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Mechanism of caspase-3 activation and Mcl-1 inhibition in the regulation of apoptosis

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Mechanism of caspase-3 activation and Mcl-1 inhibition in the regulation of apoptosis

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B.A., Keuka College, 2012

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An abstract of

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in

Graduate Division of Biological and Biomedical Science

Cancer Biology

2018

Abstract

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By Katelyn G. Ponder

Intrinsic apoptosis is regulated by the Bcl-2 family of proteins. Upon an apoptotic stimuli proapoptotic proteins, Bak and Bax will form a multimer on the outer membrane of the mitochondria releasing cytochrome c and activate the caspase cascade. Understanding this mechanism and how the proteins involved are regulated is important for advances in cancer therapies. We focused our attention on the mechanism by which caspase-3 becomes activated at the end of the apoptotic pathway and on how the anti-apoptotic, Mcl-1 could be targeted in the treatment of Multiple Myeloma.

Caspase-3 is found in the cytoplasm as an inactive zymogen dimer. Upon cleavage of the interdomain linker by an initiator caspase and subsequent removal of the N-terminal prodomain, caspase-3 is active. It is unclear if removal of the prodomain is necessary for caspase-3 activity as previous studies were done in cell free systems and have conflicting results. Using a more physiologically relevant model we found the prodomain negatively regulates the activation and activity of caspase-3. Surprisingly a single point mutation at D9 rendered caspase-3 inactive. We also found that for the complete removal of the 28 amino acid prodomain, an initial cleavage event must occur at D9.

Multiple myeloma (MM) is the second most common hematologic malignancy that arises from bone marrow plasma cells. MM cells are dependent on the anti-apoptotic protein, Mcl-1 for survival. Carfilzomib, a proteasome inhibitor, is FDA approved for single-agent use in relapsed/refractory MM. We combined carfilzomib with TG02, a multi-kinase inhibitor, and found that the combination not only had at least additive effects in MM cells lines but also in patient samples. This combination targets Mcl-1 via two different mechanisms as carfilzomib causes an increase in NOXA, an inhibitor or Mcl-1, and TG02 caused a decrease in Mcl-1 protein. Based on our preclinical studies a phase I trial was initiated to test this combination for multiple myeloma.

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Acknowledgments

I would like to thank Dr. Larry Boise for being a supportive and encouraging mentor and the current and past laboratory members for being my family away from home. To my family, pushing me to be better than I thought I could be got me to where I am today. To my husband, you are the best friend I could have asked for and coming home to you and our son kept me going. I love you.

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I. INTRODUCTION

The term apoptosis was first used to describe the mechanism of controlled cell death by Kerr, Wyllie and Currie in 1972(1). Apoptosis is a vital component of numerous cellular processes including embryonic development, regulation of the immune system and normal cell turnover(2). This process is characterized by molecular changes including cell shrinkage, pyknosis and membrane blebbing as well as biochemical changes including chromosomal DNA cleavage, intracellular substrate cleavage and phosphatidylserine externalization(1, 3, 4). This form of cell death keeps the cellular debris contained rather than contaminating the surrounding area. Cells are signaled to undergo apoptosis via multiple stimuli to remove damaged and undesired cells during development.

The ability to regulate cell death has enormous therapeutic potential as inappropriate cell death is the cause of many diseases. (i.e. cancer, auto-immune diseases). One of the Hallmarks of Cancer, described by Hanahan and Weinberg, is evading apoptosis(5). Evading apoptosis is essential for cancer cells due to the increased pressure a mutated cell has to undergo apoptosis. The process of apoptosis is complex with multiple protein-protein interactions and signaling cascades. Understanding the mechanism by which the apoptotic proteins function and the cancer cells evade apoptosis will aid in targeting these mechanisms as a cancer therapy. Many of the proteins involved in apoptosis have been elucidated, but the mechanism by which they are regulated is less understood.

A. Programmed Cell Death: Apoptosis

i. Extrinsic Apoptosis

Intrinsic and extrinsic apoptosis are the two key pathways used by cells to undergo cell death(6). The extrinsic pathway, or death receptor pathway, is initiated via an extracellular interaction between a death receptor on the cells surface and a death receptor ligand. This interaction will lead to recruitment of the death domain containing protein (FADD) and procaspase-8. These interactions form the death-inducing signaling complex (DISC)(6). The DISC complex causes the activation of procaspase-8 into the active enzyme caspase-8, which will then activate procaspase-3, which ultimately leads to cell death(1)(Figure 1).

ii. Intrinsic Apoptosis

Intrinsic apoptosis, also known as the apoptotic mitochondrial pathway, is stimulated through intracellular signals and can be initiated by several cellular stress signals (e.g. serum withdrawal, toxins, radiation). This stimulus will activate the Bcl-2 family of proteins. The Bcl-2 family of proteins are responsible for regulating the integrity of the outer membrane of the mitochondria. When Bax and Bak oligomerize they form a pore in the outer membrane of the mitochondria, thus allowing the release of cytochrome c. This process is referred to as mitochondrial outer membrane permeabilization (MOMP). When cytochrome c is released into the cytoplasm it will form a complex with APAF-1 and procaspase-9. This complex, the apoptosome, will active procaspase-9 into the active enzyme, caspase-9, which will then active caspase-3. Caspase-3 will cleave its cellular targets and ultimately lead to cell death (Figure 1). Although the activation of the extrinsic and intrinsic pathways differs, they both result in the activation of caspase-3 and subsequent cell death. There is also evidence that the proteins involved in both pathways can interact and influence the other pathway(7).

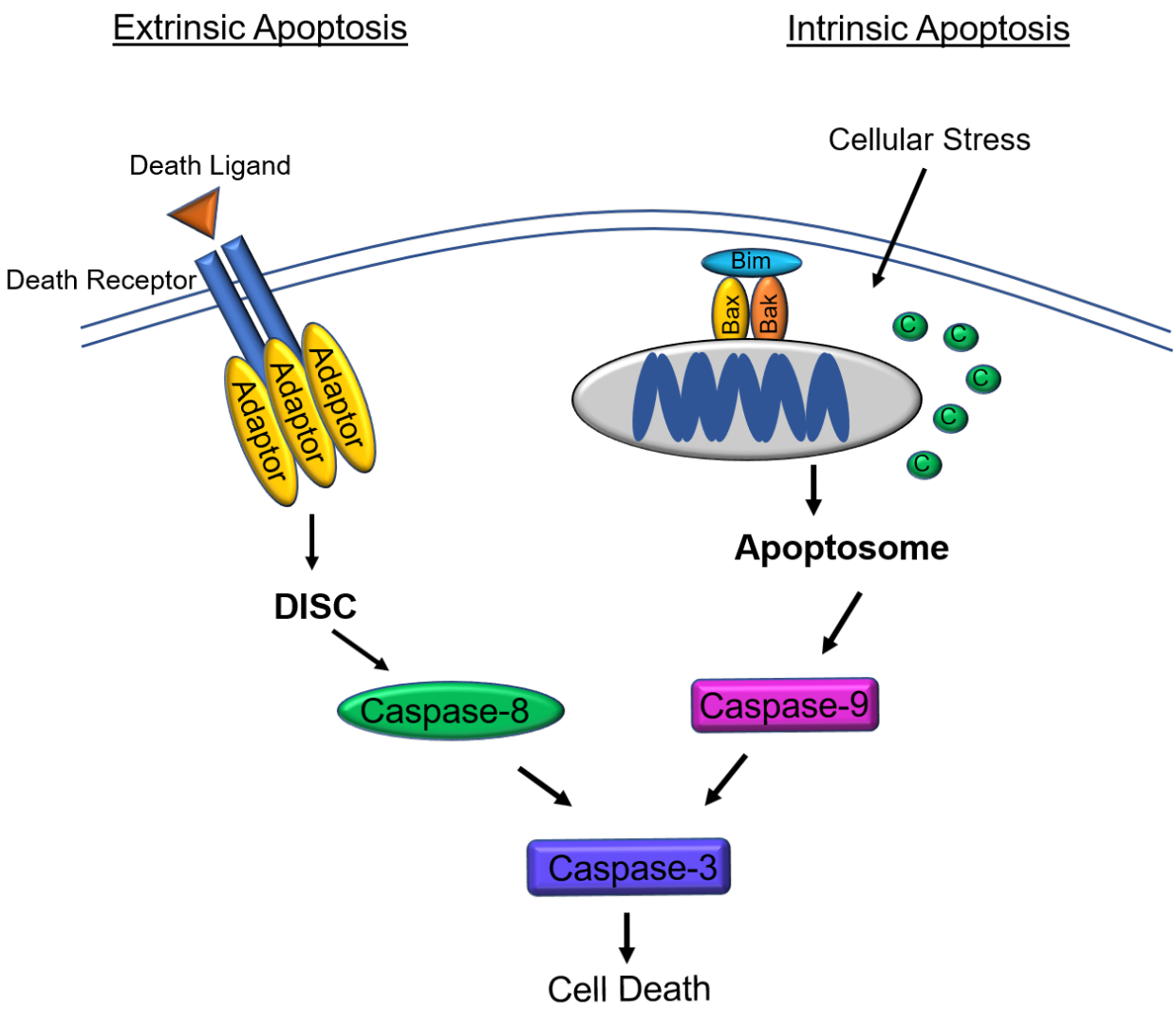

Figure 1. The extrinsic and intrinsic apoptotic pathways. The extrinsic apoptotic pathway is initiated by an extracellular death ligand binding to its death receptor. Adapter proteins bind to the intracellular region of the death receptor causing the activation of the DISC complex and subsequent activation of caspase-8 and caspase-3. The intrinsic apoptotic pathway is induced by the interactions of the Bcl-2 family of proteins causing mitochondrial outer membrane permeblization (MOMP) and the downstream activation of caspase-9. Both pathways result in the activation of caspase-3, which ultimately leads to cell death.

B. Cysteine-Aspartic Acid Proteases

Cysteine-aspartic acid proteases, caspases, are a class of enzymes that when activated will cleave cellular targets at specific recognition sequences. These cleavage events are dependent on catalytic cysteine residues found in the active site and occur after specific amino acid sequences containing aspartic acid residues(8). Caspases play regulatory roles in the immune response, cell differentiation, and cellular migration(9-11). Caspase-1, 4, and 5 are involved in regulation of the immune response while caspase-3, 6, 7, 8 and 9 acts to regulate cell death. (9, 12).

The caspases that regulate the apoptotic pathway can be divided into two categories, the initiator and executioner caspases. These caspases differ in their structure, function and the mechanism by which they are activated. The initiator caspases are activated through induced proximity while the executioner caspases are activated through cleavage events. Once the executioner caspases are active they cleave a number of cytoplasmic and nuclear substrates resulting in the morphologic changes seen during apoptosis(13).

i. Activation and Structure of Apoptotic Caspases

Caspases are composed of three domains, an N-terminal prodomain, a p20 domain and a C-terminal p10 domain. Initiator caspases (i.e. caspase-8 and caspase-9) have a longer N-terminal prodomain compared to their counterpart, the executioner caspases. Within the prodomain, initiator caspases can have either a death effector domain (DED) or a caspase recruitment domain (CARD) (11) (Figure 2A). Initiator caspases are found in the cytoplasm as inactive monomers. Through the interactions of the DED and CARD regions the caspases can aggregate with scaffolding complexes and autoactivate (14, 15). Executioner caspases (i.e.

caspase-3 and caspase-7) are composed of a shorter N-terminal prodomain that lacks both DEDs and CARDs. They are found in the cytoplasm as inactive zymogen dimers and are activated by an initiator caspase cleavage event. This mechanism of activation prevents inappropriate activation of the executioner caspases.

Activation of the executioner caspases is initiated by cleavage of the interdomain linker, found between the p20 and p10 domains, by an initiator caspase (Figure 2B). For example, caspase-9 will cleave caspase-3 at amino acid D175 located within the interdomain linker (Figure 3). This cleavage will allow for a conformational change to occur exposing the active site at C163 allowing for substrate recognition(16). The N-terminal prodomain is removed through autocleavage and caspase-3 is now active and will target and cleave cytoplasmic and nuclear proteins resulting in cell death.

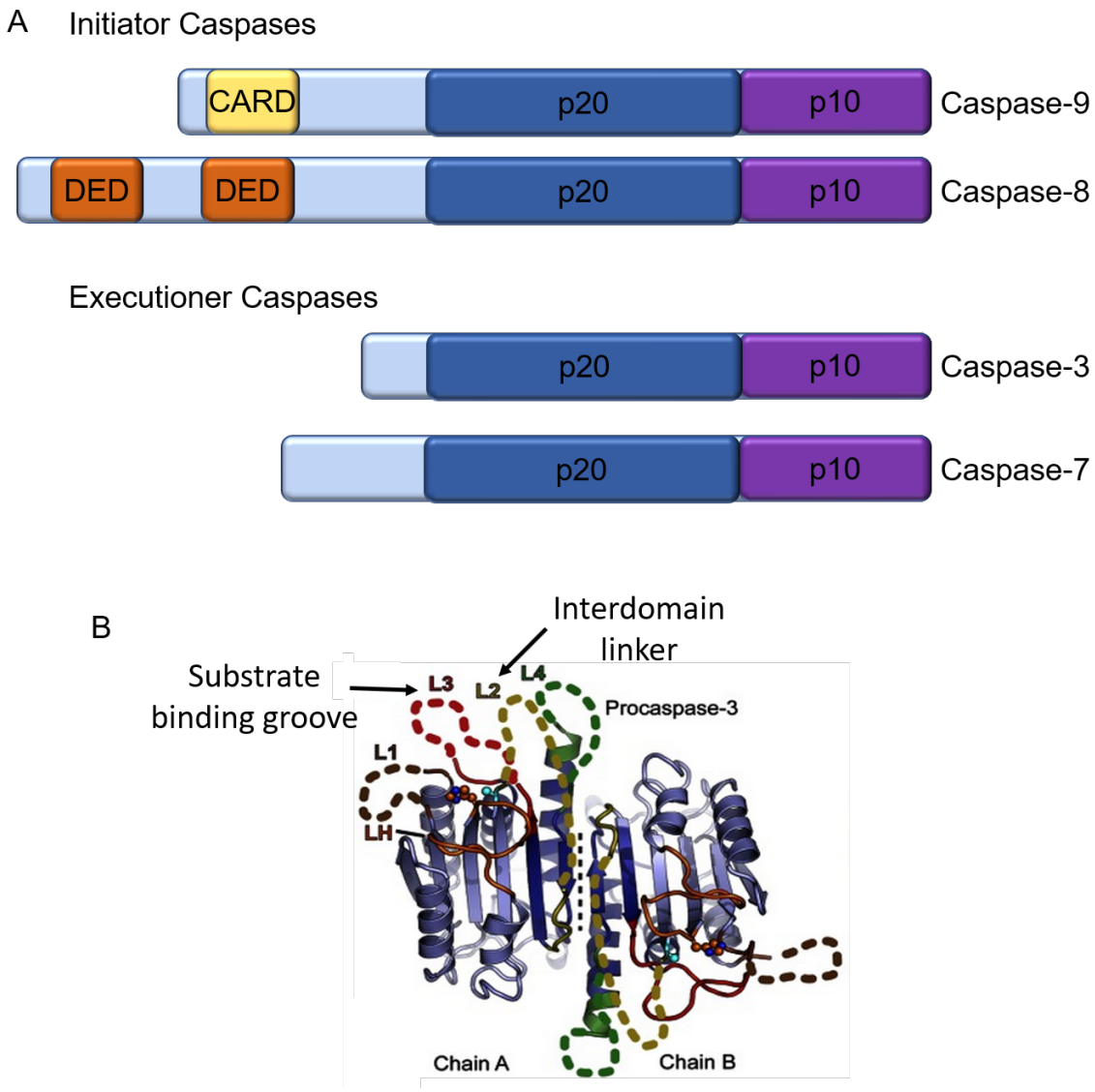

Figure 2. Structure of caspases. A. Caspases are composed of three domains, the N-terminal prodomain (light blue), the p20 domain and the C-terminal p10 domain. Initiator caspases have longer prodomains that contain caspase activating and recruiting domains (CARD) and/or death effector domains (DED). Executioner caspases have shorter prodomains that lack both CARD and DED elements. B. The crystal structure of catalytically inactive procaspase-3 (C163A) obtained from Thomsen et al(17). The dimer interface is represented by the dashed line and the interdomain linked and substrate binding groove are located at L2 and L3, respectively.

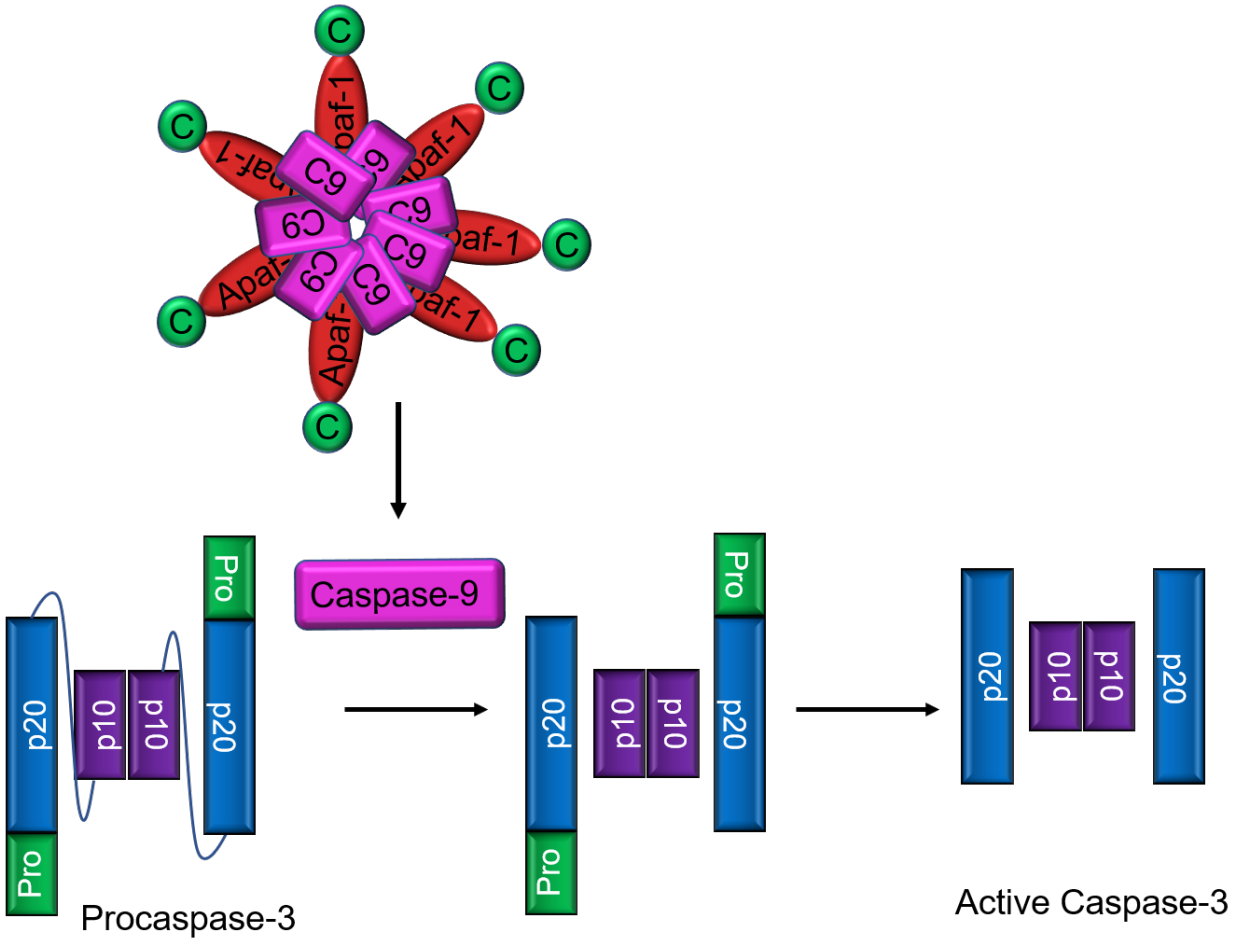

Figure 3. Structure and activation of caspase-3. The inactive form of caspase-3, procaspase-3, is composed of three domains, the prodomain (Pro), p20 and p10. After caspase-9 is activated, through induced proximity via the apoptosome, it will cleave the interdomain linker at D175. Caspase-3 can then remove its own prodomain, resulting in the active enzyme.

C. Bcl-2 Family of Proteins

The Bcl-2 family of proteins regulate the process of apoptosis. Within this family there are different classes of proteins including the pro-apoptotic, anti-apoptotic and BH3 only proteins (Figure 4). These proteins contain one or more Bcl-2 homology (BH) domains that allow interaction with one another(18). Through the interaction of the BH domains the pro and anti-apoptotic proteins will determine if a cell lives or dies via apoptosis.

Within the class of pro-apoptotic proteins are the multi-(BH) domain effectors, Bax and Bak. Once activated Bax and Bak will homo-oligomerize and permeabilize the mitochondria outer membrane, resulting in the release of cytochrome c. Bim is also a pro-apoptotic protein, but it only contains one BH domain, and is termed a direct activator BH3-only protein. Bim is able to transiently bind to Bax and Bak facilitating their activation (Figure 5).

The anti-apoptotic Bcl-2 family members are able to antagonize intrinsic apoptosis by binding to the pro-apoptotic family members. This class of proteins contain Mcl-1, Bcl-2, and Bcl-x_L. The interaction between the pro and anti-apoptotic proteins is also influenced by the BH3-only sensitizers, Noxa, Bad, Bid, Bik, and Bmf. These proteins can bind to the anti-apoptotic proteins, thus freeing the direct activators and effectors, and allowing MOMP to occur. The interactions between the Bcl-2 family of proteins will determine if the process of apoptosis is to occur. Therefore, this signaling cascade is targeted for cancer therapies as cancer cells are dependent on the anti-apoptotic proteins for survival.

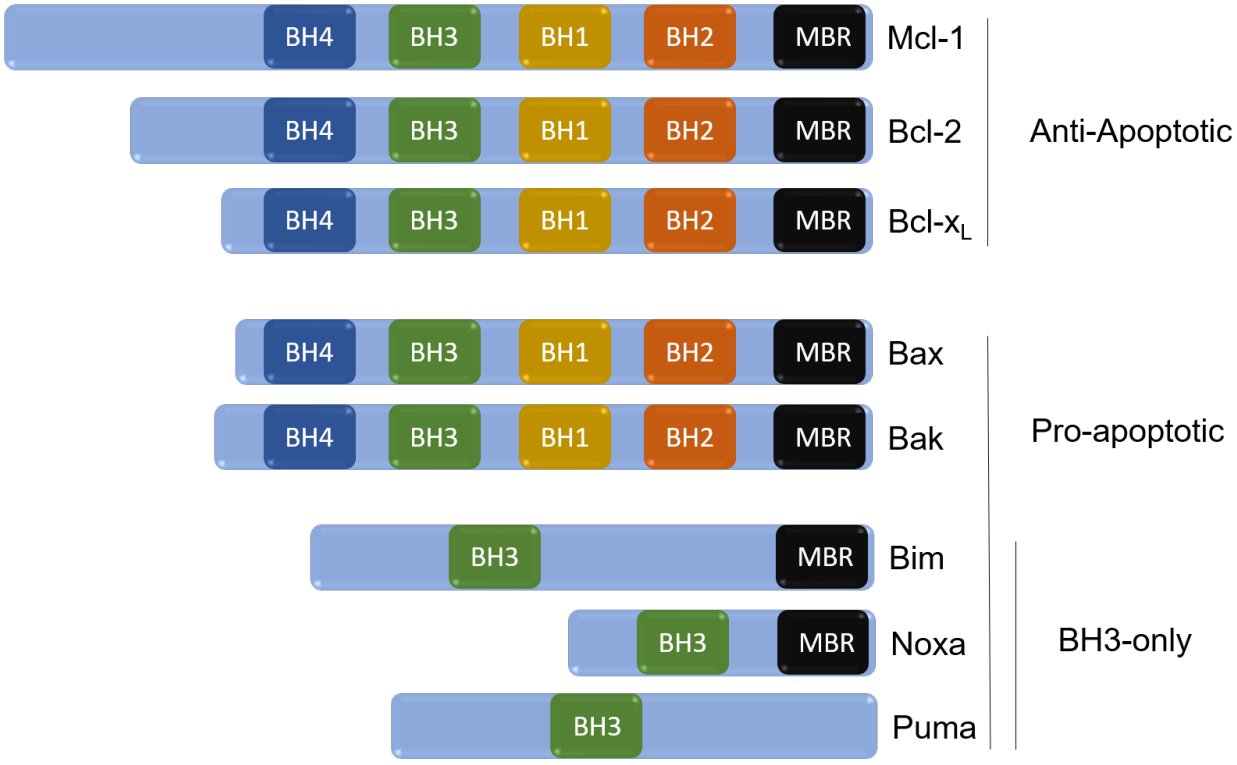

Figure 4. The structure of the Bcl-2 family members. The Bcl-2 family members are characterized by the presence of Bcl-2 homology domains (BH). The anti-apoptotic proteins contain all four BH domains. The pro-apoptotic proteins can be divided into two groups, the effector proteins that contain all four BH domains and the BH3-only proteins that contain only the BH3 domain. MBR: membrane binding region.

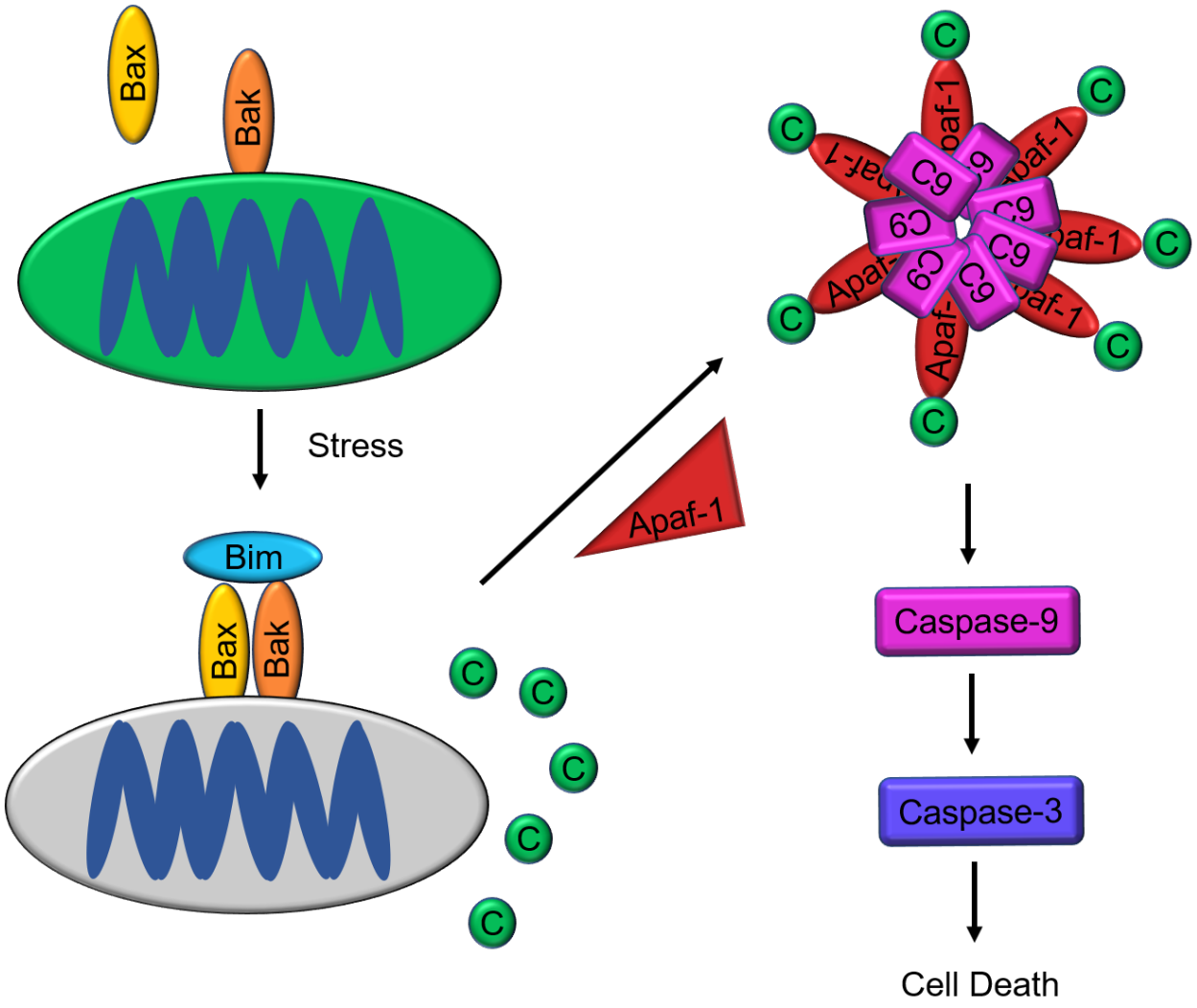

Figure 5. Intrinsic apoptotic signaling cascade. Upon a stress signal, like serum starvation, activator Bim will aid in the dimerization of Bak and Bax on the surface of the mitochondria. Bak and Bax will form a pore allowing the release of cytochrome c from the outer membrane. After mitochondrial outer membrane permeablization (MOMP) cytochrome c will bind to Apaf-1 causing a confrmoational change. Caspase-9 will then bind forming the apoptosome. Caspase-9 is activated via induced proximity. Active caspase-9 will activate caspase-3 by a cleavage event. Caspase-3 will cleave cellular targets ultimately leading to cell death.

D. Multiple Myeloma

Multiple myeloma is the second most common hematologic malignancy. It is estimated that in 2017 there will be 30,280 new cases diagnosed and 12,590 deaths in the United States(19). Currently there is not a cure for multiple myeloma, although the introduction of proteasome inhibitors and immunomodulatory drugs (IMiDs) have resulted in improved overall survival(20). Multiple myeloma is caused by the aberrant growth of bone marrow plasma cells. Plasma cells are terminally differentiated B cells that are responsible for production of antibodies(21). For multiple myeloma to occur the plasma cells need to evade the process of cell death and undergo aberrant growth. Although the myeloma cells have changed to evade the process of cell death they still maintain the plasma cell biology. Therefore, multiple myeloma cells are dependent on the anti-apoptotic Bcl-2 family of proteins for survival and on the proteasome given the high production of antibodies.

As was discussed in the Bcl-2 family of proteins section, there are two classes of Bcl-2 family of proteins, the anti- and pro-apoptotic proteins. For myeloma cells to evade apoptosis they will become dependent on the anti-apoptotic proteins. Myeloma cells are dependent on Mcl-1, an anti-apoptotic protein, for survival(22). Mcl-1 will bind and sequester Bim, a pro-apoptotic protein, thus, inhibiting the process of cell death. Bim can be released from Mcl-1 if the proapoptotic protein NOXA binds and inhibits Mcl-1. This will allow the release of Bim and cell death can occur.

Given that myeloma cells are dependent on Mcl-1 for survival drugs that can target Mcl-1 will be a beneficial treatment. This is one reason why proteasome inhibitors have been effective in treatment of multiple myeloma. Carfilzomib, a proteasome inhibitor, has been shown to increase the levels of NOXA, thus inhibiting the anti-apoptotic activity of Mcl-1. (23-25).

Proteasome inhibitors are also effective as the myeloma cells maintain their plasma cell biology of antibody production. With high rates of antibody production, the cells need the proteasome to degrade misfolded and high turn over proteins. When the proteasome is inhibited in these cells the unfolded protein response occurs which leads to cell death.

i. Proteasome Inhibitors

The proteasome is a protein complex that is responsible for degradation of high-turnover and misfolded proteins. The proteasome is composed of two 19s subunits and a 20s subunit. Within the 20s subunit there are two α rings and two β rings. The β5 subunit contains the chymotrypsin like activity of the proteasome(26). If this region of the proteasome is inhibited the proteasome will not be able to degrade the high-turnover and misfolded proteins. This will result in endoplasmic reticulum stress, the unfolded protein response and ultimately cell death. Since multiple myeloma cells are responsible for antibody production, they have a higher than normal rate of protein turnover. Therefore, using a proteasome inhibitor to induce apoptosis within the cells would be advantageous.

Carfilzomib (Kyprolis®) is an FDA approved proteasome inhibitor for single use in relapse/refractory multiple myeloma. Carfilzomib binds irreversibly to the β5 subunit of the proteasome, thus inhibiting the chymotrypsin-like activity of the proteasome(26). When the proteasome is inhibited the unfolded protein response is triggered (Figure 6). Carfilzomib has also been shown to increase the expression of NOXA. NOXA, a pro-apoptotic protein, will bind to Mcl-1 thus allowing for release of Bim to facilitate the release of cytochrome c.

Carfilzomib is now being tested in combination therapies because of its safety profile(27-29). Combination therapies are beneficial in hematologic malignancy as the combination can better

target the heterogenous population and have been shown to increase the amount of apoptosis. Given the dependence of multiple myeloma cells have on Mcl-1 using a combination that could both target Mcl-1 would be beneficial.

ii. TG02

TG02 is a novel multi-kinase inhibitor that targets CDKs 1, 2, 7 and 9 and has been shown to induce G1 cell cycle arrest and apoptosis(30). CDK9 is involved in transcriptional regulation. Therefore, inhibiting CDK9 would cause short half-life proteins, like Mcl-1, to be depleted from the cell quicker than those with a longer half-life. More interestingly, TG02 has been shown to increase the activation of proapoptotic protein Bax and decrease the levels of Mcl-1(31). Since TG02 causes a decrease in Mcl-1 levels, it would be beneficial to combine TG02 with Carfilzomib causing dual inhibition of the protein myeloma cells are dependent on for survival, Mcl-1.

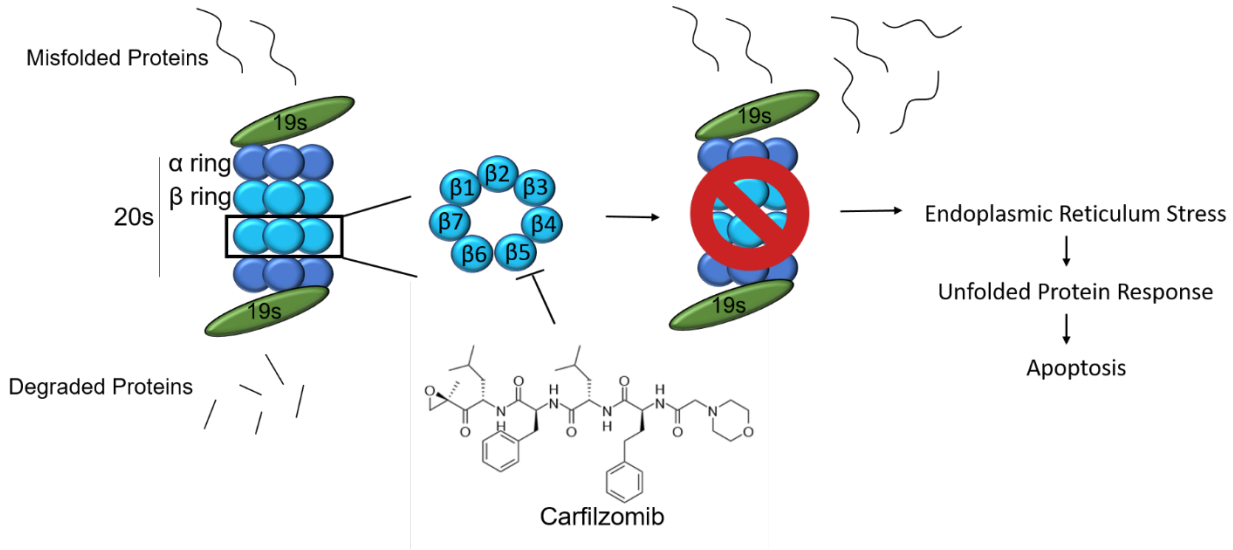

Figure 6. Carfilzomib irreversibly to the proteasome causing cell death. The proteasome is composed of two 19s subunits and a 20s subunit that has two α rings and two β rings. The proteasome is responsible for degrading misfolded proteins. Carfilzomib will irreversibly bind to the β5 subunit thereby blocking the chymotrypsin-like activity. This results in the accumulation of misfolded proteins that causes endoplasmic reticulum stress, the unfolded protein response and ultimately cell death.

E. Statement of Problem

Caspase-3 is the key executioner caspase that is activated at the end of the apoptotic cascade. Once activated, caspase-3 cleaves numerous cellular targets which will ultimately lead to cell death. Although, the mechanism why which procaspase-3 is activated is not fully understood. There are conflicting studies that demonstrate that the N-terminal prodomain has no effect on activity and others that show that removal of the prodomain causes caspase-3 to be constitutively active(32-34). As the key executioner caspase, it is important to understand how caspase-3 becomes active. Understanding how caspase-3 becomes active will allow for further understanding of its function in the process of apoptosis and in its non-apoptotic functions, like cellular adhesion and migration(9).

Knowing that multiple myeloma cells are dependent on Mcl-1 for survival provides a mechanism to target. Given that there are many ways to inhibit the function of Mcl-1 (i.e. transcriptionally, protein degradation and binding) different drug combinations could prove to be advantageous. Gaining insights on how different drugs are functioning to induce cell death in myeloma cells will aid in advancement of improved therapies. Without the understanding of which anti-apoptotic proteins the multiple myeloma cells are dependent on and the mechanism by which they can be inhibited there will be no advancement in therapeutic options.

For the discovery of new therapeutic strategies to be determined we must first understand the mechanism by which the proteins involved in the disease are regulated and activated. Regulation of the apoptotic pathway determines cellular fate. Once cells can evade this process aberrant proliferation can occur. We have focused on two regulatory apoptotic proteins, caspase-3 and Mcl-1. Gaining a complete understanding of how key apoptotic proteins, like

caspase-3 and Mcl-1, function and are regulated will aid in therapeutic advances for patients with cancer, specifically multiple myeloma.

II. THE PRODOMAIN OF CASPASE-3 REGULATES ITS OWN REMOVAL AND CASPASE ACTIVATION

(Originally published in Cell Death Discovery, October 17, 2018)

The prodomain of caspase-3 regulates its own removal and caspase activation

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Running Title: Regulatory role for the prodomain of caspase-3

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Conflict of Interest

The authors declare no conflicts of interest

Abstract

Caspase-3 is a cysteine-aspartic acid protease that cleaves cellular targets and executes cell death. Our current understanding is caspase-3 is activated by the cleavage of the interdomain linker and then subsequent cleavage of the N-terminal prodomain. However previous reports have suggested that removal of the prodomain can result in the constitutive activation of caspase-3, although other studies have not observed this. To address this question in a more physiological setting we developed an inducible doxycycline system to express a mutant form of caspase-3 that lacks the prodomain (Δ28). We found that the removal of the prodomain renders the cells more susceptible to death signals, but the caspase is not constitutively active. To elucidate the regions of the prodomain that regulate activity we created deletion constructs that remove 10 and 19 N-terminal amino acids. Surprisingly, removal of the first 10 amino acids renders caspase-3 inactive. Following serum withdrawal, the interdomain linker is cleaved, however the remaining prodomain is not removed. Therefore, there is a specific amino acid or stretch of amino acids within the first 10 that are important for prodomain removal and caspase-3 function. We created different point mutations within the prodomain and found amino acid D9 is vital for caspase-3 function. We hypothesize that an initial cleavage event at D9 is required to allow cleavage at D28 that causes the complete removal of the prodomain allowing for full caspase activation. Together these findings demonstrate a previously unknown role of the prodomain in caspase activation.

Introduction

Caspase-3 is a cysteine-aspartic acid protease that is best known for its enzymatic function at the end of the intrinsic apoptotic cascade. There are two classes of caspases that are involved in the process of apoptosis, initiator (e.g. caspase-8, -9) and executioner caspases (e.g. caspase-3, -7). Both groups are composed of a N-terminal prodomain, a large subunit (p20) and a smaller c-terminal subunit (p10) (12, 35). Notably, the initiator caspases have a longer N-terminal prodomain, compared to the executioner caspases, and they are responsible for the initial cleavage of executioner caspases that leads to their activity (10, 36). Executioner caspases are found within the cytoplasm as inactive zymogen dimers. Caspase-3, an executioner caspase, is held together as a dimer given the dimer interface is hydrophobic (37). The dimer conformation also aids in the ability of initiator caspases to process the executioner caspases (38).

The processing of the caspase-3 interdomain linker, found between the p20 and p10 domains, is completed by initiator caspase, caspase-9 (39-41). Once caspase-9 cleaves caspase-3 at the interdomain linker, caspase-3 undergoes a conformational change that exposes its active site found at amino acid C163. Previous studies have shown that caspase-3 undergoes two different cleavage events. The first, by caspase-9, within the interdomain linker and the second to remove the N-terminal prodomain (33). Once activated, caspase-3 will cleave key structural proteins, cell cycle proteins and DNase proteins, such as poly(ADP-ribose) polymerase, gelsolin, ICAD/DFF and DNA-dependent kinase (42-44). These cleavage events result in the blebbing and condensing of cells which ultimately leads to cell death (45).

The apoptotic activity of caspase-3 is well characterized, but the regulation of this process is not fully understood. Previous studies demonstrated that the complete removal of the

prodomain enhances apoptotic activity (34). However, it is unknown whether this induction results in complete activation of caspase-3 or lowers the activation threshold. No studies have determined if the induction of activity is due to loss of full length prodomain or a specific region within the prodomain. Additionally, no structural data of caspase-3 containing the prodomain has been determined. Therefore, we do not know where the prodomain is found in the inactive procaspase-3 enzyme. The prodomain is highly conserved suggesting it has a function (Fig. S1). Therefore, we undertook an investigation of the role of the prodomain in caspase-3 activation.

Materials and Methods

Cell Culture

Immortalized mouse embryonic fibroblasts (MEFs) were cultured as previously described (9). ΦNX-Ecotropic packaging cell lines were grown in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin, 1% l-Glutamine, 1% non-essential amino acids and 1% sodium pyruvate.

Cloning of Plasmids

Primers were designed for the DNA sequence of interest and polymerase chain reaction was performed using Taq PCR Kit (New England Biosystems, Waltham, MA). After amplified target DNA was run on 1% agarose DNA gel, the DNA was isolated using the GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, IL). Isolated DNA and empty vector, pBabe, were digested and the ligation reaction was conducted using the DNA insert ligation kit (Thermo Fisher Scientific, Waltham, MA) (Table S1).

Site Directed Mutagenesis

Primers for the indicated mutations were designed and mutagenesis was completed using the Quick Change XL Site Directed Mutagenesis kit (New England Biosystems, Waltham, MA) (Table S1).

Retroviral Transduction

ΦNX-Ecotropic packaging cells were transfected with a plasmid (pBabe-puro, C3 pBabe-puro, C3^{C163A} pBabe-puro, C3^{C163S} pBabe-puro, C3 Δ28 pBabe-puro, C3 Δ28^{C163A} pBabe-puro, C3 Δ10 pBabe-puro, C3 Δ19 pBabe-puro, C3 D9E pBabe-puro, C3 D9A pBabe-puro, C3 D9,28A

pBabe-puro, C3 D175A pBabe-puro, C3 D9,28,175A pBabe-puro) using Lipofectamine (Invitrogen, Carlsbad, CA). Target MEFs were then infected with retroviral supernatants using Polybrene Infection/ Transfection Reagent (Millipore, Burlington MA). Two rounds of infection within a 36-hour period was conducted. After 36 hours viral supernatants were removed from the target cells and replaced with fresh medium for 24 hours and then were selected with 2.5 μg/ml puromycin (Sigma, St. Louis, MO).

Lentiviral Transduction

293T cells were transfected with pHRCMV8.2 R, CMV-VSVG and either C3-GFP pCW-puro or C3Δ28 pCW-puro using Lipofectamine (Invitrogen, Carlsbad, CA). Target MEFs were infected as described above.

Cell Death Analysis

$0.25x10^6$ cells were seeded in 6-well plates and allowed to grow overnight. Complete medium was replaced with serum free medium to induce apoptosis. Cells were collected at 0, 24, and 48 hours and stained with annexin V-FITC (BioVision 1001–1000, Milpitas, CA) and propidium iodide (2 μg/ml Sigma, St. Louis, MO). Cells were then measured with a BD FACSCanto II as described previously(46). Data were analyzed using FlowJo (TreeStar, Ashland, OR) software.

Colometric Caspase-3 Activity Assay

$5x10^4$ cells were seeded in a 10-cm dish and allowed to grow for 24 hours. Media was removed and replaced with either serum free DMEM or complete DMEM. Cells were collected after 24 hours. Caspase activity was determined using the Caspase-3 Assay Kit (Colorimetric) (abcam ab39401, Cambridge, UK).

Caspase-3 Activation

$0.25x10^6$ cells were seeded in 6-well plates and allowed to grow over night. Cells were serum starved with DMEM media lacking fetal bovine serum. After 0, 3, 6, 12, 24 and 48 hours cells were collected and lysed. Western blotting was performed as previously described(47). Primary antibodies: rabbit anti-caspase-3 (Cell Signaling, Danvers, MA) and mouse anti-actin (Sigma, St. Louis, MO).

Immunoblotting

Western blotting was performed as previously described(47). Primary antibodies: rabbit anti-caspase-3 (Cell Signaling, Danvers, MA), XIAP (BD Biosciences, Franklin Lakes, NJ) and mouse anti-actin (Sigma. St. Louis, MO). Secondary antibodies: horseradish peroxidase-conjugated sheep anti-mouse and horseradish peroxidase-conjugated donkey anti-rabbit (Amersham, Little Chalfont, UK). Proteins were detected by chemiluminescence (Amersham, Little Chalfont, UK).

Results

To study the role of the prodomain in caspase-3 activation we stably introduced caspase mutants into immortalized caspase-3 deficient mouse embryonic fibroblasts (MEFs). As can be seen in Figure 1A, the level of expression of parental ($C3^{-/-}$C3) or mutant forms of caspase-3 were similar to that observed in wild type MEFs. Two different catalytically inactive forms of caspase-3, C163A and C163S, were expressed in caspase-$3^{-/-}$ MEFs and used to demonstrate that the catalytic site at position 163 is essential for caspase-3 function. Introduction of full length caspase-3 into the MEFs results in caspase activity (Fig. 1B) and the cells undergo apoptosis like the WT cells following serum withdrawal (Fig. 1C). However, the catalytically inactive forms, C163A and C163S, are inactive under these conditions (Fig. 1B) and do not induce cell death (Fig. 1C)(9). These results confirm that this is a functional model to measure caspase regulation and function in a physiologic setting.

Previous studies have been conducted to determine the functional role of the prodomain of caspase-3. The studies conducted were performed using transient transfection, which could provide a stress signal to cells and confound apoptotic assays. Therefore, we sought to create a stable cell line that expresses a form of caspase-3 that lacks the 28 amino acid prodomain (Δ28). We were able to create two stable cells lines, $C3^{-/-}$C3Δ28 and the catalytically inactive form $C3^{-/-}$ $C3\Delta28^{C163A}$ (Fig. S2A). We used these cells to determine the amount of cell death and the caspase activity and found that, as expected the $C3^{-/-}C3\Delta28^{C163A}$ cells did not undergo apoptosis and were not able to cleave a synthetic DEVD substrate. Unexpectedly, the $C3^{-/-}$C3Δ28 cells were able to cleave a synthetic substrate but did not undergo cell death (Fig. S2B and S2C).

Given previous studies showed removal of the prodomain increases apoptotic activity we sought to determine if, when we created this stable cell line, we selected for cells that not only

express our construct but also have a mutation or upregulation of another protein that could protect these cells from cell death. These cells have an increased expression of the x-linked inhibitor of apoptosis protein, XIAP (Fig. S2A). XIAP is an inhibitor of caspase-3 and the increased expression of XIAP is one potential explanation as to why the $C3^{-/-}$C3Δ28 MEFs do not die (48). Given the stable expression of C3Δ28 is lower than wildtype caspase-3 (Fig. S2A), we hypothesized that XIAP could be targeting C3Δ28 to the proteasome (49, 50). To test this hypothesis, we treated the cells with the protease inhibitor Carfilzomib, but did not see changes in the amount of cell death (Fig. S2D). Therefore, we believe that XIAP is working as a direct inhibitor of the caspase. Expressing caspase-3 and C3Δ28 transiently was consistent with our findings as deletion of the prodomain resulted in significantly lower expression (Fig S2E). However, cell death was observed in a transient manner (Fig. S2F). Taken together these data suggest that removal of the prodomain does not result in a constitutively active caspase, however the caspase has a lower activation threshold and these cells cannot tolerate its presence, even at physiologic levels. Therefore, removal of the prodomain makes caspase-3 easier to activate.

To be able to address the question of the role of the prodomain in caspase-3 apoptotic activity, without the stress associated with transient introduction, we turned to a doxycycline inducible system. We stably expressed two different doxycycline inducible plasmids in caspase-$3^{-/-}$ MEFs, pCW C3 GFP and pCW C3Δ28 GFP (Fig. 2A). We determined that after 48 hours of 3 μg/mL doxycycline treatment the cells express the constructs and the doxycycline treatment does not cause cell death (Fig. S3A and S3B). Following 48 hours doxycycline treatment, serum withdrawal results in caspase activation with similar kinetics as the cells with constitutive caspase-3 expression (Fig. 2B). We next compared the amount of cell death in the C3 GFP and C3Δ28 GFP MEFs. The cells were given doxycycline for 48 hours and were serum starved for 0,

24 and 48 hours. We analyzed only the cells that expressed the constructs by gating on the GFP positive cell population. When comparing the C3 GFP and C3Δ28 cells after 24 hours, significantly more cell death was observed in the C3 GFP cells than the C3Δ28 GFP cells, however, by 48 hours the death in the two populations was similar (Fig. 2C). Comparing the mean fluorescence intensity (MFI) of these cells the C3 GFP cells have a higher MFI than the C3Δ28 GFP at all three time points (Fig. 2D). Thus, the likely explanation for the difference in cell death at 24 hours is the disparity in protein expression. These data are consistent with C3Δ28 not being constitutively active, but easier to activate. Thus, the prodomain appears to regulate caspase-3 activity.

The 28 amino acid prodomain of caspase-3 is highly conserved (Fig. S1A), therefore we sought to determine if there were specific regions within the prodomain that were important for its function in regulating caspase-3 activity. We initially generated two truncation mutants of the prodomain and expressed them stably in caspase-$3^{-/-}$ MEFs (Fig. 3A). We removed the first 10 N-terminal amino acids (Δ10) and the first 19 (Δ19). Surprisingly, and in contrast to deletion of the full prodomain, neither truncation mutant was able to restore serum withdrawal induced apoptosis (Fig. 3B). Consistent with this phenotype, these deletions resulted in complete loss (C3Δ19) or reduced (C3Δ10, $p=0.058$ compared to $C3^{-/-}$C3) caspase activity upon serum withdrawal (Fig. 3C). Together these data suggest that the prodomain is removed because it contains a region that negatively regulates caspase-3 activity following caspase-9 mediated removal of the interdomain linker. Based on these findings it also appears that the removal of the prodomain requires the first 10 amino acids.

To elucidate the important amino acids within the prodomain for apoptotic regulation we created various point mutations within the prodomain. Previous studies have demonstrated that

mutating D9, D28 and D175 results in an uncleavable caspase(32), therefore we focused on the role of D9 and D28 in caspase activity and induction of apoptosis following serum withdrawal. We created the single mutations, D9A, D28A and D175A as well as double and triple mutations and stably expressed them in caspase-$3^{-/-}$ MEFs (Fig. 4A). Consistent with previous findings, mutating all 3 sites results in a caspase that is not activated by serum withdrawal and does not induce apoptosis (Fig. 4B and 4C). However, mutated individually we found that the cells have significantly less caspase activation and do not undergo apoptosis (Fig. 4B and 4C). Interestingly, loss of D9 or D28 resulted in nearly identical changes in caspase activity and induction of apoptosis. This is consistent with the possibility that the prodomain must be cleaved at D9 prior to complete removal through cleavage at D28. Therefore, we next determined how the mutations alter caspase activation.

We directly assessed caspase-3 activation following serum starvation by measuring caspase-3 cleavage via western blot analysis. When caspase-3 is not cleaved it has a molecular weight of 32 kDa which is detected on a western blot by a primary antibody that binds to the p20 domain. When the interdomain linker is cleaved and the prodomain is removed, resulting in the mature p20 fragment, the antibody detects a peptide at 17 kDa, but if the prodomain is not removed the fragment will run at 20 kDa (Fig. 5A). Blocking the interdomain cleavage through mutation of D175 results in complete loss of procaspase-3 processing following serum withdrawal (Fig. S4). Detection of cleaved caspase-3 is highest after 24 hours in all other samples (Fig. 5B). Twenty-four-hour serum starvation resulted in the generation of the mature 17 kDa p20 fragment in $C3^{-/-}$C3 cells. However, when the active site is mutated there is a shift in the mobility of the fragment to a molecular weight of 20 kDa indicating that the prodomain is not removed (Fig. 5C). This is consistent with the model that the active site at C163 is responsible

for removal of the prodomain. Interestingly deletion of the first 10 amino acids or mutation of D9 have no effect on interdomain cleavage yet prevents the full maturation of the p20 domain. The fragment is the same size as the D9,28A mutant following serum withdrawal suggests that D9 is necessary for cleavage of D28. Consistent with this finding, the D28A mutant migrates faster than the D9,28A mutant suggesting that D9 is cleaved in the prodomain.

Given the importance of amino acid D9 in prodomain removal, we wanted to further investigate the importance of cleavage at this site. The fact that D28A cleaved product migrates faster than D9A cleaved product supports a cleavage event at D9. We stably expressed a C3 D9E construct into caspase-$3^{-/-}$ MEFs (Fig. 6A). The mutation of aspartic acid to glutamic acid results in a site that can still be cleaved by the caspase, albeit less effectively (51).This mutation still supported significant caspase activity following serum withdrawal. However, there was only a minimal but significant increase in cell death compared to empty vector controls and significantly less death than observed in cells where WT caspase-3 was introduced (Fig 6B and 6C, respectively). $C3^{-/-}$C3 D9E MEFs were able to remove the prodomain, but inefficiently as indicated by the presence of bands at 17 and 20 kDa (Fig 6D). This data supports the hypothesis that there is a cleavage event at D9 that is required for full removal of the prodomain and full caspase-3 activation through cleavage at D28.

Discussion

The apoptotic function of caspase-3 has been well characterized but the mechanism by which procaspase-3 becomes active caspase-3 is not fully understood. Previous studies demonstrated in a cell free system that there are two cleavage events to form the p17 and p12 subunits (33). While one study concluded that removal of the prodomain resulted in a constitutively active caspase-3 (34) others have demonstrated that the prodomain does not influence apoptotic activity (52). These studies expressed caspase-3 transiently. We utilized an inducible system to express C3Δ28 because the stress from transiently expressing caspase-3 could be enough to activate caspase-3. Given that, at 48 hours post doxycycline induction, the cells are expressing C3Δ28 GFP at the same protein level as C3 GFP and there is no apoptosis occurring (Fig. 2B and 2C) we conclude that removal of the prodomain does not result in constitutive activation of caspase-3. Cleavage at the interdomain linker is still required. However, removal of the prodomain may allow for more efficient activation of caspase-3, suggesting that the prodomain plays a regulatory role in caspase-3 activation.

Indeed, our findings demonstrate that, in contrast to deleting the full prodomain, if one deletes part of the prodomain, the caspase cannot be efficiently activated. This suggests that a region within the prodomain is negatively regulating activation of caspase-3 following cleavage by caspase-9. Since the deletion of the first 19 residues resulted in a complete loss of function while the deletion of the first 10 residues retained activity, we propose that the negative regulatory region is located between residues 20 and 27. Deletion of the full prodomain removes this negative regulatory region. The discrepancy between the Δ10 and Δ19 activity suggested that loss of the first 10 amino acids could somehow facilitate activation. Therefore, we focused on the possibility that this region needs to be cleaved for activation.

There are two caspase specific cleavage sites within the prodomain of caspase-3. Previous studies have shown, using recombinant caspase-3 or conducting experiments *in vitro* using cytosolic extracts, that there is a rapid cleavage event at D9 followed by a slower cleavage at D28 (33, 52, 53). Furthermore an uncleavable form of procaspase-3 (D9A, D28A, D175A), has been shown to have a lower catalytic efficiency(32). To determine if these sites are important in a physiological setting we tested the activity of MEFs expressing different point mutations within the prodomain. Although previous work had shown that there are cleavage events at D9 and D28 we found that mutating just D9 was sufficient to block activation and cell death.

These findings raise some questions about the regulation of caspase activation. Why are two cleavage events required to remove the prodomain? Our data demonstrate that the cleavage at D9 is required for cleavage at D28. One possible explanation is that recognition and binding of the D9 site orients the prodomain for cleavage at D28. This would imply that binding of the prodomain at D28 in the caspase active site is not efficient on its own. Unfortunately, the prodomain was not visible in the structure of procaspase-3, thus one can only speculate on its ability to bind the active site. Regardless if the D9 recognition is only for orientation, then cleavage at the site may not be necessary. Our findings suggest this is not the case, as mutation of D9 to glutamic acid resulted in a hypomorphic allele (Fig. 6D). This is consistent with caspases being able to cleave after glutamic acid at a lower efficiency than aspartic acid(51). This suggests that in addition to orienting the prodomain for removal, cleavage at D9 must also be a regulatory event. This may also explain why the Δ10 construct displayed caspase activity following serum withdrawal. This suggests that additional negative regulatory elements may be destroyed by cleaving the prodomain at D9.

An interesting observation in our study is caspase cleavage and activity do not always correlate with cell death. This is not surprising for caspase cleavage and activity as the initial cleavage event is not a measure of caspase-3 activity. This is really a measure of caspase-9 activit. However, it is surprising that the Δ10 construct and the D9E construct display significant caspase activity with little to no change in cell death. We hypothesize that this discrepancy is due to the requirements for demonstrating activity in these assays. Caspase activity is measured using a small 4 amino acid substrate in a cell lysate while cell death requires cleavage of over 100 proteins substrates in whole cells. The activity assay would likely tolerate changes to the caspase structure due to deleting 10 amino acid residues or the D9E mutation while recognition of multiple protein substrates may be inhibited.

Finally, while our studies are limited to caspase-3, it is possible that a similar mechanism of regulation exists for the other main effector, caspase-7. The overall conservation of these prodomains is low, although this is primarily due to differences in the overall size of the prodomains. The caspase-7 prodomain is over twice the length of the prodomain of caspase-3. This may be due to other functions that have been attributed to the caspase-7 prodomain(54). However, the most conserved region between the prodomains is centered around the D9 cleavage site. Further studies are required to test this possibility.

Acknowledgements

Support for these studies was provided through RO1 GM106565 and P30 CA138292. We thank Richard Flavell for providing the MEFs.

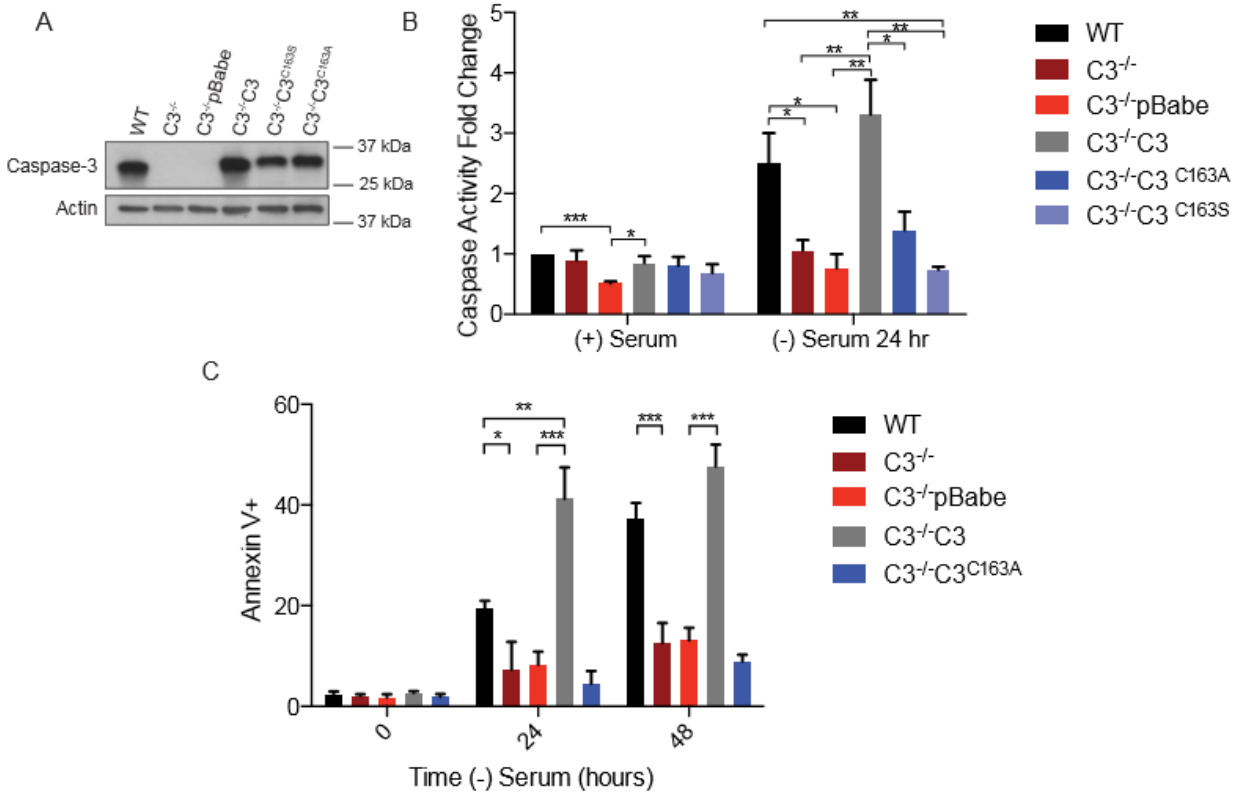


Figure 1. $C3^{-/-}C3$ and $C3^{-/-}C3^{C163A}$ MEFs have apoptotic activity and caspase activity similar to that of WT and $C3^{-/-}$pBabe, respectively. A. Protein expression of caspase-3 and loading control actin of parental MEFs and stable cell lines expressing empty vector pBabe, full length caspase-3 and two catalytically inactive forms of caspase-3, C163A and C163S. B. Caspase-3 activity was measured using a caspase activity assay. Cells were grown in full serum as a control or serum starved for 24 hours. A DEVD-chromphore substrate (DEVD-p-NA) was added to the protein lysis and light emittance was measured at 405 nm. C. Cells were serum starved for 0, 24 and 48 hours to induce apoptosis. Apoptosis was determined by positive annexin V/ propidium iodide (PI) and analyzed for cell death using flow cytometry. Data are presented as mean +/- SEM of at least 3 independent experiments. *p > 0.05, **p > 0.01, ***p > 0.001

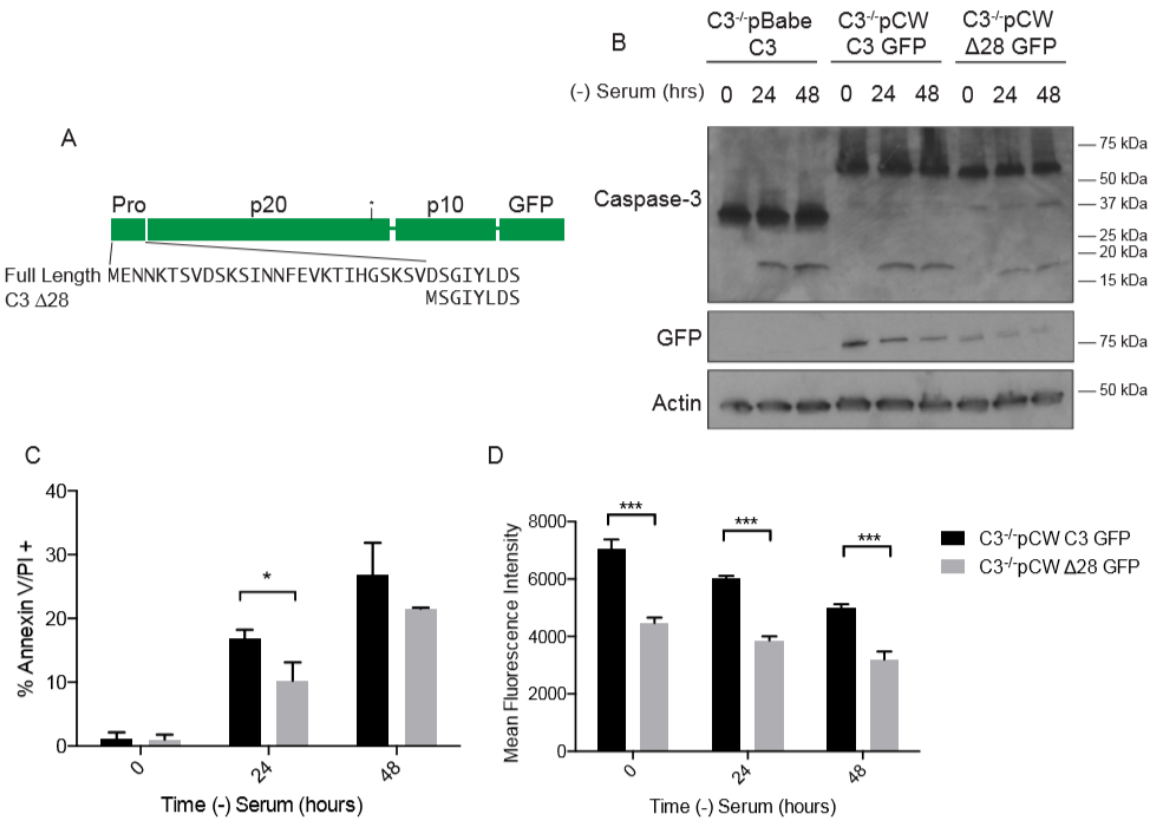


Figure 2. Complete removal of the prodomain results in apoptotic activity comparable to full length caspase-3. A. Diagram showing the C3 Δ28 construct lacks the 28 amino acid N-terminal prodomain. B. Cells expressing a doxycycline inducible full-length caspase-3 tagged with GFP (C3 GFP) and caspase-3 lacking the prodomain (Δ28 GFP) were stably expressed in caspase-3$^{-/-}$ MEFs. 48 hrs post doxycycline treatment cells were serum starved and collected at the indicated time points. Protein lysate was run on a western and probed for caspase-3, GFP and actin as a loading control. C. 48 hrs post doxycycline treatment cells were serum starved for the indicated times and cells were collected for flow cytometry analysis. Cell death was determined via positive annexin V/ propidium iodide (PI) staining. E. The mean fluorescence intensity (MFI) of the GFP signal was determined at the indicated time points. Data are presented as mean +/- SEM of at least 3 independent experiments. *p > 0.05, **p > 0.01, ***p > 0.001

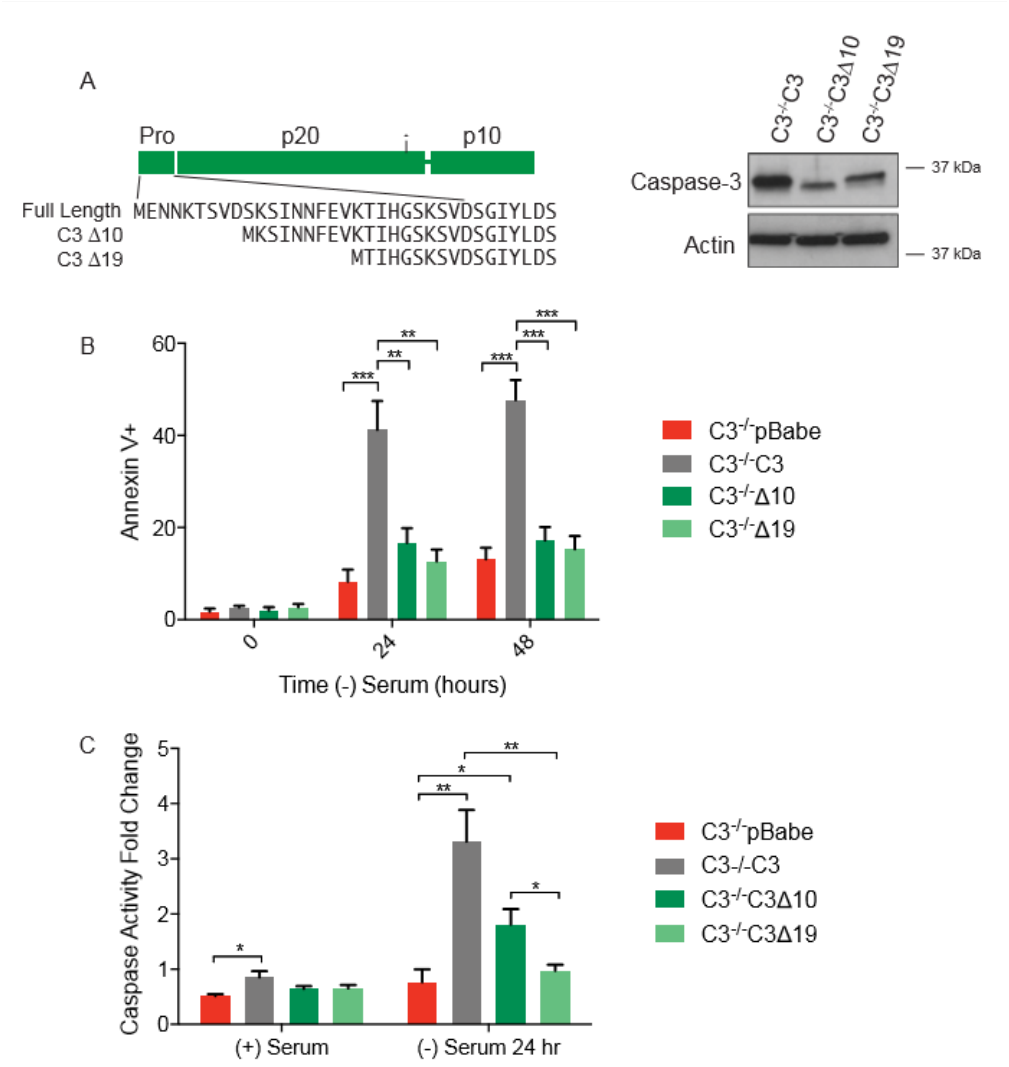


Figure 3. Removal of the first 10 amino acids decreases the apoptotic activity of caspase-3. A. Deletion mutants were created by removing the first 10 (Δ10) or first 19 (Δ19) amino acids as shown in the left diagram. Protein expression was determined using western blot analysis for caspase-3 and actin as a loading control. B. Deletion mutants were serum starved for the indicated times and subjected to cell death analysis via Annexin V/PI staining using flow cytometry. C. A caspase-3 activity assay was used to determine the ability of the indicated cells to cleave a DEVD-chromphore substrate. Data are presented as mean +/- SEM of at least 3 independent experiments. *p > 0.05, **p > 0.01, ***p > 0.001

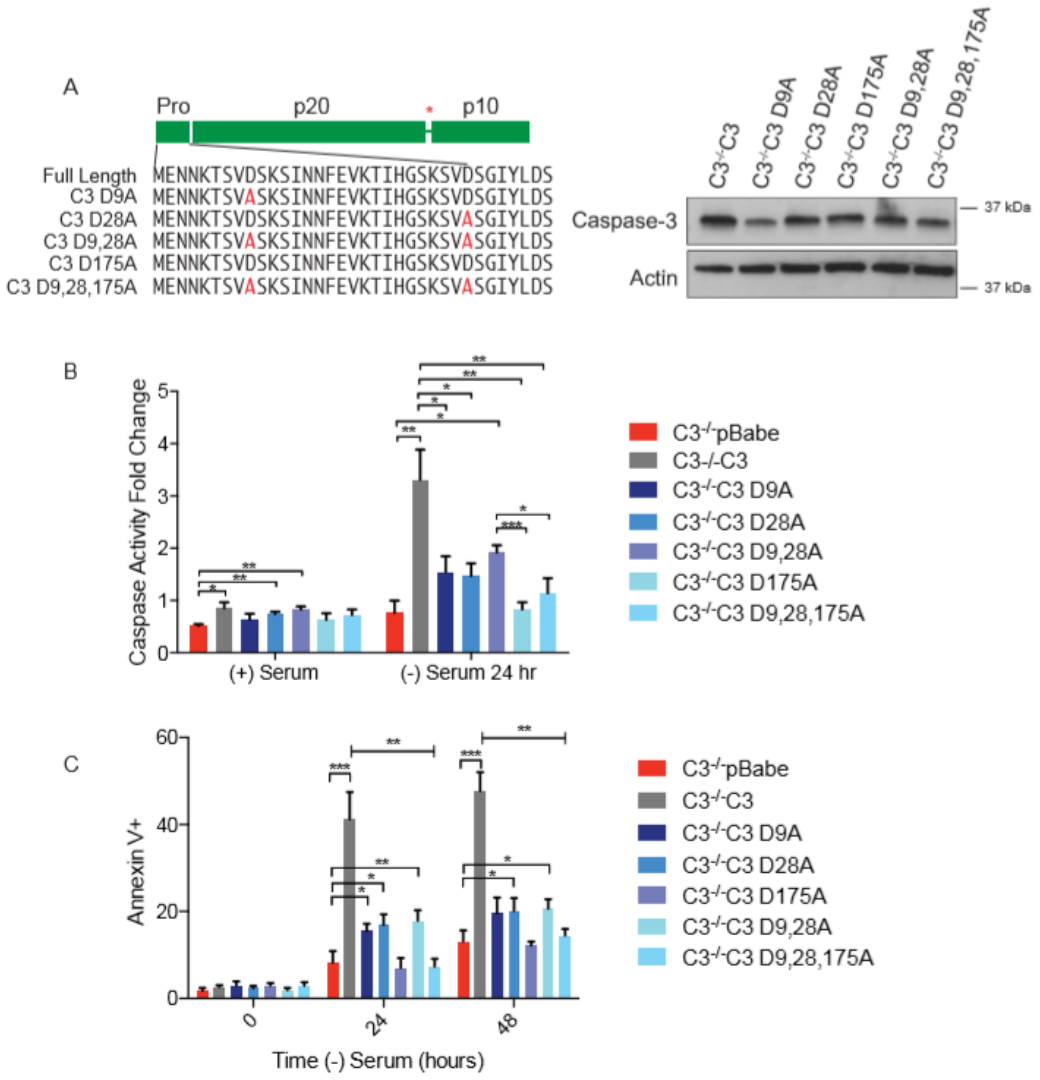


Figure 4. Mutating amino acid D9 to A9 decreases the apoptotic activity of caspase-3. A. Point mutations within the prodomain and the interdomain linker were created and indicated in red. Caspase-3 protein expression was determined by western blot analysis. Actin was used as a loading control. B. A caspase-3 activity assay was used on the indicated cell lines to determine the ability to cleave a DEVD-chromphore substrate. C. The caspase-3 point mutation cells were subjected to cell death analysis via Annexin V/PI staining using flow cytometry. Data are presented as mean +/- SEM of at least 3 independent experiments. *p > 0.05, **p > 0.01, ***p > 0.001

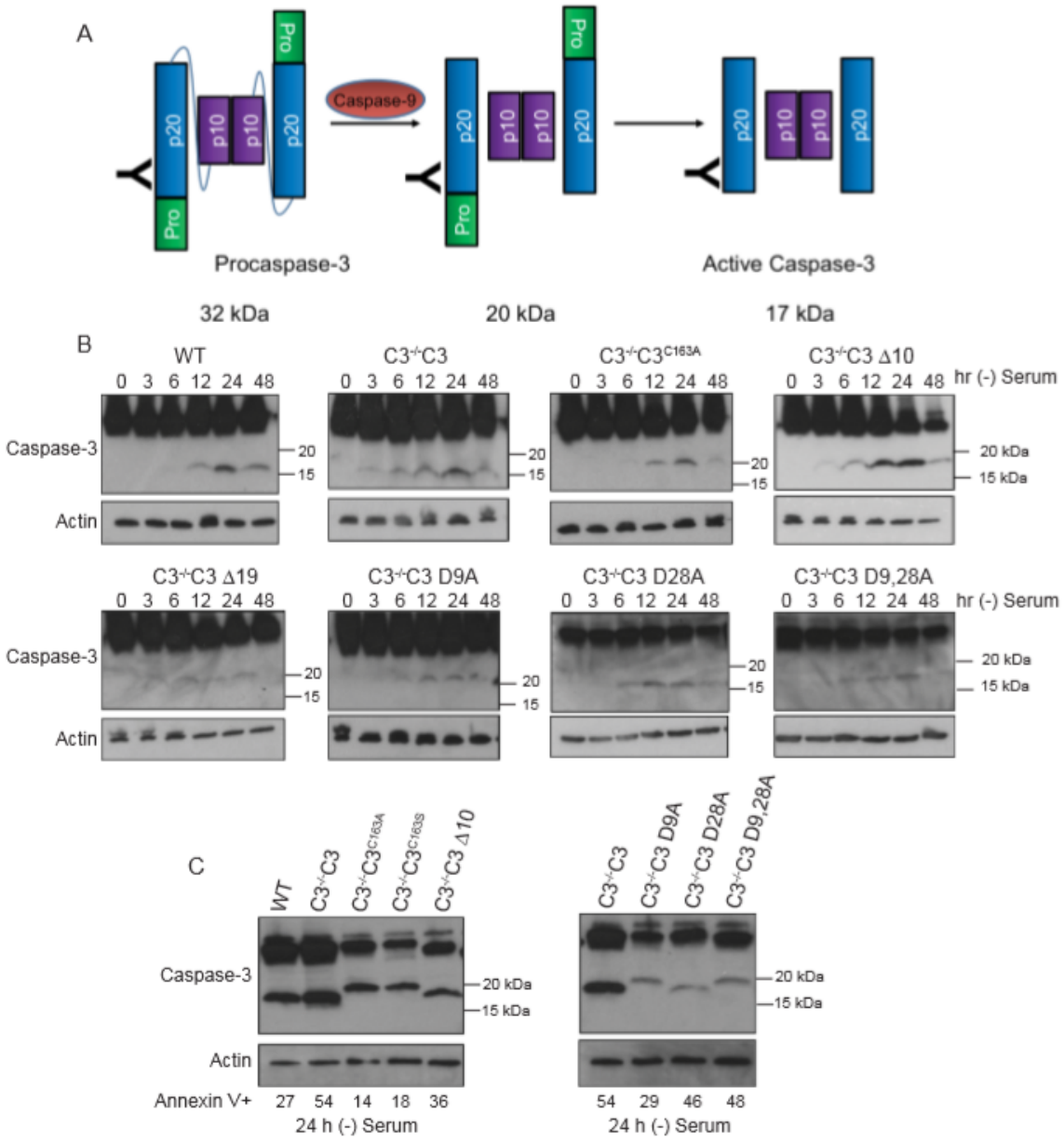


Figure 5. $C3^{-/-}C3^{C163A}$, $C3^{-/-}$C3 Δ10, $C3^{-/-}$C3 Δ19 and $C3^{-/-}$C3 D9A are unable to remove the prodomain. A. Diagram showing the resulting molecular weight bands, detected from an anti-caspase-3 antibody that binds to the p20 domain, resulting from cleavage of the interdomain linker and the prodomain. B. Cell lines were serum starved and lysates collected at the times indicated. Western blot analysis was conducted to determine procaspase-3 cleavage. C. Samples serum starved for 24 hours were run on the same gel for direct comparison. Actin was used as a loading control.

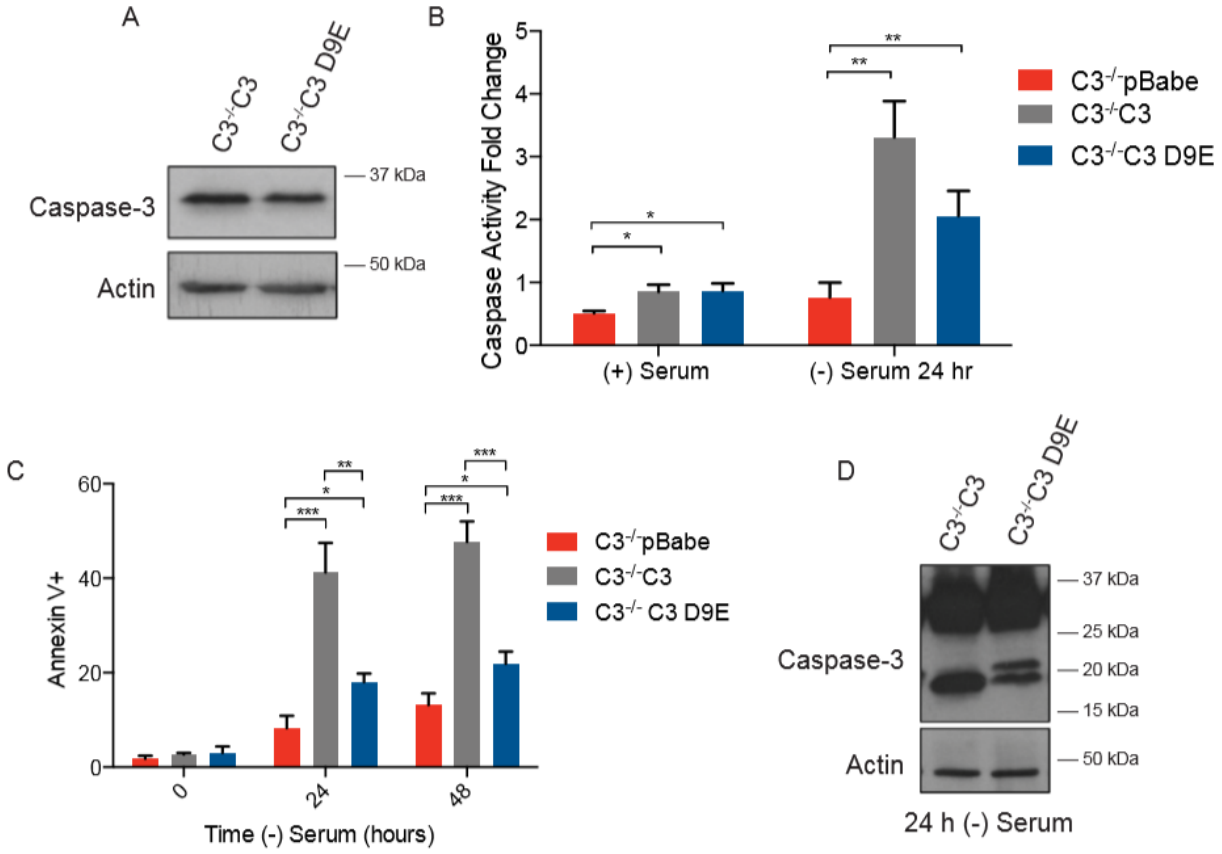
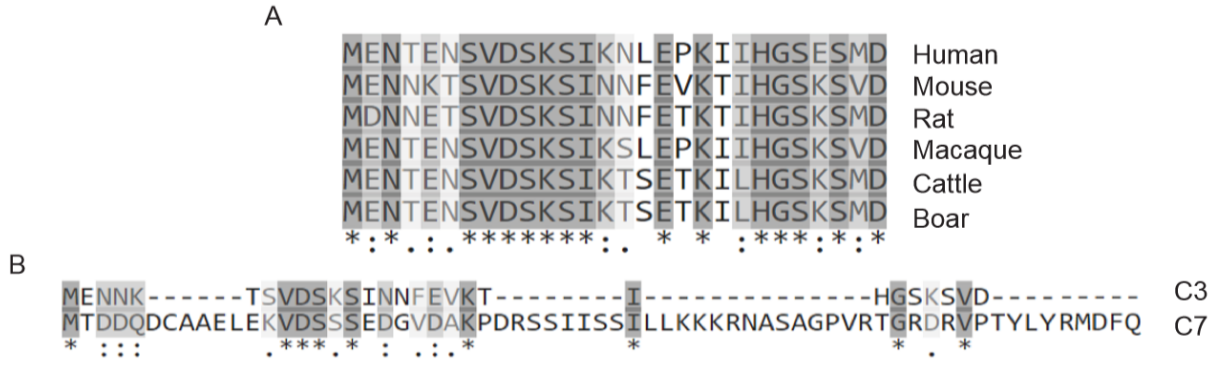
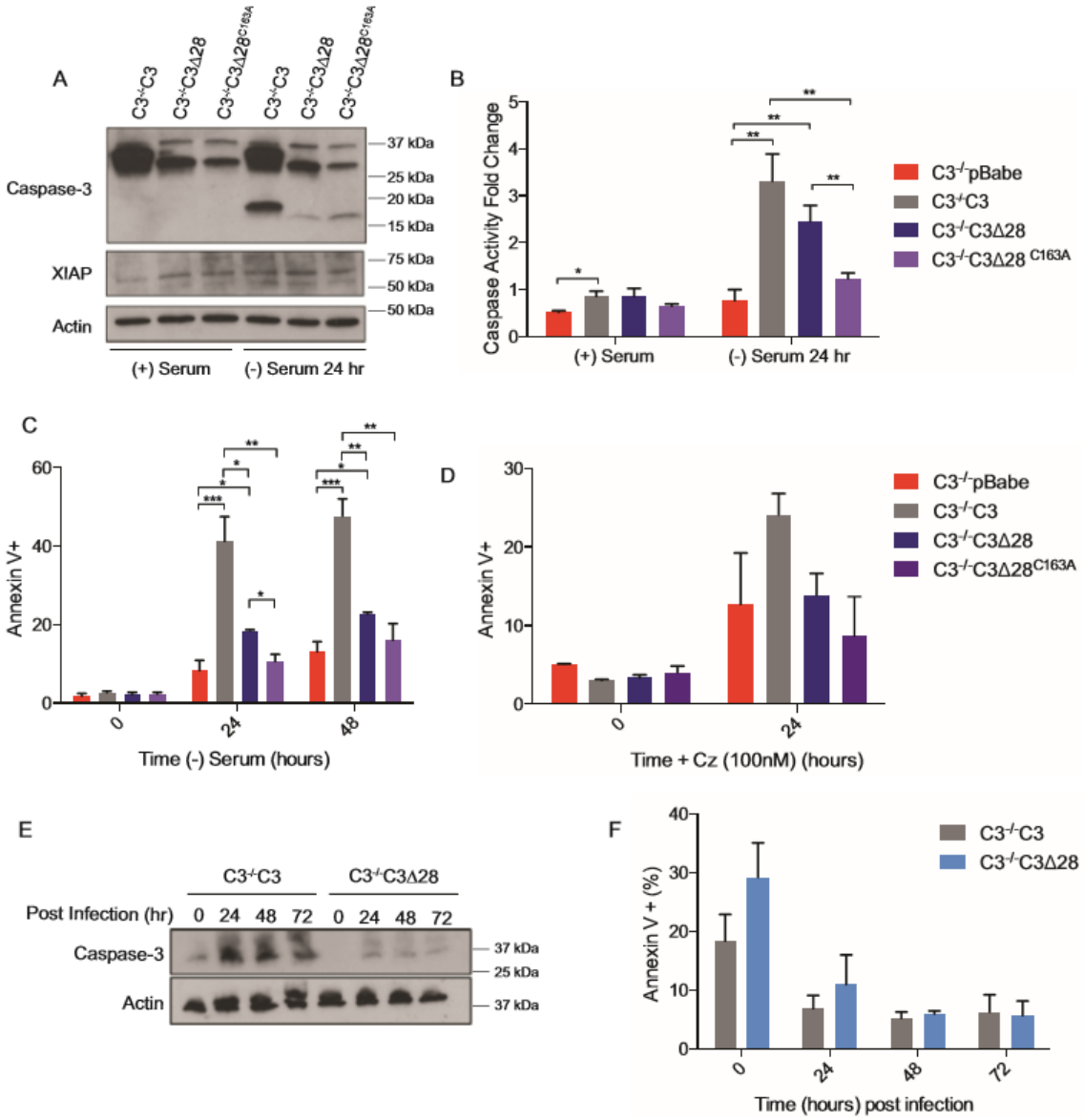


Figure 6. C3 D9E is inefficiently cleaved resulting in decreased caspase activation and cell death. A. Expression of cells stably expressing C3 and C3 D9E. B. A caspase-3 activity assay was used to determine the ability to cleave a DEVD-chromphore substrate. C. Cell death was determined using Annexin V/PI staining and flow cytometry. D. Cells were serum starved for 24 hours and the cleavage pattern of caspase-3 was determined using western blot analysis. Actin was used as a loading control. Data are presented as mean +/- SEM of at least 3 independent experiments. *p > 0.05, **p > 0.01, ***p > 0.001

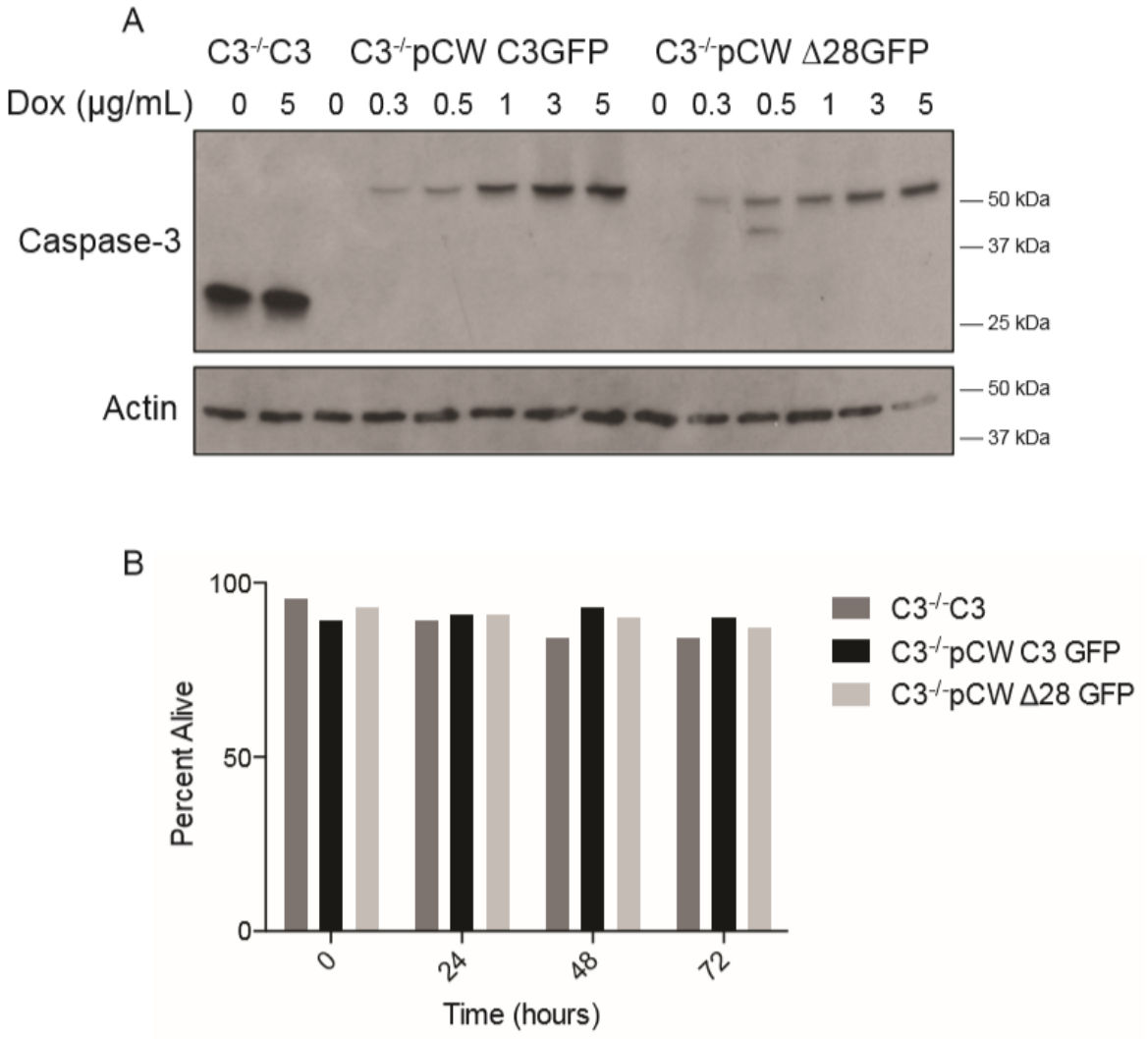


Supplemental Figure 1. The prodomain of caspase-3 is conserved, yet distinct from caspase-7. A. The percent identity of the prodomain of procaspase-3 between the 6 different species shown is 57.1%. The shaded grey areas highlight the identity. B. The prodomains of mouse procaspase-3 and -7 were analyzed in ScanProsite and only have an overall identity of 13.8%. The shaded grey areas highlight the identity between the prodomains.

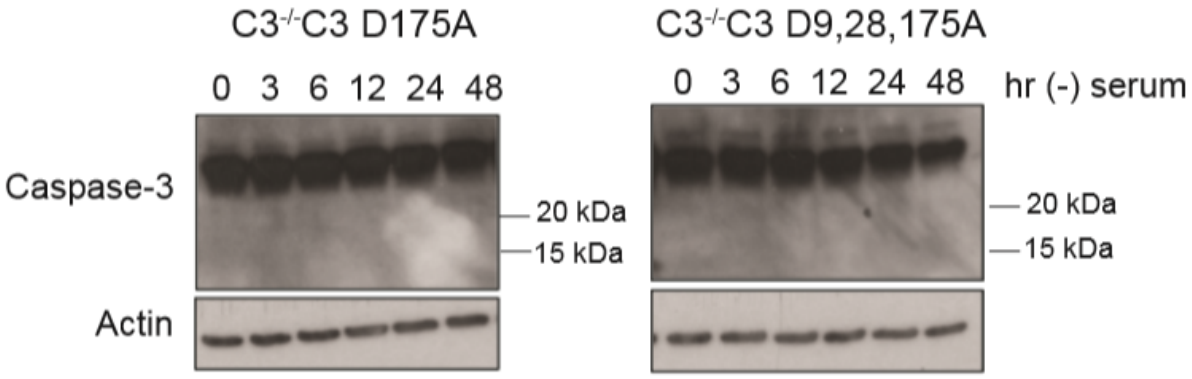


Supplemental Figure 2. Generation of stable C3Δ28 constructs and transient expression of C3Δ28 in caspase-3$^{-/-}$ MEFs. A. Cells stably expressing C3Δ28 and C3Δ28^{C163A} were generated and were used to determine the amount of cleaved caspase-3 and XIAP. B and C. Caspase activity and cell death were determined using a caspase-3 assay and annexin V/PI flow cytometry, respectively. D. Cells were treated with the proteasome inhibitor Carfilzomib (Cz) (100 nM) for the times indicated and cell death was determiend using annexin V/PI flow cytometry. (N=2) E. Caspase-3$^{-/-}$ MEFs were transiently infected with full length caspase-3 (C3)

or caspase-3 lacking the prodomain (C3Δ28). Protein expression of C3 of C3Δ28 was determined 0-72 hours post infection. F. Cells were collected at the indicated time points and cell viability was determined by annexin V/ propidium iodide (PI) and analyzed for cell death using flow cytometry. Data are presented as mean +/- SEM of at least 3 independent experiments. *$p > 0.05$, **$p > 0.01$, ***$p > 0.001$



Supplemental Figure 3. Treatment with 3 μg/mL of doxycycline for 48 hours is sufficient for expression of C3 GFP and Δ28 GFP. Caspase-3$^{-/-}$ MEFs stably expressing pCW C3 GFP and pCW Δ28 GFP were generated. A. The indicated concentrations of doxycycline (Dox) were added to induce the expression of the plasmids as shown by western blot. B. Cell viability was determined after 3 μg/mL doxycycline treatment for the indicated time points using annexin V/PI staining. C3$^{-/-}$pBabe C3 MEFs were used to determine the effect of doxycycline on a non-inducible cell line.



Supplemental Figure 4. Mutations D175A and D9,28,175A result in the inability of the interdomain linker to be cleaved. $C3^{-/-}$C3 D175A and $C3^{-/-}$C3 D9,28,175A MEFs were serum starved for the indicated times and the proteins lysates were analyzed via western blot. Actin was used as a loading control.

A

Plasmid	5' Primer
pBabe C3^{C163A}	tcatcattcaggccgcccggggtacggagc
pBabe C3 D9A	acaacaaaacctcagtggcctcaaaatccattaataattt
pBabe C3 D28A	gggagcaagtcagtggcctctgggatctatctg
pBabe C3 D175A	tgtggcattgagacagccagtggtgactgatgag
pBabe C3 D9E	acaacaaaacctcagtggagtcaaaatccattaataattt
pBabe C3 Δ10	cgccggccggatccgtgaccatgaaatccattaataat
pBabe C3 Δ19	cgccggccggatccgtgaccatgaccatacatgggagcaagtc

B

Plasmid	Template DNA	5' Primer	3' Primer
pBabe C3 Δ28	pBabe C3	taggcggattgtctgggatctatctggac	tggcccagctgctagtgataaaagtacagtt
pBabe Flag C3	pBabe C3	tggcccagctgctagtgataaaagtacagtt	tggcccagctgctagtgataaaagtacagtt
pBabe Flag C3 D175A	pBabe C3 D175A	tggcccagctgctagtgataaaagtacagtt	tggcccagctgctagtgataaaagtacagtt
pBabeC3 Δ28 GFP	pBabe C3 GFP	ggtggtaagcttatgtcgaagggcgaggagc	ggtggttagctactacctgtacagctcctcgtcc
pCW C3 GFP	pBabe C3 GFP	taggcgctagcatggagaacaacacctcagtg	gaagcggatccttacttgtacagctcgtcc
pCWC3 Δ28 GFP	pBabeC3 Δ28 GFP	taggcgctagcatgtctgggatctatctggac	gaagcggatccttacttgtacagctcgtcc

Supplemental Table 1. Primers used to generate caspase-3 mutation plasmids. A. The indicated plasmids were generated via site directed mutagenesis using the corresponding primers. B. The indicated plasmids were generated by PCR based cloning with the indicated template DNA and primers.

III. DUAL INHIBITION OF MCL-1 BY THE COMBINATION OF CARFILZOMIB AND TG02 IN MULTIPLE MYELOMA

(Originally published in Cancer Biology and Therapy, May 12 2016[55])

Dual Inhibition of Mcl-1 by the Combination of Carfilzomib and TG02 In Multiple Myeloma

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Key Words: Bcl-2, apoptosis, plasma cell dyscrasia
Abbreviations: MM: multiple myeloma; HMCL: human multiple myeloma cell lines; Cz: carfilzomib

Abstract

Carfilzomib (Kyprolis®), a second generation proteasome inhibitor, is FDA approved for single-agent use among relapsed/refractory multiple myeloma (MM). To enhance the therapeutic efficacy of carfilzomib, we sought to combine carfilzomib with other novel agents. TG02, a multi-kinase inhibitor, targets JAK2 and CDK9. The rationale for co-treatment with carfilzomib and TG02 is that both independently target Mcl-1 and most myeloma cells are dependent on this anti-apoptotic protein for survival. We observed at least additive effects using the combination treatment in MM cell lines and patient samples. To determine how the bone marrow environment affects the efficacy of the combination we conducted co-culture experiments with Hs-5 stromal cells. We also examined the mechanism of increased apoptosis by determining the affect on expression of the Bcl-2 family of proteins. We found that carfilzomib increases NOXA mRNA expression, as expected, and TG02 treatment caused a decrease in Mcl-1 protein but not mRNA levels. Consistent with this possibility, we find silencing CDK9 does not change carfilzomib sensitivity in the same manner as addition of TG02. Since changes in Mcl-1 protein occur in the presence of a proteasome inhibitor we hypothesize that regulation of Mcl-1 translation is the most likely mechanism. Taken together our data suggest that dual inhibition of Mcl-1 via decreased expression and the induction of its antagonist NOXA by the combination of carfilzomib and TG02 is active in myeloma and warrants further testing preclinically and in clinical trials. Moreover, regulation of Mcl-1 by TG02 is more complex than initially appreciated.

Introduction

Multiple Myeloma (MM) is a hematologic malignancy of bone marrow plasma cells. It is estimated that in the United States in 2015, 26,850 new cases of MM (16,090 in men and 12,760 in women) would be diagnosed, with an estimated 11,240 deaths.(56) After the introduction of the concept of proteasome inhibition and immunomodulation among myeloma therapeutic options, the survival of myeloma patients has significantly improved. Newer treatment strategies aiming at novel mechanisms or improving the efficacy of currently available treatments by combining agents to enhance synergy are warranted to ensure continuing improvement in survival among MM patients.

Currently, carfilzomib achieved an FDA approval to treat relapsed and refractory multiple myeloma patients who have received at least two prior therapies, including treatment with bortezomib and an immunomodulation therapy.(57) Carfilzomib is an irreversible proteasome inhibitor that targets the chymotrypsin-like activity of the proteasome.(58) Although carfilzomib is active in this disease, responses to proteosome inhibitors can be improved with combination therapies.(59, 60)

Plasma cells are terminally differentiated B cells that are responsible for antibody production.(21) Due to the high rates of antibody production, plasma cells also have increased rates of protein turnover. The proteasome is responsible for the selective degradation of misfolded and high-turnover proteins.(24, 61) Therefore, both normal and malignant plasma cells are highly susceptible to proteasome inhibition.(20) Apoptosis is induced when the proteasome is inhibited in part because of an accumulation of misfolded proteins which, leads to endoplasmic reticulum stress and the unfolded protein response.(25, 61-63)

Apoptosis is a signaling cascade of events that leads to the controlled death of a cell, and is regulated by the Bcl-2 family of proteins.(64) The Bcl-2 family of proteins consist of both pro-

and anti-apoptotic proteins. The anti-apoptotic proteins, including Bcl-2, Bcl-x_L and Mcl-1 work to inhibit the pro-apoptotic proteins. Upon apoptotic stimuli, pro-apoptotic proteins, Bax and Bak, will mulitmerize and induce mitochondrial outer membrane permeabilization (MOMP).[65] This process allows cytochrome c to be released from the mitochondria and the subsequent activation of the caspase cascade. When caspases are activated they will cleave vital cellular substrates leading to the death of a cell.(2)

One hallmark of cancer is the ability to resist apoptotic signals.(5) In order for MM cells to resist apoptosis they are dependent on the anti-apoptotic protein Mcl-1 for survival.(22, 66, 67) Mcl-1 is an oncogene that promotes tumorigenesis by binding to pro-apoptotic proteins Bak and Bim, thus preventing the release of cytochrome c from the mitochondria and subsequent caspase activation.(46) Given that most MM cells are dependent on Mcl-1 for survival it would be beneficial to inhibit Mcl-1 to increase the apoptotic response in these cells. NOXA is a BH3-only member of the Bcl-2 family of proteins.(68) NOXA is an inhibitor of Mcl-1. When NOXA binds to Mcl-1, the pro-apoptotic protein Bim is released resulting in MOMP and ultimately cell death.(69, 70) Carfilzomib induces not only the unfolded protein response but also the expression of NOXA.(26)

Due to the established safety profile of carfilzomib, it is now being tested in combination therapies.(27-29, 60) Treating MM with a combination treatment is regarded as beneficial for many reasons, one being that multiple myeloma has clonal heterogeneity. Using a combination of drugs would allow for more cells in the heterogeneous population to be targeted.(71) Combination treatments have been shown to synergistically increase apoptosis and demonstrate longer survival than single-agent treatments in patients.(60, 71-73)

Since MM cells are dependent on Mcl-1, we sought to combine carfilzomib with other Mcl-1 targeting drugs. TG02 is a pyrimidine-based multi-kinase inhibitor that has been shown to target CDKs, JAK2 and FLT3.(30, 31, 74) More interestingly, TG02 has been shown to increase Bax activation and decrease expression levels of anti-apoptotic protein Mcl-1.(31) Therefore, the combination of carfilzomib and TG02 would allow us to target Mcl-1 via two different mechanisms. TG02 targets proteins that are involved with transcriptional regulation, such as CDK9. Therefore, proteins with a short half-life will be selectively depleted from the cell relative to long-lived proteins. Mcl-1 is a short half-life protein, which is an explanation as to why TG02 treatment can decrease Mcl-1 protein expression. We hypothesized that using the combination of carfilzomib and TG02 would allow dual inhibition of Mcl-1, the key anti-apoptotic protein essential for survival of MM cells.

Materials and Methods

Cell Lines

U266, H929 and RPMI-8226 were obtained from ATCC. The MM.1s cell line was obtained from Dr. Steven Rosen (Northwestern University). Cells were cultured as previously described.(75)

Reagents

Propidium iodide (PI) was purchased from Sigma-Aldrich (P4170); Annexin-V–fluorescein isothiocyanate (FITC) was purchased from Biovision (1001-1000). Carfilzomib was generously provided by Onyx Pharaceuticals as part of their PRISM-NTP program. TG02 was generously provided by Tragara Pharmaceuticals.

Flow cytometric assay

Cells were collected at the various time points and treatments. 0.25–0.5 million cells were washed with phosphate buffered saline (PBS) and resuspended in 0.5 ml of FACS buffer (1% BSA in PBS containing 0.01% sodium azide) containing annexin V-FITC (BioVision 1001-1000) and propidium iodide (2 μg/ml Sigma). Cells were then measured with a BD FACSCanto II as described previously.(46) Data were analyzed using FlowJo (TreeStar) software.

Pulse dosing experiment

Cell lines indicated were co-treated with carfilzomib (IC_{10} and IC_{50} concentrations) and TG02 for one hour, washed and then treated with TG02 for an additional 23 hours. Cell death was determined via Annexin-V-FITC/PI flow cytometry.

Patient samples

Ficoll isolated buffy coat from a MM patient BM aspirate was collected and washed with PBS. Plasma cells were either treated in the presence of buffy coat cells or CD138+ plasma cells were

isolated as previously described(75) and treated. Twenty-four hour apoptosis was determined by staining with anti-CD38 (BD Biosciences 340927), anti-CD45 (BD Biosciences 348795) , and Annexin V-FITC(BioVision 1001-1000). Samples were acquired with an IRB approved protocol.

Co-culture with Hs-5 stromal cells

Hs-5 cells were plated at a density of $0.05x10^6$ cells/mL and allowed to grow for 48 h. MM cells were added to the Hs-5 cells after 48 h at a density of $0.25x10^6$ cells/mL 30 min prior to drug treatment. Conditioned medium was collected and filtered then diluted to 50% and added to MM cells. 24 h cell death was assessed via Annexin-V-FITC/PI (control and conditioned medium treated cells) or Annexin-V-FITC/anti-CD38-PE (BD Biosciences 347687) (Hs-5 co-cultured cells) flow cytometry. Data in are presented as mean +/- SEM of at least 3 independent experiments.

Real Time PCR

cDNA was prepared from RNA harvested at specified time points using the ABI high capacity cDNA kit (Applied Biosystems 4368814). Real-time PCR was performed using TaqMan gene expression master mix (ABI 4368814) with an ABI 9600 Fast thermocycler as described previously.(75) The following probes were used: Bim (*bcl2l11*) Mm00437796_m1, Bid (BID) Hs00609632_m1, NOXA (PMAIP1) Hs00560402_m1, Bmf (BMF) Hs00372937_m1, Mcl-1 (*mcl1*) Mm00725832_s1, Bcl-2 (*bcl2*) Mm00477631_m1, Bcl-x_L (*bcl2l1*) Mm00437783_m1, and GAPDH 4352932-0912031 were purchased from Applied Biosystems.

Immunoblotting

Cell lysate preparation and western blots were performed as described previously.(46) Antibodies used were as follows: rabbit anti-BID pAb (Cell Signaling Technology 2002); rabbit anti–Puma pAb (Cell Signaling 12450); rabbit anti-Bim pAb (Chemicon AB17003); rabbit anti–

Mcl-1 pAb (Stressgen PAB13415); rabbit anti–Bcl-xL pAb (13.6)26; mouse anti–Bcl-2 mAb (sc-509, Santa Cruz Biotechnology); rabbit anti-CDK9 mAb (Cell Signaling Technology C12F7); rabbit anti-phsopho Rpb1-CTD (Cell Signaling Technolog 4735) and the mouse anti-GAPDH mAb (Millipore AB2302). The ECL rabbit IgG, horseradish peroxidase-linked whole Ab (from donkey;GE Healthcare), and the anti–mouse IgG1-horseradish peroxidase conjugate (Santa Cruz sc-51625) were used as secondary antibody for Western blot.

siRNA

Silencing studies using small interfering RNAs (siRNAs) were obtained from Dharmacon RNA Technologies, selecting the ON-TARGETplus SMARTpool duplexes as the RNAi-specific technology platform. siRNA against human CDK9 and the siCON-TROL nontargeting siRNA[siRNA(-)] were used (L-003243-00-0005). siRNAs were electroporated into cells by nucleofection (Amaxa) following the manufacturer's instructions as previously described.(46)

Results

Four different human MM cell lines (HMCL), MM.1s, RPMI-8226, H929 and U266, were initially treated for 24 hours with increasing concentrations of carfilzomib or TG02. MM.1s were the most sensitive to carfilzomib treatment (IC_{50} 6.2 nM) and H929 were the most resistant (IC_{50} 31.6 nM) (Figure 1A). MM.1s were also the most sensitive to TG02 treatment (IC_{50} 101.1 nM) (Figure 1B). Co-treatment with carfilzomib and TG02 for 24 hours resulted in at least additive cell death in all four lines (Figure 1C). To better mimic the pharmacokinetics of carfilzomib in the clinic(23), pulse dosing of carfilzomib was also performed. When cells were pulsed for 1 hour with carfilzomib and TG02, then treated with only TG02 for an additional 23 hours, similar patterns of activity were observed compared with continuous dosing (Figure 1D). However, higher concentrations of carfilzomib were required to reach an IC_{50}. Activity of the combination was also observed in freshly isolated samples from relapsed/refractory MM patients (Figure 1E). Cells were either unfractionated from the buffy coat (MM53 and MM55) or CD138+ plasma cells were isolated from bone marrow aspirates and treated (MM54) and myeloma cell death was measured by annexin V staining. The combination treatment in all three patient samples resulted in at least additive effects.

We next determined how the bone marrow environment influences the activity of this combination. HMCL were co-cultured with either Hs-5 stromal cells or the conditioned medium from these cells (Figure 2). The Hs-5 stromal cells were able to protect against combination treatment induced apoptosis in MM.1s, RPMI-8226, and U266 cell lines. The protective effects of stromal cells appears to primarily impact carfilzomib, as the changes in cell death are greater with carfilzomib alone then with TG02 alone. Surprisingly, addition of Hs-5 cells or conditioned medium resulted in increased cell death in MM.1s when cells were treated with TG02 alone.

This suggests that the Hs-5 protection that is effecting carfilzomib induced cell death is not having an effect on TG02 treatment. The addition of conditioned medium was only able to protect against combination treatment induced apoptosis in RPMI-8226 cells. Despite protection by the Hs-5 stromal cells and the conditioned medium, cell death was greater in the combination treatment in all three cell lines compared to single agent treatment.

We next determined the molecular basis for the increased apoptosis of this combination. Since the rationale for this combination was based on altering the expression of members of the Bcl-2 family, we initially focused on changes in the expression of both pro- and anti-apoptotic Bcl-2 family members. HMCL were treated with carfilzomib, TG02 or the combination for 6 hours. Cells were isolated for RT-qPCR to determine mRNA levels of the Bcl-2 family. Consistent with our hypothesis, treatment with carfilzomib caused an increase in NOXA, an inhibitor of Mcl-1, at the mRNA level in RPMI-8226 and H929 cells (Figure 3A). When comparing mRNA levels of the other pro-apoptotic proteins, treatment with carfilzomib caused a decrease in Bmf expression in all cell lines except MM.1s. Bmf mRNA was significantly increased in all four cell lines with TG02 treatment, while Bid and Bim mRNA expression was significantly decreased (Figure 3A).

The mRNA levels of the anti-apoptotic proteins were also determined using quantative real time PCR. Bcl-x_L was increased with TG02 treatment in all four cell lines and Bcl-2 and Mcl-1 mRNA was decreased in three of the cell lines tested with carfilzomib treatment. We hypothesized that treatment with TG02 would result in a decrease in Mcl-1 expression, however, mRNA levels did not change following TG02 treatment (Figure 3B).

To further investigate the effect of this combination on Bcl-2 expression we determined the protein expression for the Bcl-2 family of proteins after single and combination drug

treatments. HMCL were treated with carfilzomib, TG02 or the combination for 6 hours. Cells were isolated for western blot analysis for the Bcl-2 family of proteins. Surprisingly, there was no increase in Bcl-x_L or Bim protein following TG02 treatment, despite an increase in mRNA levels (Figure 3C). Although there was no change in the Mcl-1 mRNA expression following TG02 treatment, TG02 did cause a decrease in Mcl-1 protein expression (Figure 3C). Since the loss of Mcl-1 at the protein level occurs in the presence of carfilzomib, a proteasome inhibitor, the effect of TG02 is unlikely to be due to increased protein degradation. Therefore, we hypothesized that the decrease in Mcl-1 protein following TG02 treatment was due to translational regulation. TG02 has been shown to inhibit cyclin dependent kinase 9 (CDK9).(30) To determine if TG02 was acting to inhibit CDK9, thus causing a decrease in Mcl-1 protein expression, we transiently knocked down CDK9 and treated the cells with carfilzomib, TG02 or the combination. The knockdown was verified using western blot analysis (Figure 4A). CDK9 silencing caused a decrease in Mcl-1 protein levels in MM.1s, although there was no difference in carfilzomib induced death was observed when comparing the siControl and siCDK9 transfected cells (Figure 4B). To determine if TG02 is working via CDK9 to inhibit transcription of Mcl-1 we determined the phosphorylation status of the large subunit of RNA polymerase II, Rpb1. Knockdown of CDK9 had a minimal effect on Rpb1 phosphorylation. Treatment with TG02 did cause a decrease in Rpb1 phosphorylation, thus TG02 is working to inhibit transcription but not via inhibition of CDK9. Therefore, TG02 is likely to be working through a different mechanism than CDK9 inhibition in these cells.

In order to further validate that Mcl-1 is the key player in the combination treatment induced apoptosis, we created RPMI-8226 cells that over expressed the anti-apoptotic proteins, Mcl-1, Bcl-2 and Bcl-x_L. These cells were treated for 24 hours with increasing concentrations of

carfilzomib or TG02 as well as the combination. Consistent with the possibility of the decrease in Mcl-1 protein being due to a regulation of translation mechanism, over expression of Mcl-1 in RPMI-8226 cells confers significantly less protection than over expression of Bcl-2 or Bcl-x_L when cells were treated with carfilzomib alone (Figure 5 A). 24 hour combination treatments with increasing doses of TG02 and two concentrations of carfilzomib had a similar result. The over expression of Mcl-1 confers significantly less protection (Figure 5C). This verifies that Mcl-1 protein is the key player in the combination treatment induced apoptosis.

Discussion

Multiple myeloma cells are dependent on Mcl-1 for survival.(22, 66, 67) Therefore, we sought to induce dual inhibition of Mcl-1 by combining a proteasome inhibitor, carfilzomib, with a multi-kinase inhibitor, TG02. Individually these drugs could induce apoptosis in multiple myeloma cell lines, but in combination they had at least additive effects. This effect was also seen in the presence of stromal cells, mimicking the extracellular environment, and in relapsed/refractory multiple myeloma patient samples. This demonstrates that carfilzomib and TG02 work as a combination therapy and further testing preclinically and in clinical trials is warranted.

We next sought to determine the molecular mechanism by which the combination was working. We hypothesized that dual inhibition of Mcl-1 would cause increased apoptosis in multiple myeloma cells. It is known that carfilzomib induces the expression of NOXA and that NOXA acts to inhibit Mcl-1. Consistent with our hypothesis, NOXA had increased mRNA expression in RPMI-8226 and H929 cells when treated with carfilzomib. Carfilzomib also caused a decrease in Mcl-1 mRNA expression in three out of four cell lines, but the Mcl-1 protein expression in these cells lines increased. This is consistent with previous studies demonstrating Mcl-1 regulation by ubiquitination and proteasomal degradation.(76, 77)

CDK9 is known to associate with a protein complex, TAK/P-TEFb, which functions as an elongation factor for RNA polymerase II. CDK9 phosphorylates RNA polymerase II on Serine 2, thereby activating it. Inhibition of CDK9 has been shown to cause a decrease in Mcl-1 expression attributed to the heightened dependence of labile protein on *de novo* transcription.(78) TG02 has been found to inhibit CDK9 in a number of malignant cell types(30, 31), causing a decrease in Mcl-1 protein expression. Therefore, we hypothesized that TG02 is inhibiting CDK9,

thus causing a decrease in Mcl-1 protein expression. TG02 caused a decrease in Mcl-1 protein expression, as hypothesized, however only U266 cells demonstrated a decrease in Mcl-1 mRNA expression. Since it seemed unlikely that TG02 was working via CDK9, we formally tested the role of CDK9 in Mcl-1 expression and how CDK9 inhibition influenced carfilzomib-induced death. Transiently knocking down CDK9 did not have an effect on carfilzomib induced cell death in the same manner as TG02. Paradoxically, silencing CDK9 did affect TG02 activity. Treatment with TG02 did cause a decrease in Rpb1 phosphorylation, therefore, TG02 is working to inhibit transcription but not via CDK9.

The decrease in Mcl-1 protein expression is not a result of proteasomal degradation as the decreased in Mcl-1 protein was also seen in the combination with carfilzomib. However, we have not formally ruled out a role for selective autophagy of Mcl-1. Together these results point to a mechanism of translational regulation of Mcl-1 by TG02. Inhibition of Mcl-1 translation has been previously demonstrated as a means to induce cell death in tumor cells.(79-81)

To verify that Mcl-1 is the key target in the combination treatment we created cells that over expression the anti-apoptotic proteins. Over expression of Mcl-1 in RPMI-8226 cells confers significantly less protection compared to over expression of Bcl-x_L. This further validates that Mcl-1 is the key target for this combination treatment.

We have demonstrated that the combination of carfilzomib and TG02 has at least additive effects in multiple myeloma cell lines and relapsed/refractory patient samples. We have shown that the additive effects of this combination are due to the dual inhibition of Mcl-1. Carfilzomib causes an increase in NOXA and TG02 causes a decreased in Mcl-1 protein but not mRNA. The mechanism by which TG02 causes a decrease in Mcl-1 requires additional investigation. Overall our results demonstrate a need for further testing of this combination preclinically. However,

based on these and other promising preclinical studies with bortezomib (82) a phase I trial to test this combination for the treatment of multiple myeloma has been initiated (NCT01204164). Initial results from this trial were recently presented and demonstrated that this combination has a similar safety profile as TG02 alone and has activity in carfilzomib refractory patients. [43]

Acknowledgments

We thank Shirin Kapur and Christopher Kirk for their advice regarding the use of carfilzomib. Support came from grants R01 CA127910 and 5 P30CA138292-07. LHB is supported by the TJ Martell Foundation and is a Georgia Research Alliance Cancer Scholar.

Disclosures

FB: Employee of Tragara Pharmaceuticals

AN: Advisory board member for Onyx and Spectrum Pharmaceuticals

JK: Consultant for Onyx and Millennium Pharmaceuticals, Novartis and Celgene. Research funding from Merck, Novartis and Onyx Pharmaceuticals

SL: Consultant for Onyx and Millennium Pharmaceuticals, Bristol-Myers Squibb, Novartis, Janssen and Celegene

LHB: Consultant for Onyx Pharmaceuticals and Novartis

Contributions

KP designed, performed and analyzed experiments and wrote the manuscript. SMM designed and analyzed experiments and helped write the manuscript. SH designed and performed experiments. CS performed experiments and sample acquisition. FB analyzed data and helped to write the manuscript. AN helped write the manuscript and analyzed data. JK provided assistance in writing the manuscript and data analysis. SL analyzed data and helped write the manuscript. LHB conceived the idea, designed and analyzed experiments and wrote the manuscript.

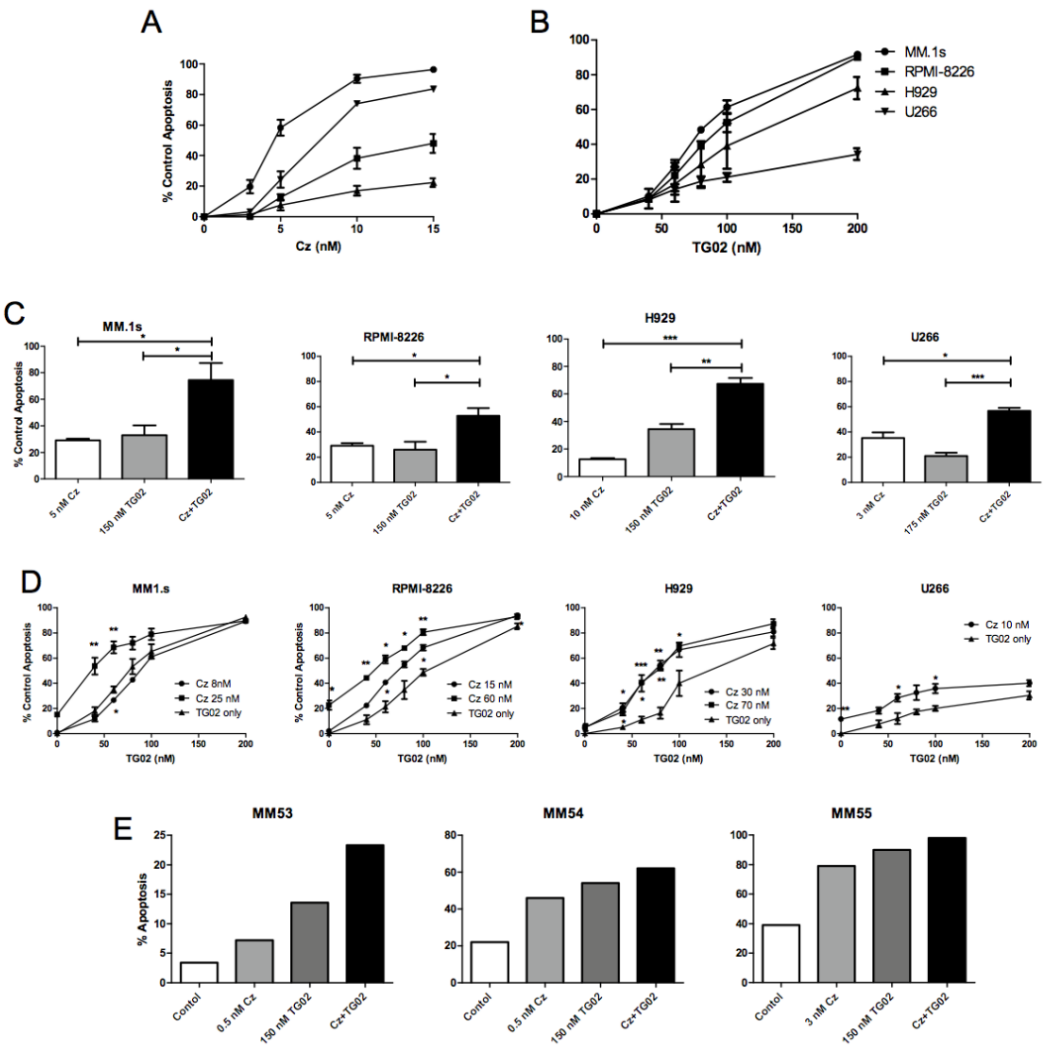

Figure 1. Continuous carfilzomib and TG02 co-treatment results in at least additive cell death. Cells were plated at a density of $0.25x10^6$ cells/mL and treated with the indicated concentrations of (A) carfilzomib, (B) TG02 or the (C) combination for 24 hours. (D) Cells were co-treated with carfilzomib (IC10 and IC50 concentrations) and TG02 for 1 hour, washed and then treated with TG02 for 23 hours. Cell death was determined via Annexin-V-FITC/PI flow cytometry. Data are presented as mean +/- SEM of at least 3 independent experiments. IC50 and IC10 significance are indicated above and below the mean values, respectively. (E) Ficoll isolated buffy coat from a myeloma patient BM aspirate was collected and washed with PBS.

Plasma cells were either treated in the presence of buffy coat cells (MM53 and MM55) or CD138+ plasma cells were isolated and treated (MM54). Twenty-four hour apoptosis was determined by staining with anti-CD38, anti-CD45, and Annexin V-FITC. * $p > 0.05$, ** $p > 0.01$, *** $p > 0.001$.

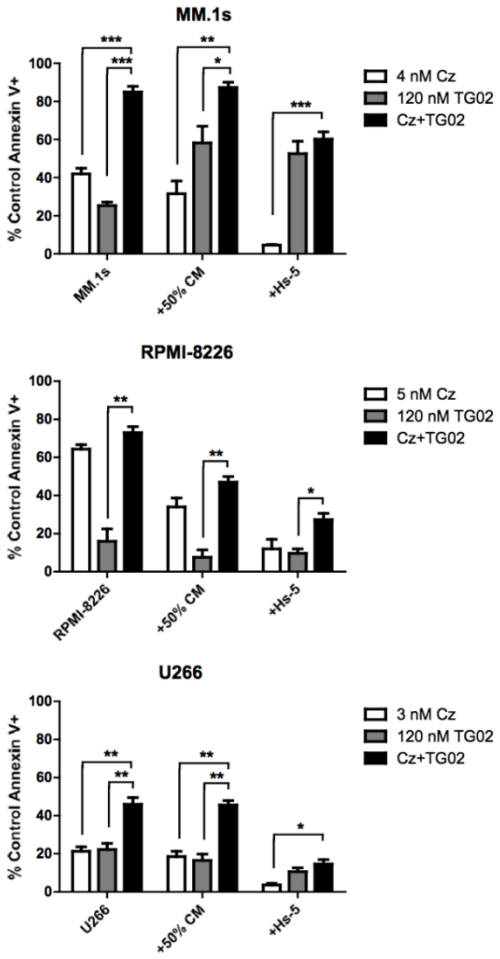

Figure 2. Co-culture with Hs-5 cells protected against combination treatment in three cell lines, while addition of Hs-5 conditioned medium was protective in only RPMI-8226. Hs-5 cells were plated at a density of $0.05x10^6$ cells/mL and allowed to grow for 48 h. MM cells were added to the Hs-5 cells after 48 h, 30 min prior to drug treatment. Conditioned medium was collected and filtered then diluted to 50% and added to MM cells. 24 h cell death was assessed via Annexin-V-FITC/PI (control and conditioned medium treated cells) or Annexin-V-FITC/anti-CD38-PE (Hs-5 co-cultured cells) flow cytometry. Data in are presented as mean +/- SEM of at least 3 independent experiments.

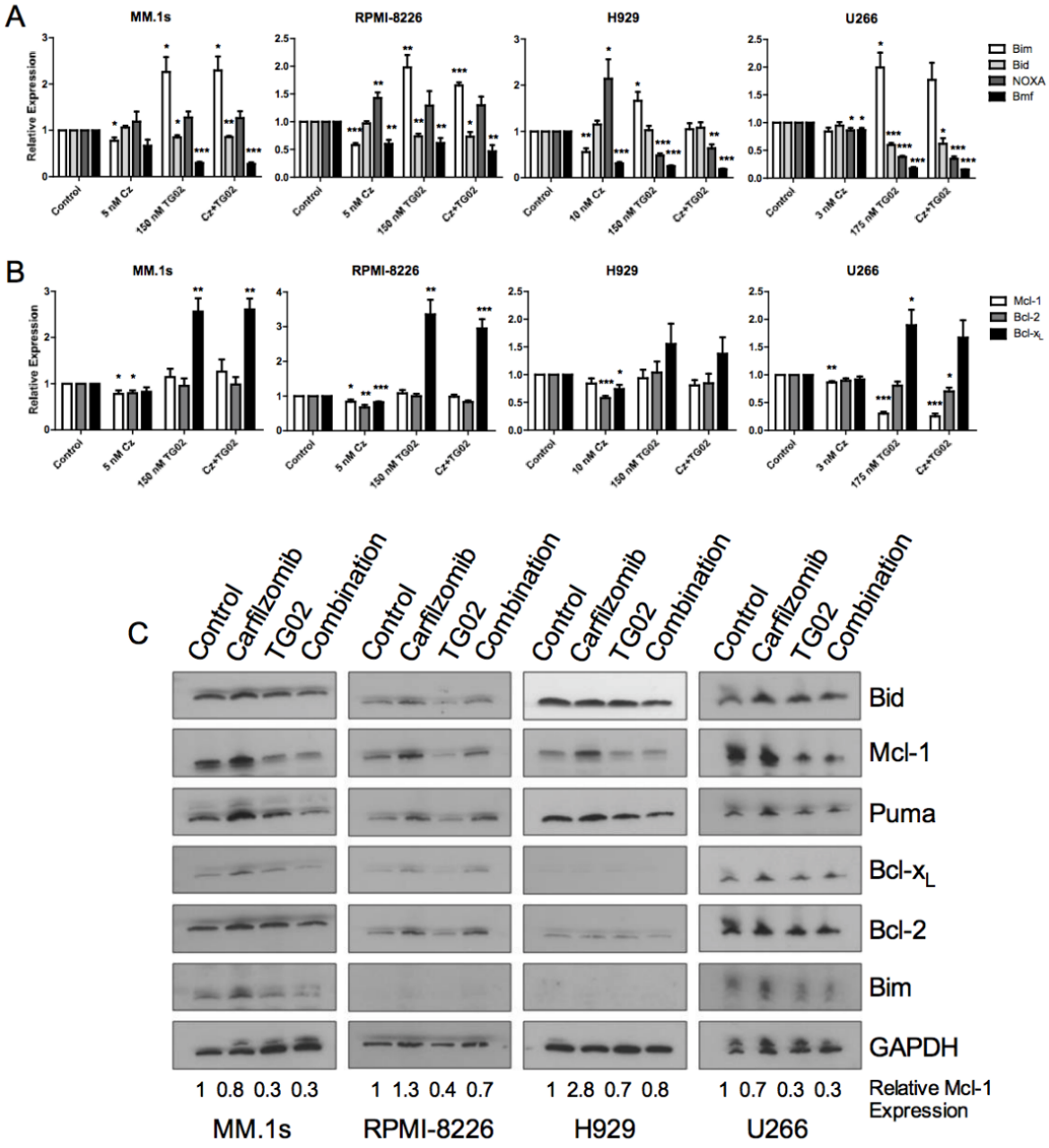

Figure 3. Treatment with carfilzomib causes an increase in NOXA mRNA and TG02 causes a decrease in Mcl-1 protein. Cells were plated at a density of $0.25x10^6$ cells/mL and treated with carfilzomib, TG02 or the combination for 6 hours. RT-qPCR results for (A) pro-apoptotic and (B) anti-apoptotic mRNA expression levels compared to control were determined and

presented as mean +/- SEM of at least 3 independent experiments. * $p > 0.05$, ** $p > 0.01$, *** $p > 0.001$. (C) Protein lysates were obtained after 6 hour carfilzomib, TG02 or the combination treatments (IC50) using RIPA buffer plus protease inhibitor cocktail. 20 μg of protein were subjected to Western blot analysis with the antibodies shown. Numbers shown represent the quantified relative expression of Mcl-1 normalized to GAPDH.

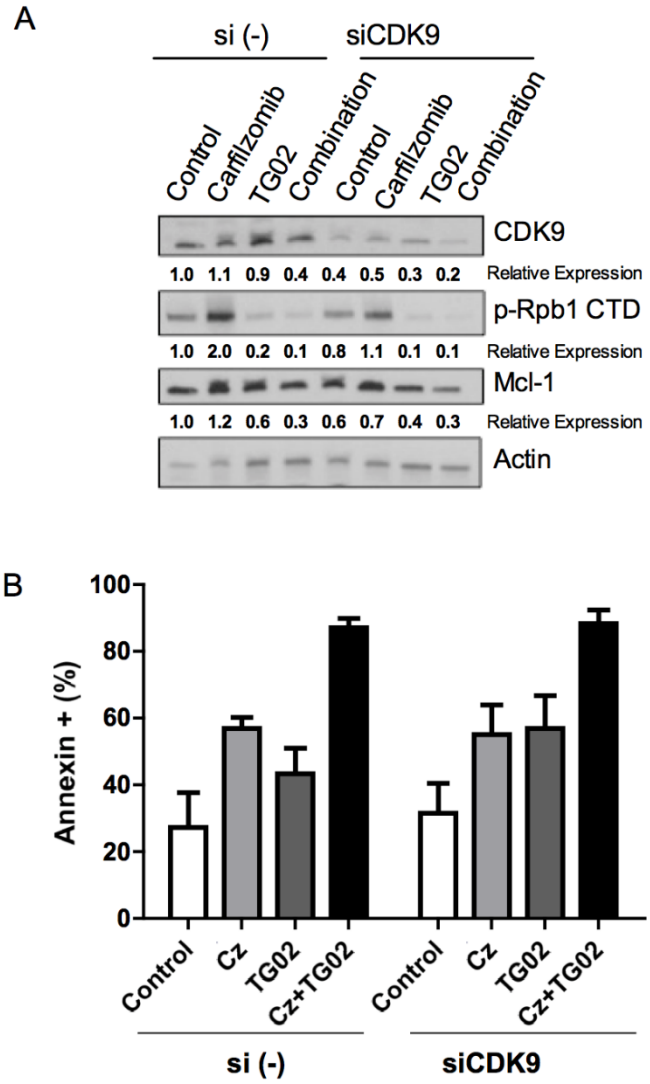

Figure 4. The decreased in Mcl-1 protein expression is not due to CDK9 inhibition. MM.1s cells were treated with siCDK9 or si (control) for 24 hours and then treated with carfilzomib, TG02 or the combination for an additional 24 hours. (A) Knockdown of siCDK9 was determined using western blot and densitometry analysis. Relative expression, normalized to actin, of the indicated proteins are shown below the corresponding bands. (B) Cell death was determined via Annexin-V-FITC/PI flow cytometry. Data are presented as mean +/- SEM of at least 3 independent experiments.

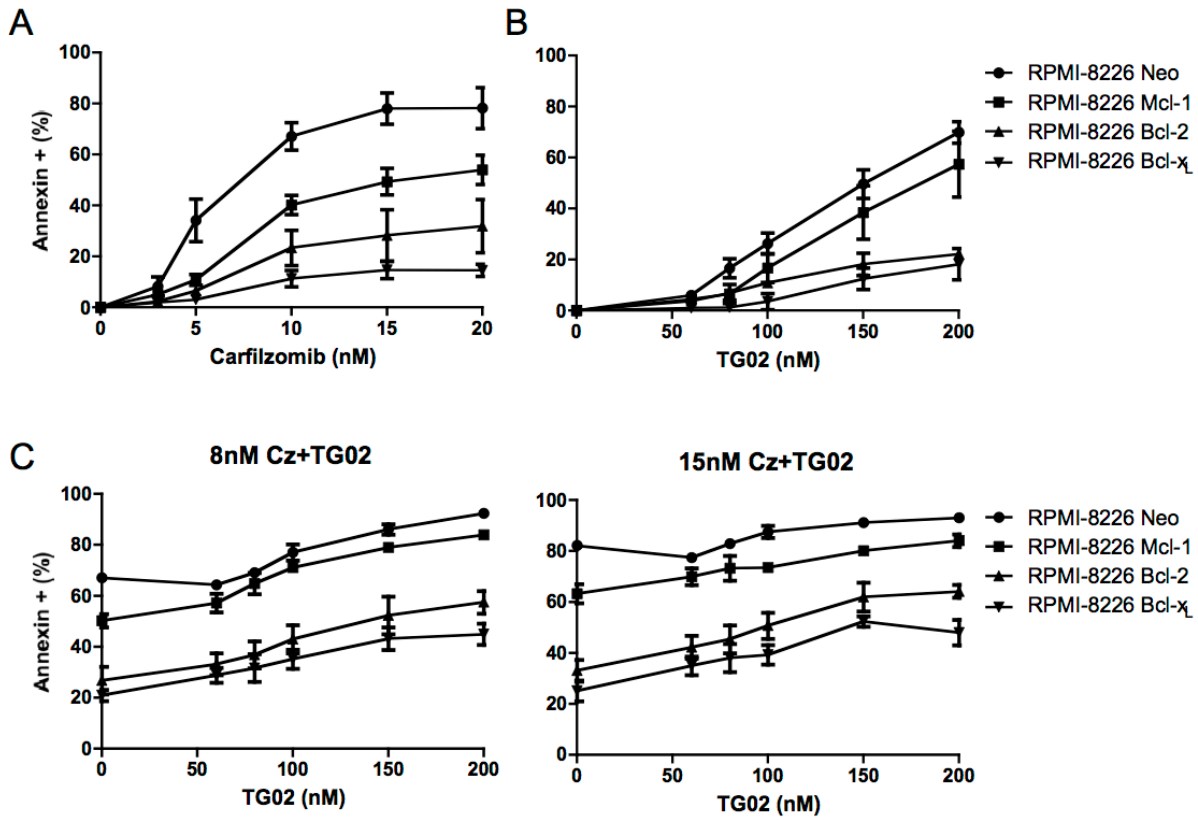

Figure 5. Over expression of Mcl-1 in RPMI-8226 cells confers significantly less protection than over expression of Bcl-2 or Bcl-x_L. RPMI-8226 cells over expressing the anti-apoptotic proteins shown were treated with increasing doses of (A) carfilzomib (B) TG02 or (C) the combination for 24 hours. Cell death was determined via Annexin-V-FITC/PI flow cytometry. Data are presented as mean +/- SEM of at least 3 independent experiments. Statistical analysis for RPMI-8226 Mcl-1 over expressing cells only shown. * $p > 0.05$, ** $p > 0.01$, *** $p > 0.001$.

IV. UNPUBLISHED RESULTS

A. INTRODUCTION

In Chapter II the sequential cleavage of the prodomain resulting in an active caspase-3 enzyme was discussed. This chapter contains unpublished data that was obtained during these studies.

B. RESULTS AND DISCUSSION

To complete the studies found in Chapter II, multiple plasmids containing point mutations and deletion constructs were created using restriction enzyme and PCR based cloning. The plasmids created, and the corresponding restriction enzymes used during the ligation are in Table 1. The plasmids created using the pBabe vector were stably expressed in $C3^{-/-}$ MEFs under puromycin selection. The pCW vector was used to create a doxycycline inducible system. These plasmids were also stably expressed in $C3^{-/-}$ MEFs under puromycin selection. Addition of doxycycline induced the expression of the corresponding caspase-3 mutant.

We determined that for the prodomain of caspase-3 to be removed at D28 there had to be an initial cleavage at D9. To further prove our findings, we placed a N-terminal flag-tag on either C3 or C3 D175A (Figure 1A). These constructs were stably expressed in $C3^{-/-}$ MEFs at levels similar to $C3^{-/-}$C3 MEFs (Figure 1B). While there was no statistically significant difference in the amount cell death, the flag-tag inhibited the ability of the interdomain linker to be cleaved (Figures 1C and D). Therefore, we could not peruse our original experiment to test if there was cleavage at D9 because the cleavage event at D175 that exposed the active site of caspase-3 was inhibited. We hypothesize that the addition of the flag-tag to the N-terminus changed the

orientation the prodomain so that it blocked the cleavage of D175. Upon further investigation, we discovered that there is no published work using a N-terminal tagged caspase-3 to do functional studies. This further supports our hypothesis that addition of an N-terminal tag to procaspase-3 changes its structure and function.

In previous work we demonstrated that mutation of a single amino acid within in the prodomain, D9A, resulted in a decrease in caspase-3 activity. Previous studies demonstrated that mutation of D9, D28 and D175 resulted in an uncleavable form of caspase-3. Therefore, we focused our initial efforts on these amino acids. These amino acids were located in regions of conservation, so we created additional point mutations to determine the effects of mutating a less conserved region. Mutating N4, F16 and V18 to alanine resulted in a significant decrease in cell death after 24 hours of serum starvation (Figure. 2A). However, these mutations did not affect the removal of the prodomain as the cleaved product is the same molecular weight as the $C3^{-/-}C3$. Given that the prodomain is able to be removed, but the cells do not die, we hypothesize that during the selection process we selected for cells that contained a mutation within caspase-3 rendering it unable to function or that we selected for cells that have an upregulation of an inhibitor of capase-3, like XIAP. XIAP binds to the cleaved form of caspase-3, thus we would see the removal of the prodomain, but caspase-3 would be able to induce apoptosis as XIAP is bound to the active site blocking substrate recognition.

Plasmid	5’ Restriction Enzyme	3’ Restriction Enzyme
pBabe C3 Δ28	BamHI	SalI
pBabe Flag C3	BamHI	SalI
pBabe Flag C3 D175A	BamHI	SalI
pBabe C3 Δ28 GFP	BamHI	SalI
pCW C3 GFP	NehI	BamHI
pCW C3 Δ28 GFP	NehI	BamHI

Table 1. Restriction enzymes used to create the corresponding plasmids. Each plasmids listed on the left, was created using PCR based cloning and the 5’ and 3’ restriction enzymes were used for ligation into either the pBabe or pCW backbone.

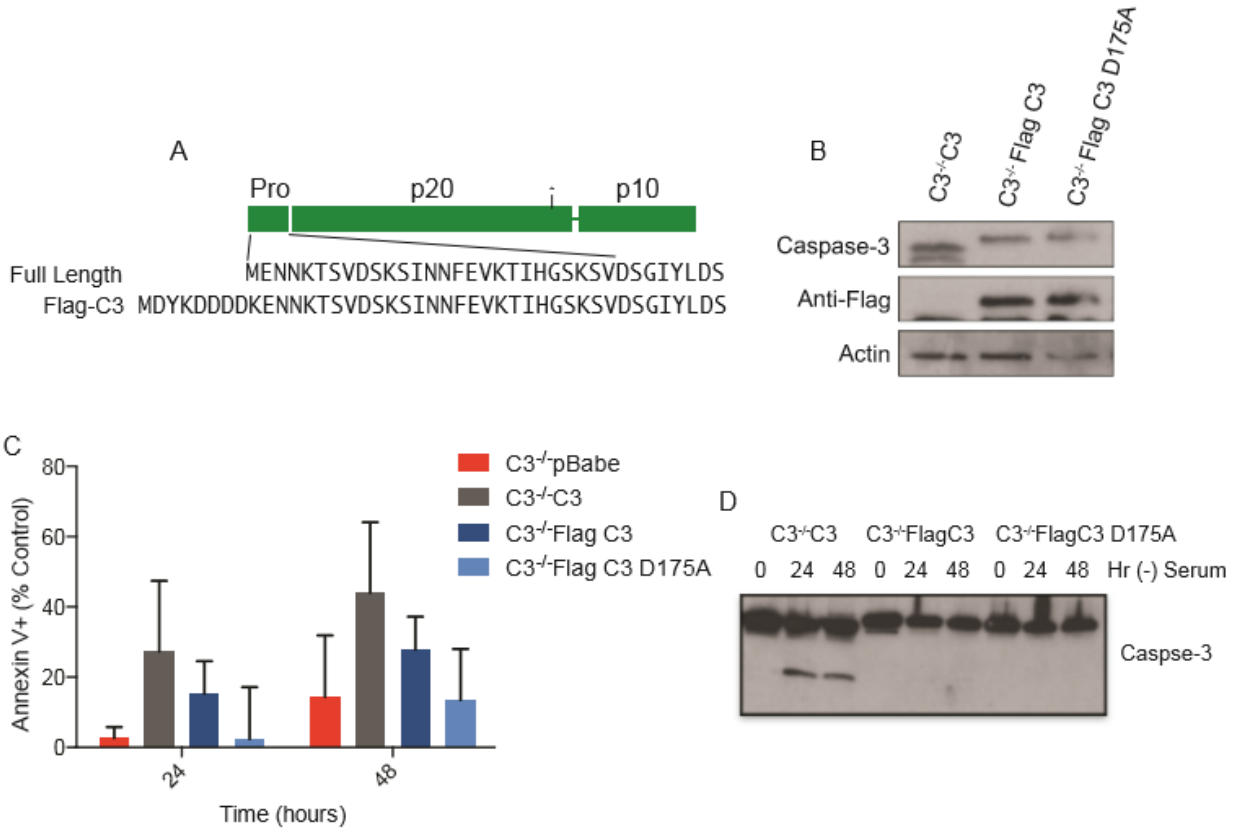

Figure 1. C3$^{-/-}$Flag C3 is inactive due to lack of interdomain linker cleavage. A. The Flag-tag was placed at the N-terminal prodomain of procaspase-3. B. C3-/- MEFs stably express C3, Flag C3 and Flag C3 D175A. C. Cell death was determined via flow cytometry with annexin V/PI staining. D. Cleaved caspase-3 was determined using western blot analysis. Data are presented as mean +/- SEM of at least 3 experiments.

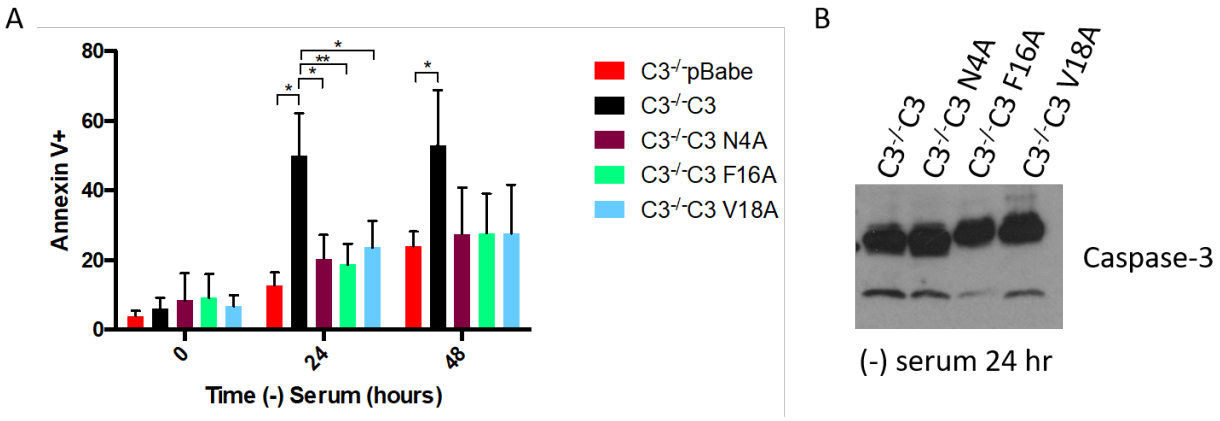

Figure 2. Point mutations result in decreased apoptosis compared to C3$^{-/-}$C3. A. Cells were serum started for the indicated time points and cell death was measured using Annexin V/PI flow cytometry. B. Cell lines were serum starved for 24 hours and lysates were collected. Western blot analysis was conducted to determine procaspase-3 cleavage. Data are presented as mean +/- SEM of at least 3 independent experiments. *p>0.05, **p>0.01

V. DISCUSSION

Understanding the mechanism by which the apoptotic proteins function and become activated will lead to improved therapeutic options for a multitude of diseases. The key proteins involved in this process include caspases and the Bcl-2 family of proteins. We sought to determine how procaspase-3 is cleaved to become the active enzyme which, when activated, will cleave cellular targets and lead to cell death. Knowing that multiple myeloma cells are dependent on the Bcl-2 family protein Mcl-1 for survival, we sought out drug combination therapies that could target this proapoptotic protein through multiple pathways to increase myeloma cell death.

A. Implications of Caspase-3 studies

The mechanism by which procaspase-3 becomes an active caspase is not fully understood. Studies have shown that in a cell free system there are two cleavage events to form the p17 and p12 subunits(33). Others have disagreed about the role of the N-terminal prodomain. While one study demonstrated that the removal of the prodomain results in a constitutively active caspase-3(34), others have shown that the prodomain does not influence apoptotic activity(52). We tested the hypothesis that the prodomain regulates apoptotic activity in a more physiologic setting. In previous studies transient expression of caspase-3 lacking the prodomain was used. Expressing this construct transiently will cause increased levels of stress to the cells which in turn can induce cell death independent of the effect of the transected plasmid. We used an inducible system, thus removing the constant stress of selection found with a transient system and found that the prodomain regulates caspase-3 activity and caspase-3 is not constitutively active.

Surprisingly we found that if a small portion of the prodomain is deleted the caspase cannot be efficiently activated. When the first 19 amino acids of the prodomain were deleted activity

was lost, but deletion of the first 10 retained activity. Therefore, we hypothesize that there is a negative regulatory region located between amino acids 20 and 27 (Figure 1). This would explain why the prodomain needs to be removed for caspase-3 to have activity. Not only does removal of a small section of the prodomain inhibit activity, but the mutation of a single amino acid blocks caspase activation and cell death. When we mutated D9 to A9 we found that both cell death and caspase activation were inhibited. Our studies demonstrated there needs to be a cleavage event first at D9 in order to have a cleavage event at D28. This begs the question of why are two cleavage events required for removal of the prodomain. One explanation is that there may be additional regulatory events that are destroyed when there is cleavage at D9. Another explanation would be that the prodomain is inherently unstructured and to have cleavage at D28 there must first be a cleavage event at D9. Initial cleavage at D9 could cause the remaining portion of the prodomain to regain structure, thus allowing for cleavage at D28 resulting in complete removal of the prodomain.

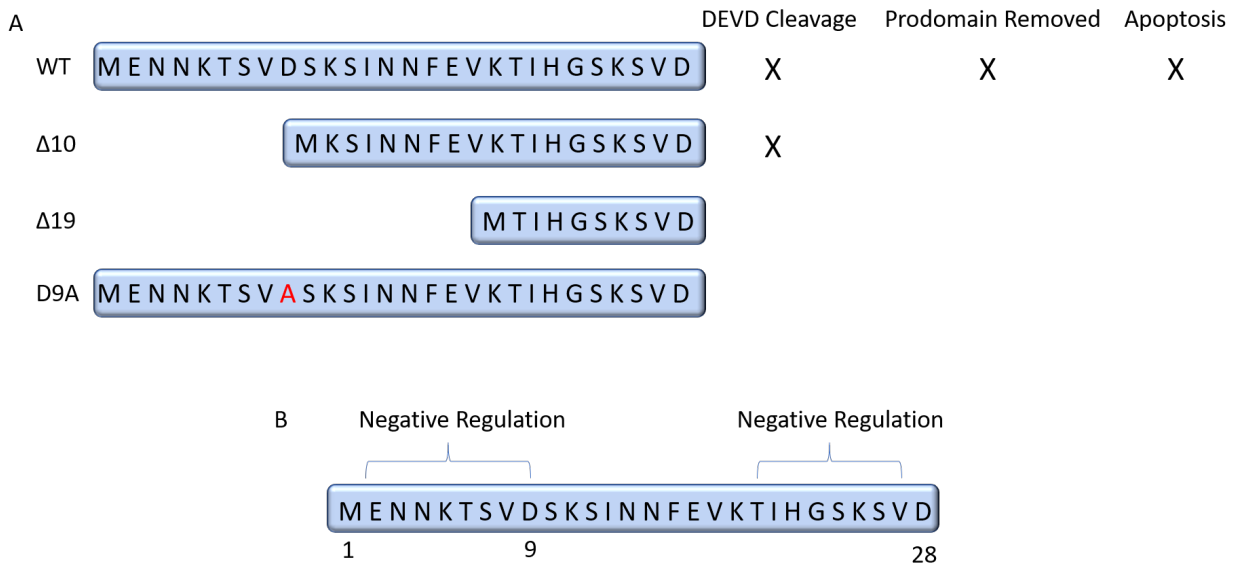

Figure 1. Predicted regulatory regions within the prodomain. A. WT MEFs are able to remove the full length 28 amino acid prodomain, cleave a synthetic DEVD substrate and undergo apoptosis. Mutants Δ10, Δ19 and D9A are unable to complete these functions, except for $C3^{-/-}$C3 Δ10 that is able to cleave a DEVD substrate. B. Predicted regulatory regions within the prodomain.

i. Caveats and opportunities for further study

During our studies we were unable to make a point mutation within the prodomain that did not have an effect on caspase-3 activation and activity. We hypothesize that due to the conservation of the 28 amino acid prodomain, mutating any amino acid could influence the orientation of the prodomain within the structure, thereby effecting its ability to be cleaved and subsequent caspase-3 activation. We have thus far tested three different amino acids within areas of less conservation, N4A, F16A and V18A. Further studies would include mutating these murine amino acids to the human counterpart, N4T, F16L and V18P. The results of these studies would indicate if the size and charge of the amino acid sequence within the prodomain is important for the prodomains structure and function.

Given that the prodomain is intrinsically disordered the current crystal structures of caspase-3 are unable to resolve the prodomain. Therefore, it is difficult to speculate how these point mutations are affecting the orientation of the prodomain. No one has tried to obtain a crystal structure of procaspase-3 with these point mutations. Since the prodomain was not resolved in the crystal structure of a C163A catalytically inactive caspase-3, the D9A mutant would also not result in the ability to resolve the prodomain. However, we found when a N-terminal flag-tag was placed on procaspase-3 that the ability of caspase-9 to cleave the interdomain linker was blocked. We hypothesize that the tag changed the orientation of the prodomain so that it was blocking the interdomain linker cleavage site. Obtaining a crystal structure with an N-terminal flag tag would demonstrate if this hypothesis is correct.

We demonstrated that for the prodomain to be removed at D28 there needs to be an initial cleavage event at D9 (Figure 2). To further support our data, we sought to analyze the cleavage

events of both the full-length caspase-3 and the D9A mutant using mass spectrometry. Unfortunately, we were unable to get the coverage we needed to make any conclusions. Future studies would include increasing the quantity of protein and using another caspase-3 antibody. Being able to compare the cleaved products from both the full-length caspase-3 and the D9A mutant would provide more evidence of a binding event at D9 or an initial cleavage event, where our current data supports the latter.

ii. Implications for future research

Since we have shown that the removal of the prodomain of caspase-3 requires two cleavage events we can hypothesize on the mechanism of removal of the other executioner caspases. Procaspase-7 has a longer prodomain than that of procaspase-3, but there is a conserved cleavage recognition site within the beginning of the prodomain that would suggest that there are also two cleavage events for the removal of the prodomain for procaspase-7. Additionally, the prodomain of caspase-7 has a nuclear localization domain. Determining the effect of cleavage on this domain and also if the prodomain of caspase-3 has additional functions, aside from regulating caspase-3 activity, would be interesting.

Overall, our data demonstrated that caspase-3 is not constitutively active and there are two sequential cleavage events within the prodomain that results in complete removal of the prodomain. We also discovered that mutation of a single amino acid within the prodomain renders caspase-3 inactive. This gives us more insight into the regulation of caspases and how caspases are activated.

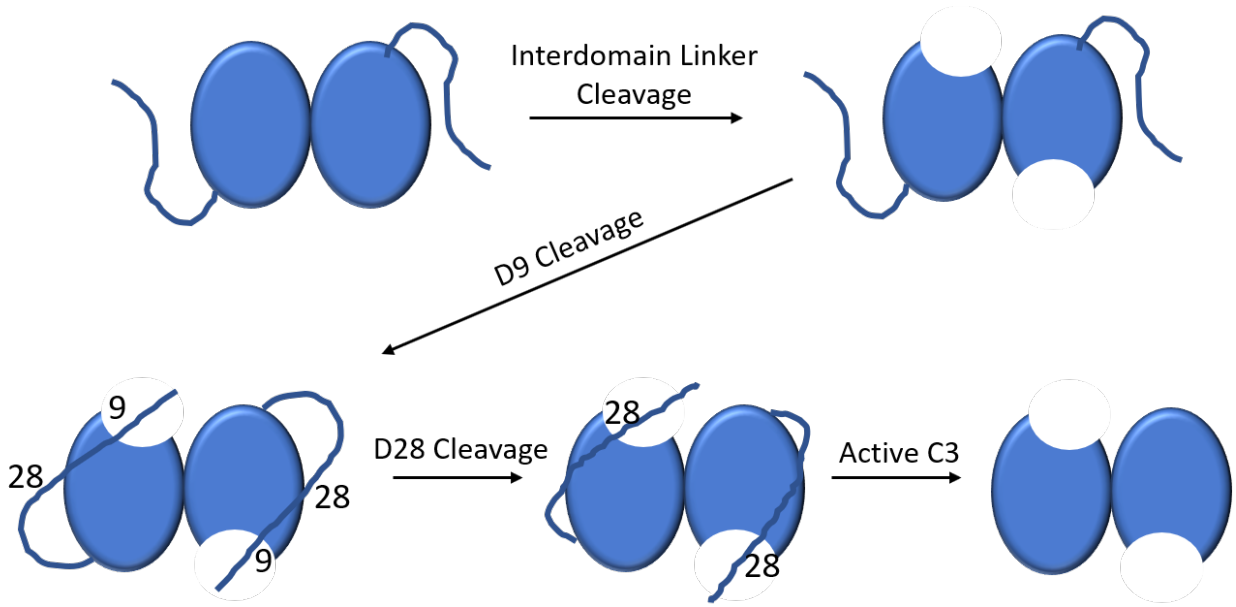

Figure 2. Model of caspase-3 prodomain removal. Procaspse-3 is found in the cytoplasm as an inactive zymogen dimer. Upon cleavage of the interdomain linker by caspase-9, caspse-3 undergoes a conformation change and the active site is exposed. This allows for binding and cleavage of the first 9 N-terminal amino acids of the prodomain. The binding and cleavage at D9 orients the prodomain for recognition and cleavage of D28. Cleavage at D28 results in the complete removal of the prodomain and the fully active caspae-3 enzyme.

B. Implications from Mcl-1 studies in multiple myeloma

Upstream of the caspase cascade, the Bcl-2 family of proteins regulate the release of cytochrome c from the outer membrane of the mitochondrial. The interplay of the pro- and anti-apoptotic proteins will determine if cytochrome c is released resulting in the activation of caspase-3. We sought to induce dual inhibition of the anti-apoptotic protein Mcl-1, as multiple myeloma cells are dependent on Mcl-1 for survival (22, 66). The combination of the proteasome inhibitor, carfilzomib, and the multi-kinase inhibitor TG02 resulted in at least additive cell death. Not only was this effect seen in the presence of stromal cells, that mimic the extracellular matrix, but was seen relapsed/refractory multiple myeloma patient samples.

A phase I clinical trial using the combination of TG02 and carfilzomib was completed. The study concluded that the safety profile of the TG02 and carfilzomib combination was similar to that of TG02 alone. 73% of patients had either stable disease or better after more than two cycles and the clinical benefit rate was 45%(83). This demonstrates not only that this combination has therapeutic potential, but that other combinations could also be beneficial.

i. Caveats and opportunities for further study

Although we discovered that the combination of carfilzomib and TG02 was effective in both multiple myeloma cell lines and patient samples, the mechanism by which TG02 caused a decrease in Mcl-1 was not determined. TG02 is working to inhibit transcription but not via CDK9. TG02 has also been shown to inhibit JAK2 and FLT3. JAK2 drives the expression of Mcl-1(30, 84). Therefore, treatment with TG02 could inhibit JAK2, thus causing a decrease in Mcl-1 expression. To test this hypothesis, we could silence JAK2 and determine the effect on

TG02 activity. Likewise, we could silence FLT3 and determine the effect on TG02 activity (Figure 3).

We were able to determine the expression levels of the Bcl-2 family of proteins after treatment with carfilzomib, TG02 and the combination in four different multiple myeloma cell lines. Although we saw increased levels of cell death with the combination treatment in patient samples, we did not determine the expression levels of the Bcl-2 family. This can be done in future studies, although isolating the plasma cells from a patient sample and conducting a 24 hour combination treatment will be difficult as the cells may not be viable enough to isolate the protein and RNA.

ii. Implications for future research

Using combination therapies has been shown to be a beneficial treatment option for hematologic malignancies. Our work demonstrated that the combination of a proteasome inhibitor, carfilzomib, and a multi-kinase inhibitor, TG02, has therapeutic potential. Given the dependence on the Bcl-2 family of proteins, future treatments for multiple myeloma will include targeting the anti-apoptotic proteins in combination with other therapies. The use of other proteasome inhibitors, like bortezomib, and kinase inhibitors could prove to have efficacy in multiple myeloma. There are currently Mcl-1 inhibitors and CDK9 inhibitors in development. Other factors may also contribute to clinical activity of combination therapies. Previous work done in our lab demonstrated that the proteasome inhibitors, carfilzomib and bortezomib, induce the heat shock factor response and found that inhibiting the master heat shock factor regulatory, HSF1, caused a sanitization to bortezomib(85). Determining which anti-apoptotic protein a

patient is dependent on and the mechanism by which the current therapies are functioning will facilitate the therapeutic potential of these combinations to use to treat their disease.

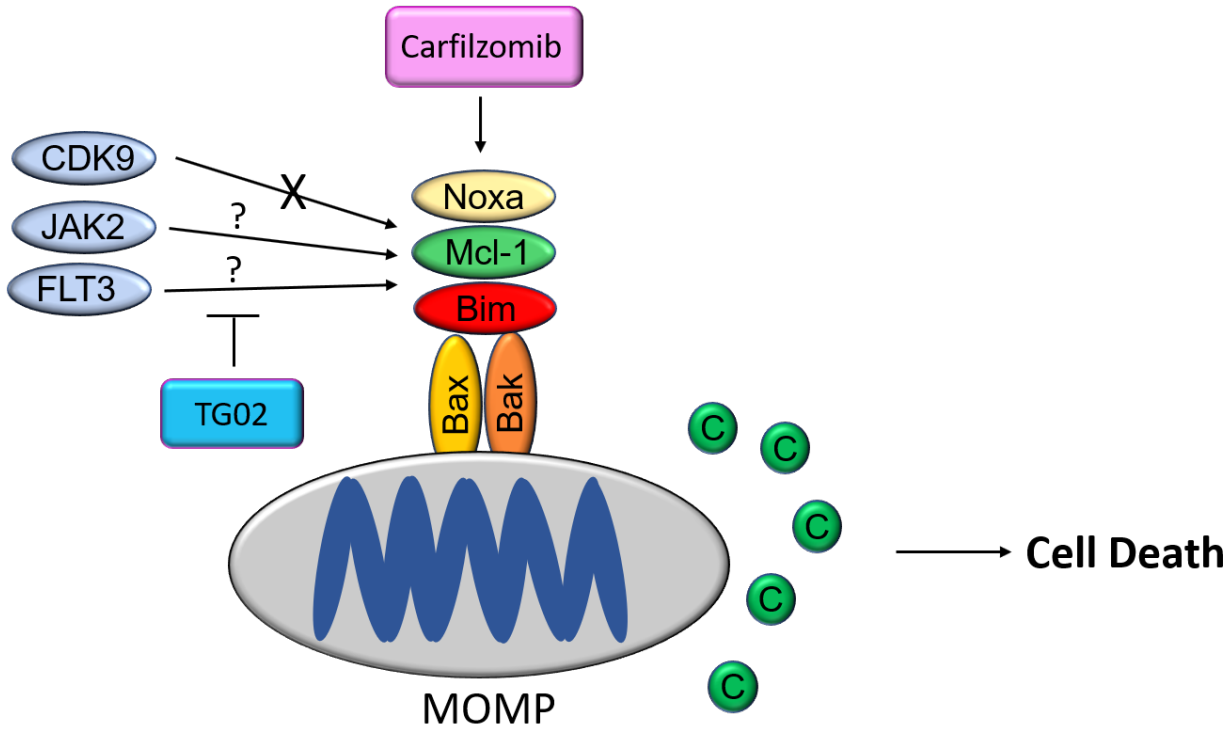

Figure 3. Mechanism of dual inhibition of Mcl-1 by carfilzomib and TG02. Proteasome inhibitor, carfilzomib induces the expression of NOXA. NOXA will bind to Mcl-1, thus allowing Bim to activate Bak and Bax and subsequent cell death. TG02, a multi-kinase inhibitor, is known to inhibit CDK9, JAK2 and FLT3. We found that TG02 caused a decrease in Mcl-1, but not though inhibition of CDK9.

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