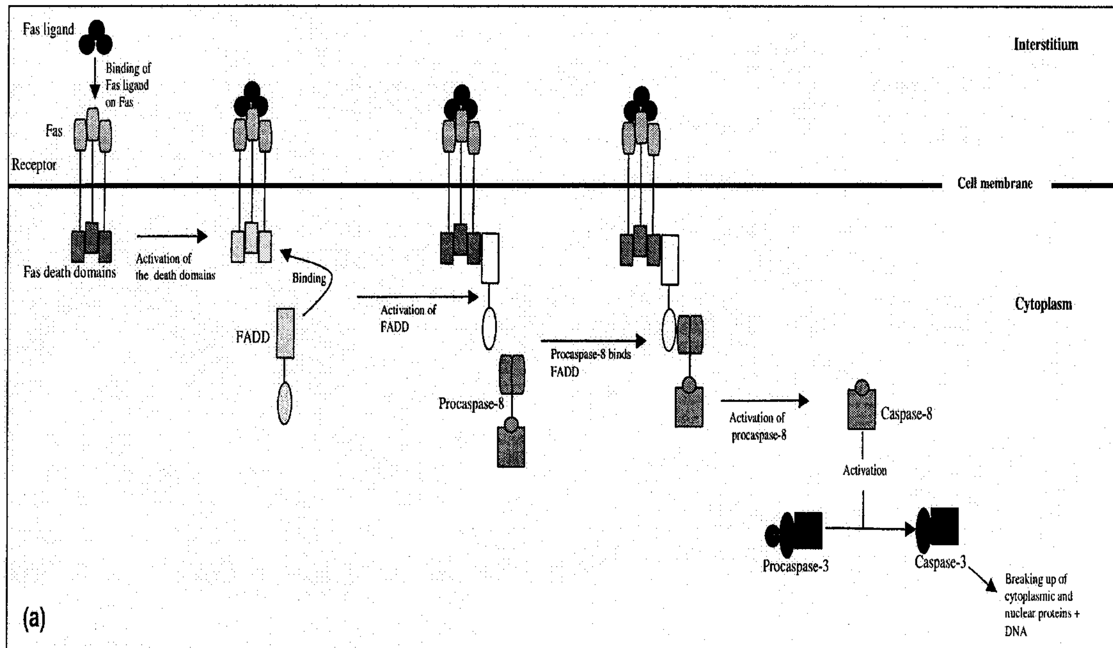
1.2.3 Mechanisms involved in the apoptosis process

1.2.3.1. Receptor-ligand mediated mechanism

Apoptosis can be triggered by binding of a ligand to a specific plasma membrane receptor (Figure1.3). Ligand binding activates the receptor which in turn leads to conversion of procaspases into active caspases. Well-known ligands are the Fas ligand and tumour necrosis factor α (TNFα) [24] which bind Fas (Figure1.3a) and $TNFR_1$ (Figure1.3b) respectively. Both receptors contain an analogous cytoplasmic domain called the 'death domain' (DD) [25] which is responsible for the signal-transduction in apoptosis. Experiments with yeast cells have revealed that two molecules are linked to the death domain: the Fas associated death domain (FADD) for Fas [26], and the $TNFR_1$ associated death domain (TRADD) for $TNFR_1$ [27]. TRADD mediates apoptosis by binding FADD, so eventually both Fas and $TNFR_1$ use FADD to transduce the death signal. Further signal transduction for both receptors occurs via caspase-8, which was originally called FADD-like Interleukin-1β-converting enzyme (FLICE) [28]. Procaspase-8 binds through its death effector domain (DED) to FADD and becomes activated to caspase-8 (figure1.2). TRADD activates caspase-8 indirectly by association with FADD. Eventually, activated caspase-8 converts procaspase-3 into the effector caspase-3 which seems to be essential for the typical morphology of apoptosis [29].

1.2.3.2. Mitochondrial mediated mechanism

The receptor-ligand mediated apoptotic pathway is not the only mechanism in the apoptotic process. Certain cytotoxic agents, such as nitrogen monoxide and radiation,



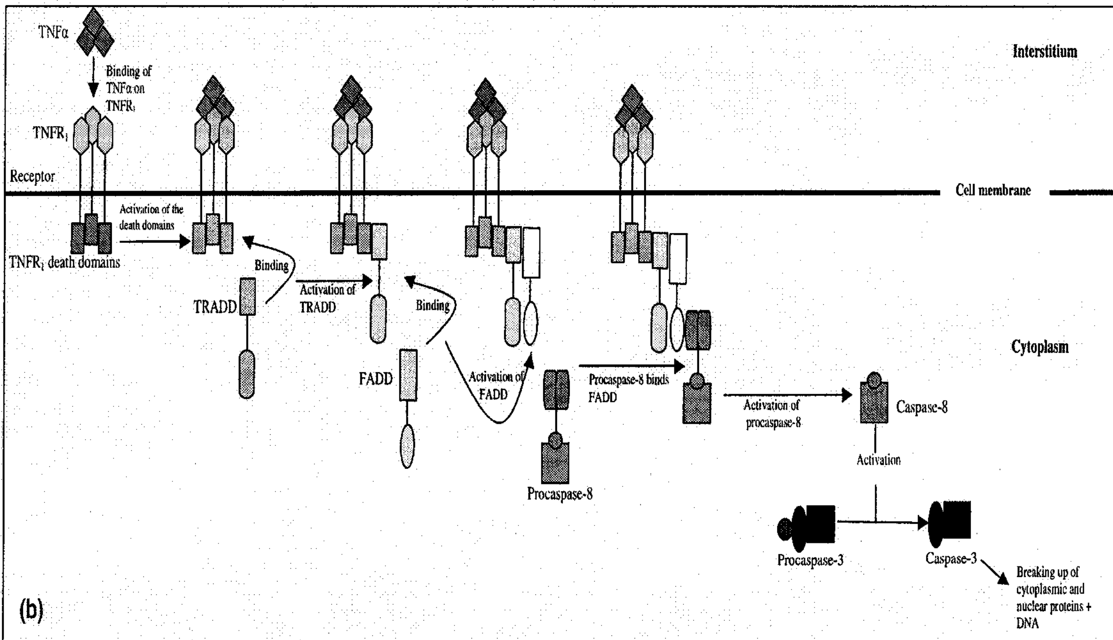


Figure 1.3 Receptor-ligand mediated mechanism in the apoptosis Process. (a) Schematic representation of caspase-3 activation by Fas ligand–Fas binding. (b) Schematic representation of caspase-3 activation by binding of TNFα to TNFR1. (Reproduced from *Morphological and Biochemical Aspects of Apoptosis, Oncosis and Necrosis* Anat.Histol.Embryol. 31, 214–223 (2002) S. Van Cruchten and W.V an den Broeck)

cause apoptosis in another manner involving the mitochondria and more specifically the mitochondrial protein cytochrome c [30], [31] (Figure1.4a). Cytochrome c is localized on the outer aspect of the inner mitochondrial membrane and in the intermembrane space [32] where it participates in the intracellular electron transport chain reaction for the production of ATP. During the apoptotic process cytochrome c is released into the cytosol [32]. There it binds to the Apoptosis protease activating factor (Apaf-1) forming a complex together with dATP. This complex subsequently activates procaspase-9 to caspase-9 [33], which in turn activates procaspase-3 leading to the characteristic morphological consequences of apoptosis.

Most authors agree that this release of cytochrome c into the cytosol is regulated by proteins of the Bcl-2 (B-cell lymphoma) family. Bcl-2, Bcl-x_L, Bcl-x_S, Bax and Bad are members of this family. Bcl-2 is an apoptosis inhibitor which is found in vertebrates. *Bcl-x* codes for two different proteins namely Bcl-$x_{L(\mathrm{ong})}$ and Bcl-$x_{S(\mathrm{hort})}$. Bcl-x_L is analogous to Bcl-2 in length and function, and also suppresses apoptosis. Bcl-x_S is 63 amino acids shorter and has lost its anti-apoptotic function. This protein is a competitor for Bcl-2 and Bcl-x_L [34]. The proteins of the Bcl-2 family are mainly localized in the outer mitochondrial membrane. The ratio of the Bax proteins to the Bcl-2/Bcl-x_L proteins will determine whether cytochrome c is released or not.

Recent studies suggest that the mitochondrial mechanism and the receptor-ligand mechanism do not act completely independently of each other (Figure1.4b). Caspase-8 which is involved in the receptor-ligand mediated apoptosis can activate the protein Bid, a member of the Bcl-2 family which is localized to the outer mitochondrial membrane.

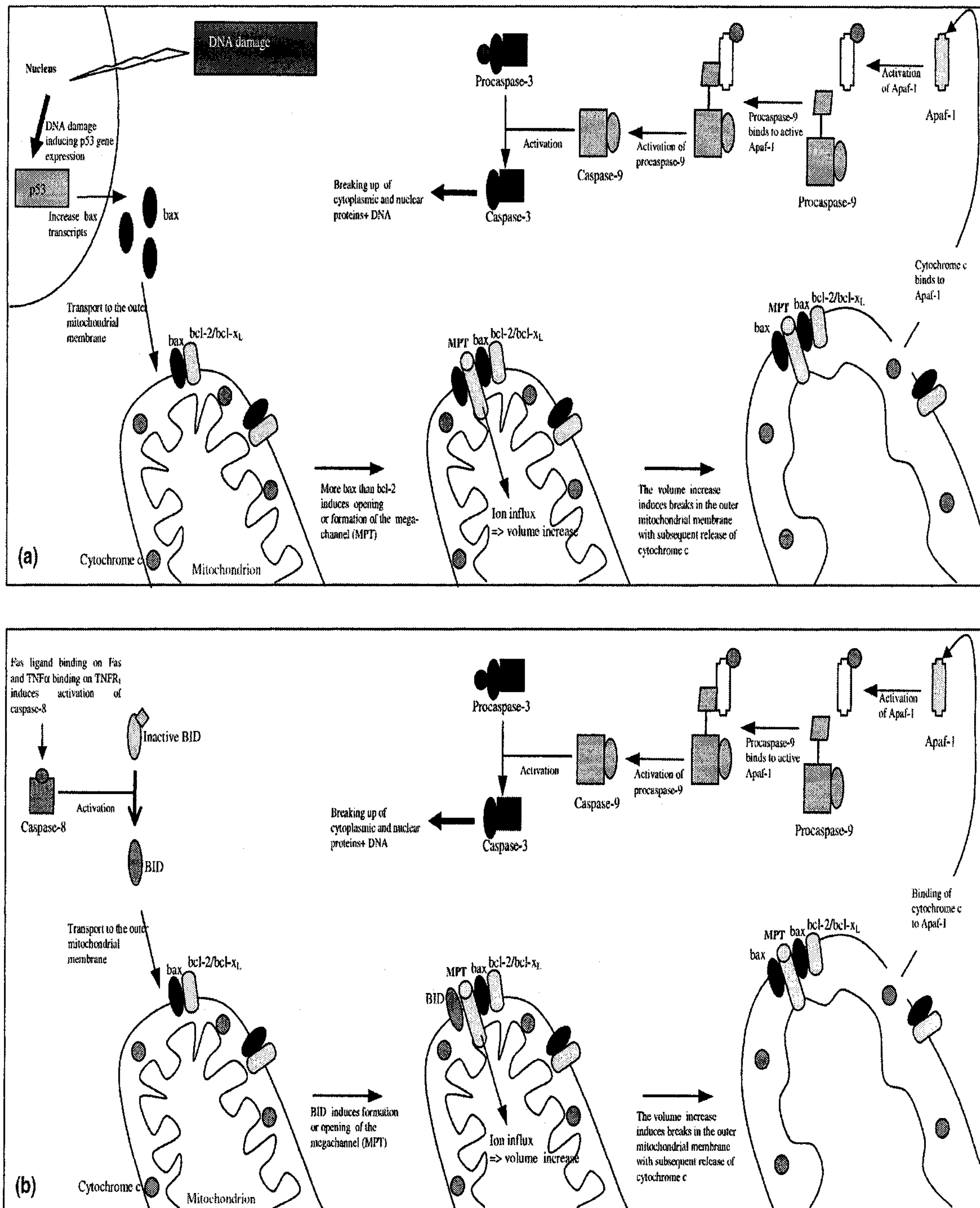


Figure 1.4. Mitochondrial mechanism of apoptosis (a) Schematic representation of the mitochondrial apoptotic mechanism induced by DNA damage. (b) Schematic representation of the mitochondrial apoptotic mechanism induced by caspase-8. (Reproduced from *Morphological and Biochemical Aspects of Apoptosis, Oncosis and Necrosis* Anat.Histol.Embryol. 31, 214–223 (2002) S. Van Cruchten and W.V an den Broeck)

Activation of Bid leads to the release of cytochrome c with subsequent activation of caspase-9 [35]. Bid might also interact with Bax [36], but further research is necessary to clarify these mechanisms.

1.3 TRAIL

1.3.1 TRAIL: structure and interaction with its receptors

TNF-related apoptosis inducing ligand (TRAIL) is a recently identified member of the TNF family, which is biologically distinct from TNFα and FasL. TRAIL is expressed as a type II membrane protein of 281 amino acids with a short intracellular amino-terminal tail and long extracellular carboxy-terminal domain (amino acids 114-281) [37, 38] (Figure 1.5). The extracellular domain can be proteolyticly cleaved to yield a soluble, biologically active form [39].

Similar to FasL and TNFα in both sequence homology and signaling characteristics, TRAIL forms a homotrimer [40] that binds three receptor molecules [41, 42]. This results in a receptor/ligand complex that initiates the signaling cascade [41, 42] (Figure 1.5). Optimal biological activity of TRAIL is dependent on the stability of the trimeric ligand and on the final tertiary configuration of the ligand complex [43]. Both the recombinant soluble form as well as the cell-associated form of TRAIL contains an unpaired cysteine residue (Cys^{230}) in their receptor-binding domain. The presence of Cys^{230} is critical for maintaining activity of the ligand as it can either bind another Cys to form a disulfide bridge or it can chelate one Zn atom per TRAIL trimer unit [43]. If the Cys forms a disulfide bond, there is reduced affinity of TRAIL for DR5 and the resulting

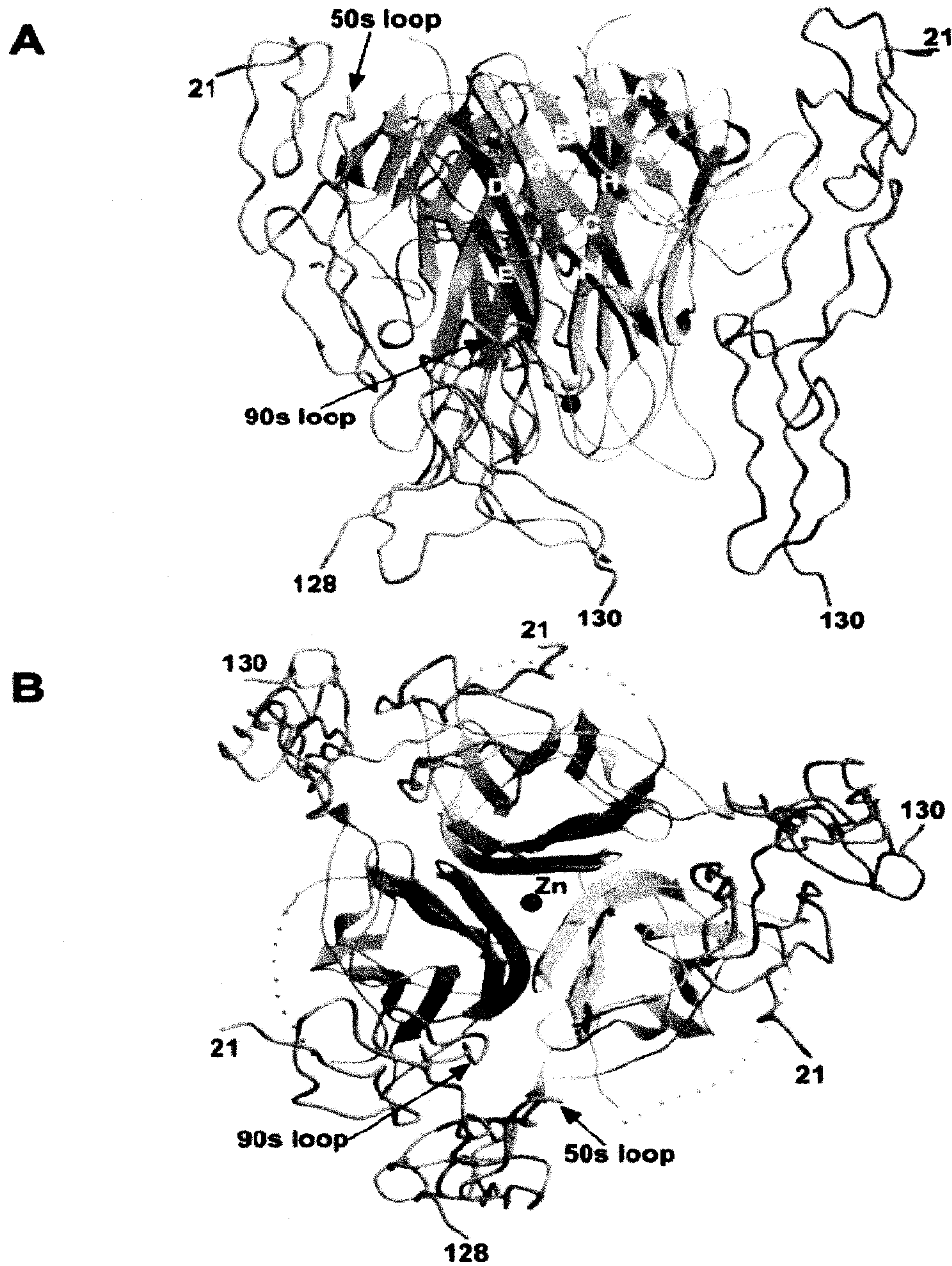


Figure 1.5 The TRAIL-DR5 complex The TRAIL trimer is drawn as ribbon rendering in gradations of blue, and the three receptors are rendered as tubes in yellow and orange colors. The bound zinc atom is colored green, and the bound chloride ion is pink. β strands and relevant loops are labeled. (A) Side view. In this orientation, the membrane of the receptor-containing cell is at the bottom of the figure. (B) View down the 3-fold axis of the complex, perpendicular to (A). (Reproduced from *Triggering Cell Death: The Crystal Structure of Apo2L/TRAIL in a Complex with Death Receptor 5* Molecular Cell, Vol. 4, 563–571, October, 1999, Sarah G. Hymowitz et al.

TRAIL was found to be poorly active [43]. Alternately, when a Zn atom was bound to a TRAIL trimeric unit, the resulting ligand retained its biological activity suggesting a role for Zn in stabilizing the ligand configuration for receptor binding [43]. Because of this specific interaction, the amount of Zn is important in determining the final selectivity and cytotoxicity in recombinant soluble TRAIL used in research and clinic [44].

Toxicity of different versions of recombinant TRAIL to hepatocytes varies depending on the preparation of the ligand [45-48]. Hepatocyte toxicity [45, 46] was reported for TRAIL prepared in the absence of Zn. It has been suggested that Zn depletion reduces solubility of the recombinant product and causes aggregation [44]. In contrast, recombinant soluble, non-tagged versions of TRAIL produced in the presence of Zn, had no adverse cytotoxic effects on human and non-human primate hepatocytes [46, 47] and keratinocytes [48]. In addition, certain forms of aggregated, poly-histidine tagged, and antibody crosslinked TRAIL can trigger apoptosis and cytotoxicity in normal cell types such as astrocytes [47, 49], keratinocytes [48], hepatocytes [45] and neurons [47]. Previous studies in our lab found that non-tagged recombinant human TRAIL proteins are saturated with zinc ions and are therefore present as homotrimers [50], whereas the tagged hepatotoxic TRAIL [45] is zinc ion poor, leading to the formation of heterodimers [46]. The heterodimers tend to aggregate and thus surpass the high threshold for TRAIL-induced signals in normal human cells [51] (Table 1).

Table1. TRAIL is the only member of TNF family that contains a metal ion: Zn

Form	Zn binding	Structure	Solubility	Biological activity	Toxicity to normal tissue
Non-tagged TRAIL	Good	Homogeneous	High	Good	Non-toxic
Tagged TRAIL	Poor	Heterogeneous	Low	Poor	Toxic

Notes:

Homogeneous: 99%trimer
Heterogeneous: 79%trimer (56%contained intersubunit disulfide)
21%free dimers (all containing disulfide)

1.3.2 The TRAIL receptor family

Five receptors have been identified that bind TRAIL. There are the two death receptors DR4 (TRAIL-R1) [52] and DR5 (TRAIL-R2/TRICK2/KILLER) [53], two decoy receptors Decoy receptor 1 (DcR1, TRAIL-R3/TRID) [53], decoy receptor 2 (DcR2, TRAIL-R4/TRUNDD/LIT) [53, 54], and a soluble receptor for TRAIL, osteoprotegrin (OPG) [55, 56].

1.3.2.1 DR4 and DR5

Two receptors DR4 [52] and DR5 [53], are type I transmembrane proteins and are implicated in pro-apoptotic signaling. They both contain a conserved death domain (DD) motif in their cytoplasmic tails and upon binding to TRAIL are responsible for transducing the death signal to the intracellular machinery (Figure 1.6). Overexpression of the two death receptors has been found to induce apoptosis independent of ligand binding due to oligomerization of the DDs, whereas deletion of the death domains on both receptors blocks the TRAIL death signal [52, 57].

1.3.2.2 DcR1 and DcR2

DcR1 [53] and DcR2 [53, 54] possess extracellular domains that have close sequence homology to the extracellular domains of DR4 and DR5. However, these receptors are unable to transduce a death signal upon binding to TRAIL and their overexpression is believed to inhibit TRAIL-induced apoptosis [57]. DcR2 contains a truncated death domain motif and DcR1 is a glycophosphatidylinostil (GPI) -anchored protein that lacks a cytoplasmic tail altogether [53, 54, 57] (Figure 1.5). As a result, both receptors lack a functioning death domain motif which is required to communicate a

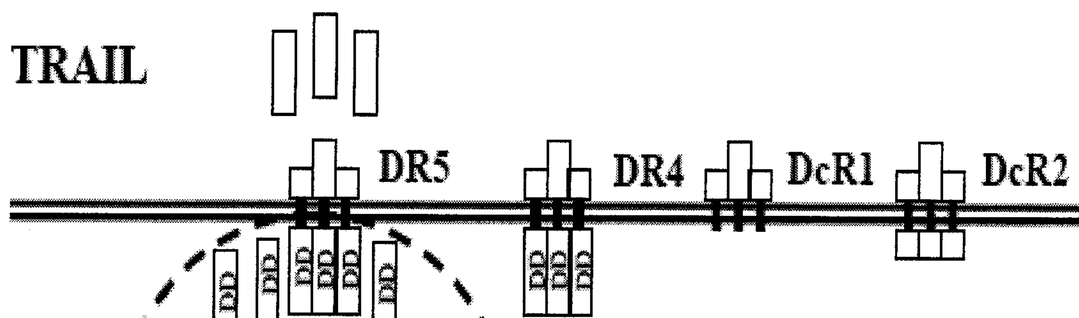


Figure 1.6 Schematic representation of TRAIL death receptor DR4 and DR5 and decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2). Two receptors DR4 and DR5, are type I transmembrane proteins and are implicated in pro-apoptotic signaling. They both contain a conserved death domain (DD) motif in their cytoplasmic tails. DcR2 contains a truncated death domain motif and DcR1 is a glycophosphatidylinostil (GPI)-anchored protein that lacks a cytoplasmic tail altogether. Therefore these two decoy receptors lack a functioning death domain motif and can not transduce death signaling into the cell.

death signal into the inside of the cell. High levels of mRNA for DcR1 and DcR2 have been found in normal cells including PBLs, spleen, heart, lung, kidney, liver, bone marrow, and placenta [53, 57, 58]. In contrast, low levels of both decoy receptors have been detected in transformed cells lines and their overexpression in sensitive cell lines confers resistance to TRAIL induced apoptosis [53, 54, 57]. Hence, it is hypothesized that DcR1 and DcR2 compete with DR4 and DR5 for the binding of TRAIL and thereby protect normal cells from TRAIL-induced apoptosis.

1.3.2.3 OPG

A soluble receptor for TRAIL, osteoprotegrin (OPG) [55, 56] has also been identified but its physiologic role as a TRAIL receptor has not firmly been established because of its low affinity for TRAIL at 37°C [59]. Initial experiments have outlined the role of OPG in regulating bone resorption and bone mass by preventing osteoclast differentiation [55]. However, a recent study showed that OPG may play an important role in survival of hormone-resistant prostate cancer cells against TRAIL-induced apoptosis [60].

1.3.3 TRAIL signalling events

1.3.3.1 TRAIL-induced extrinsic pathway of apoptosis

As noted in section 1.2.3, there are two well recognized signaling pathways that control the initiation of apoptosis: the extrinsic pathway through the death receptors [61] and the intrinsic pathway through mitochondria [62]. Both extrinsic and intrinsic pathways are required for TRAIL-induced apoptosis (Figure 1.7). The extrinsic pathway

is initiated by the binding of TRAIL to its cognate death receptors DR4 [52, 54, 57, 63] and DR5 [53, 64-66]. Upon binding to TRAIL, DR4 and DR5 recruit the intracellular adaptor protein Fas-associated death domain (FADD) [63, 65] through DD domains forming the death-inducing signaling complex (DISC). FADD contains a carboxy-terminal DD and an amino-terminal death effector domain (DED) [25, 67]. Procaspase-8 is recruited to DISC through homophilic interactions between DED domains on FADD and caspase-8 [68, 69].

Procaspase-8 has two DED prodomains and one caspase domain (Figure 1.2 B) [68, 70]. In the DISC, close proximity of procaspase-8 to other procaspase-8 molecules leads to their activation through autocatalytic cleavage [22], to form activated caspase subunits in a two-step mechanism [70] (Figure 1.2 B). In the first step, precursor or procaspase forms, p55 and p53, are cleaved into p43 and p12 fragments [70]. The p12 fragment undergoes further processing to generate an active p10 fragment [70]. The p43 fragment is also cleaved into a smaller p26 fragment which subsequently produces an active p18 fragment [70, 71]. Both active subunits of caspase-8, p10 and p18, are released into the cytosol where they can activate downstream effector caspases [68, 71] such as caspase-3. Caspase-3 is activated from p32 precursor through cleavage by activated caspase-8, into p20, p17 and p10 fragments [72]. Once activated, caspase-3 can then induce and execute programmed cell death through the cleavage and activation of substrates that inhibit cell survival such as DNA fragmentation factor (DFF) [73].

Procaspase-10 has a homologous structure to procaspase-8 and it can be recruited to and cleaved in the DISC to initiate apoptosis [74-77]. However, many cancer cells do

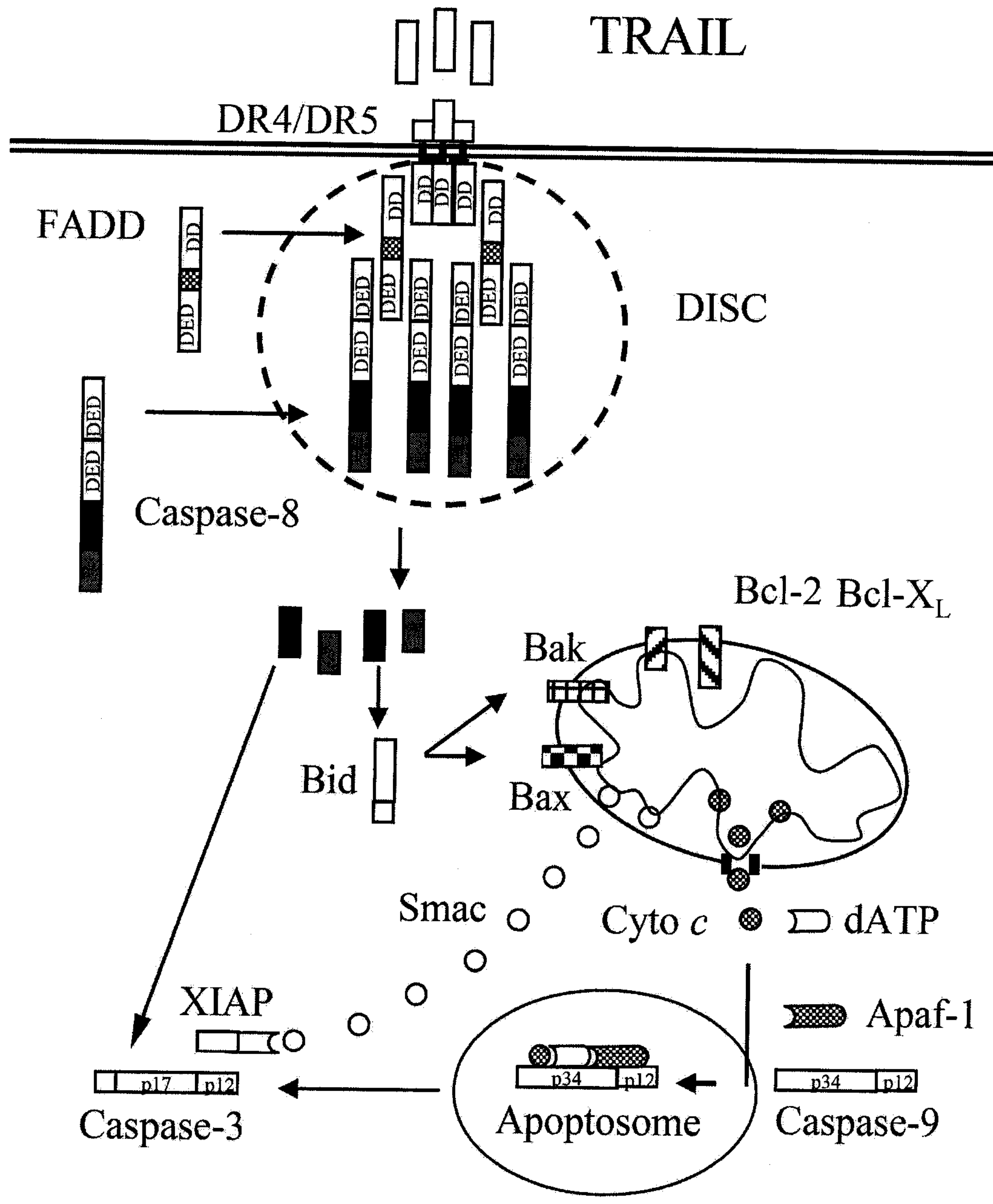


Figure 1.7 TRAIL-induced apoptosis signaling pathways. TRAIL-induced apoptosis occurs through both receptor- DISC mediated extrinsic and mitochondria-involved intrinsic pathways.

not express caspase-10 [74] and therefore caspase-8 is the most common initiator of death receptor-mediated apoptosis [61].

1.3.3.2 TRAIL-induced activation of the intrinsic mitochondrial pathway

Recent studies have shown that many cancer cells express the X-linked inhibitor of apoptosis protein (XIAP) [78, 79] which inhibits caspase-8-mediated cleavage of caspase-3 [80]. In these cells, however, the extrinsic pathway can be rescued via caspase-8-mediated cleavage of Bcl-2 inhibitory BH3-domain protein (Bid) [35] (Figure 1.7). Bid induces mitochondrial release of second mitochondria-derived activator of caspase /direct inhibitor of apoptosis binding protein with low pI (Smac/DIABLO) [81, 82]. Smac releases the XIAP-dependent inhibition of caspase-3 [83]. Bid also stimulates release of cytochrome *c* from mitochondria into the cytosol [84], where cytochrome *c* assembles with Apaf-1, dATP and caspase-9 into an apoptosome. In the apoptosome, caspase-9 is activated and in turn cleaves caspase-3 [33]. Studies have shown that TRAIL-induced apoptosis occurs through both these extrinsic and intrinsic pathways in human glioma [85] and melanoma cell lines [86].

1.3.4 TRAIL- mediated immune surveillance and its therapeutic potential against cancers

Expression of TRAIL mRNA has been detected in a variety of tissues [37]. Significantly, the cell surface expression of TRAIL protein ligand itself has been reported in natural killer (NK) cells [87-89] and contributes to NK cell-mediated protection of mice from tumor development and metastasis [88]. More importantly, TRAIL-deficient mice have been shown to be more susceptible to tumor development

[90]. In that study, researchers examined the role of TRAIL during the primary tumor development induced by a chemical carcinogen methylcholanthrene (MCA). Others have previously shown that MCA induction of fibrosarcomas is dose dependent and is primarily controlled by NK cells [91], NKT cells [92], and the effector molecules, perforin and interferon- γ (IFN-γ) [93]. Wild type (WT) and TRAIL-deficient mice were inoculated with MCA in doses ranging from 5 to 400 μg [90]. Although injection of 400 μg of MCA induced fibrosarcomas in almost all WT or TRAIL-deficient mice, there was an earlier onset of fibrosarcomas in the TRAIL-deficientmice. As the dose of MCA was reduced, a difference in tumor onset and the susceptibility of WT and TRAIL-deficient mice to tumor development was demonstrated. Notably, 100 μg of MCA induced fibrosarcomas in 7 of 10 TRAIL-deficient mice, but only in 6 of 30 WT mice, and tumor onset was earlier in TRAIL-deficient mice. These data clearly supports a suppressor function of TRAIL against primary tumor development *in vivo*. Furthermore, they demonstrated a substantial contribution of TRAIL to NK cell-mediated protection from tumor metastasis and in suppressing primary 4T1 tumor growth in the mammary gland. These studies suggest a role for TRAIL in immune surveillance against tumors *in vivo*.

Studies *in vitro* have shown that recombinant TRAIL induces the selective death of cancer cells while sparing most normal cells [90, 94]. In one study, regulation of TRAIL sensitivity in primary and transformed human keratinocytes was investigated [94]. Although TRAIL induced apoptosis in primary as well as transformed keratinocytes, a marked difference in sensitivity was observed. Primary keratinocytes (PK) were 5-fold less sensitive to TRAIL than transformed keratinocytes (TK) indicating that although PK contain all the necessary signaling components required for TRAIL-induced apoptosis

they nevertheless require higher concentrations of TRAIL than TK for efficient killing. Pre-clinical experiments in mice and non-human primates have also shown that systemic administration of recombinant human TRAIL inhibits tumor growth with little toxicity to normal tissues [49, 95]. These studies have placed TRAIL in the limelight of chemotherapy development [96, 97].

1.3.5 Mechanisms of resistance to TRAIL-induced apoptosis in cancer

Although TRAIL is regarded as a potential anticancer agent, resistance to TRAIL-mediated apoptosis induction in cancer cells remains a challenging obstacle to the use of TRAIL as a therapeutic agent. Many types of cancers are sensitive to TRAIL-induced apoptosis, but substantial numbers of cancer cells are resistant to TRAIL, especially some highly malignant tumors such as pancreatic cancer [98], melanoma [99] and neuroblastoma [100]. Although the detailed mechanisms underlying TRAIL-induced apoptosis remain to be characterized, some important components and steps in the signaling pathways of this process have been elucidated.

1.3.5.1 TRAIL receptors

Dysfunction of the death receptors DR4 and DR5 due to mutations could cause TRAIL resistance. The genes for both DR4 and DR5 have been mapped to chromosome 8p21−22, a segment noted in genome-wide searches to be one of the most common sites of loss of heterozygosity (LOH) in several types of cancers. An A-to-G alteration at nucleotide 1322 of DR4 in the human ovarian cancer cell line SKOV3 and the human bladder cancer cell line J82 [101] results in substitution of an arginine for lysine at codon 441 (K441R) in the DD of DR4. These two amino-acid changes occurred in or near the

ligand-binding domain of DR4, suggesting that these changes may cause abnormal death receptor trimerization or TRAIL binding [102]. Mutations in the DR5 gene have been identified in head and neck cancer, non-small-cell lung cancer, breast cancer, non-Hodgkin's lymphoma, and hepatocellular carcinoma. Mutated DR5 may bind with normal DR5 proteins to form a structurally abnormal DR5 trimer, which could affect binding to adaptor proteins, such as FADD [103], leading to suppression of apoptosis [104].

1.3.5.2 Bcl-2 family

During the process of TRAIL-induced apoptosis, activation of the initiator caspase-8 can transmit death signals either through direct activation of the effector caspase-3, or by mitochondrial pathway involving cleavage of the proapoptotic Bcl-2 family member Bid [105]. In this mitochondrial pathway, the ratio of expression of the proapoptotic Bax protein and the antiapoptotic Bcl-2 or Bcl-X_L proteins ultimately determines cell death or survival [106, 107].

Bcl-2 and Bcl-X_L

Overexpression of Bcl-X_L or Bcl-2 can protect some types of cells against TRAIL-mediated apoptosis, suggesting that the mitochondrial pathway predominates in these types of cells. Bcl-X_L expression correlated inversely with sensitivity to TRAIL-induced apoptosis in three pancreatic adenocarcinoma cell lines [98]. The cell line Colo357, originally sensitive to TRAIL and expressing low levels of Bcl-X_L, became resistant to TRAIL after Bcl-X_L expression was restored by means of a retroviral vector. As apoptosis induced by chemotherapy acts mainly through the mitochondrial pathway [105], downregulation of Bcl-2 or Bcl-X_L might restore sensitivity not only to chemotherapy but also to TRAIL in some types of cancer.

Bax and Bak

Lack of the proapoptotic genes of Bax or Bak can render cancer cells resistant to apoptosis induced by TRAIL or chemotherapy. In one study, Bak-deficient Jurkat cells were more resistant than wild-type Jurkat cells to apoptosis induced by TRAIL, UV, staurosporin, VP-16, bleomycin, or cisplatin. Restoring the Bak gene restored cytochrome *c* release and the sensitivity of the Bak-deficient cells to VP-16 [108].

1.3.5.3 Inhibitors of apoptosis (IAP) proteins

IAP proteins can block apoptotic events by inhibiting the catalytic activity of effector caspases (e.g., -3 and -7) or by blocking the activation of the apoptosomal caspase-9 by directly interacting with the active sites of these caspases. So far, six mammalian IAPs have been identified: cIAP1, cIAP2, X-linked inhibitor of apoptosis (XIAP), neuronal apoptosis inhibitory protein (NAIP), survivin, and BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE). XIAP is the most potent inhibitor of caspase activity [109, 110]. High expression of IAPs in cancer cells can confer resistance to TRAIL-induced apoptosis [111, 112].

1.3.5.4 DISC

Assembly of the DISC is an early molecular event in the signaling pathway of TRAIL-induced apoptosis. Several molecules, including TRAIL, TRAIL death receptors, FADD, caspase-8 or caspase-10, and c-FLIP, participate in the formation of the DISC. Most components of the DISC are essential to TRAIL-induced apoptosis, and dysfunction in any of these DISC components can lead to TRAIL resistance [100, 105], which indicates that the DISC is the critical regulator of TRAIL-induced apoptosis signalling pathway.

FADD

FADD is an adaptor molecule containing both DD and DED motifs. Its essential role in death signal transduction was first identified in FasL-induced apoptosis. Through its interactions with activated Fas at the DD and caspase-8 at the DED, FADD transmits a death signal from Fas to caspase-8 [69]. There is evidence that defects in FADD can lead to TRAIL resistance. FADD$^{-/-}$ embryonic fibroblasts stably transfected with mouse or human TRAIL death receptors were all resistant to TRAIL-induced cell death, but FADD$^{+/-}$ fibroblasts stably transfected with mouse TRAIL death receptors, and FADD$^{-/-}$ fibroblasts in which FADD had been reconstituted with a retroviral construct, were all sensitive to TRAIL-induced apoptosis [113]. This data suggests that FADD is essential in TRAIL-induced apoptosis and that dysfunction of FADD can cause TRAIL resistance, a finding supported by another study that used Jurkat cells [114]. In that study, FADD$^{-/-}$ Jurkat cells were resistant to TRAIL even at very high concentrations (1μg/mL), but wild-type Jurkat cells underwent extensive apoptosis at TRAIL concentrations as low as 10 ng/mL.

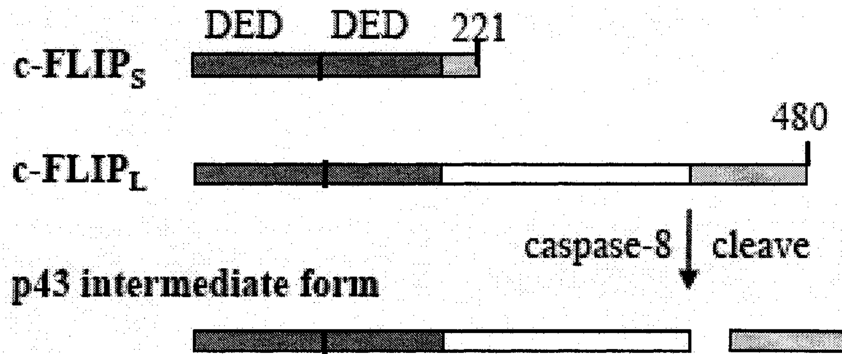
Caspase-8

Convincing evidence is accumulating, which shows caspase-8 to be a key and irreplaceable molecule in TRAIL-induced as well as Fas and TNF-α-induced apoptosis [76, 115, 116]. Down-regulation or loss of caspase-8 expression can lead to TRAIL resistance [99, 100]. This has been demonstrated using caspase-8-deficient Jurkat cells. The deficient cells were shown to be completely resistant to TRAIL, whereas the corresponding wild-type cells remained sensitive to TRAIL [116]. In addition, treatment with the specific caspase-8 inhibitor Z-IETD-FMK prevented TRAIL-induced apoptosis

in most TRAIL-sensitive cell lines [116, 117]. Other evidence implicating caspase-8 include the observation that several types of cancer cells, including neuroblastoma [100, 118], melanoma [119], and small-cell lung cancer [120], show resistance to TRAIL-induced apoptosis that correlates with down-regulation or absence of caspase-8 expression. Importantly, restoration of caspase-8 expression in these resistant cells rendered them sensitive to TRAIL [99, 100, 118, 120, 121].

cFLIP

Cellular FADD-like, IL-1β-converting enzyme-inhibitory protein (c-FLIP) was originally identified as a DED protein that is recruited to the Fas-DISC by FADD [122] where it modulates caspase-8 recruitment and activation [74]. The gene encoding c-FLIP consists of 13 exons and is found between the genes encoding caspase-8 and caspase-10 on chromosome 2 [123, 124]. The c-FLIP gene is alterativly spliced into four mRNA variants but only two forms of the protein have been detected [122, 125, 126]. The short form of c-FLIP (c-$FLIP_S$) has a molecular weight of 28 kDa and contains two DED domains [122]. In contrast, the long form of c-FLIP (c-$FLIP_L$) has a molecular weight of 55 kDa and contains two DED domains and a caspase-like domain [122] (Figure 1.8). This caspase-like domain of c-$FLIP_L$ lacks catalytic activity as a critical tyrosine residue in the active site is substituted with a cysteine [122]. Both forms of c-FLIP have been detected in many different tissues while the short form is predominantly found in lymphatic tissue [122, 123]. More recent studies show that both forms of c-FLIP are recruited to TRAIL and Fas-DISC where c-$FLIP_L$ is cleaved into a p43 intermediate form



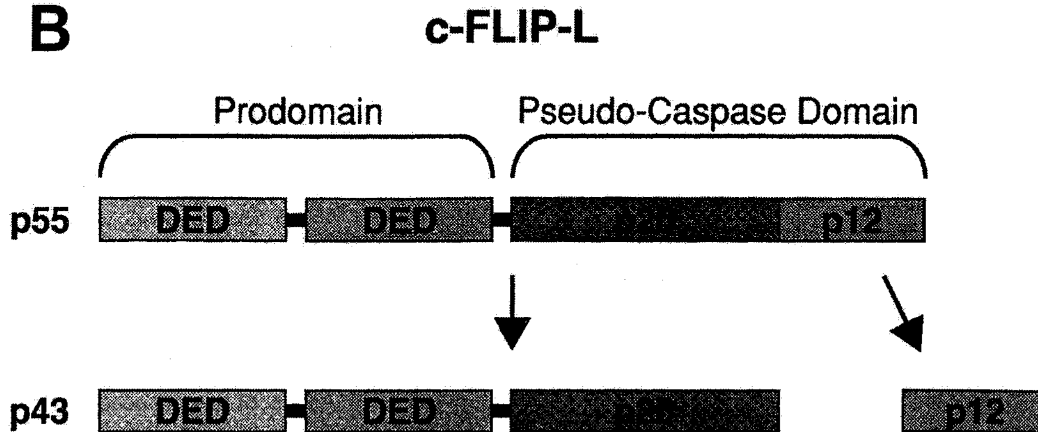


Figure 1.8 A. Schematic representation of c-FLIP structure. The short form of c-FLIP (c-FLIP$_S$) contains two DED domains (dark grey bar). In contrast, the long form of c-FLIP (c-FLIP$_L$) contains two DED domains(dark grey bar) and a caspase-like domain(white bar). c-FLIP$_L$ is cleaved into a p43 intermediate form by caspase-8 on the DISC. **B. Structure of c-FLIP$_L$ and cleavage mechanism.** Cleavage sites and the size of cleavage products are indicated. (Reproduced from Vitamines and Hormones, Volume 67, 192 (2004))

[125, 127] (Figure 1.8). Both c-FLIP$_S$ and p43 c-FLIP$_L$ remain in the DISC, where they bind to either FADD or caspase-8 through DED–DED interactions, resulting in inhibition of caspase-8 activation and inhibition of apoptosis [127].

c-FLIP is known to be an important antiapoptotic protein modulating TRAIL-DISC resistance in some cancer cell lines [128-130]. High levels of expression of c-FLIP were recently reported to correlate strongly with TRAIL resistance and malignant potential in colonic adenocarcinomas [130], melanoma [128], and hepatocellular carcinoma [129]. This is supported by the finding that downregulation of cFLIP expression using antisense RNA or siRNA can sensitize TRAIL resistance in types of cancer in which cFLIP overexpression is a key determinant of TRAIL resistance [131].

1.4 TRAIL and chemotherapeutic drugs in cancer therapy

1.4.1 Synergistic effects of chemotherapy drugs on TRAIL-induced apoptosis

The selective cytotoxicity of TRAIL for tumor cells, its involvement in NK cell-mediated cytotoxicity and the absence of toxic side effects when given *in vivo*, have made TRAIL a prime candidate as a tumor treatment. However, a large percentage of tumor cell lines are resistant to TRAIL-induced apoptosis [132-134]. Many approaches have been employed to overcome TRAIL resistance in cancer cells, notably by a combination of TRAIL therapy with chemotherapy, such as doxorubicin (DOX), cisplatin (CDDP), camptothecin (CPT), actinomycin D (Act D), and 5-FU. These agents enhance TRAIL-mediated apoptosis through a number of diverse molecular mechanisms (Table 2) which are reviewed below.

1.4.2 Molecular mechanisms of the synergistic effect

Although the exact molecular mechanisms of the synergistic effect of TRAIL and chemotherapy drugs have yet to be clarified, various studies have shown that the two therapies converge at the induction of apoptosis. For example, these agents interact with DNA to form intra-strand cross-link adducts that may activate several intracellular signal pathways including apoptosis [135]. Recently, some studies have suggested an alternative possibility, e.g. the chemotherapy agents at low non-toxic doses may target key regulatory proteins in the TRAIL signaling pathway, causing synergistic effects with TRAIL-induced apoptosis in cancer cells [85, 86] [136-148]. These studies are summarized in the table below (Table 2):

Table 2. Synergistic effect of TRAIL and chemotherapy drugs

Drugs	Effects	Cell types
Cisplatin	Induction of DR5 expression Activation of caspase-8 Cleavage of Bid Down-regulation of c-FLIPs expression Activation of caspase-3 Up-regulation of Bax expression Release of cytochrome *c*	Glioma cell lines [144] Colorectal cancer cells [141] Colorectal cancer cells [141] Glioma cell lines [85, 86] Ovarian cancer cells [149] Bladder cancer cells [150] Glioma cell lines [85] NSCLC cells
Camptothecin	Induction of DR5 expression Down-regulation of c-FLIPs expression Up-regulation of Bax expression	Glioma cell lines [85] Glioma cell lines [85] Glioma cell lines [85]
Doxorubicin	Induction of DR5 expression Activation of caspase-8, -3 Cleavage of Bid Activation of caspase-8 Cleavage of Bid Up-regulation of Bax expression Activation of caspase-3	Leukemia cell lines [151] Prostate cancer cells [152] Prostate cancer cells [153] Colorectal cancer cells [141] Colorectal cancer cells [141] Bladder cancer cells [150] Ovarian cancer cells [149]
Actinomycin D	Reduced Bcl-x_L expression Inhibition of xIAP Release of cytochrome *c* Down-regulation of c-FLIPs expression	Sarcoma [154] Prostate cancer cells [112] Prostate cancer cells [112] Colorectal cancer cells [155]
Paclitaxel	Induction of DR5 and DR4 expression Activation of caspase-3 Inhibition of Akt kinase	Prostate cancer cells [145] Ovarian cancer cells [149] Renal carcinoma cells [136]
Geldanamycin PS-341(the proteasome inhibitor bortezomib)	Blocks NF-κB activation Suppression of the Akt/PKB pathway Down-regulation of Bcl-x_L and Bcl-2 expression	Pancreatic cancer cells [8]
Sodium butyrate	Reduced Bcl-x_L expression Down-regulation of c-FLIPs expression	Sarcoma [154] Colorectal cancer cells [155]
Etoposide	Induction of DR5 expression Induction of DR5 and DR4 expression	Leukemia cell lines [151] Glioma cell lines [144] Breast cancer cells [138]
Ara-C	Induction of DR5 expression	Leukemia cell lines [151]
5-FU	Up-regulation of Bax expression Enhanced p53 expression	 Renal carcinoma cells [156]

1.4.3 Anti-cancer drugs and TRAIL-induced apoptosis in this study

In this study, we have chosen to investigate cisplatin and camptothecin as well as other anti-cancer agents with well described apoptosis induction properties, in order to find more effective therapy and investigate the mechanisms of overcoming resistance to TRAIL-induced apoptosis in pancreatic cancer.

Cisplatin (CDDP), **camptothecin** (CPT) are the common conventional chemotherapy drugs for gastrointestinal solid tumors. They have varied anti-cancer activities including the following pro-apoptotic functions. CDDP has been reported to up-regulate DR4 and DR5 mRNA expression in glioma cells, resulting in additive cytotoxicity with TRAIL [144]. Previous research in our lab using TRAIL resistant melanoma cell lines found that CDDP or CPT treatment down-regulated c-FLIPs protein expression overcoming TRAIL resistance [86]. Recent studies have provided additional evidence for combined approaches to restore TRAIL sensitivity through mitochondrial signal transduction pathways. CPT, for instance, was reported to up-regulate Bak [142], whereas CDDP increased mitochondrial membrane potential facilitating cytochrome *c* release [137], thus resulting in an additive effect on TRAIL-induced apoptosis.

Celecoxib (COX-2 inhibitor), a cyclooxygenase 2 inhibitor, was the first cyclooxygenase 2–selective nonsteroidal anti-inflammatory drug (NSAID) approved for the treatment of adult arthritis. Celecoxib exerts potent chemopreventive activity in chemical carcinogen–induced colon, bladder, and breast carcinomas [157-160] and UV-induced skin carcinogenesis [161]. Celecoxib is a clinically effective chemoprevention agent for colon cancer [162] and is being tested for its chemopreventive or therapeutic

activity against various cancers, including lung cancer, as a single agent or in combination with other agents.

Celecoxib has been found to have cytotoxic activity in tumor cells and tissues that lack the cyclooxygenase 2 enzyme [163-166]. Therefore, celecoxib appears to exert its chemopreventive and therapeutic activity through a mechanism that is independent of cyclooxygenase 2 inhibition. Celecoxib induces apoptosis in various cell types, and this activity may account for its chemopreventive and therapeutic activity [167, 168]. However, the mechanism by which celecoxib induces apoptosis remains largely uncharacterized and may differ depending on cell type. Celecoxib-induced apoptosis appears to be associated with inactivation of the protein kinase Akt in prostate, colon, and liver cancer cells [165, 169, 170] and with inhibition of endoplasmic reticulum Ca^{2+}-ATPases in prostate cancer cells [171]. Jendrossek et al. [172] reported that celecoxib induced apoptosis in Jurkat T cells via the mitochondria-mediated pathway independent of the death receptor, while in human NSCLC celecoxib mediated apoptosis occurred through the extrinsic death receptor pathway, through the induction of the expression of DR5 [173].

5-Aza-2′-deoxycytidine (ADC) is a DNA methyl transferase inhibitor which causes DNA demethylation or hemi-demethylation. As a nucleoside analogue, ADC incorporates into DNA and leads to a rapid loss of DNA methyltransferase activity thereby exerting direct cytotoxic and anti-proliferative effects on tumor cells [174]. Since tumor cells have an altered pattern of methylation of tumor-suppressor genes compared with those of normal cells [174], ADC-imposed inhibition of DNA methylation could contribute to the reversal of this altered gene regulation. Methylation is mainly dependent

on interference with DNA repair machinery and inhibition of *de novo* thymidine synthesis, but they also affect activation of pro-apoptotic signaling [174, 175].

Trichostatin A (TSA) is a potent reversible inhibitor of histone deacetylase (HDAC) activity. The nucleosomal complexes formed by the histone octamer and associated DNA are the fundamental organizational unit of eukaryotic chromatin [176, 177]. The reversible acetylation of the Σ-amino groups of lysine residues within the NH_2-terminal tails of core histones is important in the modulation of chromatin topology and regulation of gene transcription. Histone acetylation contributes to the formation of a transcriptionally competent environment by opening chromatin and allowing general transcription factors to gain access to the DNA template [178, 179]. On the other hand, histone deacetylation maintains chromatin in a transcriptionally silent state [180]. Trichostatin A is stable and can modulate gene transcription at micromolar concentrations *in vivo* in adult mice without major toxicity and does not perturb mouse embryonic or postnatal development [181]. Studies have shown that Trichostatin A induces differentiation in acute myeloid leukemia [182] and displays potent antitumor activity against prostate [183] and breast cancer cells *in vitro* and *in vivo* [184].

1.5 Purpose of the study

Pancreatic cancer, accounting for more than 30,000 deaths annually in the United States [1] and 3,300 deaths annually in Canada (Canadian Cancer Statistics, National Cancer Institute of Canada, 2004), is one of the most lethal cancers because there is no curative treatment currently available. TRAIL can selectively induce apoptosis in tumor cells *in vitro* and *in vivo* but not in most normal cells supporting its potential as an anti-cancer agent. However, its use as a single agent to treat pancreatic cancer is unlikely since many human pancreatic cancer cells are resistant to TRAIL. The molecular mechanism of the pancreatic cancer cells' resistance to TRAIL is largely unknown. The DISC is the critical regulator of TRAIL-induced apoptosis signalling, and modulation of the DISC by c-FLIP is a critical upstream event in this process. In this study, we will investigate the TRAIL-induced apoptosis signaling pathways in pancreatic cancer cells and the role of c-FLIP in TRAIL-DISC modulation.

The combination of TRAIL and chemotherapeutic drugs is potentially promising in the treatment of refractory cancers. Studies have suggested that concomitant chemotherapy drug treatment in pancreatic cancer may release the resistance in the downstream intrinsic pathways; however, none of these studies have examined the effects of the chemotherapy on the upstream DISC [7-9, 185]. The possible role of other critical molecules, particularly c-FLIP and caspase-8, in the resistance of pancreatic cancers to TRAIL needs to be more fully investigated. In this study, we investigate the molecular basis of the synergistic effect of TRAIL and anti-cancer drugs. This study could provide further insight into developing more effective therapy for pancreatic cancer.

1.6 Hypothesis

This thesis tests the following hypothesis:

1) TRAIL activates both the intrinsic and extrinsic pathways to induce apoptosis in sensitive pancreatic cancer cells. The DISC is the critical regulator of TRAIL-induced apoptosis signaling.
2) c-FLIP, by regulating the DISC, determines sensitivity to TRAIL-induced apoptosis.
3) Anti-cancer agents, by down-regulating c-FLIP, have additive effects on TRAIL-induced apoptosis.

Specific Objectives:

1) Define the sensitivity and signaling pathways for TRAIL-induced apoptosis of pancreatic cancer cell lines.
2) Establish the effect of c-FLIP modulation on TRAIL-induced apoptosis in human pancreatic cancer cells.
3) Test if chemo-modulation of c-FLIP can enhance TRAIL-induced apoptosis in human pancreatic cancer cells.

To clarify the role of c-FLIP in the modulation of DISC inhibition, c-FLIP was knocked out in a TRAIL resistant pancreatic cancer cell line using a siRNA silencing technique and over-expressed in a TRAIL sensitive pancreatic cell line using c-FLIP cDNA transfection. Treated cell lines were tested using a cell death assay for TRAIL-induced apoptosis and assessed for TRAIL-induced cleavage of the caspase cascade by Western blot analysis, and compared with untreated cell lines. Subsequently, to decrease c-FLIP expression and facilitate TRAIL curative function in cancer patients in the clinic,

some conventional chemotherapeutic drugs and anti cancer drugs were examined in search of the agents that could overcome the TRAIL resistance. The mechanisms of these agents which have synergistic effects on TRAIL sensitivity in resistant pancreatic cancer cells are investigated here.

CHAPTER 2

METHODS AND RESULTS

2.1 TRAIL-induced apoptosis in pancreatic cancer cells

2.1.1 Characterize pancreatic cell lines as sensitive or resistant to TRAIL-induced apoptosis.

Objective:

To characterize the sensitivity of several human pancreatic cancer cell lines to TRAIL-induced cytotoxicity.

Materials and Methods:

Cell lines:

Six pancreatic cell lines were purchased from the American Type Culture Collection (ATCC) (PANC-1, ASPC-1, PACA-2, BXPC-3, CAPAN-1, and CAPAN-2). Different cell lines were cultured under the following conditions (Table 3):

Table 3. Pancreatic cancer cell lines

(ATCC: American Type Culture Collection)

Cell Line	Description	Culture media
PANC-1	Pancreatic Carcinoma	1L DMEM w/ 1.5g Sodium bicarbonate, 10% (v/v) FBS
PACA-2	Pancreatic Carcinoma	1L DMEM w/ 1.5g Sodium bicarbonate, 10% (v/v) FBS , 2.5% (v/v) horse serum
BXPC-3	Pancreatic Adenocarcinoma	1L RPMI 1640, 10mM HEPES, 4.5g Glucose, 1.0 mM sodium Pyruvate, 1.5g sodium bicarbonate, 10% FBS
ASPC-1	Pancreatic Adenocarcinoma	1L RPMI 1640, 10mM HEPES, 4.5g Glucose, 1.0 mM sodium Pyruvate, 1.5g sodium bicarbonate, 10% FBS
CAPAN-1	Pancreatic Adenocarcinoma	IMDM w/ 0.15% sodium bicarbonate, 20% FBS
CAPAN-2	Pancreatic Adenocarcinoma	McCoy 5a w/ 10% FBS

Note: All media obtained from Invitrogen were supplemented with 1% penicillin/ streptomycin (Invitrogen). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Reagent:

Recombinant non-tagged, native sequence soluble form of human TRAIL (amino acids114–281) was obtained from PeproTech, Inc. (Rocky Hill, USA). Components of Acid Phosphatase (AcP) buffer solution containing 0.2 M Na-acetate [pH 5.5], 0.1% (v/v) Triton X-100 and 10 mM *p*-nitrophenyl phosphate (104 phosphatase substrate pNPP) were obtained from Sigma.

Cell viability assay:

Pancreatic cell number was assessed by the acid phosphatase assay as described previously [86, 186, 187]. This assay assesses cell viability by measuring cellular lysosomal acid phosphatase activity [188]. The substrate for the reaction is p-nitrophenylphosphate (pNPP) which, after ester hydrolysis by the enzyme, releases p-nitrophenol in addition to an inorganic phosphate [188]. The reaction is stopped by the addition of NaOH, which reacts with the p-nitrophenol product by removing the phenolic proton to produce p-nitrophenolate, a yellow colored product that absorbs at 405 nm and therefore can be quantitated spectrophotometrically [188]. A linear relationship was obtained between cell viability and acid phosphatase activity over a range of 10^3-10^5 cells (R^2= 0.996). The optimum plating density of cells was determined to be 1.0 x 10^4cells/well for maximum sensitivity of the assay.

Experimental procedure:

In performing the assay, 1.0 x 10^4 cells/well were seeded in 96-well plates overnight, and treated the following day (at about 50% confluency) with 100 μl of normal

growth medium containing (0 to 1000 ng/ml) TRAIL [85, 86] for 16 hours. After treatment, the media was removed and the cells were washed once with 100 μl of PBS and then 100 μl of AcP buffer solution was added in order to make the cells permeable to the pNPP substrate solution. Following incubation for 2 hour at 37°C, 10 μl of 1 M NaOH was added to each well to stop the reaction and the absorbance measured with a microplate reader (Bio-Rad, Mississauga, ON) at 405 nm. Eight wells were tested at each TRAIL concentration. All data is expressed as cell viability as a percentage of untreated cell viability.

Results:

The results are presented as the percentage of viable cells:

(optical density of cells treated / optical density at 405 nm of cells untreated) x 100 [189]. Two cell lines (PACA-2, BXPC-3) were grouped into **sensitive** (60-100% cell death at 100 ng/ml TRAIL), and four cell lines (PANC-1, ASPC-1, CAPAN-1, CAPAN-2) were **resistant** to TRAIL-induced apoptosis (no significant cell death at TRAIL concentration up to 100 ng/ml) (Figure 2.1). The IC_{50} for PACA-2 and BXPC-3 was close to 100 ng/ml TRAIL.

2.1.2 TRAIL induces apoptosis in sensitive pancreatic cancer cells through both extrinsic and intrinsic pathways

Objective:

To determine if the cell death caused by TRAIL was due to apoptosis, experiments were carried out in the two sensitive cell lines to test for induction of the intrinsic (caspase-9) and extrinsic (caspase-8) apoptotic pathways following TRAIL stimulation.

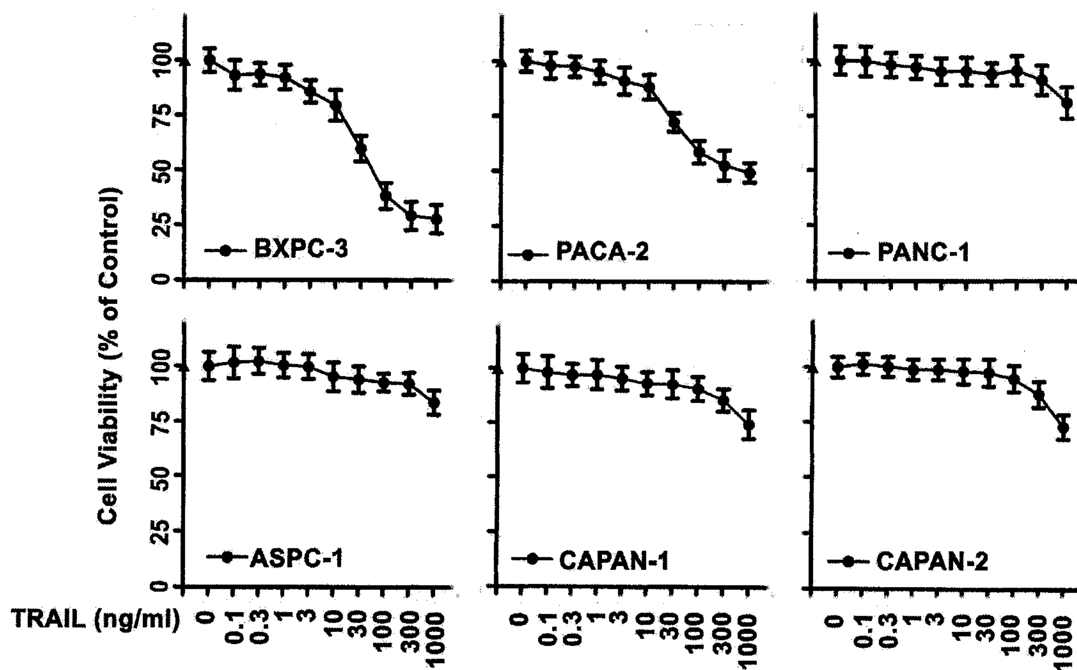


Figure 2.1 Acid phosphatase assay for TRAIL-induced cell death in six pancreatic cancer cell lines. Cell lines were grown in 96-well plates and incubated for 16 h with various doses of TRAIL as indicated. The results are presented as the percentage of control cell viability which was calculated by comparing with the untreated control value (0 h). Data represents the mean ± standard error mean (SEM, n=8).

Materials and Methods:

Cell lines:

Two sensitive pancreatic cancer cell lines PACA-2 and BXPC-3 were cultured as previously described in section 2.1.1.

Antibodies:

Mouse anti-human monoclonal antibodies specific for various molecules in the intrinsic and extrinsic signaling pathways were used as the primary antibodies in a two step-indirect method for western blot analysis. These include: anti-FADD (610399) (Transduction Laboratories, Lexington, KY), anti-caspase-8 (M032-3) (MBL, Watertown, MA, USA) and anti-c-FLIP NF6 (ALX-804-428). Rabbit anti-human polyclonal antibodies used were: caspase-3 (Stressgen, Victoria, BC), ERK1/2 (Stressgen, Victoria, BC), and caspase-9 (Cell Signalling 9502). Secondary antibodies were HRP-conjugated goat anti-rabbit antibody, HRP-conjugated goat anti-mouse antibody (Jackson, West Grove, PA) and HRP-conjugated goat anti-mouse IgG_{2b} antibody (Southern Biotechnology, Birmingham, AL). The latter binds to the caspase-8 primary antibody. For concentration of antibodies used in this study see Table 4.

Reagents:

Components of lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with 1mM PMSF and 0.2% protease inhibitor cocktail (PIC) (P8340) mixure, were all obtained from Sigma. Protein concentrations of the lysates were determined using the Bradford Assay (catalog number

500-0006) purchased from Bio-Rad. Tris buffered saline (TBS) with 0.05% Tween-20 (TBST) was obtained from Sigma-Aldrich. Enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences, Piscataway, NJ. Film was obtained from Kodak BioMax MR.

Table 4. Antibodies used in experiments

Primary Ab.	Source	Conc.	Secondary Ab.-HRP	Source	Conc.
Cas-8	MBL M032-3(Medical biology Lab)	(1:1000)	Goat Anti-Mouse IgG2b	Southern Biotechnology, Birmingham, AL	(1:5000)
Cas-3	Stressgen, Victoria, BC	(1:5000)	Goat Anti-Rabbit IgG	Jackson	(1:5000)
DFF45	Stressgen, Victoria, BC	(1:1000)	Rabbit Anti-Mouse	Jackson	(1:2000)
ERK1/2	Stressgen, Victoria, BC	(1:1000)	Goat Anti-Rabbit IgG	Jackson	(1:5000)
Cas-9	Cell Signal	(1:1000)	Goat Anti-Rabbit IgG	Jackson	(1:5000)
FADD	Transduction Laboratories, Lexington, KY	(1:500)	Rabbit Anti-Mouse	Jackson	(1:2000)
c-FLIP	NF6 Alexis Biochemicals, San Diego, CA	(1:1000)	Goat Anti-mouse IgG1	Southern Biotec.	(1:5000)
DR4	Pro Sci Cat.	(1:500)	Goat Anti-Rabbit IgG	Jackson	(1:5000)
DR5	Pro Sci Cat.	(1:500)	Goat Anti-Rabbit IgG	Jackson	(1:5000)
RIP	RIP mAb BD Pharmingen	(1:500)	Goat Anti-Mouse	Southern Biotec.	(1:5000)

Experimental procedure:

Pancreatic cells at about 50% confluence were treated with 100 ng/ml of TRAIL for 0, 0.5, 1, 3, 6 hours. These time intervals were chosen to observe the caspase cascade with an emphasis on the early apoptosis events to include DISC formation. At the given time points, cells were harvested by scraping with a disposable cell lifter (Fisher Scientific 08) and lysed through vigorous pipetting in ice cold lysis buffer. Lysates were centrifuged at 18,000 *g* for 15 minutes at 4°C in order to remove components of the plasma membranes as well as any unbroken cells and debris. Protein concentrations of the resulting supernatants were assayed and the lysates of whole cells used in Western blot analysis.

Equal amounts of protein from cells lysates (100 μg) were separated by SDS-polyacrylamide gel electrophoresis using Bio-Rad's Mini-PROTEAN 3 system with 15% or 12% separating gels and 3.5% stacking gels in 1X running buffer for 1.5 hours at 150 V. One of the lanes in each gel was reserved for a molecular weight marker, which was obtained from Bio-Rad. The separated proteins were then transferred to immunoblot membranes using Mini Trans-Blot Cell from Bio-Rad at 30 V for 990 min. After blocking unoccupied regions of the membrane in TBST, the membranes were blotted overnight with various primary antibodies. Following three successive washes (5, 5, 10 min), the membranes were incubated for one hour with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies. After incubation with the secondary antibody, the membranes were again subjected to three successive washes (10, 10, 15 min) and developed by ECL on Kodak BioMax MR Film, and then the films were scanned and saved in .tiff format. The relative difference in protein

expression was determined by visual inspection. Note: The Mini-PROTEAN 3 system was run with 2 gels at the same time. The same sequence and amount of samples were loaded on 2 gels. After transferred to the membrane, caspase-8 and -9 were detected using each membrane firstly, then stripping antibodies off and reusing the membrane for subsequent caspase-3 and DFF45 detection. Loading control ERK1/2 analysis was detected for each gel.

Results:

Cleavage of caspase-8 following exposure to TRAIL serves as an indication activation of the extrinsic apoptosis pathway, whereas cleavage of caspase-9 indicates activation of the intrinsic apoptosis pathway. Treatment of the two sensitive cell lines with TRAIL resulted in detection of caspase-8 p18 and caspase-3 p32 precursor cleavage at 3 hours and to a greater extent at 6 hours. Activated caspase-9 p35 and p37 cleavage products were detected at 3 and 6 hours (Figure 2.2 left two panels). Similar results were obtained for cleavage of DFF45, which is substrate of caspase-3. ERK1/2 was used as loading control and detected for each gel, but because results indicated that all gels were equally loaded, only a typical example was shown. These data indicate that TRAIL-induced apoptosis in sensitive pancreatic cancer cells occurs through both extrinsic and intrinsic pathways.

TRAIL (100 ng/ml)
Time (Hours)

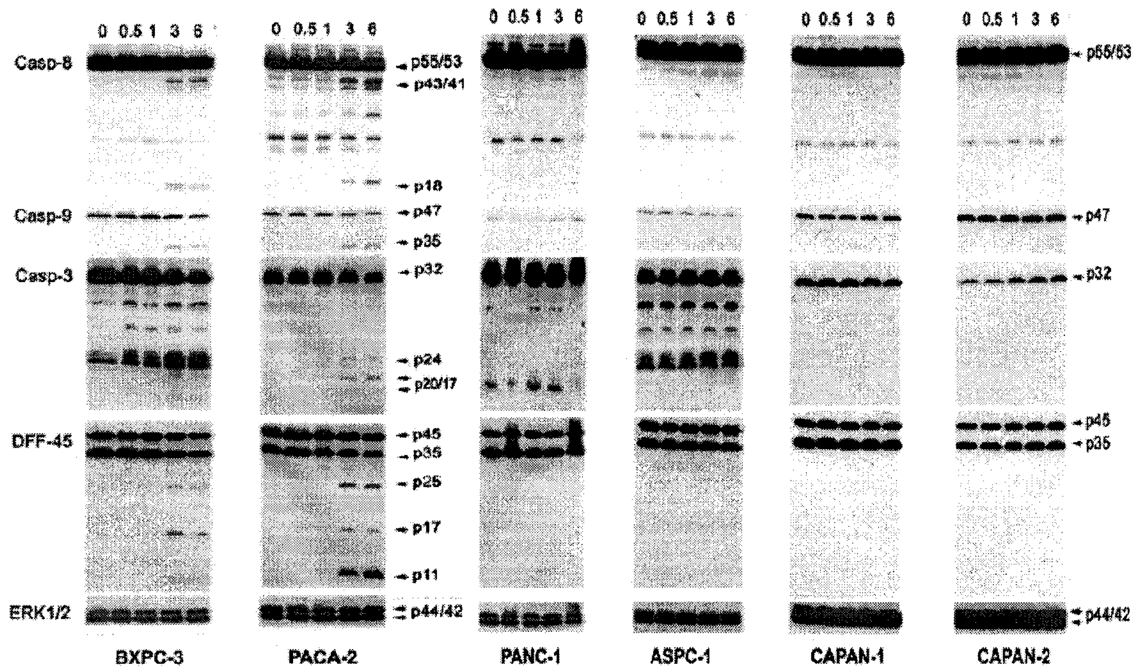


Figure 2.2 Examination of TRAIL-induced cleavage of caspases. Cells were treated with TRAIL (100 ng/ml) for 0, 0.5, 1, 3, 6 hours. Whole cell lysates were subjected to Western blot analysis (100 µg of protein per lane). Cleavage of caspases-8, -3 and -9 was detected in both sensitive cells (BXPC-3 and PACA-2). No cleavage of caspases-8, -3 and -9 was detected in four resistant pancreatic cell lines (CAPAN-1, CAPAN-2, PANC-1, and ASPC-1).

2.1.3 Investigation of DISC inhibition as the likely mechanism in TRAIL resistant pancreatic cancer cell lines.

Objectives:

To investigate if DISC inhibition is one of the mechanisms that controls TRAIL resistance in resistant pancreatic cancer cell lines.

The four resistant cell lines were investigated for activity of the intrinsic (caspase-3) and extrinsic (caspase-8) pathways following TRAIL stimulation. As well, levels of the DISC component proteins were assayed and compared between the resistant and sensitive cell lines.

Materials and Methods:

Cell lines:

Two sensitive pancreatic cancer cell lines PACA-2 and BXPC-3 and four resistant pancreatic cancer cell lines (PANC-1, ASPC-1, CAPAN-1, CAPAN-2) were cultured as previous described in section 2.1.1.

Antibodies:

Antibodies for various molecules in signaling pathways were used as in section 2.1.2. DISC protein antibodies: mouse anti-c-FLIP, mouse anti-FADD; rabbit anti-DR4 polyclonal antibody and rabbit anti-DR5 polyclonal antibody were used as primary antibodies. HRP-conjugated goat anti-rabbit antibody, HRP-conjugated goat anti-mouse antibody and HRP-conjugated goat anti-mouse IgG_{2b} antibody were used as secondary antibodies. For source and concentration of antibodies used see Table 4.

Reagents:

Reagents used here are the same as detailed in section 2.1.2

Activation of the Apoptosis Pathways and DISC Proteins in Resistant Cell Lines Following TRAIL Stimulation

The experimental procedure to examine TRAIL-induced signaling pathways is similar to that detailed in section 2.1.2. Four resistant cell lines were treated with 100 ng/ml of TRAIL for 0, 0.5, 1, 3, 6 hours. Whole cell lysates were examined by western blotting for TRAIL-induced cleavage of caspase -8, -9, -3 and DFF45.

All six pancreatic cancer cell lines were treated with TRAIL at 100 ng/ml. Whole cell extracts from the six cell lines were examined for the expression of the endogenous DISC proteins by western blotting using antibodies to DR4, DR5, FADD, caspase-8, and c-FLIP.

Results:

1) Mechanism of resistance to TRAIL

No cleavage of caspase-8, -9 and -3 was detected in the four resistant pancreatic cell lines (Figure 2.2 right four panels). The results, especially the inhibition of initiator caspase-8 activation, show that inhibition of the upstream signaling pathway is involved in TRAIL resistance in resistant pancreatic cells.

2) Expression of TRAIL DISC protein

The four resistant cell lines expressed higher levels of $c\text{-}FLIP_L$ and $c\text{-}FLIP_S$ than the sensitive cell lines (BXPC-3, PACA-2) (Figure 2.3) by visual inspection. The results

suggest that c-FLIP may be responsible for the DISC-inhibition following TRAIL stimulation in resistant pancreatic cancer cells.

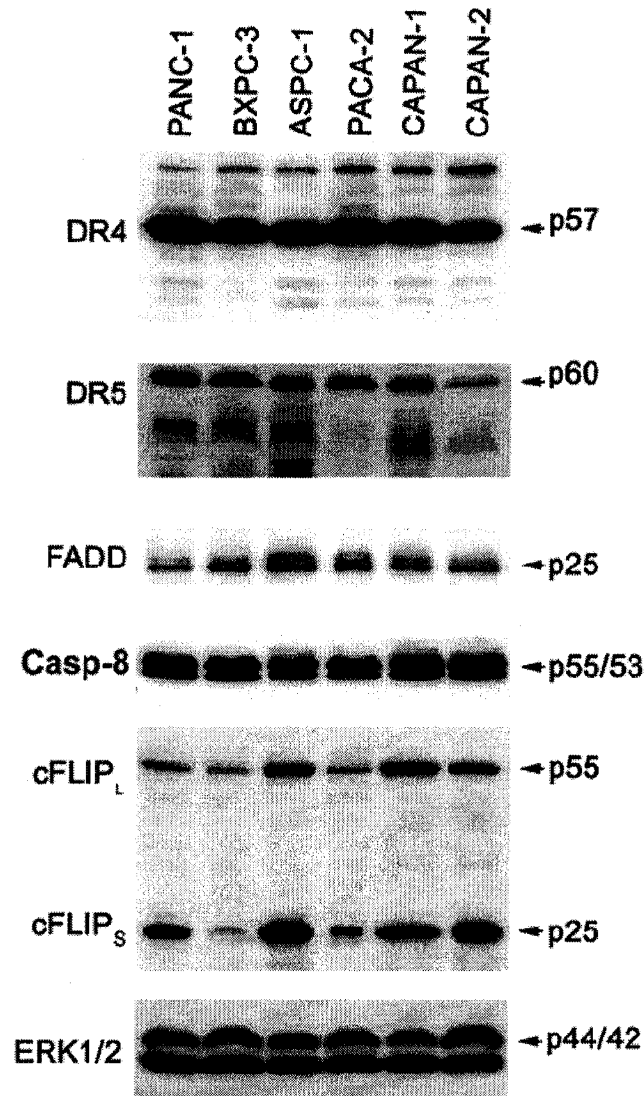


Figure 2.3 Western blot detection of TRAIL-DISC endogenous proteins. Whole cell lysates from six cell lines were subjected to Western blot analysis (100 μg of protein per lane).Compared with sensitive cells (BXPC-3 and PACA-2), resistant cells expressed higher levels of c-FLIP$_L$ and c-FLIP$_S$. Proteins detected are indicated to the left and their molecular weight to the right.

2.2 Investigating the role of c-FLIP in DISC inhibition

2.2.1 Investigating the effect of c-FLIP siRNA in resistant pancreatic cells.

Objective:

This experiment was performed to determine if c-FLIP is a key protein in modulating TRAIL-DISC inhibition in resistant pancreatic cancer cells.

c-FLIP expression will be down-regulated using transient transfection with siRNA. After determination of c-FLIP levels at 24 hours post transfection, transfectants will be treated with TRAIL and examined for early apoptosis events (caspase cleavage at 6 hours) and subsequent cell death at 16 hours.

Materials and Methods:

Cell lines:

Two pancreatic cell lines PANC-1 and ASPC-1 previously characterized as resistant to TRAIL were cultured as section 2.1.1.

Antibodies:

Antibodies for various molecules in signaling pathways were used as in section 2.1.2. Mouse anti-human mAb: anti-c-FLIP NF6 (ALX-804-428) was obtained from Alexis Biochemicals, San Diego, CA.

Study tool:

RNA interference (RNAi) is a recently developed method of gene silencing that introduces double stranded RNA (dsRNA) into cells resulting in post-transcriptional gene silencing. The small interfering RNA (siRNA) is a 21-23 nucleotide (nt) double stranded

RNA with 2 nt overhangs at each 3' ends [190]. Upon transfection into mammalian cells, they are incorporated into an RNA-induced silencing complex (RISC), a complex that cleaves mRNA complementary to the siRNA leading to silencing of that gene (Fig 2.4) [190-193].

In preliminary studies, our lab identified a synthetic siRNA duplex specific to the c-FLIP DED domain (nucleotides 535-555) that could knockdown expression of both c-$FLIP_L$ and c- $FLIP_S$. We synthesized a hairpin RNA targeting the same nucleotides and inserted it in pRNAT-U6.1/Neo plasmid using a commercial service provider, GenScript Corp (Piscataway, NJ). The insert encodes sense (5'-UCGGGGACUUGGCUGAACUUU-3') and antisense (5'-AGUUCAGCCAAGUCCCCGACA-3') oligonucleotides for the c-FLIP gene. The plasmid, pRNAT-U6.1/Neo, also carries a U6 promoter, green fluorescent protein (GFP) and a neomycin–resistance marker. The hairpin was sequenced to verify its structure (Figure2.5).

Reagents:

Reagents for the western blot are the same as section 2.1.2. Lipofectamine 2000 and OptiMEM medium were obtained from Invitrogen. Caspase inhibitor (z-VAD-FMK) is a widely used nonselective pan-caspase inhibitor which inhibits function by binding to the active site of caspases. In this experiment, the tetrapeptide caspase inhibitor z-VAD-FMK (R&D Systems, Minneapolis, MN) was prepared as a 20 mM stock in DMSO and stored at -20°C in aliquots until use.

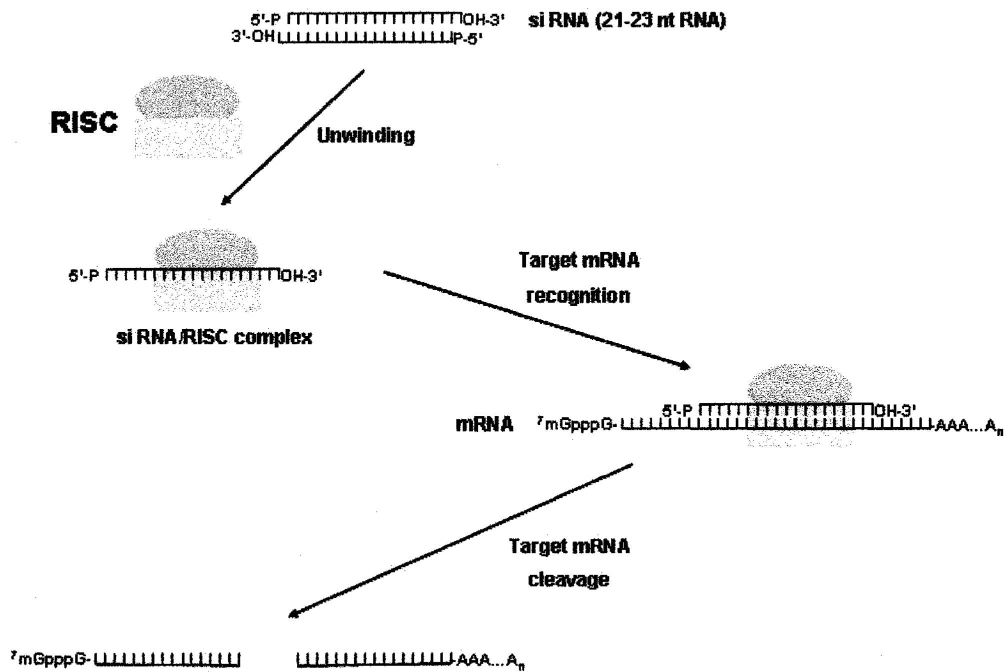


Figure 2.4 Model of siRNA gene silencing mechanism. Small interfering RNAs (siRNAs) are transfected into cells, where they are assembled into an RNA-induced silencing complex (RISC). In the RISC, siRNAs are unwound by the helicase activity of the complex and target complimentary mRNA, through base pairing, for cleavage and degradation by the RISC complex (Reproduced from *Small RNA: can RNA interference be exploited for therapy?* Lancet, 2003.362 (9393): 1041-1043.Wall, N.R. and Shi, Y.).

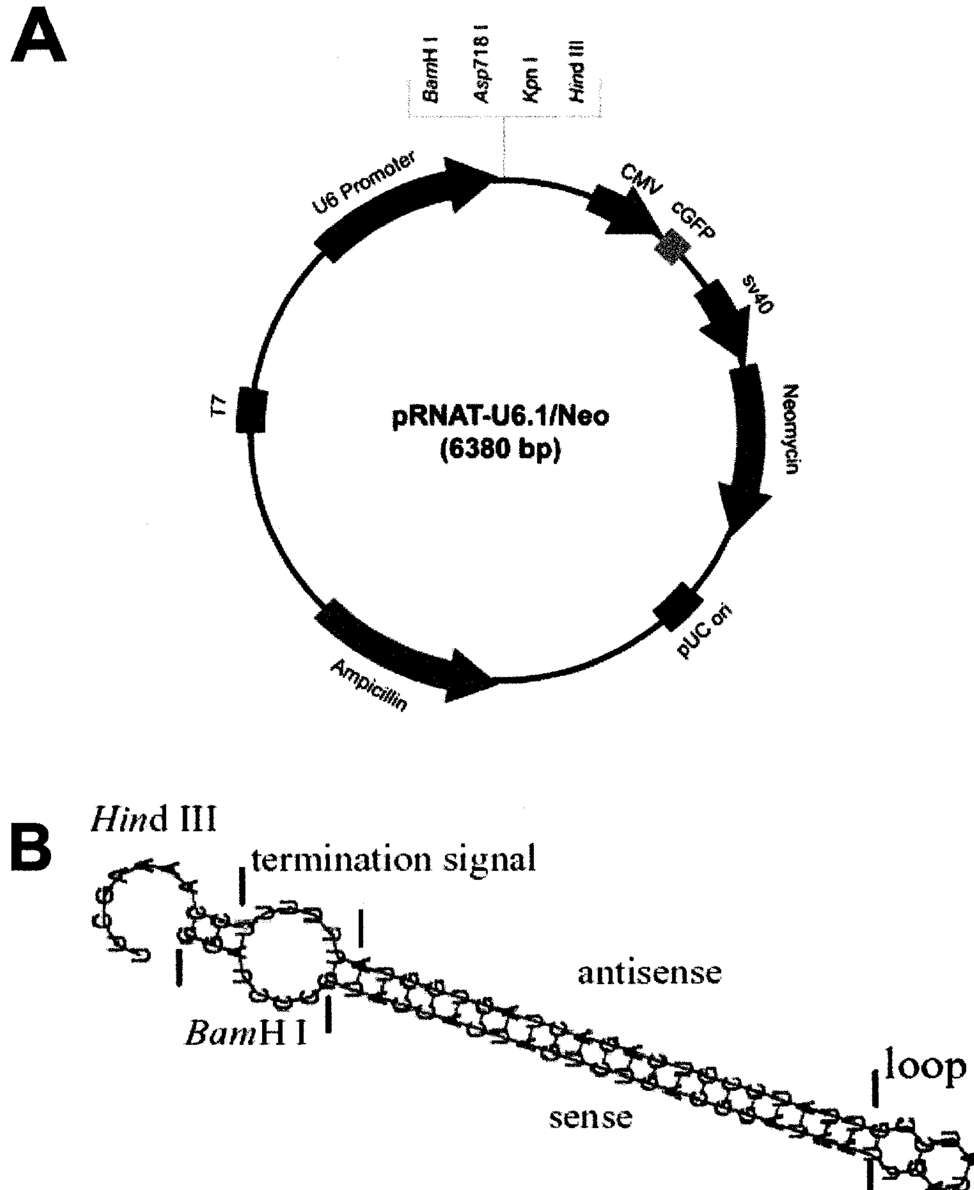


Figure 2.5 Map of plasmid pRNAT-U6.1/Neo and encoded shRNA. A. pRNAT-U6.1/Neo is a GenScript siRNA expression vector. It is designed for mammalian transfection. It carries a Neomycin resistance gene as the selectable marker. The GFP marker (coral GFP, cGFP) under CMV promoter control can be used to track the transfection efficiency. It uses U6 promoter for siRNA expression. (Reproduced from http://www.genscript.com/cgi-bin/products/marker.cgi?code=SD1211) **B.** The insert encoding a short hairpin targeting c-FLIP nucleotides 535-555 was inserted in pRNAT-U6.1/Neo vector.

SiRNA transient transfection of PANC-1and ASPC-1

The day before transfection, 0.5 x 10^6 viable cells were plated per well on 6-well plates and were allowed to grow in antibiotic-free growth medium for 24 hours to a confluency of 90% before being treated with siRNA. Then cells were transfected with pRNAT-U6.1/ FLIPsiRNA plasmid using the Lipofectamine 2000 method following the manufacturer's protocol (Invitrogen). Fluorescence microscopy was used 24 hours later to detect GFP as a measure of transfection efficiency.

Detection of c-FLIP and TRAIL-induced apoptosis after siRNA transfection

24 hours after siRNA transfection, whole cell extracts from PANC-1 and ASPC-1 transfectants were prepared and examined for the expression of c-FLIP by western blot. The experimental procedure was the same as used in section 2.1.2.

PANC-1 and ASPC-1 were transfected with siRNAs, then 24 hours later were treated with 100 ng/ml TRAIL. 6 hours later whole cell extracts were prepared and examined for the cleavage of caspase-8 and caspase-3 by western blot.

TRAIL induced killing in the presence and absence of caspase inhibitor :

After siRNA transfection for 24 hours, PANC-1 and ASPC-1 transfectants were seeded on 96-well plates (1.0 x 10^4 cells/well), then treated the following day (at about 50% confluency) with 100μl of normal growth medium containing (0, 10, 30, 100, or 300 ng/ml) TRAIL for 16 hours. In a set of parallel cultures, 20 μM of the pan-caspase inhibitor (z-VAD-FMK) was added 2 hours before TRAIL treatment. Cell viability was assessed using the acid phosphatase assay.

Results:

The transfection efficiency of PANC-1 c-FLIP siRNA transfectants, assessed by observing green fluorescent protein under fluorescence microscopy, showed higher than 75% transfection efficiency (Figure 2.6). Western blot analysis showed that transfection of c-FLIP siRNA markedly reduced expression of c-$FLIP_L$ and c-$FLIP_S$ in PANC-1 (Figure 2.7 A.) and ASPC-1 (Figure 2.8 A.). Western blot results showed that both caspase-8 and caspase-3 were activated in transfectants following TRAIL treatment (Figure 2.9). This suggested that c-FLIP knockdown can sensitize resistant pancreatic cancer cells to TRAIL-induced apoptosis via the caspase-8-initiated caspase cascade. TRAIL killing was assessed in the presence and absence of caspase inhibitor. The data showed a dose-dependent cell killing with effective doses ranging from 10 to 300 ng/ml. The cell killing was completely inhibited by the pan-caspase inhibitor (z-VAD-FMK) (Figure 2.7 B., 2.8 B.).

2.2.2 Investigation of the effect of over-expression of c-$FLIP_L$, c-$FLIP_S$ or double over-expression on TRAIL-induced apoptosis in a previously sensitive pancreatic cancer cell line

Objective:

To further clarify the role of c-$FLIP_L$ and c-$FLIP_S$ in TRAIL-DISC modulation in pancreatic cancer cells.

c-FLIP expression will be up-regulated in PACA-2, a TRAIL sensitive cell line, by the generation of stable transfectants overexpressing c-$FLIP_L$. Transient transfection of parental and c-$FLIP_L$ transfectants with c-$FLIP_S$ will then be performed. After

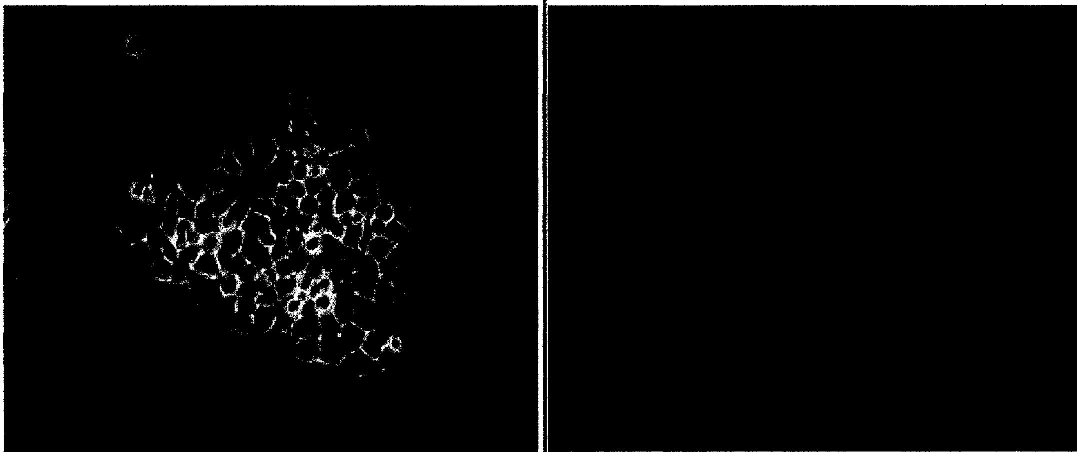


Figure 2.6 The transfection efficiency of c-FLIP siRNA vector. The transfection efficiency of c-FLIP siRNA vector was assessed by observing green fluorescence emitted by GFP as shown under fluorescence microscopy. (More than 75% transfection efficiency)

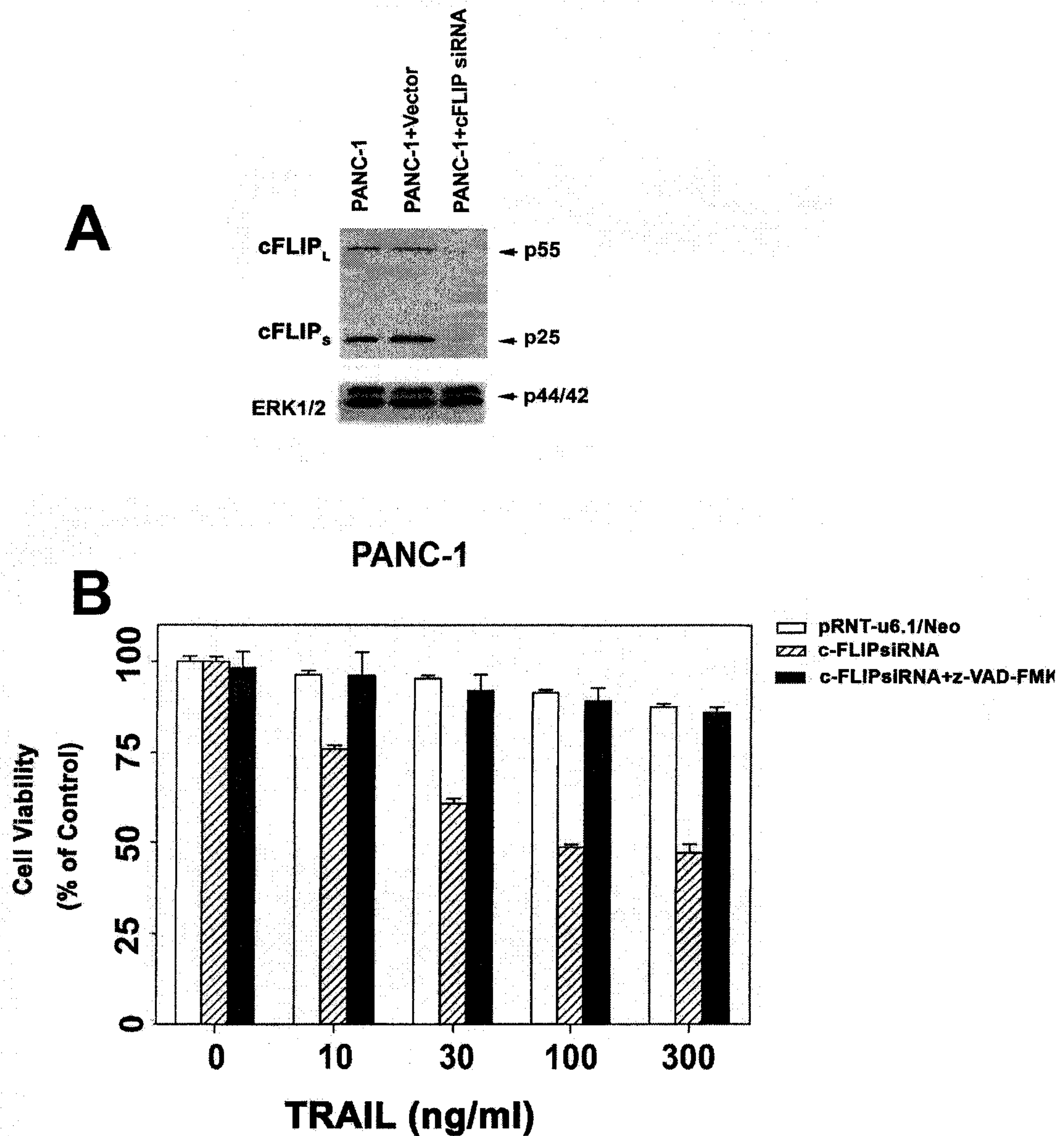


Figure 2.7 SiRNA induced inhibition of c-FLIP in PANC-1. (A)Western blot assay showed decreased expression of c-FLIP levels by visual inspection. ERK1/2 was used as an equal loading control. Whole cell lysates were subjected to Western blot analysis (100 μg of protein per lane). (B) Cell viability was performed using an acid phosphatase assay. The transfectants were treated with (0, 10, 30, 100, 300 ng/ml) TRAIL for 16 hours with or without pretreatment with 20μM z-VAD-FMK+ 100 ng/ml TRAIL for 16 hours. Changes in percentage of cell viability by treatments were calculated by comparing with untreated control value (100%). A representative experiment is shown as a bar graph ± SD (*n*=8).

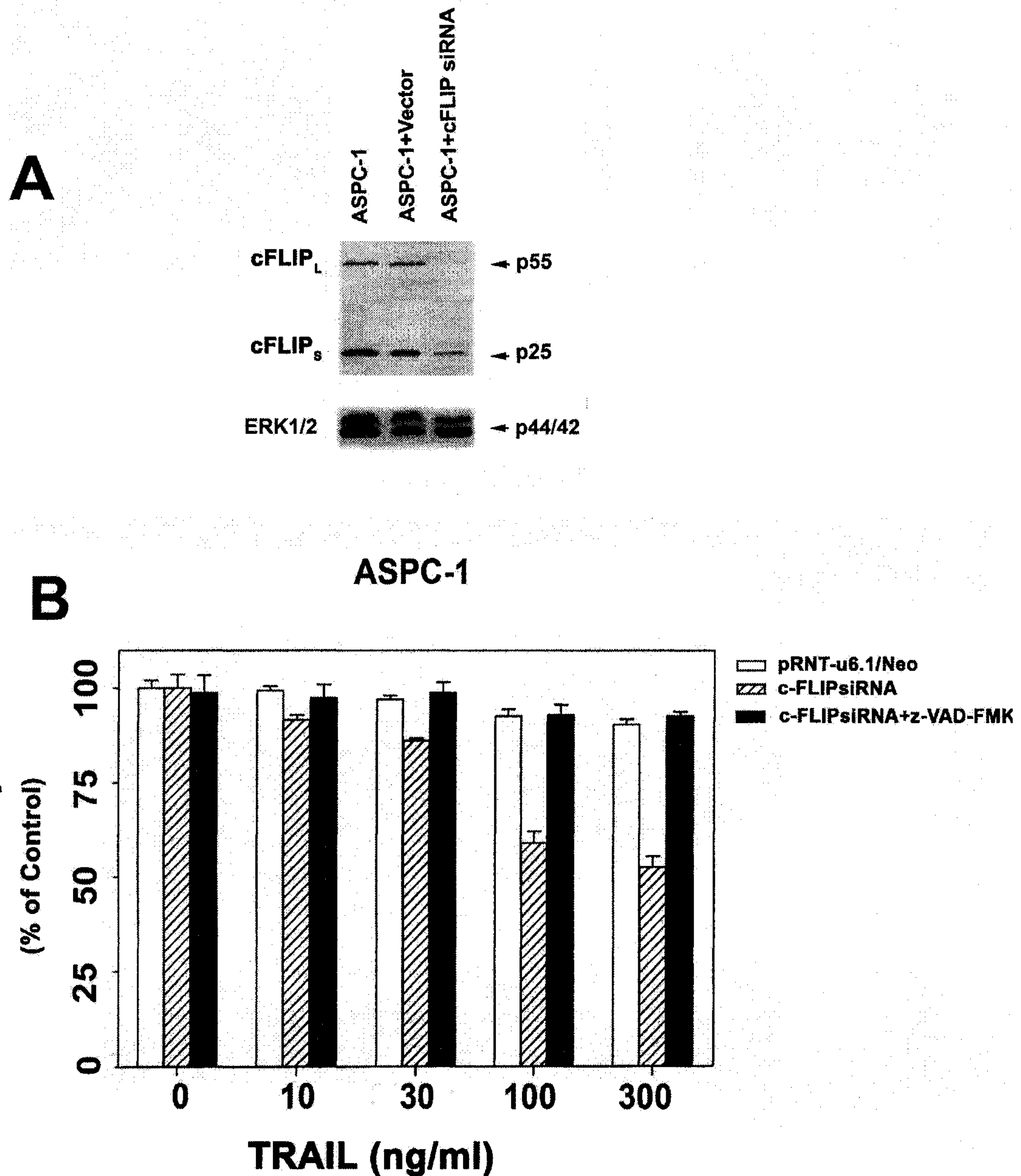


Figure 2.8 SiRNA induced inhibition of c-FLIP in ASPC-1. (A)Western blot assay showed decreased expression of c-FLIP levels by visual inspection. ERK1/2 was used as an equal loading control. Whole cell lysates were subjected to Western blot analysis (100 μg of protein per lane). (B) Cell viability was performed using an acid phosphatase assay. The transfectants were treated with (0, 10, 30, 100, 300 ng/ml) TRAIL for 16 hours with or without pretreatment with 20μM z-VAD-FMK+ 100 ng/ml TRAIL for 16 hours. Changes in percentage of cell viability by treatments were calculated by comparing with untreated control value (100%). A representative experiment is shown as a bar graph ± SD (*n*=8).

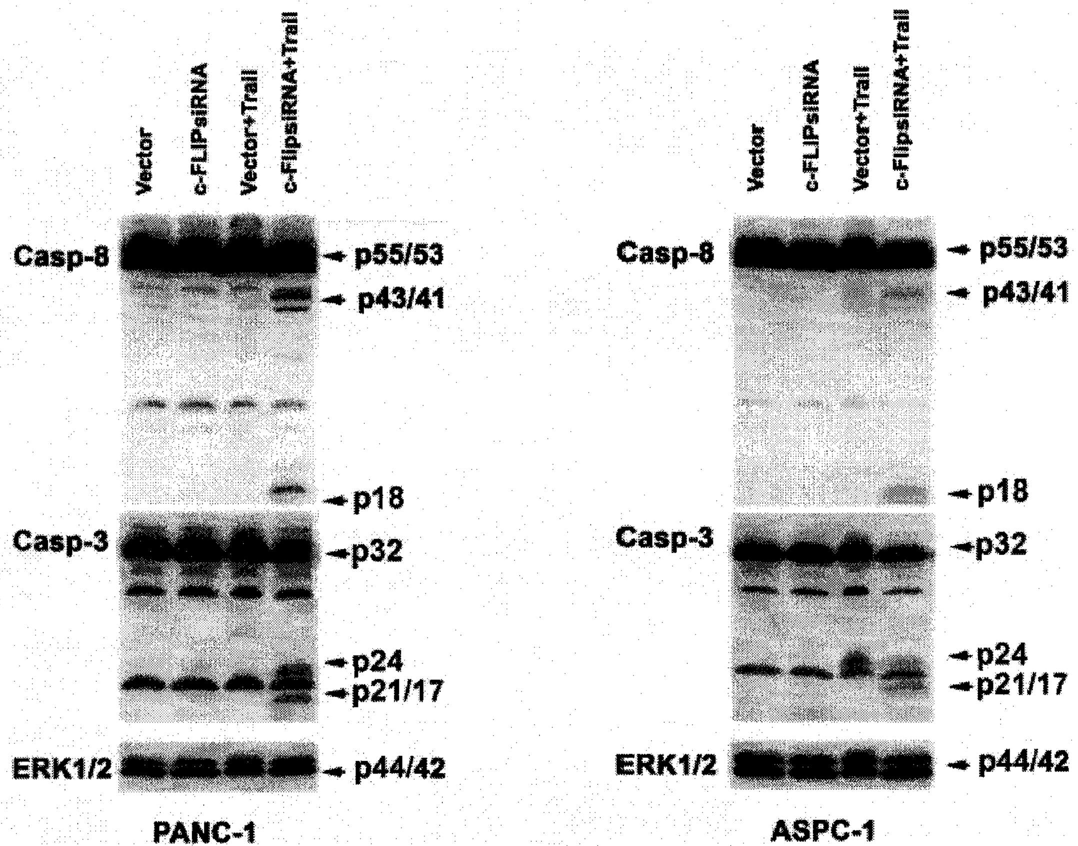


Figure 2.9 Western blot detection of caspase-8 and caspase-3 cleavage. Compared to cells transfected with vector, cleavage of caspase-8 and -3 was detected in c-FLIP knock down cells after treatment of 100 ng/ml TRAIL for 6 hours. Whole cell lysates were subjected to Western blot analysis (100 μg of protein per lane).

confirming up-regulation of c-FLIP, all three transfectants will be tested for caspase cleavage and cell viability after TRAIL treatment.

Materials and Methods:

Cell line:

The TRAIL sensitive pancreatic cancer cell line PACA-2 was cultured as previous described in section 2.1.1.

Antibodies:

Antibodies were used as in section 2.2.1(see Table 4).

Reagents:

The expression vector for c-FLIPlong (pEF rs FLAG-cFLIP) was kindly provided by Dr. Peter H. Krammer, German Cancer Research Center, Germany. The control vector pcDNA3 was obtained from Invitrogen, Carlsbad, CA. The vector contains a puromycin selective marker or a FLAG-tagged c-$FLIP_S$ or c-$FLIP_L$ gene. Puromycin powder obtained from Sigma-Aldrich was prepared as a 50 mg/ml in double distilled water and stored at -20°C in aliquots until use. Reagents for acid phosphatase assay were the same as detailed in section 2.1.1. Reagents for western blot are the same as section 2.1.2.

Generation of c-$FLIP_L$ cDNA stable transfected cell line

The day before transfection, 0.5 x 10^6 viable PACA-2 cells were plated per well on 6-well plates. Cells were allowed to grow in antibiotic-free growth medium for 24 hours to a confluency of 90% before being treated with cDNA using Lipofectamine 2000.

Twenty four hours later, cells were switched to DMEM with 10% FBS and 3 μg/ml of puromycin. The growth medium with selection drug was changed every week until colonies appeared. The drug resistant clones were maintained in DMEM culture media containing 3 μg/ml of puromycin for 2 months. c-$FLIP_L$ expression levels were detected by western blots. This cell line was named PACA-2 c-$FLIP_L$.

Transient transfection of c-$FLIP_S$ cDNA into PACA-2 and PACA-2 c-$FLIP_L$ overexpressing cell lines

The transfection procedure was the same as detailed in section 2.2.1 "siRNA transient transfection procedure". After transient transfection of c-$FLIP_S$ cDNA into PACA-2 c-$FLIP_L$ over-expressing cells and wild type cells, we obtained c-$FLIP_L$, c-$FLIP_S$ and both c-$FLIP_L$ and c-$FLIP_S$ over expressing cells.

Detection of the changes in expression level of c-FLIP and TRAIL signaling proteins

Twenty four hours after cDNA transfection, whole cell extracts from all these transfectants (c-$FLIP_L$, c-$FLIP_S$ and both c-$FLIP_L$ and c-$FLIP_S$ over expressing cells) were examined for the expression of c-FLIP and compared to untransfected cells using western blot (see section 2.1.2).

Untreated PACA-2 cells and c-$FLIP_L$ over expressing PACA-2 cells were treated with 100 ng/ml TRAIL for 0, 0.3, 1, 3, 5 hours. Then whole cell extracts were prepared and examined for the cleavage of caspase-8 by western blot.

Meanwhile, untreated or cells expressing with c-$FLIP_L$, c-$FLIP_S$ or both were treated with 100 ng/ml TRAIL for 5 hours. Then whole cell extracts were prepared and

examined for the cleavage of caspase-8 by western blot compared to untreated parental cells and untreated transfectants.

Assessment of TRAIL killing of c-FLIP overexpressors:

Twenty four hours after cDNA transfection, PACA-2 wild type and transfectants (c-FLIP$_L$, c-FLIP$_S$ and both c-FLIP$_L$ and c-FLIP$_S$ over-expressing cells) were seeded on 96-well plates (1.0 x 10^4 cells/well). The following day (at about 50% confluency), cells were treated with 100 μl of normal growth medium containing (0, 10, 30, 100, 300 ng/ml) TRAIL for 16 hours. Then cell viability was assessed using the acid phosphatase assay. The experimental procedure is the same as detailed in section 2.1.1.

Results:

1) The expression of c-FLIP$_L$ and c-FLIP$_S$

Western blot analysis showed that expression of c-FLIP$_L$ in PACA-2 c-FLIP$_L$, c-FLIP$_S$ transiently transfected in PACA-2 or both c-FLIP$_L$ and FLIP$_S$ in double transfectants had protein levels significantly higher than wild type as shown by western blot (Figure 2.10).

2) Caspase-8 cleavage following TRAIL treatment

Western blot analysis for caspase-8 activation demonstrated that over-expression of c-FLIP$_L$ diminished TRAIL-induced activation of caspase-8 in a time dependent manner (Figure 2.12). Compared to wild-type cells, less cleavage of caspase-8 was detected in c-FLIP$_L$ or c-FLIP$_S$ over-expressing cells, and no cleavage of caspase-8 was detected in the c-FLIP$_L$ and c-FLIP$_S$ double over-expressing cells (Figure 2.13).

2) Cell viability after TRAIL treatment

The transfectants were treated with TRAIL followed by the acid phosphatase assay to determine cell viability. The results showed that over expression of c-$FLIP_L$ or c-$FLIP_S$ protected PACA-2 cells from TRAIL-induced apoptosis, and over-expression of both c-$FLIP_L$ and c-$FLIP_S$ almost completely inhibited TRAIL-induced apoptosis in PACA-2 cells (Figure 2.11).

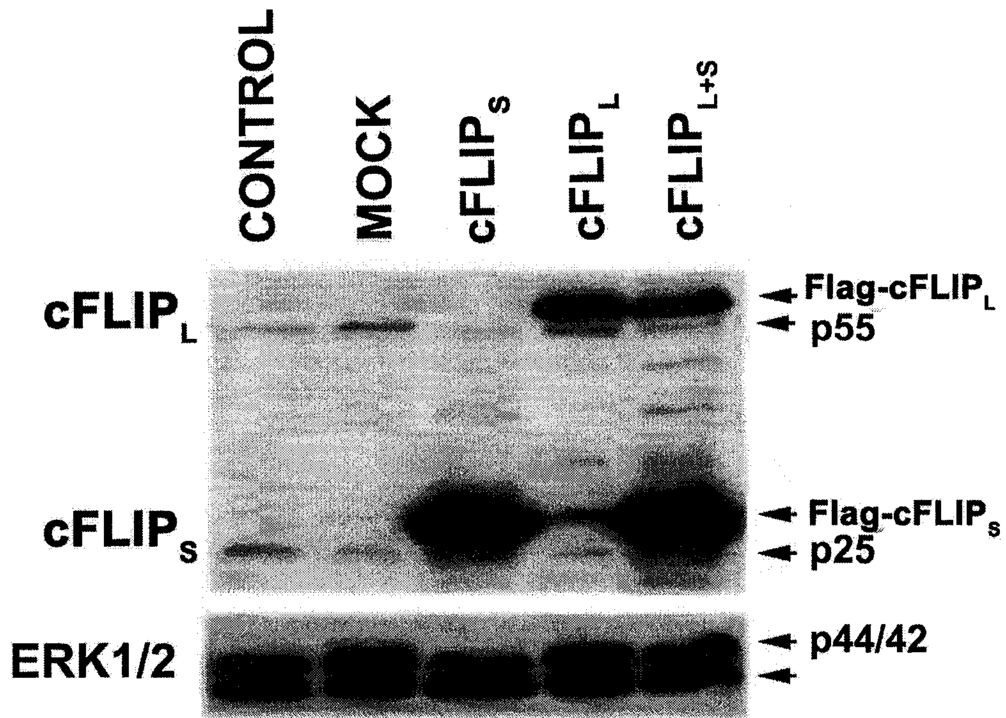


Figure 2.10 Western blot detection of c-FLIP. Transient transfection of c-$FLIP_S$ cDNA into wild type or stably transfected c-$FLIP_L$ over-expressing PACA-2 cells, increased expression of c-$FLIP_L$, c-$FLIP_S$, or both ten-fold. ERK1/2 was used as an equal loading control. Whole cell lysates were subjected to Western blot analysis (100 μg of protein per lane).

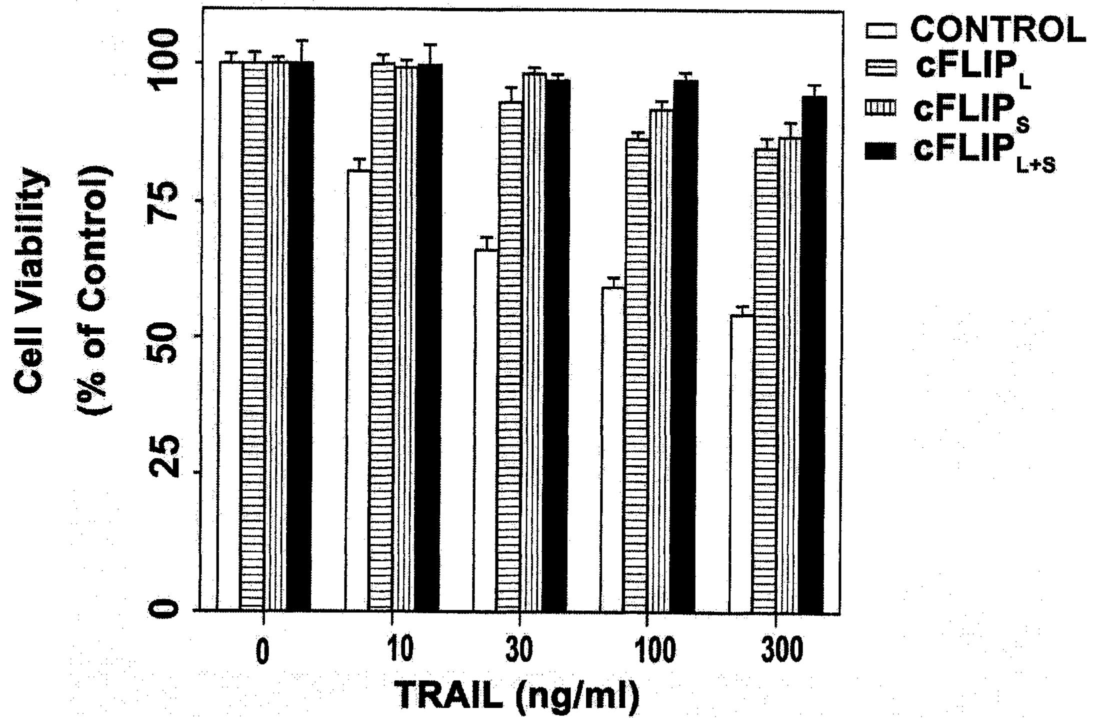


Figure 2.11 Effect of c-FLIP over-expression on TRAIL-induced apoptosis in TRAIL-sensitive PACA-2 cells. PACA-2 wild type cells and transfectants (c-FLIP$_L$, c-FLIP$_S$ and both c-FLIP$_L$ and c-FLIP$_S$ over expression cells) were treated with (0, 10, 30, 100, 300 ng/ml) TRAIL for 16 hours. Cell viability was performed using the acid phosphatase assay. Changes in percentage of cell viability by treatments were calculated by comparing with untreated control value (100%). A representative experiment is shown as a bar graph ± SD (*n*=8).

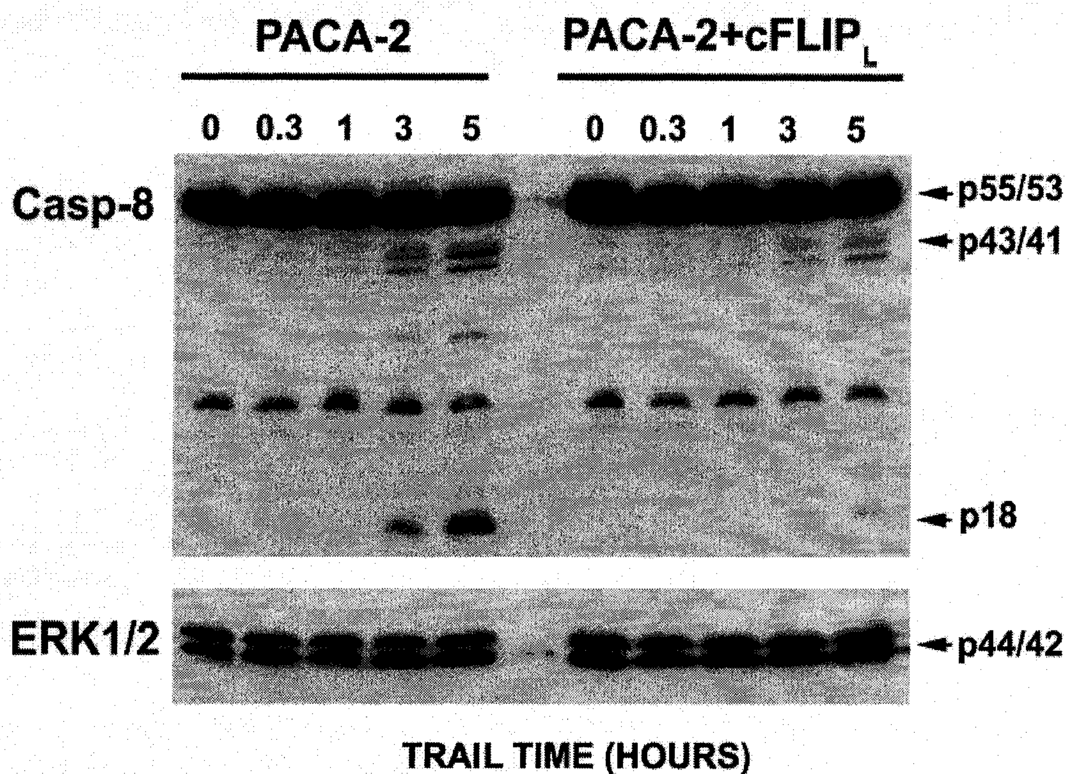


Figure 2.12 Western blot detection of caspase-8 activation in PACA-2 wild type cells and c-FLIP$_L$ over-expressing cells. PACA-2 wild type cells and c-FLIP$_L$ over-expressing cells were treated with 100 ng/ml TRAIL for 0, 0.3, 1, 3, 5 hours. Then whole cell extracts were examined for the cleavage of caspase-8 by western blot (100 μg of protein per lane).

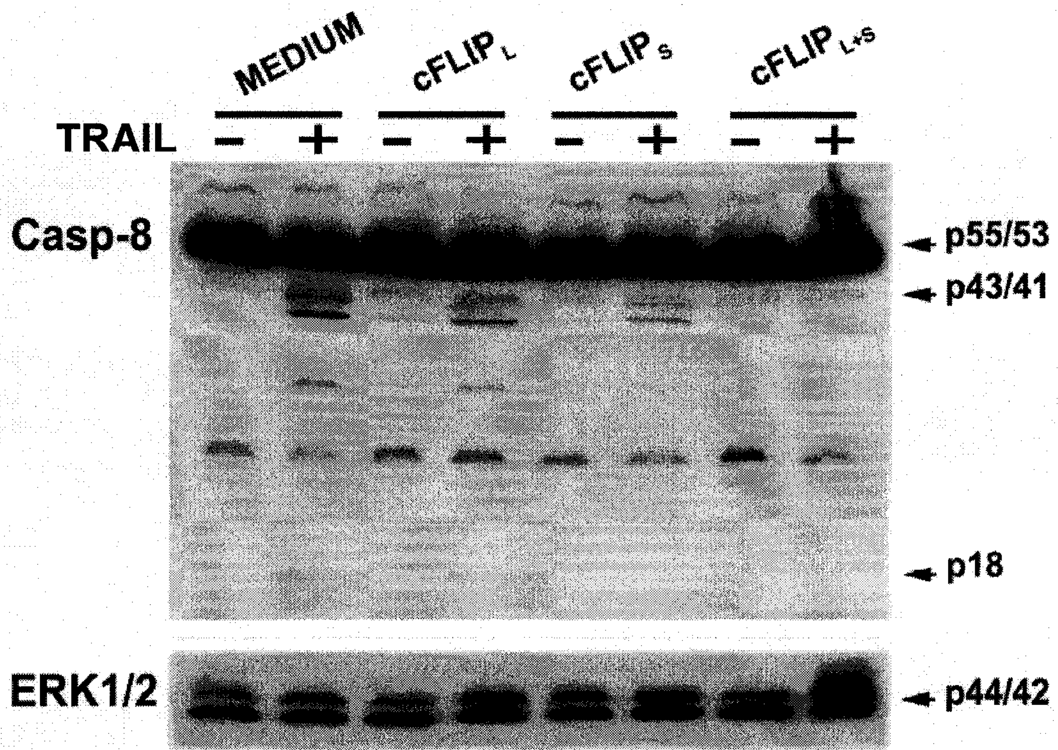


Figure 2.13 Western blot detection of caspase-8 activation. Wild type cells, single over-expressors of c-FLIP$_L$, c-FLIP$_S$ and double over-expressors of both c-FLIP$_L$ and c-FLIP$_S$ were treated with 100 ng/ml TRAIL for 5 hours prior to harvesting. Then whole cell extracts from transfectants together with untreated cells were examined for the cleavage of caspase-8 by western blot and compared to wild-type cells. Less cleavage of caspase-8 was detected in c-FLIP$_L$ or c-FLIP$_S$ over-expressing cells, and no cleavage of caspase-8 was detected in cells over-expressing both c-FLIP$_L$ and c-FLIP$_S$ (100 μg of protein per lane).

2.3 Investigation of the ability of chemotherapeutic and anti-cancer drugs to sensitize resistant pancreatic cancer cells to TRAIL-induced apoptosis.

2.3.1 Anti-cancer drugs and pancreatic cancer

Objective:

To screen for anti-cancer drugs that can sensitize TRAIL-induced apoptosis in resistant pancreatic cancer cells. Two resistant pancreatic cell lines PANC-1 and ASPC-1 will be pretreated with five selected pharmaceutical agents and the effect of the subsequent addition of TRAIL on cell viability will be assessed.

Materials and Methods:

Cell lines:

Two resistant pancreatic cell lines PANC-1 and ASPC-1 were cultured as previous.

Anticancer drugs:

CDDP and CPT (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) were prepared as 100 mg/ml stock in dimethyl sulfoxide (DMSO) with the final concentration of DMSO not exceeding 0.1% (v/v). Celecoxib was purchased from LKT Laboratories (St. Paul, MN). It was dissolved in DMSO at a concentration of 50 mM and stored at -80 °C. Trichostatin A (TSA) and 5-Aza-2′-deoxycytidine (ADC) (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) were prepared as 100 µm stock in ethanol and 0.25 mg/ml stock in water respectively. Stock solutions were diluted to the indicated concentrations with growth medium immediately before use.

Procedure:

TRAIL-resistant pancreatic cancer cell lines (PANC-1 and ASPC-1) grown in 96-well plates (10,000 cells/well) overnight were treated with CDDP, CTP, celecoxib, TSA or ADC for 16 hours at the concentrations indicated [85, 86, 173, 174, 181, 182]. An aliquot of cells was further treated with TRAIL (100 ng/ml) or TRAIL (100 ng/ml) with 20 μM z-VAD-FMK for 16 hours. Cell death was determined by acid phosphatase assays as previously described.

Results:

1) Acid phosphatase assays showed only a limited cytotoxic effect of TSA and ADC. Further treatment of these cell lines with 100 ng/ml TRAIL for an additional 16 hours did not increase cell death (data not shown).

2) Acid phosphatase assays showed only a limited cytotoxic effect of CDDP, CPT and celecoxib. Further treatment of these cell lines with 100 ng/ml TRAIL for an additional 16 hours resulted in significantly increased cell death. These sensitizing effects were completely inhibited by pretreatment of z-VAD-FMK (Figure 2.14)

2.3.2 Investigation of the mechanism of pharmaceutical enhancement of TRAIL sensitivity in previously resistant pancreatic cancer cells

Objective:

To explore the mechanism of additive effects and determine whether anti-cancer agents known to be additive with TRAIL may have the same effect as c-FLIP siRNA. Treatment of two resistant pancreatic cancer cell lines with a combination of sensitizing pharmaceuticals and TRAIL will be compared with treatment with individual agents for caspase cleavage and c-FLIP levels.

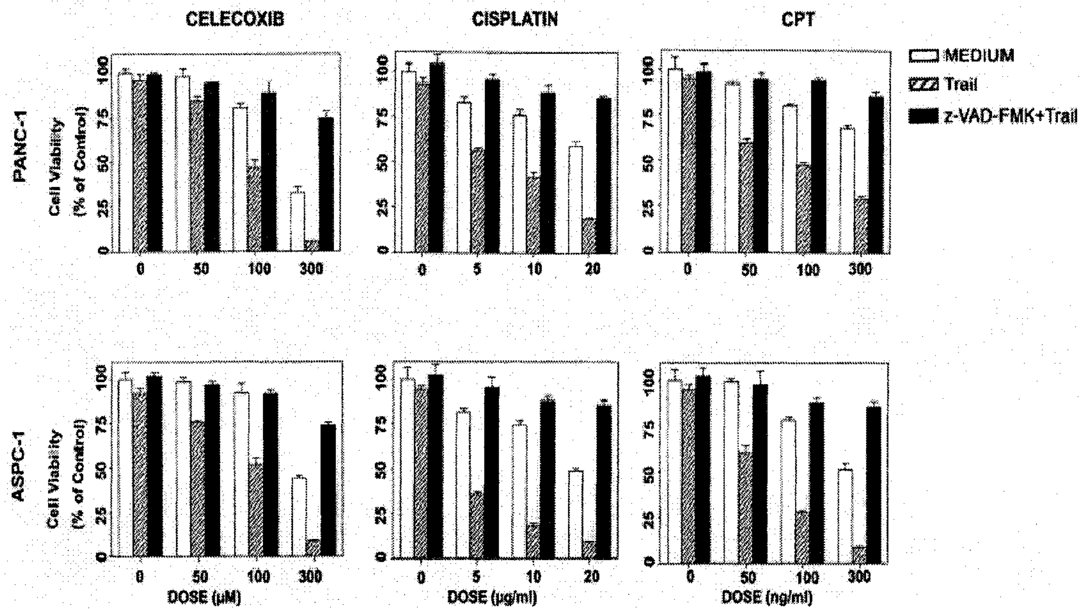


Figure 2.14 Synergistic effects of CDDP, CPT, and celecoxib on TRAIL-induced apoptosis in resistant pancreatic cancer cells. Cell viability was performed using the acid phosphatase assay. Data showed only a limited cytotoxic effect of low doses of CDDP, CPT and celecoxib treated for 16hours. In contrast, further treatment of these cell lines with 100 ng/ml TRAIL for an additional 16 hours resulted in significantly increased cell death and these sensitizing effects are almost completely inhibited by pretreatment of 20 μM z-VAD-FMK. Changes in percentage of cell viability by treatments were calculated by comparing with untreated control value (100%). A representative experiment is shown as a bar graph ± SD (*n*=8).

Materials and Methods:

Cell lines:

Two resistant pancreatic cell lines PANC-1 and ASPC-1 were cultured as previous.

Antibodies:

Antibodies were used as in section 2.2.1 (see Table 3).

Anticancer drugs:

According to the results in section 2.3.1, CDDP, CPT and Celecoxib were used at low non-toxic dose concentrations, eg. celecoxib 50 μM, CDDP 5 μg/ml, or CPT 50 ng/ml.

Procedure:

The experimental procedure to examine the TRAIL signaling pathway is the same as in the section 2.1.2. TRAIL-resistant pancreatic cancer cell lines (PANC-1 and ASPC-1) were treated with TRAIL (100 ng/ml), low doses of CDDP (5 μg/ml), CPT (50 ng/ml) or Celecoxib (50 μM) for 16 hours. An aliquot of cells treated with Celecoxib, CPT, and CDDP were further stimulated with 100 ng/ml of TRAIL for 6 hours. Whole cell lysates were examined by western blot for TRAIL-induced cleavage of caspase-8 and expression of the endogenous DISC proteins using antibodies to DR4, DR5, FADD, caspase-8, and c-FLIP.

Results:

1) Western blot analysis for detection of cleavage of caspase-8

In the presence of low doses of Celecoxib (50μM), CDDP (5 μg/ml), or CPT (50 ng/ml), the cleavage product of caspase-8 was detected in the resistant cells PANC-1 (Figure 2.15) and ASPC-1 (Figure 2.16) only when subsequently treated with TRAIL.

2) Western blot analysis of the effects of Celecoxib, CDDP and CPT on TRAIL-DISC protein expression

After treatment with low doses of Celecoxib, CDDP or CPT for 16 hours, compared to untreated cells, FADD was expressed at consistent levels in PANC-1 but a little bit low in ASPC-1 cells. DR4 and DR5 were expressed at consistent levels in ASPC-1 cells. But in PANC-1 cells, DR4/5 was upregulated after treatment with celecoxib, CDDP or CPT. In PANC-1 and ASPC-1 cells, expression of c-FLIP$_L$ and c-FLIP$_S$ were markedly down regulated after treatment with low doses of Celecoxib, CDDP or CPT. (Figure 2.17).

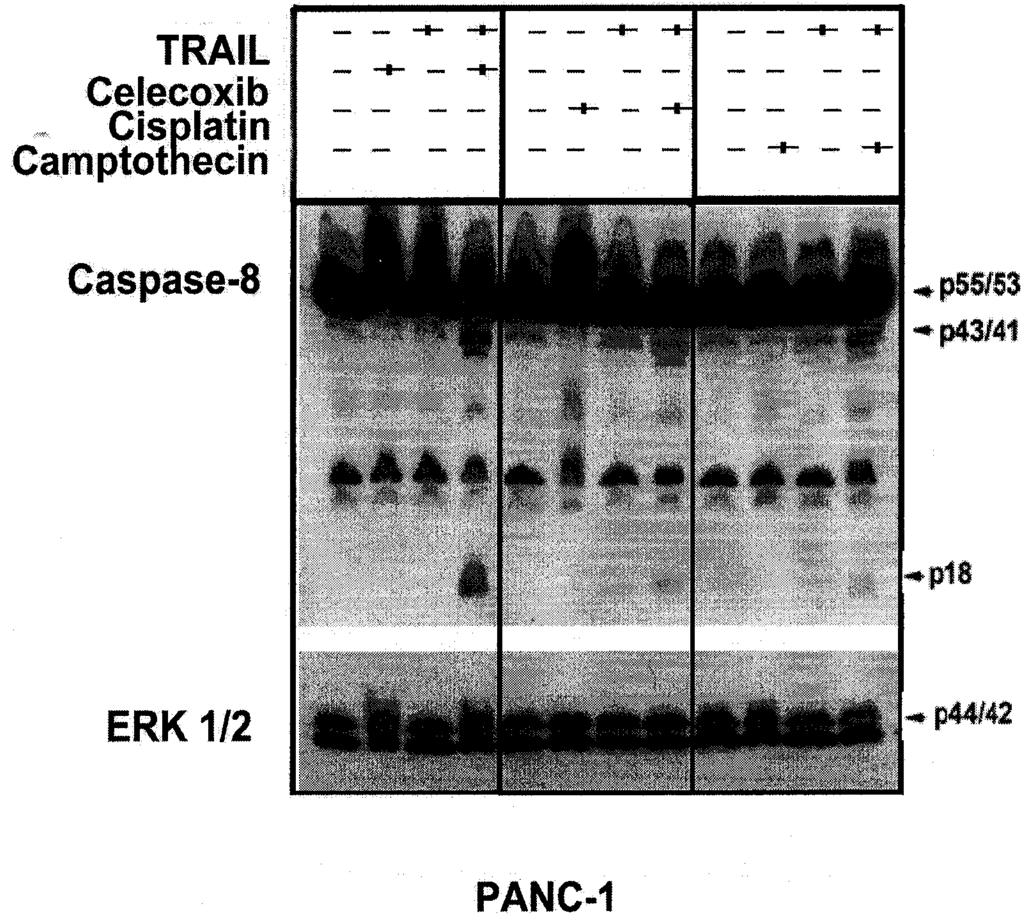


Figure 2.15 TRAIL-induced cleavage of caspase-8 after pretreatment with Celecoxib, CPT, and CDDP in PANC-1 cells. Cells were treated with TRAIL (100 ng/ml), low doses of CDDP (5μg/ml), CPT (50 ng/ml) or Celecoxib (50 μM) for 16 hours. Cells treated with Celecoxib, CPT, and CDDP were further stimulated with 100 ng/ml of TRAIL for 6 hours. Whole cell lysates were subjected to Western blot analysis (100 μg of protein per lane).

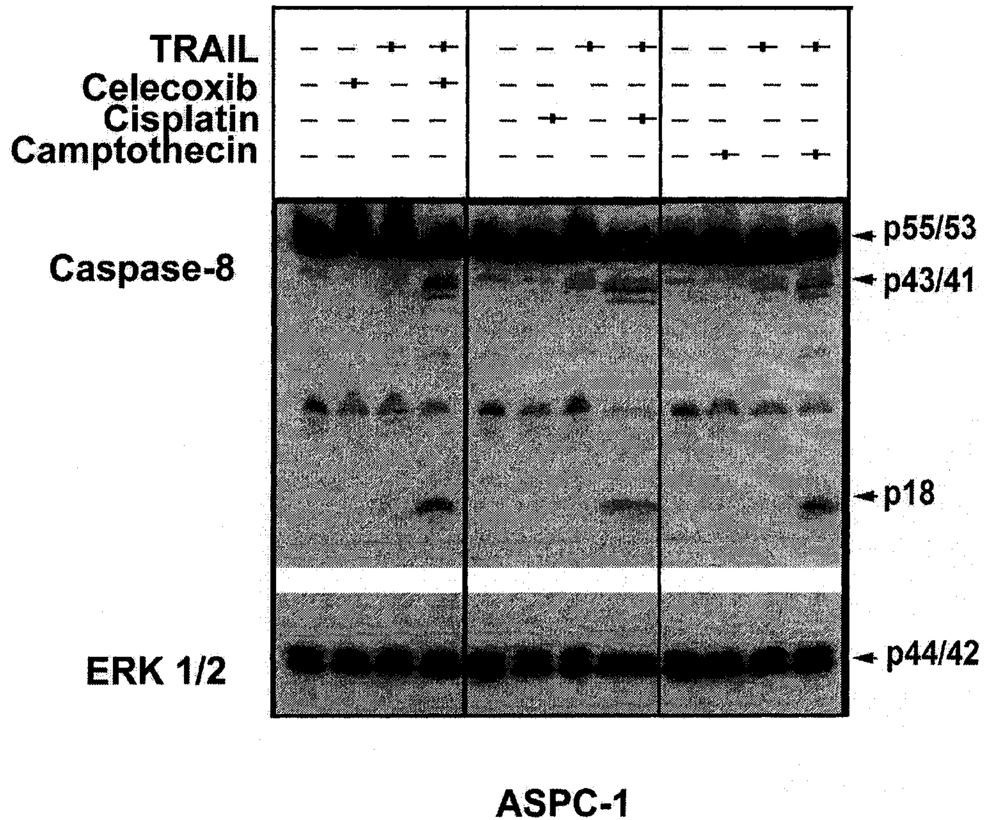


Figure 2.16 TRAIL-induced cleavage of caspase-8 after pretreatment with Celecoxib, CPT, and CDDP in ASPC-1 cells. Cells were treated with TRAIL (100 ng/ml), low doses of CDDP (5μg/ml), CPT (50 ng/ml) or Celecoxib (50 μM) for 16 hours. Cells treated with Celecoxib, CPT, and CDDP were further stimulated with 100 ng/ml of TRAIL for 6 hours. Whole cell lysates were subjected to Western blot analysis (100 μg of protein per lane).

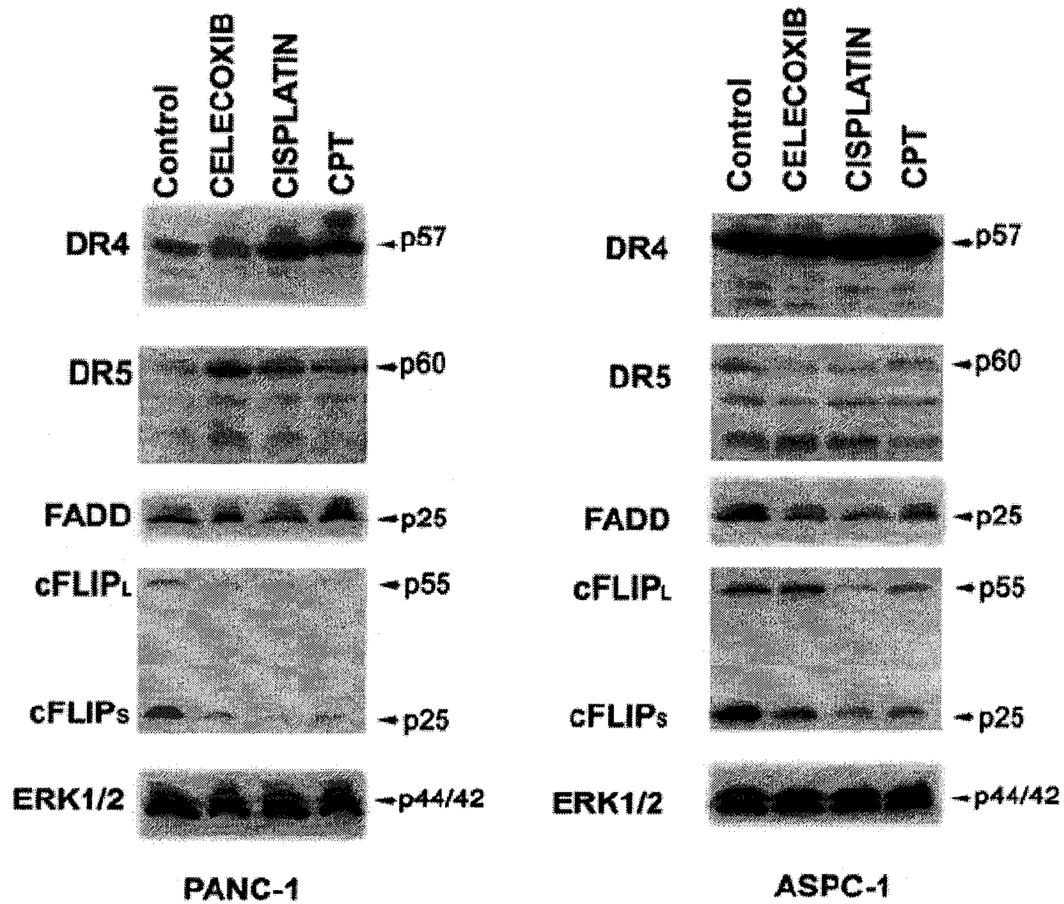


Figure 2.17 Effect of Celecoxib, CPT, and CDDP on TRAIL DISC protein expression. TRAIL-resistant PANC-1 and ASPC-1 cells were treated with Celecoxib (50 μM), CDDP (5 μg/ml), and CPT (50 ng/ml) for 16 hours and, together with untreated cells, subjected to Western blots assay. Compared to untreated cells, FADD was expressed at consistent levels in PANC-1 but a little bit low in ASPC-1 cells. DR4 and DR5 were expressed at consistent levels in ASPC-1 cells. But in PANC-1 cells, DR4/5 was upregulated after treatment with celecoxib, CDDP or CPT. In PANC-1 and ASPC-1 cells, expression of c-FLIP$_L$ and c-FLIP$_S$ were markedly down regulated after treatment with low doses of Celecoxib, CDDP or CPT. Whole cell lysates were subjected to Western blot analysis (100 μg of protein per lane).

CHAPTER 3

DISCUSSION AND CONCLUSIONS

3.1 Introduction

Pancreatic cancer is the fourth most common cause of cancer death in the world and is one of most lethal cancers. Clinical investigation has shown that current chemotherapeutic regimens are ineffective in the majority of pancreatic cancers because of *de novo* resistance to chemotherapy and the lack of selectivity resulting in damage to both cancer cells and normal cells. TRAIL is regarded as a potential selective anticancer agent because it causes apoptosis in cancer cells but not in normal cells. Studies have shown that many types of cancers are sensitive to TRAIL-induced apoptosis, but substantial numbers of cancer cells are resistant to TRAIL, especially some highly malignant tumors such as pancreatic cancer [98], melanoma [99] and neuroblastoma [100]. Resistance to TRAIL can occur at different points in the signaling pathways of TRAIL-induced apoptosis. There is some evidence that TRAIL resistance is mediated at the DISC level [100, 105]. Assembly of the DISC is an early molecular event in the signaling pathway of TRAIL-induced apoptosis, and dysfunction in any of DISC components can lead to TRAIL resistance [100, 105]. Understanding the mechanisms

underlying such resistance and developing strategies to overcome this resistance is important for the successful use of TRAIL for future clinical pancreatic cancer therapy.

3.2 Review and discussion of experimental data

3.2.1 TRAIL induces apoptosis in sensitive pancreatic cancer cells through both the extrinsic and intrinsic pathways and DISC inhibition is the likely mechanism in TRAIL resistant pancreatic cancer cell lines.

Based on previous research, the mechanism by which TRAIL induces apoptosis in various sensitive cancer cells is well understood: TRAIL binds death receptors DR4 [52, 57, 63] and DR5 [53, 65, 66], which in turn recruit FADD [63, 65], caspase-8, and -10, leading to the assembly of the DISC. In the DISC, caspase-8 and -10 are cleaved and these activated components cleave caspase-3 to execute apoptosis via the extrinsic pathway. Alternatively, caspase-8 can cleave Bid to induce apoptosis through the intrinsic pathway; this involves mitochondrial release of apoptotic factors for caspase-9-mediated cleavage of caspase-3 [194].

It has been reported that TRAIL can induce apoptosis in some pancreatic cancer cells in a dose- and time- dependent manner, as shown by morphological analysis, caspase activation and poly (ADP-ribose) polymerase (PARP) cleavage [6]. However, the mechanism of TRAIL-induced apoptosis has not been clarified in pancreatic cancer cells. In our study, firstly using an acid phosphatase assay to assess cell viability, six pancreatic cancer cell lines were characterized into sensitive (PACA-2, BXPC-3) or resistant (PANC-1, ASPC-1, CAPAN-1, CAPAN-2) to TRAIL-induced cytotoxicity. Next,

activation of both the extrinsic and intrinsic pathways in response to TRAIL was demonstrated using a Western blot assay. After treatment with TRAIL, in two sensitive cell lines, activation of the extrinsic pathway was demonstrated through the appearance of caspase-8 subunit p18 and caspase-3 activated p20 and p17 subunits at 3 hours and increasing at 6 hours. Similarly, activation of the intrinsic pathway was demonstrated through the appearance of caspase-9 p35 cleavage products at 3 and 6 hours. As was mentioned in the introduction, since high expression of IAP proteins has been found in cancer cells, which is most potent inhibitor of caspase activity and control the processing and activation of caspase, a processed caspase is not necessarily catalytically active [78-80]. So in this study, we have detected cleavage of DFF45 at the same time, which is substrate of caspase-3. Above data indicate that TRAIL induces apoptosis in sensitive pancreatic cancer cells through both the extrinsic and intrinsic pathways.

Previous studies have shown that TRAIL induces apoptosis through the DISC [52, 53, 57, 63, 65, 66]. Many cancer cells express the TRAIL specific death receptors DR4 and DR5 yet are resistant to TRAIL, indicating that some mechanism must exist to modulate the DISC or other downstream factors. In our study, neither cleavage of caspase-8 nor caspase-3 was detected in the four resistant cell lines; these results suggest that inhibition of the apoptosis initiator caspase-8 on the DISC might be involved in the resistant mechanism, thus preventing TRAIL-induced apoptosis in the resistant cell lines. So DISC inhibition is the likely mechanism of resistance to TRAIL.

In the experiments presented here, I have tested what causes DISC inhibition and explored two methods (siRNA and anti-cancer agents) to release the DISC inhibition to see if TRAIL-induced apoptosis can go to completion.

3.2.2 c-FLIP siRNA targeting the c-FLIP gene sensitizes resistant pancreatic cells to TRAIL-induced apoptosis.

To explore the molecular mechanisms of pancreatic cancer cell resistance, we analyzed the levels of all DISC proteins (DR4, DR5, FADD, caspase-8, c-FLIP). Western blot analysis results revealed that there were no obvious differences in expression levels of DR4, DR5, FADD or caspase-8. However, the four resistant cell lines expressed higher levels of c-FLIP$_L$ and c-FLIP$_S$ than the sensitive cell lines (BXPC-3, PACA-2). The results suggested that c-FLIP may be involved in DISC-inhibition following TRAIL stimulation in resistant pancreatic cancer cells.

Next we carried out a series of experiments to investigate the role of c-FLIP in TRAIL-DISC modulation. In this study siRNA gene silencing technology was utilized. Two resistant pancreatic cancer cell lines (PANC-1 and ASPC-1) which have higher expression of c-FLIP were transfected with the pRNAT-U6.1/ FLIPsiRNA plasmid, which stably synthesizes siRNA targeting nucleotides 535-555 (DED domain) of the c-FLIP gene. From previous work in our lab we identified this siRNA can knockdown expression of both c-FLIP$_L$ and c-FLIP$_S$. The transfection efficiency of c-FLIP siRNA was more than 75% based on expression of the co-transfected green fluorescent protein under fluorescence microscopy. Western blot analyses showed that transfection of c-FLIP siRNA markedly reduced the expression of c-FLIP$_L$ and c-FLIP$_S$ in PANC-1 and ASPC-1. Then the transfectants were treated with TRAIL followed by an acid phosphatase assay to determine cell viability. The data showed a dose-dependent cell killing with effective doses ranging from 10 to 300 ng/ml. The cell killing was almost completely inhibited by a pan-caspase inhibitor (z-VAD-FMK) suggesting that the cell killing might be apoptotic

in nature. Studies have shown that apoptotic cells could be analyzed by flow cytometry for sub-G1 content. The sub-G1 method relies on the fact that at a late stage in the apoptotic cascade, a large numbers of small fragments of DNA are created. If cells are fixed in the ethanol and subsequently rehydrated the small fragments of DNA are able to be eluted. This means that after staining with a quantitative DNA-binding dye, cells that have lost DNA will take up less stain and will appear to the left of the G1 peak or a hypodiploid in a DNA histogram [195]. So in this study, apoptosis in PANC-1 c-FLIP siRNA transfectants was confirmed through detecting subG1 peak by flow cytometry analysis, as compared control cells. Exposure to different dose TRAIL and pretreatment with z-VAD-FMK with additional TRAIL, PANC-1 wild type cells showed no obvious subG1 peak, but c-FLIP knock down cells showed subG1 peak in dose dependent way, and these effects were mostly inhibited by z-VAD-FMK (data not shown). Activation of caspase-8 and caspase-3 was demonstrated by western blot assay in PANC-1 and ASPC-1 c-FLIP siRNA transfectants treated with 100 ng/ml TRAIL. These results suggest that c-FLIP knockdown more likely could sensitize resistant pancreatic cancer cells to TRAIL-induced apoptosis via the caspase-8-initiated caspase cascade.

3.2.3 Over-expression of c-FLIP$_L$, c-FLIP$_S$ or double over-expression inhibits TRAIL induced apoptosis in sensitive pancreatic cancer cells through inhibition of the activation of caspase-8.

Previous studies [128-130] have shown that several types of malignant tumors express high levels of c-FLIP and that overexpression of c-FLIP can confer resistance to TRAIL. High levels of c-FLIP have also been recently reported to correlate strongly with

malignant potential in colonic adenocarcinomas [130], melanoma [128], and hepatocellular carcinoma [129]. Our results in the current study support the above research. Using the Lipofectamine 2000 method we generated c-FLIP$_L$, c-FLIP$_S$ and double over expressing cells. Our original idea was that we wanted to generate over-expression stable cell lines to do this research. We know stable cell line could provide enough cells or protein so that the experiments could be much easier than transient transfection. Here it should note that we just got c-FLIP$_L$ over-expression stable cell line through puromycin selection. We tried to generate c-FLIP$_S$ over-expression stable cell line but we failed finally. We did not know the exact reason. Also we can not generate double over-expression cell line because the vectors containing c-FLIP$_L$ or c-FLIP$_S$ have the same antibiotic selective marker.

Next the acid phosphatase assay showed that over expression of c-FLIP$_L$ or c-FLIP$_S$ protected PACA-2 cells from TRAIL-induced cytotoxicity, and over-expression of both c-FLIP$_L$ and c-FLIP$_S$ almost completely inhibited TRAIL-induced cytotoxicity in the previously TRAIL sensitive PACA-2 cells. Similarly, apoptosis in PACA-2 wild type, c-FLIP$_L$, c-FLIP$_S$ and double over expressing cells was also confirmed through detecting subG1 peak by flow cytometry analysis. Changes in subG1 peak which was induced by TRAIL treatment in PACA-2 cells were markedly inhibited by over expression of c-FLIP$_L$ or c-FLIP$_S$ and almost completely inhibited by double over expression (data not shown). Western blot analysis demonstrated that over-expression of c-FLIP$_L$ diminished TRAIL-induced activation of caspase-8. Compared to untransfected cells, less cleavage of caspase-8 was detected in c-FLIP$_L$ or c-FLIP$_S$ over-expressing cells, and no cleavage of caspase-8 was detected in double c-FLIP$_L$ and c-FLIP$_S$ over-expressing cells. The

above results strongly suggest that c-FLIP plays a critical role in TRAIL-DISC modulation.

The use of two different methodologies for modulating c-FLIP, *i.e.* down regulation of c-FLIP expression in resistant pancreatic cancer cells by transfecting siRNA and up-regulation of c-FLIP expression in sensitive pancreatic cancer cells by transfecting cDNA, is compelling proof that TRAIL resistance in pancreatic cancer cell lines is mediated by c-FLIP.

3.2.4 Chemotherapeutic drugs sensitize resistant pancreatic cancer cells to TRAIL-induced apoptosis through down-regulation of c-FLIP.

RNAi-based therapeutics harness an endogenous cellular regulatory mechanism in which small double-stranded RNA molecules, or siRNA, bind to and mediate the destruction of specific mRNA molecules, preventing their translation into protein [190-193]. Despite its great potential, moving siRNA forward into the clinic is beset by problems with siRNA stability and *in vivo* delivery [196]. Although siRNA when given to mice intravenously, effectively down-regulates an endogenous target without toxicity to normal tissue in the animals [197, 198], there are still practical limitations of siRNA therapy to patients in the clinic. Until now, few studies have reported successful systemic siRNA delivery even in rodent models, and strategies to achieve this are highly sought after [196]. So, the question remains as to how to decrease c-FLIP expression and facilitate TRAIL curative function in pancreatic cancer patients. A synergistic effect of the combination of TRAIL with various types of chemotherapy has been reported for

various other cancer cells [138, 139, 143, 144, 149, 199-202], but the molecular mechanisms of the synergistic effect are controversial (see section 1.4.2). In previous work in our lab in lung cancer and glioma cells [85], we found that some chemotherapeutic agents can decrease c-FLIP expression and overcome TRAIL resistance. We therefore examined several conventional chemotherapeutic and anti-cancer agents in search of clinically useful agents that can overcome the TRAIL resistance in pancreatic cancer cells and subsequently explored the mechanisms of synergistic effects.

In the present study, the acid phosphatase assay for cell viability was performed to screen conventional chemotherapy and anti-cancer agents (CDDP, CPT, celecoxib, TSA or ADC) in two resistant pancreatic cancer cell lines (PANC-1 and ASPC-1) for their effect, both as individual agents and in combination with TRAIL. We found that pretreatment with Celecoxib, CPT, CDDP in PANC-1 and ASPC-1 cells markedly enhanced TRAIL-induced apoptosis in a dose dependent manner and these effects were completely inhibited by pretreatment with the pan-caspase inhibitor z-VAD-FMK. This indicated that these three anti-cancer drugs could sensitize TRAIL-induced apoptosis in resistant pancreatic cancer cells and the cytotoxic effects are apoptotic in nature. Western blot assay showed that low doses of Celecoxib, CDDP and CPT can release caspase-8 inhibition and markedly enhances TRAIL-induced apoptosis in previously resistant PANC-1 and ASPC-1 cells.

To investigate the molecular mechanisms of these two cell lines by which the agents modulate the TRAIL signaling pathways we determined if the chemotherapy agents at the low doses modulate expression of the TRAIL-DISC proteins. Western blots

showed that the low dose treatment with each of these agents significantly down-regulate c-$FLIP_S$ and c-$FLIP_L$. It is noteworthy that cell killing induced by the combination of Celecoxib, CPT or CDDP with TRAIL is more than that induced by TRAIL treatment after the siRNA silencing. This suggests that there exist other mechanisms to enhance the cell death besides c-FLIP downregulation. Possible mechanisms involved here might include upregulation of DR5 and/or DR4 or downregulation of BcL-xl and /or BcL-2 or other mechanisms. Indeed we observed that low doses of celecoxib, CDDP, and CPT increase the DR5 expression in PANC-1 cells which is consistent with previous reports in human NSCLC [173], celecoxib had been found to mediate apoptosis occurred through the induction of the expression of DR5 [173]. We also found CDDP and CPT increase the DR4 expression in PANC-1 cells. Another intracellular adaptor protein FADD seemed to be involved in the resistant mechanisms. Studies have shown that FADD is an adaptor protein responsible for the recruitment of pro-caspase-8 to the death receptor [69], so its down-regulation will affect the DISC formation and in consequence the apoptotic behavior of the cells. In this study our results contrast with this. In ASPC-1 cells, we observed that low doses of celecoxib, CDDP, and CPT treatment decreased FADD expression. There might be some other unknown mechanisms involved in FADD modulation here, which need to be further investigated. But taken together, down-regulation of c-FLIP might still to be the major mechanisms involved in the synergistic effects of combination treatment. We hope above data might provide support for a novel combination therapeutic approach in patients with pancreatic cancer.

3.3 Conclusions and future work

TRAIL is a recently identified member of the TNF death ligand family, which is found in normal tissues [37, 38]. *In vitro* and *in vivo* studies have shown that TRAIL induces apoptosis in tumor cells but not in most normal cells and may prove to be a valuable cancer therapy agent [46, 49, 95]. To explore this therapeutic potential in pancreatic cancer, we investigated six human pancreatic cancer cell lines and showed that some cell lines are sensitive whereas others are resistant to TRAIL killing.

Based on the previous study in lung cancer cells and glioma cells in our lab, we found that part of cancer cells resistance to TRAIL treatment may come from heterogeneity among the cells within the same cell line. In another word, the cells in the same cell line may not derive from the same clone which might explain the phenomena that there are still around 30-40% cells alive after exposure to the highest dose (1000ng/ml) Trail treatment in the two sensitive cell line (PACA-2, BXPC-3). In the future, we may use specific techniques to purify the colony cells and sort these cells to different groups which may provide us more appropriate research model.

Recent studies demonstrate that c-FLIP [125, 203, 204] is a key intracellular adaptor that is recruited to the DISC and modulates the TRAIL-DISC inhibition. We investigated the role of c-FLIP-mediated pathways in TRAIL induced apoptosis in pancreatic cancer cells to clearly define that c-FLIP is a critical protein that controls pancreatic cancer cells resistance to TRAIL-induced apoptosis. Furthermore, we demonstrated that chemotherapy agents and anti-cancer agents such as cisplatin, camptothecin and celecoxib can target c-FLIP-mediated pathways, thus overcoming the

resistance to TRAIL-induced apoptosis in the resistant pancreatic cancer cells. In this study we just primarily detected the resistant mechanisms in two resistant cell lines (PANC-1 and ASPC-1). It does not mean the same mechanisms are necessarily involved in other two resistant cell lines or other resistant pancreatic cancer cells. Further investigation still need to be done. Hopefully these findings in current study may lead to more effective therapy for pancreatic cancer in the clinic.

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