CHARACTERIZATION OF A SMALL MOLECULE SMAC MIMETIC'S

ROLE IN INDUCING APOPTOSIS IN HUMAN CANCER CELLS

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Dedication

I would like to dedicate my thesis to my parents, Gonul and Atilla Yalcin, my brother, Kivanc Yalcin, my sister in-law Meryem Yalcin and my husband Paul Chin.

CHARACTERIZATION OF A SMALL MOLECULE SMAC MIMETIC'S ROLE IN INDUCING APOPTOSIS IN HUMAN CANCER CELLS

by

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CHARACTERIZATION OF A SMALL MOLECULE SMAC MIMETIC'S ROLE IN INDUCING APOPTOSIS IN HUMAN CANCER CELLS

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The University of Texas Southwestern Medical Center at Dallas, 2008

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Inhibitor of apoptosis proteins (IAPs) regulates apoptosis by inhibiting caspases. This inhibition mechanism is an escape from death used by some human cancers. Second mitochondria-derived activator of caspases (Smac), a mitochondria-released protein during apoptosis, binds to IAPs BIR domains with four amino acid residues (AVPI) and releases the inhibition caused on caspases by IAPs. With the idea of designing a Smac mimicking drug, that will induce apoptosis in cancer cells, we synthesized a small molecule Smac mimetic compound. I tested the ability of the Smac mimetic compound to induce apoptosis on several human cancer cells in combination with chemotherapeutic agents. Unexpectedly, in 25% of the cancer cells we tested, Smac mimetic treatment alone caused apoptosis. Of the cancer cells that were sensitive to Smac

mimetic, MDA-MB231 human breast cancer cells and HCC44, HCC461, H2126 lung cancer cells had the highest sensitivity. In addition, a majority of the lung cancer cell lines I tested were sensitive to TNF and/or TRAIL in combination with Smac mimetic. We identified the target of Smac mimetic to be XIAP, cIAP1, and cIAP2 in both Smac mimetic induced and TNF/Smac mimetic induced apoptosis. Moreover, we were able to mimic the Smac mimetic effect by triple knockdown experiments of IAPs in TNF induced cell death. Furthermore, we identified the target of Smac mimetic to be XIAP in the TRAIL pathway. This work identifies the targets and mechanism of Smac mimetic induced cell death in cancer cells.

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Prior Publications

Yalcin, A., Koulich, E., Mohamed, S., Liu, L., D'Mello, S. R. (2003). "Apoptosis in cerebellar granule neurons is associated with reduced interaction between CREB-binding protein and NF-kappaB." J Neurochem 84(2): 397-408.

Petersen, S. L., Wang, L., Yalcin-Chin, A., Li, L., Peyton, M., Minna, J., Harran, P., Wang, X. (2007). "Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis." Cancer Cell 12(5): 445-56.

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List of Definitions

ATP – Adenosine-5'-triphosphate

dATP - 2’-deoxyadenosine 5’-triphosphate

BCL - B-cell leukemia/lymphoma

IAP – inhibitor of apoptosis

NF-κB – nuclear factor of kappa light polypeptide gene enhancer in B-cells

TNF – tumor necrosis factor

TRAIL – tumor necrosis factor related apoptosis-inducing ligand

CHAPTER ONE
INTRODUCTION

Apoptosis a.k.a. programmed cell death is a crucial process for development and homeostasis in healthy multi-cellular organisms (Kerr et al. 1972). Apoptosis has to be regulated fastidiously, hence too much or too little of it can be detrimental to the organism. Too much cell death may cause neurodegenerative diseases and too little may lead to autoimmune disorders or cancer (Thompson 1995). Therefore, apoptosis must be precisely controlled and balanced physiologically. There are two main apoptosis pathways, intrinsic (mitochondria) and extrinsic (receptor). Mainly named to describe from where the signal to die originates. Regardless, wherever the signal originates, the central event in both pathways involves activation of family of cysteine-proteases named caspases (Yuan et al. 1993).

Caspases are cysteine-dependent, aspartate-specific proteases. Caspases are synthesized as zymogens, when activated the pro-form is cleaved to large and small subunits. There are 14 family members in mammals and they are functionally divided into two groups: apoptotic and inflammatory. (Stennicke and Salvesen 2000) (Degterev et al. 2003). Caspase 1, 4, 5, 11, 12, 13 and 14 are inflammatory caspases activated in response to inflammatory processes. The apoptotic caspases are further divided into initiator and executioner caspases. Caspase 2, 8, 10 and 9 are the initiator caspases. Initiator caspases have long pro-

domains consisting of caspase recruitment domain (CARD) or death effector domain (DED) (Thornberry 1998). CARD and DED domains are protein-protein interaction domains and recruit initiator caspases to large complexes such as the apoptosome for caspase 9 and death inducing signaling complex (DISC) for caspase 8 and 10. (Inflammatory caspases also form large complexes called the "inflammasome" with specific adaptor proteins (Martinon et al. 2002).)

Through dimerization and autolysis, initiator caspases are activated. Mouse knockout studies identified caspase 8 to be required for all receptor death pathways and caspase 9 to be required for mitochondrial pathway (Wang and Lenardo 2000). Caspase 3, 6 and 7 are the effector caspases and are executers of the death of the cell.

Caspase activation is a hallmark of apoptosis. Apoptosis involves the activation of initiator caspases, which then cleave and activate the executioner caspases. The executioner caspases have many targets which include proteins that are responsible for the characteristic structural trademarks of apoptosis: nuclear condensation, DNA fragmentation and membrane blebbing (Taylor et al. 2008).

Intrinsic and Extrinsic pathways

As previously alluded, the origin of the apoptotic signal determines which initiator caspase will be activated (figure 1.1). Internal apoptotic stresses, like DNA damage, signal through the mitochondria. Mitochondria release

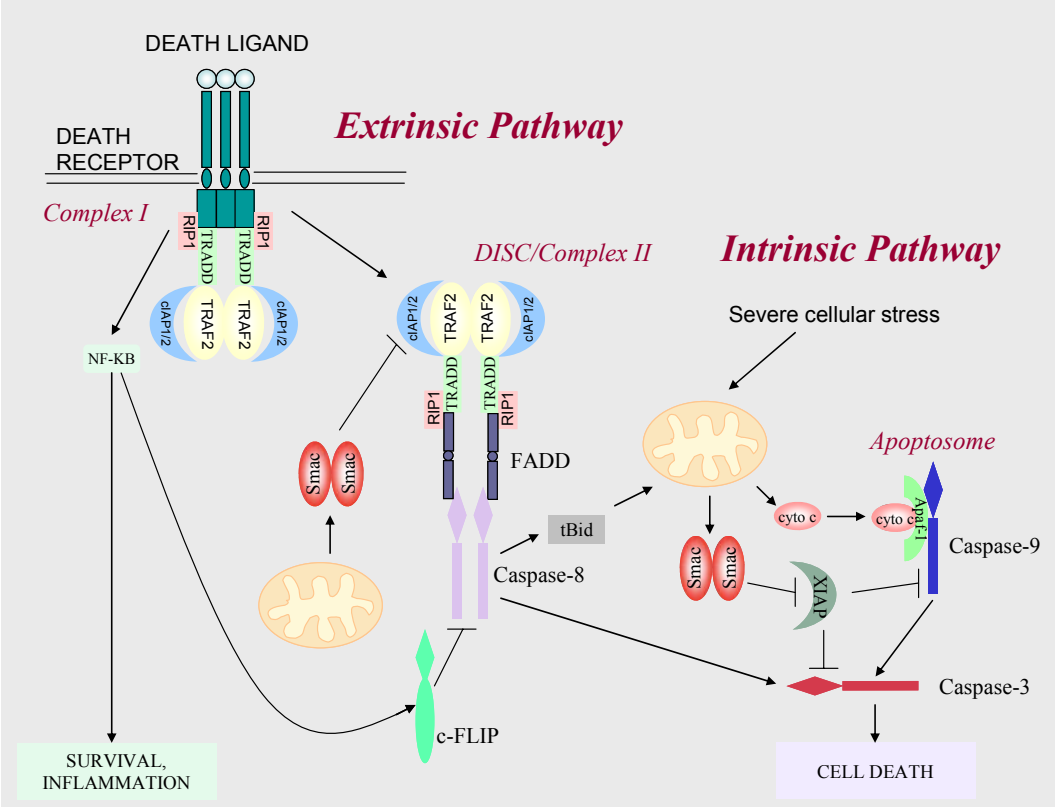

Figure 1.1. Intrinsic and Extrinsic pathways

Intrinsic pathway is initiated through severe cellular stresses while the extrinsic pathway is initiated at the cell membrane. For detailed review, see text.

cytochrome c to the cytosol to initiate the intrinsic pathway (Liu et al. 1996; Li et al. 1997). Cytochrome c binds to Apaf-1, a cytosolic protein, which normally exist in monomer state in a healthy cell. Binding of cytochrome c induces oligomerization of Apaf-1 to a heptameric complex called the apoptosome in the presence of dATP/ATP (Liu et al. 1996; Zou et al. 1997). The apoptosome serves as a structural platform for the initiator caspase, caspase 9, to be recruited to the complex (Zou et al. 1999). Recruitment of caspase 9 to the apoptosome leads to caspase 9 activation which then allows caspase 9 to activate executioner caspases like caspase 3 (Li et al. 1997; Zou et al. 1997; Wang 2001).

The extrinsic pathway is initiated at the cell membrane by the death receptors (Tartaglia et al. 1993). All death receptors are members of the tumor necrosis factor (TNF) superfamily receptors (Smith et al. 1994; Ashkenazi and Dixit 1998; Ashkenazi 2002; Jin and El-Deiry 2005). Collectively, they are: Fas (CD95), TNFR1, TR1 (DR4), and TR2 (DR5). When ligands of the death receptors bind to their receptors: FasL/Fas, TNFα/TNFR1 and TRAIL/DR4 DR5, they cause trimerization and activation of their receptors (figure 1.2). FasL and TRAIL pathways cause recruitment of Fas-associated via death domain (FADD) through its DD (death domain) to the receptor, followed by recruitment of caspase 8 to this complex through its death effector domain (DED) (Jin and El-Deiry 2005). This complex is the so-called DISC complex. Once caspase 8 is activated it will further cleave the downstream effector caspases.

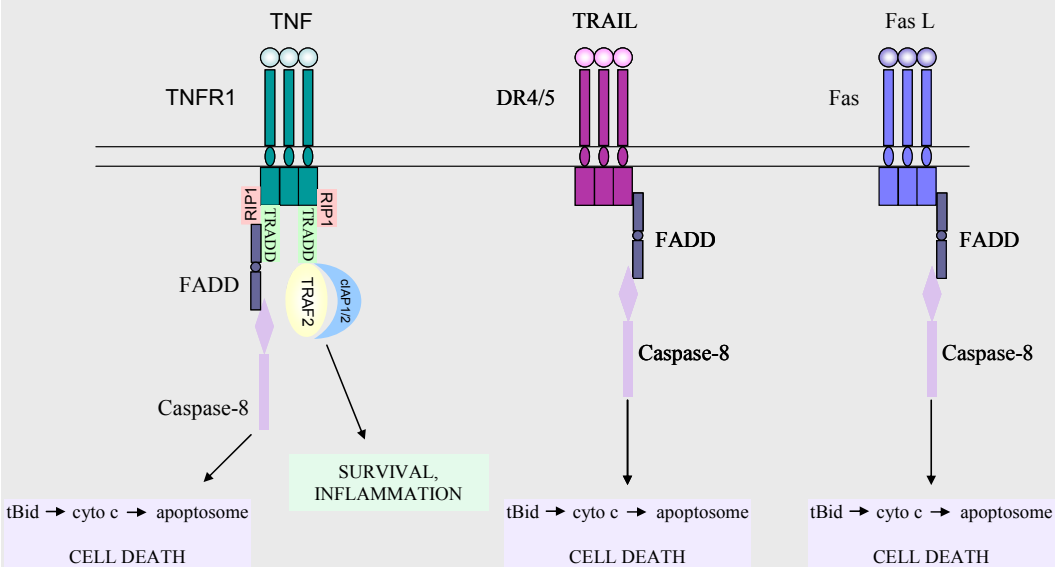

Figure 1.2. TNFR1, TRAILR1/R2 and FAS complexes

Illustration of the different complexes formed by death receptors.

The TNFα pathway differs from the Fas and TRAIL pathways in terms of its pleiotropic effects; when activated in most cells, this pathway needs a second hit to be a true death receptor (Varfolomeev and Ashkenazi 2004). In TNFR1 pathway, when the receptor is activated it recruits adaptor proteins like TNFR1-associated death domain protein (TRADD). TRADD binds to the receptor through its DD domain. It in turn recruits TNF-receptor-associated factor 2 (Traf 2), receptor-interacting serine-threonine kinase 1 (RIPK1), cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2. This complex, named complex I, will prevent the apoptotic signal and sustain the survival and inflammation signal through NF-κB (Chen and Goeddel 2002; Muppidi et al. 2004; Karin 2006).

In addition to complex I activation, TNFα binding to the receptor can also cause JNK activation (Varfolomeev and Ashkenazi 2004). About two hours after the binding of the ligand, TNFR1 is internalized. This is thought to be a signal to dissociate complex I and form complex II (Micheau and Tschopp 2003). Complex II is formed through internalization of the receptor and recruitment of FADD and caspase 8. Caspase 8 will be activated in the complex II a.k.a. DISC. Once caspase 8 is activated, it further activates downstream effector caspases like caspase 3.

The extrinsic pathway can also cross talk to the intrinsic pathway by caspase 8 cleaving Bid, a BCL-2 family protein (Luo et al. 1998; Wang 2001). Caspase 8 cleaved Bid is called tBid, which stands for, truncated Bid. tBid can

relocate to the mitochondria and cause release of second mitochondrial activator of caspases (Smac) and cytochrome c (Wang 2001). Smac is a pro-apoptotic protein that relieves caspases from their endogenous inhibitors (Du et al. 2000). Release of Smac and cytochrome c to the cytosol will strengthen the caspase activation signal, thus ensuring the continuation of the death pathway.

Regulation of cell death

Both intrinsic and extrinsic pathways are under exclusive regulation to control unwanted cell death. The balance between pro- and anti-apoptotic signals is controlled by complex regulatory networks. Main anti-apoptotic regulatory pathways identified are NF-κB, AKT, Bcl-2 and IAP pathways.

The intrinsic pathway is under the control of Bcl-2 family proteins (Wang 2001; Danial and Korsmeyer 2004; Youle and Strasser 2008). They collectively control cytochrome c release from the mitochondria. Bcl-2 family members are grouped into three classes. One class inhibits apoptosis and contains members such as Bcl-2, Bcl-XL and myeloid cell leukemia sequence 1 (Mcl-1). The members of this class contain four conserved regions, Bcl-2 homology (BH) 1-4 domains and are the anti-apoptotic members. The second class promotes apoptosis and consists of proteins, Bax and Bak. These proteins contain BH1-3 domains. A third class of BH3-only proteins like Bad, Bid, and Bim have a conserved BH3 domain that can bind and regulate the anti-apoptotic Bcl-2

proteins to promote apoptosis. Bax and Bak have a crucial role in apoptosis; they oligomerize and form a large pore in the mitochondrial outer membrane for the apoptogenic proteins like cytochrome c to pass through (Danial and Korsmeyer 2004; Youle and Strasser 2008).

It is critical to tightly control the caspase activation inside the cell (Taylor et al. 2008). As discussed, the first level of regulation comes from the strictly controlled signaling to activate them. However, a second level of regulation comes from specific protein inhibitors. Cellular specific inhibitors of caspases are a family of proteins called Inhibitor of Apoptosis Proteins (IAPs) (Salvesen and Duckett 2002). IAPs are the most important negative regulators of caspases. They regulate both initiator and effector caspases (Silke et al. 2002). They inhibit caspases under a variety of stimuli: death receptor activation, growth factor withdrawal, ionizing radiation, viral infection and genotoxic damage.

Inhibitor of apoptosis proteins (IAPs)

The first identified IAPs were baculoviral genes that compensated for the loss of function mutation of baculoviral pan-caspase inhibitor, p35 (Crook et al. 1993). IAPs are believed to be used by baculoviruses to allow viral propagation without causing the host cell to die (Hawkins et al. 1998; Uren et al. 1998). Since the first discovery of baculoviral IAPs several IAPs have been identified. The first mammalian IAP identified was neuronal inhibitor of apoptosis protein

(NIAP) (Roy et al. 1995). After NIAP, seven mammalian IAPs were identified: X-linked inhibitor of apoptosis (XIAP/ MIHA/ hILP/ BIRC4/ ILP-1) (Deveraux et al. 1997), cellular IAP1/Human IAP2 (cIAP1/ HIAP2/ MIHB/ BIRC2); cellular IAP2/Human IAP1 (cIAP2/ HIAP1/ MIHC/ API2/ BIRC3) (Rothe et al. 1995), testis-specific IAP (Ts-IAP/ hILP2/ BIRC8/ ILP-2) (Richter et al. 2001), BIR-containing ubiquitin conjugating enzyme (BRUCE/ Apollon/ BIRC6) (Hauser et al. 1998), Survivin (TIAP/ BIRC5) (Ambrosini et al. 1997) and Livin (KIAP/ ML-IAP/ BIRC7) (Vucic et al. 2000).

All IAPs contain at least one 70-80 amino acid Baculovirus IAP Repeat (BIR) domain but can contain as many as three BIR domains (Hinds et al. 1999) (figure 1.3). XIAP, cIAP1, cIAP2 and Livin also contain a C-terminal Really Interesting New Gene (RING) domain, which constitutes its E3 ubiquitin ligase activity (Yang et al. 2000) . XIAP, cIAP1 and cIAP2 through their RING domains can auto-ubiquitinate and regulate their activities. cIAP1 and cIAP2 also possess CARD domains, however, so far no known function have been identified.

The first observation for the function of IAPs demonstrated that they inhibited apoptosis when over-expressed. XIAP prevents caspase 3 maturation and binds to caspase 3 and 7; however it does not bind to caspases 1, 6 and 8 (Deveraux et al. 1997). Further studies dissecting different domains of XIAP identified that the BIR2 domain can bind to caspase 3 and 7 and BIR3 domain can bind to caspase 9 (Chai et al. 2000). Later studies using the BIR2 domain of

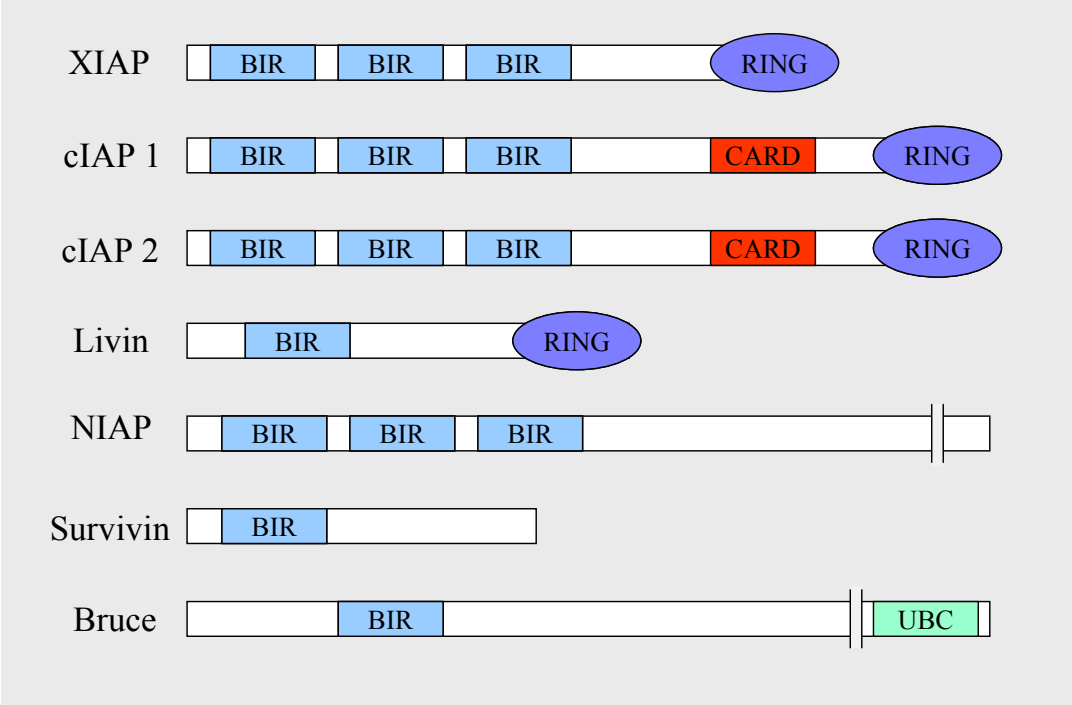

Figure 1.3. Mammalian Inhibitor of Apoptosis Proteins.

Structures of mammalian IAPs, highlighting the locations of the BIR (light blue), CARD (red), RING (dark blue), and UBC (green) domains

XIAP and caspase 3 and 7 show that the linker region between BIR1 and BIR2 is the IAP region that makes contact with caspase 3 and 7. Consequently, effector caspases are blocked through steric hindrance by the BIR2 domain and/or by the linker region blocking the substrate entry site (Chai et al. 2001).

XIAP appears to be the only true caspase inhibitor; other IAPs although they bind, they do not have the inhibitory function (Eckelman and Salvesen 2006; Eckelman et al. 2006). Previous studies identifying the caspase inhibiting activity of cIAP1 and cIAP2 were later discovered to be inaccurate. In fact, the GST tags of purified cIAP1 and cIAP2 induced their oligomerization, and the oligomerization of the recombinant proteins led to their inhibitory activity. However, in those *in vitro* studies recombinant cIAPs may not have had the correct conformation or post-translational modifications necessary to inhibit caspases, so the verdict is still out about the caspase inhibitory functions of cIAPs. Nevertheless, cIAP1 and cIAP2 are accepted to be antagonists of caspases, since when over-expressed they protect the cells under a variety of apoptotic stimulus.

Catalytic auto-cleavage of caspase 9 occurs at Asp 315 to yield a p35 (big) and p12 (small) subunit. The newly generated N-terminus of caspase 9 starts with ATPF. This peptide sequence is a conserved BIR3 binding motif. Co-crystallization studies of BIR3 and caspase 9 revealed that when BIR3 binds to caspase 9, it holds caspase 9 in an inactive confirmation and inhibits its heterodimerization. This prevents caspase 9 proteolytic activity. Further

cleavage of the caspase 9 p12 subunit by caspase 3 at Asp 330 yields a p10 subunit generating a more active caspase 9 by removing the ATPF sequence (Zou et al. 2003). This creates a feedback mechanism where caspase 9 activation will activate caspase 3 and activated caspase 3 will further activate caspase 9. This cleavage removes the XIAP inhibition on caspase 9.

So far, the BIR1 domain of XIAP has not been demonstrated to interact with any substrate; however, it contains an Akt phosphorylation site at Ser 87 which reduces its auto-ubiquitination (Dan et al. 2004). In addition, the RING domains of IAPs can auto-regulate themselves or other substrates. Apoptotic stimuli like etoposide and glucocorticoids induce rapid degradation of XIAP and cIAP1 dependent on their RING domains (Yang et al. 2000). XIAP and cIAP1 can also target caspase 3 and 7 to proteasomal degradation (Suzuki et al. 2001).

Ubiquitination and degration of XIAP is phosphorylation dependent. Akt is a serine/threonine kinase that is known to promote cell survival. Through phosphorylation of XIAP's BIR1 domain, Akt is known to prevent cisplatin-induced cell death (Dan et al. 2004). Several other proteins play critical roles in regulating IAPs including; XIAP associated factor 1 (XAF 1) (Liston et al. 2001), Checkpoint kinase 1 (Chk1) (Galvan et al. 2004), Smac and OMI/HtrA2 (Verhagen et al. 2000; Yang and Du 2004).

A variety of cancer cells and tumor biopsies contain elevated levels of IAP expression (LaCasse et al. 1998). XIAP over-expression has been detected in

many tumor cell lines, and cIAP gene amplification has also been found in a variety of cancers including, renal cell carcinomas, glioblastomas, gastric carcinomas, and non-small cell lung carcinomas. These observations support the notion that IAPs can function as oncogenes. Subsequently, the major obstacles in chemotherapy are primary and acquired resistance. Upregulation of IAP has been found to increase resistance to chemo- and radiation therapies. For this reason, IAP targeting is a new exciting therapy strategy.

Smac

Concurrently with cytochrome c, Smac, a 25 kD mitochondrial protein, escapes from the mitochondria to the cytosol during certain apoptotic stimuli (Du et al. 2000; Verhagen et al. 2000). Release of Smac is also thought to occur through Bak/Bax channels like cytochrome c. Smac is a pro-apoptotic protein that is an important regulator of apoptotic signaling. Smac is targeted to the mitochondria through its 55 amino acid mitochondrial targeting sequence. The targeting sequence undergoes cleavage once inside the outer membrane space where Smac resides. This ensures that mature Smac never sees the cytosol unless a mitochondrial stress is present. This is of critical importance to the cell since Smac can induce premature apoptosis.

Smac functions as a general IAP inhibitor. The first four amino acid residues of Smac Ala-Val-Pro-Ile (AVPI) are absolutely required for Smac

function. Deletion of these four residues abolishes Smac's interaction with IAPs (Du et al. 2000). Smac is an endogenous homodimer, and homodimerization is essential for its activity (Chai et al. 2000) (figure 1.3). Mature Smac (mitochondrial-derived) binds to BIR3 domain of XIAP. Structural analysis of the Smac-XIAP binding revealed that the four amino acid N-terminal AVPI peptide is able to recognize the surface grove of the BIR3 domain of XIAP (Liu et al. 2000). The XIAP binding sequence of Smac, AVPI, is similar to the XIAP binding sequence of caspase 9, ATPF (Srinivasula et al. 2001). Similarly, the binding sequence on caspase 9 is only exposed when caspase 9 is cleaved. In addition to binding the BIR3 domain of XIAP, Smac can bind to the BIR2 region of IAPs, presumably disrupting the IAP-active caspase 3 and 7 interactions through steric hindrance (Chai et al. 2001). Since, IAPs only bind and inhibit active/mature caspases, the release of active caspases by competion from Smac results in unimpeded caspase activation (Wang 2001). The inhibition of IAPs at multiple stages (initiation and execution) ensures that Smac can induce rapid execution of apoptosis when released from the mitochondria.

The mutual exclusion and direct competion between Smac and activated caspases exposes an elegant control mechanism on the apoptotic pathways. When cytochrome c is released from the mitochondria, it binds and causes oligomerization of Apaf-1. Oligomerization Apaf-1 recruits and activates caspase 9 in the apoptososme which leads eventually to caspase 3 activation. However, in

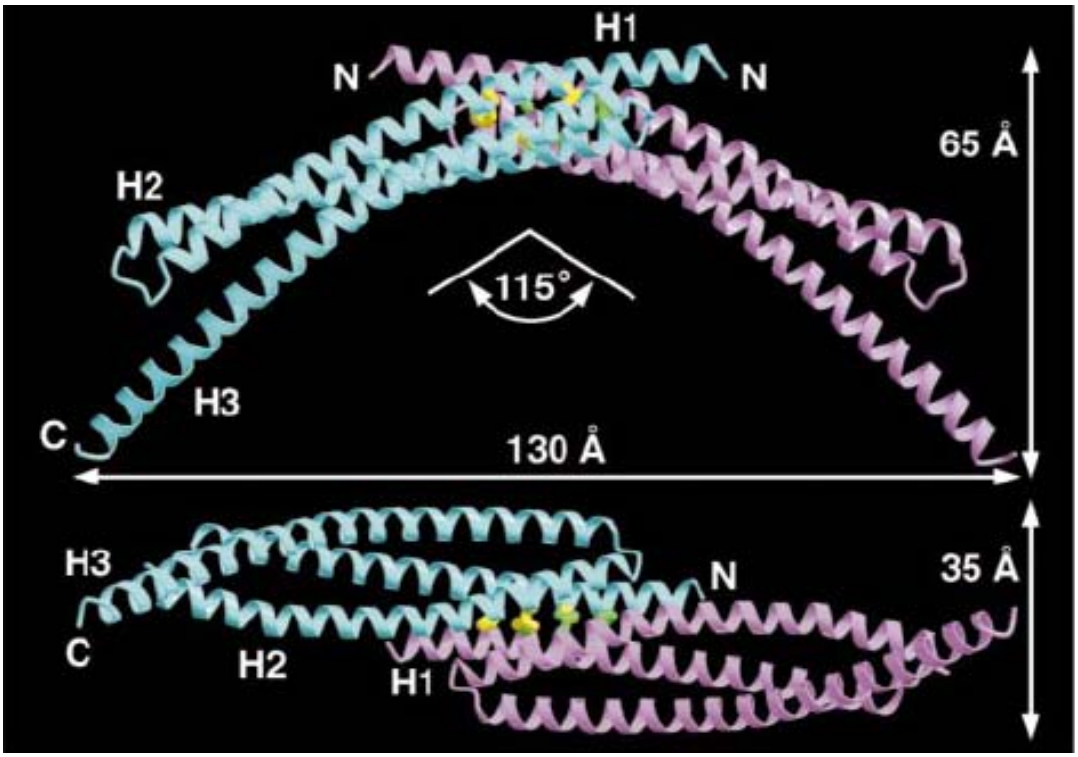

Figure 1.4. Structure of Smac homodimer

The arch-shaped structure of a Smac dimer, with the two monomers colored cyan and pink, respectively. The two views of the structure are related by a 908 rotation around a horizontal axis. (Adapted from Wu et. al. 2000)

the presence of IAPs, the active caspases in the apoptosome are inhibited (Bratton et al. 2001; Srinivasula et al. 2001). In addition, this inhibition of the caspases can be permanent because of the RING domains of IAPs (Yang et al. 2000; Suzuki et al. 2001). This system provides a safety net in case of incidental or transient mitochondrial leakage of cytochrome c (12 kD) (Chai et al. 2000). In the case of severe or persistent mitochondrial damage, mitochondrial release of Smac together with cytochrome c ensures that IAP inhibition of caspases will be removed and apoptosis will proceed (Wang 2001).

Still, regulation provided by Smac is not only relevant to the intrinsic pathway. Because intrinsic and extrinsic pathways converge at caspase 3 activation, high levels of IAPs can abort the receptor pathway by inhibiting caspase 3. Consequently, the receptor pathways may rely on tBid to release Smac from mitochondria to counter IAP inhibition (Li et al. 1998; Luo et al. 1998).

Smac Mimicry

Deregulated apoptosis is a hallmark of cancer and will always be an attractive target for development of new chemotherapies. Tumors escape cell death by upregulating or overactivating anti-apoptotic genes and by down-regulation of pro-apoptotic genes. Acquired resistance of cancer cells diminishes the effectiveness of cancer therapies. Most regulatory pathways in apoptosis are redundant and most cancer therapies are non-selective between normal and tumor cells leading to many disappointments in cancer therapy.

Smac function is dependent on its N-terminal four amino acid sequence. This characteristic of Smac enabled the idea that small molecules that mimic this activity could be created as potential drugs (Arnt et al. 2002; Fulda et al. 2002; Pardo et al. 2003; Yang et al. 2003). With this idea of Smac mimicry, several labs and pharmaceutical companies started testing different peptidyl and non-peptidyl Smac mimetics.

Smac protein was first discovered in Dr Xiaodong Wang's lab (Du et al. 2000). Smac was co-identified and named DIABLO (direct IAP binding protein with low pI) in Dr. David L. Vaux's lab in Australia (Verhagen et al. 2000). After its initial discovery, within few months there was enough structural information on the binding of Smac and IAPs that the first Smac mimicking peptides followed quickly after (Chai et al. 2000; Wu et al. 2000). By 2002, several labs started testing of these compounds in comparison to native Smac protein. Smac peptides composed of seven to ten amino acid peptides were the first ones to be tested and were able to activate caspase 3 in *in vitro* caspase 3 assays (Chai et al. 2000). These peptides were able to bind to IAPs, and they synergized with other chemotherapeutic agents like paclitaxel, etoposide and doxorubicin (Arnt et al. 2002). Animal studies demonstrated that they were able to reduce the tumor burden in combination with TRAIL (Fulda et al. 2002). TRAIL was the wonder cytokine when it was first discovered because of it specificity to kill tumor cells versus normal cells (Huang and Sheikh 2007). Later it was discovered that when

administered alone, over time it became ineffective in the once sensitive tumor lines. However, when delivered together with Smac peptides, resistant cell lines became sensitive to TRAIL once again (Fulda et al. 2002).

Smac mimetic compounds attracted great interest because of their potential to synergize with a number of different apoptosis inducing stimulus. Nevertheless, there were two issues to be solved. First, the bioavailability and metabolic stability of these compounds was poor. Much higher doses of these peptides were needed; in other words, they were not very good at mimicking endogenous Smac protein. The other obstacle was cell permeability.

In 2004, our lab developed the true Smac mimetic (Li et al. 2004) (figure 1.4). Our Smac mimetic compound 3 penetrated the cell membrane and bound to XIAP with an affinity equal to Smac protein itself. Furthermore, as a true Smac mimetic, it could also bind to cIAP1 and cIAP2 and hence promote TNFa and TRAIL induced apoptosis at nanomolar concentrations.

We believe Smac mimicry has enormous potential to be a highly specific chemotherapeutic agent. One of the most important challenges for cancer therapies today is that they also tend to kill normally growing cells as well as cancer cells, which results in undesirable side effects. Compound 3 can exert its function in nanomolar ranges and is highly specific to tumor cells. Moreover, no toxicity is observed at thousand fold higher doses in normal cells.

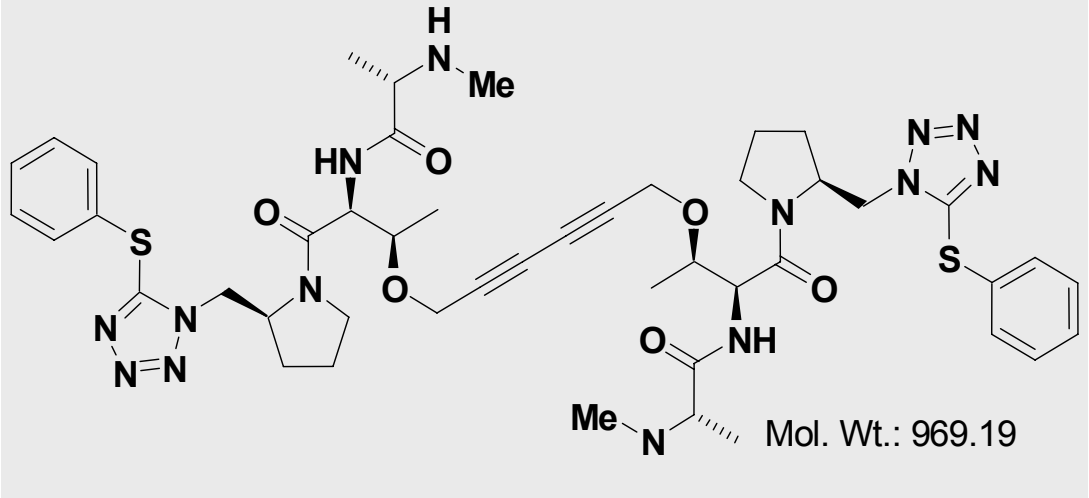

Figure 1.5. Structure of Smac mimetic dimer (compound 3)

Chemical structure of Smac mimetic.

In my thesis, I studied the mechanism and potential targets of Smac mimetic, compound 3, induced cell death. I identified the *in vitro* and *in vivo* targets of Smac mimetic. Studying the sensitivity and resistance of cancer cell lines to Smac mimetics, guided me to identify acquired resistant cell lines to Smac mimetic. We identified that some cell lines are sensitive to Smac mimetic alone. These studies led us to categorize cancer cells and understand the reason for sensitivity versus resistance to Smac mimetic. I, then, focused my studies on the Smac mimetic synergy with TRAIL and was able to demonstrate that the only target of the Smac mimetic in the TRAIL death receptor pathway was XIAP.

CHAPTER TWO
SMAC'ING DOWN CANCER

2.1 Smac mimetic induced cell death

IAPs inhibit active caspases. Of all eight mammalian IAPs, XIAP is the most potent caspase inhibitor. It interacts with initiator caspase 9 and executioner caspases 3 and 7 through its BIR3 and BIR2 domains, respectively (Chai et al. 2000; Wang 2001). The role of cIAP1 and cIAP2 are less clear, although they are known to associate with the TNFR1 signaling complex (Rothe et al. 1995; Uren et al. 1996).

The IAP inhibition on caspases is counteracted by the N-terminal four amino acids of Smac (Du et al. 2000; Verhagen et al. 2000). Synthetic Smac N-terminal peptides were found to by-pass the mitochondrial regulation on Smac and sensitize both human cancer cells in culture and tumor xenographs in mice to apoptosis when combined with TRAIL or chemotherapeutic agents (Arnt et al. 2002; Fulda et al. 2002; Pardo et al. 2003; Yang et al. 2003; Li et al. 2004). In 2004, Dr Xiaodong Wang in collaboration with Dr Patrick Harran and Dr Jeff De Brabander, designed and synthesized a Smac mimetic compound that can pass through the cell membrane and bind XIAP with an affinity equal to that of Smac protein itself (Li et al. 2004)(figure 1). In addition, this small molecule can also bind and eliminate cIAP1 and cIAP2 activating both TRAIL and TNFα induced apoptosis at low nanomolar concentrations in cancer cells.

The design and synthesis of this compound originated from examining the co-crystal structure of Smac in complex with the XIAP BIR3 domain. This structure demonstrated that the Smac N-terminus AVPI interacts with a groove formed on the BIR3 surface on XIAP (Liu et al. 2000). However, structural variations to the C-terminal end of the tetra-peptide AVPI, for example AVPF of caspase 9 was a better performing Smac mimetic *in vitro*. Therefore computer-simulated conformations of AVPF were selected as a guide to design the non-peptidyl replacements for the C-terminal half (PF). The hybrid mimetics that resulted were purified and evaluated in competition assays with endogenous Smac at the Smac binding site on XIAP BIR3 (Huang et al. 2003). Oxazoline 1 was the most potent competitor, although, none of the compounds performed better than AVPF. However, when oxazoline 1 was examined for its ability to induce caspase 3 activation using *in vitro* caspase 3 activity assay in soluble Hela cell extracts, its potency exceeded that of synthetic AVPF. Yet, when compared to Smac protein, these compounds were orders of magnitude less active. Modifications of 1 reached tetrazoyl thioethers of type 2. Attempts at a particular manipulation of the alkyne in 2 produced a by-product eventually characterized as C_2-symmetric diyne 3. The dimer 3 was the end product of an oxidative homodimerization known as a Glaser coupling (figure 2.1). Both dimer 3 and its corresponding monomer 2 have similar affinity for XIAP BIR3 domain. However, in a caspase 3 activation assay that measures release of endogenous IAP

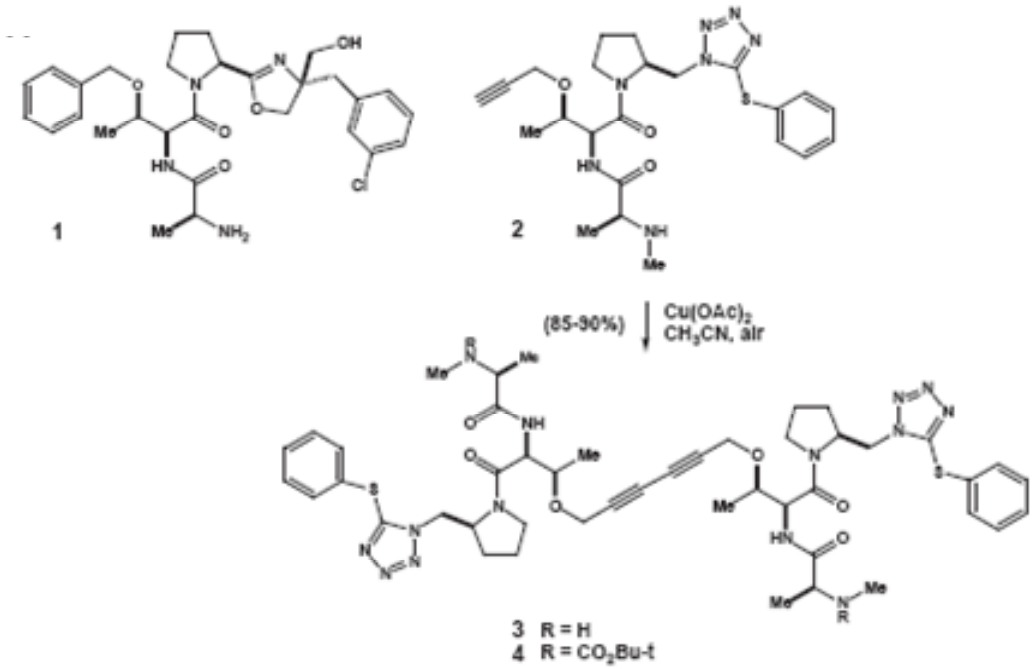

Figure 2.1. Chemical structures of the small molecules described in this study

Chemical synthesis steps for compound 3, Smac mimetic dimer. Compound 4 is negative compound

activity in Hela cell extract, dimer 3 was a much more active Smac mimetic. One explanation for this observation is the bivalency of Smac protein. Native Smac homodimer binds similarly to XIAP. Compound 3 may interact simultaneously with adjacent BIR domains in XIAP like Smac. The resultant two-point bound complex may be considerably more stable than single-site affinities would foresee.

Dr. Lin Li, from Dr. Xiaodong Wang's Lab, tested the activity of compound 3 *in vivo* in T98G cells. T98G is a human glioblastoma cell line that is known to be resistant to several apoptotic stimuli, like DNA damage–induced apoptosis. Compound 3 alone at high concentrations (1 μM) did not induce apoptosis or caspase 8 activation in T98G cells (figure 2.2). However, when used in combination with TRAIL (50 ng/mL), 100 nM of compound 3 caused extensive cell death (figure 2.2). In fact, caspase 8 activation and apoptosis are observed at concentrations of compound 3 as low as 100 pM when combined with 50 ng/mL TRAIL. TRAIL alone at the same concentration, did not induce caspase 8 activation, nor did it induce apoptosis in this cell line. Caspase 3 activity in response to compound 3 plus TRAIL was measured by Western blot analysis of cleaved poly adenosine diphosphate ribose polymerase (PARP), a caspase 3 substrate. Cleavage of endogenous PARP appears in the presence of 30 pM of 3 and 50 ng/mL TRAIL (lane 10, figure 2.2).

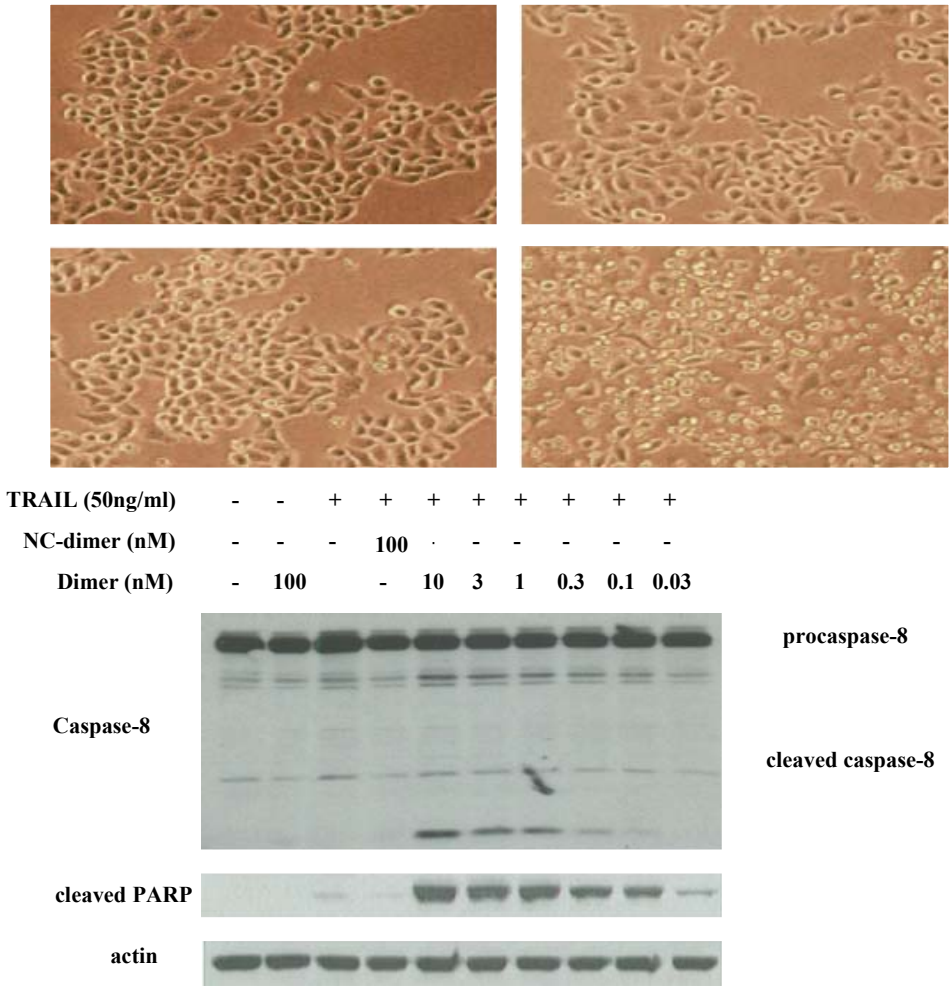

Figure 2.2. Activation of cell death and caspases in response to TRAIL and Smac mimetic in T98G cells.

Light microscopy images of T98G cells treated with: mock (left upper), TRAIL 50ng/ml (right upper), Smac mimetic 1uM (right lower) and TRAIL 50ng/ml in combination with Smac mimetic 100nM (right lower).

Lower western blot analysis: T98G cells were treated with TRAIL (50 ng/ml) alone or Smac mimetic (100nM) alone (for 8 and 12 hours, respectively) or were treated first with various concentrations of Smac mimetic for 4 hours and then with TRAIL for 8 hours. Cell extracts were prepared and subjected to Western blot analysis with the use of antibodies specific for caspase 8, PARP and actin.

Control compound 4 has no effect in this assay. Importantly, unlike the situation in cancer cells, compound 3 alone at 10 μM or in combination with TRAIL had no detectable effects on primary cultures of human skin fibroblasts (figure 2.3).

To verify that dimer 3 targets IAPs, a biotinylated variant was synthesized. Although the display and spacing of monomers within this construct differs slightly from compound 3, this molecule functioned equally well to relieve IAP inhibition of caspase 3 in Hela cell extracts. When biotinylated 3 was added to T98G cell extracts and recovered with streptavidin beads, Western blots of associated proteins showed the presence of XIAP, cIAP1, and cIAP2. Pre-incubating T98G cells with excess dimer 3 blocked these affinity purifications (pull-downs), and a biotinylated control had no detectable IAP affinity. These results suggested that compound 3 facilitates TRAIL-induced apoptosis by neutralizing the effects of multi-BIR domain containing IAPs.

The Smac mimetic, Dimer 3, design, synthesis and activity to synergize with TRAIL and TNFα was published in Science magazine issue September 3rd 2004. Around this time, all of us, in the laboratory of Dr Xiaodong Wang, were excited by a surprising phenomenon. Dr. Lin Li observed that some cancer cells she had tested were sensitive to Smac mimetic alone. This was an unexpected activity of Smac mimetic, since from our understanding of how Smac functions

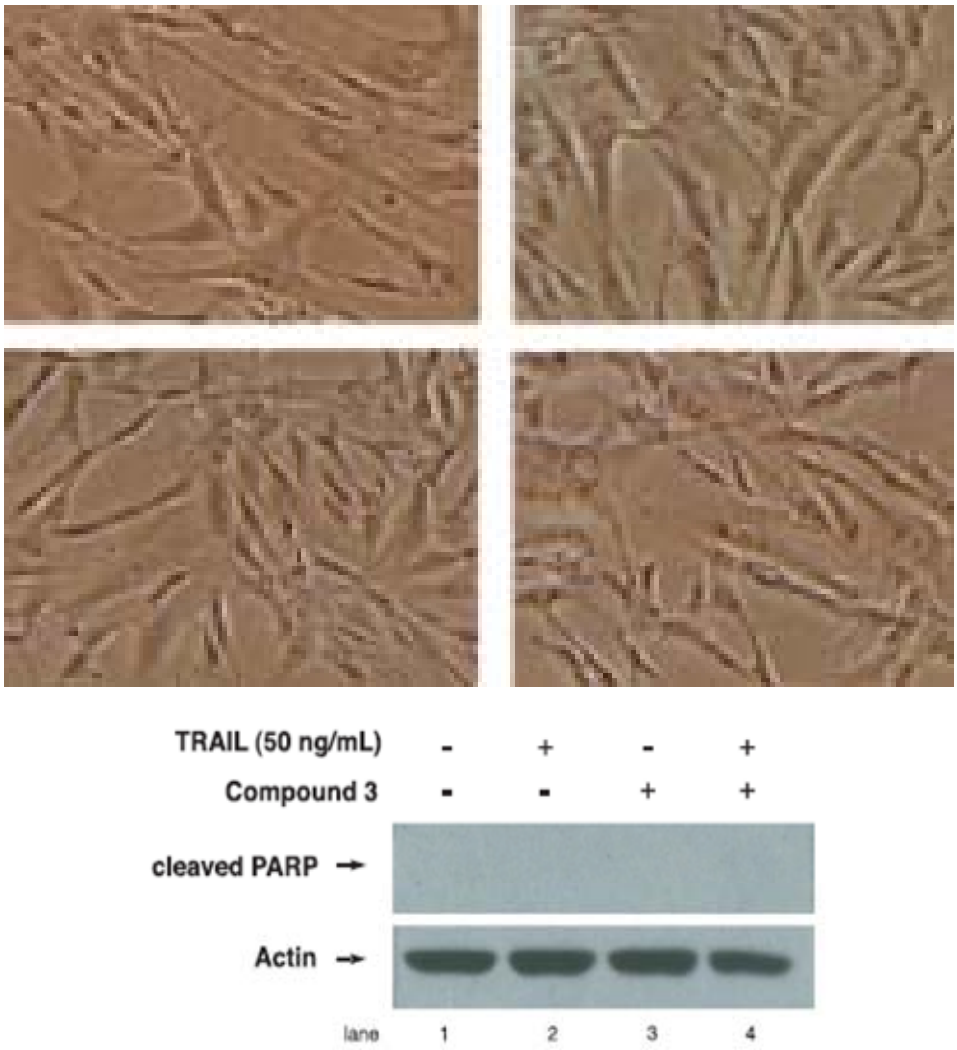

Figure 2.3. Activation of cell death and caspases in response to TRAIL and Smac mimetic in primary human fibroblast cells

Light microscopy images of primary human fibroblast cells treated with: mock (left upper), TRAIL 50ng/ml (right upper), Smac mimetic 10uM (right lower) and TRAIL 50ng/ml in combination with Smac mimetic 10uM (right lower).

Lower western blot analysis: primary human fibroblast cells were treated with TRAIL (50 ng/ml) alone or Smac mimetic (10uM) alone (for 8 and 12 hours, respectively) or were treated first with Smac mimetic for 4 hours and then with TRAIL for 8 hours. Cell extracts were prepared and subjected to Western blot analysis with the use of antibodies specific for PARP and actin.

this activity was unattainable unless there already was activated caspases in these cancer cells. At this point, I became intrigued by the question: what is the mechanism of Smac mimetic induced cell death?

2.2 An unbiased approach for identification of targets of Smac mimetics

Dr. Lin Li found that of twenty cell lines she tested, approximately 25%, were sensitive to Smac mimetic alone. The IC50 values for these sensitive cell lines ranged between 40 - 1000nM. MDA-MB 231 breast cancer cells had the highest sensitivity with 40nM (figure 2.4). Around four hours after Smac mimetic treatment, caspase 8, 9 and 3 were all activated (figure 2.5). Moreover, we could observe activated caspases at 1nM of Smac mimetic (figure 2.6). As I mentioned earlier, the only way for Smac (or Smac mimetic) to exert its function is to have activated caspases which are inhibited by IAPs. This was not the case, since we did not observed any active caspases (figure 2.5, lane 1).

The aim of my project was to understand the mechanism of Smac mimetic induced cell death and to explore new proteins and pathways involved in Smac mimetic induced cell death. To attack this question we decided to take two approaches. First we wanted to utilize the biotinylated Smac mimetic compound to do unbiased pull-downs to compare the differences between sensitive and resistant cells biochemically. Secondly, we could utilize RNAi technique against different known components of the apoptotic pathways to determine which component is responsible for sensitivity to Smac dimer.

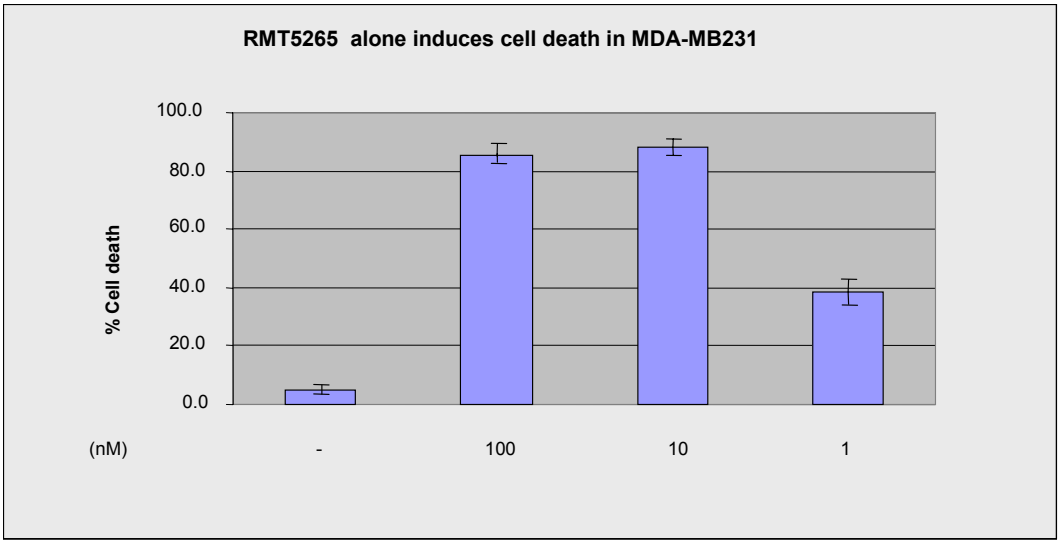

Figure 2.4 Smac mimetic alone induces cell death in MDA-MB 231 cells.

MDA-MB231: breast cancer cells were treated with the compound at the indicated concentrations of Smac mimetic (RMT5265) for 24 hours. Cell death was measured by Methylene blue staining.

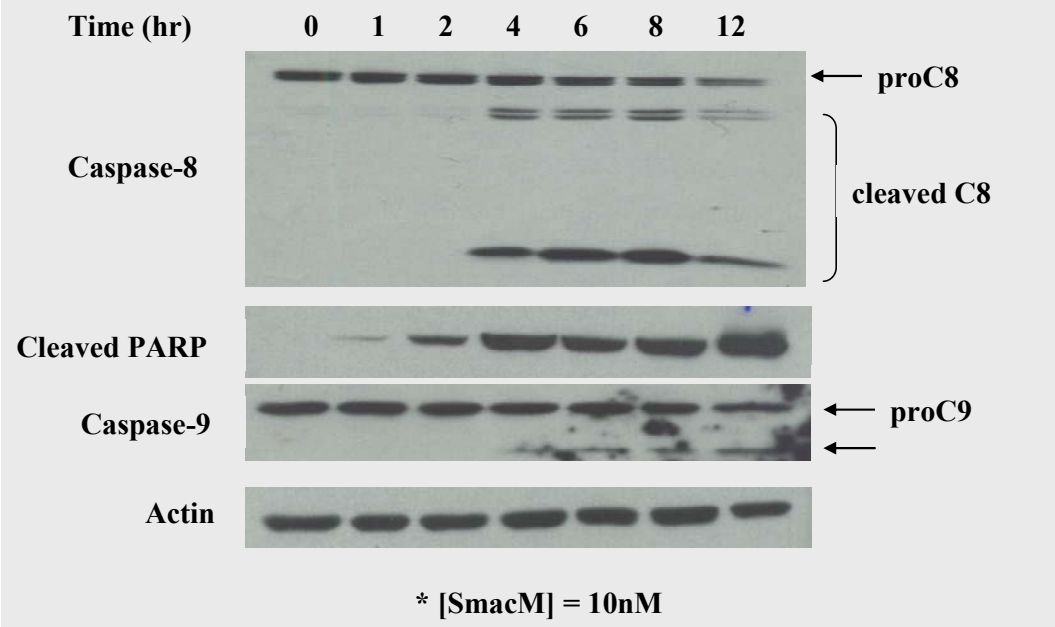

Figure 2.5. Smac mimetic alone induces caspase activation in MDA-MB 231 cells

MDA-MB 231 cells were treated with Smac mimetic (100nM) alone for 1, 2, 4, 6, 8 and 12 hours, respectively. Cell extracts were prepared and subjected to Western blot analysis with the use of antibodies specific for caspase 8, PARP, caspase 9 and actin.

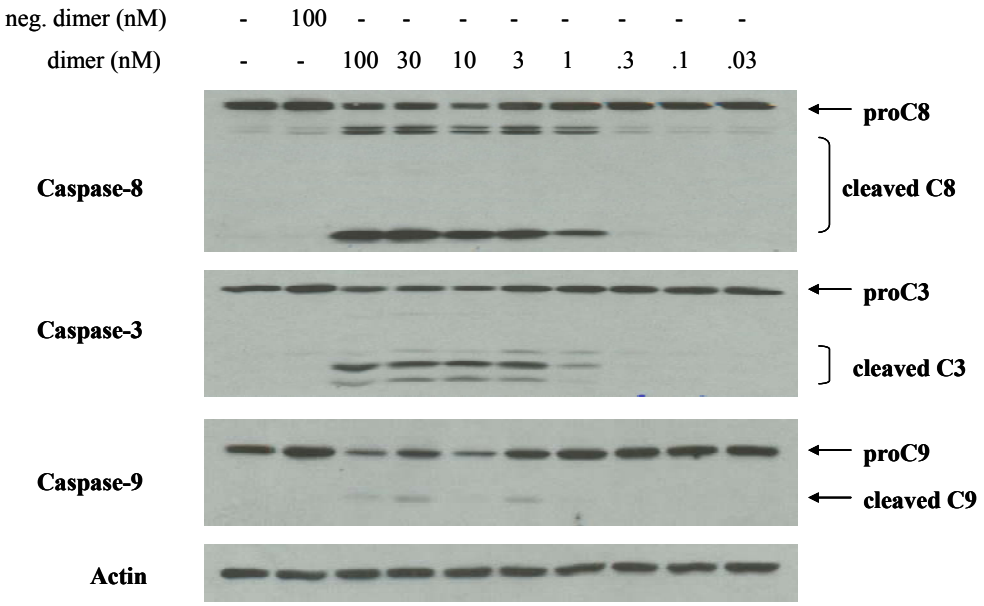

Figure 2.6. Smac mimetic alone induces caspase activation in MDA-MB 231 cells

MDA-MB 231 cells were treated with Smac mimetic at 100nM, 30nM, 10nM, 3nM, 1nM, 0.3nM, 0.1nM and 0.03nM alone for 8 hours. Cell extracts were prepared and subjected to Western blot analysis with the use of antibodies specific for caspase 8, caspase 3, caspase 9 and actin.

2.2.1 In vitro pull-down assays

The initial pull-down experiments were performed by Dr. Lin Li and she was able identify that Smac mimetic (SM) can bind to the known binding partners of Smac namely XIAP, cIAP1 and cIAP2 by western blot analysis. However at this point one of my suspicions was there might be an additional target/targets of Smac mimetic in these sensitive cells making them sensitive to such low doses of Smac mimetic alone treatment. For the initial pull-downs I used resistant cells such as T98G or Hela. However, there was a quick realization that because these cells have totally different backgrounds, identifying differential bands would be very difficult and may give too many false positives. For this reason I took the cells most sensitive to SM, MDA-MB231 cells, and selected them using SM to see if I could make a resistant cell line. I slowly increased the dose of SM from 1nM up to 10uM. Eventually, resistant (R) MDA-MB231 clones were identified. Because they were derived from the same background, they could help identify true positives when compared with the parental cells (M cells, for sensitive parental MDA-MB231 cells). One caveat for doing such a drug-selection is instead of inducing these cells to evolve to be resistant (selecting mutants); you could be selecting individual cells within the heterogeneous tumor cell line. In the latter case one might not necessarily see a SM related survival.

Furthermore, to identify all potential targets of Smac mimetic I needed to optimize the biotinylated Smac mimetic pull-down. Since I did not have an idea

about the identity of the target that was causing this sensitivity to SM; the pull-down had to be clean and precise enough to be able to see specific bands on the SDS-PAGE gel when stained with silver stain. This would allow me to cut the specific bands and identify the identity through mass spectrometry analysis. The initial pull-down silver staining using biotinylated Smac mimetic (B-SM) was very dirty. (Figure 2.7).

Not only we could not see any specific bands, but also the efficiency of the pull-down was around 5% (calculated from the amount of XIAP that is depleted from the supernatant) (figure 2.8). I used XIAP as the positive control for the pull-downs since it is the primary target of Smac. To test the effect of differential ionic strength needed for this binding and to increase the efficiency, I tested different detergents and salt concentrations using XIAP interaction as a control. The only condition that had an effect on the binding was 0.1% SDS which completely abolished the binding to XIAP (figure 2.8). Concurrently, we hypothesized that the biotin moiety being to close to the active compound may create a steric hindrance to the binding. Thus, we decided modify the B-SM to expand the linker region which resulted in a new B-SM with a 16 carbon linker chain (figure 2.9).

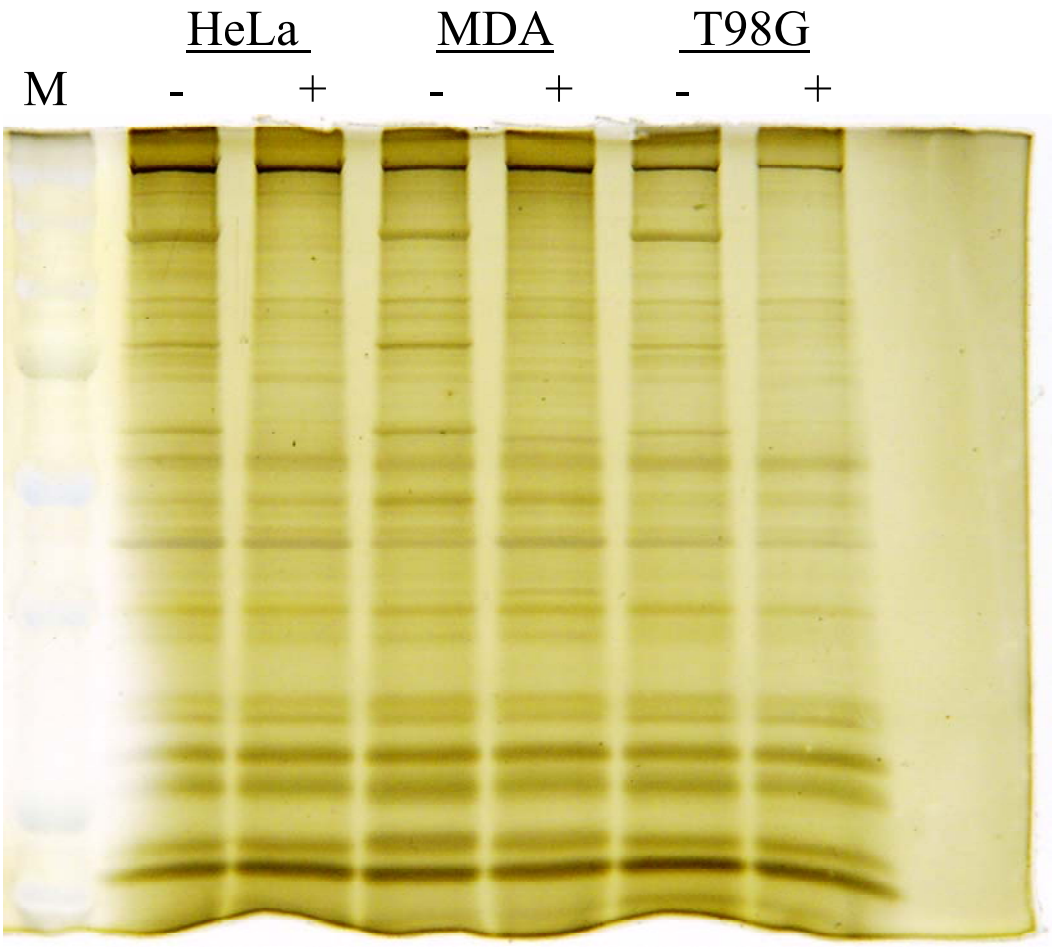

Figure 2.7. Biotinylated Smac mimetic Pull-downs using Hela, MDA-MB 231 and T98G cells.

Biotinylated Smac mimetic was immobilized onto streptavidin-conjugated beads and incubated with Hela, MDA-MB 231, and T98G cell extracts. The recovered beads were boiled, and released proteins were resolved by gel electrophoresis. The gel was silver stained and photographed. Lane 1 Protein size marker, 2, 4, 6 precipitations using a negative control compound. Lane 3, 5 and 7 precipitation using biotinylated Smac mimetic.

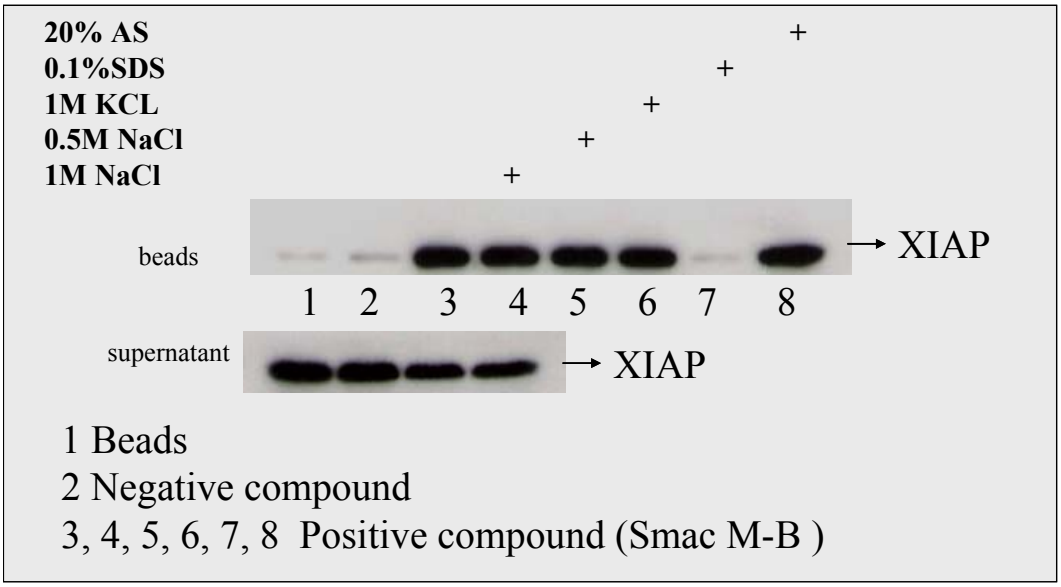

Figure 2.8. SDS can abolish XIAP-Smac mimetic interaction.

Biotinylated Smac mimetic (RMT5265B) was immobilized onto streptavidin-conjugated beads and incubated with MDA-MB 231 cell extracts in presence of 1M NaCl, 0.5M NaCl, 1M KCl, 0.1 % SDS and 20% ammonium sulfate. The recovered beads were boiled, and released proteins were resolved by gel electrophoresis. The membrane was probed with antibody against XIAP.

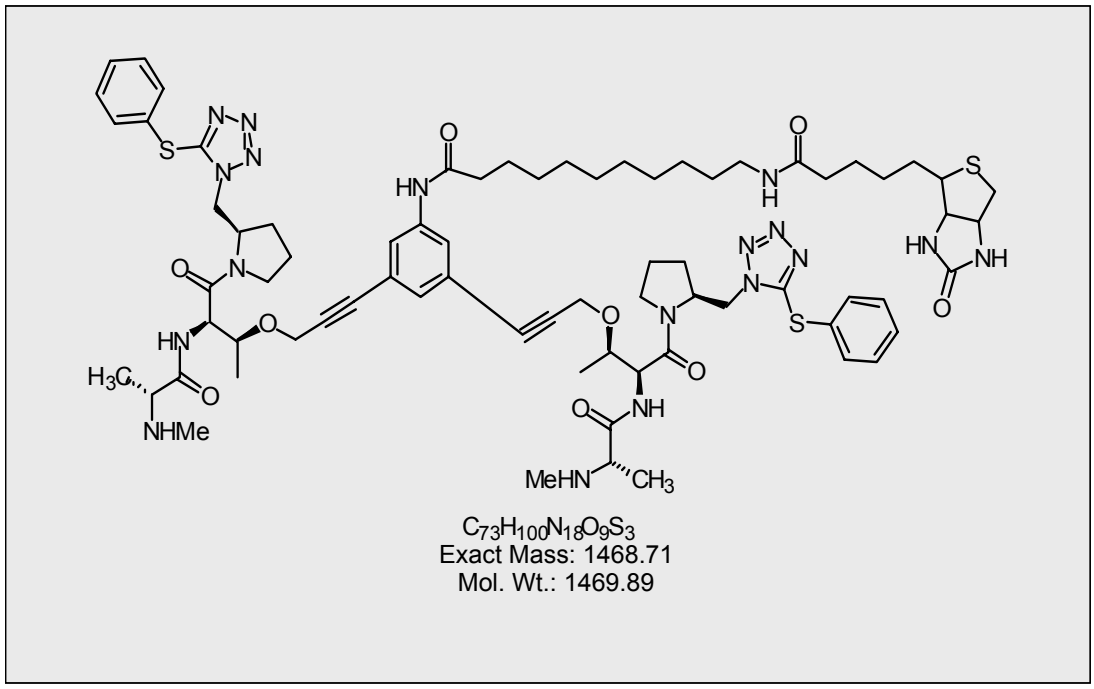

Figure 2.9. Structure of Smac mimetic-Longer linker

Chemical structure of Biotinylated Smac mimetic dimer 3 with modified longer linker (LL).

I tested the activity of the new B-SM in the caspase 3 *in vitro* assay to determine how it compared to the SM dimer. B-SM LL (longer linker) performed as well as SM dimer in the caspase 3 assay (figure 2.10). After testing the efficiency of the new B-SM we were excited to see that all (100%) of XIAP was depleted from the whole cell lysate (WCL) (figure 2.11).

During the synthesis of the new B-SM, we experimented with ways to improve the pull-down. We devised a protocol to increase specificity that included pre-clearing the lysate, diluting the lysate concentration, and finally eluting with the excess non-biotinylated SM. There were only two bands visible in the silver stain gel, one was around 57kD and the other was around 70kD (figure 2.12). I cut these two bands and send them to sequencing (mass spectroscopy). The identified proteins from the 57kD band were XIAP, TRAF1 and TRAF2. The 70kD band was identified to contain both cIAP1 and cIAP2. Unfortunately, both bands were present in sensitive and resistant MDA-MB231 cell experiments.

Meanwhile in collaboration with Drs. John Minna and Mike Peyton we screened 50 lung cancer cell lines for the SM alone cell death activity. Dr. Mike Peyton found that when a cut off dose of 1uM was selected approximately 22% of the lung cancer cells were sensitive to SM (figure 2.13). Three of these cell lines had equal or better IC50 values than MDA-MB231 cells. Cell lines H2126 and

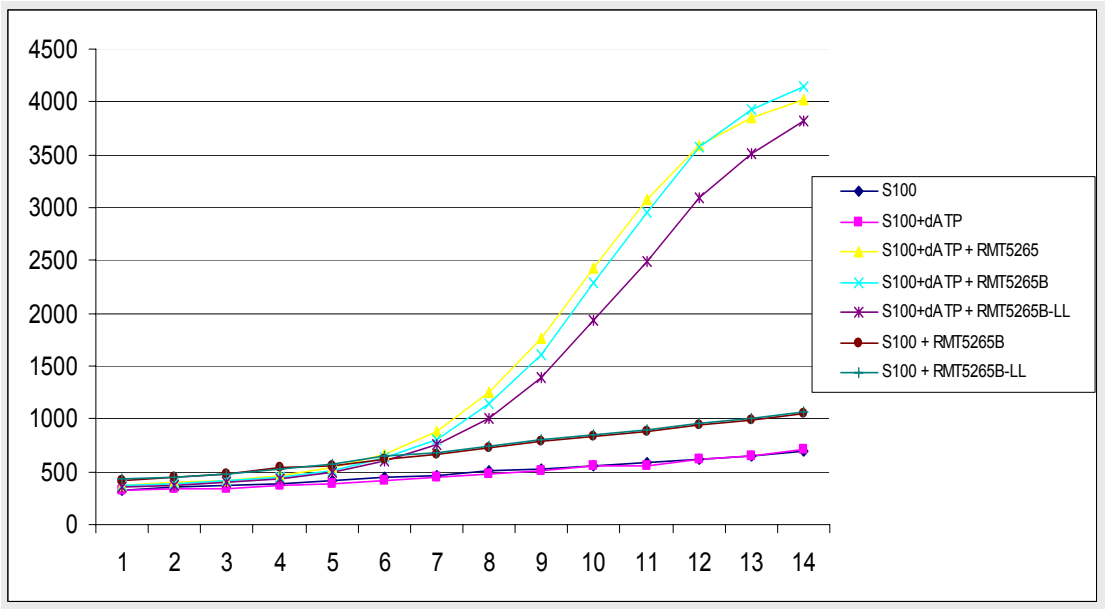

Figure 2.10. Longer linker biotinylated Mimetic (RMT5265B-LL) *in vitro* caspase 3 activation assay.

Hela S100 was activated with or without 1 mM dATP and in the presence of 100 nM of either Smac mimetic, biotinylated Smac mimetic, or biotinylated Smac mimetic-longer linker (LL). The activation of caspase 3 was monitored using a fluorogenic substrate (Ac-DEVD-AMC) (y-axis: rfu, relative fluorescence units and x-axis: indicating each measurement point, increments are 10 minutes apart).

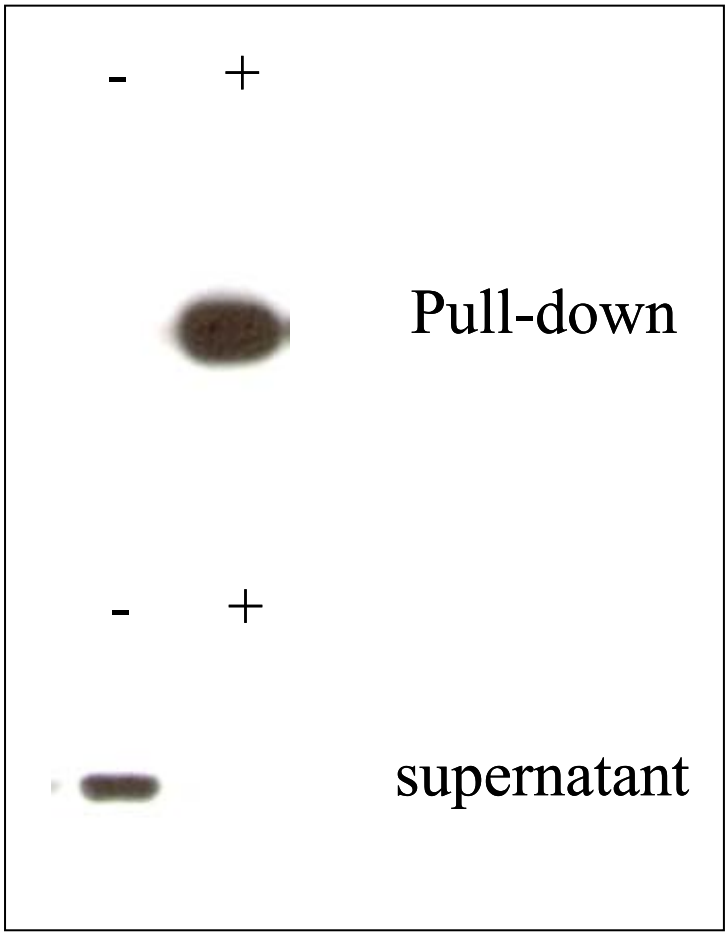

Figure 2.11. Biotinylated Smac mimetic-LL pull-down assays measuring the efficiency.

Biotinylated Smac mimetic-LL was immobilized onto streptavidin conjugated beads and incubated with MDA-MB 231 cell extracts. The recovered beads were boiled and released proteins and supernatants were resolved by gel electrophoresis. The membrane was probed with antibody against XIAP.

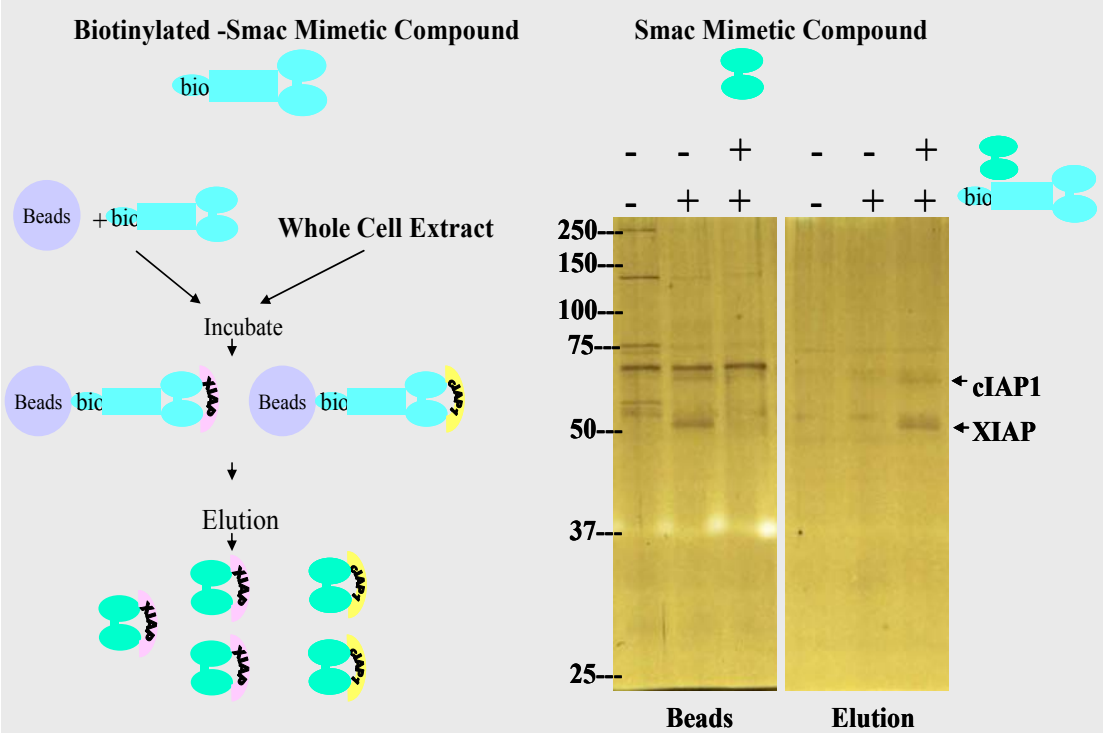

Figure 2.12. Biotinylated Smac mimetic-LL pull-down and elution.

Pull-down assays using biotinylated Smac mimetic-LL and whole cell extracts from MDA-MB 231 cells. First lane negative compound, second and third lane positive compound. Third lane is eluted with compound 3 in excess.

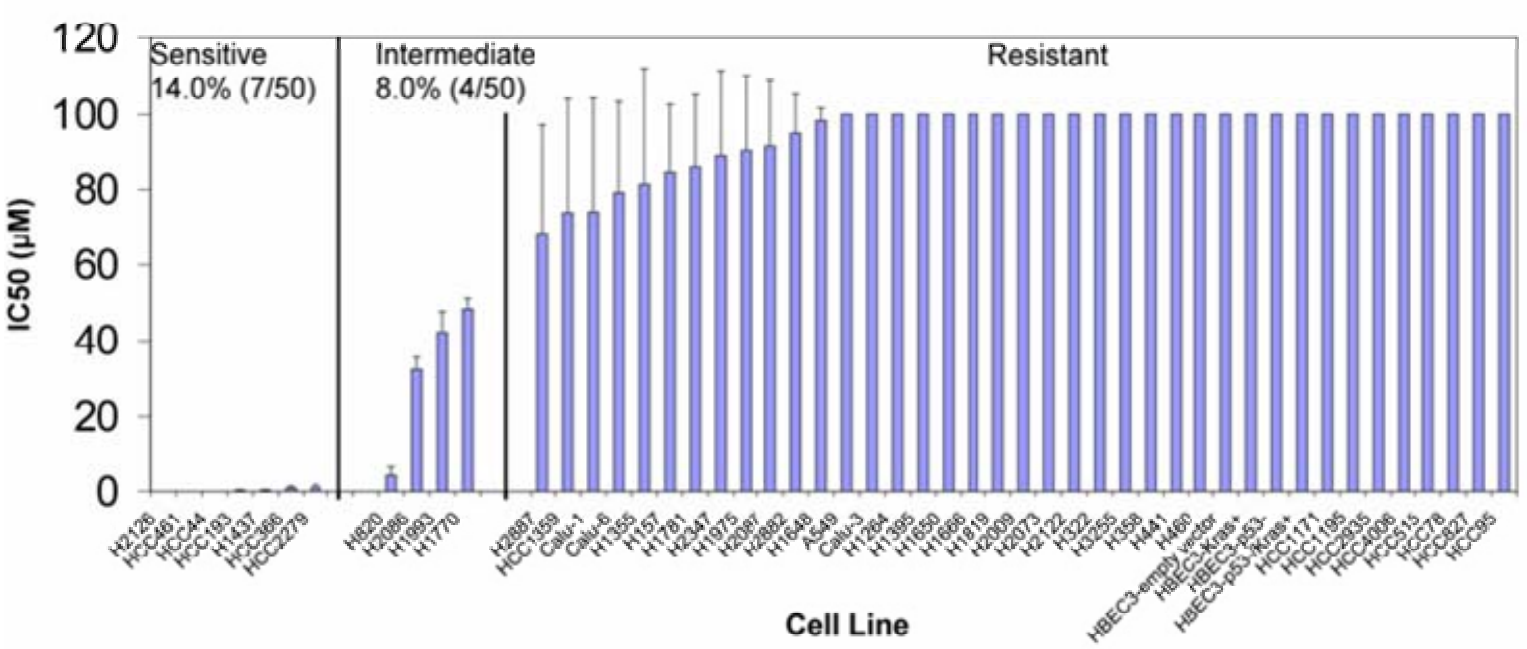

Figure 2.13. Fifty lung cancer cell lines tested for Smac mimetic sensitivity.

A panel of 50 non small cell carcinoma 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 assay.

HCC461 had IC50 values of 6nM; cell line HCC44 had an IC50, which was 40nM. He also observed that 40% of the lung cancer cells that he screened were highly resistant to SM at even 100uM doses. I took the top three most sensitive cells (H2126, HCC461, and HCC44) together with the top three most resistant cells (H2009, HCC827 and HCC515) to compare in my pull-down experiments. Since I could not see a difference between the MDA-MB231 sensitive and resistant cells we decided to switch to lung cancer cells where the difference in the IC50s of the sensitive and resistant cells was over fifteen thousand fold. I first verified that the sensitive cells responded to SM (figure 2.14). I also treated them with SM in combination with pan-caspase inhibitor, Z-VAD, to verify that the cell death we see is apoptosis (figure 2.14). The time course of caspase activation was similar to MDA-MB231 cells (figure 2.15). I repeated the same set of experiments with resistant cells and verified that these cells were in fact resistant to SM alone (figure 2.16). To further characterize the resistant cells, I treated them with SM in combination with TRAIL (figure 2.17). Like Hela and T98G cells H2009, HCC827 and HCC515 also responded to combination treatment, but not to SM alone or to TRAIL alone.

At this moment, we were eager to see the pull-down experiment with the lung cancer cells. We saw an additional band around 38kD size (figure 2.18).

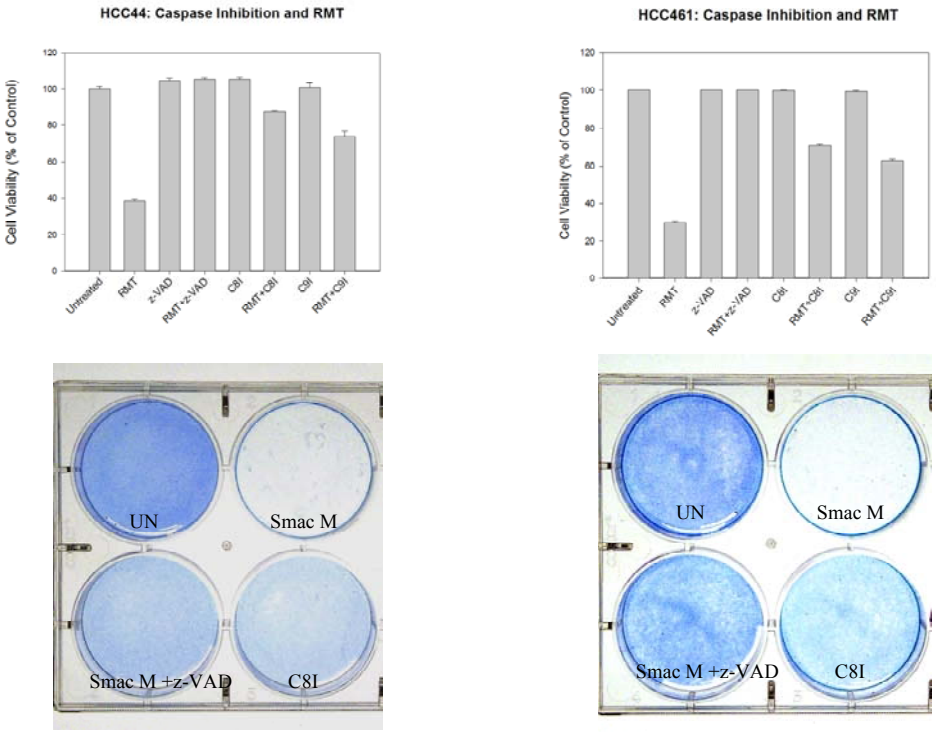

Figure 2.14. HCC44 and HCC461 survival assays
Upper panel: cells were treated with Smac mimetic (RMT), z-VAD, caspase 8 and 9 inhibitors. 24 hours later viability was measured using Cell-Titer Glo assay. Lower panels: cells were treated for 96 hours to assess long term survival of the Smac mimetic treatment and then stained with methylene blue. UN- untreated, RMT- Smac mimetic, z-VAD-Pan caspase inhibitor, C8I-Caspase 8 inhibitor. C9I-Caspase 9 inhibitor

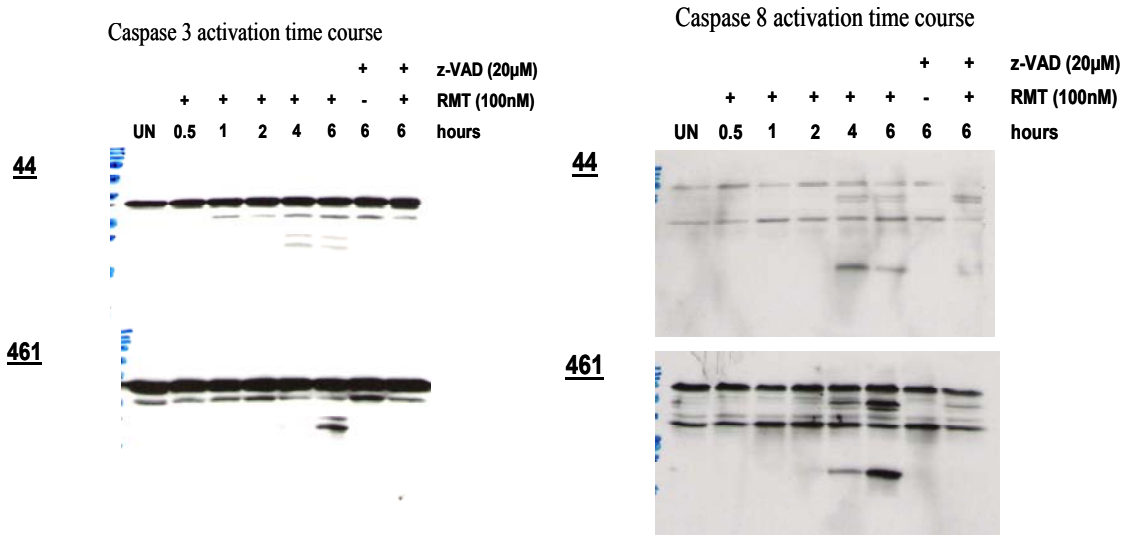

Figure 2.15. HCC44 and HCC461 caspase activation time course

HCC44 and HCC461 cells were treated with 100 nM of Smac mimetic (RMT) for 0.5, 1, 2, 4, and 6 hours, respectively. z-VAD was pretreated 1 hour before addition of Smac mimetic. Cell extracts were prepared and subjected to Western blot analysis with the use of antibodies specific for caspase 8 and caspase 3.

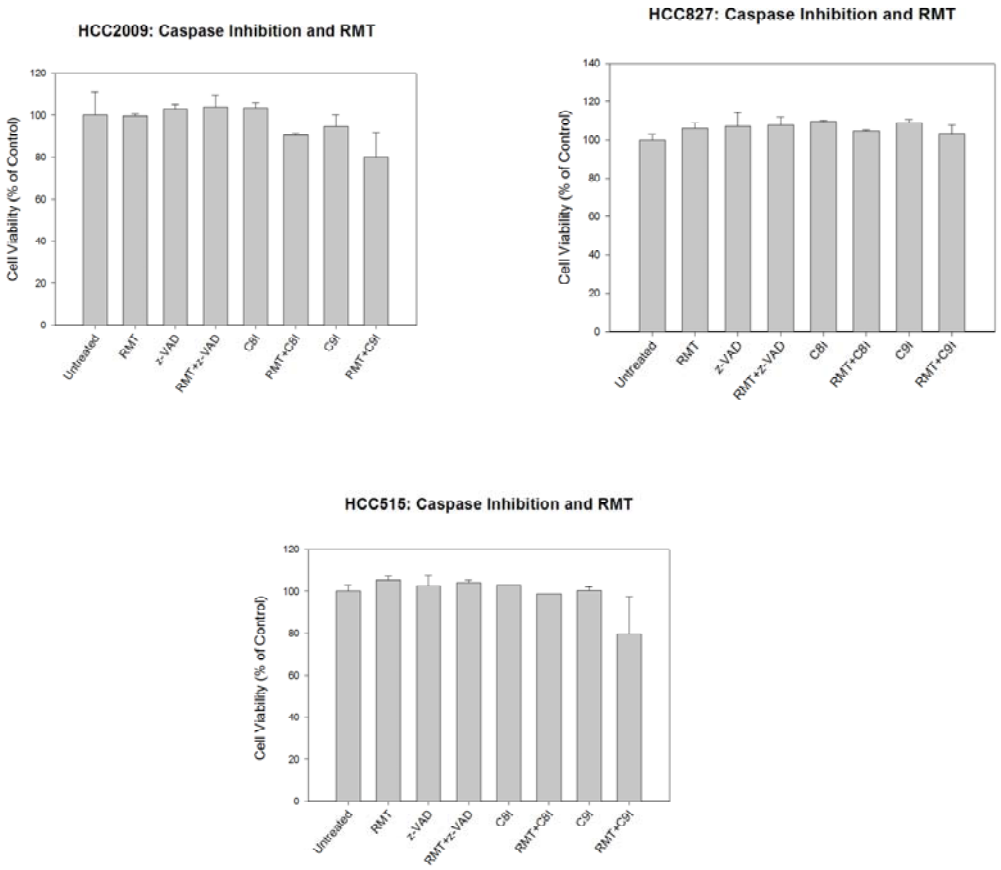

Figure 2.16. HCC515, HCC827 and H2009 survival assays

Cells were treated with Smac mimetic (RMT), Z-VAD, caspase 8 and 9 inhibitors. 24 hours later viability was measured using Cell-Titer Glo assay.

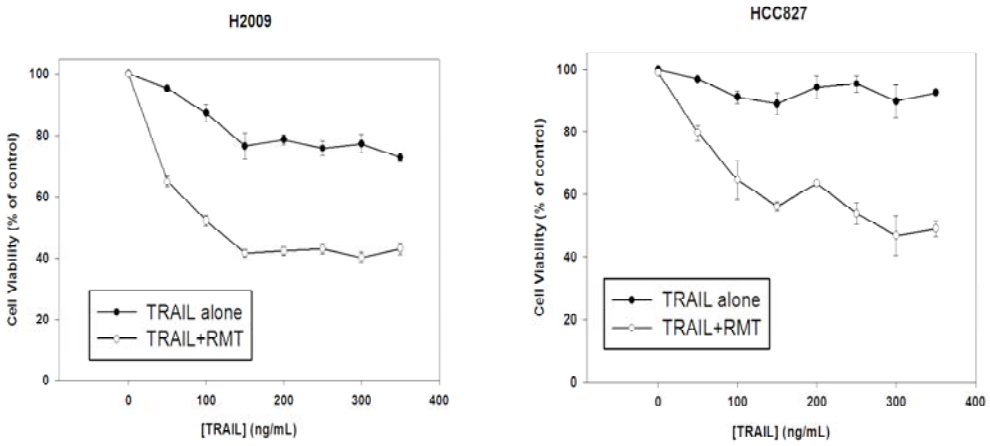

Figure 2.17 Survival assay in H2009 and HCC827 cells upon treatment with Smac mimetic and TRAIL.

H2009 and HCC827 cells were treated with various doses of TRAIL alone or together with Smac dimer 3 for 24 hours. Cell death was quantified by methylene blue staining. Values represent the average of three independent experiments.

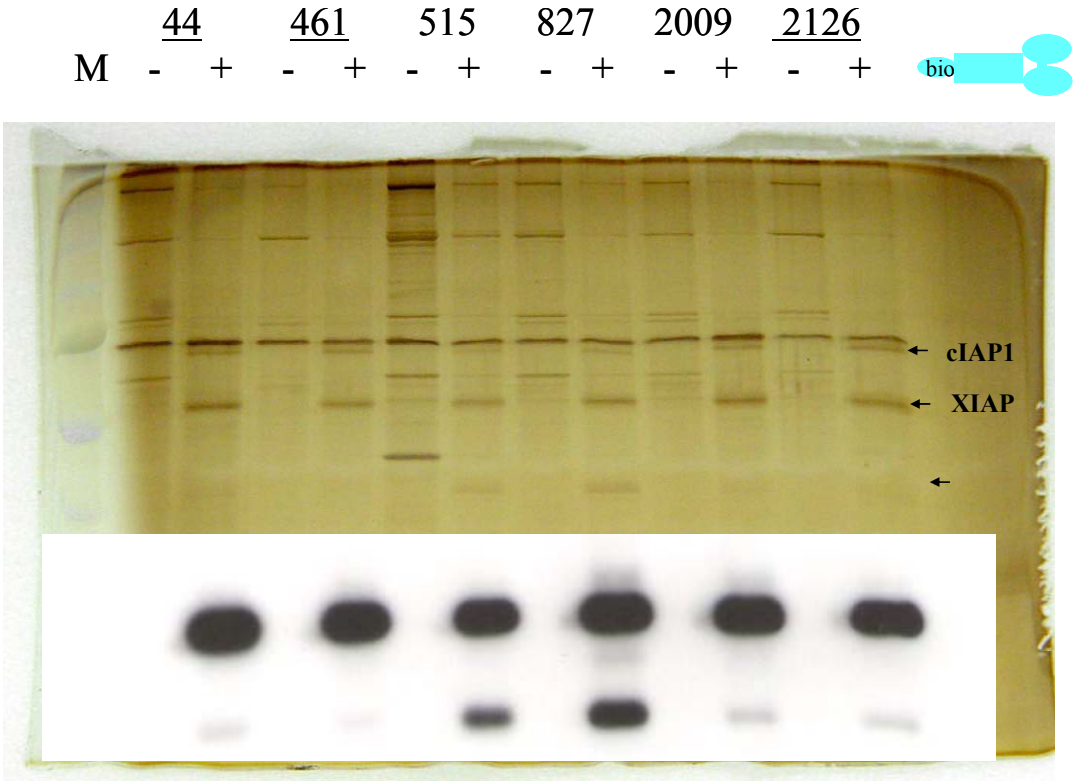

Figure 2.18. Pull-down assays using biotinylated Smac mimetic and whole cell extracts from HCC44, HCC461, HCC515, HCC827, H2009, H2126 cells.

Pull downs are performed either in the presence of negative Smac mimetic compound (-) or Smac mimetic (+). Cell lines underlined are sensitive cells. Top; silver stained PAGE. Bottom; western blot with anti-XIAP antibody.

When western blotted with the XIAP antibody the 38 kD band was visualized again, suggesting that it was a product of XIAP. Sequencing of the 38 kD band verified that it actually was XIAP (table 1). Another interesting observation was that in resistant cells, the 57kD band appeared like a doublet rather than a single band (figure 2.18). To be able to sequence for modifications by mass spectroscopy, i.e. phosphorylation, a larger amount of protein is necessary. For this reason, I optimized conditions to scale up the pull-down assay. When we got back the mass spectroscopy results from the cell lines, it caught our attention that the XIAP in resistant cells had a phosphorylation on Serine 65 residue (table 2). From the results of the mass spectroscopy, we observed several other phosphorylations on TRAF2 and cIAP1, but none of them were specific to sensitive or resistant cells.

To determine if the Serine 65 phosphorylation of XIAP was the reason to sensitivity to SM, we decided to make a phosphorylated Serine 65 polyclonal antibody. We synthesized two peptides corresponding to the phosphorylated and non-phosphorylated versions of the sequence around the Ser 65. The phospho-Serine 65 XIAP antibody was produced in Antibody Core facility of UT Southwestern by Dr Wayne Lai. It was purified through binding and elution from the phosphorylated peptide followed by flow through the non-phosphorylated peptide cross-linked column.

Gel band 38 kD

Sample ID	Protein Identifed
827	IAP-like protein ILP

Gel band 57 kD

Sample ID	Protein Identifed
827	IAP-like protein ILP Chain A, Nmr Structure of A 9 Residue Peptides From SmacDIABLO Baculoviral IAP repeat-containing protein 2 TNF receptor-associated factor 2 Tubulin, beta, 2
2009	IAP-like protein ILP Chain A, Nmr Structure of A 9 Residue Peptides From SmacDIABLO TNF receptor-associated factor 2 Chain F, Structure of Tnf Receptor Associated Factor 2 Baculoviral IAP repeat-containing protein 2 TNF receptor-associated factor 1 Tubulin 5-beta
44	IAP-like protein ILP Chain A, Nmr Structure of A 9 Residue Peptides From SmacDIABLO TNF receptor-associated factor 2 Baculoviral IAP repeat-containing protein 2
461	IAP-like protein ILP TNF receptor-associated factor 2 Chain A, Nmr Structure of A 9 Residue Peptides From SmacDIABLO Baculoviral IAP repeat-containing protein 2 HRPE773 Caspase 14, precursor Lysozyme (E.C.3.2.1.17) Chain H, Cathepsin D at Ph 7.5 Chain B, Human Bleomycin Hydrolase, C73sDELE455 MUTANT heat shock protein 27

Gel band 70 kD

Sample ID	Protein Identifed
827	Baculoviral IAP repeat-containing protein 2 IAP-like protein ILP
2009	Baculoviral IAP repeat-containing protein 2 IAP-like protein ILP
44	Baculoviral IAP repeat-containing protein 2 IAP-like protein ILP
461	Baculoviral IAP repeat-containing protein 2 IAP-like protein ILP

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  1 MTFNSFEGSK TCVPADINKE EEFVEEFNRL KTFANFPSGS PVSASTLARA
 51 GFLYTGEGDT VRCFSCHAAV DRWQYGDSAV GRHRKVSPNC RFINGFYLEN
101 SATQSTNSGI QNGQYKVENY LGSRDHFALD RPSETHADYL LRTGQVVDIS
151 DTIYPRNPAM YSEEARLKSF QNWPDYAHLT PRELASAGLY YTGIGDQVQC
201 FCCGGKLKNW EPCDRAWSEH RRHFPNCFFV LGRNLNIRSE SDAVSSDRNF
251 PNSTNLPRNP SMADYEARIF TFGTWIYSVN KEQLARAGFY ALGEGDKVKC
301 FHCGGGLTDW KPSEDPWEQH AKWYPGCKYL LEQKGQEYIN NIHLTHSLEE
351 CLVRTTEKTP SLTRRIDDTI FQNPMVQEAI RMGFSFKDIK KIMEEKIQIS
401 GSNYKSLEVL VADLVNAQKD SMPDESSQTS LQKEISTEEQ LRRLQEEKLC
451 KICMDRNIAI VFVPCGHLVT CKQCAEAVDK CPMCYTVITF KQKIFMS
```

Table 2.1. Mass Spectroscopy results from the *in vitro* pull-down assays

Each band (38, 57, 70 kD) was sequenced and identified proteins were listed in this table (top panels).
XIAP sequenced peptides are shown as example in the bottom panel.

Gel band 57 kD

Sample ID	Protein Identifed	Phosphopeptides
827	IAP-like protein ILP	TCVPADINKEEEFVEEFNR CFSCHAAVDR
2009	IAP-like protein ILP	TCVPADINKEEEFVEEFNR CFSCHAAVDR
44	IAP-like protein ILP TNF receptor-associated factor 2	TCVPADINKEEEFVEEFNR FHAIGCLETVEGEK

Gel band 70 kD

Sample ID	Protein Identifed	Phosphopeptides
827	Baculoviral IAP repeat-containing protein 2	MKYDFSCELYR LGDSPIQK EIDSTLYK
2009	Baculoviral IAP repeat-containing protein 2	MKYDFSCELYR YDFSCELYR EIDSTLYK
44	Baculoviral IAP repeat-containing protein 2	MKYDFSCELYR YDFSCELYR LGDSPIQK EIDSTLYK
461	Baculoviral IAP repeat-containing protein 2	MKYDFSCELYR LGDSPIQK

Table 2.2. Identified Phospho-proteins.

Phospho-proteins identified in the *in vitro* pull-down assays. (Baculoviral IAP 2: cIAP1, IAP like protein ILP: XIAP and TNF receptor associated factor 2: TRAF2.)

Unfortunately, when I performed western blot analysis of the same samples, we found out that this phosphorylation also existed in the sensitive cells hence was not the difference we had hoped (figure 19).

2.2.2 In-vivo pull-down assays

One concern we had about these *in vitro* pull downs was they might not be reflective of the endogenous events that resulted in certain cells to be sensitive. Especially, given that SM in combination with TRAIL can induce, at nanomolar doses, death of the cells previously resistant to 100uM doses of SM. This was the first clue that the extrinsic pathway was involved in the SM sensitivity. However, using *in vitro* pull down in this scenario may never lead us to identify the target of SM. This idea made us wonder if this B-SM will be able to get inside the cell and if so, could these pull downs be performed *in vivo* to identify the *in vivo* targets.

The 1.47 kD B-SM was able to pass through the plasma membrane and induce apoptosis with the same dose as SM (100nM). I revised the pull down protocol to *in vivo* conditions. The *in vivo* pull-downs worked and gave us more clues towards identifying the endogenous target of SM (figure 2.20, 2.21, 2.22). The first observation was differential amounts of XIAP were pulled down from different cells, and the trend was more XIAP being pulled down in sensitive cells (table 3). However, in resistant cells, XIAP was still able to bind to SM, and more importantly, one of the sensitive cell lines HCC44, like the resistant cells, had very little XIAP binding.

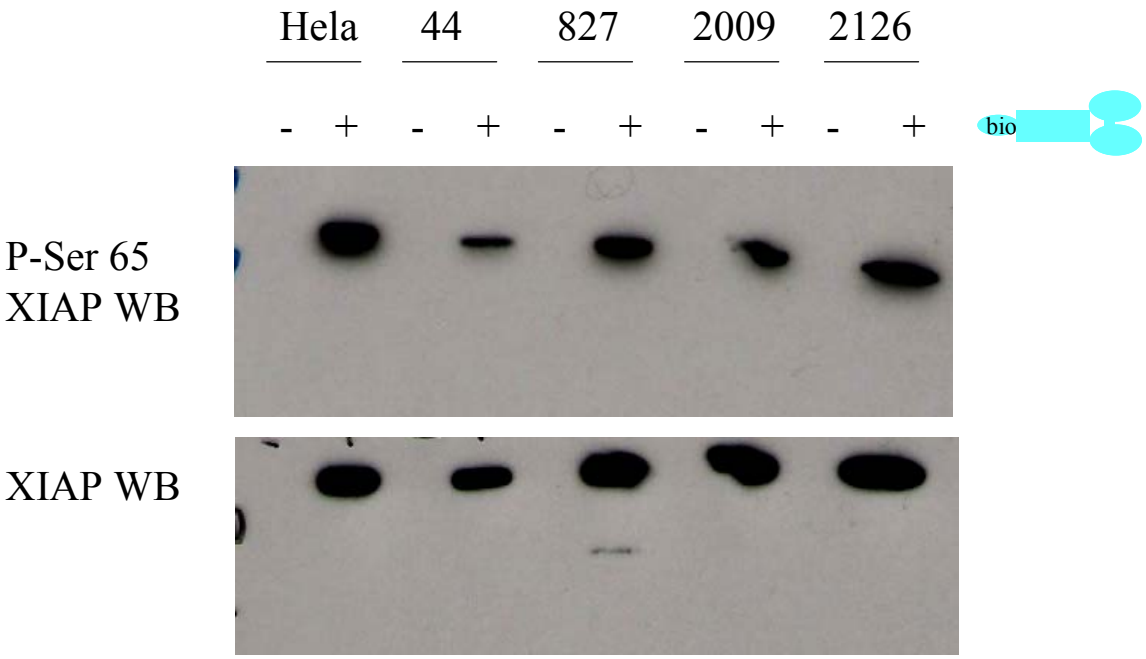


Figure 2.19 Phosphorylation of XIAP serine 65.

Samples from pull-downs were re-run and western blotted with anti-phospho-ser65-XIAP and XIAP antibodies.

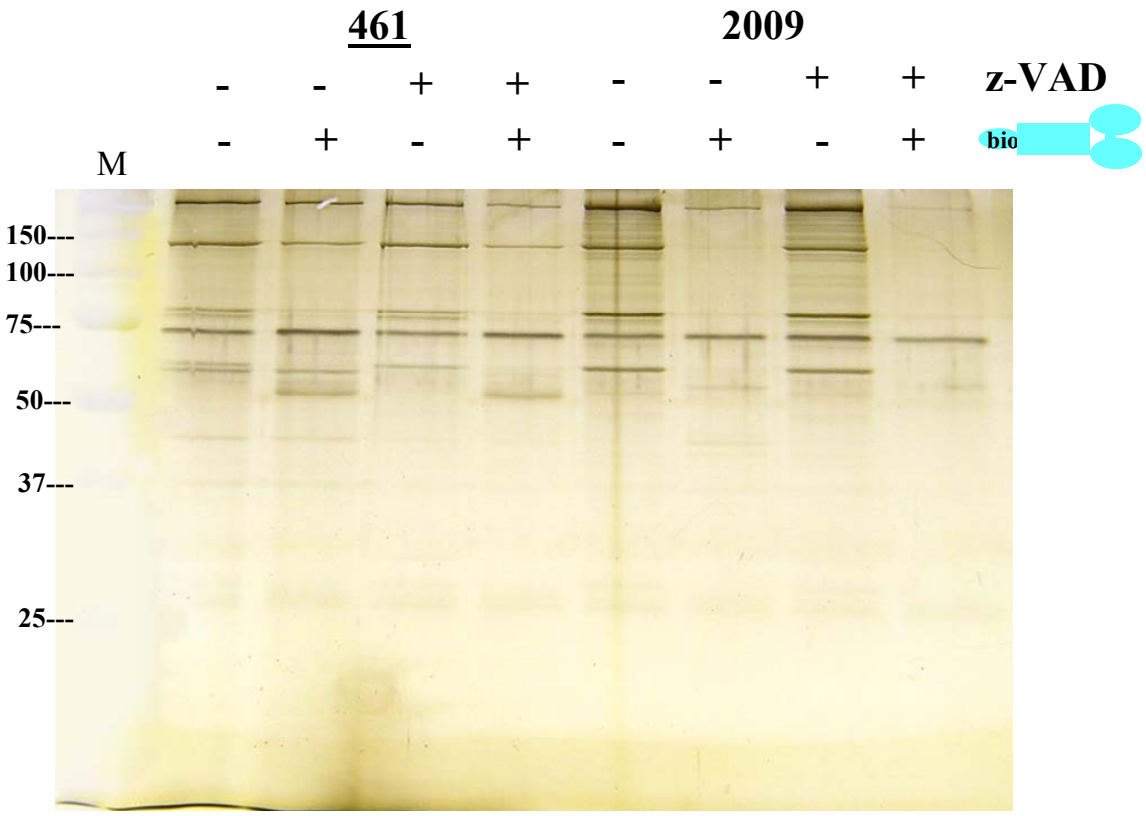


Figure 2.20. *In vivo* pull-down assays in HCC461 and H2009 cells.

Cells were treated either in the absence (-) or presence (+) of biotinylated Smac mimetic and z-VAD. After one hour of treatment, cells were lysed and pull-down assay was performed by adding streptavidin beads to the lysate. Underlined is a sensitive cell

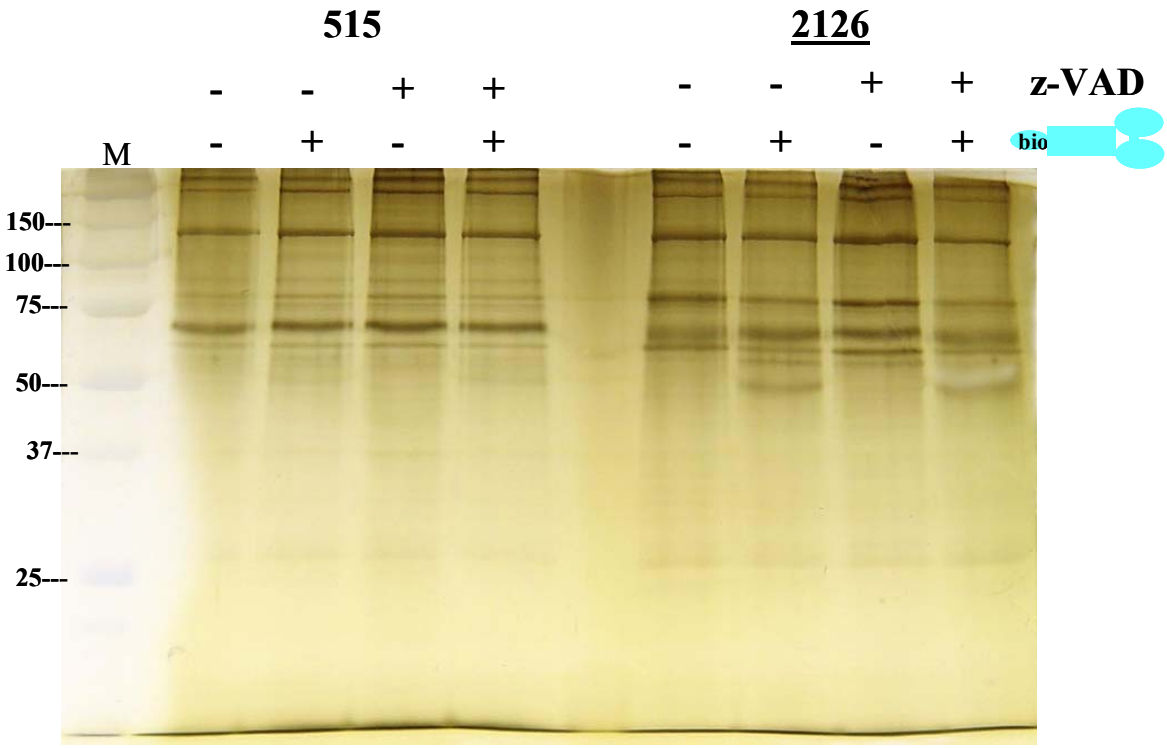


Figure 2.21. *In vivo* pull-down assays in HCC515 and H2126 cells.

Cells were treated either in the absence (-) or presence (+) of biotinylated Smac mimetic and z-VAD. After one hour of treatment, cells were lysed and pull-down assay was performed by adding streptavidin beads to the lysate. Underlined is a sensitive cell

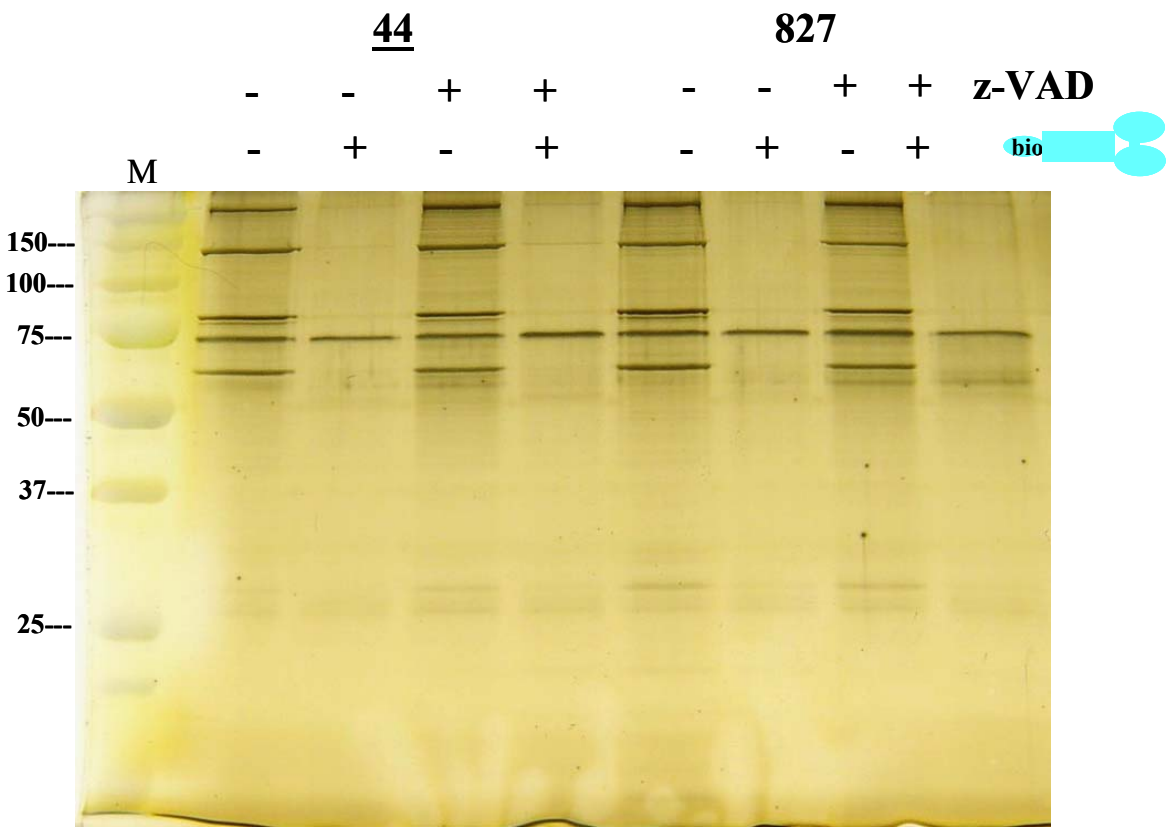


Figure 2.22. *In vivo* pull-down assays in HCC44 and HCC827 cells.

Cells were treated either in the absence (-) or presence (+) of biotinylated Smac mimetic and z-VAD. After one hour of treatment, cells were lysed and pull-down assay was performed by adding streptavidin beads to the lysate. Underlined is a sensitive cell

cell line	44	461	2126	515	827	2009
XIAP	+	+	+	+	+	-

Table 2.3. Summary of *in vivo* pull-downs

XIAP binding was assigned relative units; big plus sign for high, small plus sign for less and minus sign represents very little binding.

We concluded that this small difference can not account for the more than fifteen thousand fold difference in sensitivity. The second observation was that there was no cIAPs pulled down *in vivo*. At this point of time, we did not know why cIAPs were not interacting with SM *in vivo*, but the answer came soon after.

Dr. Lai Wang, a post-doctoral fellow in our lab, discovered that within fifteen minutes of SM treatment cIAP1 disappeared completely (figure 2.23). Levels of cIAP2 also reduced, but not to the same extend as cIAP1. Using recombinant cIAP1 and cIAP2 he was able to show that SM can induce ubiquitination and degradation of cIAPs. This degradation of cIAPs upon SM treatment was blocked by ring domain mutants, which were deficient in E3 ubiquitin ligase activity. Hence this was an auto-ubiquitination. The degradation of cIAPs also could be blocked by a proteasome inhibitor, MG-132, suggesting that the proteasome pathway was involved. Smac protein has been known to induce cIAP auto-ubiquitination and degradation upon binding. SM *in vivo* was causing the same effect on cIAPs as Smac protein itself. This result once again reinforced that SM is a true Smac mimicking compound.

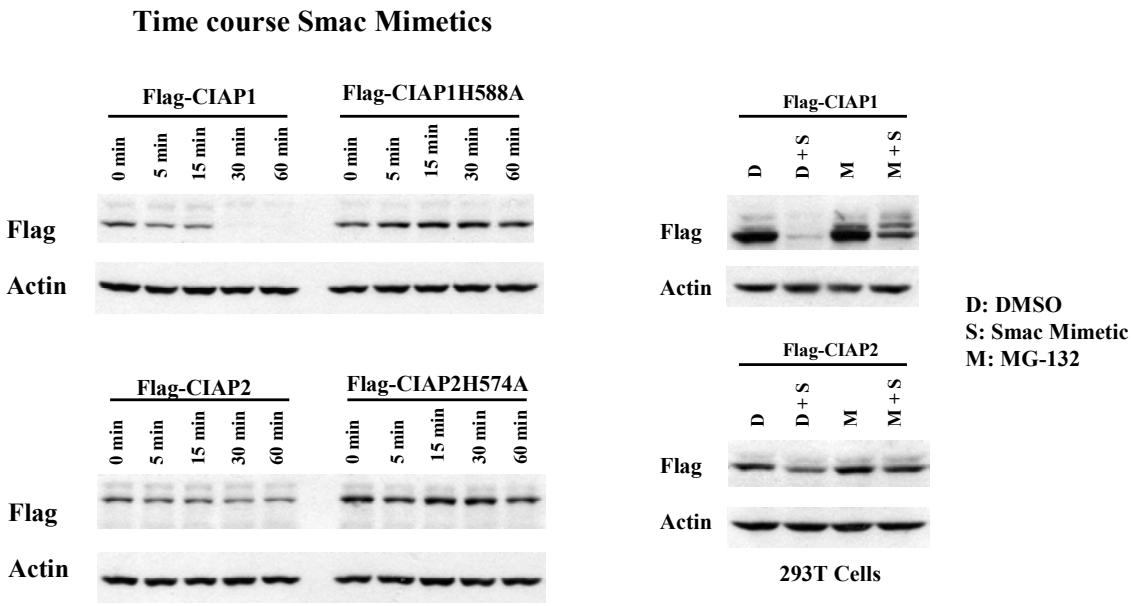


Figure 2.23. cIAP1 and cIAP2 auto-ubiquitination and degradation upon Smac mimetic treatment.

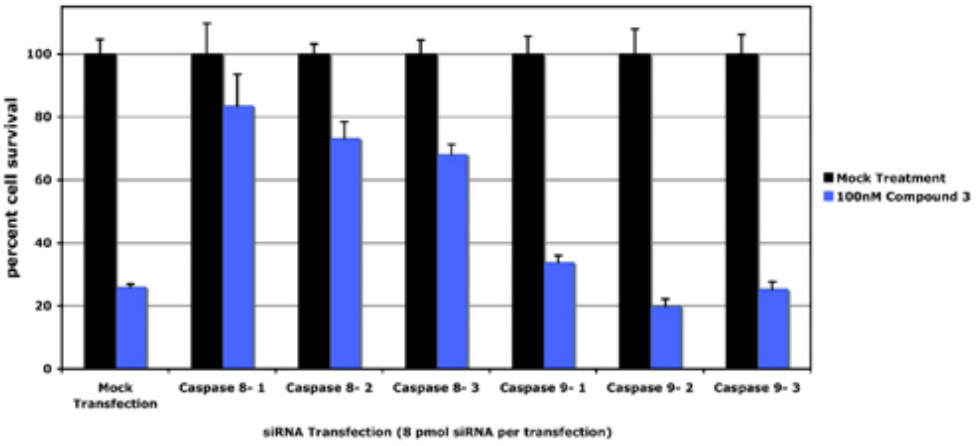
Left panels: HEK-293T cells were transfected with either wild-type or ring finger mutant forms of Flag-cIAP1 or Flag-cIAP2 for 48 hours and then treated with Smac mimetic. Cell lysates were collected at the indicated time points. Flag and β-actin antibodies were used for western-blot analysis.

Right panels: HEK-293T cells were transfected with either wild-type or ring finger mutant forms of Flag-cIAP1 or Flag-cIAP2 for 48 hours and then treated with Smac mimetic and MG-132 as indicated. Cell lysates were collected at the indicated time points. Flag and β-actin antibodies were used for western-blot analysis.

2.3 The RNAi approach: TNF is the key

(The experiments described in this section (2.3) are published, Cancer Cell, 2007)

The reason why some cells were sensitive to SM came from *in vivo* experiments utilizing RNAi. Although previous evidence suggested that SM may be inducing cell death by manipulating the extrinsic pathway, we needed an absolute answer to the question. When we did RNAi against caspase 8 and caspase 9, we realized only caspase 8 knock down (KD) was able to block SM induced cell death (figure 2.24). Caspase 9 KD had no effect on SM induced cell death. At this point, it was clear that the extrinsic pathway was involved, and the only way to determine the target/targets of SM was to use *in vivo* experiments. Since caspase 8 KD could block SM induced cell death and since caspase 8 is essential for all the receptor pathways, we decided to do RNAi to all the death receptors to find out which pathway was involved. Of the death receptor pathways, TNFR1, TRAILR1, TRAILR2 and CD95, only TNFR1 was able to rescue the cell death phenotype (figure 2.25). However, this interesting phenomenon begged the question, how was TNFR1 activation occurring? Consequently to find out how the receptor was activated, we did ELISA to test the media collected from the cell culture plates of sensitive and resistant cells and looked for its ligand TNF. Remarkably, in sensitive cells TNF was found in the media and in resistant cells this was not the case (figure 2.26).



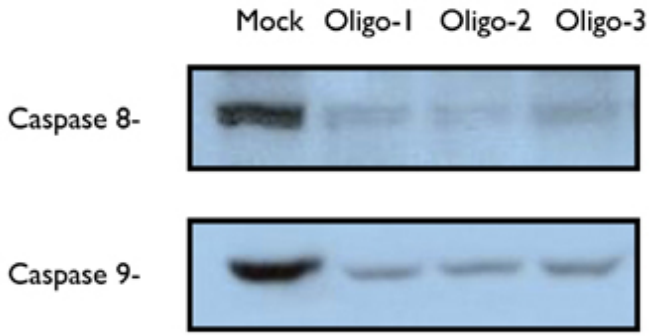


Figure 2.24 siRNA candidate approach to inhibit cell death caused by Smac mimetic.

Top panel: cells were assayed for the ability of particular transiently transfected individual siRNAs to produce a rescue phenotype in HCC461 cells following 100 nM Smac mimetic. Cell viability was determined by measuring Cell-Titer Glo assay.
Bottom panel: efficiency of total protein level knockdown per siRNA was determined by western blot.

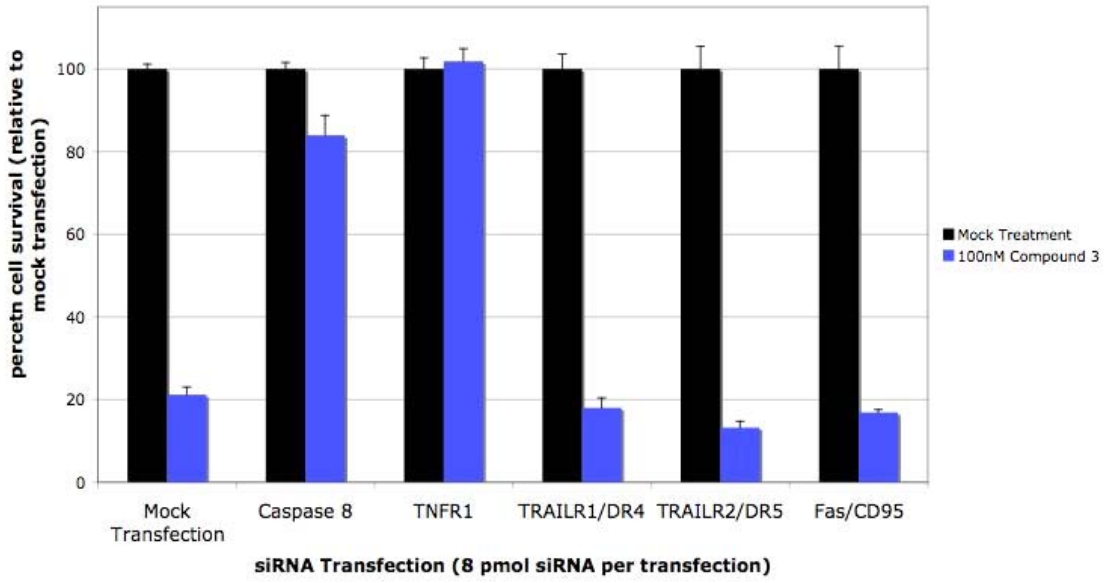


Figure 2.25. siRNA candidate approach to identify the pathway affected by Smac mimetic.

Cells were assayed for the ability of particular transiently transfected individual siRNAs to produce a rescue phenotype in HCC461 cells following 100 nM Smac mimetic. Cell viability was determined by Cell-Titer Glo assay.

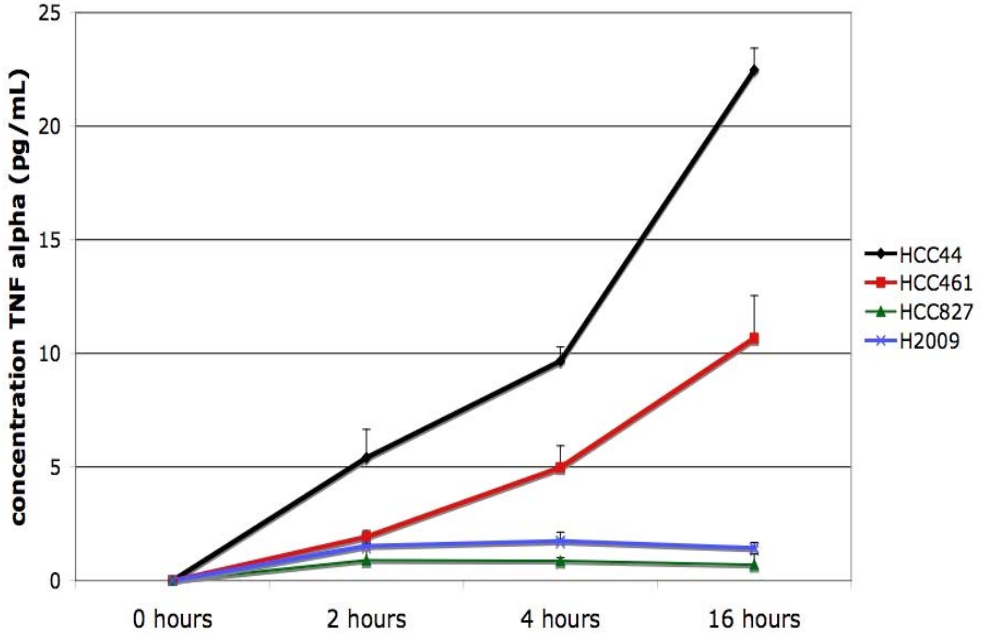


Figure 2.26. Sensitive cells secrete TNF.

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. Samples were removed at the indicated time points and used for quantitative sandwich enzyme immunoassay analysis (ELISA).

This apparent autocrine secretion of TNF was the difference that we had been looking for, explaining why some cells are resistant while others are sensitive to SM. Subsequently, if this were true, adding blocking antibodies against TNF to the media would be able to block this cell death. To test this and confirm that TNF was the only secreted death receptor ligand we added TNF, TRAIL and FasL antibodies to the media. The TNF antibody was the only one that blocked the cell death caused by SM (figure 27).

2.4 Targets of Smac Mimetic

(The experiments discussed in this section (2.4) were performed by Dr. Lai Wang. Figure 28 is my contribution.)

We knew now that SM was targeting the cIAPs in the TNFα pathway. Since cIAP1 and cIAP2 are known to be part of TNFR1 complex, we wanted to determine what downstream effect SM had on the DISC complex. The degradation of cIAPs also existed in the originally categorized resistant cells. The only difference between these cells and the sensitive cells was the TNF auto-secretion. As a result, we re-categorized these once called resistant cells as sensitive cells meaning they were all sensitive to SM treatment; they just needed an exogenous ligand to activate the death pathway (For the sake of simplicity and to avoid confusion these cells will still be referred to as resistant cells).

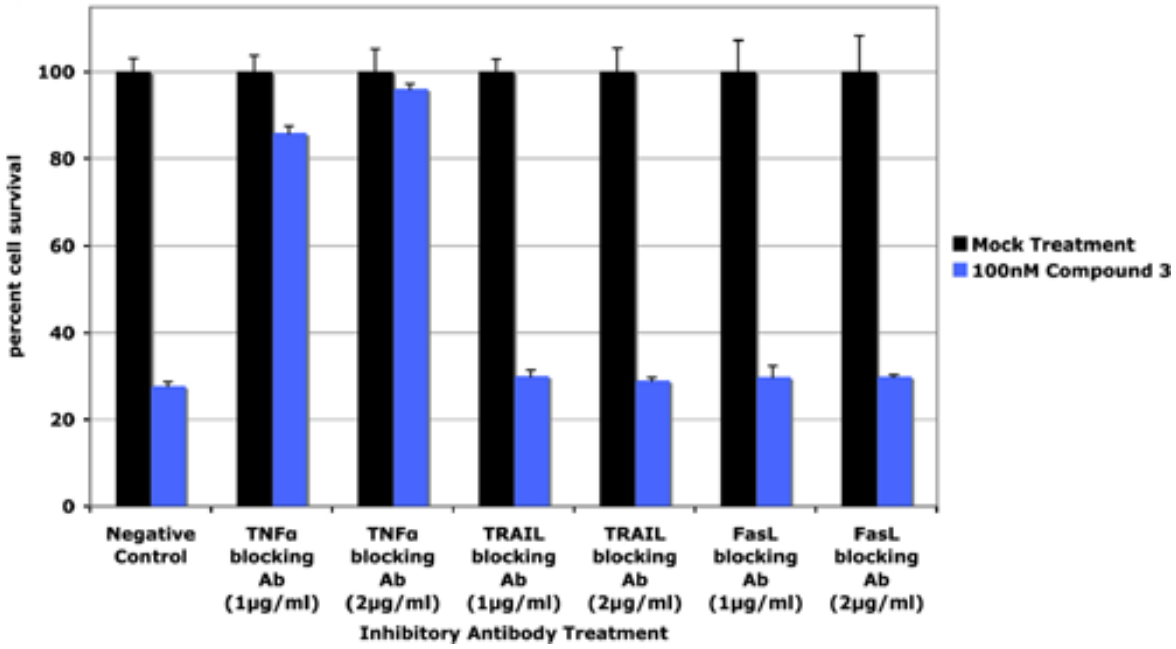


Figure 2.27. Treatment with blocking antibody to TNFα blocks Smac mimetic induced cell death.

Pretreatment (1 hr) of neutralizing antibodies (1–2 ug/mL) against TNFα (R&D Systems), TRAIL (Biolegend), and FasL/CD95 (Biolegend) prior to 100 nM compound 3 treatment for 24 hours. Cell viability was determined with Cell Titer Glo.

As discussed in Chapter 1, TNFα signals for survival and inflammatory responses; thus, for SM to induce a death signal through the TNF pathway, it must have been inducing the formation of the caspase 8 complex (DISC). When we immunoprecipitated the caspase 8 complex we saw that it was formed in the cells treated with SM or SM in combination TNFα in sensitive and resistant cells respectively. To see the specific effect of SM on this complex and to better control the dosing of TNF we decided to study the complex formation events in resistant cells.

As expected around two hours after SM and TNFα treatment, caspase 8 complex formed (figure 2.28). To mimic the effect of SM, we did RNAi to the cIAP1, cIAP2 and cIAP1/2. Following the siRNA transfections, we immunoprecipitated the caspase 8 complex. Only the cIAP1/2 KD cells were able to form the caspase 8 complex, with TNF alone treatment (figure 2.29). Although cIAP1/2 seemed like the only targets of SM from the immunoprecipitation experiments, when we tried to mimic the SM effect in cell viability assays, we realized that inhibition of all three IAPs: XIAP, cIAP1 and cIAP2 was necessary. Only when we did a triple knockdown of all three IAPs could we mimic the cell death phenotype caused by SM (figure 2.30).

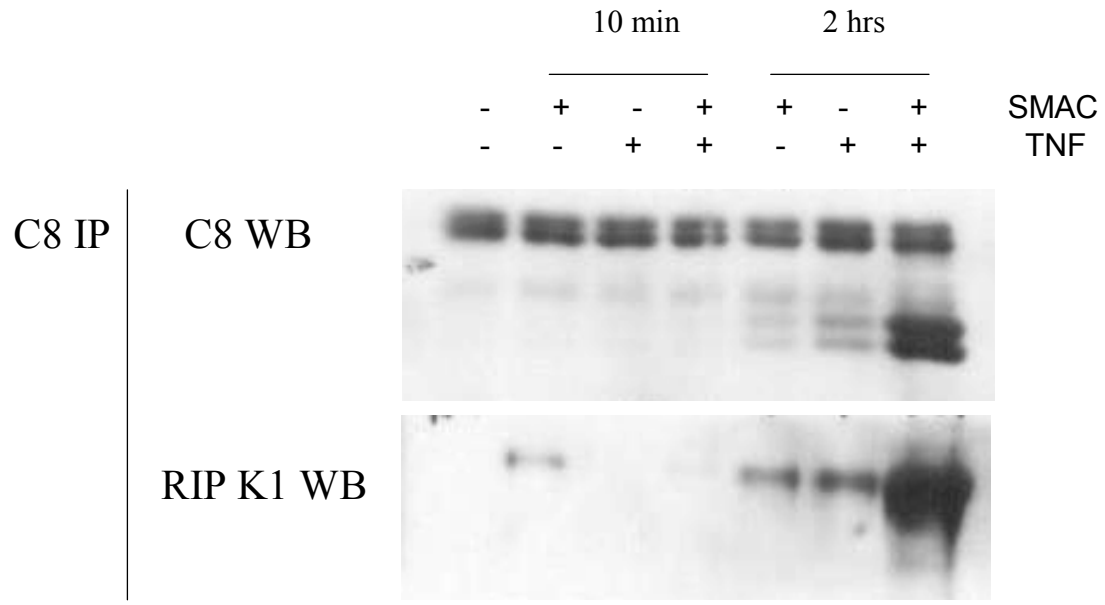


Figure 2.28. Caspase 8 complex Immunoprecipitation.

All cells were treated with z-VAD and either Smac mimetic (SMAC), TNFα, or Smac mimetic and TNFα for the indicated time points. Cells were lysed and pull-down experiments were performed using caspase 8 antibody. Following PAGE, western blots were done using anti-caspase 8, and anti-RIPK1 antibodies.

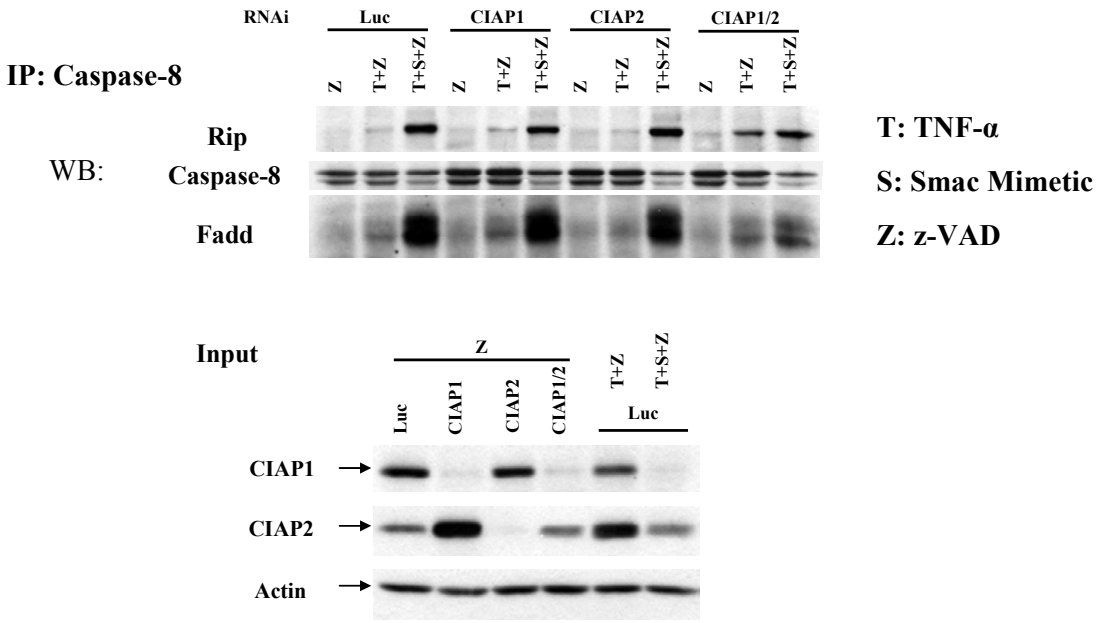


Figure 2.29. cIAP1 and cIAP2 Knock-down mimics Smac mimetic induced caspase 8 complex formation

Upper panel: Panc-1 cells were transfected with luciferase (Luc), cIAP1, and/or cIAP2 siRNA. Forty-eight hours post transfection; cells were treated as indicated for 4 hours. Cells were then harvested and analyzed for the caspase-8 immunocomplex.

The RNAi efficiency of cIAP1/2 siRNAs. Forty-eight hours post transfection; cell lysates from cells transfected with respective siRNA(s) were collected and subjected to western-blot analysis of cIAP1, cIAP2, and Actin levels.

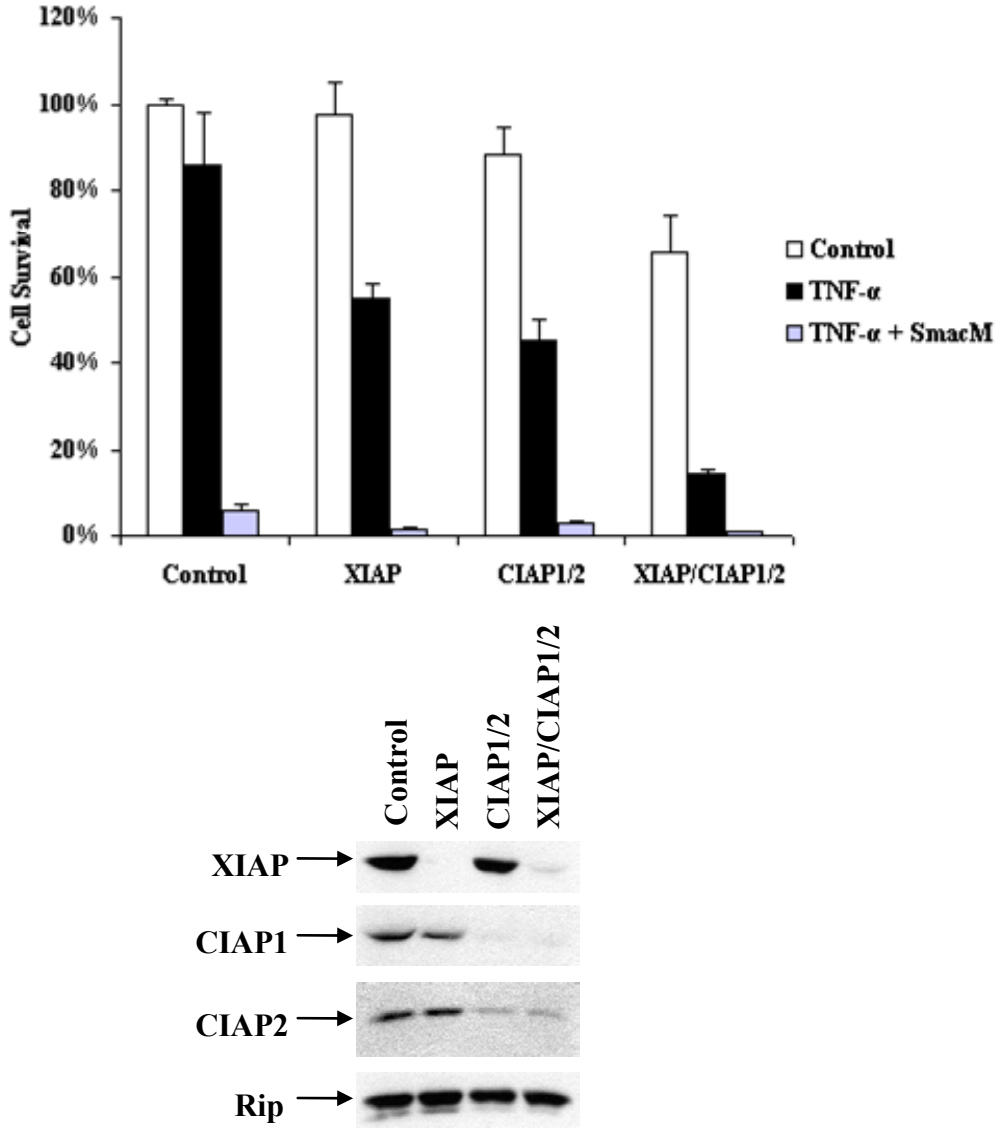


Figure 2.30. cIAP1, cIAP2 and XIAP triple knock-down mimics the Smac mimetic effect.

Upper panel: cells were transfected with siRNA as indicated for 48hrs then treated with either TNFα or TNFα and Smac mimetic for 24 hrs, cell viability was measured by Cell-Titer Glo Assay.

Lower panel: Knock-down efficiency was measured using Western blots with XIAP, cIAP1, cIAP2 and RIPK1 (Rip) antibodies.

CHAPTER THREE
SMAC AND TRAIL THE DEADLY DUO

3.1 Mechanism of Smac mimetic induced sensitivity in Trail resistant cells

The biological role of tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is not well understood. TRAIL has been suspected to have a role similar to FasL in immune response and tumor surveillance. Dysfunction of the Fas pathway results in autoimmune response against DNA and rheumatoid factor and can lead to accumulation of cancer cells. Similarly, the knockout mice of TRAIL confirmed a tumor surveillance role in natural killer cells (Cretney et al. 2002). Since its first discovery, the specificity of TRAIL to kill transformed cells versus normal cells attracted enormous attention from the cancer field (Nicholson 2000; Fischer and Schulze-Osthoff 2005).

TRAIL a.k.a. APO2L was first identified through its homology to FasL and TNFα (Wiley et al. 1995; Pitti et al. 1996). TRAIL is a type II transmembrane protein which gets cleaved from the membrane to form the soluble homotrimer active cytokine. TRAIL has five receptors: TRAILR1/DR4 (Pan, G. et al. 1997), TRAILR2/DR5 (APO2/ KILLER/TRICK2) (Marsters et al. 1997; Marsters et al. 1997; Pan, G. et al. 1997), TRAILR3/DcR1 (TRID/LIT) (Sheridan et al. 1997), TRAILR4/DcR2 (TRUNDD) (Pan, G. et al. 1997) and osteoprotegerin (OPG) (Emery et al. 1998). Cytoplasmic tails of CD95 (FasL receptor), TNFR1 and TRAILR1 contains significant homology, this region is the

so-called death domain DD. TRAILR3 and TRAILR4 have been identified through EST database and cDNA bank searches. They are the so-called Decoy receptors (Dc). They lack a functional DD domain thus they can not induce apoptosis (Marsters et al. 1997; Pan, Guohua et al. 1997). TRAILR3/DcR1 overexpression protects TRAIL sensitive cells from apoptosis. Although it has a partial DD, TRAILR4/DcR2 can also act as a decoy receptor when overexpressed. OPG is a soluble extra-cellular protein which regulates the development and activation of osteoclasts during bone remodeling. OPG ligand (TRANCE/ RANKL) upon binding to its receptor can activate NF-κB and cause osteoclastogenesis. OPG binds to TRAIL with much less affinity than OPG ligand.

Immunoprecipitation studies of the TRAIL receptor complexes lead to the identification of FADD (MORT1) in the DISC complex (Sprick et al. 2000). FADD contains DD and DED domains which it uses to bind to TRAILR1 and caspases 8 and 10, respectively. Briefly, TRAIL binding to its receptors causes recruitment of FADD through its DD domain (Ashkenazi 2002). FADD in turn recruits caspase 8 and/or 10 to the complex. Activation of caspase 8 in this complex, leads to cleavage of caspase 3 and Bid in TRAIL sensitive cells. FLIP is a negative regulator of the TRAIL pathway. FLIP is homologous to the N-terminus of caspase 8 but lacks protease activity (Inohara et al. 1997; Irmler et al. 1997). It has a tyrosine in the place of the active cysteine residue in caspases.

Overexpression of FLIP leads to less caspase 8 recruitment to the receptor DISC complex. FLIP overexpression is thought to be one of the reasons why some cells are resistant to TRAIL (Kim et al. 2003). It is critical to understand why some cells are naturally resistant to TRAIL in order to overcome resistance in those cells. Since, TRAIL is very selective towards killing cancer cells and sparing the normal. The biochemical analysis of regulatory DISC components in TRAIL sensitive versus resistant cells can explain the key differences and provide mechanistic information that can be used to sensitize the resistant ones.

The mitochondrial amplification loop through cleavage of Bid by caspase 8 is suspected to be another key determinant of susceptibility to TRAIL (Danial and Korsmeyer 2004). Truncated Bid (tBid) as mentioned earlier induces Smac release to the cytosol. If this is the case, we can speculate that XIAP-Smac interaction is to be the key regulator of TRAIL pathway, which is supported by the observed synergy between SM and TRAIL (Li et al. 2004).

A second signaling complex also forms upon TRAIL binding to its receptor which consists of RIPK1, TRAF2 and IKK (Falschlehner et al. 2007). However this complex is mainly thought to form with TRAILR4/DcR2. TRAILR1, in some cells, has been shown to induce NF-κB activation through this complex and, like TNFR1 complex, thought to promote survival. Unlike TNFR1 and CD95, the internalization of the TRAILR1 is not required for apoptosis (Kohlhaas et al. 2007).

In our initial screens, we found approximately 50% of the cell lines we tested were sensitive to SM in combination with TRAIL. As discussed in Chapter 2, SM can synergize with TRAIL and induce cell death with nanomolar concentrations (figure 2.17). This synergy can potentially be very beneficial in terms of creating new specific alternatives to current chemotherapies since both of the treatments have tumor-cell specific effects.

3.2 Target of Smac mimetic in Trail pathway: Mimicking Smac mimetic effect in vivo

In the past one year, I turned my attention to studying the mechanism of this synergy to identifying the target of SM in the TRAIL pathway. Since we were able to use the RNAi technique to identify the targets of SM in the TNFα pathway, I decided to use the same approach in the TRAIL pathway.

First, I wanted to test which of the IAPs, or if all three as in the TNFα pathway, were able to mimic the SM effect in the TRAIL pathway. To do this I transiently transfected H2009 cells with XIAP, cIAP1, cIAP2 and combinations of all three siRNAs. Then, I treated them with TRAIL alone (figure 3.1). XIAP siRNA alone was able to mimic the SM effect. The cIAP1 siRNA treatment also did have slightly reduced viability. We attributed this effect to the ability of cIAP1 siRNA to reduce the XIAP amount (figure 3.2).

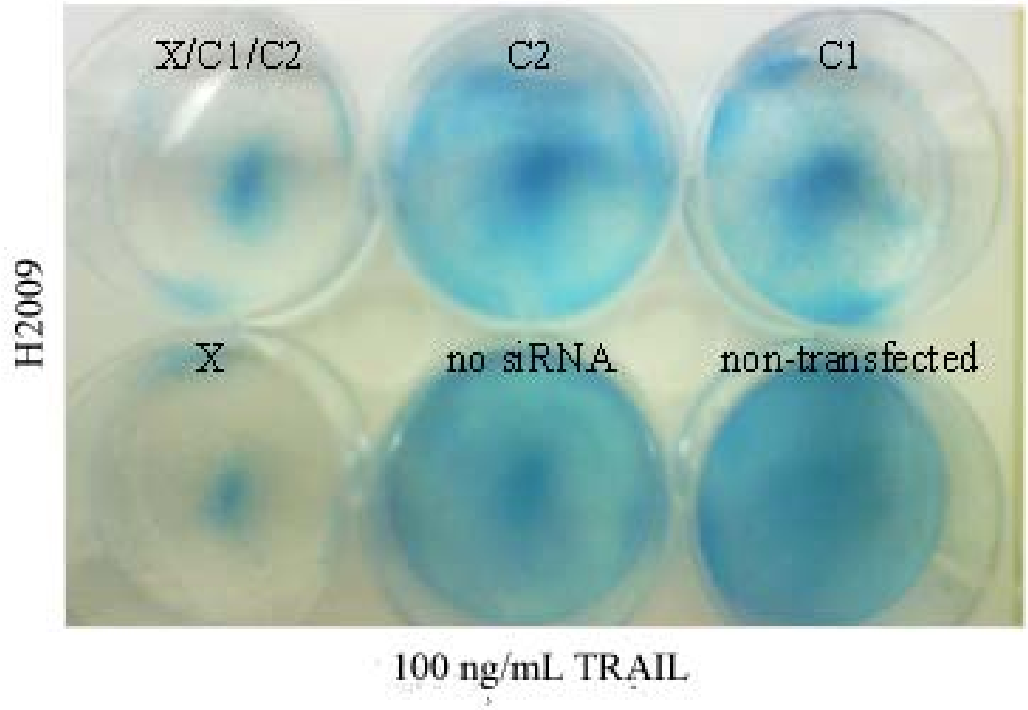


Figure 3.1. Transient XIAP siRNA mimics TRAIL and Smac mimetic induced cell death.

Cells were transfected with siRNAs against IAPs [XIAP (X), cIAP1 (C1), cIAP2 (C2)] for 48 hrs then treated with TRAIL for 24 hrs. Viability was assessed with methylene blue.

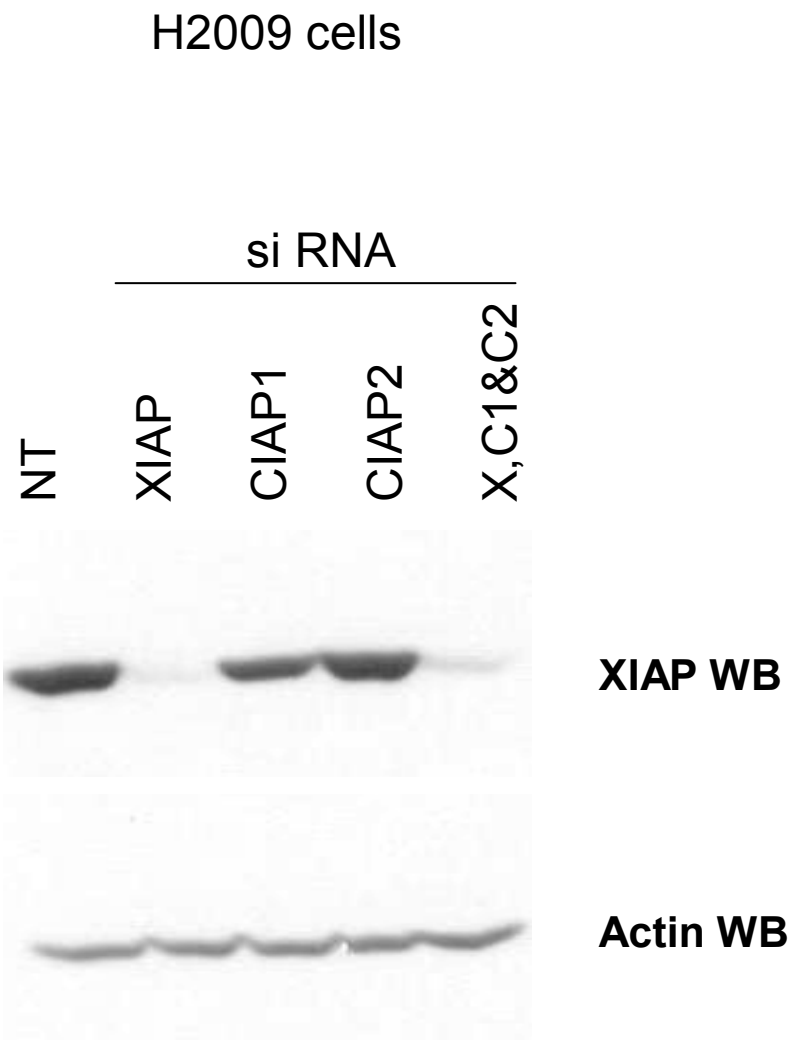


Figure 3.2. Knock-down efficiencies for Figure 3.1

Knock-down efficiency was measured using Western blots with XIAP and actin antibodies.

To further validate the role of XIAP in TRAIL pathway, I generated tetracycline inducible XIAP shRNA stable cell lines. Addition of tetracycline to the media of the stable KD cells induced significant reduction in the XIAP expression (figure 3.3). Consistent with the observations made with transient transfections, upon tetracycline treatment the once resistant cells became sensitive to TRAIL alone (figure 3.3). To eliminate off-target effects and also to increase the efficiency of the KD further, a second XIAP shRNA oligo, which performed better in transient transfections, was cloned. This new six-copy inducible XIAP shRNA KD cells showed the same response to TRAIL (figure 3.4). Importantly these cells when treated with TNF alone did not show any cell death phenotype (figure 3.4). Thus, this effect was specific to the TRAIL pathway.

To extend our effort to characterize the effect of XIAP on the TRAIL pathway, we also generated a rescue plasmid. This rescue plasmid contained a wild type XIAP transgene where the shRNA targeting sequence was silently mutated to make it resistant to shRNA KD. This rescue transgene was also under control of the inducible tetracycline promoter; hence when induced, stable cells would lose the endogenous copy and gain the wild type transgene. The colonies that expressed the same amount as endogenous XIAP were selected (figure 3.5). TRAIL alone induced cell death observed in the KD cells was partially rescued with the wild-type XIAP transgene (figure 3.5). Once again these cells were resistant to TRAIL treatment like the parental cells.

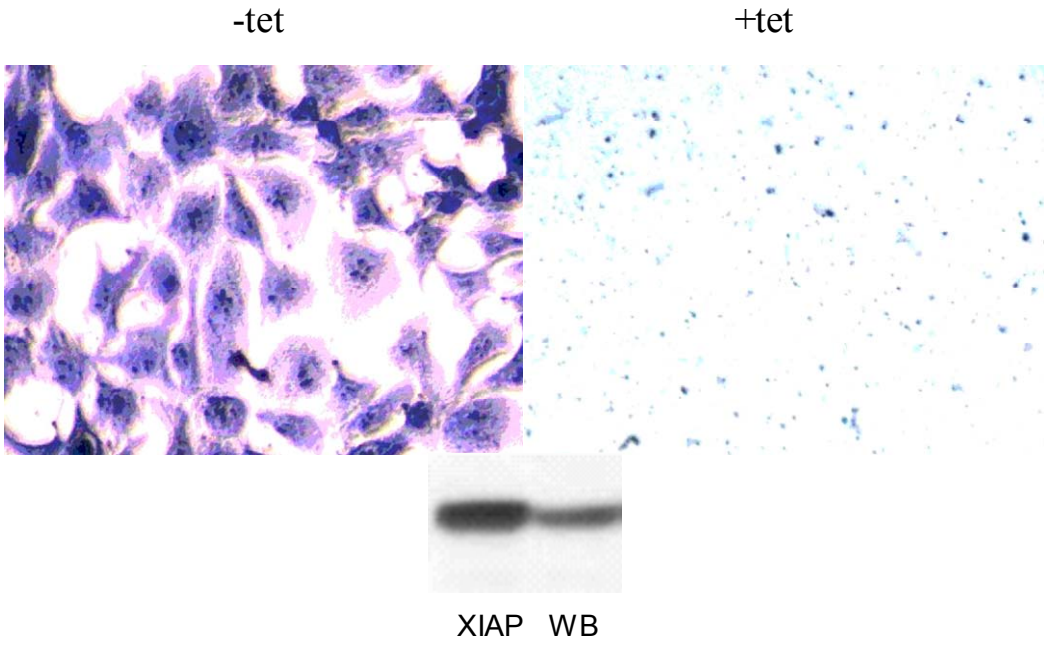
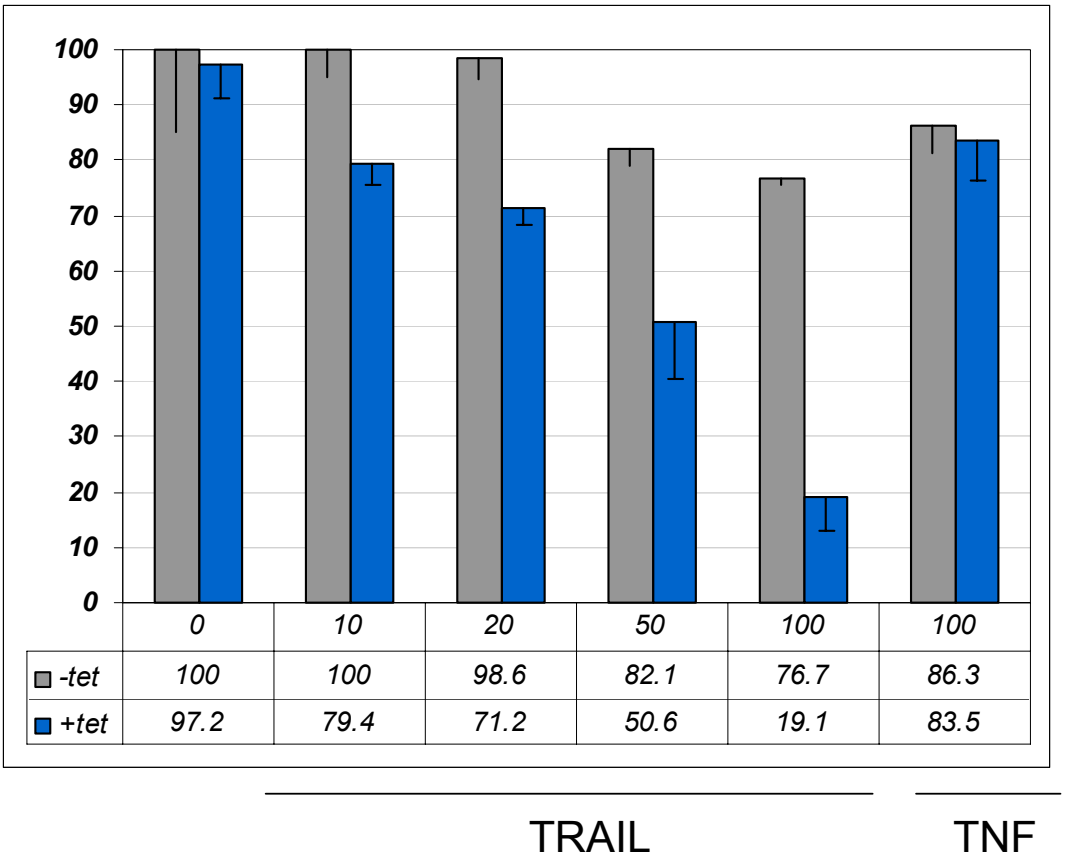


Figure 3.3. TRAIL induced cell death in tetracycline inducible shXIAP colony #61 (oligo #1) cells. XIAP KD mimics Smac mimetic effect.

Tetracycline (tet) inducible six copy short hairpin RNA (shRNA) to XIAP stable cells were treated with Tet for 48 hours followed by TRAIL treatment. Light microscopy pictures were taken after staining with methylene blue. Knock-down efficiency after 48 hrs is shown in bottom panel.



	0	10	20	50	100	100
-tet	100	100	98.6	82.1	76.7	86.3
+tet	97.2	79.4	71.2	50.6	19.1	83.5

Figure 3.4 TRAIL induced cell death in tetracycline inducible shXIAP colony #14 (oligo # 2) cells. XIAP KD mimics Smac mimetic effect.

Tetracycline (tet) inducible six copy short hairpin RNA (shRNA) to XIAP stable cells were treated were treated with tet for 48 hours followed by increasing doses of TRAIL 10, 20, 50, or 100 ng/mL or TNF 100 ng/mL. Cells were stained with Cell Titer Glo.

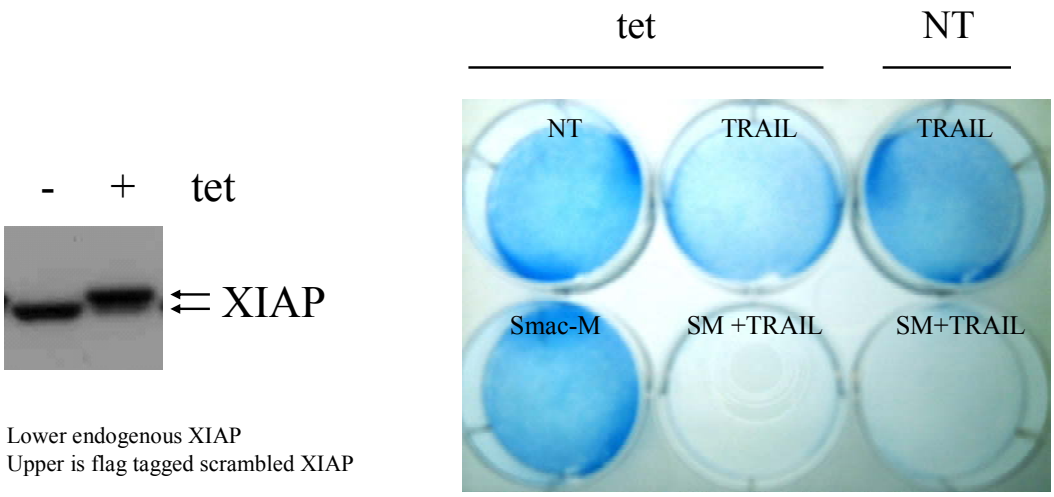


Figure 3.5. Smac mimetic effect created with XIAP KD can be partially rescued by wt XIAP.

Stable cells were generated, as described in appendix, with tetracycline (tet) inducible six copy short hairpin RNA (shRNA) to XIAP and a tetracycline (tet) inducible silently mutated (in shRNA target sequence) wild type flag tagged XIAP.

Right panel: Tetracycline, TRAIL and SM treated cells were used for methylene blue staining.
Left panel: After treatment 72 hours in the absence or presence of tetracycline, cell lysates were collected and subjected to western-blot analysis using XIAP antibody (right panel).

3.3 Characterization of Smac Mimetic induced DISC complex in TRAIL pathway

To understand how SM treatment/XIAP KD sensitizes these cells to TRAIL, I examined caspase 8 activation. The DISC complex was immunoprecipitated using caspase 8 antibodies. The complex formation can be visualized upon 1 hour after treatment with SM and TRAIL. Interestingly, this complex was also formed to a lesser extent in the TRAIL treatment only (figure 3.6). However, SM induced complex was stronger, in terms of activating caspase 8 (figure 3.6, lane 7). Although TRAIL plus SM induced cell death is not dependent on RIPK1 (data not shown), it is noteworthy to mention that more RIPK1 was associated with the DISC complex upon SM treatment. Importantly, cIAP1 was also observed to be a complex with caspase 8 (figure 3.7). Caspase 8 has never been shown to interact with cIAPs endogenously. This interaction of caspase 8 with cIAP1 was disrupted upon TRAIL only treatment hence was not important for the SM and TRAIL induced cell death. Also as mentioned earlier, we did not see cell death upon cIAP1 siRNA and TRAIL treatment.

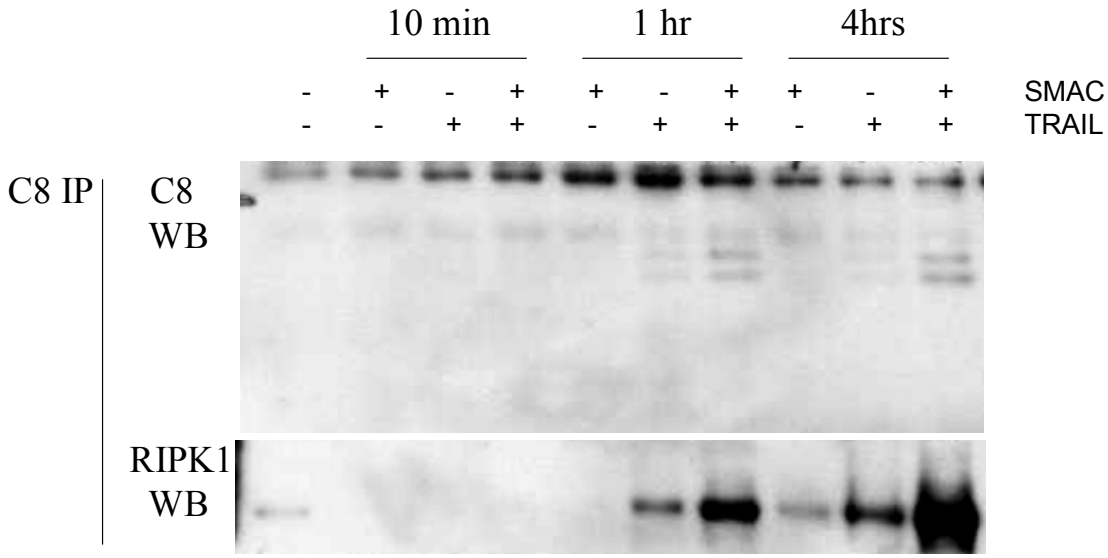


Figure 3.6. Caspase 8 complex immunoprecipitation following TRAIL and Smac mimetic treatment.

Cells were treated with Smac mimetic (SMAC), TRAIL or Smac mimetic and TRAIL (plus z-VAD in all treatments) for the indicated time points. Cells were lysed and pull-down experiments were performed using caspase 8 (C8) antibody. Following PAGE, western blots were done using anti-caspase 8, and anti-RIPK1 antibodies.

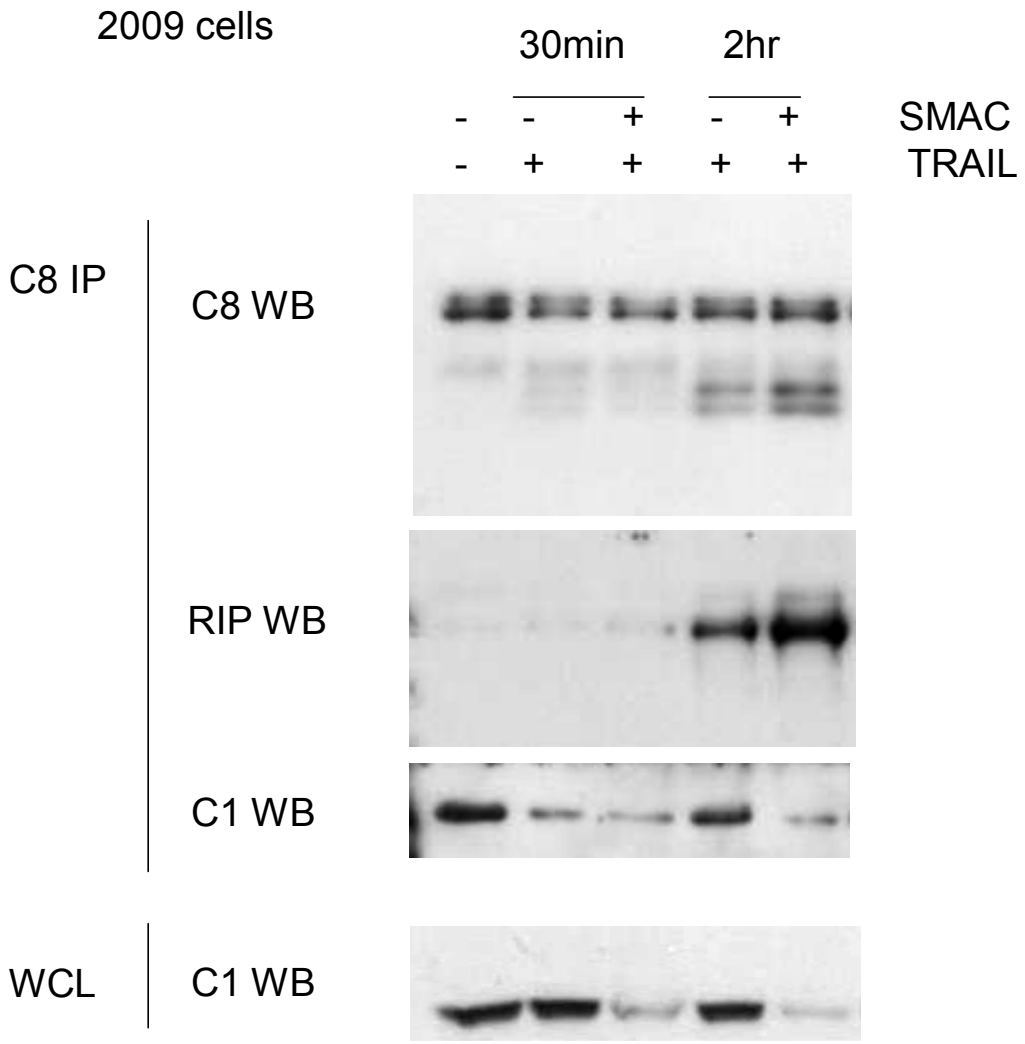


Figure 3.7. Caspase 8 complexes following TRAIL and Smac mimetic treatment.

Cells were treated with Smac mimetic (SMAC), TRAIL or Smac mimetic and TRAIL (plus z-VAD in all treatments) for the indicated time points. Cells were lysed and pull-down experiment was performed using caspase 8 antibody. Following PAGE, western blots were done using anti-caspase 8 (C8), cIAP1 (C1) and anti-RIPK1 (RIP) antibodies.

CHAPTER FOUR
DISCUSSION AND CONCLUSION

Defective and deregulated programmed cell death is a hallmark of cancer cells that contributes to tumorigenesis, tumor progression and resistance to therapy. The apoptotic pathways that are manipulated by cancer cells have been studied extensively over the last decade and this body of knowledge is now paving the way to create novel cancer therapies (Nicholson 2000; Denicourt and Dowdy 2004; Fischer and Schulze-Osthoff 2005). As a result, numerous of these new drugs are in clinical trials, and some are now being used in treatment of patients. Yet, a majority of the anti-cancer therapies have a common problem, they are cytotoxic agents. They tend to efficiently kill cancer cells, but unfortunately, they kill the normal cells as well. The lack of specificity is actually only one of the problems cancer therapy faces. Another major problem is even when an ideal looking chemotherapeutic comes along, tumor cells, eventually, tend to acquire resistance to them.

Cancer cells are experts in finding ways to impede caspase activation. In fact, most of them found a way to inhibit caspases even after they are activated using IAP upregulation or gene amplification. Smac protein functions by relieving the IAP mediated inhibition on active caspases. This inhibition is not simply binding and eliminating IAP inhibition on caspases. For cIAPs, Smac can

activate their ubiquitin ligase function causing auto-ubiquitination and eventual degradation (Yang and Du 2004). That Smac function is dependent on a four amino acid sequence and that this four amino acid sequence can mimic mature Smac protein is a distinctive trait.

Smac is a mitochondrial protein that only gets released when the apoptotic signal is severe, thus assuring that the apoptosis proceeds only when the cell is absolutely committed (Wang 2001). This is important for both intrinsic and extrinsic pathways, as it puts IAPs and Smac to the spotlight.

4.1 Smac Mimetic-induced cell death

The idea of mimicking endogenous Smac with small molecules started with the first discovery of Smac. The Smac mimicry idea is to use small molecule AVPI derivatives in combination with apoptosis inducing agents to by-pass the mitochondrial regulation on Smac and sensitize cancer cells to apoptosis. With a little serendipity, we discovered our Smac mimetic dimer (Li et al. 2004). The dimer compound was initially synthesized unintentionally and later characterized to be a by-product of one of the steps in the synthesis. Fortunately, the Smac mimetic dimer was much more active than the monomer Smac mimetic, and we attributed this to the bivalency of the native Smac protein.

Studying the cell death pathway induced by our Smac mimetic led us to identify the endogenous targets. Knowing all *in vitro* and *in vivo* targets was

important as very few of the drugs in the market today have all their targets known. It is this aspect that unavoidably makes these drugs non-specific cytotoxic agents. They usually bind to several molecules and inhibit or activate them with different concentrations, hence cause undesirable side-effects.

Smac mimetic binds specifically to three IAPs: XIAP, cIAP1 and cIAP2. It was also able to pull-down TRAF1 and 2, but we speculate these bindings to be through IAPs as cIAP1 and 2 were initially identified through purification of the TNFR2-TRAF signaling complex. The specificity of Smac can be appreciated in this experiment: normal human fibroblast cells were treated with about a thousand fold of the Smac mimetic dose used in to induce apoptosis in sensitive cells, either alone or with TRAIL, and there was no associated cytotoxicity (Li et al. 2004). In fact, even in some cancer cells this amount of Smac mimetic alone did not cause any cytotoxicity. Only when combined with TRAIL or other chemotherapeutic agents was the cell death phenotype observed. This specificity comes from the unique interaction that Smac and IAPs have.

The existence of Smac sensitive cells was a surprise to us initially, since at that time we were only associating this sensitivity to Smac. However, it has been suspected for some time that some cancer cells may turn on their apoptotic pathways, but these signals are halted by downstream blocks. In fact, overcoming this apoptotic signaling gives them the survival/growth advantage. However, in our sensitive cell lines, the TNFα survival advantage turns out to be their fatal

flaw, as this same pathway that they use to survive can be switched to apoptosis by Smac mimetics (Petersen et al. 2007). For these cancers, Smac mimetic can be used as a single agent treatment. Moreover, the presence of TNFα can potentially be detected and used as a bio-marker to determine if a certain tumor is going to respond to the Smac mimetic alone.

Finally, Smac mimetic is the perfect tool to dissect the function of cIAPs. The role of XIAP was apparent after the biochemical and structural studies demonstrated direct interactions with caspases and Smac. *In vitro* functional studies in combination with *in vivo* assays gave XIAP the title of most potent IAP and created a prototypical role for all IAPs. The reason for this prototypical role was that XIAP was using its BIR domains in caspase interactions and all IAPs had BIR domains. However, the function of cIAPs was more confusing and seemed somewhat redundant. They were thought to have redundant functions with XIAP, since the initial studies show that they also can bind to caspases in the same manner as XIAP. Secondly, in the initial KO study when XIAP was knocked out, the cIAPs expression was found to increase, which supported once again the redundancy hypothesis (Harlin et al. 2001). Later on, caspase binding and thus inhibition was attributed to the GST tags, and the KO studies became controversial. When another group made the same KO mice they did not see this upregulation phenotype (Olayioye et al. 2005). At this point cIAPs were only known to associate with the TNF receptor complexes; however, their function in

this complex was not clear, since they were not able to bind to caspase 8. Using Smac mimetic, we were able to show how they functioned in this complex. Degradation of cIAPs was necessary to form the caspase 8–RIPK1-FADD complex which was required for activation of caspase 8 (Dr Lai Wang and Xiaodong Wang, in press) (Figure 4.1).

Last year we published our results showing that the single agent Smac mimetic sensitivity was caused by autocrine TNFα secretion (Petersen et al. 2007). We also identified that Smac mimetic induced pathway was RIPK1 dependent. There were two other papers published at the same time using different Smac mimetics (Varfolomeev et al. 2007; Vince et al. 2007). One of these papers used a monomer Smac mimetic. They were also able to show that cIAPs are regulated by allosteric activation of their RING finger ubiquitin ligase domains through binding of Smac mimetic. This activity has also been seen before with Smac protein.

Interestingly, Varfolomeev et. al. also discovered that cIAPs are ubiquitin ligases for the key kinase in the non-canonical NF-κB pathway, NIK (NF-κB-inducing kinase). They found that co-expression of NIK with cIAP1 or cIAP2 leads to degradation of NIK whereas co-expression with a RING domain mutant of cIAP1 did not. Moreover, they saw that treatment of NIK stable cells with their Smac mimetic induced degradation of endogenous cIAP1 and showed upregulation of the NIK.

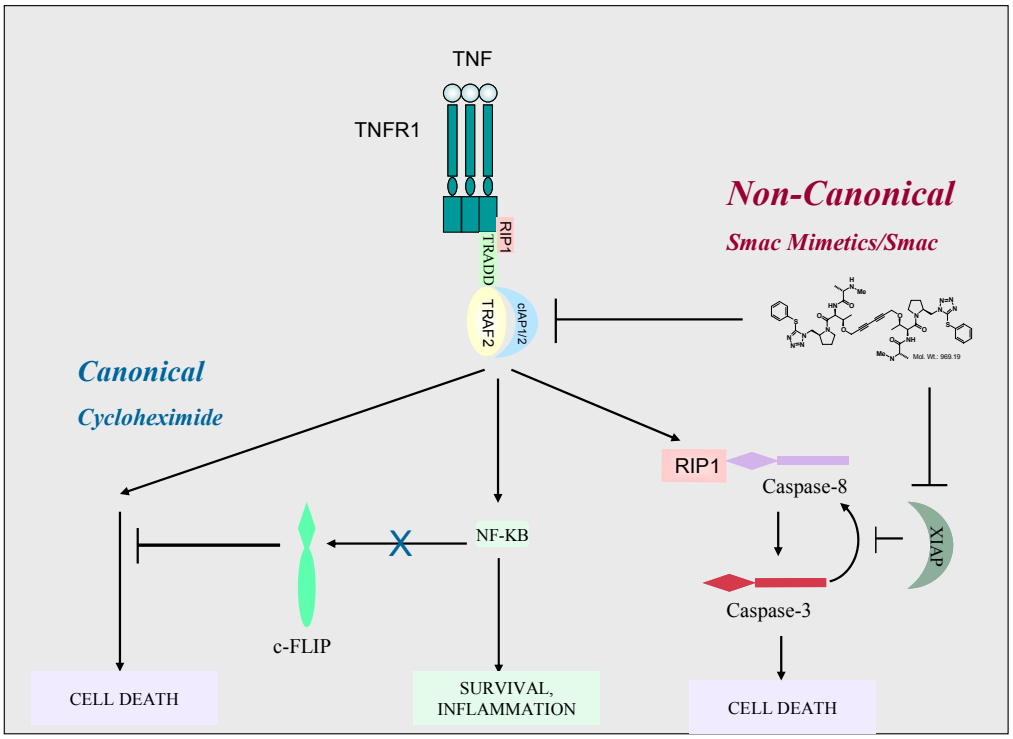


Figure 4.1. Model of TNFα and Smac mimetic induced caspase 8 activation.

Upon TNFα stimulation, Smac mimetic stimulates the release of RIPK1 from TNFα receptor (Lai Wang and Xiaodong Wang, in press) (data not shown) by inducing auto-ubiquitination and degradation of cIAP1 and cIAP2 and the formation of a RIPK1/FADD/caspase-8 death inducing signaling complex. Degradation of cIAPs and inhibition of XIAP is necessary to mimic the full Smac mimetic effect.

Smac mimetic induces the non-canonical pathway, see text for details.

The canonical TNF pathway was discussed in the introduction chapter. Briefly, TNF will induce formation of complex I which activates c-FLIP through NF-κB. c-FLIP heterodimerizes with caspase 8 and inhibits recruitment of caspase 8 to the complex. Therefore, canonical pathway is regulated by levels of c-FLIP.

Thus, they concluded that Smac mimetic was activating the non-canonical NF-κB pathway. This NF-κB activation was speculated to be the reason for the secreted TNFα by the sensitive cells (Wu et al. 2007).

4.2 Model for Smac mimetic and TRAIL induced cell death

TRAIL receptors belong to the TNF superfamily of receptors. Since the discovery of the death receptors, ligands of these pathways have been studied extensively for their potential as cancer therapies. TNFα was the first member of this family to be considered as an anti-tumor drug (Kettelhut et al. 1987). However, despite the tremendous potential, TNFα caused systemic inflammation resembling septic shock in patients. This failure of TNFα was a big disappointment. The use of TNFα turned out to be only limited to limb perfusions. Unfortunately, FasL also showed high toxicity as it caused massive hepatocyte apoptosis and lethality in mice (Kosai et al. 1998).

The ability of TRAIL to induce cancer cell specific effects and avoid cytotoxic side effects was extremely exciting. TRAIL appeared to be the wonder receptor scientists were hoping for. The one reason that TRAIL was not "the" magic drug is not all cancers were sensitive to TRAIL induced apoptosis. Furthermore, even when a certain cancer cell was sensitive to TRAIL, over time they acquired resistance. So TRAIL still needed to be used as a combination therapy. Recombinant human Apo2L/TRAIL (rhApo2L/TRAIL) which is

designed to activate both TRAIL receptors DR4 and DR5 is in Phase II clinical trials by Genentech. rhApo2L/TRAIL is being evaluated as a combination therapy in non-Hodgkin's lymphoma and metastatic non-small cell lung cancer.

Smac mimetic synergy with TRAIL could one day be "a" magic drug cocktail. Smac mimetic synergizes with TRAIL, at picomolar doses, to induce caspase activation (Li et al. 2004). Since both TRAIL and Smac mimetic effects are tumor cell specific and show no cytotoxicity in normal cells, studying the mechanism of this pathway is critical for future therapies.

Using siRNAs against IAPs we were able show that XIAP is the target of Smac mimetics in the TRAIL pathway. Although the target of Smac mimetic was previously not determined in the TRAIL pathway, this was not a surprise to us, since previously XIAP anti-sense oligos were shown to synergize with TRAIL (McManus et al. 2004). Interestingly, cIAP1 and cIAP2 siRNAs did not have an affect in this TRAIL pathway. Even tough Smac mimetic caused degradation of cIAPs, we noticed that TRAIL alone treatment led to dissociation of cIAP1 from the caspase 8 complex, freeing caspase 8 to be activated. Around two hours after the treatment, cIAP1 was again detected in this complex. cIAP1 and cIAP2 have been shown to be associated with the receptor complex, but never have been shown to interact with caspase 8. However, we now know cIAPs can also interact with RIPK1 (Dr Lai Wang and Xiaodong Wang, in press). Yet, I was not able to detect RIPK1 in this complex without treatments. RIPK1 recruitment to the

complex was seen after treatment with TRAIL and TRAIL plus Smac mimetic which coincided with cIAP1 recruitment at the two hour time point. The removal of cIAP1 initially from caspase 8 in the TRAIL only treated samples could be causing some caspase 8 activation and as a result some cell death (20%). Yet, the amount of activated caspase 8 was enhanced when treated with Smac mimetic in combination with TRAIL leading to full activation of the pathway and dramatic cell death.

The results described above support a model of TRAIL induced apoptosis (Figure 4.2). TRAIL binding to its receptor causes activation of the receptor and formation of caspase 8-RIPK1-FADD complex. This complex can activate caspase 3, but in the presence of XIAP, caspase 3 activation will be inhibited. Smac mimetic targets XIAP in this pathway and removal of XIAP will free-up caspase 3. This activated caspase 3 can feedback to caspase 8 resulting in activation of more caspase 8. This feedback mechanism will enhance the death signaling and result in dramatic cell death.

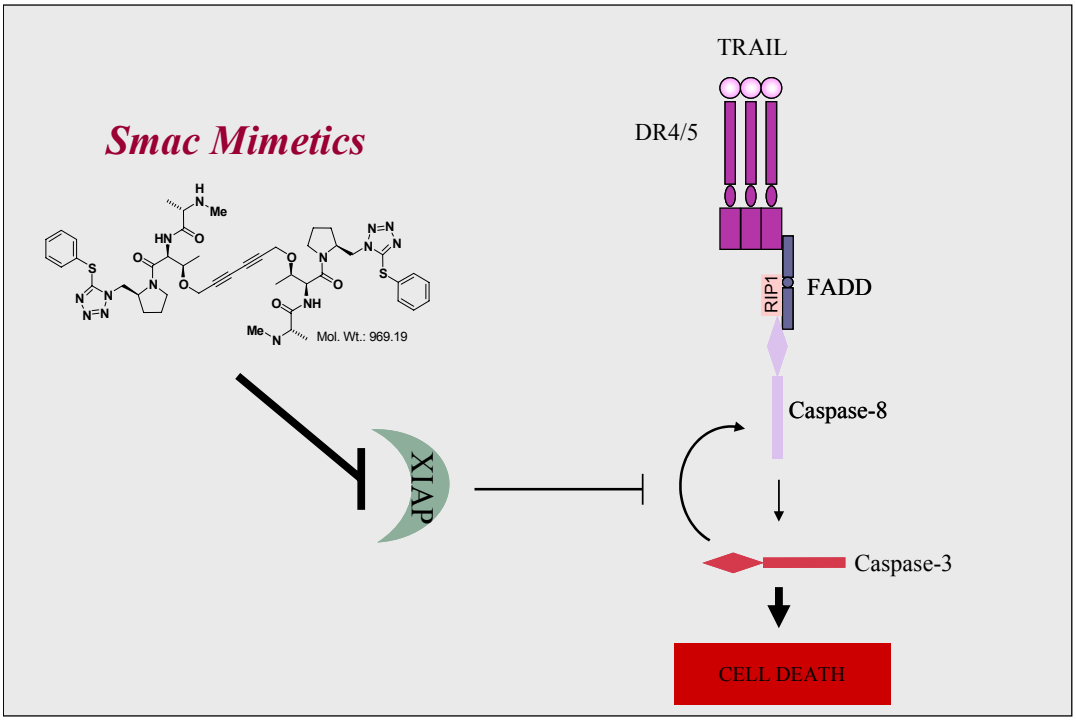


Figure 4.2. Model for Smac mimetic induced TRAIL pathway.

TRAIL binding to its receptor causes activation of the receptor and formation of caspase 8-RIPK1-FADD complex. This complex can activate caspase 3, but in the presence of XIAP, caspase 3 activation will be inhibited. Smac mimetic targets XIAP in this pathway and removal of XIAP releases inhibition of caspase 3. Activated caspase 3 can feedback to caspase 8 resulting in activation of more caspase 8. This feedback mechanism will enhance the death signaling and result in dramatic cell death.

4.3 The big picture

After studies showing TRAIL can also activate NF-κB, the TRAIL and TNF pathways are now thought to be more similar in nature that initially presumed. This is supported by the fact that most cancer cells are resistant to TRAIL alone treatment (Ishimura et al. 2006). One point worth making is that complexes in the TRAIL pathway are formed simultaneously after the initial stimulus of the receptor, whereas in the TNF pathway, the caspase 8 complex forms approximately two hours after stimulation which coincides with the receptor internalization (Micheau and Tschopp 2003). This temporal difference in apoptotic complex formation could be attributed to the fact that TRAILR1 internalization is not required for apoptosis. In a recent study, it was shown that the caspase 8 complex still formed, and apoptosis was not inhibited after blocking the endocytosis of the TRAILR1 (Kohlhaas et al. 2007).

With our studies, we were able to use Smac mimetic to dissect the key differences between these pathways. In the TNF pathway, the activation of caspase 8 depends on removal of cIAPs from the complex. Removal of all IAPs is necessary to induce cell death. On the other hand, in the TRAIL pathway, inhibition of XIAP is sufficient to induce cell death. In the TRAIL pathway, if the target of Smac mimetic is XIAP only, just knocking down XIAP can have the same effect as Smac mimetic treatment. We were able to show this trying to

recapitulate the Smac mimetic treatment. In that case XIAP anti-sense oligos could do the job of Smac mimetics.

In the end, cancer cells can be categorized into three different kinds: sensitive to Smac mimetic as single agents, sensitive to Smac mimetics co-treated with exogenous TNFα and resistant to co-treatment with Smac mimetics and TNFα/TRAIL. This third group may be similar to the first group in the sense of they may already have a survival advantage. In fact, these cells can be sensitized when an additional treatment of IKK inhibitors or PI3-K inhibitors were added on top of the TNFα and Smac mimetic treatment (Sean Petersen and Xiaodong Wang unpublished observation). In other words, these cells have another survival pathway to escape from cell death.

In conclusion, the ability of Smac mimetic to synergize with any kind of apoptotic signal inducing agent underscores the importance of its mechanism. One very important side of this story is that it is the only compound that has potential to put TNFα back in the picture as a chemotherapeutic. If the dosing of TNFα is what is creating the toxicity, then toxicity can be tuned down using Smac mimetic, and TNFα can once again be used in patients. One can also imagine that this could also be the case for FasL. In our collective efforts to combat cancer, Smac mimetic has the potential to be a very unique drug.

APPENDIX A

Materials and Methods

Reagents

Smac mimetic and the biotinylated compound were synthesized as previously described (Li et al. 2004) . z-VAD and MG-132 was obtained from Calbiochem. The following antibodies were used for western-blot: caspase-3 (Cell Signaling, 9662), caspase-8 (Cell Signaling, 9746), caspase 9 (Cell Signaling, 9502), caspase-8 (BD Bioscience, 556466), cIAP1 (R&D, AF8181), cIAP2 (BD Biosciences, 552782), FADD (Stressgen, AAM-212E), Flag (Sigma, A8592), HA (Roche, 12013819001), Pan cIAP (R&D, MAB3400), PARP (Cell Signaling, 9542), RIPK1 (BD Biosciences, 610458), RIPK1 (BD Biosciences, 551041), TNFRI (Santa Cruz Biotech, SC-8436), XIAP (BD Biosciences, 610717).

Plasmids and siRNA Oligos

p3xFLAG-CMV-7 mammalian expression plasmids for cIAP1 and cIAP2 as well as their E3 ligase mutants H588A andH574A, respectively, were kindly provided by Dr. Chunying Du (Stowers Institute). cIAP1 with a C-terminal Flag tag and its truncation constructs were amplified with specific primers accordingly and subcloned into pCI-neo plasmid. p3xFLAG-CMV-7 mammalian expression plasmids for XIAP were kindly provided by Lin Li (Joyant Inc.).XIAP construct

was amplified with specific primers accordingly and subcloned into pcDNA3.1(+)/TO. For XIAP-shRNA construct, six copies of XIAP shRNA (sequence as 5'- AATGCAGAGTCATCAATTA -3' and 5'-CAGAATGGTCAGTACAAAG-3') were cloned into pSuperior.puro vector using the protocol as previously described (Zhong et al., 2005). To generate XIAP scramble expression constructs, XIAP cDNA with an N-terminal Flag epitope was first subcloned into a modified pcDNA4/TO vector (Invitrogen) containing neomycin resistant gene. The shRNA targeting site on XIAP was then mutated at six different sites without affecting amino acid sequence.

All siRNAs were purchased from Dharmacon caspase-8 and caspase-9, individual oligos were designed and tested (caspase-8-1 target sequence, 5-TGAAGATAATCAACGACTATT-3; caspase-8-2 target seqTence, 5-TGGATTTGCTGATTACCTATT-3; caspase-8-3, obtained from Dharmacon [J-003466-14], caspase-9-1 target seqTence, 5-GATGCCTGGTTGCTTTAATTT. caspase-9-2, obtained from Dharmacon [J-003309-05]; caspase-9-3, obtained from Dharmacon [J-003309-06]). For TNFR1 and TNFa, individual oligos were purchased from Dharmacon (TNFR1: TNFR1-1, J-005187-05; TNFR1-2, J-005197-06; TNFR1-3, J-005197-08) (TNFa-1, J-010546-09; TNFa-2, J-010546-10, TNFa-3, J-010546-12 For cIAP1, cIAP2, XIAP, and, individual oligo was used (cIAP1 target sequence 5'-TTCGTACATTTCTCTCTTA-3'; cIAP2 target sequence 5'-AATGCAGAGTCATCAATTA-3'; XIAP target sequence 5'-

CCAGAATGGTCAGTACAAA-3'.

Recombinant Proteins

GST-TNF-α bacterial expression plasmid was kindly provided by Dr. Zhijian Chen (UT Southwestern Medical Center at Dallas) and GST-TNF-α protein was generated as previously described (Ea et al., 2006). GST-cIAP1 and GST-cIAP2 was expressed in E. coli strain BL21 (DE3) and purified using Glutathione Sepharose 4B beads (Amersham Biosciences).

Cell Culture

Cell lines HCC44, HCC461, H2126, HCC515, HCC827, H2009, and 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). T98G glioblastoma, and HEK-293T cells were obtained from ATCC and were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Sigma) and 100- units/ml penicillin/streptomycin (Hyclone).

Methylene Blue Staining

For Supplementary Figure 2A, cell survival was accessed by methylene blue staining. Briefly, cells were treated as indicated in figure legend. After 48 hours, cells were washed twice with PBS (Invitrogen) and stained with 2%

methylene blue (w/v) in 50% ethanol for 15 minutes with shaking at room temperature (RT). Cells were then washed twice with PBS and air-dried before being photographed.

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 100 ml media per well. Cells were allowed to grow to near confluence and treated with compound 3 or vehicle (H20) by adding 100 ml media with compound 3, diluted to 23 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 manufacturer's protocol, media was aspirated from each well and 100 ml fresh media added. Cells were allowed to equilibrate to room temperature when 100 ml of the Cell Titer-glo reagent was added. Cells were placed on an orbital shaker for 2 min and then were incubated for an additional 10 min. 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 three independent samples.

Generation of Stable Cell Lines

To generate XIAP overexpression cells, linearized plasmids were transfected into H2009 cells as previously described. Twenty-four hours post transfection, cells were split and placed in complete medium containing 1 mg/ml G418 (Calbiochem). After 2 to 3 weeks, clones were lifted and tested for expression of the transgene. To generate H2009 tetracycline repressor expression cells, an expression plasmid containing tetracycline repressor (TetR) was transfected into H2009 cells and cells were selected with 10 μg/ml blasticidine (Invitrogen). Inducible XIAP-shRNA construct was then stably introduced into H2009-TetR cells, selected with 1 μg/ml puromycin (Calbiochem). Finally, inducible wild type XIAP rescue cells were generated by transfecting XIAP-shRNA stable cells with XIAP scramble expression constructs and selected with 1 mg/ml G418.

Fluorogenic Caspase-3 Activity Assay

Cells were treated and subsequently pelleted. Cell pellet was resuspended in Buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1mM DTT, 0.1 mM PMSF, and Complete Protease Inhibitor; Roche). The resuspended cell pellet was incubated on ice for 20 minutes before cells were broken by freezing in liquid nitrogen followed by thawing in 37°C water bath repetitively for 3 times. The resulting broken cell mixture was centrifuged at 20,000 g for 30 minutes. Protein concentration of the supernatant was determined by Commassie Plus Protein Assay Kit (Pierce). Twenty micro-grams of cell lysates were incubated with 10 μM fluorogenic caspase-3 substrate (Calbiochem) in a 20 μl system. Fluorescence reading was carried out as previously described (Nijhawan et al., 2003).

Preparation of Cell Lysates with Lysis Buffer

Cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na3VO4, 25 mM β-glycerol-phosphate, 0.1mM PMSF, and Complete Protease Inhibitor) and vortexed for 20 seconds. The resuspended cell pellet was incubated on ice for 20 minutes and centrifuged at 20,000 g for 30 minutes. Supernatant was collected for further analysis.

Cell Survival Assay

Cell survival analysis was performed using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) following manufactory instruction with minor modification. In brief, 25 microliter of Cell Titer-glo reagent was added to the cell culture medium. Cells were place on a shaker for 10 minutes and were then incubated at room temperature for an additional 10 minutes. Luminescent reading was carried on Tecan SPECTRAFluor Plus reader (Tecan).

Elisa Analysis of Autocrine TNFa Secretion

Cells were plated onto 6-well dishes and allowed to grow to near confluence (approximately 80%). Media was then aspirated and the cells were washed two times with cold PBS. Fresh media (1 ml) was added and 100 ml aliquots were removed at each time point (three independent wells were tested at each time point). Samples were kept at _20_C until ready for use. Elisa analysis was performed using a quantitative sandwich enzyme immunoassay from R&D Systems (TNFa Quanti-glo Chemiluminescent Elisa, QTA00B), as per manufacturer's instructions.

Plasmid and siRNA Transfection

In general, plasmid DNA and Lipofectamine 2000 (Invitrogen) were diluted in Opti-MEM medium (Invitrogen) separately and were left in RT for 5 minutes before they were mixed together. The plasmid DNA and lipid mixture were incubated at RT for another 20 minutes to 1 hour. In the meantime, cells were trypsinized and resuspended in complete cell growth medium without antibiotics before added to the incubated DNA/lipid mixture. For siRNA transfection, siRNA was used instead of plasmid DNA.

Immunoprecipitation

For caspase-8 immunoprecipitation, caspase-8 antibody (Santa Cruz Biotech, SC-1636) was coupled to protein A Dynal beads (Invitrogen) in PBS in the presence of 5 mg/ml bovine serum albumin (Sigma) for 2 hours at room temperature. The pre-coupled beads were then incubated with cell lysates over-night at 4ºC. The next day, beads were washed with lysis buffer. The immunoprecipitants were eluted off the beads using low pH elution buffer (Pierce). Acid elution was neutralized by adding 1/20 volume of 1 M Tris-HCl, pH 9.4.

For Flag or HA pulldown, cell lysates were incubated with M2 Flag agarose beads (Sigma), or HA agarose beads (Sigma), respectively, over-night at 4ºC. The following day, beads were washed with lysis buffer containing

additional 300 mM NaCl. The immunoprecipitants were eluted by directly boiling the beads in SDS sample buffer.

Biotinylated Smac Mimetic Pull Down

Cell lysates were incubated with avidin Dynal beads (Invitrogen) in the presence of 100 nM biotinylated Smac mimetic overnight at 4ºC. The following day, beads were washed with lysis buffer. The precipitants were eluted by directly boiling the beads in SDS sample buffer or eluted using 1uM non biotinylated Smac mimetic.

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