SMAC MIMETIC INDUCED-CELL DEATH: MODE OF ACTION

AND OVERCOMING RESISTANCE

Approved by Supervisory Committee:

Xiaodong Wang, Ph.D._____

Zhijian Chen, Ph.D._____

Steve McKnight, Ph.D._____

Michael Roth, Ph.D._____

Dedication

I would like to dedicate my thesis to the memory of my mother, Angela, and my father, Gary, whom daily I strive to make proud.

SMAC MIMETIC INDUCED-CELL DEATH: MODE OF ACTION AND OVERCOMING RESISTANCE

By

Sean L. Petersen, Ph.D.

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SMAC MIMETIC INDUCED CELL DEATH: MODE OF ACTION IN SENSTIVE CANCER CELL LINES AND OVERCOMING RESISTANCE

Sean L. Petersen, Ph.D.

University of Texas Southwestern Medical Center at Dallas, 2009

Supervising Professor: Xiaodong Wang, Ph.D.

Cancers are characterized by uncontrolled growth and proliferation. One of the key regulators that act to prevent tumor development is programmed cell death, or apoptosis. Defects in the ability of tumors to undergo apoptosis are as important in cancer progression as the loss of signaling controls that limit growth and proliferation. Hence, a fundamental approach, that to date has not been fully exploited, is to attempt to reestablish proper apoptotic signaling allowing cells that have lost normal regulatory controls to essentially commit suicide.

Among key regulators of cell death is the inhibitor of apoptosis (IAP) family of proteins that act to suppress activation of enzymes, the caspases, responsible for carrying out the cell death program. An endogenous protein called second mitochondrial activator

of caspases (smac) is released from the mitochondria upon genotoxic stress, such as DNA damage, and binds to IAPs, un-inhibiting caspases, allowing apoptosis to occur. In many cancers this process is defective, with the observation that IAPs are often over expressed and that cancer cells become resistant to genotoxic stress and do not release smac from the mitochondria.

The nature of the interaction between smac and IAPs presents itself as an ideal target. A four amino acid motif of smac binds to and displaces caspases from the IAPs. As a means to bypass the need to induce genotoxic stress, a small molecule mimetic of the four amino acid motif was synthesized and shown to be able to synergize with pro-apoptotic stimuli to induce apoptosis, and was also shown to have single agent efficacy.

My research has aimed to identify the mechanism of why some cell lines are sensitive to single agent smac mimetic. I identified autocrine TNF production, both basal and smac mimetic induced, as a key feature of cells able to respond to single agent treatment. Additionally, I was able to identify key components responsible for cell death to occur by conducting a limited, targeted siRNA knockdown screen of TNF signaling components to identify receptor interacting protein kinase I (RIPK1) as a key component involved in caspase-8 activation.

Furthermore, I was also interested in understanding why a majority of cells do not respond to smac mimetic, either as a single agent or in combination with TNF. I determined at least two mechanisms whereby this was achieved. Firstly, one of the key mechanisms of smac mimetic action is to induce the degradation of cellular IAP1 (cIAP1) and cIAP2. This degradation is key for the proper formation of an active RIPK1-caspases 8 complex. Some cells are highly sensitive to TNF induced up-regulation of

cIAP2, which becomes refractive to degradation following the initial smac mimetic treatment, owing to loss of cIAP1. The return of cIAP2 blocks formation of the RIPK1-caspase 8 complex and the presence of cIAP2 accounts for resistance in some cell lines.

Secondly, there is another class of cells that do not express cIAP2, basally or in response to TNF, that are nonetheless resistant. These cells are defective in responding to TNF and are thus unable to properly recruit RIPK1 to the TNF receptor. These cells are also defecting in nuclear factor-kappaB (NF-κB) signaling and possess, in relative terms, far less RIPK1 than do sensitive cells and simply do not have enough RIPK1 to incorporate into the death inducing complex.

As a last goal, I wanted to determine if it was possible to force cells that are resistant to become sensitive. Given the key role that TNF plays in smac mimetic sensitivity, it seemed like a good bet that interfering with signaling downstream of the receptor might allow events at the receptor to still occur, but block downstream pro-survival events from happening. Utilizing both siRNAs and chemical inhibition of NF-κB I was able to sensitize previously resistant cells to smac mimetic and TNF treatment. Additionally, I was able to demonstrate that targeting parallel pathways that regulate cIAP2 also sensitized cells. Specifically, targeting protein kinase B (AKT) and targeting the epidermal growth factor receptor with erlotinib (Tarceva) were both highly effective.

These results will hopefully expand the therapeutic use of smac mimetic as well as other established chemotherapeutic. Such combinatorial therapy offers the hope of limiting the toxicity of current therapies and expanding the pool of patients that might be responsive.

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LIST OF ABBREVIATIONS

ATP	Adenosine Tri-phosphate
PKB	Protein Kinase B
TNF	Tumor Necrosis Factor
TNFR1	TNF Receptor I
TRAIL	TNF related apoptosis inducing ligand
FADD	Fas Associated Death Domain
TRADD	TNFR1 associated death domain protein
TRAF2	TNF receptor associated factor 2
cIAP1	cellular Inhibitor of Apoptosis 1
cIAP2	cellular Inhibitor of Apoptosis 2
XIAP	X-linked Inhibitor of Apoptosis
c-FLIP	cellular FLICE-like inhibitory protein
NF-kB	Nuclear Factor-kappaB
I-kB alpha	Inhibitor of kappaB alpha
IKK	I-kB kinase
DMSO	dimethyl sulfoxide
SM	Smac Mimetic
CHX	cycloheximide
RIPK1	Receptor Interacting Protein Kinase 1
PKC	Protein Kinase C
DD	Death domain
DED	Death effector domain
CARD	caspases activation and recruitment domain

CHAPTER ONE: Background

1.1 Introduction

Cancer is second only to heart disease as a cause of premature death in industrialized countries. Rates of cancer can be correlated with both an aging population and with behavioral factors, such as diet and smoking. Thus, the search for effective treatments is paramount as the population ages. Traditional therapeutic intervention consist of toxic compounds that target fast growing cells by either causing extensive DNA damage or by disrupting DNA replication. Such compounds are essentially poisons and the hope is that you kill the cancer before you kill the patient. Over the last decade many novel chemotherapeutic agents have been developed that target specific signaling pathways that tumors exploit, in the hopes of reducing toxicity and increasing efficacy. Many of these compounds have had tremendous results in particular cancers, but often have only limited scope of use. Thus, the search for effective compounds that target critical features of cancer cells, but leave normal cell unharmed is ongoing.

1.2 The nature of cancer

Cancer can be described as a process whereby the normal checkpoints that regulate growth and proliferation have gone awry. Under normal conditions growth and proliferation are tightly controlled by a variety of processes, including signals that regulate entry into the cell cycle and signals that promote programmed cell death, a form of cell suicide that maintains cellular homeostasis. There is a critical balance between growth and death. In the adult, for every new cell an old one must die. When this balance is disturbed cancers can arise.

In their seminal review of cancer, Hanahan and Weinberg described the hallmark features that distinguish cancer cells from normal cells (Figure 1-1). They elucidated six characteristics of cancer: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasiveness and metastasis (Hanahan and Weinberg 2000). By the time a tumor has been detected, it will usually possess all of these characteristics. How these characteristics are acquired is thought to be multi-step process that takes many years to come about. An initiation event must occur, a mutation in a gene or its regulatory promoter, which gives a cancer cell a competitive advantage over other cells. Over time as this cell propagates other mutations are acquired and its ability to out compete its neighbors becomes greater until finally it overruns the system. Cancer progression can be thought of in term of evolution in microcosm. Those cells and their derivatives that acquire the characteristics described by Hanahan and Weinberg will grow faster under more adverse conditions and outgrow their competitors that are limited in their need for growth factors and blood supply.

Thus, the difficulty in treating cancer is apparent; tumors are essentially superior cells in the sense of their competitive advantage of being able to propagate and bypass normal limitations to growth. Additionally, because these cells are predisposed to acquiring further mutations the likelihood that some subset will survive treatment is high and for most cancers remission is inevitable. When a cancer does return it is usually resistant to therapy that has worked previously and thus is immensely more difficult to eradicate a second time. Hence, there is a need for highly effective therapies that can

completely eradicate the tumor or, conversely, for therapies that are able to manage the tumor.

Tumor management is an argument that is gaining support, with some interesting evidence. Is it is better to try and control cancer rather than "cure" it, given that a cure is likely an unrealistic goal for the majority of cancer types. This idea stems from the fact that, though many tumors are responsive initially to aggressive chemotherapy that eventually a population of resistant cells will arise that can no longer be treated. These resistant cells are for the most part much more unhealthy than their sensitive counterparts, but once sensitive cells are eliminated the resistant cells are able to proliferate unimpeded. Thus, the idea is that by not eradicating the population of sensitive cells, but rather controlling them, limiting them to a manageable size might be better for long-term survival. One caveat would be the risk of such tumors metastasizing to other parts of the body.

1.3 Common Therapeutic Interventions

Modern chemotherapy for the treatment of cancer can be traced back to at least the 1940s, when during World War II autopsies from soldiers killed by nitrogen mustard gas showed depletion of both bone marrow and lymph nodes (DeVita and Chu 2008). Goodman and Gilman conducted seminal work for the Department of Defense demonstrating in mouse lymphoma models that application of nitrogen mustard effectively caused regression of the cancer (Freireich 1984). Several related alkylating agents were soon developed, and were effective at remising lymphoma in human patients. However, the effects were short lived. The proof-of-principle was, however, established that chemical treatment had the potential to fight cancer.

Thereafter, many investigations were conducted to find other chemical compounds that could affect the growth and spread of cancer. Early examples of effective chemotherapeutic were folate antagonists and 6-mercaptopurine (6-MP), which were able to target blood disorders (Papac 2001).

The first compound discovered that could target solid tumors was also the first example of a rational approach to cancer drug development. In the 1950s, Charles Heidelberger exploited a metabolic observation that cancers cells utilize uracil to a much greater degree than do normal cells. Heidelberger was able to target a wide variety of cancer types by attaching fluorine to uracil (5- fluorouracil), inhibiting mRNA transcription.

Perhaps the most successful therapeutics to date are the platinum based agents, the topoisomerase inhibitors and the microtubule assembly promoters, which are still the most widely used and the most effective chemotherapeutic agents on the market. The prototype platinum based drug is cisplatin, which was originally investigated to look at how bacteria respond to electric fields but was discovered to be able to prevent bacteria from dividing. Although the exact mechanism of how cisplatin works is undetermined it presumably involves the ability of cisplatin to cross link DNA to interfere with DNA replication. Thus, it targets fast growing cells indiscriminately to include intestinal cells and immune cells. A common feature of all these drugs is that they act on fast growing cells, which presents something of a problem. It creates a situation where it is a race to see if the cancer can be killed before the patient. Thus, development of more targeted therapeutics was undertaken to target specific signaling pathways that cancers exploit to proliferate uncontrollably.

One of the most successful examples of rationally designed inhibitors is imatinib (Gleevec), which specifically target the BCR-ABL fusion protein whose kinase activity promotes leukemia. This compound was specifically designed to bind the active site of the protein and block its activity. It has shown success at treating chronic phase CML. Unfortunately, as with so many other chemotherapeutics, rates of recurrence and resistance are high.

The development of monoclonal antibodies that bind to and inhibit the activity of specific target protein which function as oncogenes has also shown tremendous promise. Examples include herceptin, which targets the Her2/Nue receptor. The HER2/neu receptor is often mutated in breast cancer, which causes breast tissue to proliferate uncontrollably. Herceptin is able to bind the receptor and essentially turn it off.

Given the high incidence of tumor recurrence and subsequent resistance to therapy much more work is required to effectively treat most cancers on a long-term basis. Thus different approaches must be taken to find effective targets that can be exploited. Most targeted therapeutics attack the growth promoting mechanism cancer cells exploit. An alternative approach, that has gained ground in recent years, is to attack cancer from the opposite direction (Fesik 2005). To re-impose the natural response of a cell that is growing out of control to commit suicide, or apoptosis.

1.4 Apoptosis

Apoptosis, or programmed cell death, is a fundamental process required by all multi-cellular organisms for proper development and cellular homeostasis. Apoptosis plays important roles in neurodevelopment of the brain in the embryo, tissue sculpting of the limbs, and sexual development (Figure 1-2) (Prindull 1995, Meier, Finch and Evan

2000). The fact that cell death occurs had been widely known since the 1800s, however, little attention was paid to it until 1972 when Kerr and colleagues described the significance of regulated cell deletion and the morphological characteristics of apoptosis. Cell death is a choreographed process wherein the cell undergoes nuclear and cytoplasmic condensation, DNA fragmentation, cell shrinkage with membrane blebbing eventually leading to break up of the cell into membrane enclosed particles, which are then taken up by phagocytes or neighboring cells (Kerr, Wyllie and Currie 1972).

Apoptosis was initially characterized in the nematode worm, Caenorhabditis elgans (*C. elgans*). *C. elgans* is an ideal model organism to study programmed cell death in because of its strictly defined pattern of development (Brenner 1974). Each worm develops in an exact and invariant manner producing 1090 cells, of which 131 are culled leaving an adult worm of 959 cells. Sulston was able to show that the developmental lineage of each cell was identical and that the same cells were always purged (Sulston 1976). A genetic approach using randomized mutagenesis of the worm germ-line was then undertaken to identify genes required for the purging of cells during development. Horvitz identified *ced*-3 (cell death abnormal, *ced*) and *ced*-4 as being absolutely necessary for programmed cell death to occur (Ellis and Horvitz 1986). Later, *ced*-9 was identified as being able to prevent cell death from occurring, over expression of which blocked cell death under apoptosis inducing conditions (Hengartner and Horvitz 1994). A fourth regulator, *egl-1* (egg laying defective, *egl*), was identified as an activator of apoptosis that blocked the actions of *ced-9* (Conradt and Horvitz 1998). Thus a very linear pathway leading to programmed cell death was elucidated (Figure 1-3) (Lettre and Hengartner 2006). This pathway is highly conserved throughout evolution, and

mammalian cells possess homologues of all the *c. elgans* genes, but with much greater diversity and more elaborate control mechanisms.

The main executioners of apoptosis are the caspases, homologues of *ced-3*, which are proteases, dependent upon a cysteine residue acting as a nucleophile to cleave proteins possessing characteristic aspartic acid containing motifs (Thornberry and Lazebnik 1998). To date 14 caspases have been identified in higher vertebrates, which can be divided into two groups: those involved in inflammation (-1, -4, -5, -11, -12, -13, -14) and those that carry out apoptosis (-2, -3, -6, -7, -8, -9, -10) (Figure 1-4) (Taylor, Cullen and Martin 2008). All caspases have several distinguishing features; they are all synthesized as inactive zymogens containing a prodomain followed by p20 (large) and p10 (small) subunits (Hengartner 2000). Caspases must be cleaved for apoptosis to occur which then activates their own protease activity. Caspases are also unique in their targeting of substrates with Asp-Xxx sequences (Thornberry et al. 1997).

The apoptotic caspases can be further divided into executioner and initiator caspases. The executioner caspases (-3, -6, -7) are the actual dealers of death in the cell and cleave substrates that produce the biochemical and physical phenotypes associated with apoptosis. The initiator caspases possess long prodomains containing either a death effector domain (DED), as in the case of caspase-8, or a caspase activation and recruitment domain (CARD), as in the case of caspase-9, both of which mediate protein-protein interactions that recruit adaptor proteins necessary for apoptosis to occur (Danial and Korsmeyer 2004).

Both caspase-8 and -9 converge on caspase-3 but by different pathways, ultimately leading to the same fate. Caspase-9 is activated in response to signals

originating from the mitochondria (intrinsic), while caspase-8 is the downstream effector of signals originating from the cell surface (extrinsic) (Figure 1-5) (Taylor et al. 2008).

1.5 Intrinsic apoptosis activation

Apoptosis can be initiated in response to genotoxic stress induced by DNA damage resulting from gamma irradiation or from chemical agents, from growth factor withdrawal or cytokine withdrawal. In the case of DNA damage the cell will initiate a DNA damage response and attempt to fix the problem. If the damage is too severe the cell will initiate signals that lead to apoptosis (Roos and Kaina 2006).

The mitochondrion, in addition to its role in ATP production, is the primary gatekeeper of pro-apoptotic proteins responsible for the activation of caspases in response to genotoxic stress (Danial and Korsmeyer 2004). A key event in apoptosis is mitochondrial outer membrane permeabilization (MOMP) that allows for the release of key components required for caspase-9 activation and subsequent activation of caspase-3.

In 1996 Xiaodong Wang discovered the mammalian homologue of *ced*-4, as well as other key players involved in mitochondria-induced apoptosis. Biochemical reconstitution of caspase activity found apoptotic protease activating factor (Apaf-1) as the homologue of *ced*-4, cytochrome c (previously thought to be only an electron carrier) and caspase-9 as being required for caspase activation in vitro (Li et al. 1997, Liu et al. 1996, Zou et al. 1997). It was discovered that mitochondrial outer membrane permeabilization allowed for the release of cytochrome c into the cytosol, which is a key event in activating caspase-9. Caspase-9 must oligomerize to self-process into an active form. Oligomerization is dependent upon Apaf-1 binding cytochrome c, which makes Apaf-1 competent to bind casapse-9 by forming a large multimeric complex in the shape

of a wheel called the apoptosome. Both caspase-9 and Apaf-1 possess a CARD domain, which mediates protein-protein interaction betwixt the two. Apaf-1, when not bound to cytochrome c masks its CARD domain with a pair of WD40 domains. Cytochrome c binding dislodges the WD40 domains and allows for CARD-CARD interaction between caspase-9 and Apaf-1 mediated by binding of dATP. Apoptosome formation increases the local concentration of caspase-9 allowing self-processing to occur (Li et al. 1997).

Additional factors are also released from the mitochondria that both neutralize other inhibitory mechanism and are required for the downstream effects of apoptosis. These include apoptosis inducing factor (AIF) and EndoG, which are required for DNA fragmentation (Parrish et al. 2001, Susin et al. 1999, Li, Luo and Wang 2001). Another factor that is released is second mitochondrial activator of caspases (Smac), which functions to bind members of the inhibitor of apoptosis (IAP) family of proteins, which function to prevent unregulated activation of caspases in the cytosol (especially caspase-3, -7 and -9). Both smac and the IAPs will be discussed in section 1.10.

Elaborate mechanisms are in place to regulate when and under what circumstances mitochondrial permeabilization occurs (Figure 1-6) (Jin and El-Deiry 2005). The actions of the Bcl-2 family of proteins are paramount in this regard, which will be discussed below.

1.6 Control of intrinsic apoptosis by the Bcl family of proteins

In *c. elgans*, *egl-1* is a pro-apoptotic factor that binds to *ced-9* displacing *ced-4* and allowing it to oligomerize with *ced-3* (Conradt and Horvitz 1998). The equivalent mammalian homologue was identified as a BH-3 only Bcl-2 family member.

Because release of factors from the mitochondria can be fatal if released unintentionally, elaborate control mechanisms are in place to insure that these factors are released only when needed. The Bcl-2 family of proteins are the key regulators of the mitochondria in relationship to apoptosis (Adams and Cory 2007). The first member of the family to be identified was Bcl-2 (B-cell lymphoma 2). Bcl-2 was initially characterized as an oncogene owing to the fact that it was over expressed in 90% of human follicular lymphoma patients examined. It was also shown that all cases of over expression were correlated with a translocation event that juxtaposed Bcl-2 with the immunoglobin heavy chain locus (Bakhshi et al. 1985, Cleary and Sklar 1985, Tsujimoto et al. 1985). Instead of being a traditional oncogene Bcl-2 did not promote growth, but instead prevented death. Expression of human Bcl-2 was able to prevent apoptosis from occurring in c. elgans (Vaux, Weissman and Kim 1992).

The Bcl-2 family comprises three different groups of protein with very different functions either promoting or inhibiting apoptosis, the balance of which determine entry into the apoptotic program. The five known pro-survival members are Bcl-2, Bcl-x_L, Bcl-w, A1 and Mcl-1, all of which contain four characteristic BH (Bcl-2 homology) domains except for Mcl-1, which has only three. Each of these proteins is critical for cellular homeostasis given that loss of any one results in lethality.

The rest of the family can be classified as pro-apoptotic either directly involved in mitochondrial permeabilization or as factors the block the pro-survival arm of the family. These can be divided into the Bax (Bcl-2 associated protein X) like proteins (Bax, Bak and Bok) and the BH-3 only proteins (Bim, Bid, Puma, Noxa, Bmf, Bad, Hrk and Bik). Bax and Bak are absolutely required for initiation of apoptosis (Ruiz-Vela et al. 2005). In

uncompromised cells, BAX is inactive and located in the cytosol, but in response to genotoxic stress, BAX is inserted into the mitochondria as a homo-oligomerized multimer, resulting in downstream mitochondrial permeabilization (Danial and Korsmeyer 2004). Additionally, BAK interacts with a resident voltage-dependent anion channel protein 2 (VDAC2), which keeps BAK inactive, keeping it in check at the mitochondrion. Following the initiation of apoptosis, VDAC2 is displaced and BAK undergoes homo-oligomerization, with subsequent cytochrome c release (Danial and Korsmeyer 2004).

The BH-3 only proteins act as counters to the pro-survival Bcl-2 proteins. For example, BAD in its phosphorylated form is associated with 14-3-3, a phosphoserine binding module, that sequesters it in the cytosol, when dephosphorylated BAD is able to translocate to the mitochondria to participate in cytochrome c release. BAD is phosphorylated by protein kinase B, a pro-survival signaling pathway. Additionally, BAD had been shown to directly interact and neutralize the cell protective effects of Bcl-2 (Zha et al. 1996).

A key feature of the Bcl-2 family in determining whether a cell will undergo apoptosis or not, is the stoichiometric balance of the various pro- and anti-apoptotic elements. The tumor suppressor p53 plays a major role in shifting the balance towards apoptosis given its role as a transcription factor that is responsible for surveillance of the cell environment to ensure homeostasis, under conditions of cell damage or uncontrolled growth p53 will initiate a cascade of events leading to apoptosis. Additionally, it had been found that a majority of tumors have p53 mutations that inactivate it, and indeed, expression of wild type p53 caused leukemia cells with endogenous p53 mutation, to

undergo apoptosis (Yonish-Rouach et al. 1991). Furthermore, since p53 is a transcription factor and that many chemotherapeutics work by activation of p53, it was determined that p53 is able to up regulate many pro-apoptotic genes including Bax (Miyashita et al. 1994, Zhan et al. 1994).

1.7 Extrinsic apoptosis activation

Apoptosis can also occur in response to signals originating from cell surface receptors. The receptors that constitute this pathway are a subfamily (the death receptors) of the TNF receptor (TNFR1) super family of receptors that are involved in a number of physiological processes to include regulation of the immune system, general survival signaling through NF-kB and initiation of apoptosis (Aggarwal 2003).

Currently there are six receptors belonging to the death receptor family (TNFR1, CD95 (Fas, APO-1), TRAMP (APO-3, DR-3), TRAIL-R1 (DR-4), TRAIL-R2 (DR-5) and DR6), they are all cell surface cytokine receptors that can induce cell death following ligation of their cognate ligands or receptor specific agonistic antibodies. All are type-I transmembrane proteins with N-terminal extracellular domains responsible for ligand binding and up to six intracellular C-terminal cysteine rich domains responsible for transduction of the signal into the cell. Death receptors are also characterized by containing a highly conserved death domain (DD), which act as a platform for the recruitment of other adaptor proteins required for cell death (Itoh and Nagata 1993, Tartaglia et al. 1993). Adaptor proteins often also contain death effector domains (DED) which facilitate recruitment of proteins such as caspase-8 that execute the cell death program from the receptor. Cell death initiated from death receptors is mediated by caspase 8. The path of caspase-8 activation is very similar among all the death receptors

with the exception of TNF, which will be discussed separately owing to its complicated signal potential. CD95/Fas and TRAIL are rather linear in comparison and suffice as an introduction.

CD95 was initially identified as a cell surface antigen targeted by apoptosis inducing antibodies (Trauth et al. 1989, Yonehara, Ishii and Yonehara 1989) and activated by membrane bound CD95 ligand (CD95L/FasL), but not soluble CD95L. Cloning of the ligand shortly thereafter also confirmed that this receptor belonged to the TNF super family and the mode of death was apoptosis (Suda et al. 1993). CD95 seemed to be primarily used by cytotoxic T-cells to kill peripheral blood T lymphocytes (Suda et al. 1995). Unfortunately, use of CD95L as an anti-tumor agent did not live up to hopes as mice treated with CD95L quickly died from massive liver failure (Ogasawara et al. 1993). Upon ligand binding, CD95 recruits Fas-associated protein with DD (FADD), a protein that contains both a DD and a DED, thus bridging the receptor to caspase-8, which also contains a DED, to form a death inducing signaling complex (DISC) (Figure 1-7) (Kischkel et al. 1995, Wajant 2002). Typically, both ligand and receptor functions as either dimers or trimers. This is particularly important for the receptor. Receptors are pre-assembled but incompetent for signal transduction until ligand binding. Receptor trimerization and possible incorporation into lipid rafts probably function to increase the local concentration of caspase-8 allowing for auto-processing (Boatright et al. 2003, Donepudi et al. 2003). This seems to be very similar to apoptosome formation, which also increases the local concentration of caspase-9 allowing for autocatalytic processing.

TNF related apoptosis inducing ligand (TRAIL) was initially identified from an EST screen based on homology to CD95L (Pitti et al. 1996, Wiley et al. 1995) and found

to be capable of binding five different receptors only two of which carry a death domain (TRAIL-R1 and TRAIL-R2). The others are considered decoy receptors and do not transduce a signal, but instead bind TRAIL as competitors of the bonafide receptors. Both TRAIL receptors function very similarly to CD95. Both recruit FADD and caspase-8 to induce apoptosis. As opposed to CD95L, TRAIL seems to have far fewer side effects and is fairly potent at inducing apoptosis in model systems and the are many trials underway to see if TRAIL is therapeutically viable (Johnstone, Frew and Smyth 2008).

Though CD95 and TRAIL are fairly linear pathways their effects are context dependent. In some cells caspase-8 activation by the receptor is enough to induce apoptosis (type I), whereas in other cells caspase-8 activation alone is not sufficient to induce apoptosis. In these cells caspase-8 must also cleave Bid, a member of the Bcl-2 family of proteins, which produces a truncated form of the protein, tBid. tBid then acts on another Bcl-2 family protein, Bax, to cause mitochondrial permeabilization and release of cytochrome c, thus activating the intrinsic apoptosis pathway (Esposti 2002, Li et al. 1998).

1.8 TNF/TNFR1/NF-kB signaling pathway

TNF is different from CD95 and TRAIL signaling due to its pleiotropic effects. TNF through its receptor TNFR1 is able to signal either survival or death depending on the context. Though TNF was first identified in 1975, as a factor produced by the immune system able to induce cytotoxicity in tumor cell as well as in certain animal models (Carswell et al. 1975), knowledge of its effects had been known since the end of the 19th century and was one of the first described therapeutics for the treatment of cancer (Coley 1991). Coley had observed that a patient with malignant facial tumor was cured

by a happenstance of fate; following an operation to remove part of the tumor, a bacterial infection set in and the tumor disappeared. Following that observation Coley developed filtrated bacterial extracts as a treatment, though the work could not be repeated (Balkwill 2009). Further work on the subject, found that lipopolysacharride, produced by bacteria, could also shrink tumors. It was not until 1975 when Carswell separated the effects of endotoxin from the response of the cell. It was the cell, specifically macrophages, that produced the factor called, tumor necrosis factor (TNF), and not something in the bacterial filtrates that caused tumors to shrink (Carswell et al. 1975).

With the cloning of TNF in 1984 (Pennica et al. 1984), it became possible to further characterize the effects of the ligand and the receptors it bound. TNF was found to bind two distinct receptors TNFR1 and TNFR2 (Aggarwal, Eessalu and Hass 1985) and to be related to lymphotoxin α, a factor produced by lymphocytes that has similar properties to TNF but whose function is largely unknown still (Gray et al. 1984). TNFR1 and TNFR2 were later cloned allowing for further elucidation of the signaling pathway (Heller et al. 1990, Loetscher et al. 1990, Schall et al. 1990, Smith et al. 1990). Mouse knockout studies revealed critical roles for TNF in host immune defense and response to injury and its involvement in autoimmune disease (Beutler 1999). In particular, monoclonal neutralizing antibodies against TNF have shown significant promise in the treatment many autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease (Taylor et al. 2000, Blam, Stein and Lichtenstein 2001).

The major signaling pathway that TNF activates is nuclear factor- kappaB (NF-κB), a signaling pathway that up-regulates a vast number of pro-inflammatory, pro-survival and anti-apoptotic genes in response to a large array of physical and chemical

insults (Gilmore 2009). Dysregulation of NF-κB has been implicated in a number of human disease pathologies to include cancer (Courtois and Israel 2004, Courtois and Smahi 2006). NF-κB is a transcription factor family that consists of five members with conserved N terminal Rel homology domains (RHD) that mediate DNA binding and homo-/heterodimerization with each other: they are p50, p52, p65, c-Rel and RelB. Normally NF-κB is held in the cytosol bound to Iκ-B proteins, which act to prevent translocation of NF-κB into the nucleus. At the receptor level, ligand binding causes the recruitment of Iκ-B kinases (IKK) and their activation, activated IKKs phosphorylate IκB triggering its ubiquitination and subsequent proteasome mediated degradation. Removal of IκB allows NF-κB to move into the nucleus and up-regulate target genes (Figure 1-8) (Hayden and Ghosh 2008).

There are many receptors and ligands that regulate NF-κB, but all seem to share some similarity in the components utilized by the system. For TNF/TNFR1, ligand binding causes a conformational change allowing for the recruitment of the adaptor, TNFR1 associated death domain protein (TRADD), which binds TNFR1 through its DD. TRADD then further recruits other proteins necessary for signaling to occur. For NF-κB activation TNF receptor associated factor 2 (TRAF2) and TRAF1 are recruited which then recruit cIAP1, cIAP2 and receptor interacting kinase 1 (RIPK1) (Wang et al. 1998, Kelliher et al. 1998, Li et al. 2006, Ea et al. 2006). This complex referred to as complex I is critical for IKK phosphorylation and subsequent NF-κB activation (Micheau and Tschopp 2003). Concurrently, and at a much slower rate, another signaling complex is formed that has the potential to signal apoptosis. TRADD in this case is able to recruit

caspases-8 and FADD (Micheau and Tschopp 2003). This complex is insufficient to cause cell death so long as the NF-κB signal is active and strong enough (Figure 1-9).

TNF can signal apoptosis, but only in limited circumstances. The primary NF-κB pathway must be shutdown or the levels of genes regulated by NF-κB must be blocked. For example, NF-kB up-regulates c-FLIP, cIAP1, cIAP2, XIAP and Bcl-2, all major mediators of resistance to apoptosis (discussed further below). Typically, TNF mediated apoptosis is induced with co-treatment of cycloheximide (CHX) (Kreuz et al., 2001). CHX is a protein translation inhibitor that very quickly affects the levels of c-FLIP in the cell. Reduced c-FLIP levels allows caspases-8 to be more easily activated (Micheau et al. 2001, Kreuz et al. 2001).

1.9 Regulation of caspase activation by IAPs, Smac and c-FLIP

The final barriers to activation of apoptosis consist of members of the inhibitor of apoptosis (IAP) family of proteins and cellular FLICE-inhibitory protein (c-FLIP), which directly bind to and prevent caspases activation. In general, IAPs are specific to caspases-3, -7 and -9, while c-FLIP antagonizes caspase-8 activation. The importance of IAPs in relationship to apoptosis and to cancer became evident upon the realization that IAPs are often up regulated in cancer and that over expression of IAPs block cell death (Salvesen and Duckett 2002, Deveraux and Reed 1999). Additionally, c-FLIP has also been implicated in tumor progression and resistance to apoptosis by actively preventing caspase-8 activation (Thome and Tschopp 2001, Igney and Krammer 2002)

The first IAP was identified as a baculovirus gene able to inhibit cell death in virally infected *Spordotera frugiperda* insect cells (Crook, Clem and Miller 1993). The function of the virally encoded gene was to prevent apoptosis for occurring in infected

cells allowing the virus to propagate (Hawkins et al. 1998, Uren et al. 1996). Subsequently, IAP genes were identified in virtually all eukaryotic organisms able to carry out apoptosis (Uren, Coulson and Vaux 1998).

There are eight mammalian members of the IAP family: X-linked IAP (XIAP/BIRC4) (Deveraux et al. 1997), cellular IAP1/ (cIAP1/ BIRC2) (Rothe et al. 1995), cIAP2 (BIRC3) (Rothe et al. 1995), neuronal IAP (NAIP/BIRC1) (Roy et al. 1995), BIR-containing ubiquitin conjugating enzyme (BRUCE/ Apollon/ BIRC6) (Hauser et al. 1998), Survivin (BIRC5) (Ambrosini, Adida and Altieri 1997), Livin (BIRC7) (Vucic et al. 2000) and testis-specific IAP (Ts-IAP / BIRC8) (Richter et al. 2001) (Figure 1-10) (Srinivasula and Ashwell 2008). All IAPs are characterized as having from 1 to 3 evolutionarily and structurally conserved baculovirus IAP repeat (BIR) domains, which based on the solution structure of the domain coordinates a zinc ion utilizing a histidine and three cysteine residues (Hinds et al. 1999). BIR domains mediate interactions with target proteins, particularly with caspases (Shi 2004, Shiozaki and Shi 2004) as well as other signaling components such as TAK1 and TRAF2 (Varfolomeev et al. 2006, Lu et al. 2007, Samuel et al. 2006). Multiple BIR copies allow for increased affinity for target proteins and an expanded range of targets with which an IAP can interact (Srinivasula and Ashwell 2008). Additionally, XIAP, cIAP1 and cAIP2 contain a C-terminus Really Interesting New Gene (RING) domain, which possesses E3 ubiquitin ligase activity (Yang et al. 2000, Vaux and Silke 2005). The RING domains within these proteins have been implicated in both auto-ubiquitination and ubiquitination of target proteins such as caspase-3 and -9 (Morizane et al. 2005, Silke et al. 2005, Suzuki, Nakabayashi and Takahashi 2001). It has also been shown that cIAP1s RING domain is required for cIAP2

ubiquitination (Conze et al. 2005). Finally, cIAP1 and cIAP2 each have a CARD domain, which gives them the potential to interact with proteins that carry a CARD domain. However, no functional role for the cIAP1 and cIAP2 CARD domains has been identified.

XIAP, cIAP1 and cIAP2 are the best characterized of the IAPs that play a role in inhibition of apoptosis. Only XIAP has been shown to directly inhibit the catalytic activity of caspases-3, -7, and -9 (Chai et al. 2001, Riedl et al. 2001, Shiozaki et al. 2003). Although, cIAP1 and cIAP2 can bind to caspases-3, -7 and -9, they cannot suppress catalytic activity (Eckelman and Salvesen 2006).

The mechanism of how XIAP inhibits caspase activation has been well studied. XIAP has 3 BIR domains (1-3). BIR2 has been shown to inhibit active caspase-3 and -7, while BIR3 targets active caspase-9 (Shi 2002). Initial studies of how XIAP inhibits caspases-3 and -7 revealed that the BIR2 domain and a number of residues N terminal to it were critical (Sun et al. 1999). X ray crystallographic structures of BIR2 bound to caspases-3 and -7 solved by several groups showed that an 18 residue portion of XIAP directly N terminal to BIR2 bind the substrate binding pocket of the active proteolytic site of caspases-3 and -7, blocking substrate access (Chai et al. 2001, Huang et al. 2001, Riedl et al. 2001) (Figure 1-11) (Shiozaki and Shi 2004) . Inhibition of Caspase-9 is achieved by an entirely different way, mediated by the BIR3 domain of XIAP. An x-ray crystal structure of caspase-9 bound to BIR3 revealed that XIAP is able to keep caspases-9 in its monomeric state. XIAP does this by binding to the caspase-9 homodimerization binding surface. (Shiozaki et al. 2003).

Functionally, XIAP does not seem to be essential to prevent apoptosis on its own

as XIAP knockout mice show no overt phenotype (Harlin et al. 2001). However, it does seem to protect certain cells, such as post-mitotic neurons and cardiomyocytes, from certain stressors (Potts et al. 2003, Potts et al. 2005). cIAP1 and cIAP2 knockout mice also do not show overt phenotypes, though a double knockout has not yet been generated. However, in cell culture triple knockdown utilizing siRNA showed that these cells are highly sensitive to pro-apoptotic stimuli (Wang, Du and Wang 2008).

cIAP1 and cIAP2 do not directly inhibit caspases, but can still bind to caspases-3, -7 and -9 (Eckelman and Salvesen 2006). They do, however, still affect apoptosis by other means since over expression does block induced apoptosis in a yeast system where cIAP1 and cIAP2 can prevent caspase activation (Wright, Han and Hockenbery 2000, Jin and Reed 2002) and that over expression of cIAP1 and cIAP2 has been found to be a prime determinant of tumorigenesis and of drug resistance in cancer (Eckelman, Salvesen and Scott 2006). Exactly how cIAP1 and cIAP2 prevent apoptosis is still unclear although they have been shown to be able to ubiquitinate several targets that promote general cell survival.

cIAP1 and cIAP2 do, however, have other functions important in the regulation of survival and death. Particularly important is their role in NF-κB activation through TNF signaling (Rothe et al. 1995, Shu, Takeuchi and Goeddel 1996). It had been shown that for TNFR1 to efficiently activate NF-κB, that upon TNF binding to TNFR1: TRAF2, TRAF1, cIAP1 and cIAP2 are recruited to the receptor facilitating the further recruitment of RIPK1 leading to activation of IKKα and IKKβ. Recently, it has been shown that cIAP1 and cIAP2 actually function to hold RIPK1 at the receptor and that loss of both IAPs results in the release of RIPK1 from the receptor and incorporation into a caspase-8

activating complex (Wang et al. 2008).

Given the strict control IAP proteins exert over the cell death process, a mechanism of relieving inhibition must be available to allow regulated cell death to occur. Two groups initially identified second mitochondrial activator of caspases (Smac) (Du et al. 2000) (also called Diablo (Verhagen et al. 2000) as a protein capable of relieving inhibition of caspases by dislocating IAPs. Smac is sequestered within the mitochondria and is released along with cytochrome c upon intrinsic apoptosis activation. Smac sequestration is a key regulatory control given that inappropriate release can cause cell death and that expression of Smac missing its mitochondrial targeting sequence is sufficient to cause cell death (Yang et al. 2000).

Smac upon entry into the cytosol dimerizes, a critical feature of its ability to inhibit IAPs, and binds its targets, utilizing 4 highly conserved N terminal residues Ala-Val-Pro-Ile (AVPI) which are absolutely critical, as deletion of these residues abolishes its ability to promote apoptosis (Du et al. 2000). X ray crystallographic structure of XIAP-Smac shows that the AVPI residues bind to the BIR3 domain of XIAP (Liu et al. 2000, Wu et al. 2000) thus dislocating caspases-9. In addition, Smac is able to interact with the BIR2 domain and disrupt caspases-3 and -7 (Chai et al. 2000) (Figure 1-12 and 1-13) (Shi 2002, Shiozaki and Shi 2004).

Interestingly, Smac is also able to induce the degradation of cIAP1 and cIAP2, and indeed, when short peptides of the AVPI motif are introduced, that too, is sufficient to induce degradation (Du et al. 2000). It has been demonstrated that both cIAP1 and cIAP2 possess functional RING domains and that they are capable of auto-ubiquitinating themselves for destruction by the proteasome.

Finally, c-FLIP is a homologue of caspase-8 lacking its proteolytic domain. Two main isoforms of the protein exist having different functionalities of inhibiting caspase-8 activation. $FLIP_L$ consists of two N-terminal DEDs and a C-terminal CARD lacking enzymatic activity, whereas $FLIP_S$ lacks its CARD and has both N-terminal DEDs and a short C-terminal stretch of amino acids not found in $FLIP_L$ (Donepudi et al. 2003). Both $FLIP_L$ and $FLIP_S$ can be recruited to the caspase-8 containing DISC, but function differently. $FLIP_S$ prevents the initial cleavage of caspase-8 while $FLIP_L$ inhibits the final cleavage between the pro-domain and the p20 subunit of the p43/41 fragment (Wajant 2003b).

c-FLIP has also been shown to play a significant role in tumor progression and resistance to death receptor induced apoptosis (Irmler et al. 1997). Many cancers have been found to over express c-FLIP and relative levels of c-FLIP correlate with final outcomes. Additionally, over expression of c-FLIP in cell culture has been shown to make cells more resistant to apoptotic stimuli (Xiao et al. 2003, Mitsiades et al. 2002), while deletion or siRNA knockdown of c-FLIP promoted sensitivity (Siegmund et al. 2002, Hyer et al. 2002). It has also been demonstrated that c-FLIP is the primary repressor of caspase-8 activation induced by TNF, as evidenced by TNF induced apoptosis with the use of protein translation inhibitors to suppress c-FLIP production (Wang et al. 2008).

1.10 Smac Mimetics as cancer therapeutics

The vast majority of chemotherapeutic interventions against cancer are based on the idea of targeting growth promoting signaling pathways. Only recently has attention been given to the idea of re-establishing the apoptotic pathway in order to kill cancer

cells (Fesik 2005). The realization that most cancers not only have aberrant growth signaling, but also have elevated levels of antiapoptotic gene products such as IAPs, c-FLIP and Bcl-2 spurred that development of compounds that could suppress the effects of these proteins. One of the more successful examples of this is the development of Bcl-2 inhibitors (Oltersdorf et al. 2005, Labi et al. 2008).

In particular, given the important role that IAPs, specifically XIAP, cIAP1 and cIAP2, play in regulation of apoptosis and the nature of the interaction between Smac and XIAP, a number of groups have developed small molecule mimetics of the four amino acid residues of Smac that interact with the BIR domains of the IAPs (Vince et al. 2007, Sun et al. 2004, Mastrangelo et al. 2008, Sun et al. 2008b, Lu et al. 2008, Sun et al. 2009, Peng et al. 2008, Zhang et al. 2008, Huang et al. 2008, Sun et al. 2008a, Nikolovska-Coleska et al. 2008, Varfolomeev et al. 2007).

In addition, collaboration between the laboratories of Dr. Xiaodong Wang and Dr. Patrick Harran were one of the first to synthesize a smac mimetic in 2004 (Li et al. 2004). The idea behind the project was to be able to inhibit IAPs while not necessarily having to disrupt the mitochondria to induce apoptosis in conjunction with other pro-apoptotic stimuli that normally would have little effect on the cells involved. Proof of principle had been demonstrated early on through the use of small peptides of the AVPI sequence introduced into cancer cells along with other pro-apoptotic stimuli (Arnt et al. 2002, Fulda et al. 2002, Pardo et al. 2003, Yang et al. 2003). These lead to the development of a number of potentially, therapeutically viable small molecule inhibitors, including our own (Li et al. 2004).

1.11 Design and synthesis of a small molecule smac mimetic

The crystal structure of Smac in complex with the BIR3 domain of XIAP shows the Smac interacts with a groove formed on the surface of BIR3 of XIAP (Wu et al. 2000). Testing of small peptides mimicking the AVPI motif showed that structural variations in the C-terminal end of the tetrapeptide are acceptable and that a peptide of AVPF was superior at disrupting XIAP-caspase-9 interaction. Computer simulations using the AVPF peptide as a guide resulted in the synthesis of 180 synthetic compounds and were tested for their ability to disrupt caspase-9-BIR3 interaction (Liu et al. 2000). Of these oxazoline (compound 1) (Figure 1-14) was the most potent competitor but when it was tested for in vitro caspase-3 activation in soluble HeLa extracts its potency was far less than that of Smac itself. To overcome this deficiency further modification of the base structure was performed and resulted in modifications of the original oxazoline to a tetrazoyl thioether (compound 2) (Figure 1-14). Manipulation of the alkyne of the tetrazoyl thioether produced a by-product eventually characterized as C2-symmetric diyne (compound 3) (Figure 1-14) (Li et al. 2004). Comparisons between the isolated compounds versus the original AVPF peptide showed that compound 3 was equivalent to AVPF at binding to BIR3 domain of XIAP (Figure 1-15), of activating caspase-3 in vitro (Figure 1-16) and at displacing Smac from XIAP under non-denaturing conditions (Figure 1-17) and at disrupting the interaction of XIAP and caspase-9 using GST-tagged caspse-9 (Figure 1-18) (Li et al. 2004).

1.12 Smac mimetic is able to synergize with TRAIL and TNF to induce apoptosis

Testing of compound 3 in cell culture using T98G glioma cells demonstrated the therapeutic potential of smac mimetic. T98G cells are highly resistant to cell death, neither compound 3 alone or TRAIL alone had any effect, yet when compound 3 was

used in conjunction with TRAIL (50ng/ml) cell death was highly induced (Figure 1-19). Western blot analysis of caspase-8 activation and PARP cleavage, an indicator of caspase-3 activation, indicated that T98G cells underwent apoptosis with concentration of compound 3 as low as 100pM (Figure 1-20) (Li et al. 2004). To validate that smac mimetic was actually interacting with IAPs; a biotinylated version was created and used in T98G cell extracts to attempt to pull down XIAP. Interestingly, not only was XIAP pulled down, but cIAP1 and cIAP2 as well. Though not necessarily surprising given the high homology between the BIR domains of all three proteins, it did indicate possibility that smac mimetic might work well with TNF, since cIAP1 and cIAP2 are involved in TNF signaling (Figure 1-21) (Li et al. 2004).

Given the synergy between smac mimetic and TRAIL, TNF was also examined for synergistic potential. Identical results were seen with TNF co-treatment with smac mimetic. Cell death was induced with 100nM compound 3 and TNF (10ng/ml), while TNF alone had no effect (Figure 1-22) (Li et al. 2004). The effects of smac mimetic were very similar to that seen with CHX co-treatment, which is the standard method for inducing TNF mediated apoptosis (Figure 1-23 and 1-24) (Li et al. 2004).

1.13 Screening of a large panel of cells reveals cell lines that show synergy between smac mimetic and TNF, as well as single agent sensitive cell lines

Following the initial discovery that T98G cells showed synergistic response to smac mimetic and TNF, a panel of available cancer cell lines was evaluated for similar properties. Dr Lin Li performed this screen on approximately 20 different cell lines, 9 of 19 showed synergy (Table 1-1). Subsequently, as more cell lines were tested several

showed single agent smac mimetic sensitivity. These included non-small cell lung cancer cell lines HCC44, HCC461, breast cancer cell line MDA-MB-231 and skin cancer cell line SK-MEL-5. All showed sensitivity to smac mimetic in the low nanomolar range (Figure 1-25). Thus, three different types of cell could be identified: those that are single agent sensitive, those that also require TNF (H2009) and those that are completely resistant (HCC827).

1.14 Discussion

Small molecule mimetics of Smac were demonstrated to be effective at synergizing with pro-apoptotic stimuli to induce apoptosis. Surprisingly, stimuli that normally activate the extrinsic apoptotic pathway worked quite well given that endogenous smac is not required for extrinsic apoptotic activation. However, it was demonstrated that smac mimetic does bind to cIAP1 and cIAP2, which are involved in TNF signaling, and that IAP inhibition might be important for TNF induced cell death.

The finding that some cells show single agent sensitivity was quite surprising and quite perplexing and became the focus of further investigation. If the mechanism of sensitivity could be determined then that could expand the use of smac mimetic and potentially lead to identifying other components necessary to induce cell death.

This is the point at which I became involved with smac mimetic and my initial goal was to determine the mechanism of action and the nature of single agent sensitivity.

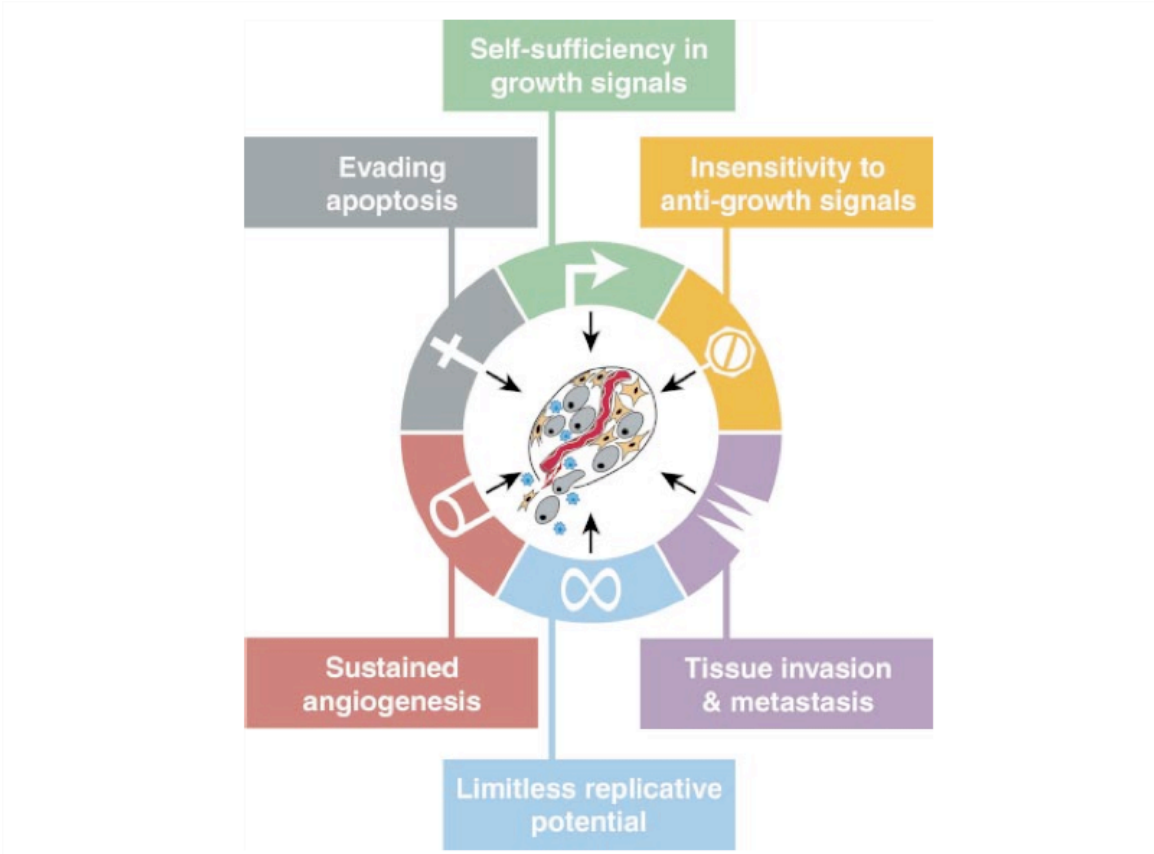

Figure 1-1. Acquired Capabilities of Cancer.
Adopted from (Hanahan and Weinberg 2000).

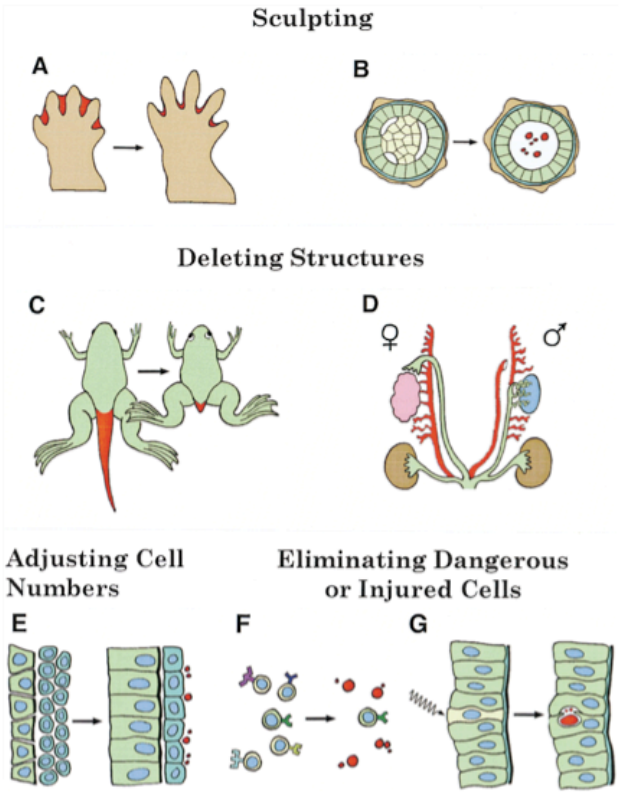

Figure 1-2. Some functions of programmed cell death in animal development
(A and B) Sculpting. (C and D) Deleting unwanted structures. (E) Controlling cell numbers. (F and G) Eliminating nonfunctional, harmful, abnormal, or misplaced cells. Adopted from (Jacobson, Weil and Raff 1997).

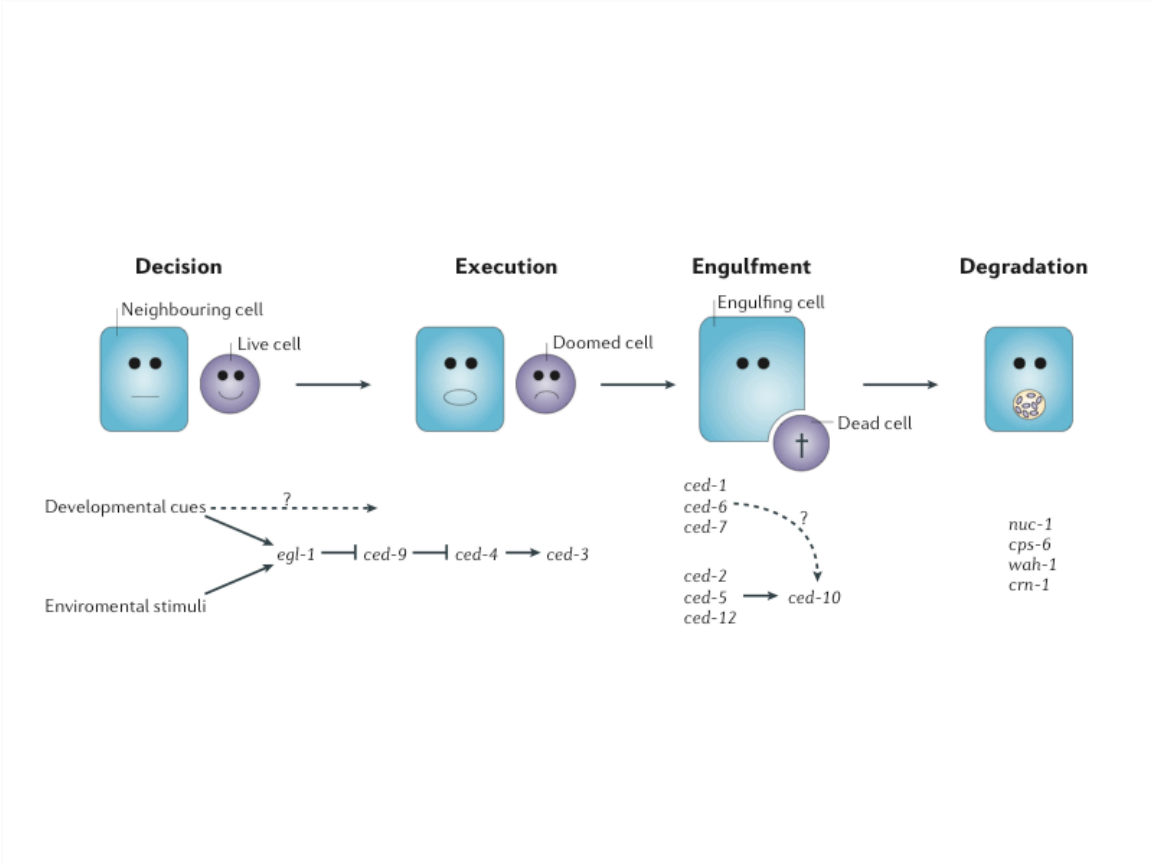

Figure 1-3. Schematic representation of *c. elgans* apoptosis pathway.
The induction of egg-laying defective (egl)-1 transcription, either by developmental cues or external signals, is key in the decision of a cell to undergo apoptosis in C. elegans. The cell death abnormal (ced)-9 gene normally blocks apoptosis by inhibiting ced-4 activity. However, once activated, egl-1 inhibits the anti-apoptotic gene ced-9, thereby allowing the pro-apoptotic genes ced-4 and ced-3 to interact and execute cell suicide. The dead cell is then recognized and engulfed by a neighboring cell: apoptotic-corpse clearance requires the participation of two partially redundant genetic pathways (ced-1?ced-6?ced-7 and ced-2?ced-5?ced-12) that converge at the level of ced-10. Finally, several genes (abnormal nuclease-1 (nuc-1), CED-3 protease suppressor-6 (cps-6), worm AIF homologue-1 (wah-1) and cell-death-related nuclease-1 (crn-1)) encode proteins with nuclease activity that participate in the degradation of the dead cell within the engulfing cell. Adopted from (Lettre and Hengartner 2006).

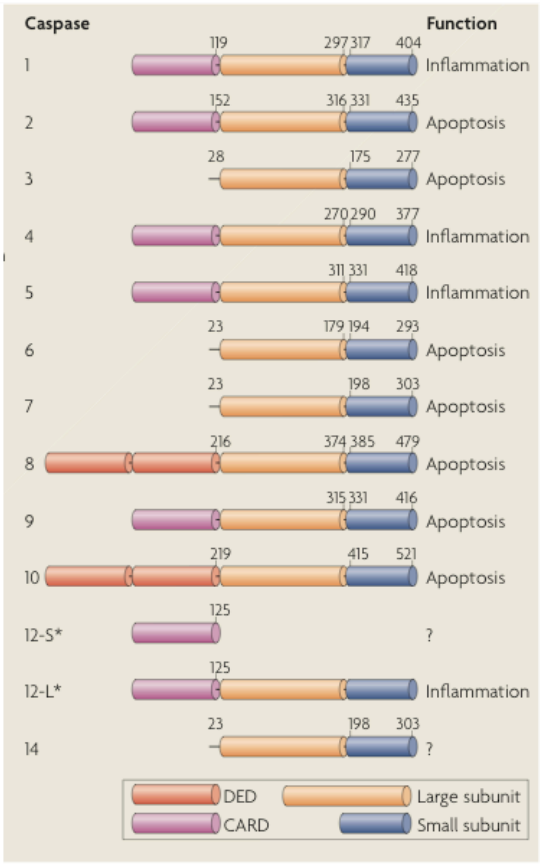

Figure 1-4. The caspase family of proteases.
Caspases (cysteine aspartic acid-specific proteases) are highly specific proteases that cleave their substrates after specific tetrapeptide motifs (P4-P3-P2-P1) where P1 is an Asp residue. The caspase family can be subdivided into initiators, which are able to auto-activate and initiate the proteolytic processing of other caspases, and effectors, which are activated by other caspase molecules. The effector caspases cleave the vast majority of substrates during apoptosis. Adopted from (Taylor et al. 2008).

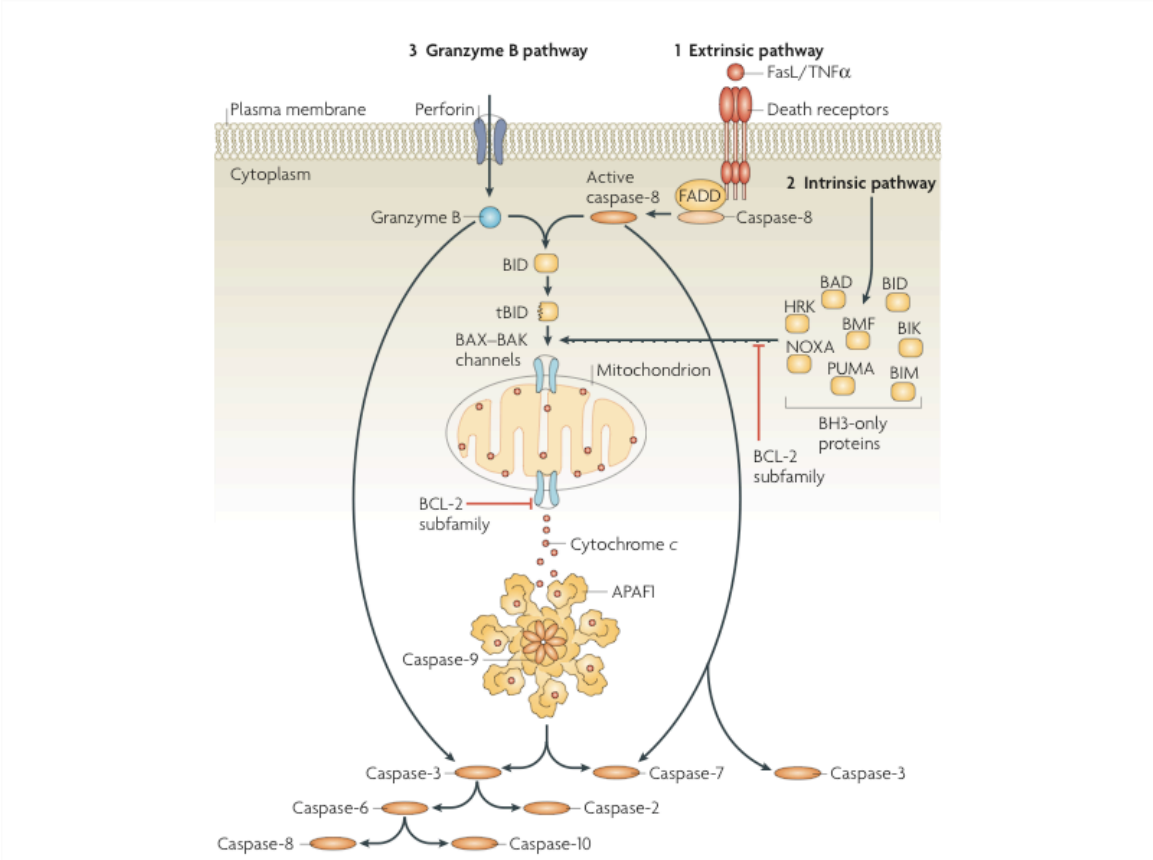

Figure 1-5. Extrinsic and intrinsic paths of apoptosis.
Caspase activation by the extrinsic pathway (route 1) involves the binding of extracellular death ligands (such as CD95 or TNF) to transmembrane death receptors. In the intrinsic pathway (route 2), diverse stimuli that provoke cell stress or damage typically activate one or more members of the BH3-only protein family. BH3-only proteins act as pathway-specific sensors for various stimuli. BH3-only protein activation above a crucial threshold overcomes the inhibitory effect of the anti-apoptotic BCL-2 family members and promotes the assembly of BAK–BAX oligomers within mitochondrial outer membranes. The granzyme B-dependent route to caspase activation (route 3) involves the delivery of this protease into the target cell through specialized granules that are released from cytotoxic T lymphocytes (CTL) or natural killer (NK) cells. Adopted from (Taylor et al. 2008).

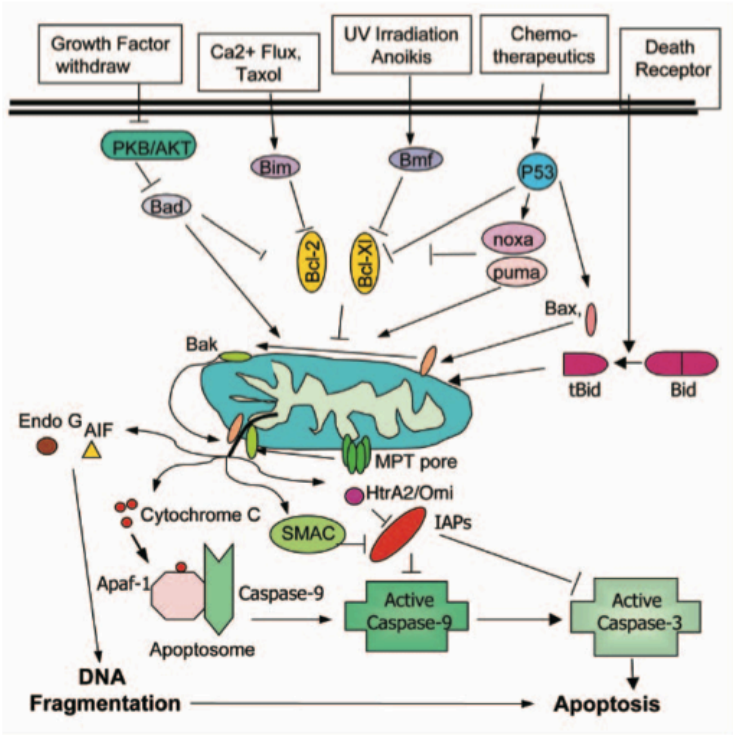

Figure 1-6. Overview of the intrinsic apoptosis activation pathway.
See text for details. Adopted from (Jin and El-Deiry 2005).

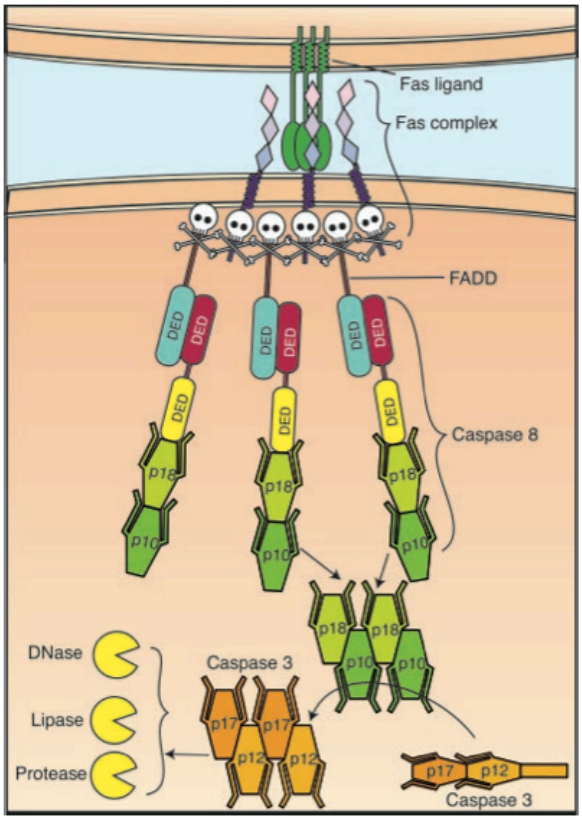

Figure 1-7. CD95 death receptor activation pathway.
See text for details. Adopted from (Wajant 2002).

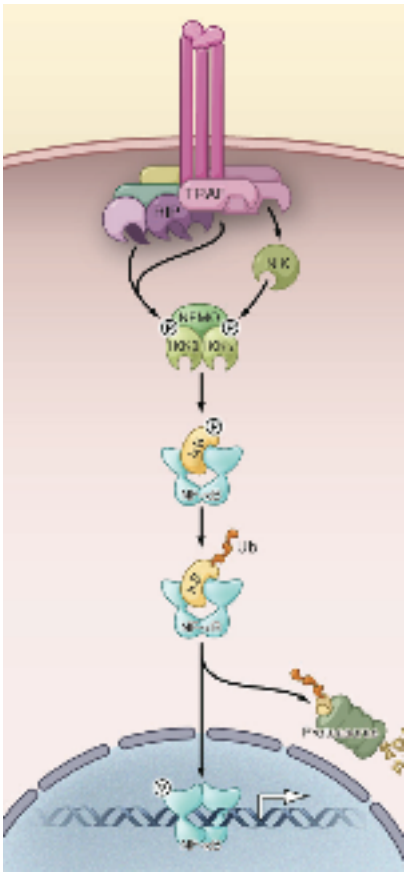

Figure 1-8. NF-kB Signaling Pathways.
Following receptor ligation and recruitment of receptor proximal adaptor proteins, signaling to IKK proceeds through TRAF/RIP complexes, generally in conjunction with TAK1, leading to canonical NF-kB signaling, or through TRAFs and NIK leading to the noncanonical NF-kB pathway. IKK activation results in I-kB alpha phosphorylation and degradation in the canonical pathway or p100 processing to p52 in the noncanonical pathway. Phosphorylated NF-kB dimers bind to I-kB DNA elements and induce transcription of target genes. Adopted from (Hayden and Ghosh 2008).

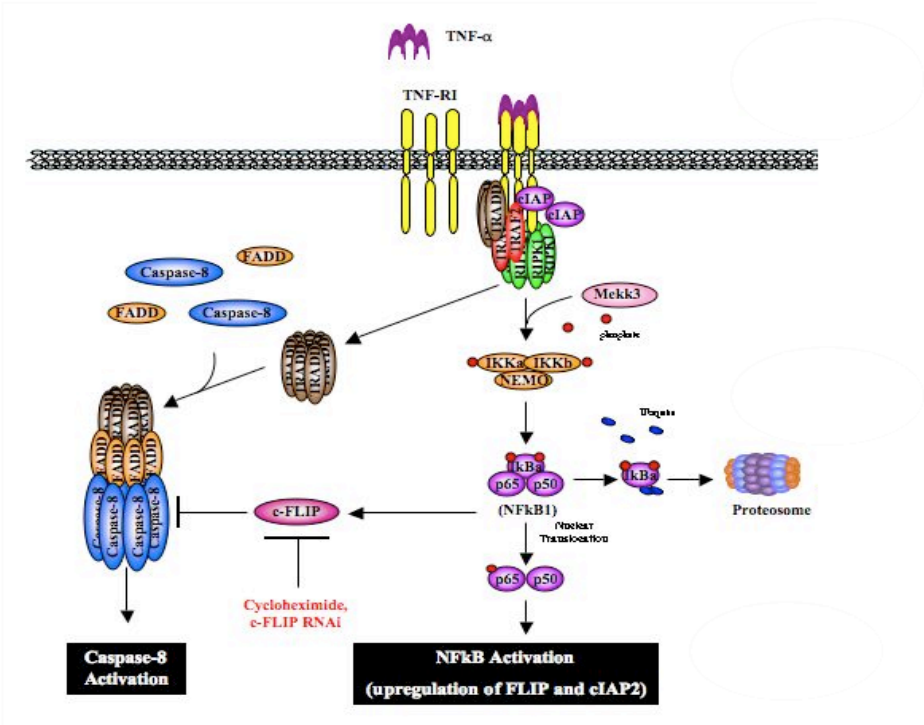

Figure 1-9. Overview of TNF/TNFR1 signaling pathway.
TNF can signal two distinct outcomes, either survival or death. See text for details.

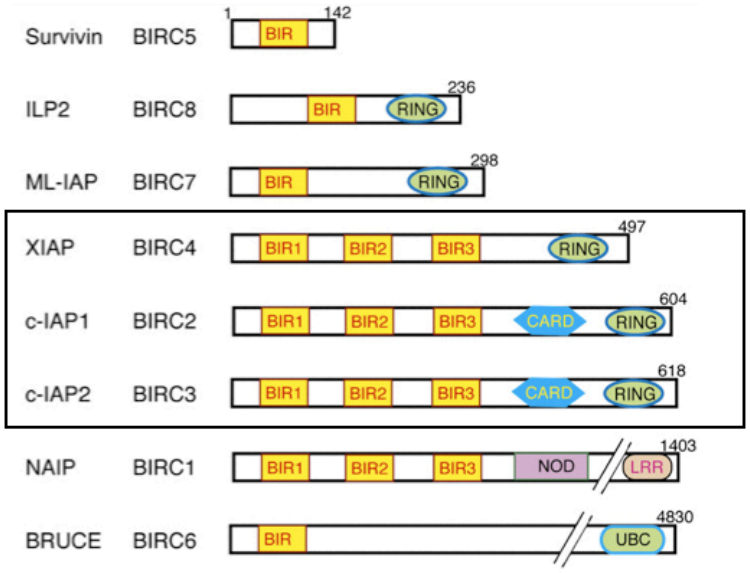

Figure 1-10. Schematic representation of the human and drosophila IAP family of proteins.
The number of residues in each IAP as well as the functional motifs that they contain is shown. Abbreviations: CARD, caspase-associated recruitment domain; UBC, ubiquitin-conjugation; NOD, nucleotide-binding oligomerization domain; and LRR, leucine-rich repeats. Adopted from (Srinivasula and Ashwell 2008).

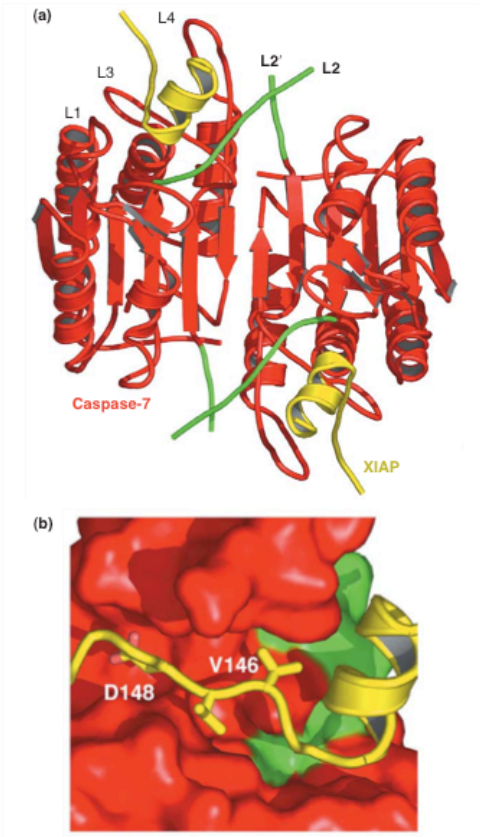

Figure 1-11. Molecular mechanism of X-linked inhibitor of apoptosis (XIAP)-mediated inhibition of caspase-7.
(a) The structure of caspase-7 bound to an XIAP fragment. A short peptide segment (yellow) immediately preceding the second baculoviral IAP repeat (BIR2) domain of XIAP binds to the substrate-binding groove of caspase-7 (red). This prevents substrate binding and subsequent catalysis. (b) Close-up of the binding interactions between the XIAP fragment and the substrate-binding groove of caspase-7. Two residues of XIAP that make important contacts with caspase-7 are labeled. Adopted from (Shiozaki and Shi 2004).

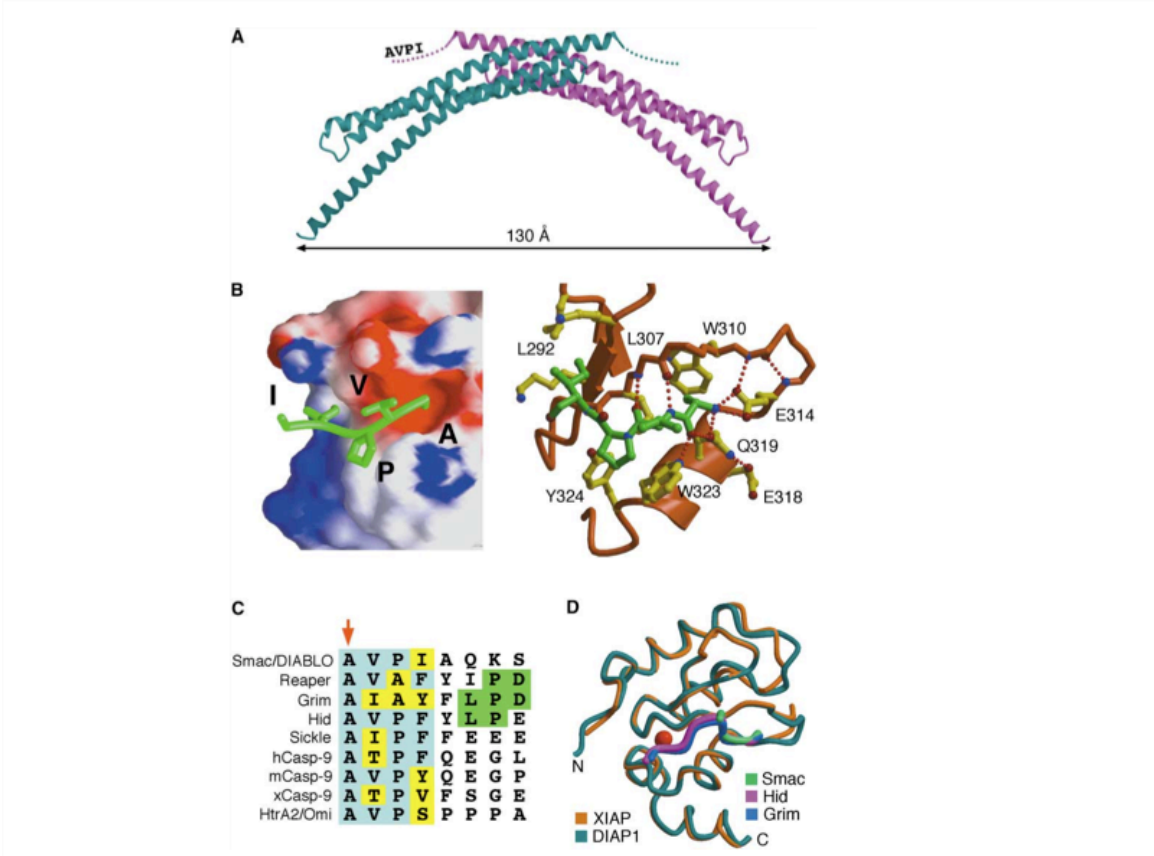

Figure 1-12. A Conserved IAP-Binding Tetrapeptide Motif.
(A) Structure of the mature Smac. The disordered N-terminal residues are shown as dotted lines. (B) Close-up view of the Smac N-terminal tetrapeptide binding to the BIR3 surface groove. The BIR3 domain is shown either by electrostatic potential (left panel) or in ribbon diagram (right panel) to highlight the interactions. The amino and carbonyl groups of the N-terminal Ala make several hydrogen bonds to conserved residues in XIAP. (C) A conserved motif of IAP-binding tetrapeptides. The tetrapeptide motif has the consensus sequence A-(V/T/I)-(P/A)-(F/Y/I/V/S). The Drosophila proteins have an additional binding component (conserved 6th–8th residues). (D) A conserved IAP-binding mode from mammals to fruit flies. The structure of DIAP1-BIR2 is superimposed with that of the XIAP-BIR3 domain, with their corresponding bound peptides Hid (pink), Grim (blue), and Smac/DIABLO (green). Adopted from (Shi 2002).

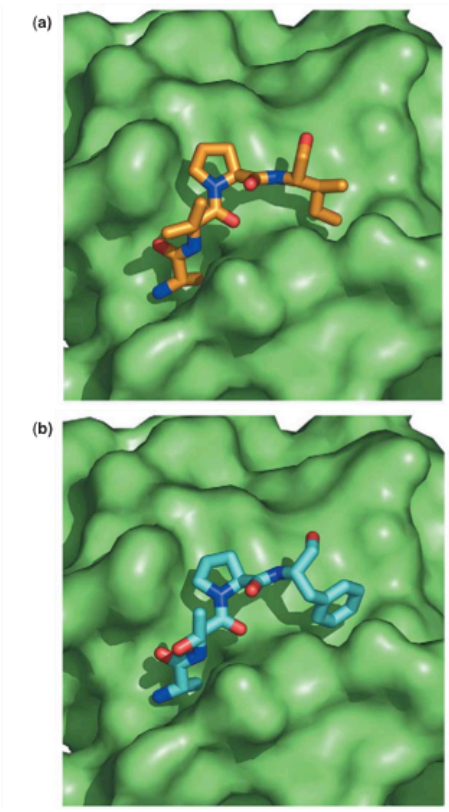

Figure 1-13. Molecular mechanism of second mitochondria-derived activator (Smac)-mediated removal of caspase-9 inhibition by X-linked inhibitor of apoptosis (XIAP).
(a) The N-terminal tetrapeptide motif of Smac (also known as DIABLO, for direct IAP-binding protein with low pI) (orange) bound to a surface groove of the third baculoviral IAP repeat (BIR3) domain of XIAP (green). (b) The N-terminal tetrapeptide motif of the p12 subunit of caspase-9 (cyan) bound to the same surface groove of the BIR3 domain of XIAP (green). The N-terminal IAP-binding tetrapeptide motif (orange in a) of Smac competes with a similar motif in caspase-9 (cyan in b) for binding to the same conserved surface groove on XIAP-BIR3, thus removing XIAP-mediated inhibition of caspase-9. Adopted from (Shiozaki and Shi 2004).

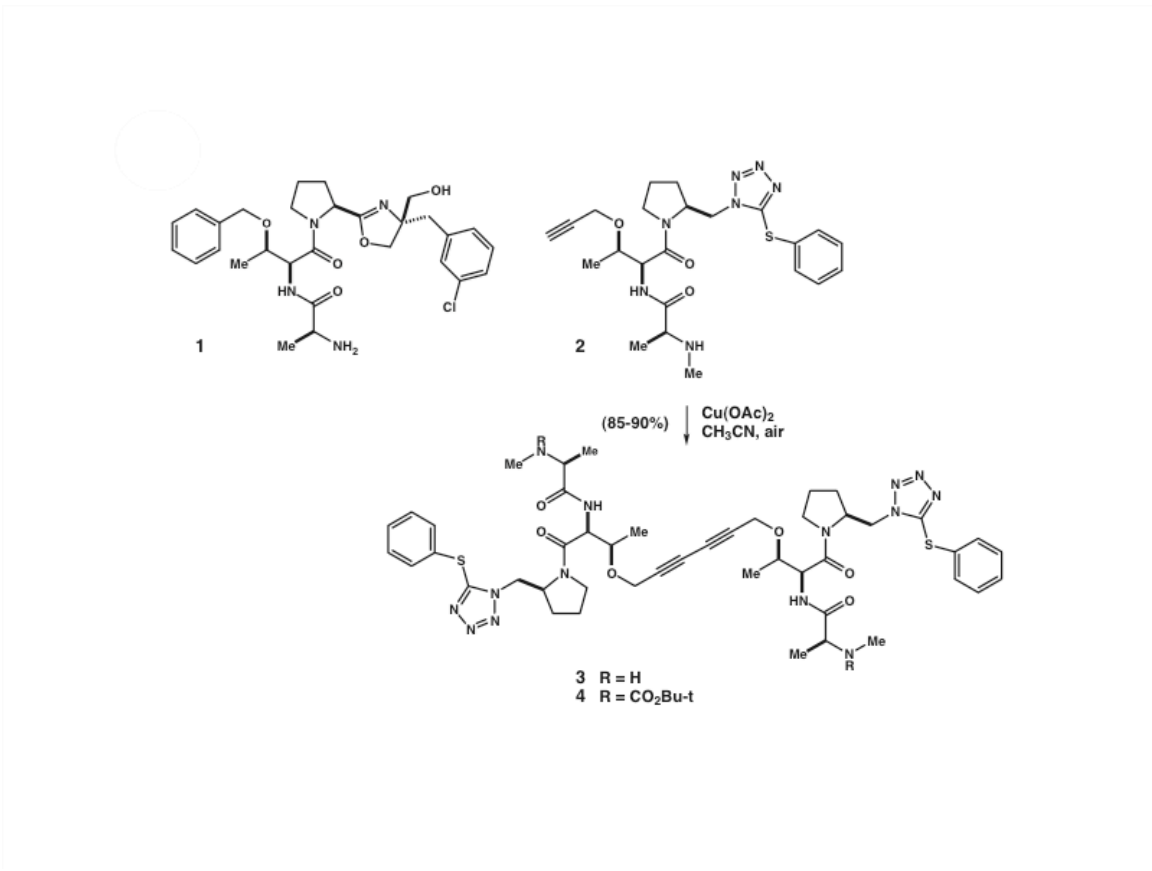

Figure 1-14. Chemical structures of the small molecules synthesized. (Adopted from (Li et al. 2004)

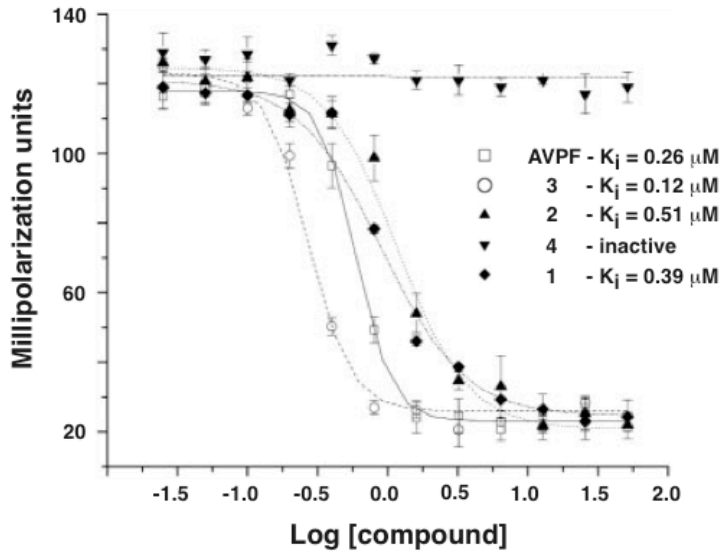

Figure 1-15. Fluorescence polarization assay for the interaction of Smac and mimetics with the Bir3 domain of human XIAP. (Adopted from (Li et al. 2004)

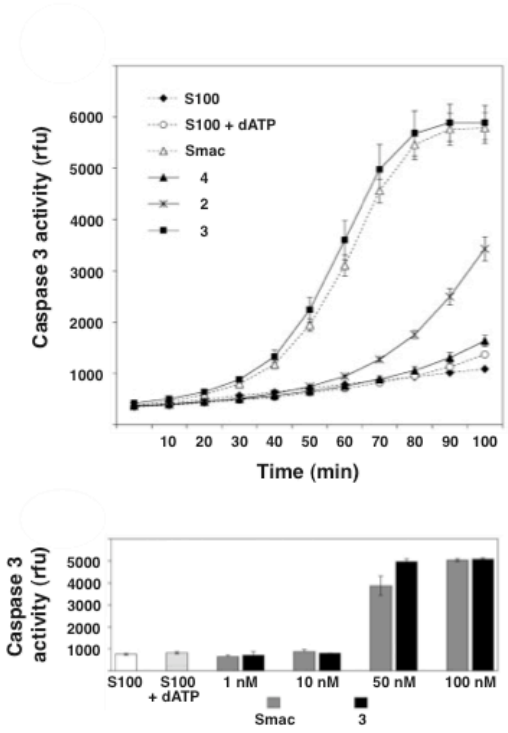

Figure 1-16. Polyacrylamide gel electrophoresis under nondenaturing conditions and Coomassie Blue staining were used to evaluate the binding of 3 to recombinant full-length human XIAP. (Adopted from (Li et al. 2004)

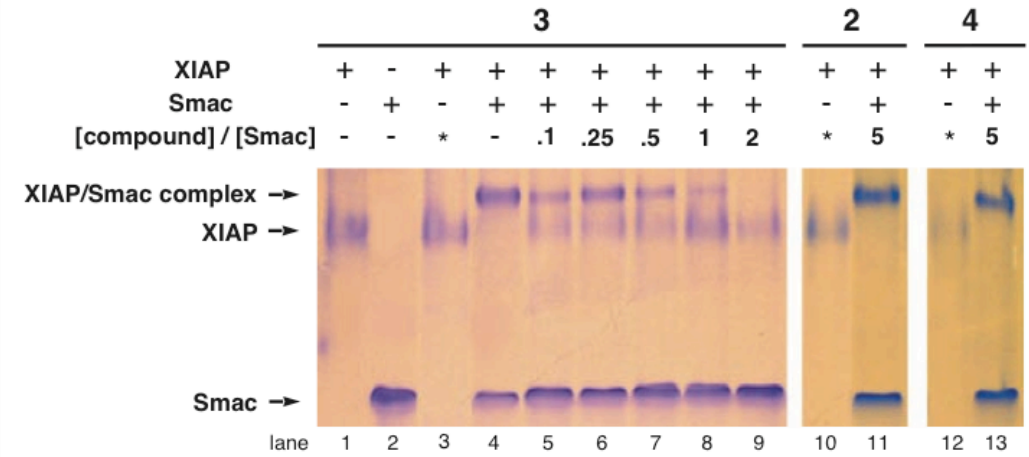

Figure 1-17. Time course comparison of caspase 3 activation by recombinant Smac and small molecule mimetics. (Bottom) Bar graph representation of the same experiment except with varying concentrations of Smac and compound 3. (Adopted from (Li et al. 2004)

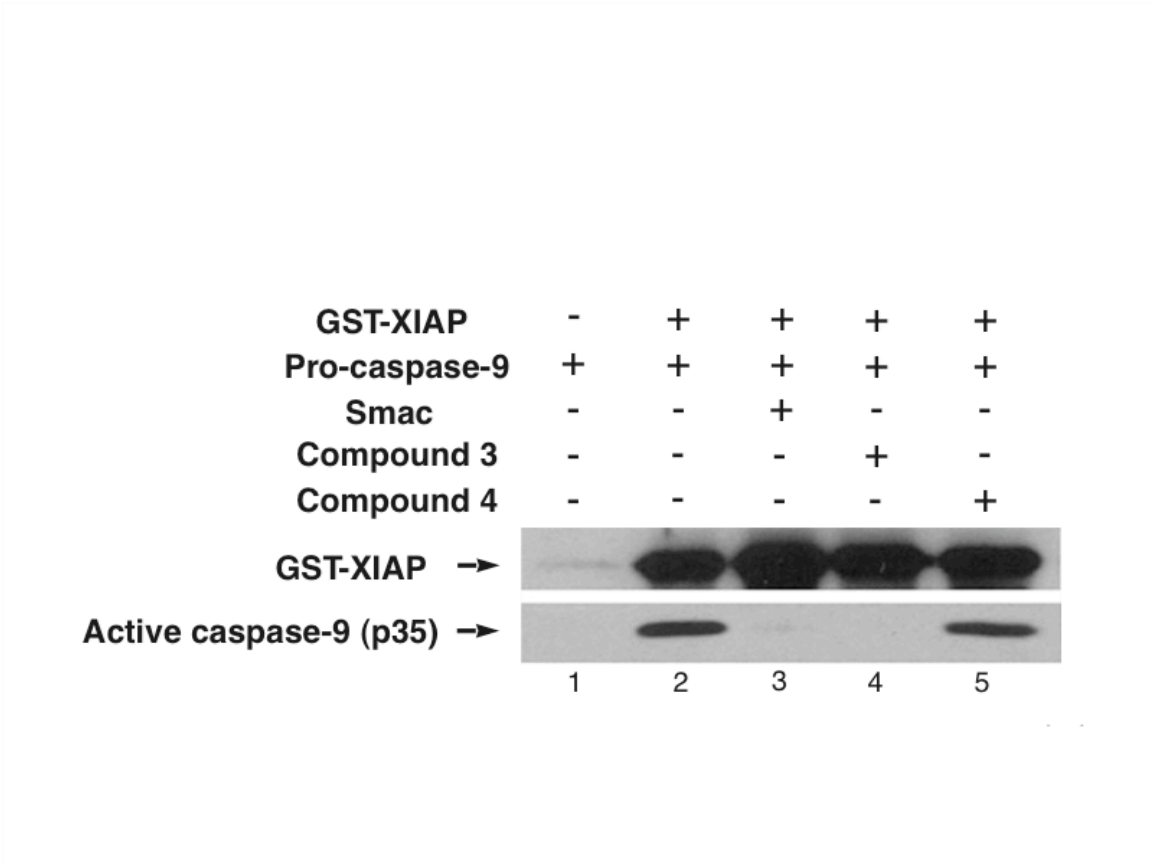

Figure 1-18. Smac and compound 3 compete with glutathione S-transferase (GST)–tagged human XIAP for active caspase 9 binding. (Adopted from (Li et al. 2004)

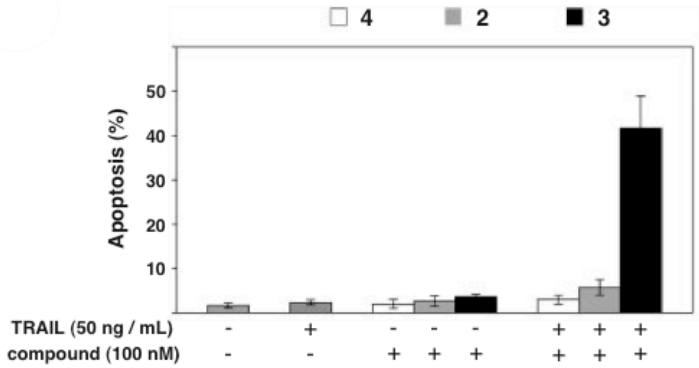

Figure 1-19. Compound 3 and TRAIL act synergistically to induce apoptosis in human glioblastoma (T98G) cells. (Adopted from (Li et al. 2004).

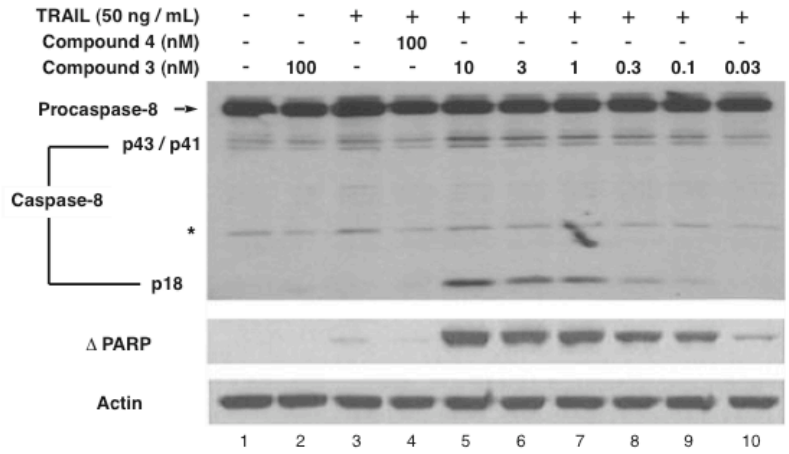

Figure 1-20. Activation of caspase 8 and caspase 3 by smac mimetic in combination with TRAIL. (Adopted from (Li et al. 2004).

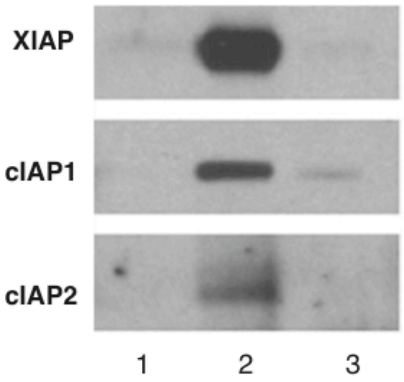

Figure 1-21. Affinity purification of IAP proteins using a biotinylated form of compound 3. (Adopted from (Li et al. 2004).

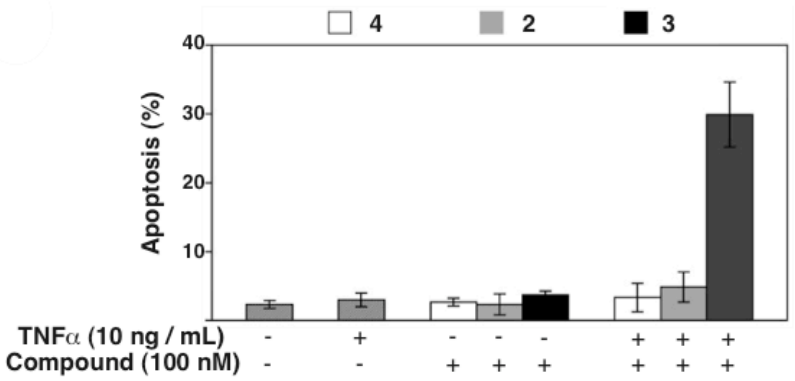

Figure 1-22. Compound 3 and TNF act synergistically to induce apoptosis in cell culture. (Adopted from (Li et al. 2004).

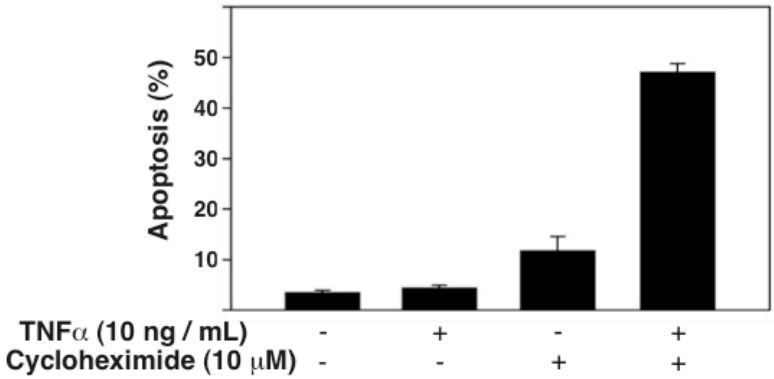

Figure 1-23. Response of cells to TNF and cycloheximide. (Adopted from (Li et al. 2004).

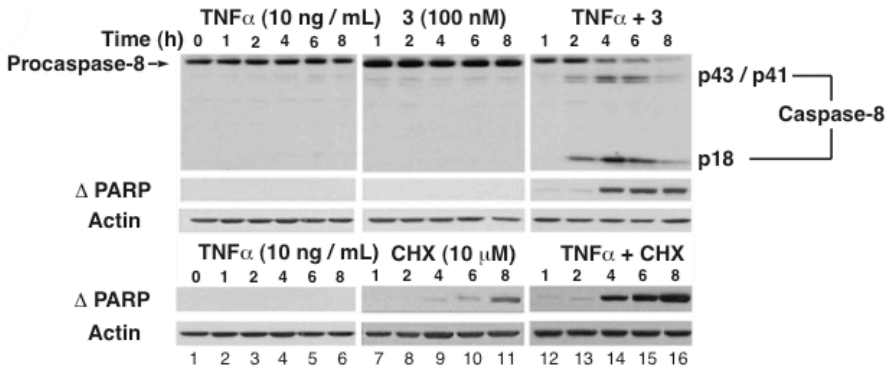

Figure 1-24. Comparison between smac mimetic response to TNF and CHX.
(Top) Time course of caspase 8 and caspase 3 activation in Hela cells treated with TNF and/or smac mimetic (Bottom) Time course of caspase 3 activation in Hela cells treated with TNF, CHX, or both. (Adopted from (Li et al. 2004).

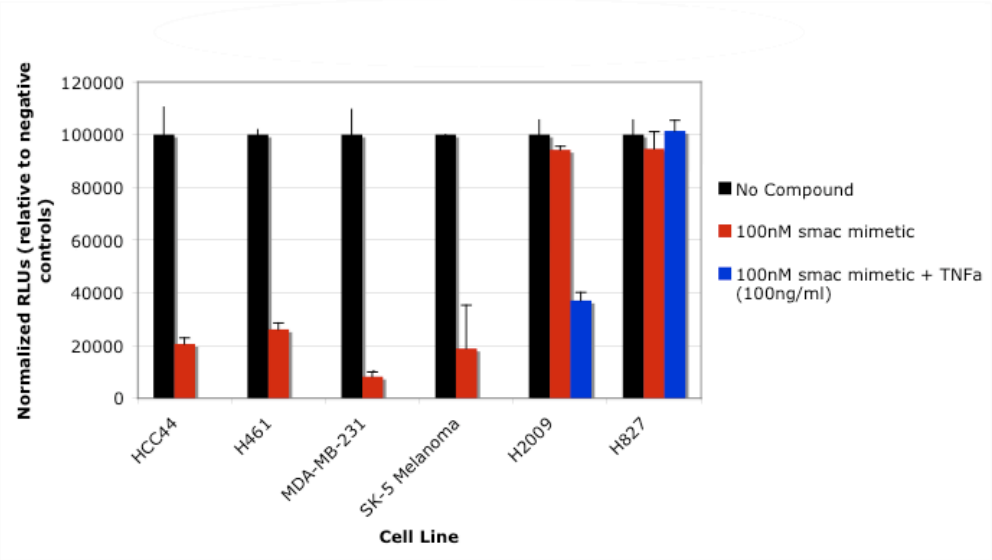

Figure 1-25. Single agent sensitive cell lines.
Cells were treated with 100nM smac mimetic overnight and assayed for cell viability. As comparison, a representative cell line is included that responds to smac mimetic and TNF. Another cell line is representative of a completely resistant cell line.

Cell Line	[TNF]	TNF + SM (100nM)	tumor type
Miapaca-2	39	0.015	pancreas
H460	>200	>200	lung
PC-3	>48	>48	prostate
BxPC-3	>200	0.210	pancreas
SK-MES-1	>48	>48	lung
PANC-1	>200	0.001	pancreas
HT-29	>48	>48	colon
HTC-116	33	0.75	colon
A-375	>48	23	skin
BT474	>200	0.002	breast
LewisLung	>200	>200	lung
MDA-MB-435	>48	>48	breast
DU-145	>48	>48	prostate
T98G	>48	8	brain
HelaS3	>48	>48	cervical
MCF-7	>48	22.1	breast
K562	>48	>48	leukemia
CCRF-CEM	4.7	0.002	leukemia
H-226	>48	>48	lung

Table 1-1. Screening of a panel of cancer cell lines for synergy between smac mimetic and TNF. (Courtesy of Dr Lin Li).

CHAPTER TWO: Autocrine TNFα signaling renders human cancer cells susceptible to smac-mimetic-induced apoptosis

2.1 Introduction

Cancer is second only to heart disease as the leading cause of death in industrialized countries. Although mortality rates have declined in recent years due to earlier detection and more options in treatments, the outlook for certain cancers remains bleak (Workman and Kaye 2002). For example, lung cancers, despite treatment advances, have a general long-term survival rate of only ~15%. Conventional cancer chemotherapeutics, such as paclitaxel, cisplatin, topotecan or etoposide, have a small therapeutic index between cancer and normal cells. Complicating matters further is the almost inevitable onset of resistance and subsequent relapse.

In recent years focus has shifted to less toxic therapeutics that target specific signaling pathways driving inappropriate cell growth and proliferation. Examples include imatinib and erlotinib, rationally designed tyrosine kinase inhibitors that block the ATP binding site of the constitutively active BCR-ABL tyrosine kinase translocation mutation found in chronic myeloid leukemia (CML) (Druker 2002) and the tyrosine kinase domain mutant EGFR found in non-small cell lung cancers. Monoclonal antibodies such as Herceptin™, which targets the HER2/neu (erbB2) receptor that is often amplified in breast cancer, have also been developed (Izumi et al. 2002, Slamon et al. 2001).

In addition to aberrant growth signals, many cancers also have dysfunction in the ability to undergo apoptosis (Hanahan and Weinberg 2000, Danial and Korsmeyer 2004).

Over expression of anti-apoptotic genes have been correlated with tumorigenesis and resistance to chemotherapy. A classic example is seen in non-Hodgkin's lymphoma, where constitutive Bcl-2 over-expression blocks apoptosis in the presence of pro-apoptotic stimuli (Kirkin, Joos and Zornig 2004, Miyashita and Reed 1993). Additionally, over expression of various members of the inhibitor of apoptosis (IAP) family of proteins has also been found in cancers able to evade apoptosis (Gordon et al. 2002, Nachmias, Ashhab and Ben-Yehuda 2004, Sui et al. 2002). An alternative approach to the treatment of cancer, therefore, would be to re-establish the cell death program and set conditions such that cells can undergo apoptosis given an appropriate stimulus (Fesik 2005). The potential of such tactics can be seen by the emergence of small molecule inhibitors targeting various components of the regulatory system of apoptosis. Olterdorf and co-workers recently identified a potent small molecule inhibitor targeting Bcl-2, Bcl-X_L and Bcl-w that was able to synergize with other chemotherapeutics as well as having single agent actions against lymphoma and small cell lung cancer with significant cure rates in animal models (Oltersdorf et al. 2005).

In addition, small molecule inhibitors of second mitochondria-derived activator of apoptosis (Smac) have recently been developed that promote apoptosis in synergy with other pro-apoptotic stimuli (Bockbrader, Tan and Sun 2005, Chauhan et al. 2007, Glover et al. 2003, Li et al. 2004, Mizukawa et al. 2006, Sun et al. 2004, Sun et al. 2008b, Wilkinson et al. 2004, Wu et al. 2003, Zobel et al. 2006). Smac is an ideal candidate for small molecule mimetic design and therapeutic application because of its unique function in regulating apoptosis. Under normal conditions Smac is sequestered in the mitochondria and is released into the cytosol only upon induction of apoptosis or mitochondrial

dysfunction (Du et al. 2000, Chai et al. 2000). Once in the cytosol Smac is able to bind its targets, which consist of a family of related proteins known as the inhibitors of apoptosis (IAPs) (Liu et al. 2000, Srinivasula et al. 2001, Wu et al. 2000). IAPs function to prevent unregulated activation of the apoptotic cell death program by binding to and inhibiting multiple proteins necessary for apoptosis to occur. For example, XIAP (X chromosome encoded IAP) binds to activated caspases 3, 7 and 9 and inhibits their activities (Crook et al. 1993, Deveraux et al. 1997, Roy et al. 1997). A key feature of regulated apoptosis is the release of Smac from mitochondria and the subsequent release of caspases from inhibition. A mimetic of Smac is able to relieve caspases from inhibition without need for mitochondrial disruption to promote apoptosis in cancer cells given an additional pro-apoptotic stimulus.

Previously, our laboratory demonstrated the feasibility of such an approach by preparing a small molecule mimetic of Smac and showed that it permeates cells readily and acts in a similar fashion as natural Smac in the cell while bypassing mitochondrial regulation (Li et al. 2004). The small molecule mimetic is a synthetic, two headed structure designed to resemble the N-terminal amino acid residues (AVPI) of Smac protein that interact with BIR domains of XIAP (Wu et al. 2000). The compound was shown to bind specifically to at least three members of the IAP family: XIAP, cellular IAP 1 (cIAP1), and cIAP2. It was able to promote apoptosis synergistically with pro-apoptotic stimuli (TRAIL or TNFα) that on their own have no effect on cell death in the cancer cell lines examined (Li et al. 2004).

In addition to inhibiting caspase activation, an ancillary function of both cIAP1 and cIAP2 is that both proteins are implicated in nuclear factor kappa B (NFκB)

activation (Chen, Cao and Goeddel 2002, Shu et al. 1996) and suppressing caspase-8 activation during TNFα signaling (Deveraux et al. 1998, Wang et al. 1998). TNFα is a pleiotropic ligand of tumor necrosis factor receptor 1 (TNFR1) and TNFR2 that can signal both cell survival and cell death (Aggarwal 2003, Bhardwaj and Aggarwal 2003, Wajant 2003a). The pro-survival and pro-death signaling aspects of TNFα are mediated through two separate protein complexes (Micheau and Tschopp 2003). The pro-survival complex, in addition to TNFα bound to TNFR1, also contains the adaptor protein tumor necrosis factor receptor 1 associated death domain protein (TRADD), TNF receptor associated protein 2 (TRAF2), receptor interacting protein kinase 1 (RIPK1), and cIAP1 and cIAP2. This complex recruits and activates I kappa kinases (IKK), leading to the activation of NFκB (Devin et al. 2000, Shu et al. 1996). A pro-death complex is also formed, but so long as there is a pro-survival signal being generated, it is unable to induce cell death. The death-inducing complex assembles following internalization of the TNFR1 receptor and consists of TRADD and RIPK1, which then recruit Fas associated protein with death domain (FADD) and caspase-8 to generate the death inducing signaling complex (DISC) (Micheau and Tschopp 2003).

Here we report the finding that various human cancer cell lines that undergo apoptosis upon Smac mimetic treatment without the need for exogenous pro-apoptotic stimuli or co-treatment with chemotherapeutic agents. We have elucidated the molecular mechanism underlying this single agent sensitivity. Our work reveals a functionality of the Smac mimetic relating specifically to TNFα signaling that is distinct from simple interference with caspase inhibition. The Smac mimetic is able to exploit certain cancer

cells that secrete TNFα and usurp this pro-survival signal to promote cell death via formation of a RIPK1 dependent death-inducing complex.

2.2 A Smac mimetic induces cell death in human non-small cell lung cancer cell lines and specifically targets IAPs

Initial testing of various cell lines to the synergistic effects of Smac mimetic treatment with conventional chemotherapeutics revealed a subset of cells that responded to the Smac mimetic alone (data not shown). A larger panel of non-small cell lung cancer (NSCL) cell lines was therefore screened for responsiveness to the Smac mimetic (Figure 2-1). Of the 50 cell lines examined, 14% responded to low nanomolar concentrations and an additional 8% had IC50s in the low micromolar range. The majority of cells (78%) were unresponsive to Smac mimetic concentrations up to 100uM. Closer inspection of two sensitive lung cancer cell lines (HCC44 and HCC461) showed that these cells respond robustly to 100nM treatment of Smac mimetic, with approximately 80% of the cells killed by the Smac mimetic after 24 hours of treatment (Figure 2-2). To ensure that these effects were specific, a compound with its critical alanine group acetylated and therefore inactivated as a Smac mimetic, was also used (Li et al. 2004). Neither HCC461 nor Hcc44 cell lines were sensitive to the treatment of this inactive compound. Two other lung cancer cell lines (H2009 and HCC827) were resistant to 100nM Smac mimetic treatment (Figure 2-2).

To ensure that the Smac mimetic was specifically targeting IAPs in these cells, a biotinylated form of the Smac mimetic was synthesized and used for *in vitro* pull-down to determine what precisely the Smac mimetic was interacting with in these cells. Whole cell lysates were incubated with the biotinylated Smac mimetic and interacting proteins

were pulled down with avidin-coated beads and analyzed by SDS-PAGE followed by silver staining (Figure 2-3) and mass spectrometry. As compared to the affinity matrix alone, the biotinylated Smac mimetic specifically pulled down two protein bands visualized by silver staining. Mass spectrometric analysis of these bands revealed that the band around the 70-kDa marker contained cIAP-1 and c-IAP2, while the band near 50-kDa consisted of XIAP, TRAF2, and TRAF1. TRAF-1 and TRAF-2 were most likely co-purified with cIAP1 and cIAP2, since they have been shown previously to interact and form multi-subunit complexes involved in NFκB activation (Rothe et al. 1995). It is evident from the above result that the Smac mimetic is highly specific in interacting with IAP proteins. Interestingly, although there was no significant difference in XIAP/TRAF-1/TRAF-2 pulled down from sensitive (HCC44 and HCC461) or non-sensitive (HCC827 and H2009) cell extracts, there seemed to be more cIAPs from the sensitive cells pulled down by the biotinylated Smac mimetic.

2.3 Smac mimetic alone induces tumor regression in HCC461 xenograft model

Observing the robustness of HCC461 cell death induced by the Smac mimetic alone in cell culture, we decided to examine whether such a property remained unchanged in a xenograft mouse tumor model. HCC461 cells were grown in matrigel and injected subcutaneously into athymic nude mice. Tumors were allowed to grow for 7 days (average tumor size; 200-300mm^3) and tumor-bearing mice were thereafter given six separate intravenous injections of either Smac mimetic or saline over the following 11 days (D7, 9, 11, 13, 15, 17). As shown in Figure 2-4, mice treated with saline did not survive past day 30 (average tumor size; 2200mm^3), while mice treated with the Smac mimetic showed marked and sustained tumor regression (average tumor size; <50mm^3).

At day 40 tumors began to gradually reappear, but these were still significantly smaller at day 50 (average tumor size: 500mm^3) than control tumors at day 30. Of the mice treated with the Smac mimetic, 2 out of 5 (40%) were tumor free at the end of the experiment. This result is quite different than that obtained from a breast cancer xenograft mouse model treated with the same Smac mimetic compound at the same doses (5 mg/kg) and dosing schedule. In the breast cancer model, Smac alone had little effect and the mice needed to be dosed together with Trail to achieve similar therapeutic effect (data not shown). To ensure that the xenograft response was specific to cell lines that showed sensitivity in cell culture, an additional resistant NSCL cancer cell line, HCC15, was also tested for responsiveness to the smac mimetic alone. As shown in figure 2-5, this cell line that was resistant to Smac mimetic treatment in cell culture remained so *in vivo* under identical conditions as were used to test HCC461.

2.4 Smac mimetic induced apoptosis

HCC461 was examined in greater detail to determine how exactly the Smac mimetic caused cell death in cell culture. Time course measurements of caspase activation revealed that apoptosis was the mechanism of cell death and that within 6 hours of Smac mimetic treatment the executioner caspases-3 was activated (Figure 2-6). Prior to that, the initiator caspase-8 (Figure 2-6) and caspase-9 (data not shown) were activated at approximately 4 hours. Furthermore, inhibition of caspase activation by a pan caspase inhibitor, z vad fmk (z vad), prevented cleavage of caspases-3, -8 and -9 following 6 hours of Smac mimetic treatment (Figures 2-6 and data not shown, respectively). Caspase inhibition was also correlated with response to Smac mimetic where a one-hour z vad pre-treatment was able to rescue cells from cell death following

24 hours of Smac mimetic treatment (Figure 2-7). The effects of z-vad were also tested for long-term survival in the presence of Smac mimetic. Cells were treated with Smac mimetic, z vad, or both for 5 days to ensure that rescue by z vad was permanent and that rescue was not a transient artifact of z-vad exposure. As shown in Figure 2-8, cells treated with Smac mimetic plus z-vad were still viable after 5 days whereas cells treated with the Smac mimetic alone were killed.

2.5 Smac mimetic induced apoptosis occurs through engagement of TNFR1 by TNFα

Because both caspase-8 and caspase-9 are activated in the same time frame, a candidate short interfering RNA (siRNA) screen was used in HCC461 to determine which caspase was the initiator of apoptosis and ultimately what signaling pathways mediates Smac mimetic induced apoptosis. Experiments using siRNAs to knockdown caspase-8 or caspase-9 revealed that transient knockdown of caspase-8 rescued cells from cell death, while knockdown of caspase-9 had no effect on cell viability (Figure 2-9). Given that caspase-8 activation is most likely caused by certain members of the TNF super-family of receptors, further screening of those receptors was conducted (Figure 2-10). The canonical members of the death receptor family are TNF receptor 1 (TNFR1), TRAIL receptor 1 (TRAIL-R1/DR4), TRAIL receptor 2 (TRAIL-R2/DR5), and Fas/CD95 (Aggarwal 2003). siRNA knockdown of TNFR1 rescued cells from Smac mimetic mediated cell death, whereas knockdown of the other receptors had little or no effect (Figure 2-10 and 2-11). Further knockdown of the ligands of TNFα, TRAIL, and FasL confirmed that TNFR1 is a critical component since knockdown of TNFα was also able to rescue cells from apoptosis (Figure 2-12 and 2-13). Knockdown efficiencies of the

siRNAs used were determined by Western blotting utilizing three individual siRNA oligos (Figure 2-14).

Since TNFR1-TNFα signaling appears to be a requirement for the Smac mimetic induced apoptosis, we reasoned that these cells might be secreting TNFα into the culture medium. Elisa analysis of the conditional media from these cells revealed that sensitive cell lines (HCC44 and HCC461) were secreting TNFα into the culture medium over time, whereas, resistant cell lines (HCC827 and H2009) were not (Figure 2-15). To validate that TNFα secretion was critical for the Smac mimetic induced cell death, HCC461 cells were pre-treated with a neutralizing TNFα antibody (1-2 μg/ml) (Oettinger, D'Souza and Milton 1999) and then treated with the Smac mimetic. As shown in Figure 2-16, the TNFα neutralizing antibody completely rescued cells from apoptosis whereas neutralizing antibodies against TRAIL and FasL (1-2 μg/ml) had no rescuing effect.

2.6 Smac mimetic mediated cell death is dependent on RIPK1

Although the Smac mimetic alone requires endogenous TNFα signaling to induce cell death, there is a seeming incongruity between the fact that the Smac mimetic interferes with IAP-caspase interactions and that under normal conditions, TNFα is a pro-survival signal that up-regulates anti-apoptotic genes including cIAP1 and cIAP2 (Wang et al. 1998). Indeed, given cIAP1 and cIAP2's purported roles in NFκB signaling, there is no apparent disruption of basal NFκB activation, which is a key mediator of survival signaling, upon Smac mimetic treatment in these cells (Li et el., 2004). However, the Smac mimetic is still able to initiate apoptosis even under conditions of constitutively active TNFα signaling by autocrine secretion. Therefore, to determine how the Smac

mimetic is able to initiate apoptosis under such conditions, experiments using siRNAs to target components of the TNFα signaling pathway were carried out to determine which components were necessary for cell death to occur.

Targeting of components known to be part of the TNFα signaling pathway revealed that caspase 8 activation and subsequent cell death was RIPK1 dependent. siRNA knockdown of RIPK1 completely rescued cells from apoptosis, while knockdown of the adaptor protein TRADD seemed to enhance cell death and knockdown of FADD had only a partial rescue effect (Figure 2-17). The effect of RIPK1 knockdown was verified by 4 different siRNA oligos that target different segments of the mRNA. RIPK1 dependence in Smac mimetic mediated cell death was unexpected given the traditional view that RIPK1 is involved more in NFκB survival signaling than in signaling apoptosis. This finding is consistent with a recent report showing that shRNA knockdown of RIPK1 prevented TNFa induced apoptosis in human tumor cells (Jin and El-Deiry 2006). However, the precise function of RIPK1 in survival and in cell death is not yet clear. Over-expression of RIPK1 was shown to be able to sensitize 293 cells to apoptosis by TNFα treatment (Hsu et al. 1996), whereas RIPK1 deficient Jurkat T cells do not activate NFκB when treated with TNFα and are sensitized to TNFα induced apoptosis (Ting, Pimentel-Muinos and Seed 1996, Kelliher et al. 1998). Knockdown efficiencies for RIPK1, FADD and TRADD were examined by Western blot (Figure 2-18).

Given the apparent role of RIPK1 in Smac mimetic mediated cell death, it might be possible the Smac mimetic is somehow able to allow the formation of a RIPK1 containing signaling complex that activates caspase-8, hence overriding the otherwise

pro-survival signal of TNFα. Immunoprecipitation of caspase-8 following Smac mimetic treatment revealed the formation of a RIPK1-FADD-Caspase 8 complex that did not contain TRADD or TRAF2 (Figure 2-19). Formation of the complex was enhanced by pre-treatment with z vad, likely due to the fact that z vad prevents full activation of caspase-8 and is able to trap caspase-8 into the RIPK1-FADD-Caspase-8 complex, which would normally disassociate following full caspase-8 activation. Similar co-immunoprecipitation experiments of caspase-8 in cells that are unable to respond to the Smac mimetic (HCC827), showed that they do not readily form a RIPK1-FADD-Caspase-8 complex when the Smac mimetic and TNFα are added (data not shown), indicating that formation of this complex is a requirement for cell death to occur.

2.7 Single agent sensitivity to the Smac mimetic in other cell lines is similarly due to TNFα signaling

Similar experiments as above described, done in another lung cancer cell line (HCC44) as well as two other Smac mimetic sensitive cancer cell types including the MDA-MB-231 cells that have been previously shown to be sensitive to single agent Smac mimetic treatment (Bockbrader et al. 2005) and melanoma SK MEL-5 cells, showed that in all cases responsiveness to the Smac mimetic was a result of TNFα engagement of TNFR1 and that apoptosis was RIPK1 dependent. siRNA knockdown of caspase-8, TNFR1, TNFα, and RIPK1 as well as addition of neutralizing TNFα antibody to the culture media rescued these cell lines from cell death (Figures 2-20, 2-21 and 2-22). Additionally, in the case of cell lines that are resistant to Smac mimetic alone, but do respond to co-stimulation by TNFα (H2009 and T98G), siRNA knockdown of caspase-8, TNFR1 and RIPK1 revealed that in cases where exogenous TNFα is added,

the mechanism of response is the same as that for cells able to secrete TNFα (Figure 2-23 and data not shown).

2.8 Discussion

The perennial challenge in cancer drug discovery is specificity, namely, how to eradicate cancer cells without harming normal tissues. Most currently used therapeutics indiscriminately target fast growing cells regardless of origin. Among the hallmark characteristics that cancer cells display are the ability to evade apoptotic signals, such as oncogene transformation resulting in unregulated growth and proliferation, hypoxia, lack of proper growth factor stimulation or inhibition, and genomic instability, which will normally induce apoptosis. As knowledge of the selective molecular mechanisms by which cancer cells are able grow, proliferate and evade apoptosis is being gained, therapeutically viable targets present themselves. The now classic example is Gleevec™. Selective growth advantages that the constitutively activated BCR-ABL kinase translocation mutation provides CML also presents an ideal single agent target that if successfully blocked would restore the proper balance of signaling to the cell. Similarly, many cancers have also been shown to up-regulate anti-apoptotic genes, which when over expressed confer resistance to apoptosis, making such targets ideal candidates for therapeutic intervention. Here we identify autocrine TNFα production as another potential means by which cancer cells gain selective growth advantage and resistance to apoptosis and a means by which a single agent Smac mimetic can exploit that signal to restore sensitivity to apoptosis.

The above results suggest that targeting of the IAPs, via Smac mimetic, is a promising strategy for the treatment of cancer. Specifically, we show that targeting the

IAPs through the use of a small molecule mimetic of Smac had the unintended, but fortuitous, effect of also altering the functional state of the TNFα receptor from one that signals through the NFκB survival pathway to one that also promotes the formation of a RIPK1 dependent, death inducing complex, that is able to override the pro-survival signal and initiate apoptosis. Furthermore, the Smac mimetic is able to do this by exploiting the tumor cells own pool of TNFα, in the form of autocrine secretion. It seems likely that cells which produce autocrine TNFα might rely on such a signal for survival, particularly to activate pro-survival and anti-apoptotic genes regulated by NF-κB, and that such dependence might be the difference between Smac mimetic sensitivity and Smac mimetic resistance. Namely, that because IAPs play potentially significant and unique roles in TNF mediated activation of NF-κB, that such cells would be particularly sensitive to the Smac mimetic. Other cell lines may rely on other cytokines and growth factors for such signaling and be resistant because IAP do not play a significant role in signaling in these other systems. Given the identification of cancer cells that secrete TNFα and that rely on TNFα for survival signaling, it is possible that cancers fitting such a profile would be ideal targets of single agent smac mimetic therapy.

In addition, Smac mimetic alone treatment of human tumor (HCC461) xenografted into mice reduced the size of tumors over an extended period of time. Tumors treated with Smac mimetic showed immediate response by reducing in size to nearly undetectable levels and staying that way for an extended period of time. Only 30 days after the final treatment did the tumors start to re-emerge and within the treatment group, 40% remained tumor free at the end of the experiment. This experiment suggests that the single agent sensitivity observed in cultured cells maintained this property in

animals. Unfortunately, although the secreted TNFα was readily detectable with a simple Elisa method in the culture media when these single agent sensitive cells are cultured in vitro, no circulating TNFα could be detected in the sera from HCC461 tumor-bearing mice using the same method (not shown). Consistently, admission of the TNFα neutralizing antibody to HCC461 tumor-bearing mice also did not block tumor shrinkage after Smac mimetic treatment (not shown). It is possible that too little TNFα got into the circulation and/or most of it may act locally at the tumor site. A more sensitive method able to detect low levels of TNFα in sera and in the tumor will be needed if this feature is going to be potentially developed into a biomarker for single agent sensitivity in human patients. Another key aspect of the treatment was the observation that animals treated with the Smac mimetic showed no signs of associated cytotoxicity and were in general good health.

While only 22% of non-small cell lung cancer cell lines examined responded to Smac mimetic treatment alone, some portion of the other 78% of cell lines that have no response should respond to combined Smac mimetic/TNFα (as shown for H2009) treatment while another portion will not respond at all (as in the case of HCC827, data not shown). Why some cells respond and other do not is a key issue facing cancer therapy in general. Unique differences in the nature of survival signaling and the mechanism of evasion of apoptosis make this a remarkably difficult task. It seems likely that as the ability of profiling unique tumors sensitivity to a variety of agents expands, the utilization of various combination therapies should specifically resolve this issue.

2.10 MATERIALS AND METHODS

Reagents

Compound 3 and compound 4 were synthesized as previously described(Li et al. 2004) and the biotinylated variant used for affinity purifications was identical to that reported earlier, except the linker connecting the biotin motif to the Smac mimetic was five carbons longer (synthesized using commercial (+)-biotinamidohexanoic acid N-hydroxysuccinimide ester - see supporting information for ref 15). The compound was diluted to 100uM stocks. Lung cancer cell lines were obtained from the laboratory of Dr John Minna (UT Southwestern). Antibodies used are: Caspase-3 (Cell Signaling, 9662), Caspase-8 (Cell Signaling, 9746), TNFR1 (Abcam, ab19139), TNF alpha (Cell Signaling, 3707), FADD (Cell Signaling, 2782), RIPK1 (BD Pharmingen, 551041), TRADD (Cell Signaling, 3684), TNF alpha (R&D, MAB210), FasL (Biolegend, 306408), TRAIL (Biolegend, 308207).

Cell Culture

Cell lines HCC44, HCC461, HCC827, H2009, MDA-MB-231 were cultured in HyQ RPMI-1640 medium (Hyclone) supplemented with 5% fetal bovine serum (FBS, Hyclone) and 100-units/ml penicillin/streptomycin (Gibco). SK MEL-5 cells were cultured in Minimum Essential Medium (Gibco) supplemented with 10% FBS, 100 units/ml penicillin/streptomycin and 2μg/ml L-glutamine (Gibco).

Cell Survival Assay

Cells were plated onto 96 well assay plates (white with clear bottom (3610), Corning Costar) at different cell densities, depending on cell type, in 100μl media per well. Cells were allowed to grow to near confluence and treated with compound 3 or vehicle (H_20) by adding 100μl media with compound 3, diluted to 2X the desired final concentration, to each well. Cells were incubated overnight and assayed the following day utilizing the Cell Titer-glo Luminescent Cell Viability Assay (Promega), which measures cell viability based on ATP levels present in live cells. As per manufactures protocol, media was aspirated from each well and 100μl fresh media added. Cells were allowed to equilibrate to room temperature when 100μl of the Cell Titer-glo reagent was added. Cells were placed on an orbital shaker for two minutes and then where incubated for an additional 10 minutes. Luminescent measurements were done on a Tecan SPECTRAFluor Plus 96 well plate reader. For IC50 determination, half maximal luminescent readings, relative to the vehicle treated cells, were considered to be representative of the IC50 for each cell line tested. For assays measuring rescue effects, all values were normalized to the mock treated or mock transfected conditions to account for variability in the cytotoxicity of transfecting siRNA into cells and for possible cytotoxic effects that knockdown of the particular gene used might have. All values are represented graphically as mean ± SD for 3 independent samples.

Western blot analysis of caspase activation and of siRNA knockdown efficiency

HCC461 cells were plated onto 6 well cell culture dished (Corning Costar) at differing cell densities, depending on the application, in 2ml media. For caspase activation determination, cell were plated to near confluence and allowed to attach overnight. Cells were then treated as indicated in Figure 2A. Cells were treated to a final concentration of

100nM compound 3 at each time point. For cell treated with z vad fmk (Sigma), 10μM z vad was added 1 hour prior to compound 3. For siRNA knockdown efficiency, HCC461 cells were plated in 2ml antibiotic-free media at a density of 8 x 10^4 cells per well and allowed to attach overnight. siRNAs were then transfected as described below. Cells were lysed in lysis buffer (20mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Triton X100, 10% glycerol, 0.5mM DDT and Complete protease inhibitor (Roche)) and were then incubated on ice for 20 minutes and spun down at 10,000 RPM for 10 minutes. The soluble fraction was kept and protein concentration determined by Bradford assay. Protein concentrations were normalized to 50μg total and SDS-PAGE was done followed by western blotting of target antibody.

Biotinylated smac mimetic pull down

Cells were plated on 15 cm dishes and grown to confluence. Cells were harvested and lysed as previously described. Biotinylated smac mimetic (100nM) was pre-incubated with 20 μl (bead volume) streptavidin dynabeads (Invitrogen) for 1 hour at 4C^o. Beads were then washed 2X in lysis buffer. Smac mimetic bound dynabeads were then incubated with cell lysates overnight at 4C^o. The next day, beads where washed 4X with cell lysis buffer and SDS-loading buffer was added. Samples were run on SDS-PAGE and silver stained with Bio-Rad silver stain plus kit. Bands for each lane were cut out for mass spectrometry.

siRNA Transfection

siRNA transfections were done in both 6 well and 96 well dish formats. For 6 well dishes, the day prior to transfection cells were plated at a density of 8 x 10^4 cells per well in antibiotic-free media. The next day, Lipofectamine 2000 was used to transfect cell, as per manufactures protocol. Briefly, 3µl Lipofectamine 2000 was combined with 120 pmol (6µl of a 20µM stock) siRNA in a volume of 500µl Opti-mem media (Gibco) and incubated for 20 minutes, the complexes of Lipofectamine 2000 and siRNAs were then added directly to each well and the cells were incubated until nearly confluent, approximately 48-72 hours later depending on growth conditions. For 96 well dishes, the day prior to transfection cells were plated at a density of 1.2 x 10^3 cells per well in antibiotic-free media. Lipofectamine 2000 was used, as above, by mixing 0.2µl Lipofectamine 2000 and 8pmol (0.4µl of a 20µM stock) siRNA in a total volume of 50µl. All siRNAs were purchased from Dharmacon. For caspase 8 and caspase 9 individual oligos were designed and tested (caspase 8-1 target sequence 5-UGAAGAUAAUCAACGACUAUU-3, caspase 8-2 target sequence 5-UGGAUUUGCUGAUUACCUAUU-3, caspase 8-3 was obtained from Dharmacon (J-003466-14), caspase 9-1 target sequence: 5-GAUGCCUGGUUGCUUUAAUUU, caspase 9-2 was obtained from Dharmacon (J-003309-05), caspase 9-3 was obtained from Dharmacon (J-003309-06)). For TNFR1 and TNF alpha individual oligos were purchased from Dharmacon (TNFR1: TNFR1- 1 J-005187-05, TNFR1- 2 J-005197-06, TNFR1- 3 J-005197-08; TNFa: TNF alpha- 1 J-010546-09, TNF alpha- 2 J-010546-10, TNF alpha- 3 J-010546-12). For all pooled siRNAs Dharmacon's siGENOME SMARTpool pre-designed pools of 4 oligos were used and validated by western blot. These included siRNAs for Caspase 8, TNFR1, TNFa, FADD, TRADD and RIPK1.

Methylene blue viability assay

Cells were plated onto 6 well dishes at low density and grown to 25% confluence. Cells were then treated as indicated in Figure 2D. 5 days following treatment cells were washed 2X with cold PBS after which 2mL of methylene blue reagent was added (2% methylene blue (w/v) in 50% ethanol) for 15 minutes. Cells were then washed with water until all excess dye was removed. The plate was then photographed.

Elisa analysis of autocrine TNFa secretion

Cells were plated onto 6 well dished and allowed to grow to near confluence (approximately 80%). Media was then aspirated and the cells were washed 2X with cold PBS. 1mL of fresh media was added and 100uL aliquots were removed at each time point (three independent wells were tested at each time point). Samples were kept at -20C^{o} until ready for use. Elisa analysis was performed using a quantitative sandwich enzyme immunoassay from R&D Systems (TNFα Quanti-glo Chemiluminescent Elisa, QTA00B), as per manufacturer's instructions.

Caspase-8 antibody immunoprecipitation

Cells were grown on 15cm plates, treated as indicated, and harvested in 5X volume lysis buffer (as previously described). Cells were left on ice for 20 minutes and centrifuged at 20000-x g for 20 minutes. 20μl (bead volume) protein A agarose beads were coupled to 2μg of caspase 8 antibody (Santa Cruz, SC-6136) in 250μl PBS (supplemented with 5mg/ml BSA). After 2 hours incubation at room temperature the beads were washed 2X

in lysis buffer whereupon, 2mg of cell lysates (2mg/mL) were added and incubated over night at 4C^{o}. The following day, beads were washed 4X with lysis buffer and protein eluted off the beads using low pH elution buffer (Pierce 21004). Elution buffer was neutralized by adding 1:20 1M Tris-HCl, pH 9.4. Samples were then analyzed by SDS-PAGE followed by Western blot.

In vivo matrigel model of HCC461 and HCC15 tumor model into nude mice

Harlan Athymic Nude-Foxnlnu 5-6 week old mice were injected subcutaneously with $1x10^{7}$ HCC461 or HCC15 tumor cells in the left flank. 7 days later when tumors had reached 200-300 mm^{3} in size, mice were randomized into treatment groups of 5 mice per group. Compound 3 was first dissolved in H_2O at 20mg/ml as the stock solution, and then diluted in PBS to 5mg/kg based on the weight of individual mice. The compound and the vehicle (saline) were administered intravenously in a total volume of 0.2ml every other day for 6 treatments total (q2d x 6). Mice were weighed and tumors measured using vernier calipers two times per week. Tumor volumes were calculated according to (length x width^{2})/2. All animal experiments were performed in the vivarium of Joyant Pharmacology Department. The animal protocol was approved by IACUC, which is valid until 1 May 2009. All animal experiments performed in Joyant conform to the relevant regulatory standards. All values are represented graphically as the mean ± SEM.

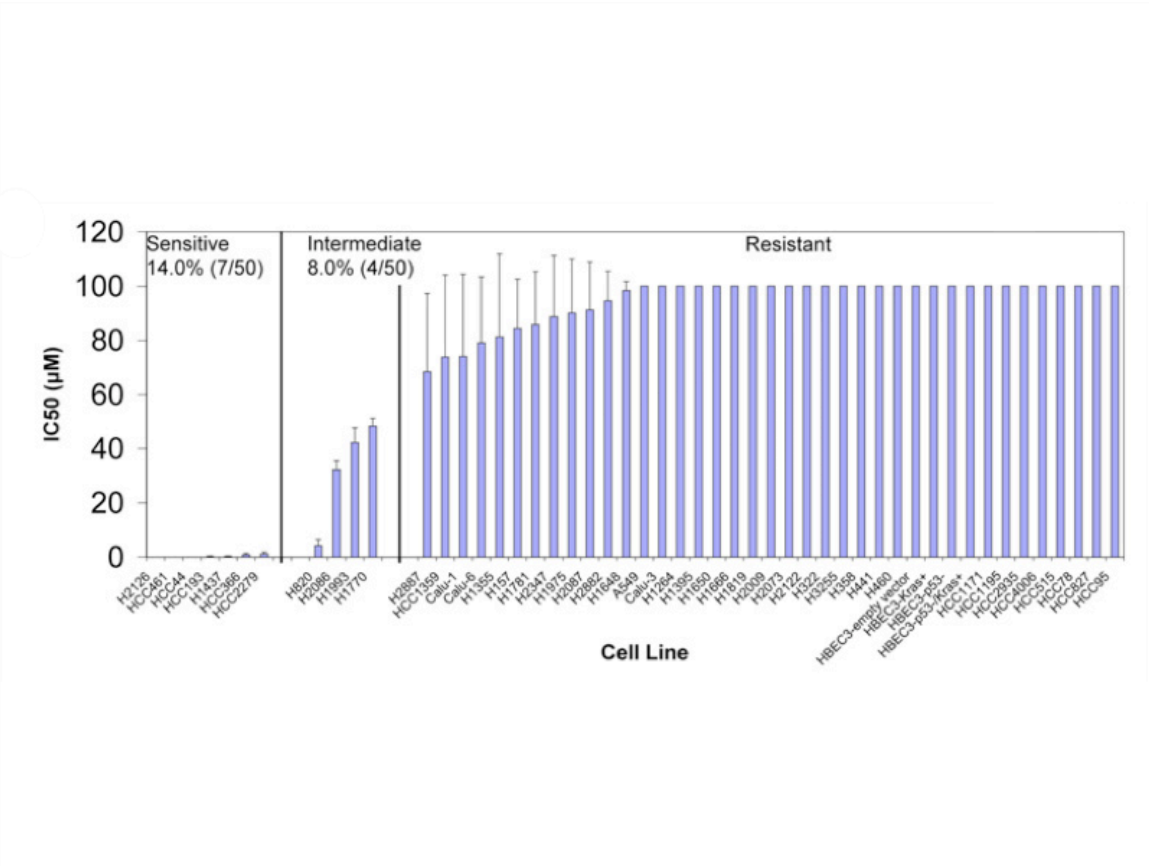

Figure 2-1. Screening of a panel of 50 non-small cell lung cancer cell lines.
A panel of 50 non-small carcinoma cell lung cancer cell lines was tested for responsiveness to a Smac mimetic treatment alone. IC50s were determined for each cell line based on cell survival as measured by ATP levels in live cells using Cell Titer-glo (Promega). IC50 determination was based on concentrations of compound 3 that yielded half maximal luminescence relative to untreated cells.

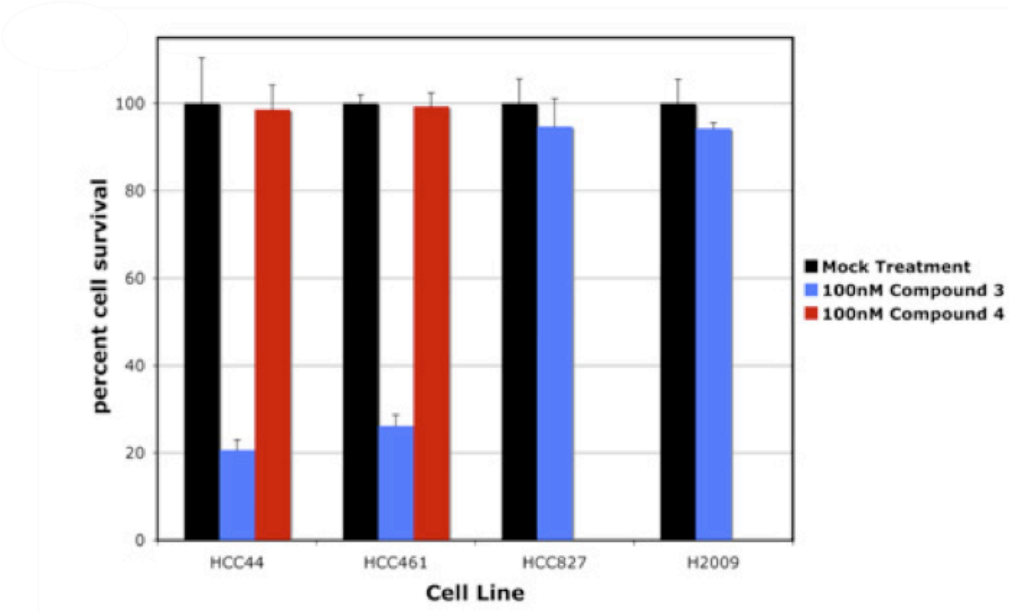

Figure 2-2. Some cell lines display single agent sensitivity to smac mimetics
Treatment of selected group of cell lines to 100nM of compound 3 and to compound 4 (negative control compound with similar structure by differing in function group configuration as described in Li et al., 2004). Smac mimetic sensitive cell lines (HCC44 and HCC461) and Smac mimetic resistant cell lines (HCC827 and H2009) were chosen. Graphical representation for IC50s and cell survival indicate the mean ± SD of at least 3 independent testing conditions.

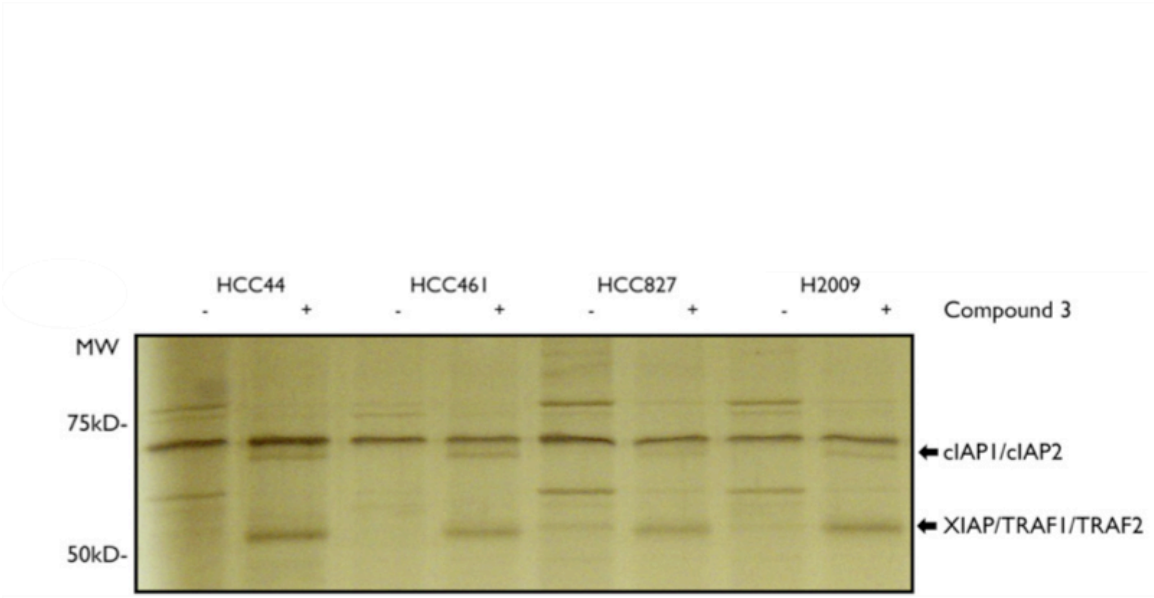

Figure 2-3. Biotin conjugated smac mimetic can pull down IAPs
In vitro pull-down utilizing a biotinylated form of compound 3. The biotinylated compound was able to pull down protein bands at around 50kD and at around 70kD not seen in control lanes (avidin beads only), which were cut out of the gel and analyzed by mass spectroscopy. Proteins identified are indicated.

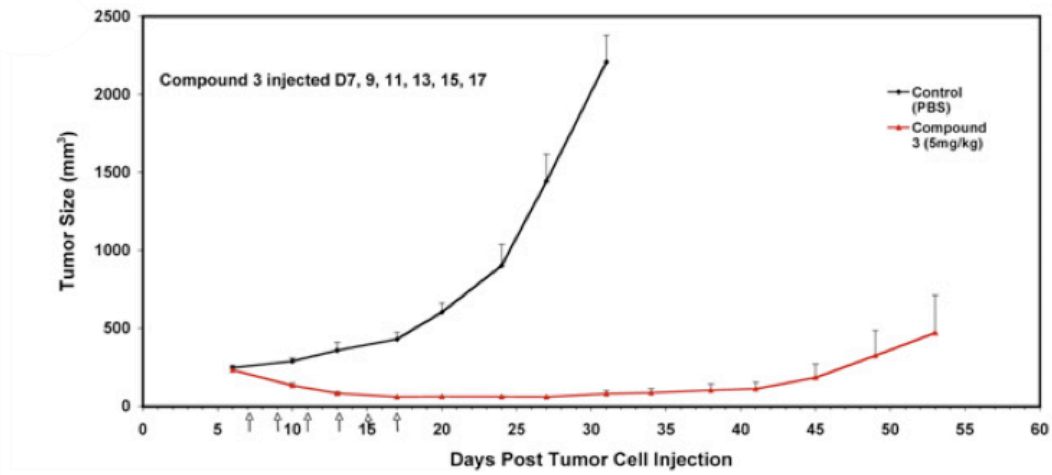

Figure 2-4. Single agent sensitivity is maintained in mouse xenograft
In vivo response of mouse xenografts of HCC461 cells to compound 3. Harlan athymic nude mice were injected subcutaneously with HCC461 cells in a matrigel randomly separated into treatment groups (n=5) and given 6 intravenous injections of compound 3 or saline every other day. Tumors were measured twice per week until the end of the experiment. Within in the compound 3 treatment group 2/5 (40%) remained tumor-free at the end of the experiment.

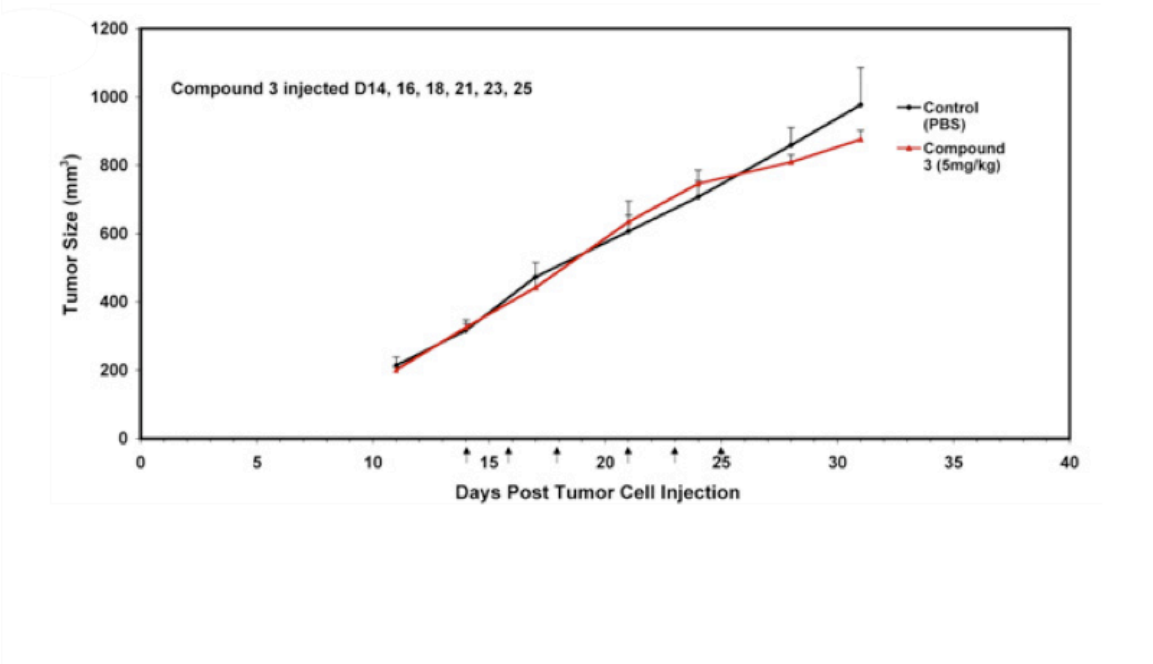

Figure 2-5. Resistant cells remain resistant in mouse xenograft
In vivo response of mouse xenografts of a Smac mimetic-resistant HCC15 cells to compound 3. Conditions were identical to those for HCC461 xenografts. For tumor size measurements, graphical representations indicate the mean ± SEM of 5 individual samples per condition.

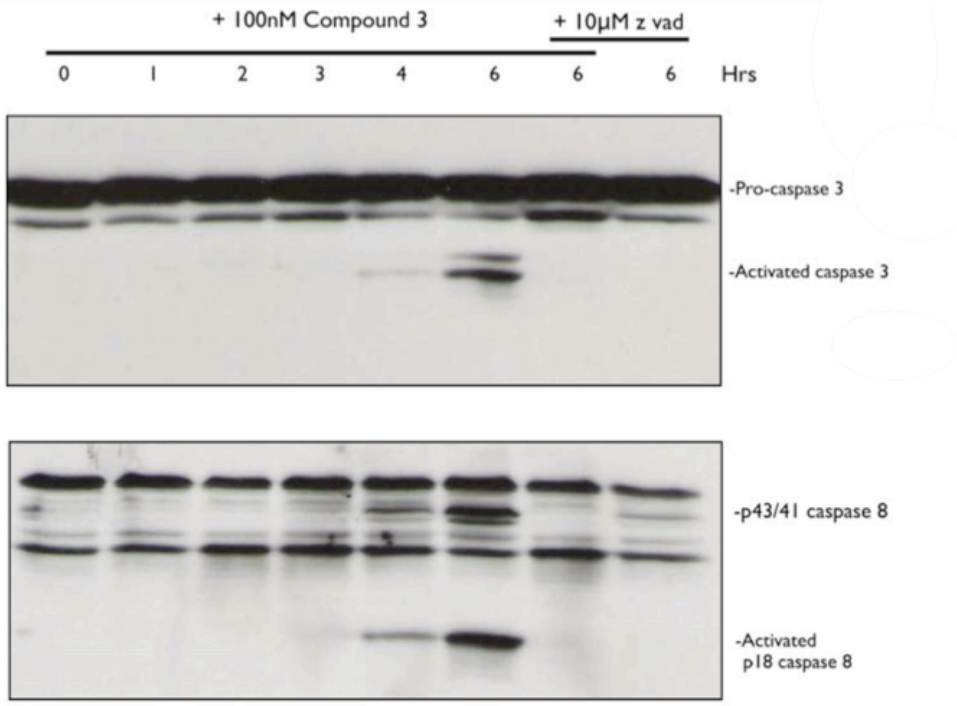

Figure 2-6. Analysis of caspase activation in HCC461 cell lysates following a time course of 100nM compound 3 treatments.
For caspase inhibition, 10μM z vad fmk (Sigma) was added 1 hour prior to compound 3. (A) Western blot analysis of caspase-3 activation. (B) Western blot analysis of caspase-8 activation.

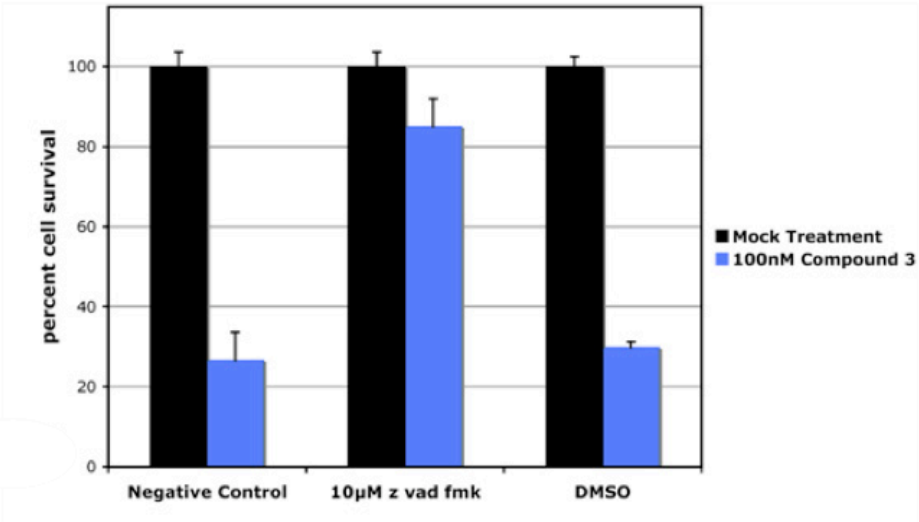

Figure 2-7. Rescue of Smac mimetic cell death by 10µM z vad.

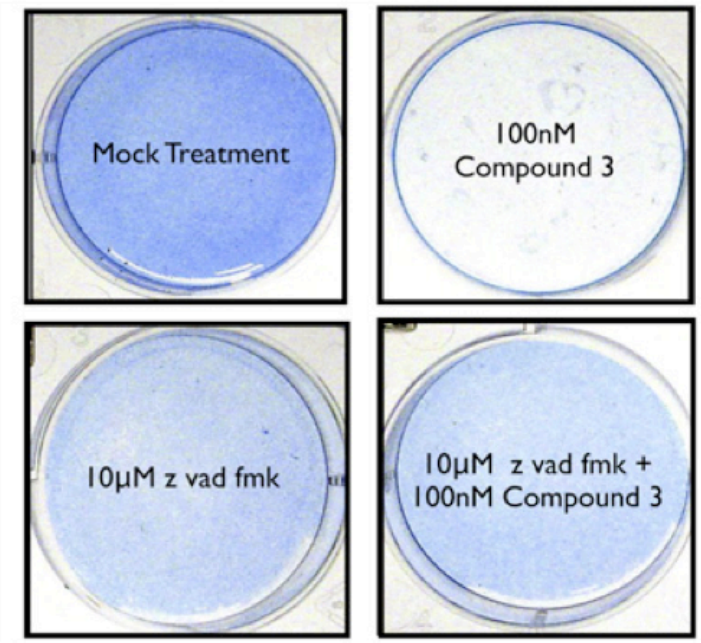

Figure 2-8. Long-term survival of cells treated with z vad plus compound 3.
Cells were treated as indicated for 5 days to ensure that rescue from cell death was not a transient artifact of z vad treatment. Cell viability was determined using methylene blue staining. Each graphical representation indicates the mean ± SD of at least 3 independent testing conditions.

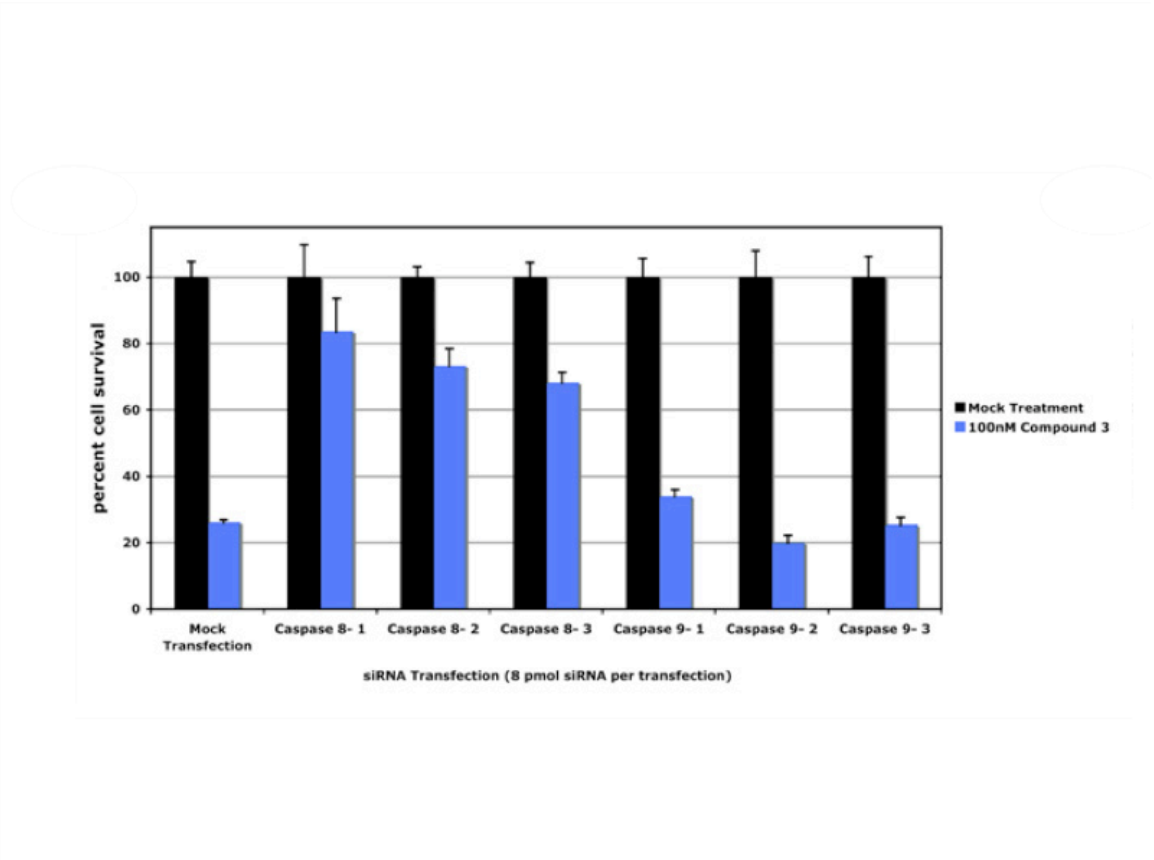

Figure 2-9. Caspase 8 and caspase 9 siRNA transfection to determine extrinsic or intrinsic apoptosis activation by smac mimetic.
Cells were assayed for the ability of particular transiently transfected individual siRNAs (Dharmacon) to produce a rescue phenotype in HCC461 cells following 100nM compound 3 treatments. Cell viability was determined by measuring total ATP levels. Each siRNA treatment and corresponding compound treatment (n=4, per transfection and compound 3 treatment) was normalized to the mock-transfected control to account for cytotoxicity.

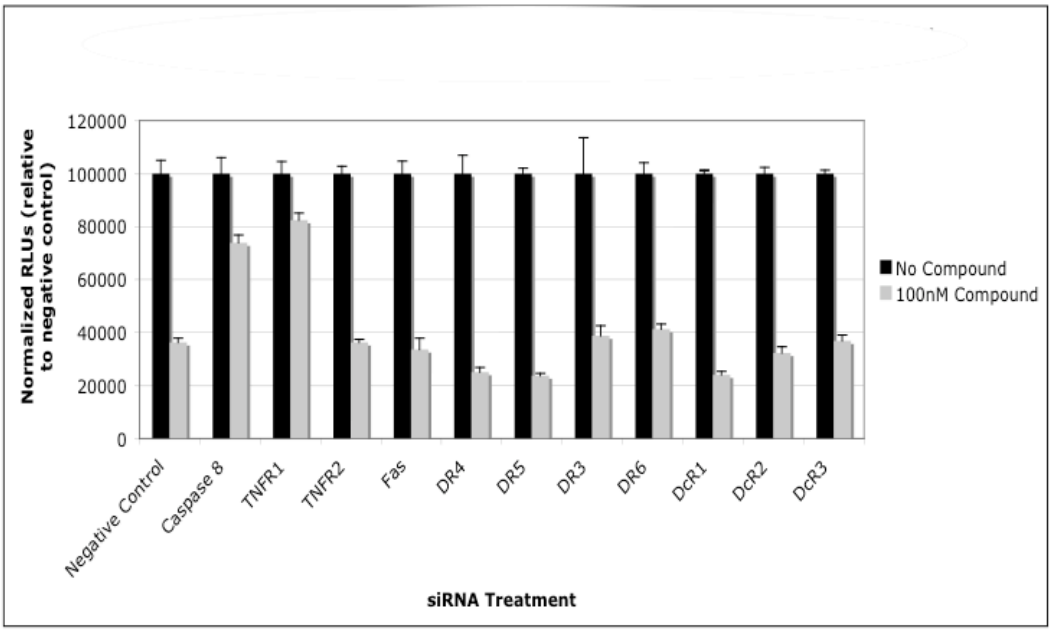

Figure 2-10. Initial siRNA screen targeting death receptors to identify those that rescue cells from smac mimetic induced cell death.
Cells were assayed for the ability of particular transiently transfected individual siRNAs (Dharmacon) to produce a rescue phenotype in HCC461 cells following 100nM compound 3 treatments. Cell viability was determined by measuring total ATP levels. Each siRNA treatment and corresponding compound treatment (n=4, per transfection and compound 3 treatment) was normalized to the mock-transfected control to account for cytotoxicity.

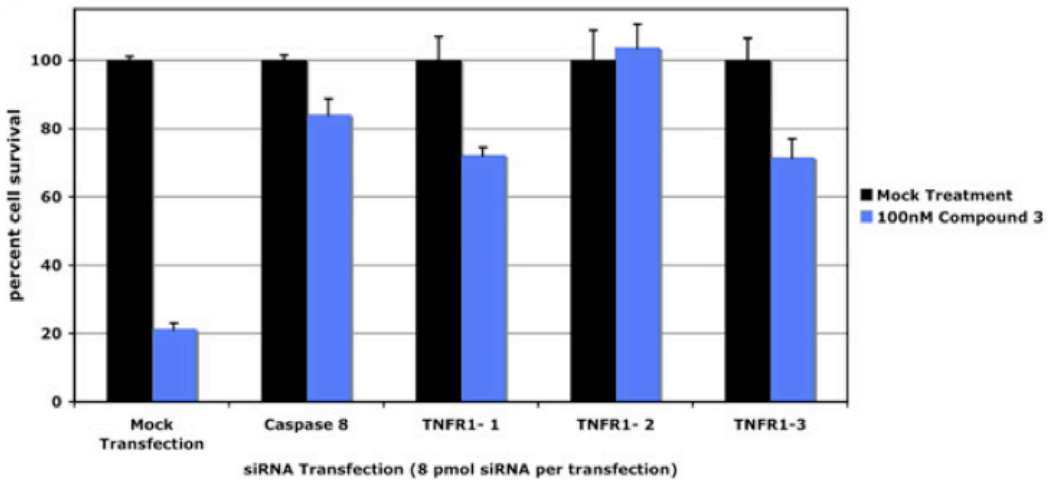

Figure 2-11. Validation of TNFR1 requirement for smac mimetic induced cell death. Cells were assayed for the ability of particular transiently transfected individual siRNAs (Dharmacon) to produce a rescue phenotype in HCC461 cells following 100nM compound 3 treatments. Cell viability was determined by measuring total ATP levels. Each siRNA treatment and corresponding compound treatment (n=4, per transfection and compound 3 treatment) was normalized to the mock-transfected control to account for cytotoxicity.

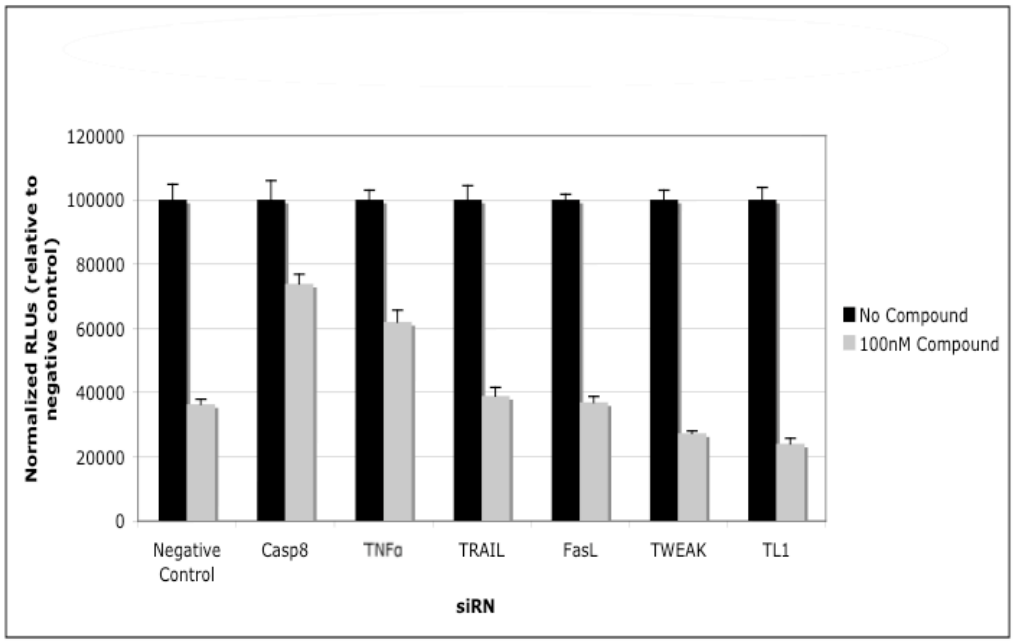

Figure 2-12. Initial siRNA screen of death ligands.
Cells were assayed for the ability of particular transiently transfected individual siRNAs (Dharmacon) to produce a rescue phenotype in HCC461 cells following 100nM compound 3 treatments. Cell viability was determined by measuring total ATP levels. Each siRNA treatment and corresponding compound treatment (n=4, per transfection and compound 3 treatment) was normalized to the mock-transfected control to account for cytotoxicity.

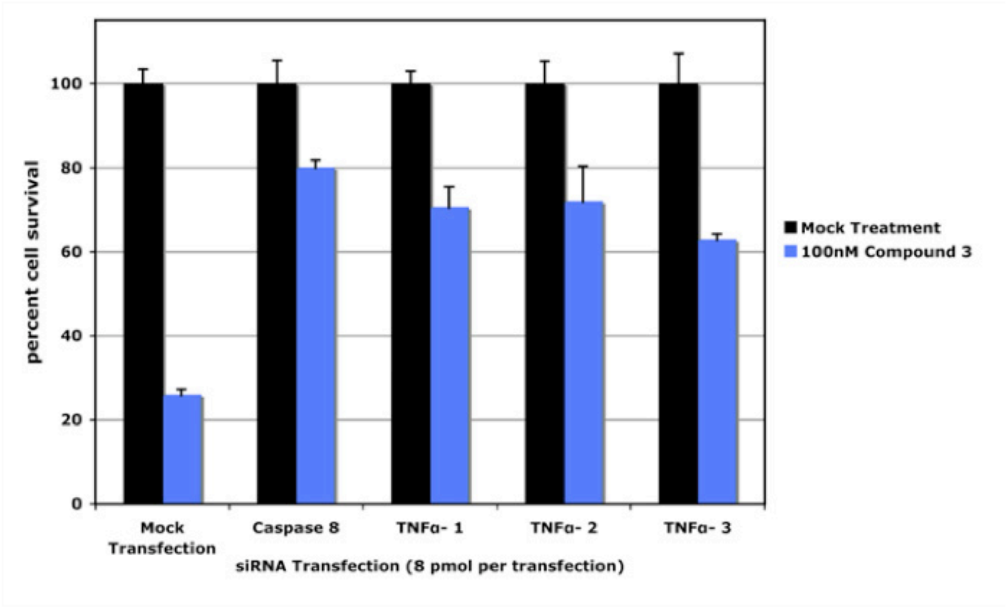

Figure 2-13.Validation of TNF as the ligand required for smac mimetic induced cell death.

Cells were assayed for the ability of particular transiently transfected individual siRNAs (Dharmacon) to produce a rescue phenotype in HCC461 cells following 100nM compound 3 treatments. Cell viability was determined by measuring total ATP levels. Each siRNA treatment and corresponding compound treatment (n=4, per transfection and compound 3 treatment) was normalized to the mock-transfected control to account for cytotoxicity.

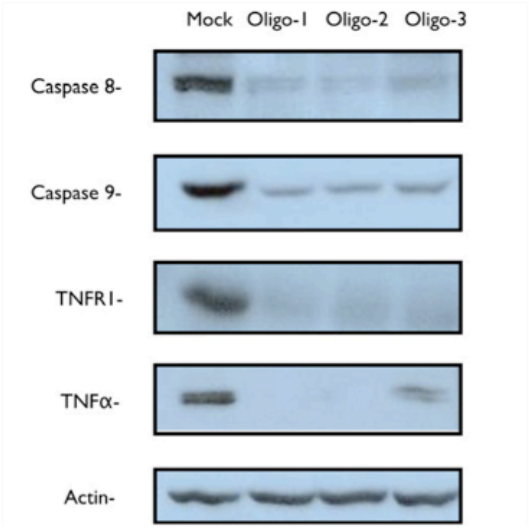

Figure 2-14. Efficiency of siRNA knockdowns as determined by western blot.

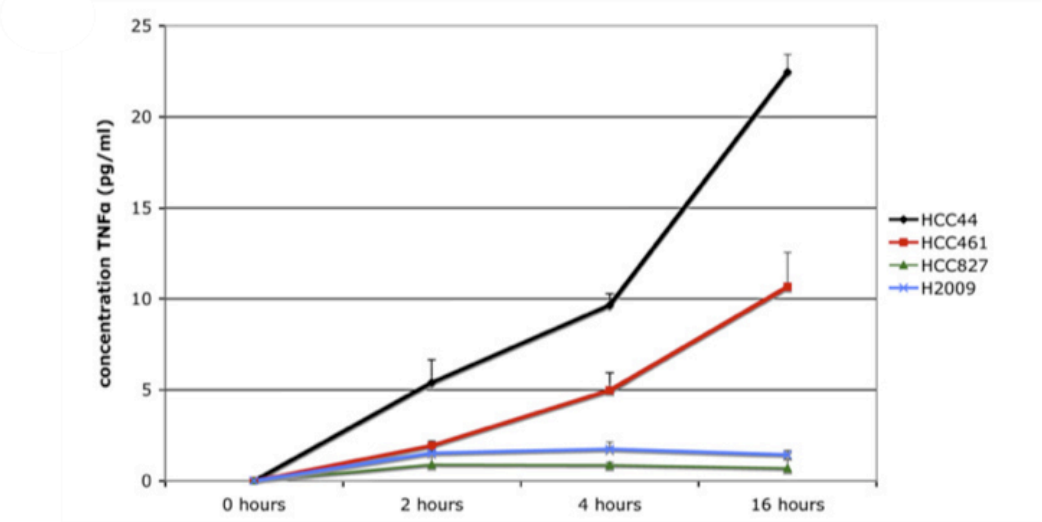

Figure 2-15. Sensitive cells secrete TNF alpha in an autocrine fashion.
Smac mimetic sensitive cell lines (HCC44 and HCC461) and Smac mimetic resistant cell lines (HCC827 and H2009) were tested for the presence of TNFα in conditioned cell culture media for each cell line. Sample were removed at the indicated time points and used for quantitative sandwich enzyme immunoassay analysis (R&D Systems) to determine the concentration of TNFα present, as described in the methods section. Sensitive cells were secreting low levels of TNF in the cell culture medium, while there was no detectable TNF levels present in resistant cell lines.

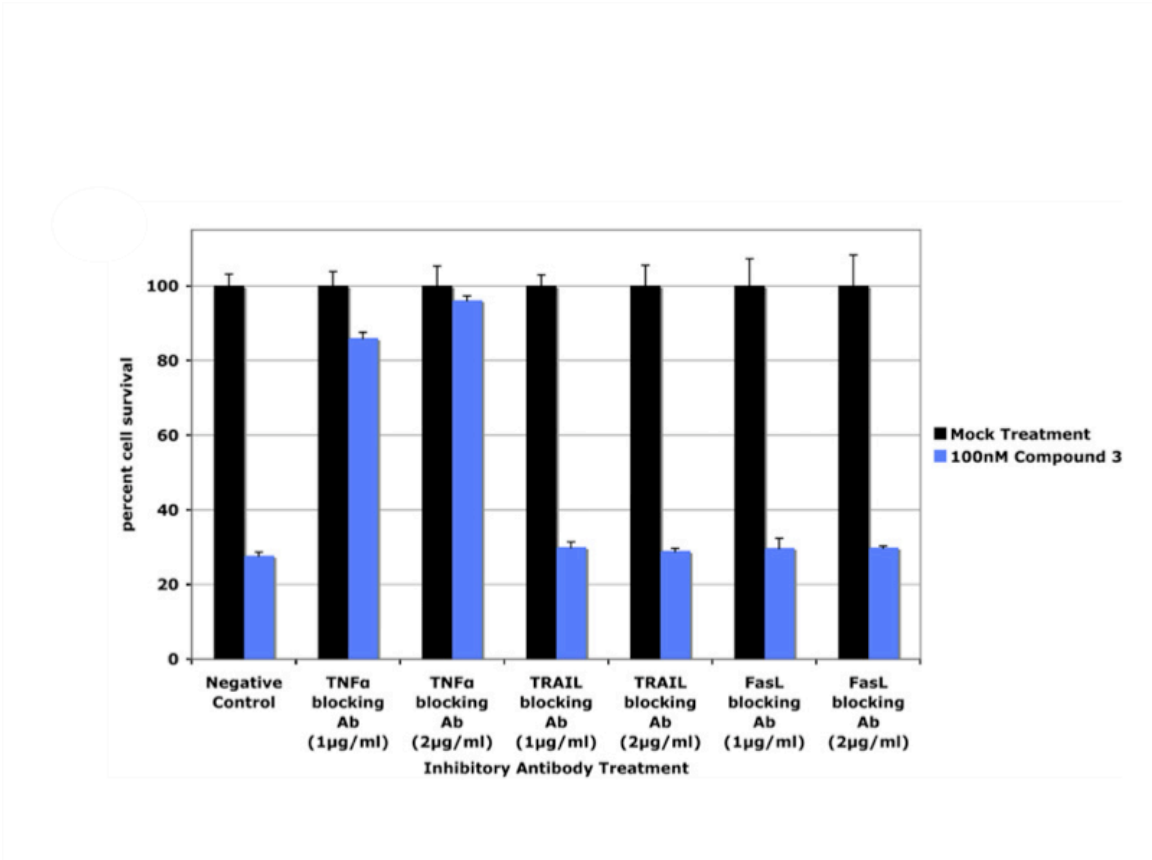

Figure 2-16. Smac mimetic induced cell death can be blocked by TNF neutralizing antibodies.

Pre-treatment (1 hour) of neutralizing antibodies (1-2 ug/mL) against TNFα (R&D Systems), TRAIL (Biolegend) and FasL/CD95 (Biolegend) prior to 100nM compound 3 treatments. Cell viability was determines as previously described. Each graphical representation indicates the mean ± SD of at least 3 independent testing conditions.

Pre-treatment (1 hour) of neutralizing antibodies (1-2 ug/mL) against TNFα (R&D Systems), TRAIL (Biolegend) and FasL/CD95 (Biolegend) prior to 100nM compound 3 treatments. Cell viability was determines as previously described. Each graphical representation indicates the mean ± SD of at least 3 independent testing conditions.

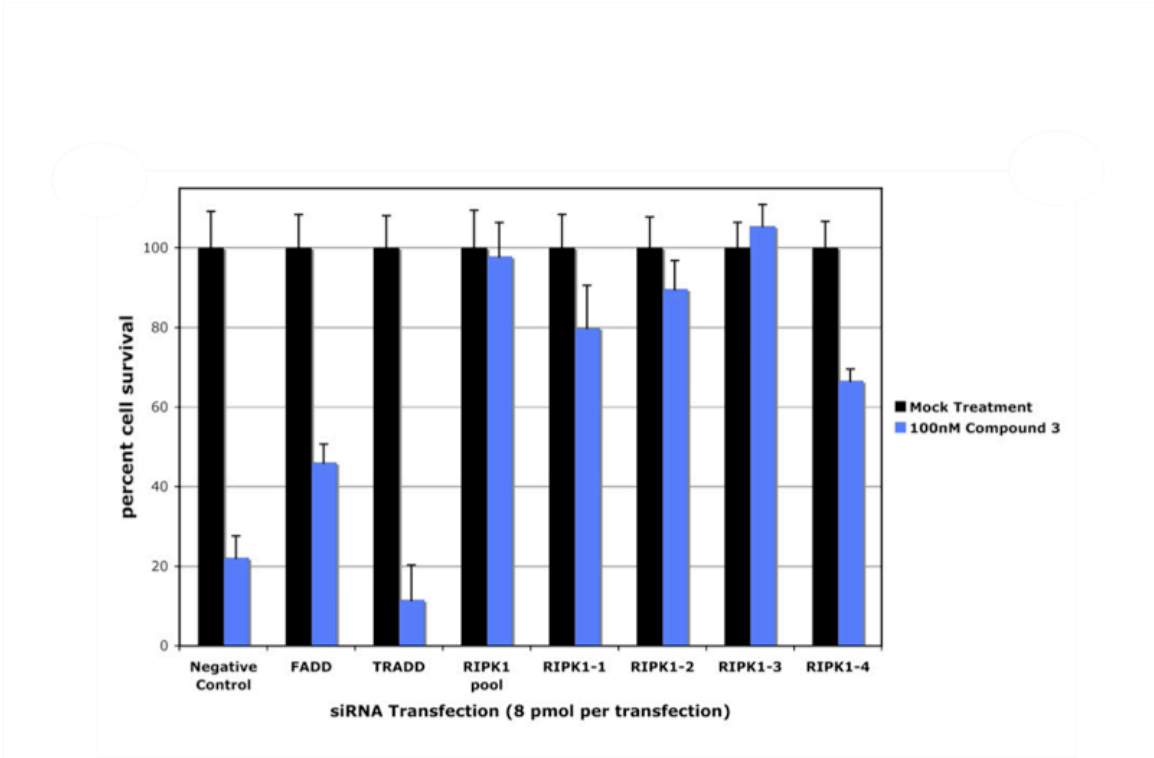

Figure 2-17. Smac mimetic mediated apoptosis is RIPK1 dependent.
siRNAs targeting known components of the TNF signaling pathway (FADD, TRADD and RIPK1). RIPK1 dependence was verified by the use of single siRNA oligos targeting different regions of the mRNA. siRNA transfection and viability assays were done as previously described.

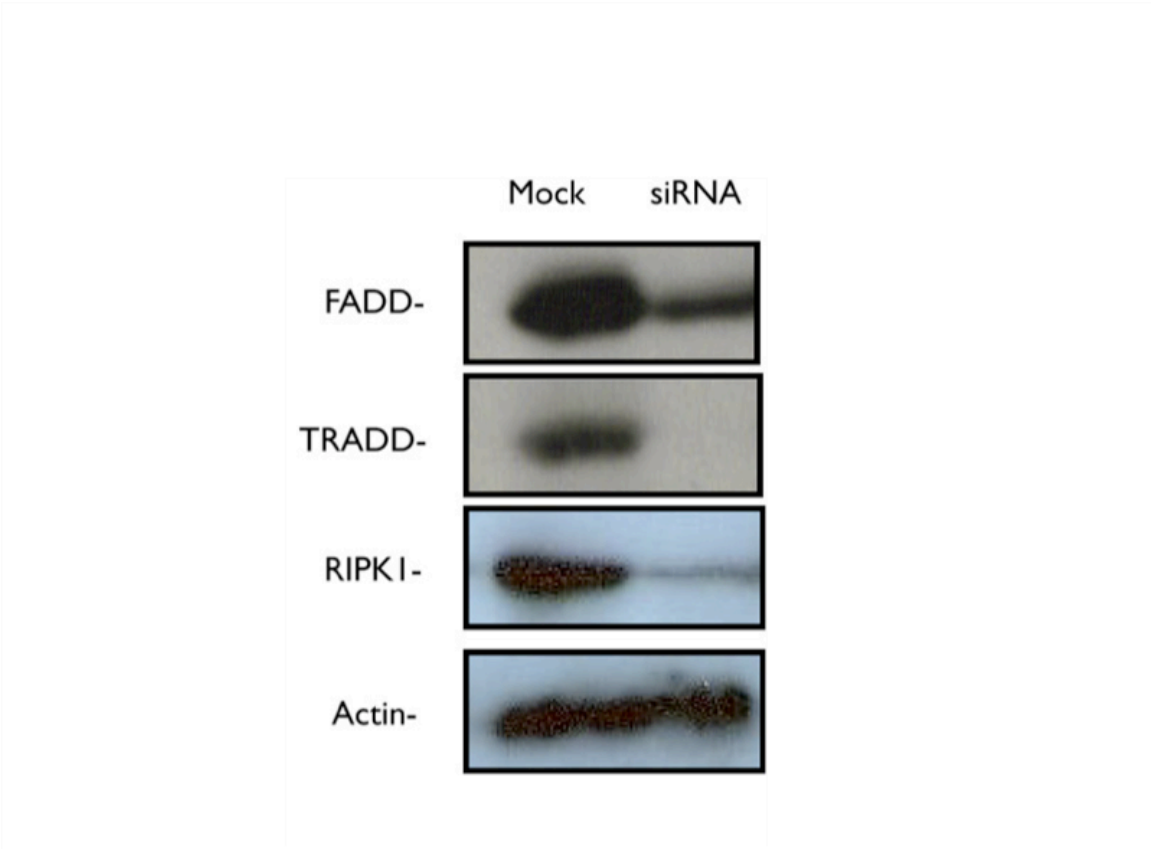

Figure 2-18. Validation of siRNA knockdown efficiency.
Western blot analysis of protein levels following siRNA transfection.

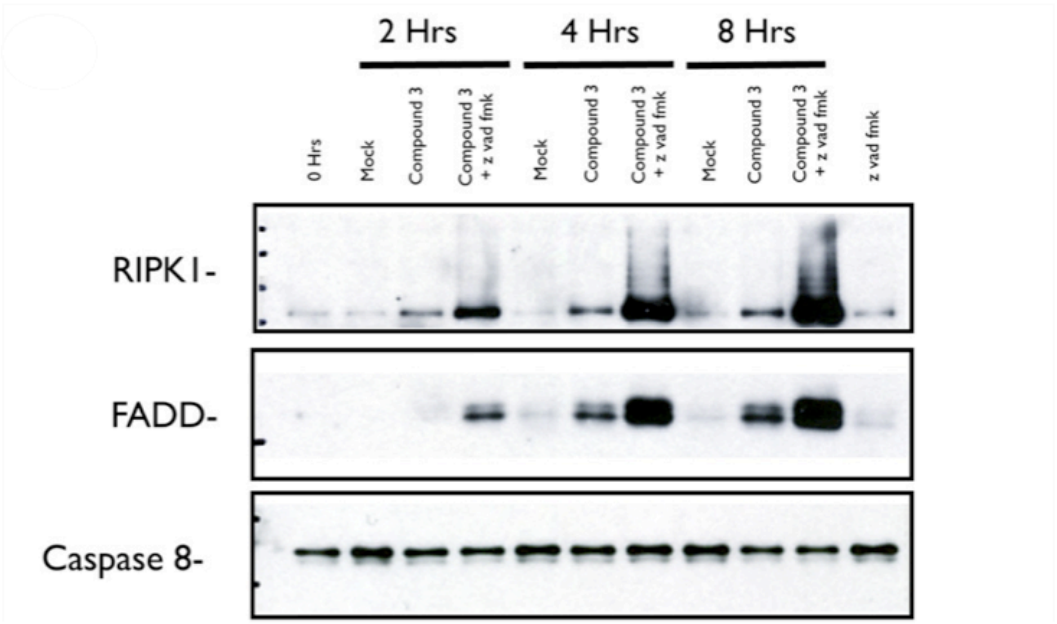

Figure 2-19. Smac mimetic promotes the formation of a RIPK1-FADD-caspase-8 death-inducing complex.
RIPK1, FADD, caspase-8 complex co-immunoprecipitation using caspase-8 antibody (Santa Cruz, SC-6136). Cells were treated in the indicated manner and co-immunoprecipitations were done as described in the methods. Z vat was added as a means of capturing caspase-8 in complex with RIPK1 and FADD by preventing full activation and subsequent dissociation of the complex from caspase-8.

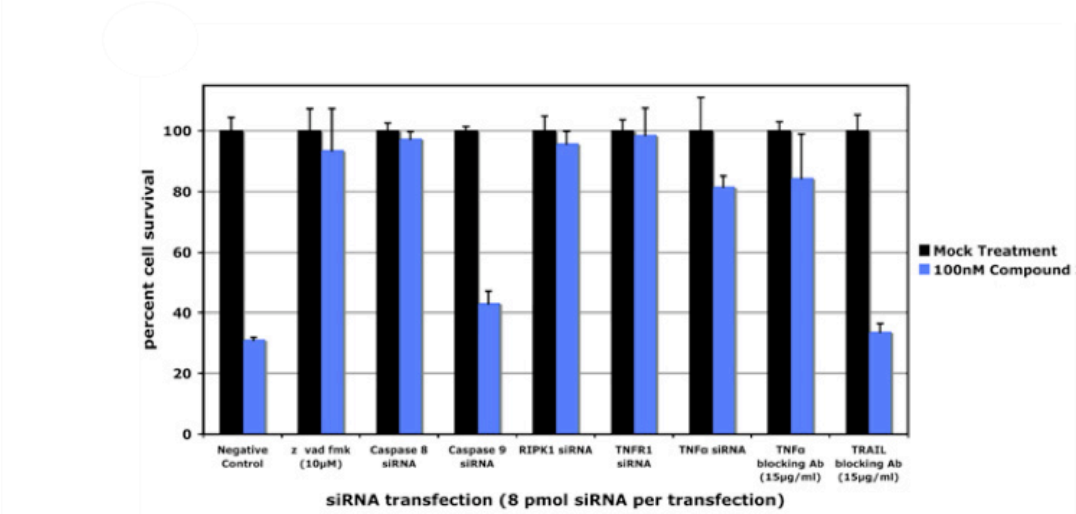

Figure 2-20. Verification of TNFR1, RIPK1 and caspases-8 dependence for smac mimetic induced cell death in HCC44.

Cell lines were tested for rescue by siRNA transfection and neutralizing antibody pre-treatments. siRNA targeting caspase-8, TNFR1, TNFα and RIPK1 (Dharma on signore SMART pool pre-designed pools of 4 logos), as well as neutralizing antibody against TNF (R and D Systems), were able to rescue these cell lines from Smac mimetic mediated apoptosis, while caspase-9 siRNA transfection (Dharmacon siGENOME SMARTpool) and pre-treatment with TRAIL (Biolegend) neutralizing antibody had no rescue effects.

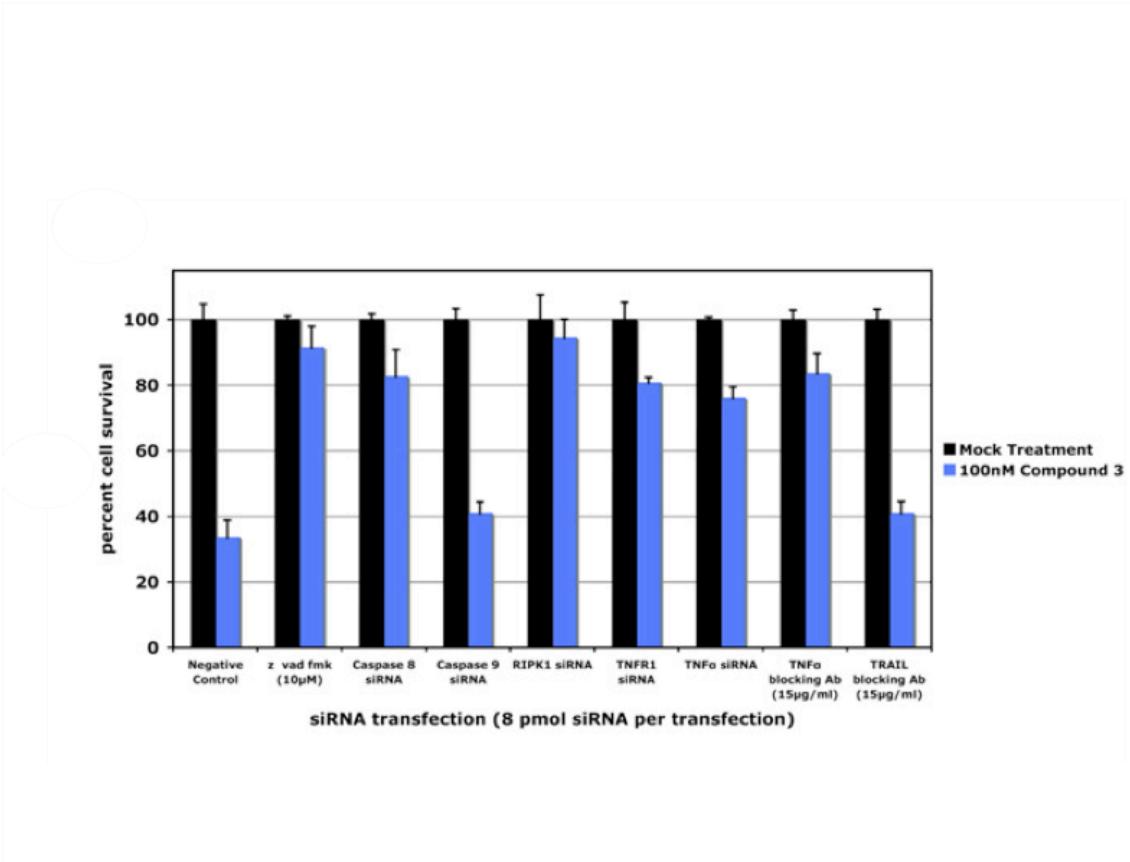

Figure 2-21. Verification of TNFR1, RIPK1 and caspases-8 dependence for smac mimetic induced cell death in MDA-MB-231.

Cell lines were tested for rescue by siRNA transfection and neutralizing antibody pre-treatments. siRNA targeting caspase-8, TNFR1, TNFα and RIPK1 (Dharma on signore SMART pool pre-designed pools of 4 logos), as well as neutralizing antibody against Tuna (R and D Systems), were able to rescue these cell lines from Smac mimetic mediated apoptosis, while caspase-9 siRNA transfection (Dharmacon siGENOME SMARTpool) and pre-treatment with TRAIL (Biolegend) neutralizing antibody had no rescue effects.

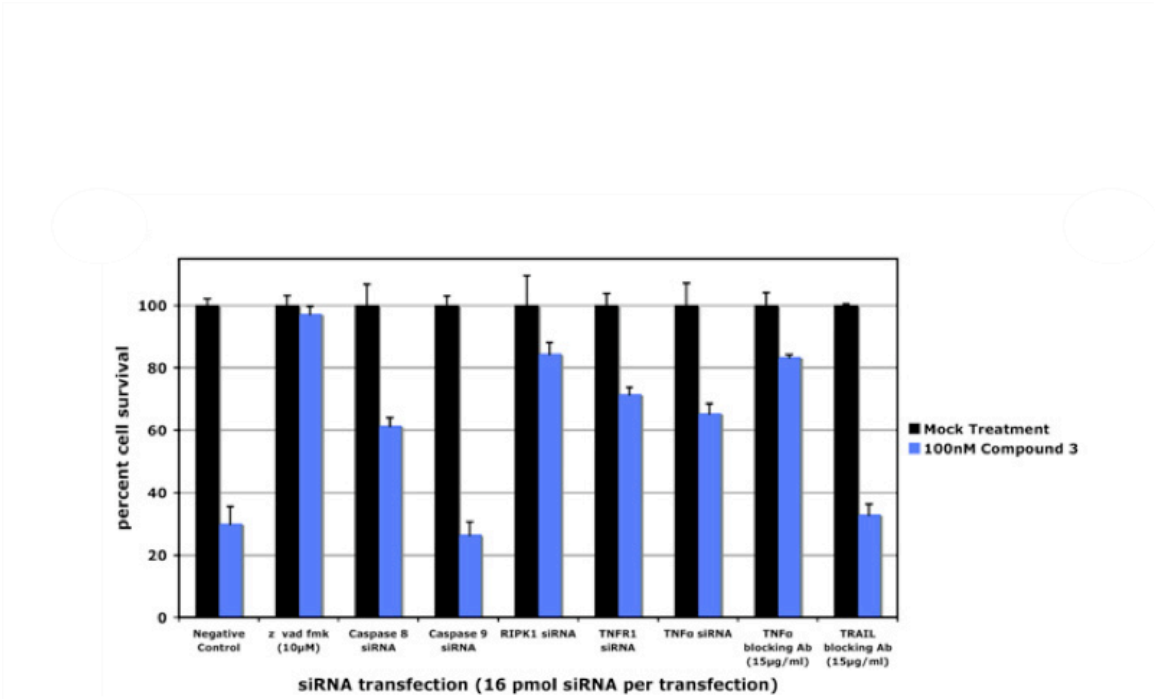

Figure 2-22. Verification of TNFR1, RIPK1 and caspases-8 dependence for smac mimetic induced cell death in SK-5 MEL cells.
Cell lines were tested for rescue by siRNA transfection and neutralizing antibody pre-treatments. siRNAs targeting caspase-8, TNFR1, TNFα and RIPK1 (Dharmacon siGENOME SMARTpool pre-designed pools of 4 oligos), as well as neutralizing antibody against TNFa (R and D Systems), were able to rescue these cell lines from Smac mimetic mediated apoptosis, while caspase 9 siRNA transfection (Dharmacon siGENOME SMARTpool) and pre-treatment with TRAIL (Biolegend) neutralizing antibody had no rescue effects.

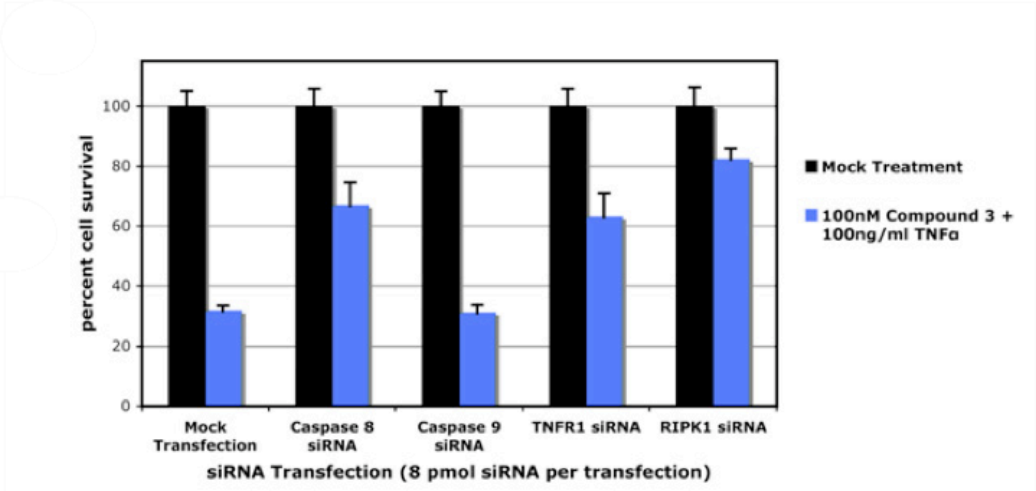

Figure 2-23. Validation of RIPK1 andcaspase-8 requirement in a cell that is sensitive to exogenous TNF and smac mimetic.
H2009 cells were shown to respond to 100nM compound 3 and 100ng/mL TNFα. siRNA knockdown of caspase 8, TNFR1 and RIPK1 were able to rescue these cells from compound 3/TNFα co-treatment.

CHAPTER THREE: Overcoming resistance to smac mimetic induced cell death in human cancer cell lines

3.1 Introduction

The development of targeted therapeutics against specific pathways in cancer etiology has greatly expanded the repertoire of effective drugs against many cancer types (Sawyers 2002). Examples include EGFR (Messersmith and Ahnen 2008), VEGF (Sledge 2005) and HER2/neu (Chen, Lan and Hung 2003). However, significant obstacles remain including variability of response to chemotherapeutics among patients and tumor types, relapse following successful initial treatment, and initial and acquired resistance (Doyle 1993, Gottesman 2002, Verrills and Kavallaris 2003). Ideally, the identification of the mechanism by which tumors are able to resist treatment and the development of specific inhibitors of resistance signaling pathways could broadly expand the effective use many chemotherapeutic agents.

Cancers are characterized by uncontrolled cellular growth and proliferation (Hanahan and Weinberg 2000). Normally, a cell will respond by activating a process whereby it undergoes programmed cell death, or apoptosis, to ensure cellular homeostasis (Danial and Korsmeyer 2004). One of the hallmark features of cancer is the ability to evade apoptosis by a variety of means, particularly by up-regulation of anti-apoptotic genes such as certain members of the Bcl-2 family of proteins (Adams and Cory 2007, Kirkin et al. 2004)and the inhibitor of apoptosis (IAP) family of proteins (Ambrosini et al. 1997, Deveraux and Reed 1999, Gordon et al. 2002, Nachmias et al. 2004). IAPs, particularly cellular IAP1 (cIAP1), cIAP2, and x-linked IAP (XIAP), function to prevent

unregulated cell death by binding to and preventing processing of the initiator caspase-9 as well as the executioner caspases-3 and -7 (Liu et al. 2000, Srinivasula et al. 2001, Wu et al. 2000). IAPs are defined by having 1 to 3 baculovirus IAP Repeat (BIR) domains that are involved in caspase interaction (Hinds et al. 1999, Shi 2004). Additionally, XIAP, cIAP1 and cIAP2 possess a Really Interesting New Gene (RING) domain, which has E3 ubiquitin ligase activity that controls auto-ubiquitylation resulting in proteasome-dependent auto-degradation of cIAP1 and cIAP2, but not necessarily XIAP (Morizane et al. 2005, Silke et al. 2005, Suzuki et al. 2001). IAP inhibition of apoptosis is relieved by Second Mitochondria-derived Activator of Caspases (Smac) that, along with cytochrome c, is released from the mitochondria upon induction of the intrinsic activation pathway of apoptosis in response to genotoxic stresses such as ionizing radiation or DNA damaging agents (Chai et al. 2000, Du et al. 2000, Verhagen et al. 2000). Smac relieves inhibition of apoptosis by at least two mechanisms: by specifically binding to XIAP, disrupting its interaction with caspase-3, -7 and -9, and by inducing auto-ubiquitination and proteasome degradation of cIAP1 and cIAP2. Smac acts as a dimer to disrupt IAP-caspase interaction and induce auto-ubiquitination by binding to IAPs utilizing a conserved region of the protein containing just 4 amino acids (AVPI) (Chai et al. 2000, Liu et al. 2000, Wu et al. 2000).

Because IAPs have been shown to be up-regulated in many cancers and because the interaction of Smac with IAPs involves just 4 amino acids an ideal point of attack is to introduce either short peptides or small molecules that mimic the AVPI sequence motif and relieve IAP inhibition without having to disrupt the mitochondria (Li et al. 2004). Over the past several years many groups have designed and synthesized various versions

of small molecule smac mimetics with the hopes of sensitizing cancer cells to apoptosis. Previous reports have demonstrated that smac mimetics are effective at inducing cell death both as a single agent and with other pro-apoptotic stimuli. It has been shown that single agent Smac mimetic sensitivity depends on autocrine tumor necrosis factor (TNF) signaling and that many other cell types are sensitive to exogenous addition of TNF (Varfolomeev et al. 2007, Vince et al. 2007, Petersen et al. 2007, Bertrand et al. 2008).

The primary mechanisms by which smac mimetics induce cell death involves both direct binding and inhibition of XIAP and induced proteasome degradation of cIAP1 and cIAP2 (Bertrand et al. 2008, Varfolomeev et al. 2007, Vince et al. 2007, Wang et al. 2008). Upon TNF ligation to TNF receptor I (TNFR1); a complex of cIAP1, cIAP2, TNFR-associated factor 2 (TRAF2) and TRAF1 is recruited along with receptor interacting protein kinase I (RIPK1) to promote nuclear factor-κB (NF-κB) signaling (Chen et al. 2002, Devin et al. 2000, Shu et al. 1996). Degradation of cIAP1 and cIAP2 allows for the release of RIPK1 from TNFR1 and subsequent incorporation into a complex with caspase-8 and Fas-associated death domain (FADD) that is capable of promoting cell death and overriding the pro-survival effects of NF-κB signaling (Petersen et al. 2007, Wang et al. 2008), that is distinctly different from the canonical activation pathway (Micheau and Tschopp 2003).

This would imply that any cancer cell expressing TNFR1 should respond to smac mimetic/TNF treatment and, in fact, TNFR1 expression is quite ubiquitous among various cancer cell types (Wajant, Pfizenmaier and Scheurich 2003). However, most cell lines tested to date do not appear to be entirely responsive to such treatment, begging the questions: how are they able to evade the potent effects of smac mimetic treatment and

can resistance be overcome? Is resistance an active process or simply a matter of limiting levels of essential components required for an active RIPK1-FADD-Caspase 8 complex?

It has been observed that Smac mimetics are able to induce degradation of cIAP1 and cIAP2 in all cell lines regardless of sensitivity. In most cells cIAP1 is predominant over cIAP2 owing to the regulatory role of cIAP1 in actively ubiquitinating cIAP2 to keep cIAP2 levels suppressed as demonstrated in cIAP1 knockout mice that display highly elevated levels of cIAP2 in the absence of cIAP1 (Conze et al. 2005, Srinivasula and Ashwell 2008). Lack of any overt developmental or physiological phenotypes implies that cIAP2 can replace the functionality of cIAP1, especially given the high homology between the two.

Both cIAP1 and cIAP2 are TNF responsive genes regulated by NF-kB (Hong et al. 2000). cIAP2, in particular, is extremely sensitive to TNF, which presents something of a paradox and a potential mechanism of resistance to smac mimetics. The requirement of introducing TNF either endogenously, through autocrine secretion or Smac mimetic induced non-canonical NF-κB activation (Varfolomeev et al. 2007, Vince et al. 2007, Petersen et al. 2007), or exogenously for Smac mimetics to work implies the possibility that following the initial degradation of cIAP1 and cIAP2 by Smac mimetics that the presence of TNF allows for the return of cIAP2 and that this might be enough to cause resistance given that once cIAP1 is degraded that there would no longer be any control of cIAP2 by Smac mimetic via cIAP1 E3 ligase activity.

Inappropriate NF-kB signaling has been implicated in tumor progression by promoting up-regulation of pro-growth and –survival genes as well as anti-apoptotic genes (Courtois and Smahi 2006). Initial attempts at chemically inhibiting NF-κB by

targeting I-κB kinases (IKK) specifically showed tremendous early promise by sensitizing cancer cells to TNF (Yamamoto and Gaynor 2001). However, a side effect of total inhibition of NF-κB was to cause cytotoxicity in normal cells as well as inducing a cytotoxic immune response resulting in a cytokine storm that can be fatal.

Regulation of cIAP2 occurs via several additional mechanisms beyond NF-κB. It has been reported that cIAP2 and XIAP are up regulated in a Ras dependant manner through receptor tyrosine kinase activation by promoting autocrine production of TGF-alpha (Liu et al. 2005). There is also evidence that cIAP2 is regulated by other signaling pathways other than NF-kB, or in conjunction with NF-kB, such as phosphoinositide-3 kinase (PI-3K) (Seol 2008, Terragni et al. 2008), protein kinase C delta (PKCδ) (Wang, Wang and Evers 2003) and cyclooxygenase 2 (COX-2) dependant cyclic adenosine monophosphate (cAMP) upregulation (Nishihara et al. 2003).

We report here that following the initial degradation of cIAP1 and cIAP2 by Smac mimetic, cells able to up-regulate cIAP2 are highly resistant to Smac mimetic/TNF; and that interfering with basal or TNF induced cIAP2 induction sensitizes these cells. Interference of NF-κB signaling using sub-threshold concentrations of IKK inhibitor, as well as siRNA against both cIAP2 and IKKγ/NEMO, were effective at limiting cIAP2 up-regulation while not adversely effecting overall survival in the presence of TNF. Additionally, we observed synergistic potentiation between Smac mimetic/TNF and inhibitors of PI-3K, as well as erlotinib (Tarceva), an epidermal growth factor receptor (EGFR) kinase specific inhibitor. Both treatments have no effect without smac mimetic and effectively block cIAP2 production both basally and in response to TNF, indicating inter-dependence between these pathways and NF-κB in the production of cIAP2.

Finally, there are other cell lines that do not express cIAP2 either endogenously or in response to TNF, yet these cells are completely resistant. Interestingly, these cell also have lower relative levels of RIPK1, which is insufficient to form the RIPK1-death inducing complex. Low RIPK1 has been shown to correlate with TRAIL sensitivity and we find that cells that do not express cIAP2 are also particularly sensitive to TRAIL.

3.2 Resistant cell line NCI-H1299 shows elevated cIAP2 levels following smac mimetic/TNF treatment

It has been demonstrated that certain non-small cell lung cancer cells lines are responsive to low dose (100nM) smac mimetic treatments in the presence of autocrine or exogenously added TNF (Li et al. 2004, Petersen et al. 2007). Comparison of one such cell line NCI-H2009 with a completely resistant cell line NCI-H1299 showed a marked difference in how these cell lines respond to Smac mimetic treatment. For a cell line to be considered sensitive, cell death should occur at a smac mimetic concentration of 100nM in the presence of 50ng/ml TNF. H2009 underwent apoptosis at 100nM with only modest improvement at 1uM (Figure 3-1). A time course of response to Smac mimetic or TNF alone and in combination, showed that both cIAP1 and cIAP2 are degraded by Smac mimetic and that neither cIAP1 nor cIAP2 are particularly sensitive to up-regulation by TNF (Figure 3-2). As previously mentioned, one of the functions of cIAP1 and cIAP2 at TNFR1 is to hold RIPK1 at the receptor promoting NF-kB signaling. Co-immunoprecipitation of TNFR1 showed that TNF binding to TNFR1 causes recruitment of cIAP1 and RIPK1 to TNFR1 and that upon Smac mimetic co-treatment both IAPs are

lost and RIPK1 comes off the receptor (Figure 3-3). RIPK1 is then free to enter into a caspase-8- FADD containing complex that is required for cell death to occur (Figure 3-4).

In contrast, H1299 cells are considered resistant and show only modest sensitivity to smac mimetic at higher concentrations (1uM) in the presence of 50ng/ml TNF (Figure 3-5). Compared to H2009, cIAP2 in H1299 is initially degraded, but within 5 hours following Smac mimetic treatment alone and within 3 hours following Smac mimetic/TNF treatment, cIAP2 returned to basal and higher levels, respectively (Figure 3-6). These cells also display strong response to TNF alone, very quickly up-regulating cIAP2 well above baseline (Figure 4-6). cIAP1 is only mildly responsive to TNF and does not return following Smac mimetic/TNF co-treatment. Additionally, these cells are incapable of forming a RIPK1-Caspase 8- FADD complex and cIAP2 is highly recruited to TNFR1, presumably compensating for the loss of cIAP1 (Figure 3-23 and 3-24).

3.3 Loss of cIAP2 facilitates sensitivity of H1299 cells to low dose smac mimetic treatment

Given the dramatic return of cIAP2 following smac mimetic treatment and given that cIAP2 is not degraded with further addition of smac mimetic following the initial treatment (data not shown), it might be possible that cIAP2 is the causative agent behind resistance. To determine whether this is the case siRNAs targeting cIAP1and cIAP2 were used to examine the behavior of each under limiting condition of the other. siRNA against cIAP2 was effective at preventing the accumulation of cIAP2 following Smac mimetic/TNF treatment, while having only moderate effects on cIAP1 and XIAP (Figure 3-7). As has been previously reported (Conze et al. 2005), loss of cIAP1 resulted in

accumulation of cIAP2 (Figure 3-8). Furthermore, knockdown of cIAP1 effectively blocked the initial degradation of cIAP2, supporting cIAP1s role as the main E3 ubiquitin ligase for cIAP2 in the cell (Figure 3-8). Loss of XIAP had no discernable effect on the behaviors of either cIAP1 or cIAP2 (Figure 3-8).

Knockdown of cIAP2 sensitized previously resistant cells to smac mimetic/TNF, much more than either cIAP1 or XIAP knockdown, which did show some minor sensitivity (Figure 3-9). Leakiness by cIAP1 and XIAP knockdown can probably be accounted for by the key role XIAP has in directly inhibiting caspase-3 and the fact that cIAP1 levels are typically much higher than cIAP2. Co-immunoprecipitation of caspase-8 in mock-transfected cells only weakly pulled down RIPK1 with no FADD present. Under cIAP2 deficient conditions robust formation the RIPK1-Caspase 8-FADD complex occurred (Figure 3-10). Co-immunoprecipitation in cIAP1 or XIAP deficient cells did not show formation of the complex (data not shown).

Because cIAP2 protein levels return to normal following Smac mimetic treatment one would expect that inhibiting translation should sensitize resistant cell lines. Co-treatment with low-dose (0.5ug/ml) cycloheximide (CHX) attenuated how cIAP2 responds to smac mimetic, not completely blocking full return but somewhat limiting it (Figure 3-11). The effect of low dose CHX is different from that seen with higher doses as the caspase 8-homologue FLICE-inhibitory protein (c-FLIP) is not effected and therefore apoptosis does not occur with low dose CHX (500ng/ml) and TNF treatment, whereas cells are sensitized to TNF at higher concentrations of CHX (5ug/ml) (Figures 3-11 and 3-12). However, with the addition of Smac mimetic, cells become highly sensitive to low concentrations of CHX (Figure 3-12).

3.4 NEMO siRNA sensitizes resistant cell line H1299 by blocking TNF induced up-regulation of cIAP2

To ensure that the effect of CHX was specific to the NF-κB pathway, siRNAs targeting the I kappa-B kinase-gamma (IKK-γ/NEMO), the regulatory subunit of the IKK complex) was used to block NF-κB activation and subsequent cIAP2 induction following TNF treatment. Four independent oligos targeting NEMO each was able to sensitize H1299 cells to smac mimetic/TNF (Figure 3-13) and all oligos were able to effectively knockdown NEMO (Figure 4-14). For all subsequent knockdowns oligo #3 was used. Further examination of whether NEMO knockdown mimicked CHX in the sense of sensitizing cell to TNF alone revealed that although there was some sensitivity, the effect when Smac mimetic was added was significantly greater (Figure 3-15). Additionally, to verify that cell death induced by NEMO knockdown plus smac mimetic/TNF was dependent on RIPK1, cells were co-transfected with siRNAs against both RIPK1 and NEMO, which had the effect of blocking cell death (Figure 3-15). Double knockdown of RIPK1 and NEMO was effective as shown by immunoblotting (Figure 3-16). This form of cell death therefore matches that seen in cell lines sensitive to single agent, which are RIPK1 dependent, and is different from that observed for CHX/TNF, which is RIPK1 independent (Wang et al. 2008).

A time course of Smac mimetic/TNF treatment revealed identical results as those seen for cIAP2 siRNA and low dose CHX: cIAP2 was not induced upon smac mimetic/TNF treatment (Figure 3-17) and remained suppressed throughout all treatments. Nor did loss of NEMO overtly affect cIAP1 or XIAP. Furthermore, only under NEMO

deficient condition was the RIPK1- Caspase-8- FADD complex able to form (Figure 3-18).

3.5 Chemical inhibition of NF-kB with BMS-345541 sensitizes H1299 cells to smac mimetic/TNF

To determine the feasibility of utilizing chemical inhibitors of the NF-κB pathway to block up-regulation of cIAP2 in response to TNF it was decided to target IKK phosphorylation, preventing phosphorylation of Iκ-B alpha and its subsequent proteasome degradation, using an IKK-2/IKK-β specific inhibitor, BMS-345541 (Burke et al. 2003, MacMaster et al. 2003, McIntyre et al. 2003, Townsend et al. 2004). One of the issues with inhibiting NF-κB is the dependence cells have on it for maintaining proper levels of both pro-survival and anti-apoptotic gene products, which when disrupted results in severe sensitivity to pro-apoptotic stimuli. In particular, cells become sensitive to the effects of TNF under such condition, similar to what is observed for treatment with CHX. This, of course, presents problems because of increased immune response to minor infection leading to the possibility of a cytokine storm induced form of septic shock. However, because cIAP2 up-regulation is such an acute response to TNF, occurring within hours, it might be possible to use sub-threshold concentrations of the inhibitor and not aversely affect other key proteins involved in survival, such as c-FLIP.

Smac mimetic/TNF treatment after pre-treatment with 5uM BMS-345541 prevented up-regulation of cIAP2, both basally following smac mimetic treatment alone and induced by TNF (Figure 3-19). Pre-treatment did not adversely affect cIAP1, XIAP or c-FLIP (Figure 3-19). Therefore, a panel of various NF-kB inhibitors was screened for

sensitivity with Smac mimetic and TNF, but not to TNF alone (Figure 3-20). From the screen we chose BMS-345541 as a representative for further examination. BMS-345541 synergized with Smac mimetic/TNF in a dose dependent manner to induce cell death with an optimal killing concentration at 5uM (Figure 3-21). Only at higher concentrations did pre-treatment with BMS-345541 induce sensitivity to TNF alone (Figure 3-21). The differences between low- (5uM) and high- (20uM) dose IKK inhibition is demonstrated by effects on phosphorylation IκB-α. The normal response to smac mimetic/TNF is to cause phosphorylation of IκB-α and its accumulation over time. BMS-345541 at low dose delays and attenuates phosphorylation, while at a high dose completely blocks it .

RIPK1 is a component absolutely necessary for both proper NF-κB activation and for Smac mimetic induced cell death. Knockdown of RIPK1 attenuates both cIAP2 levels in response to smac mimetic/TNF and Iκ-B alpha phosphorylation in a similar manner as low dose IKK inhibition, but since RIPK1 is required for Smac mimetic cell death, the effect on viability is minimal (Figures 3-21 and 3-22). Determining RIPK1 dependence in IKK inhibitor/Smac mimetic/TNF induced cell death is problematic due to the requirement of RIPK1 for NF-κB signaling. When adding BMS-345541 to RIPK1 deficient cells, they become particularly sensitive to TNF, reducing the effective dose at which the IKK inhibitor causes TNF sensitivity from 20uM to 2.5uM (Figures 3-22). The effect of low dose IKK inhibitor in RIPK1 deficient cells on cell death and Iκ-B alpha phosphorylation is equivalent to that seen with high dose IKK inhibitor under the same conditions (Figures 3-22 and 3-23).

However, the loss of cIAP2 as a result of IKK inhibitor co-treatment resulted in robust formation of the RIPK1-caspase 8-FADD complex (Figure 3-25). Since one of the

functions of cIAP1 and cIAP2 is to hold RIPK1 at TNFR1, loss of cIAP2 should effect the ability of RIPK1 to come off the receptor and its incorporation into the caspase-8-FADD containing complex. TNFR1 co-immunoprecipitation showed that upon TNF treatment cIAP1 and cIAP2 were recruited to the receptor and that with Smac mimetic co-treatment cIAP2 appears to be able to compensate for the loss of cIAP1 (Figure 3-24). When IKK inhibitor is added there are then no IAPs associated with the receptor (Figure 3-24) and RIPK1 is able to dissociate from the receptor and enter into the caspase-8-FADD complex to induce apoptosis as seen by casapse-8 co-immunoprecipitation with and without BMS-345541 added (Figure 3-25).

3.6 Inhibition of alternative pathways regulating cIAP2 sensitizes resistant cells to Smac mimetic/TNF

Inhibiting NF-kB does not appear to effect baseline levels of cIAP2, indicating that other signaling pathways operate to maintain the initial state of cIAP2. H1299 cell have been documented to carry a mutation in N-ras (Q61K) and to also display constitutive protein kinase B (AKT) activation (Figure 3-26). To test whether inhibition of AKT might affect cIAP2 levels, the phosphoinositide-3 kinase (PI-3K) inhibitor, LY294002, was used to inhibit AKT phosphorylation (Figure 3-26). Such inhibition effected cIAP2 levels in a similar fashion as IKK inhibition, blocking basal cIAP2 return and limiting TNF induced cIAP2 up-regulation (Figure 3-26). Pre-treatment with LY294002 synergized with smac mimetic/TNF in a dose dependent manner showing optimal sensitivity at 50uM, while having no effect when co-treated with TNF or smac mimetic alone (Figure 3-27). LY294002 co-treatment was also able to promote

significant formation of the RIPK1-caspase 8-FADD complex (Figure 3-30), further validating the idea that cIAP2 is the sole arbiter of resistance to smac mimetic/TNF treatment.

To determine if a clinically viable agent to which our cell lines are resistant could synergize with Smac mimetic to induce apoptosis, erlotinib (Tarceva), an epidermal growth factor receptor (EGFR) kinase specific inhibitor, was used. Erlotinib did not alter AKT phosphorylation, so its mechanism of action must be different from inhibition of PI-3K (or from it intended mechanism of inhibiting EGFR mutants, since H1299 has a wild type receptor). It is possible that erlotinib is affecting the Ras oncogene that is mutated in this cell line blocking regulation of cIAP2. Elotinib did nonetheless affect return of basal levels of cIAP2 following Smac mimetic treatment as well as affecting cIAP2 levels following Smac mimetic/TNF treatment (Figure 3-28). Erlotinib was able to synergize with smac mimetic/TNF at 5uM and did not have any effects with smac mimetic or TNF alone (Figure 3-29). Correlated with cell death was the ability of erlotinib to promote robust formation of the RIPK1-casapse-8-FADD complex (Figure 3-30). Both co-treatments are RIPK1 dependent, under conditions where RIPK1 is knocked down using siRNA; both LY294002 and erlotinib lost their ability to synergize with smac mimetic/TNF (Figure 3-31).

3.7 Resistance to smac mimetic/TNF in cells that do not express cIAP2 is a result of deficiency in TNF responsiveness and recruitment of RIPK1 to TNFR1

Not all cell lines express cIAP2 but are still highly resistance even though cIAP1 in these cell lines is efficiently degraded by smac mimetic. As an example, NCI-H460

large cell lung cancer cell lines were examined. H460 cells do not show synergy with BMS-345541 (10uM) or with LY294002 (50uM) suggesting that their mode of resistance is different (Figure 3-32). Curiously, H460 cells do not up-regulate cIAP2 in the presence of TNF, but do express cIAP1 (Figure 3-33). This suggested that perhaps these cells are somehow deficient in TNF signaling in general and that either they are deficient in TNFR1 or are missing key components involved in signaling TNF. These cells are not completely devoid of NF-kB response as indicated by modest phosphorylation of Iκ-B alpha, however, total levels of Iκ-B alpha were not altered by Smac mimetic/TNF treatment (Figure 3-34). Furthermore, these cells are incapable of forming the RIPK1-caspase 8-FADD complex, even in the presence of IKK inhibitor (Figure 4-35). Co-immunoprecipitation of TNFR1 showed that cIAP1 is able to dock to the receptor, but that RIPK1 was hardly recruited at all (Figure 3-36).

A comparison between the cell lines used in this study indicate that H460 cells are relatively deficient in RIPK1 (Figure 3-37), while having equivalent amounts of the other key components required for smac mimetic induced cell death, such as TNFR1, caspase-8, and FADD (Figure 3-37). Hence resistance in these cells can be accounted for by the inability to recruit sufficient RIPK1 to the receptor. An interesting corollary is that low levels of RIPK1 in H460 makes them particularly sensitive to TRAIL induce apoptosis, while H1299 and H2009 are fairly unresponsive (data not shown).

3.8 Discussion

Among several resistant cell lines it was observed that following the initial Smac mimetic induced degradation of cIAP1 and cIAP2 that total protein levels of cIAP2, but

not cIAP1, somewhat rebounded over time (approx 5 hrs following treatment). Furthermore, it is known that cIAP2 is regulated by NF-kB and that TNF treatment strongly up-regulates cIAP2. A time course of Smac mimetic/ TNF treatment revealed that cIAP2 levels rebound within 3 hours of treatment and within 5 hours are much higher than initial levels. Interestingly, cIAP1 once it is degraded does not rebound and is not inducible by TNF. A critical feature of how cIAP2 is regulated is that cIAP1 is responsible for cIAP2 ubiquitination and its subsequent degradation. Hence, once cIAP1 is completely eliminated, even though there is still plenty of Smac mimetic available, cIAP2 is no longer subject to smac mimetic induced degradation.

These observations imply two things. First, the potential that cIAP2 is responsible for resistance by being up regulated by TNF and can perform the functional role of cIAP1, as well as its own, which is to recruit RIPK1 to TNFR1 for NF-κB activation. Second, that Smac mimetic is opposed by the very thing that it requires to work, namely, TNF signaling. If the cell is "too" responsive to TNF then the cell is able to overcome the cell death promoting effects of smac mimetic.

To determine whether cIAP2 is truly important for resistance two approaches were taken, siRNA and chemical inhibition of NF-kB signaling. Using siRNA against cIAP2, cells became sensitive to smac mimetic/ TNF treatment and cIAP2 levels remained suppressed following treatment. Chemical inhibitors of NF-kB signaling are somewhat more difficult to use because of the inherent toxicity of both suppressing NF-kB and using TNF. Typically, cell death is induced by the use of TNF and cycloheximide (CHX), a translational inhibitor. The effect of CHX is to suppress the production of anti-apoptotic genes, thus allowing TNF to signal death as opposed to survival. Inhibitors of

NF-kB essentially do the same thing but in a less global manner, specifically inhibiting the regulation of various NF-kB responsive anti-apoptotic genes, in particular c-FLIP and the IAPs. The key is to find a dose that suppresses the quick up-regulation of cIAP2 without disrupting the longer-term regulation of other anti-apoptotic genes such as c-FLIP.

Using an IKK2 specific inhibitor, BMS-345541, resistant cells were made sensitive at fairly low doses, such that sensitivity was restricted to conditions of Smac mimetic/ TNF treatment only. Low dose inhibitor treatment had the effect of suppressing up-regulation of cIAP2. Treatment with TNF alone had no effect and did not affect levels of c-FLIP. Indeed, it is possible to use very low doses of CHX and Actinomycin D, a transcriptional inhibitor, which have no effects with TNF alone but do induce cell death with the addition of smac mimetic. This further indicates that accounting for the quick up-regulation of cIAP2 is critical to promote sensitivity.

Looking more closely at how resistant cells form the death inducing RIPK1-caspase-8-FADD complex there is an initial low-level recruitment of RIPK1, but not FADD, to caspase-8 due to the initial degradation of cIAP1 and cIAP2, which is quickly stopped once enough cIAP2 returns to block further RIPK1 recruitment. When using either siRNA against cAIP2, or BMS-345541, formation of the complex was greatly enhanced with strong recruitment of both RIPK1 and FADD to caspase-8 within 3 hours and maximizing at 5 hours, the point when these cells begin to undergo apoptosis.

RIPK1-FADD-caspase 8 complex formation mirrors events at TNFR1. Upon TNF stimulation cIAP1, cIAP2 and RIPK1 are recruited to the receptor. The addition of smac mimetic causes degradation of cIAP1 and cIAP2 and release of RIPK1 from the receptor.

Within a few hours cIAP2 returns to the receptor at higher than basal levels and assumes the role of cIAP1, as well as its own, preventing further release of RIPK1 from the receptor. When IKK inhibitors are used, neither cIAP1 nor cIAP2 are present, allowing for sustained RIPK1 recruitment to the receptor followed by enhanced formation of the death-inducing complex.

IKK inhibitors as therapeutic agents have not had success due to cytotoxic side effects regarding overblown inflammatory responses. However, it is conceivable that some of these agents might be effective at sub-threshold levels in conjunction with Smac mimetic, since the desired effect is the acute response of cIAP2 to NFκB stimulation following Smac mimetic treatment and not the need to permanently shut down NF-kB signaling.

There are, however, many pathways that activate NF-kB and considerable cross-talk between different pathways and it might be possible to bypass the need to directly inhibit NF-kB by targeting parallel or convergent pathways, such as PI3K. Testing a variety of inhibitors for synergy with Smac mimetic and TNF, it was observed that inhibition of PI3K had robust synergism, while having no effect at all alone or with TNF. Curiously, the mode of action of various PI3K inhibitors, in particular LY294002, in this cell line was identical to that seen when using IKK inhibitors. Specifically, they had strong suppressive effects on cIAP2 up-regulation following smac mimetic/ TNF treatment and allowed for enhanced RIPK1-FADD-caspase 8 complex formation. Exactly why PI3K inhibitors have the same effect is curious because the PI3K inhibitors do not seem to effect overall NF-kB activation in response to TNF, only cIAP2 is affected. This cell line does show constitutive AKT phosphorylation, which is completely blocked by

PI3K inhibitors. cIAP2 can be regulated by mechanisms other than NF-κB and it appears that in this cell line the AKT pathway is responsible for basal cIAP2 protein production and that AKT is responsible for restoring cIAP2 levels back to baseline following treatment with smac mimetic alone and can also limit how effectively TNF up-regulates cIAP2 as well.

To see if any therapeutically viable drugs that target the PI3K pathway might synergize with smac mimetic/ TNF, Tarceva, an EGFR kinase inhibitor, and PD153035 (data not shown), also an EGFR specific kinase inhibitor, were used and had identical result to more general PI3K inhibitors. Interestingly, the cell line used was completely resistant to Tarceva alone, which offers the possibility of expanding the use of Tarceva clinically. This also shows proof of principle that combinatorial treatment that target alternate signaling pathways might synergize with Smac mimetic in unexpected ways and show the possibility of expanding the use of Smac mimetic as well as more established therapeutically viable drugs.

Finally, other cell lines that do not express or up-regulate cIAP2 are also resistant and Smac mimetic seem unlikely to work in these cell lines. Importantly, however, is the fact that these cell lines are particularly sensitive to TRAIL and that lack of both RIPK1 and cIAP2 correlate with this sensitivity and might be used as a biomarker for TRAIL response (unpublished observation), whereas cell lines that express cIAP2, and relatively more RIPK1, are less sensitive to TRAIL. It is probable that the issue has more to do with cIAP2 rather than RIPK1. cIAP2 seems to be required for efficient RIPK1 recruitment, since over expression of RIPK1 does not promote more efficient RIPK1-caspase-8-FADD complex formation.

3.9 Methods

Reagents

Smac mimetic was synthesized as previously described (Li et al. 2004). The compound was diluted to 100uM stocks. Lung cancer cell lines were obtained from the laboratory of Dr John Minna (UT Southwestern). Antibodies used are: Caspase-8 (Cell Signaling, 9746), caspase-8 (Santa Cruz, sc-6136) TNFR1 (Abcam, ab19139), TNFR1 (Santa Cruz, sc-7895), FADD (Santa Cruz, sc-56093), RIPK1 (BD Pharmingen, 551041), cIAP2 (BD Pharmingen, 552782), cIAP1 (R and D, AF8181), pan IAP (R and D, MAB3400), c-FLIP (Alexis, ALX-804-428), NEMO (Santa Cruz, sc-8256), phospho AKT (ser473) (Cell Signaling, 4058), AKT (Cell Signaling, 9272), phospho I-kB (Cell Signaling, 9246), I-kB (Cell Signaling, 9242). Chemicals used are: DMSO (Sigma, D8418), BMS-345541 (Sigma, B9939), LY294002 (Sigma, L9908), erlotinib (a gift from UT Southwestern High-throughput Screening Core), CHX (Calbiochem, 239765).

Cell Culture

Cell lines HCC1299, H460 and H2009, were cultured in HyQ RPMI-1640 medium (Hyclone) supplemented with 5% fetal bovine serum (FBS, Hyclone) and 100-units/ml penicillin/streptomycin (Gibco).

Cell Survival Assay

Cells were plated onto 96 well assay plates (white with clear bottom (3610), Corning Costar) at different cell densities, depending on cell type, in 100μl media per well. Cells

were allowed to grow to near confluence and treated with smac mimetic or vehicle (H_20) by adding 100μl media with compound 3, diluted to 2X the desired final concentration, to each well. Cells were incubated overnight and assayed the following day utilizing the Cell Titer-glo Luminescent Cell Viability Assay (Promega. Cells were allowed to equilibrate to room temperature at which time 25μl of a 1:1 mixture of Cell Titer-glo reagent and 1% triton X-100 PBS was added. Cells were placed on rocking shaker for five minutes and incubated for an additional 5 minutes on the bench top. Luminescent measurements were done on a Tecan SPECTRAFluor Plus 96 well plate reader. For assays measuring toxicity effects, all values were normalized to the mock treated or mock transfected conditions to account for variability in the cytotoxicity of transfecting siRNA into cells and for possible cytotoxic effects that knockdown of the particular gene used might have. All values are represented graphically as mean ± SD for 3 independent samples.

Western blot analysis of time course treatments and of siRNA knockdown efficiency

Cells were plated onto 6 well cell culture dishes (Corning Costar) at differing cell densities, depending on the application, in 2ml media. For all time courses, cells were plated to near confluence and allowed to attach overnight. Cells were then treated as indicated in. Cells were treated to a final concentration of 100nM smac mimetic or 50ng/ml TNF at each time point. For cell treated with z vad fmk (Sigma), BMS-34554 (Sigma), Erlotinib, CHX (Sigma) or DMSO compounds were added 1 hour prior to smac mimetic or TNF treatment. For siRNA knockdown efficiency, H1299 cells were plated in 2ml antibiotic-free media at a density of 2 x 10^4 cells per well and allowed to attach

overnight. siRNAs were then transfected as described below. Cells were lysed in lysis buffer (20mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Triton X100, 0.5mM DDT and Complete protease inhibitor (Roche)) and were then incubated on ice for 20 minutes and spun down at 10,000 RPM for 10 minutes. The soluble fraction was kept and protein concentration determined by Bradford assay. Protein concentrations were normalized to 50μg total and SDS-PAGE was done followed by western blotting of target antibody.

siRNA Transfection

siRNA transfections were done in both 6 well and 96 well dish formats. For 6 well dishes, the day prior to transfection cells were plated at a density of 2 x 10^4 cells per well in antibiotic-free media. The next day, Lipofectamine RNAiMax was used to transfect cell, as per manufactures protocol. Briefly, 3μl Lipofectamine RNAiMax was combined with 120 pmol (6μl of a 20μM stock) siRNA in a volume of 250μl Opti-mem media (Gibco) and incubated for 20 minutes, the complexes of Lipofectamine RNAiMax and siRNAs were then added directly to each well and the cells were incubated until nearly confluent, approximately 48-72 hours later depending on growth conditions. For 96 well dishes, the day prior to transfection cells were plated at a density of 1 x 10^3 cells per well in antibiotic-free media. Lipofectamine RNAiMax was used, as above, by mixing 0.3μl Lipofectamine 2000 and 12pmol (0.6μl of a 20μM stock) siRNA in a total volume of 20μl. All siRNAs were purchased from Dharmacon. For RIPK1, cIAP1, cIAP2 and XIAP oligos have been previously validated and those oligos were used here (Petersen et al. 2007, Wang et al. 2008). Target sequences for: cIAP1 (5-UUCGUACAUUUCUCUCUUA-3), cIAP2 (5-AAUGDAGAGUCAUCAAUUA-3),

XIAP (5-CCAGAAUGGUCAGUAACAAA-3), RIPK1 (5—3). For NEMO, a set of four oligos were purchased from Dharmacon and tested. Target sequences for: Oligo#1 (5-AACAGGAGGUGAUCGAUAAUU-3), Oligo#2 (5-GAAGCGGCAUGUCGAGGUCUU-3), Oligo#3 (5-GAAUGCAGCUGGAAGAUCUUU-3), Oligo#4 (5-GGAAGAGCCAACUGUGUGAUU-).

Caspase-8 and TNFR1 antibody immunoprecipitation

Cells were grown on 15cm plates, treated as indicated, and harvested in 5X volume lysis buffer (as previously described). Cells were left on ice for 20 minutes and centrifuged at 20000-x g for 20 minutes. 20µl (bead volume) protein G agarose beads (GE Healthcare), 2µg of caspase 8 antibody (Santa Cruz, SC-6136) or 3µg TNFR1 antibody (Santa Cruz, sc-7895) were mixed with 2mg of cell lysates (2mg/mL) and incubated over night at 4C^{o}. The following day, beads were washed 4X with 500mM NaCl lysis buffer and protein was eluted off the beads using 2X SDS loading buffer and boiled for 5 minutes. Samples were then analyzed by SDS-PAGE followed by Western blot.

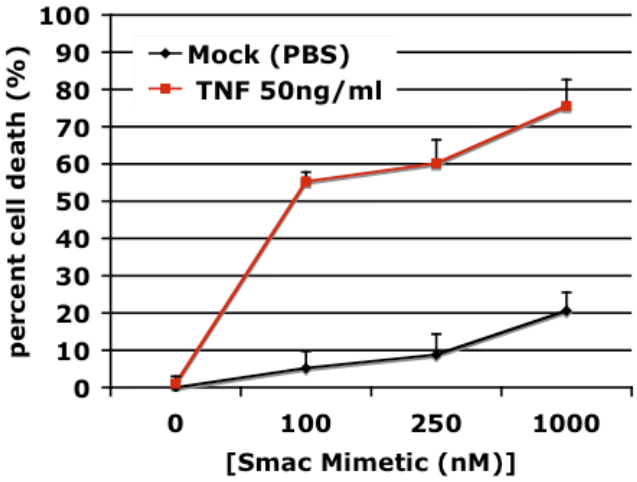

Figure 3-1. Dose response of H2009 cells to increasing concentrations of smac mimetic with and without 50ng/ml TNF.
Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

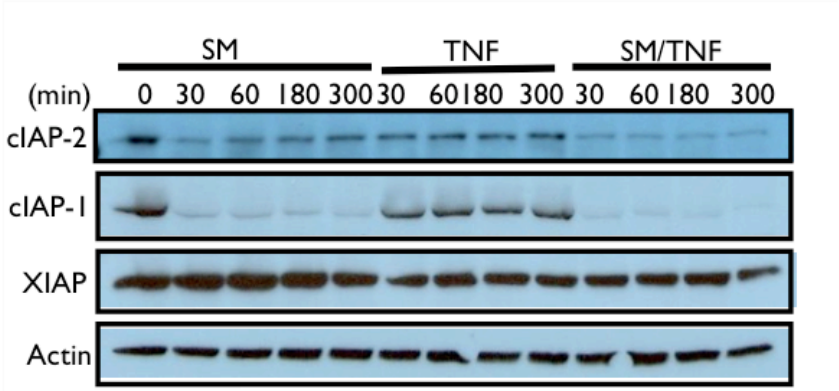

Figure 3-2. Degradation of cIAP1 and cIAP2 in response to smac mimetic in H2009. Western blot analysis of cIAP1, cIAP2 and XIAP during a time course of response of H2009 to smac mimetic, TNF and smac mimetic/TNF from 0 to 300 minutes.

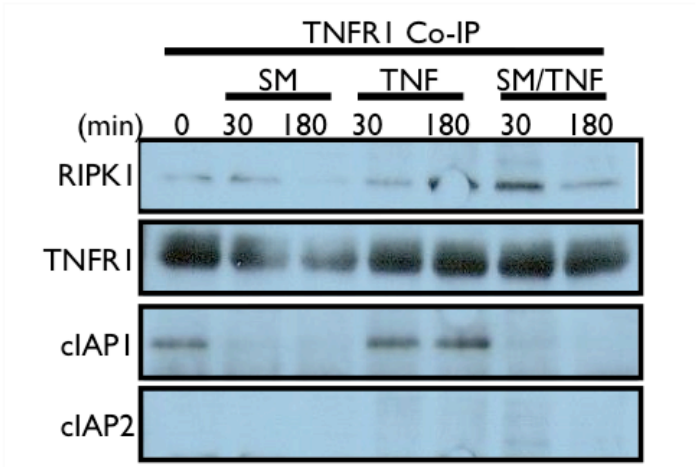

Figure 3-3. RIPK1 and cIAP1 are recruited to TNFR1 by TNF treatment, but RIPK1 is released in the presence of smac mimetic.
TNFR1 co-immunoprecipitation of H2009 cells treated with 100nMsmac mimetic (SM), 50ng/ml TNF or both (SM/TNF), interactions of RIPK1, cIAP1 and cIAP2 interacting with TNFR1 analyzed by western blot.

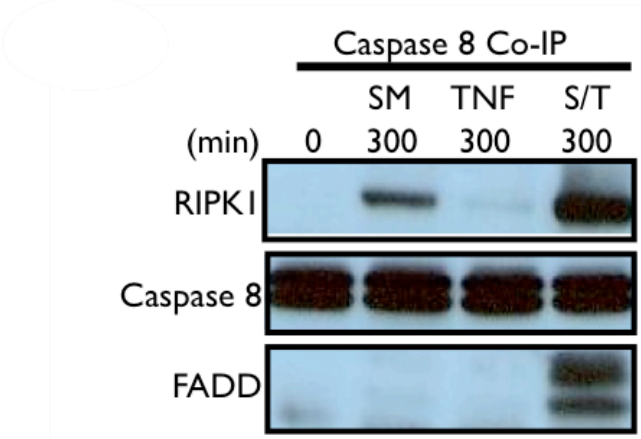

Figure 3-4. Formation of the RIPK1-FADD-caspase-8 complex by smac mimetic and TNF treatment.
Caspase-8 co-immunoprecipitation of H2009 as in 4-3, showing formation of RIPK1-caspase-8-FADD death inducing complex formation in response to 100nM smac mimetic and 50ng/ml TNF.

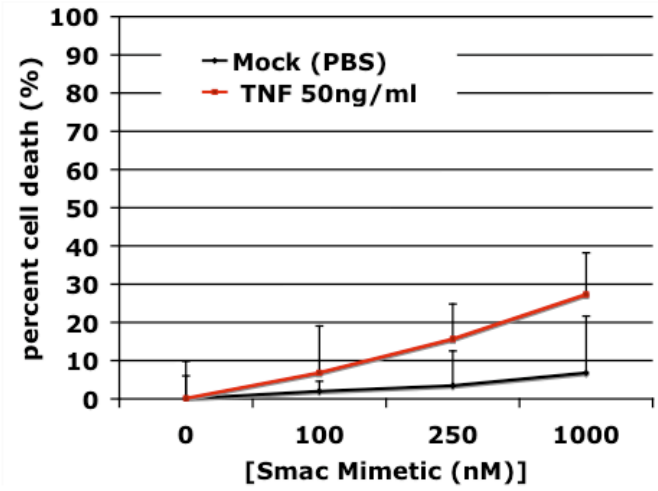

Figure 3-5. Dose response of H1299 cells to increasing concentrations of smac mimetic with and without 50ng/ml TNF.
Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

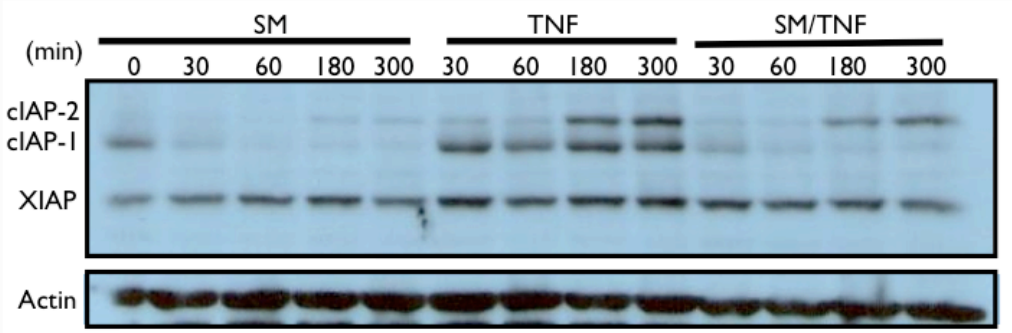

Figure 3-6. Degradation of cIAP1 and cIAP2 in response to smac mimetic in H2009, cIAP2 is up regulated by TNF.
Western blot analysis of cIAP1, cIAP2 and XIAP during a time course of response of H2009 to smac mimetic, TNF and smac mimetic/TNF from 0 to 300 minutes.

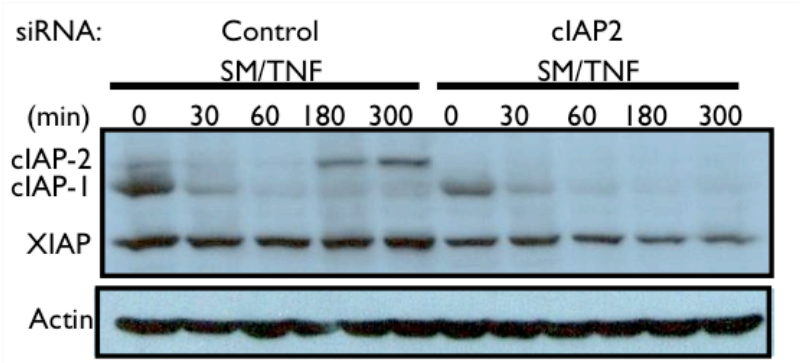

Figure 3-7. siRNA against cIAP2 can block TNF induce up regulation of cIAP2. Comparison of cIAP2 degradation in response to smac mimetic/TNF (SM/TNF) between mock transfected versus cIAP2 siRNA transfected cells.

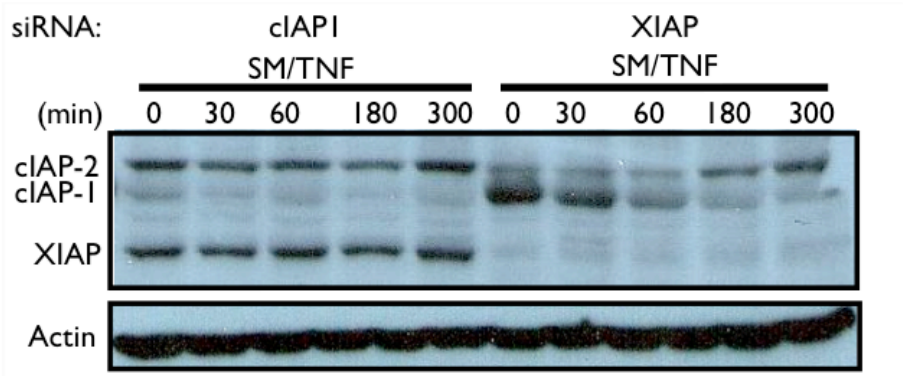

Figure 3-8. siRNA against cIAP1 prevents the initial degradation of cIAP2 by smac mimetic treatment.

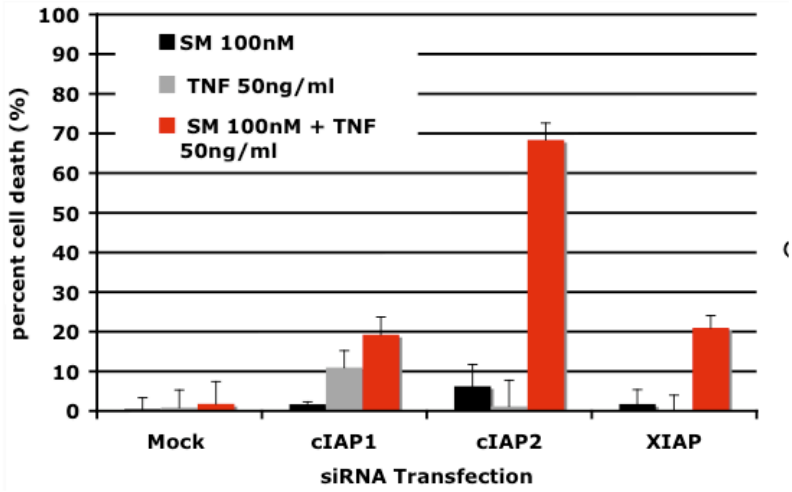

Figure 3-9. siRNA knockdown of cIAP2 sensitizes resistant cell line H1299 to smac mimetic/TNF.
Cell death of cIAP1, cIAP2 and XIAP siRNA cells in response to 100nM smac mimetic (SM), 50ng/ml TNF or both. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

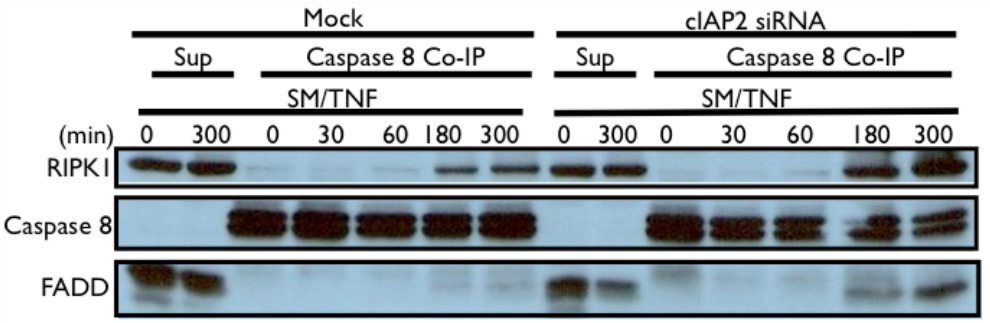

Figure 3-10. Formation of the RIPK1-FADD-caspase-8 complex is enhanced under cIAP2 siRNA knockdown conditions.
Formation of the RIPK1-caspase-8-FADD complex as shown by caspases-8 co-immunoprecipitation in cIAP2 siRNA cells in response to smac mimetic/TNF as compared to mock-transfected cells.

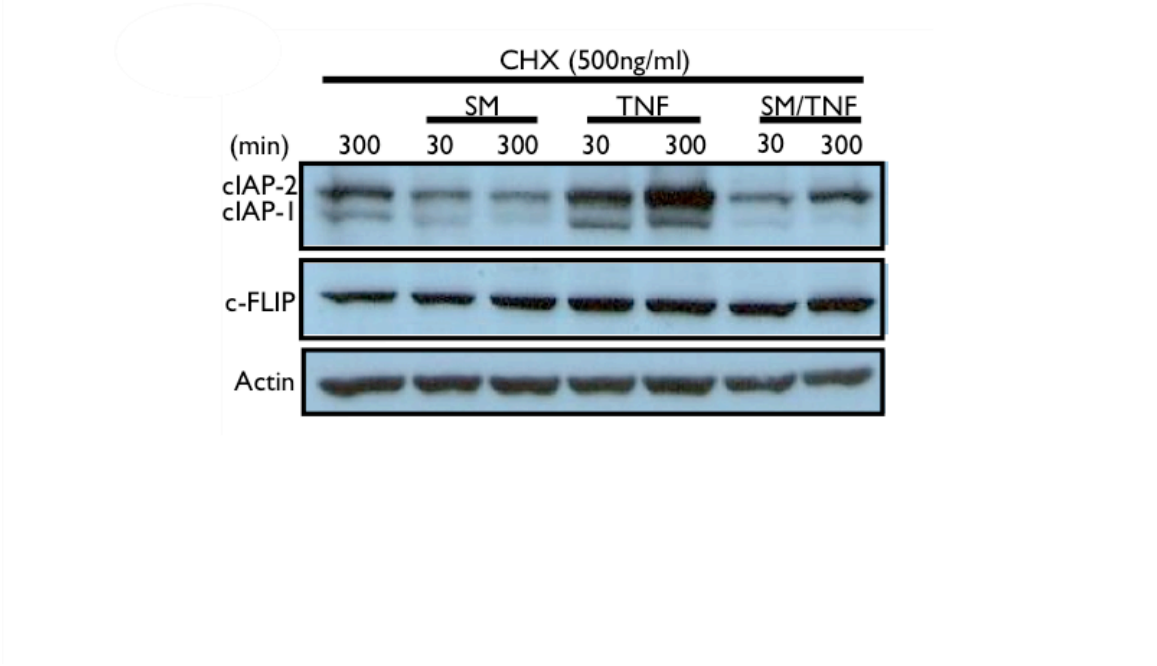

Figure 3-11. Low dose CHX (500ng/ml) attenuates cIAP2 up regulation by TNF
Low dose 500ng/ml cycloheximide (CHX) effect how cIAP2 and c-FLIP respond to 100nM smac mimetic, 50ng/ml TNF or both.

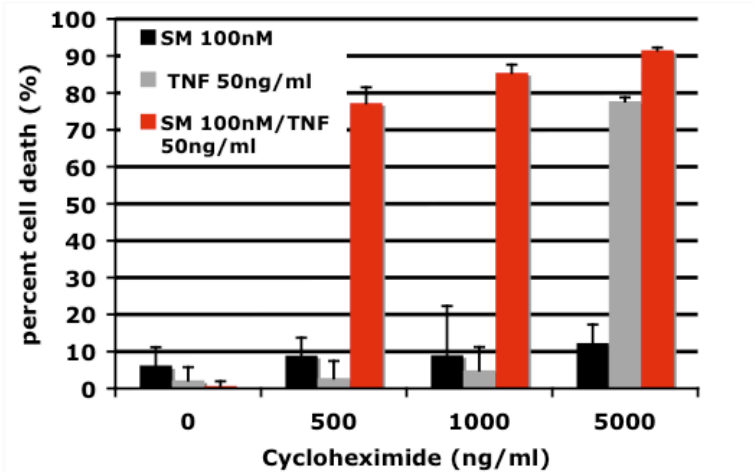

Figure 3-12. Low dose CHX (500ng/ml) sensitizes resistant cells to smac mimetic/TNF, but not to TNF alone.
Cell death under increasing concentration of CHX. Cells were pre-treated with CHX for 1 hour and then treated overnight with 100nM smac mimetic (SM), 50ng/ml TNF or both.

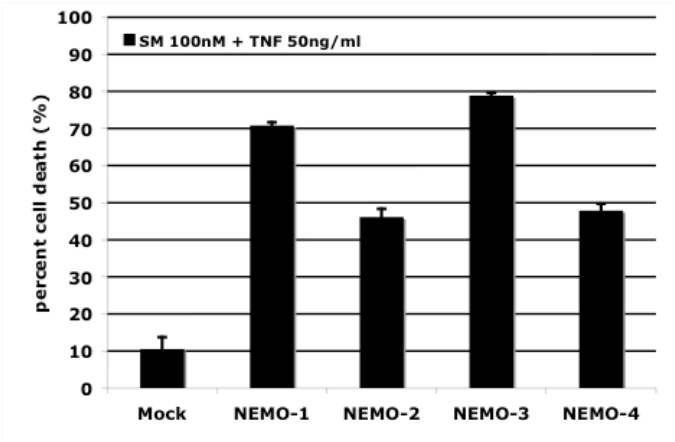

Figure 3-13. siRNA knockdown of NEMO sensitizes resistant cells.
Testing of 4 individual NEMO siRNA oligos for the ability to sensitize resistant cells to 100nM smac mimetic and TNF. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

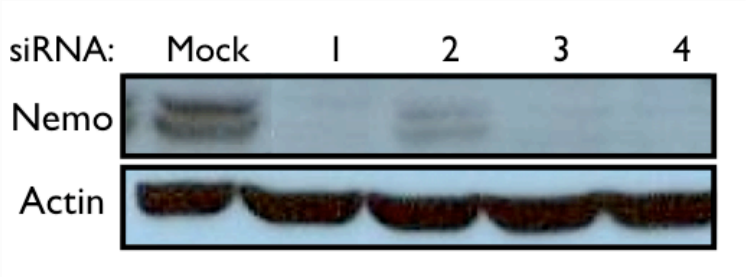

Figure 3-14. Western blot analysis of efficiency of NEMO siRNA knockdown for each individual siRNAs.

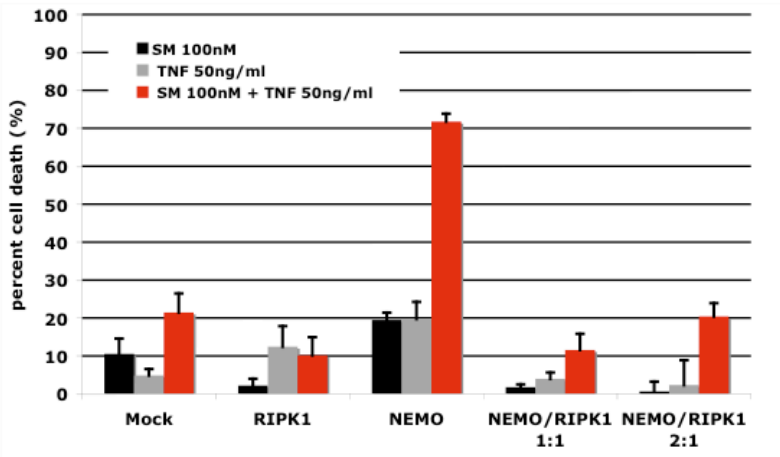

Figure 3-15. Cell death facilitated by NEMO knockdown is RIPK1 dependent. Determination of RIPK1 dependence in NEMO knockdown induced sensitivity. Cells were co-transfected with both NEMO and RIPK1 at both a 1:1 and 2:1 ratio. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

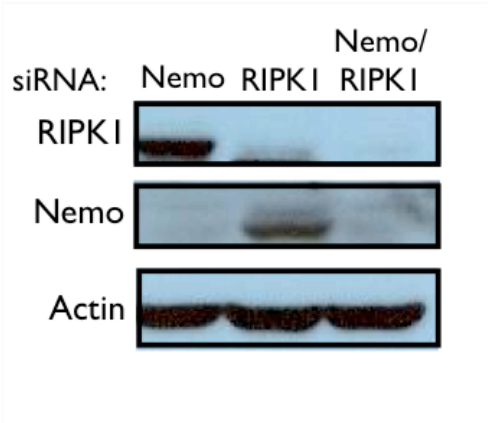

Figure 3-16. Western blot analysis of efficiency of NEMO and RIPK1 double siRNA knockdown.

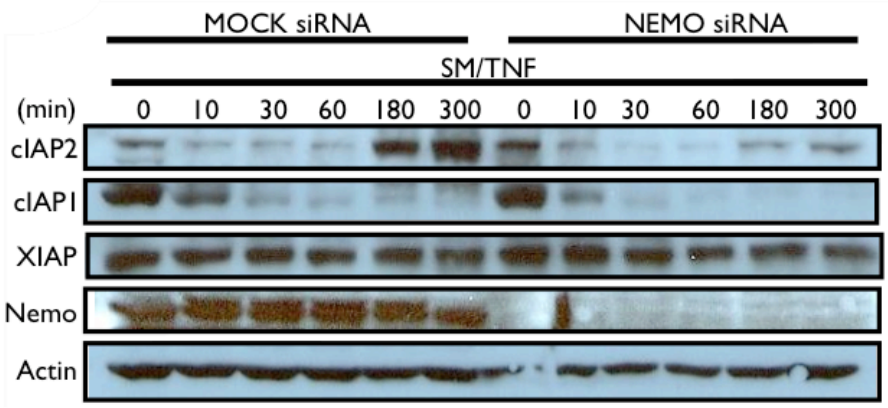

Figure 3-17. NEMO knockdown cells show deficiency in up regulating cIAP2 in response to TNF.
Time course comparison of cIAP2 protein levels in mock transfected and NEMO siRNA transfected cells in response to 100nM smac mimetic and 50ng/ml TNF (SM/TNF).

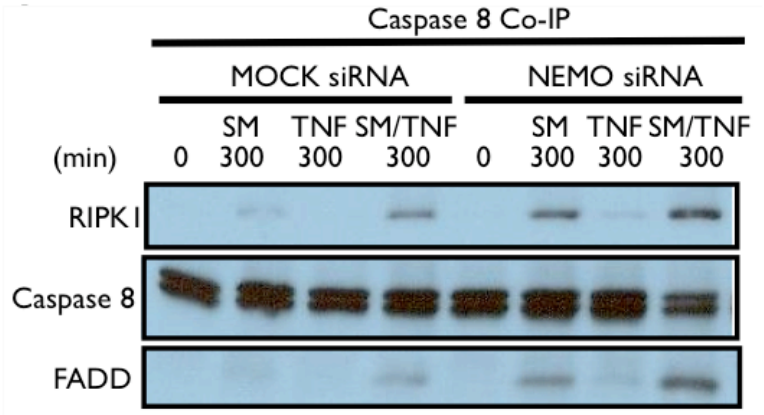

Figure 3-18. Formation of the RIPK1-FADD-caspase-8 complex is facilitated in NEMO knockdown cells as compared to wt cells.
Western blot analysis of caspases-8 co-immunoprecipitation comparing formation of RIPK1-caspase-8-FADD complex in mock transfected and NEMO siRNA transfected cells after 300 minutes of 100nM smac mimetic, 50ng/ml TNF or both.

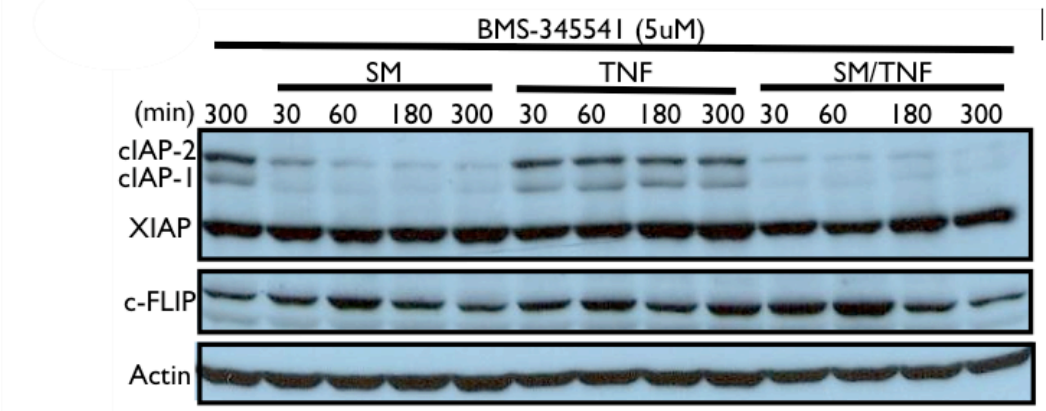

Figure 3-19. Inhibition of NF-kB using BMS-345541 prevents TNF induced up regulation of cIAP2.
Time course of response of cells pre-treated with 5uM BMS-345541. Cells were then treated with 100nM smac mimetic (SM), 50ng/ml TNF or both (SM/TNF) and cIAP2 levels were analyzed by western blot.

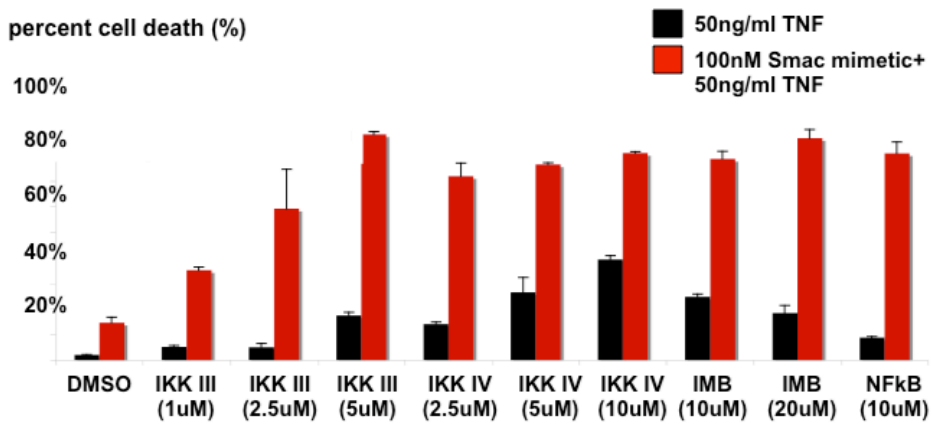

Figure 3-20. Screen of a variety of NF-kB inhibitors on their ability to synergize with smac mimetic/TNF.

Dose response of resistant cells pre-treated with various NF-kB inhibitors for 1 hour. Cells were then treated with 100nM smac mimetic, 50ng/ml TNF or both overnight. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

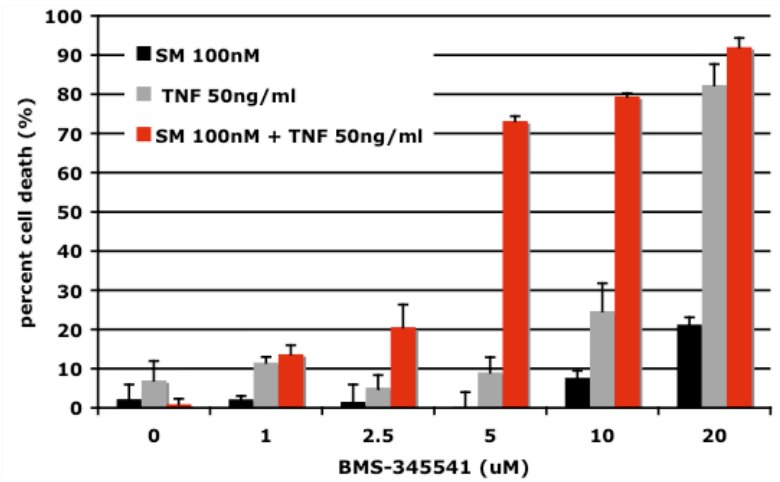

Figure 3-21. BMS-345541 synergizes with smac mimetic/TNF to cause cell death.
Dose response of resistant cells pre-treated with BMS-345541 (1uM to 20uM) for 1 hour. Cells were then treated with 100nM smac mimetic, 50ng/ml TNF or both overnight. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

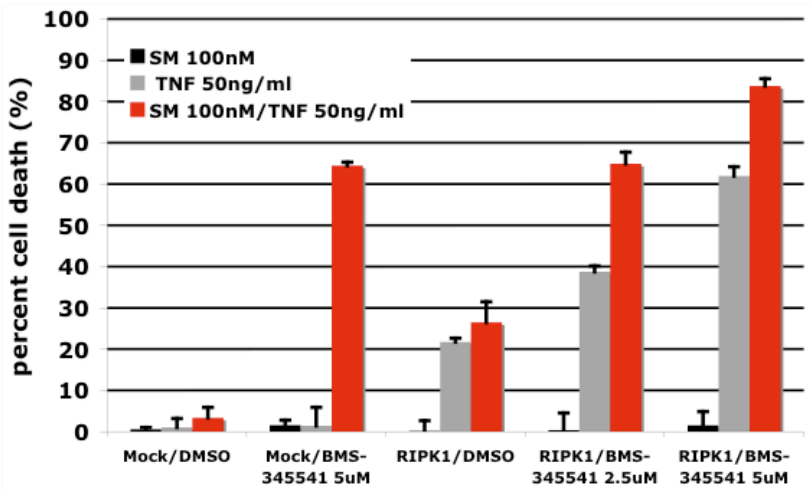

Figure 3-22. BMS-345541 treatment in RIPK1 knockdown cells causes greater sensitivity to TNF by completely shutting down NF-kB signaling.
Evaluation of RIPK1 dependence of BMS-345541 induced sensitivity. RIPK1 Knockdown cells were pre-treated with varying concentrations of BMS-345541 followed by treatment with 100nM smac mimetic, 50ng/ml TNF or both. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

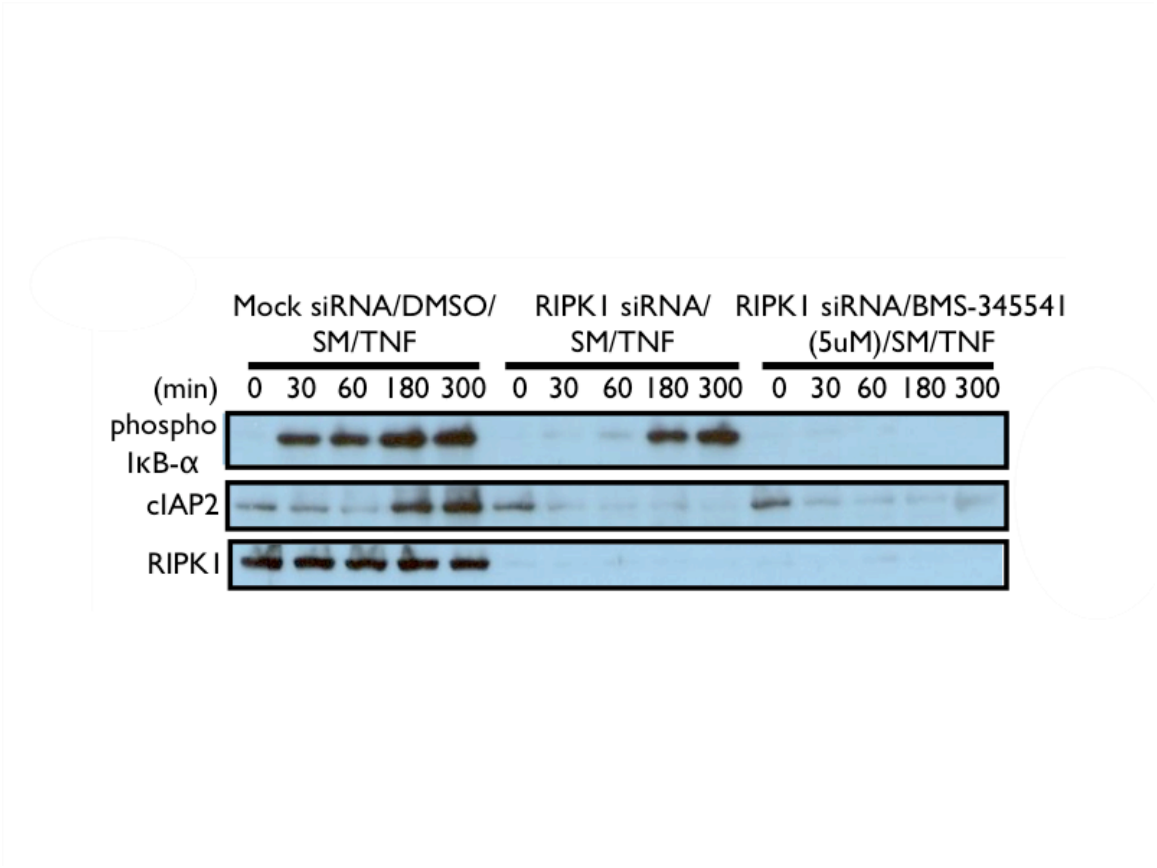

Figure 3-23. BMS-345541 treatment in RIPK1 knockdown cells completely block I-kB alpha phosphorylation and blocks cIAP2 up regulation in response to TNF.
Western blot analysis of phosphorylation of Iκ-B-α in RIPK1 knockdown cells with and without pre-treatment with 5uM BMS-345541 followed by overnight treatment with 100nM smac mimetic, 50ng/ml TNF or both.

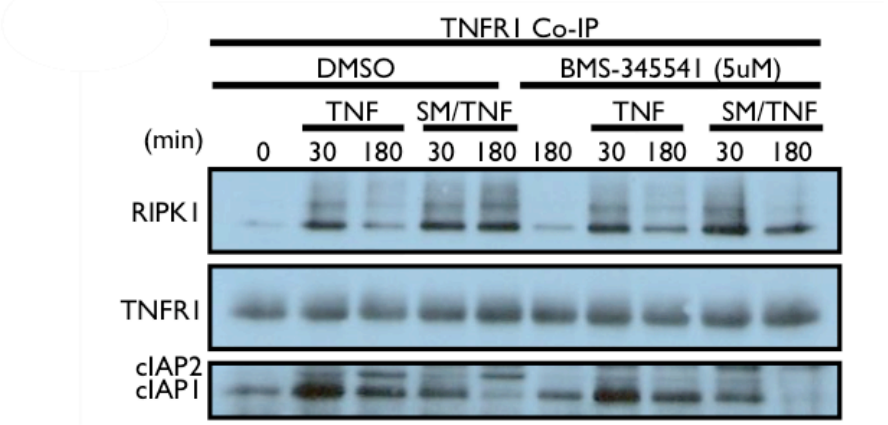

Figure 3-24. BMS-345541 treatment facilitates release of RIPK1 from TNFR1 by suppressing cIAP2.
TNFR1 co-immunoprecipitation comparing DMSO and BMS-345541 pre-treated cells followed by treatment with 100nM smac mimetic (SM), 50ng/ml TNF or both for the times indicated. Elutes were analyzed by western blot for recruitment of RIPK1, cIAP1 and cIAP2 to the receptor.

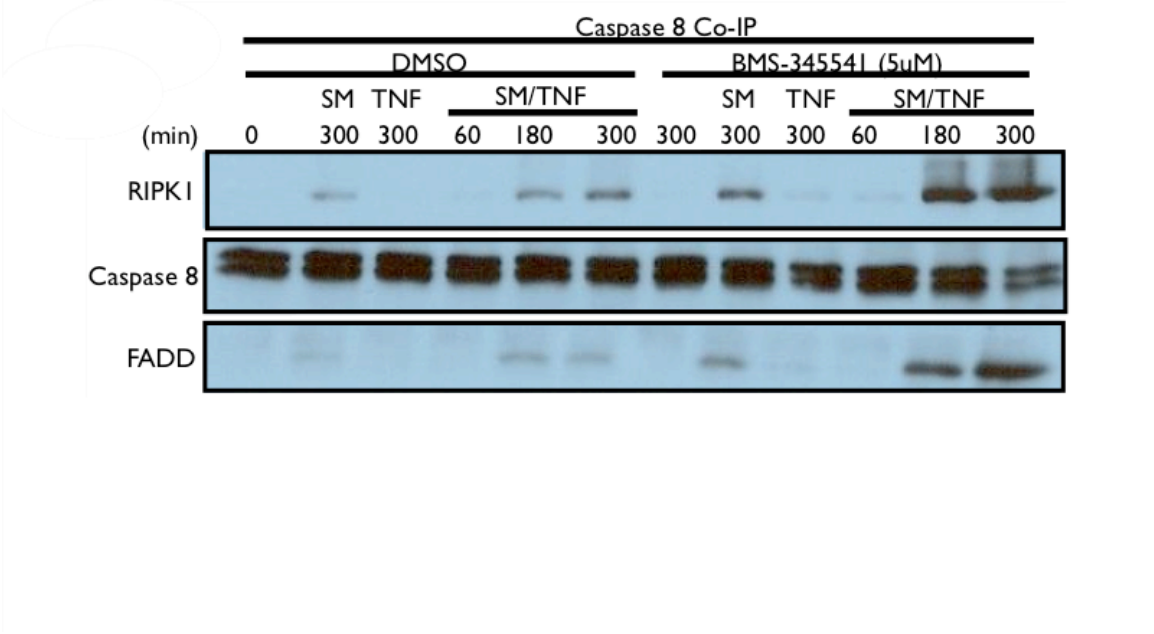

Figure 3-25. BMS-345541 treatment promotes robust formation of the RIPK1-FADD-caspase-8 complex.
Caspase-8 co-immunoprecipitation comparing DMSO and BMS-345541 pre-treated cells followed by treatment with 100nM smac mimetic (SM), 50ng/ml TNF or both for the times indicated. Elutes were analyzed by western blot for formation of the RIPK1-caspase-8-FADD complex.

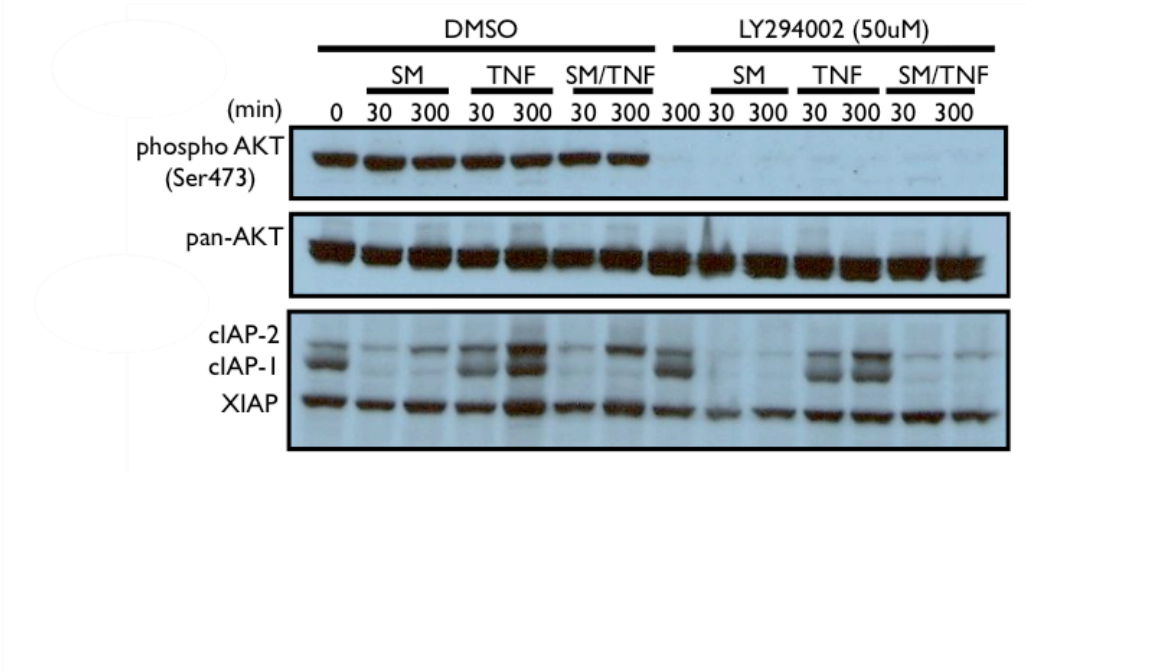

Figure 3-26. Treatment of resistant cells with LY294002 blocks constitutive AKT phosphorylation and prevents cIAP2 upregulation.
Effects of inhibition of AKT. Cells were pre-treated with 50uM LY294002 or DMSO for 1hour and then treated with 100nM smac mimetic, 50ng/ml TNF or both for the indicated times and then analyzed by western blot for inhibition of AKT phosphorylation. The same cells were also analyzed by western blot for cIAP2 response under conditions of AKT inhibition.

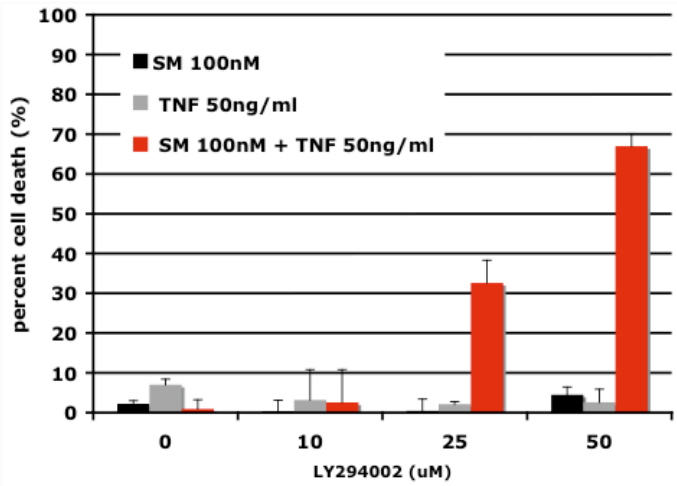

Figure 3-27. Treatment with LY294002 sensitized resistant cells to smac mimetic/TNF.
Dose dependent response of resistant cells to pre-treatment with increasing concentrations of LY294002 followed by overnight treatment with 100nM smac mimetic, 50ng/ml TNF or both. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

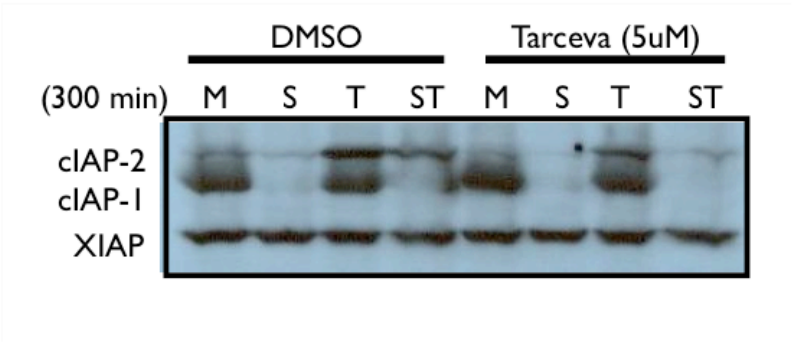

Figure 3-28. Treatment with erlotinib (Tarceva) can block the smac mimetic/TNF induced up regulation of cIAP2.
Response of resistant cells to pre-treatment with 5uM Tarceva (erlotinib) followed by 100nM smac mimetic (S), 50ng/ml TNF (T) or both (ST) as indicated. Effects on cIAP2 were analyzed by western blot. (E) Dose dependent response of resistant cells to pre-treatment with increasing concentrations of Tarceva followed by overnight treatment with 100nM smac mimetic, 50ng/ml TNF or both. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

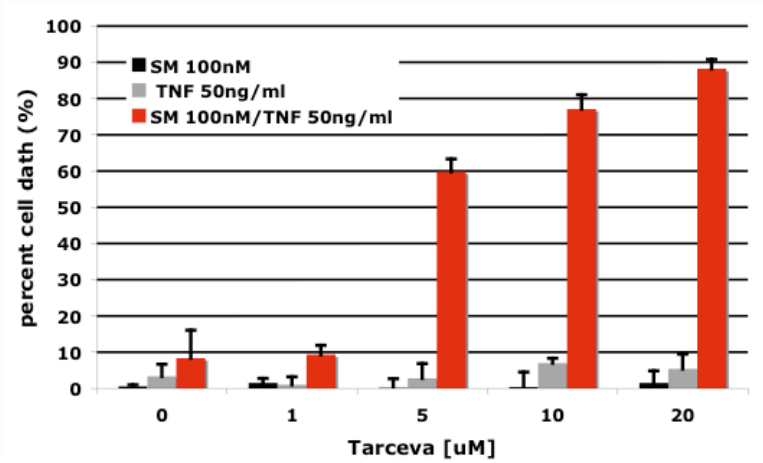

Figure 3-29. Treatment with erlotinib (Tarceva) sensitizes resistant cells to smac mimetic/TNF.
Dose dependent response of resistant cells to pre-treatment with increasing concentrations of erlotinib followed by overnight treatment with 100nM smac mimetic, 50ng/ml TNF or both. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

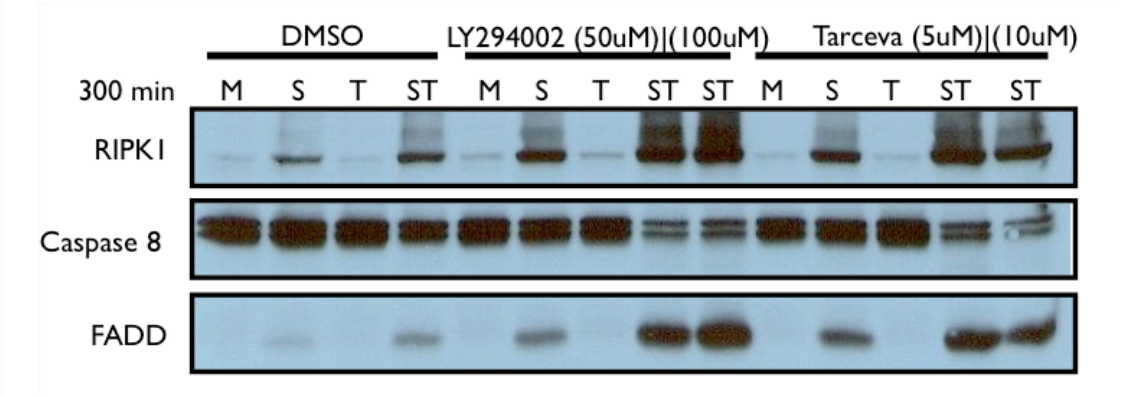

Figure 3-30. Treatment with both LY294002 and erlotinib (Tarceva) promote robust formation of the RIPK1-FADD-caspase-8 complex.
Caspase-8 co-immunoprecipitation comparing DMSO, 50uM LY294002 and 5uM Tarceva pre-treated cells followed by treatment with 100nM smac mimetic (SM), 50ng/ml TNF or both for the times indicated. Elutes were analyzed by western blot for formation of the RIPK1-caspase-8-FADD complex.

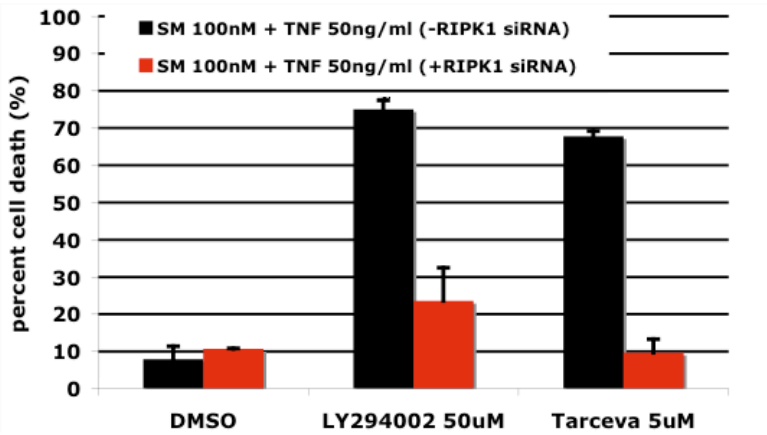

Figure 3-31. Sensitization by co-treatment with LY294002 and erlotinib (Tarceva) are RIPK1 dependent.
Evaluation of RIPK1 dependence of LY294002 and Tarceva induced sensitivity. RIPK1 knockdown cells were pre-treated with 50uM LY294002 and 5uM Tarceva followed by treatment with 100nM smac mimetic, 50ng/ml TNF or both. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

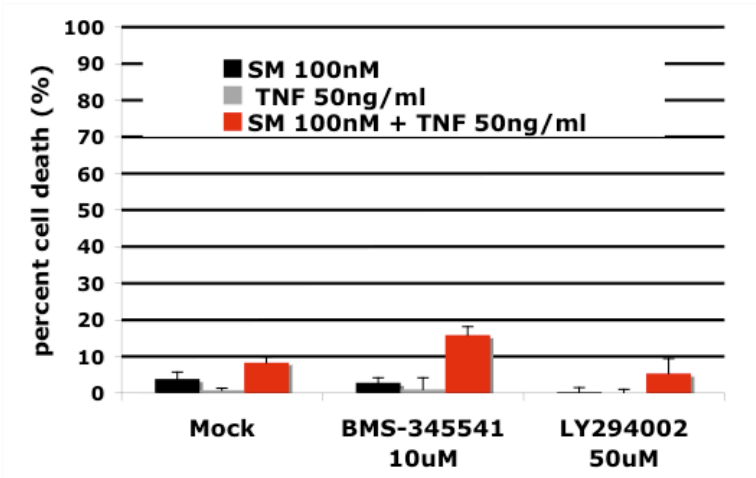

Figure 3-32. Cell line H460 is resistant to co treatments with BMS-345541 and LY294002 with smac mimetic/TNF.
Dose response of H460 cells pre-treated with 10uM BMS-345541 and 50uM LY294002. Cells were then treated with 100nM smac mimetic, 50ng/ml TNF or both. Percent cell death is based on cell titer glo (Promega) cell viability assay in 96 well plates. Graphical representations indicate the mean ± SD of at least 3 independent testing conditions.

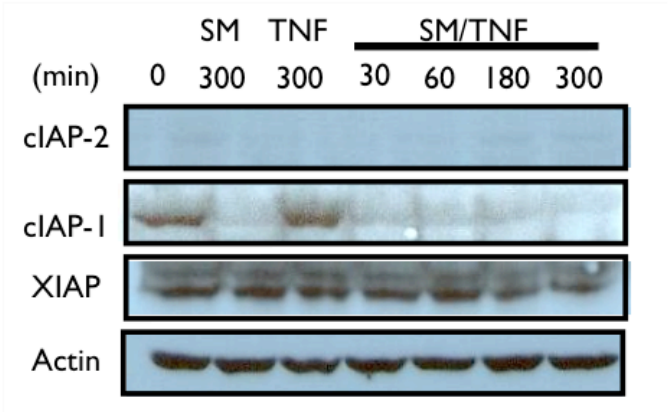

Figure 3-33. H460 cells do nor express cIAP2, but are still resistant.
Cells were treated with 100nM smac mimetic, 50ng/ml TNF or both for the indicated times and then analyzed by western blot for cIAP2 expression.

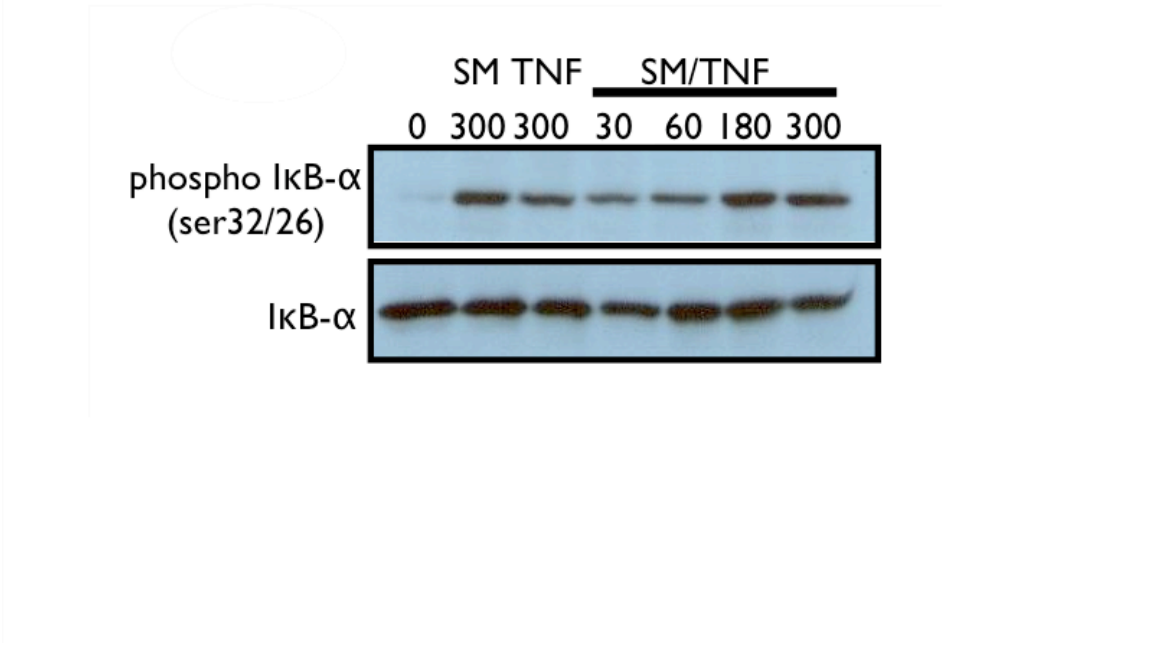

Figure 3-34. H460 cells are deficient at NF-kB activation in response to TNF. Cells were treated as in 4-32 and analyzed for I-kB response following treatment with smac mimetic and TNF.

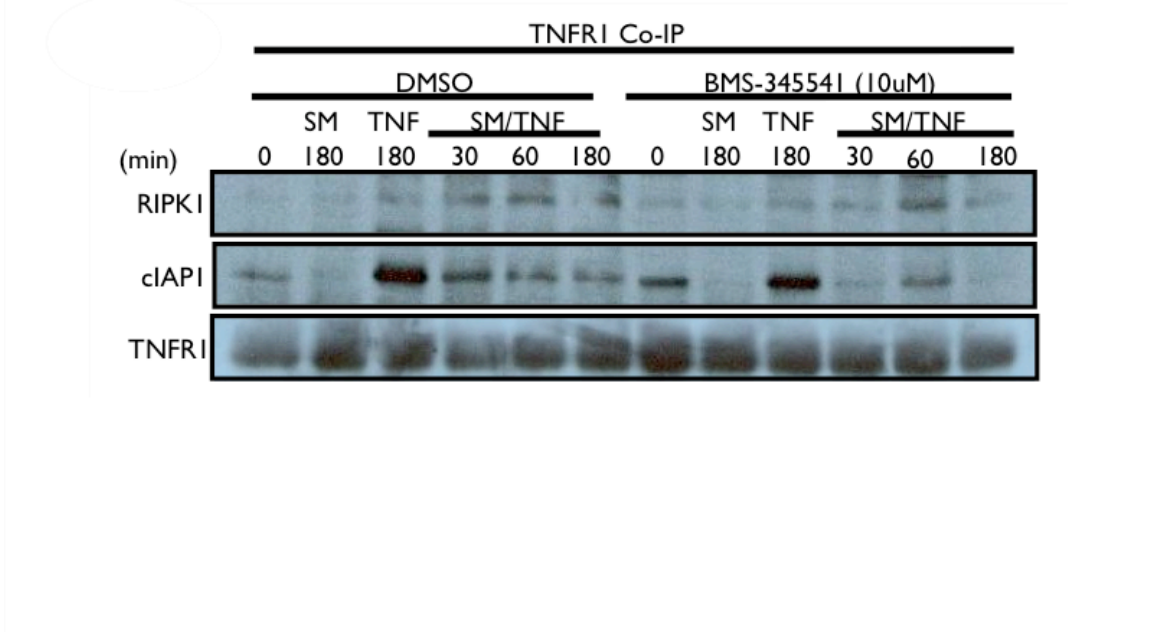

Figure 3-35. H460 cells do not efficiently recruit RIPK1 to TNFR1.
TNFR1 co-immunoprecipitation comparing DMSO and BMS-345541 pre-treated cells followed by treatment with 100nM smac mimetic (SM), 50ng/ml TNF or both for the times indicated. Elutes were analyzed by western blot for recruitment of RIPK1, cIAP1 and cIAP2 to the receptor.

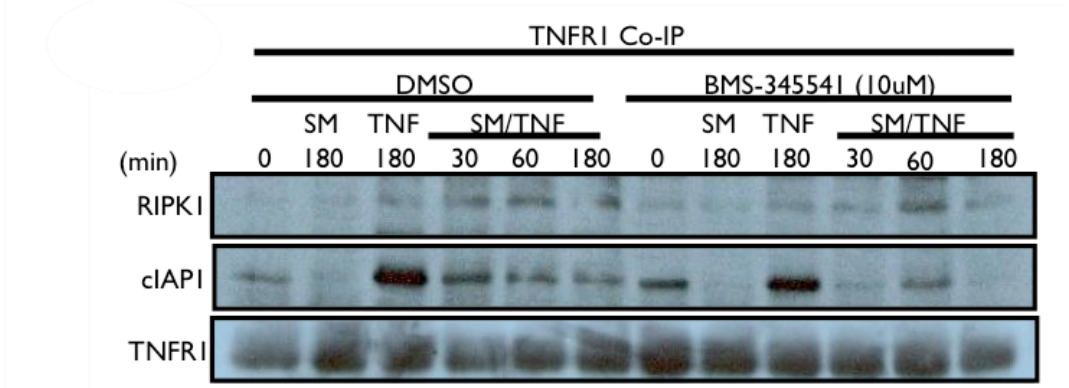

Figure 3-36. H460 do not form the RIPK1-FADD-caspase-8 complex, even in the presence of BMS-345541.
Caspase-8 co-immunoprecipitation comparing DMSO and BMS-345541 pre-treated cells followed by treatment with 100nM smac mimetic (SM), 50ng/ml TNF or both for the times indicated. Elutes were analyzed by western blot for formation of the RIPK1-caspase-8-FADD complex.

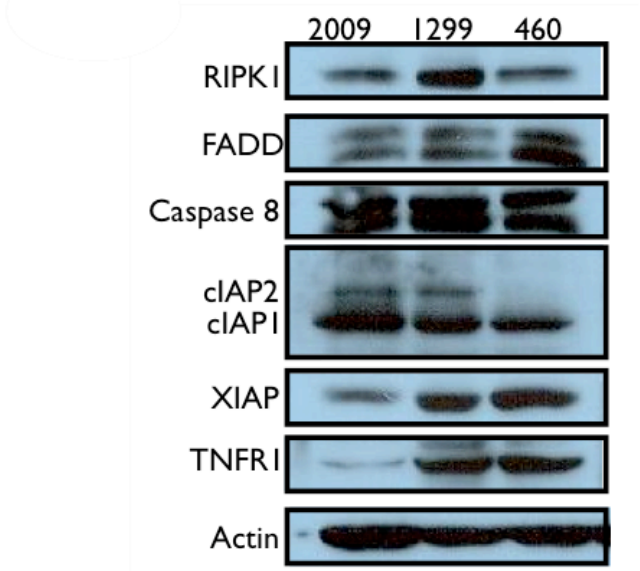

Figure 3-37. Comparison of expression of RIPK1, FADD, caspases-8, cIAP1, cIAP2 and XIAP between H2009, H1299 and H460.

CHAPTER FOUR: Unresolved issues and future directions

There are several outstanding questions that require further evaluation. Namely, what exactly are the roles of cIAP1 and cIAP2 in regulating caspase activation? They both have been shown to be able to bind, but not necessarily inhibit caspase-3, -7 and -9. Direct interaction with caspase-8 has not been demonstrated. However, they both contain a CARD domain that allows for interactions with other CARD containing proteins. No functional role has yet been described for the CARD domain of cIAP1 and cIAP2 and given the ability of cIAP2 to block Smac mimetic-induced apoptosis, it may well be that cIAP2 in addition to acting at the TNFR1 might also play a role in actively inhibiting caspase-8 activation.

As an initial attempt to determine whether cIAP1 or cIAP2 could interact with caspase-8, an endogenous caspase-8 co-immunoprecipitation was done looking for such an interaction. As can be seen in Figure 4-1, caspase-8 can pull down both cIAP1 and cIAP2. Interestingly, when smac mimetic is added, cIAP1 and cIAP2 are initially degraded and no interaction exists. Over time, however, as cIAP2 levels rebound even more cIAP2 is seen to be interacting with caspase-8, further indicating that cIAP2 is capable of replacing the activity of cIAP1. More interesting is that the return of cIAP2 and its interaction with caspase-8 correlates with sensitivity to smac mimetic and TNF (Figure 4-1).

However, only a very small fraction of total cIAP2 interacts with caspase-8 (Figure 4-2) and doesn't seem likely to be enough to be able completely inhibit all the

caspase-8 that could potentially be activated in response to smac mimetic/TNF. A more detailed stoichiometric analysis will need to be done in the future to see if that is truly the case and what exactly the molar ratios are between caspase-8 and the IAPs.

A further complication is that doing the reverse co-immunoprecipitation utilizing cIAP2 antibodies to pull down caspase-8 does not appear to work (Figure 4-3). It could be that there is too little actual cIAP2 present and that only a small fraction of the total is binding caspase-8. It may, therefore, require much more starting material to see the interaction, more than what was used initially(1mg total protein).

As a future attempt to solve this problem a tagged cIAP2 expression vector in cell line H460, which does not express cIAP2, but does express plenty of caspase-8, will be utilized. This will allow for affinity purification of caspase-8 and other interacting partners with cIAP2 to be identified. Furthermore, several key amino acid residues of cIAP2 have been identified as being critical for both ubiquitylation and degradation that could be mutated to see if they affect binding.

Finally, it was also observed that Smac mimetic causes the recruitment of c-FLIP (long) to caspase-8 and this does not happen under TNF only conditions (Figure 4-4). It does not appear that this recruitment necessarily blocks caspase-8 activation because it still occurs in BMS-345541 treated cells that robustly undergo apoptosis in response to Smac mimetic and TNF. It seems likely that c-FLIP recruitment might play some functional role in activating caspase-8 down stream of the formation of the RIPK1-FADD-caspase-8 death-inducing complex.

Another issue is the relationship between cIAP2 expression and RIPK1 expression and their roles in smac mimetic sensitivity. It has been observed that cells that

do not express cIAP2 have lower RIPK1 levels relative to those cells that express cIAP2 and that those cells are less responsive to TNF induced NF-kB activation. This suggests that cIAP2 is essential for activation of NF-kB or at least activation that results in up regulation of RIPK1. Of course, too much cIAP2, especially if it is induced by TNF or other signaling pathways results in resistance, but also results in elevated RIPK1 expression. Thus, there is a fine balance between cIAP2 expression and responsiveness to TNF. Those cells that express cIAP2, but which do not up-regulate it in response to TNF are those that are most sensitive to smac mimetic. The question therefore, is how can cIAP2 be induced at a low level in cells that does not endogenously express it that results in RIPK1 up regulation and can smac mimetic then be effective at killing those cells. A nice corollary to these observations and questions is that low RIPK1 and no cIAP2 expression seems to confer sensitivity to TRAIL induced apoptosis without need for Smac mimetic.

Finally, there is the issue of treating people/ animals with TNF. TNF as a therapeutic agent has seem only limited application, such as limb perfusion for the treatment of solid tumors in the extremities where it can be localized to a specific part of the body and not enter the general circulation. Given that smac mimetic works with very low dosed of TNF in the case of autocrine secretion. It may well be possible that enough TNF is circulation at the site of potential tumor targets that the issue is moot, given that very often at tumor sites there is already an inflammatory response and that certain cytokines are already being released. However, it might be possible to use very low doses of TNF and low dose NF-kB inhibitors, all operating at sub-threshold levels, with smac mimetic as a potentially powerful combinatorial chemotherapy.

To summarize, over the last three years tremendous progress has been made in understanding how smac mimetic work and how to possibly make it a more effective therapeutic against virtually every type of cancer imaginable. Particularly responsive tumors would be those that produce TNF in an autocrine fashion, but not necessarily restricted to such types as long as TNF is present in the tumor microenvironment from neighboring cells or macrophages recruited to the area as part of an inflammatory response.

Additionally, it was found that smac mimetic induced cell death is entirely dependent on RIPK1. Work done by Lai Wang, Ph.D. showed that a key event is the degradation of cIAP1 and cIAP2 which function at TNFR1 to hold RIPK1 at the receptor promoting NF-kB activation. Loss of cIAP1 and cIAP2 promoted the release of RIPK1 from the receptor into a complex with caspase-8 and FADD, which is sufficient to cause apoptosis (Figure 4-5).

Additionally, mechanisms of resistance to smac mimetic were identified. One was that those cells that are highly responsive to TNF, and had constitutive AKT activation, highly up regulated cIAP2. Once cIAP1 is degraded, there is no longer a mechanism for cIAP2 to be ubiquitinated and degraded, thus cIAP2 levels rebound within a few hours of smac mimetic/TNF treatment. cIAP2 can replace the functionality of cIAP1 and RIPK1 is precluded from entering the death-inducing complex. Two, there are cells that do not express cIAP2 but are nonetheless resistant. These cells do not respond very well to TNF and have relatively low levels of RIPK1. It seems that lack of cIAP2 negatively affects NF-kB response and regulation of RIPK1. Thus, very little RIPK1 is recruited to TNFR1 and caspase-8 cannot be activated.

Finally, at least in cells that induce cIAP2, resistance can be overcome by a variety of mechanisms that have potential therapeutic value. Firstly, low dose inhibition of NF-kB can prevent up regulation of cIAP2 and make cells sensitive to smac mimetic/TNF. The dosing is key here, in that, it must be low enough to prevent cIAP2 up regulation, but not so high as to cause total inhibition of signaling. Inhibitors of NF-kB that have thus far been evaluated for clinical use have had problems of causing cytotoxic shock from an extreme immune response. Low dose treatment might be viable with smac mimetic since one does not need to completely shut down the NF-kB signal. Second, targeting alternate pathways of cIAP2 regulation also sensitizes cells to smac mimetic/TNF, independent of NF-kB signaling. This indicates that cIAP2 regulation is more complicated than simply being responsive to TNF. Clearly, TNF and other pathways, such as PI-3K, are required. Third, using a clinically used kinase inhibitor, Tarceva, cells were sensitized to smac mimetic/TNF. These cells are completely resistant to Tarceva treatment alone and obviously this opens the possibility of expanding the use of Tarceva to more patients.

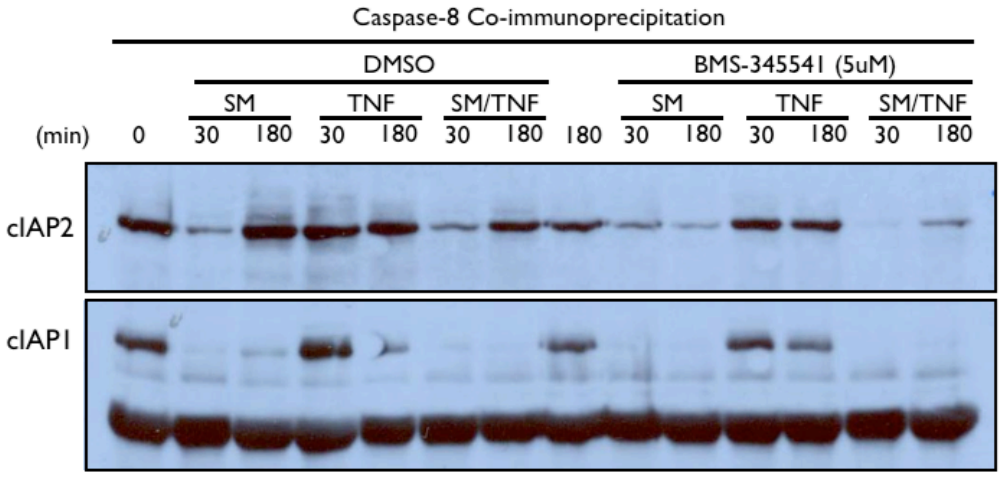

Figure 4-1. cIAP1 and cIAP2 interact with caspase-8 and cIAP2 interaction correlated with resistance to smac mimetic/TNF.
Caspase-8 co-immunoprecipitation comparing DMSO versus BMS-345541 treatment. Cells were pretreated for I hour and then treated as indicated. Western blot of cIAP1 and cIAP2 were done to see if an interaction occurred.

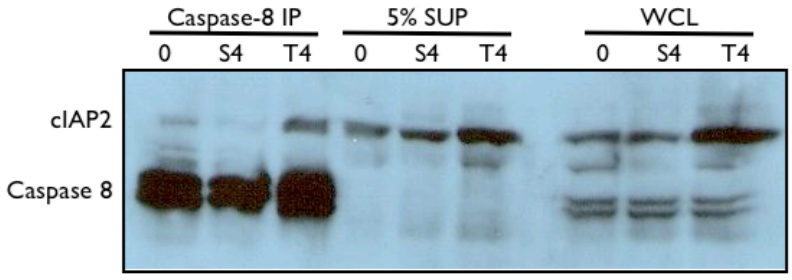

Figure 4-2. The majority of cIAP2 does not interact with caspase-8.
Caspase-8 co-immunoprecipitation and western blotting of cAIP2. To determine relative interaction between cIAP2 and caspase-8 and the amount of free cIAP2 whole cell lysates and the supernatant were also analyzed by western blot.

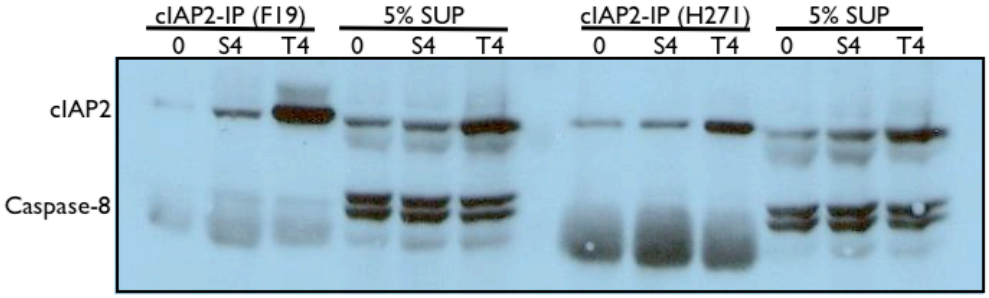

Figure 4-3. Co-immunoprecipitation of cIAP2 cannot pull-down caspase-8.
Two different cIAP2 antibodies were used to try and pull down caspase-8 endogenously.

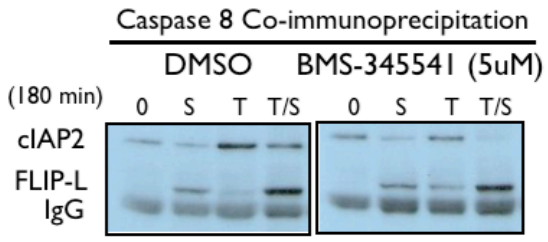

Figure 4-4. c-FLIP is recruited to caspase-8 in response to smac mimetic, but not to TNF.
Caspase-8 co-immunoprecipitation showing the interaction of c-FLIP. C-FLIP appears to be recruited under smac mimetic treatment condition and not under TNF only condition.

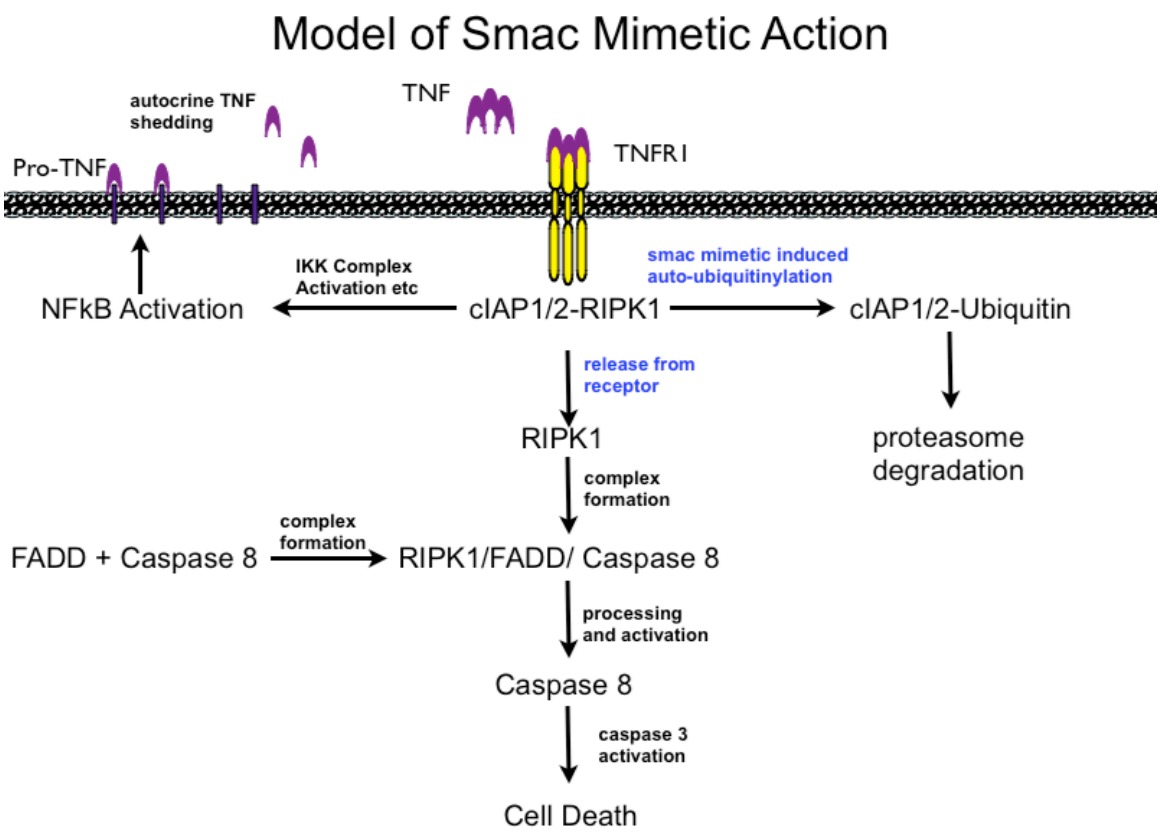

Figure 4-5. Model of Smac Mimetic Action.
Diagram of smac mimetic mode of action.

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