MECHANISMS OF MITOCHONDRIA-MEDIATED APOPTOSIS

INDUCED BY CYTOTOXIC STRESS

BY

Shary N. Shelton

Submitted to the graduate degree program in Pharmacology, Toxicology & Therapeutics and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Committee:

_____
John D. Robertson, Ph.D. (Chair)

_____
Dianne Durham, Ph.D.

_____
Bruno Hagenbuch, Ph.D.

_____
Hartmut Jaeschke, Ph.D.

_____
Thomas L. Pazdernik, Ph.D.

Date Defended:_____

The Dissertation Committee for Shary N. Shelton certifies that this is the approved version of the following dissertation:

MECHANISMS OF MITOCHONDRIA-MEDIATED APOPTOSIS INDUCED BY CYTOTOXIC STRESS

_____
Chairperson - John D. Robertson, Ph.D.

Committee:

Dianne Durham, Ph.D.

Bruno Hagenbuch, Ph.D.

Hartmut Jaeschke, Ph.D.

Thomas L. Pazdernik, Ph.D.

Date approved:_____

Acknowledgements

I feel very fortunate to have had this opportunity and would like to thank many people who contributed to the completion of this dissertation. I am very thankful and proud to have completed this work in the laboratory of Dr. John D. Robertson. I thank him for allowing me to join his laboratory and for his continued instruction and support. I was able to learn and develop scientifically under his supervision both in didactic settings and by following his lead as an example. In addition, I would like to thank Drs. Dianne Durham, Bruno Hagenbuch, Hartmut Jaeschke, and Thomas L. Pazdernik for seving on my dissertation committee. I would also like to thank all of the faculty members within the department for their instruction and encouragement during this time.

Others I wish to thank are the other graduate students within the department. In particular, however, I would like to thank Dr. Mary Shawgo for her day-to-day support, the many thought-provoking conversations, and her friendship. I would also like to thank my family for their continued support and patience during this time, including Farnsworth the dog.

Lastly, I would like to acknowledge the financial support of the National Institutes of Health, the IDeA Networks of Biomedical Research Excellence Program of the National Center for Research Resources, the National Institute of Environmental Health Sciences Toxicology Taining

Grant, and the Biomedical Research Training Program at the University of Kansas Medical Center.

Table of Contents

List of Figures and Tables

List of Abbreviations

17-AAG: 17-allylaminogeldanamycin

ALT: Alternative lengthening of telomeres

Apaf-1: Apoptotic protease activating factor-1

ATP: Adenosine 5'-triphosphate

Bak: Bcl-2 antagonist killer

Bax: Bcl-2 associated X protein

Bcl-2: B-cell lymphoma-2

Bcl-X_L: B-cell lymphoma-extra long

Bid: BH3-interacting domain death agonist

BMH: *Bis*-maleimidohexane

CAM: Cell adhesion molecule

CARD: Caspase Recruitment Domain

Casp: Caspase

Ced: Cell death abnormal

Diablo – Direct IAP binding protein with low pI

DISC: Death inducing signaling complex

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

Egl: Egg laying defective

ETOP: Etoposide

FADD: Fas associated death domain

FBS – Fetal bovine serum

FITC: fluorescein isothiocyanate

G0: Gap phase zero

G1: Gap phase one

G2: Gap phase two

h: hour(s)

hsp: Heat-shock protein

Htr2a – High temperature requirement protein A2

IAP: Inhibitor of apoptosis

min: Minutes

Mcl-1 – Myeloid cell leukemia-1

MLL: Mixed lineage leukemia

mL: Milliliter

mM: Millimolar

MOMP: Mitochondria outer membrane permabilization

OMM: Outer mitochondrial membrane

p53: Protein 53

PI: Propidium Iodide

pRb: Protein retinoblastoma

pSUPER: pSuper vector

PS: Phosphatidylserine

Puma – p53-upregulated modulator of apoptosis

RAIDD: RIP-associated ICH-1/CED-3 homologous protein with a death domain

RNA: Ribonucleic acid

RNAi: RNA interference

ROS: Reactive Oxygen Species

S: DNA synthesis phase of cell cycle

sec: second(s)

shRNA: short hairpin RNA

siRNA: Short interfering RNA

SMAC: Second mitochondrial-derived activator of caspases

TGFβ: Transforming growth factor-β

TP53: Tumor protein 53

TRAP: tumor necrosis factor receptor-associated protein

μg: Microgram

μL: Microliter

WT: Wild type

XIAP – X-linked inhibitor of apoptosis

Abstract

Defects within the apoptotic pathway are thought to contribute to tumorigenesis and therapeutic resistance. Most cytotoxic anti-cancer drugs are thought to activate the mitochondria-mediated apoptotic pathway; however, the precise mechanistic details remain unclear and, in some instances, controversial. The prevailing view of mitochondria-mediated apoptosis is that the process occurs through a series of events that are orchestrated in a linear step-wise fashion. From this perspective, initiating events include the activation of a BH3-only family member which then activates Bax and/or Bak resulting in their homo-oligomerization and pore formation spanning the outer mitochondrial membrane to induce mitochondrial outer membrane permeabilization (MOMP). Subsequently, cytochrome *c* and other pro-apoptotic factors are released from the intermembrane space of the mitochondria into the cytosol through this pore. Cytosolic cytochrome *c* interacts with Apoptotic protease activating factor-1 (Apaf-1) and initiator caspase-9 where this complex serves as the activating platform for initiator caspase-9. Active caspase-9 then activates downstream effector caspase-3 and -7, which produce the biochemical and morphological features associated with apoptosis.

For my dissertation research, I have investigated the underlying molecular requirements necessary for mitochondria-mediated apoptosis

induced by a DNA topoisomerase II inhibitor, a novel heat shock protein 90 (Hsp90) inhibitor, and elevated temperature in Jurkat T-lymphocytes. Combined, the data suggest that different cytotoxic stressors use similar as well as distinct mechanisms to execute mitochondria-mediated apoptosis. In addition, the data provide new insights into the mechanistic details utilized by these three different stressors, which may prove useful for future drug design and therapeutic approaches.

In the first Specific Aim, I investigated the molecular requirements necessary for mitochondria-mediated apoptosis in response to the DNA-damaging drug etoposide. The data suggest that the BH3-only family member Bid is important during DNA-damage induced apoptosis where it functions to facilitate MOMP. These studies also found that the active form of the protein, tBid, is generated downstream of initiator caspase-9 activation by executioner caspase-3 and/or -7. Taken together, these data suggest that effector caspases, Bid, and mitochondria forge an amplification loop to irreversibly commit a cell to apoptosis.

The antiproliferative effects of a novel C-terminal inhibitor of Hsp90, designated KU135, were characterized in the second Specific Aim. This study compared the effects of an N-terminal Hsp90 inhibitor, 17-AAG, to the effects of the C-terminal Hsp90 inhibitor, KU135. KU135 was found to have more potent antiproliferative effects than 17-AAG, and KU135 activated the mitochondria-mediated apoptotic pathway, whereas 17-AAG

was primarily cytostatic. Finally, 17-AAG caused robust induction of the cytoprotective proteins Hsp90 and Hsp70 which could account for its lack of an apoptotic response.

The molecular requirements for heat-induced apoptosis were examined in the third Specific Aim. The data showed that heat-induced apoptosis is a mitochondria-mediated event and that caspase-9, and not caspase-2, is the most apically activated caspase. The BH3-only protein, Bid, was found to be important during heat-induced apoptosis where it was needed to facilitate MOMP. However, we found that tBid was generated downstream of caspase-9 suggesting that it may function to promote cytochrome *c* release during this process as part of a feed forward amplification loop.

Chapter 1: Introduction and Background

1.1 The Relationship Between Apoptosis and Cancer

It is estimated that there were 1,479,350 new cancer cases and 562,340 new cancer-related deaths during 2009 in the United States. There are currently over 100 types of known cancers with lung, breast, colon, and prostate being the most common (American Cancer Society). These cancers are thought to develop through a series of events eventually causing healthy cells to develop into a cancerous cells. Events that contribute toward tumorigenesis include genetic modifications to the cell that favor uncontrolled proliferation and a lack of cell death. In this way, a cancerous cell possesses a survival advantage. It has been suggested that the large variety of genotypes of different cancer cells fall into one of six categories, with one genotype from each category being necessary for cell transformation. These categories include: self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, and evasion of apoptosis (Hanahan and Weinberg, 2000). This idea suggests a model where carcinogenesis is a process in which a cell progresses from a state of normalcy to one of malignancy acquiring at

least six mutations or sufficient mutations that fall within all six of the required categories.

The vast majority of adult cells are in a state of quiescence (Gap phase 0 or G_0) and require a stimulus to exit this phase and enter the cell cycle. The growth signals that cause this shift in the cell's behavior are provided through the microenvironment; however, some oncogenes have acquired the ability to function as continuous growth stimuli. An example is the Ras proto-oncogene that is activated through binding of a ligand, such as growth factors, to tyrosine kinase receptors (Hanahan and Weinberg, 2000). By activating Ras, one of the pathways activated is the Raf/MEK/ERK MAP kinase pathway, which promotes cell survival by providing a mitogenic growth stimulus. It is estimated to be mutated and constitutively active in 25% of all cancers (Downward, 2006); furthermore, it is often used as a mechanism of transformation in research settings and has been one of the most studied receptor tyrosine kinase pathways relative to cancer. Cancer cells not only induce neighboring cells to produce growth factors, but also often produce the growth factors to which they themselves respond generating an aberrant autocrine-signaling loop (Hanahan and Weinberg, 2000). Having self-sufficiency in growth signals is one of the six capabilities defined above as being required for tumorigenesis.

Another capability of tumorigenesis is becoming insensitive to anti-growth signals (Hanahan and Weinberg, 2000). These signals are often conveyed to remove a cell that is actively participating in the cell cycle to a state of rest or to move the cell into a permanent differentiated state (Weinberg, 1995). An example of an anti-growth signaling pathway is the TGFß pathway that maintains the tumor suppressor protein retinoblastoma (pRb) in a hypophosphorylated state, which then functions to halt progression of the cell cycle through the restriction point within gap phase 1 (G_1). This pathway is disrupted in many different types of human cancers, which, in turn, allows the phosphorylation of pRb and subsequently the cell to progress through the restriction point within G_1 (Weinberg, 1995).

The ability to go through an infinite number of DNA replication cycles is another acquired trait of tumor cells (Hanahan and Weinberg, 2000). A typical cell has a finite number of divisions it can undergo because of the loss of terminal sequences at the end of chromosomes, which is known as the end-replication problem that occurs during DNA synthesis. However, cancer cells are able to continuously replicate their DNA without entering into a state of cellular senescence or a state of crisis as a consequence (Hayflick, 1997; Shay and Bacchetti, 1997). This ability largely depends on the enzyme telomerase that maintains telomere length at the end of chromosomes by adding six nucleotide bases to the newly

replicated strand of DNA (Bryan and Cech, 1999). It has been estimated that 85-90% of all malignancies up-regulate the expression of telomerase. It has also been suggested that cells that do not up-regulate telomerase maintain their telomere length through an alternate form of maintenance known as alternative lengthening of telomeres (ALT) (Shay and Bacchetti, 1997; Bryan and Cech, 1999).

Angiogenesis is not an innate ability of proliferating cells; in fact, the lack of a blood supply is actually a way of interfering with a proliferating mass of cells (Hanahan and Weinberg, 2000). The cells within the center of a tumor mass will often die and subsequently initiate an inflammatory response due to the lack of a blood supply at the heart of the tumor. Tumor cells must acquire the ability to generate a source for oxygen and nutrients through angiogenesis (Bouck et al., 1996; Hanahan and Folkman, 1996; Folkman, 1997). An initiating step during angiogenesis is the production of vascular endothelial growth factor (VEGF), which then binds to its cognate tyrosine kinase receptor present on endothelial cells (Veikkola and Alitalo, 1999). Many cancers have up-regulated the expression of VEGF and/or down-regulated known inhibitors of VEGF such as thrombospondin-1, thereby facilitating and promoting angiogenesis (Volpert et al., 1997; Hanahan and Weinberg, 2000).

Metastases account for 90% of human cancer cell deaths (Sporn, 1996). Advanced primary tumor masses can acquire the ability to

metastasize to close and/or distant secondary sites. This process requires the ability of the tumor cells to release themselves from the site of origin to target tissues by down-regulating or mutating cell-cell interacting proteins such as cell adhesion molecules (CAMs) and/or cadherins, which are often altered in metastatic cells (Hanahan and Weinberg, 2000). An example is E-cadherin, which is expressed ubiquitously on epithelial cells; however, epithelial-derived metastatic cancers have often down-regulated E-cadherin (Christofori and Semb, 1999). This down-regulation prevents the anti-growth signal that is usually communicated between two cells through E-cadherin bridges thereby facilitating the release of the cells from the site of origin to target sites (Christofori and Semb, 1999).

Finally, the expansion of cancer cells is not only determined by the rate of proliferation but also the rate of cell death. Resistance to apoptosis is thought to be a hallmark of most, if not all, types of cancer (Hanahan and Weinberg, 2000). This type of cell death can be triggered by intracellular and extracellular stimuli. For example, apoptosis is stimulated extracellularly by death receptor ligands and/or the lack of survival signals, for example, hormones. It is also stimulated intracellularly in response to DNA damage, oncogene activation, or hypoxia (Evan and Littlewood, 1998). Apoptosis was first associated with cancer in 1972 through experiments using hormone withdrawal to stimulate death in hormone-dependent tumors (Kerr et al., 1972).

An example of an anti-apoptotic mutation observed in more than 50% of cancers is a mutation in the protein 53 gene (p53). This protein functions as a tumor suppressor by halting the cell cycle in response to DNA damage, or triggers apoptosis if the damage is beyond repair (Harris, 1996b; Harris, 1996a). By mutating p53, the cell acquires two survival advantages with the first being progression through the cell cycle, irrespective of the amount of DNA damage. The second survival advantage is avoidance of apoptotic cell death. p53 is a unique tumor suppressor in that mutation of only one allele will render the cell mutant just as if it were a homozygous mutation. Therefore, this tumor suppressor does not behave as suggested by Knudson's two-hit hypothesis, which suggests that both tumor suppressor gene copies must be mutated for the cell to exhibit a loss-of-function phenotype (Knudson, 1971). p53 functions as a tetramer meaning that only 1 of 16 functional protein complexes would be produced in the heterozygous mutant. Furthermore, because of the dual function of this protein, mutations within this gene generate more than one survival and/or resistance effect.

1.2 Overview of Chemotherapeutic Agents and Cell Death

Chemotherapy was introduced for the treatment of cancer over fifty years ago with the nitrogen mustard DNA alkylating agent

mechlorethamine (Bonte et al., 1956; Johnstone et al., 2002). There are currently several different classes of chemotherapeutic agents, many of which kill tumor cells by inducing apoptosis as the therapeutic end-point, although they have distinct drug targets (Lowe and Lin, 2000). Resistance to therapy, due to down-regulation of key components of the pro-apoptotic machinery or up-regulation of key anti-apoptotic proteins, is thought to be quite common. Furthermore, resistance due to aberrant regulation of the apoptotic pathway is also thought to be a major component of multi-drug resistant cancers, although this argument is still under debate and other mechanisms are clearly involved, for example, upregulation of the efflux drug transporter P-glycoprotein (Brown and Wouters, 1999; Johnstone et al., 2002).

There are several classes of anticancer agents including: DNA damaging agents, antitubulin agents, kinase inhibitors, hormone inhibitors, antibody based therapies, and vascular targeting agents (Thurston 2007). This is not an exhaustive list, and new strategies are being developed, such as heat-induced cell death (Pissuwan et al., 2006) and the targeting of heat shock protein 90 (Hsp90) (Whitesell et al., 1994; Schulte et al., 1995). Nonetheless, the largest groups are those that bind and/or damage DNA either directly or indirectly (Thurston 2007). Chemotherapeutic agents are also often given in combination to achieve additive or synergistic effects, to enhance efficacy, and /or to decrease the

toxic adverse effects of the different drugs by lowering the administered dose compared to the dose when given independently. It is currently estimated that 50% of combination chemotherapy regimens contain at least one type of DNA-damaging agent within the drug class of topoisomerase II inhibitors (Hande, 1998; Martincic and Hande, 2005). Because the majority of anti-cancer agents are not selective for neoplastic cells, there are short-term and long-term toxic adverse effects associated with the majority of these drugs due to the damaging and killing of cells that are not pathological.

1.3 Overview of Topoisomerase II Inhibitors and Cell Death

Topoisomerase II is an enzyme that functions to decatenate DNA by cleaving both strands of DNA, pulling one strand through the other, and resealing the cleaved strands (Wang, 2002). This process is energy-dependent and requires the presence of the divalent cation Mg^{2+}. There are two isoforms of this enzyme, α and β, and both function during DNA replication, chromatid condensation, and transcription to unravel supercoiled DNA. Research is ongoing to determine if the functions are entirely redundant, although the phenotype of the topoisomerase II β knockout mice suggests these enzymes have distinct functions, at least in part (Yang et al., 2000).

As mentioned above, one class of DNA-damaging agents are the topoisomerase II inhibitors. This class of anticancer drugs contains both epipodophyllotoxins such as etoposide and anthracyclines such as doxorubicin with the therapeutic end-point of each being cell death. Although both types of drugs induce apoptosis, they each do so by similar, as well as distinct, mechanisms of action. While epipodophyllotoxins are considered to be cell cycle-specific (late S/G_2), anthracyclines are thought to not target the cell-cycle during a specific phase, although there is evidence to suggest they are selective for cells in the S/G_2 phase of the cell cycle (Chu and Sartorelli 2007; AccessMedicine). Furthermore, epipodophyllotoxins are thought to be specific for the topoisomerase II α isoform, whereas it is not known whether the anthracyclines discriminate between the two isoforms.

Epipodophyllotoxins are semisynthetic derivatives of podophyllotoxin which is extracted from *Podophyllum peltatum* (mayapple root). It is currently thought that podophyllotoxins directly bind to the active site of topoisomerase II and prevent the re-ligation of the cleaved strands of DNA (Nitiss, 2009) (Figure 1). This causes an accumulation of DNA double-strand breaks that then cause the cell to undergo mitochondria-mediated apoptosis. Etoposide is an example of an epipodophyllotoxin that is currently used clinically to treat various cancers, including leukemia, lymphoma, lung cancer, and breast cancer. Resistance to etoposide is

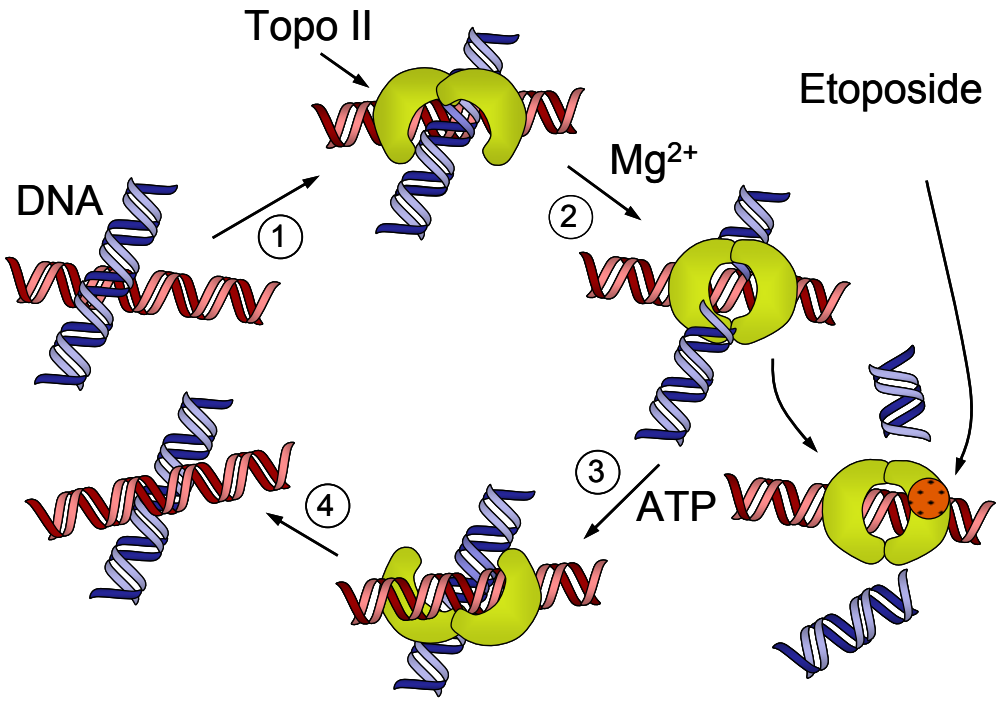


Figure 1: Topoisomerase II-epipodophyllotoxin drug complex.

Etoposide is used as an example of an epipodophyllotoxin that binds to the catalytically active site of topoisomerase II and prevents the religation of topoisomerase II-cleaved double stranded DNA; therefore, this drug induces double stranded DNA breaks.

known to occur through an increased expression of the efflux transporter P-glycoprotein, a decrease in expression of topoisomerase II α, or through mutation of the catalytically active site (Nitiss and Beck, 1996). The short-term adverse effects of these drugs include bone marrow suppression, alopecia, and gastrointestinal distress, which are characteristic of several

classes of chemotherapeutic agents that target rapidly dividing cells within the body, whereas the most serious long-term adverse effect is development of secondary leukemias involving mixed lineage leukemia (MLL) chromosomal translocations (Felix et al., 2006).

1.4 Overview of Hsp90 as an Anti-cancer Drug Target

The heat shock proteins (Hsps) were first discovered by Ritossa in 1962 due to their induction in response to elevated temperatures (For Review: (Ritossa, 1996)). It is now known that the majority of proteins within this family are present constitutively within the cell where they function to chaperone other proteins in their proper folding and localization within the cell. In addition, they also prevent protein aggregation and mediate proteolytic turnover. Heat shock proteins have been conserved throughout evolution ranging from bacteria to humans where the family members fall into five main classes depending on their molecular weight (i.e. Hsp27, Hsp60, Hsp70, Hsp90 and Hsp100) (Kregel, 2002). Heat shock proteins are transriptionally up-regulated in response to proteotoxic and stressful environments (e.g. heat, toxins, and hypoxia), where it is widely believed that this is a compensatory mechanism implemented to maintain protein homeostasis and is termed the “heat shock response” (Whitesell and Lindquist, 2005).

Although the heat shock family of proteins function as molecular chaperones to assist in protein folding, it has been suggested that they bind different clients at different times depending on the phase of protein maturation (Whitesell and Lindquist, 2005). In addition, chaperones often function in protein complexes that include other heat shock proteins, adaptors, and co-chaperones. The composition of these protein complexes is thought to depend on the specific client involved as well as its level of maturation (Whitesell and Lindquist, 2005). An example is the estrogen receptor which initially is chaperoned by a complex containing Hsp70 and Hsp40; however as the receptor matures it will be passed to a complex containing Hsp70 and Hsp90 and the final mature protein will be associated with a complex where the only heat shock protein within the complex is Hsp90 (For Review: (Whitesell and Lindquist, 2005)). This model suggests that the chaperoning properties of Hsp90 are needed late in the process of protein maturation and may offer stability for mature but labile client proteins (Whitesell and Lindquist, 2005).

Many cancers including solid tumors and hematological malignancies have been reported to have elevated levels of several heat shock proteins, where over-expression of Hsp27, Hsp70, and Hsp90 have been correlated with a poor therapeutic outcome(Morimoto and Santoro, 1998; Bagatell and Whitesell, 2004) . In response to a stressful stimulus, the transcriptional up-regulation of heat shock proteins is regulated by the

transcription factor HSF1 (heat shock factor-1) in healthy cells; however, it is currently not known if the mechanism of up-regulation is the same in cancer cells (Bagatell and Whitesell, 2004). It seems reasonable to suspect that activation of HSF-1 resulting in the cytoprotective heat shock response would occur in cancer cells due to the harsh conditions of their microenvironment which often include hypoxia, acidosis, and nutrient deprivation (Bagatell and Whitesell, 2004). HSF-1 is maintained in an inactive state through its binding to Hsp90, but it is thought that as misfolded proteins increase during a stress response, they compete with HSF-1 for Hsp90 which eventually causes the release of HSF-1 from the Hsp90 protein complex which, in turn, facilitates its activation (Zou et al., 1998).

Hsp90 represents a family of ATPase-containing molecular chaperones that comprise 1-2% of total cellular protein, although this can increase to as much as 6% under conditions of cellular stress. There are four isoforms of Hsp90 including Hsp90α, Hsp90β, Grp94 (glucose regulating protein 94), and TRAP-1 (tumor necrosis factor receptor-associated protein-1). The functional difference between these isoforms has yet to be determined; however, there are differences in their localization and expression. Hsp90α and Hsp90β are both located within the cytoplasm of the cell; however, it is thought that the α isoform is inducible, whereas the β isoform is present constitutively (Hahn, 2009).

Grp94 and TRAP-1 are localized to the endoplasmic reticulum and the mitochondira, respectively (Hahn, 2009).

Hsp90 is known to have between 100-200 client proteins and is often present at elevated levels in cancer cells where it is thought to stabilize oncogenic proteins involved in all six hallmarks of cancer (Figure

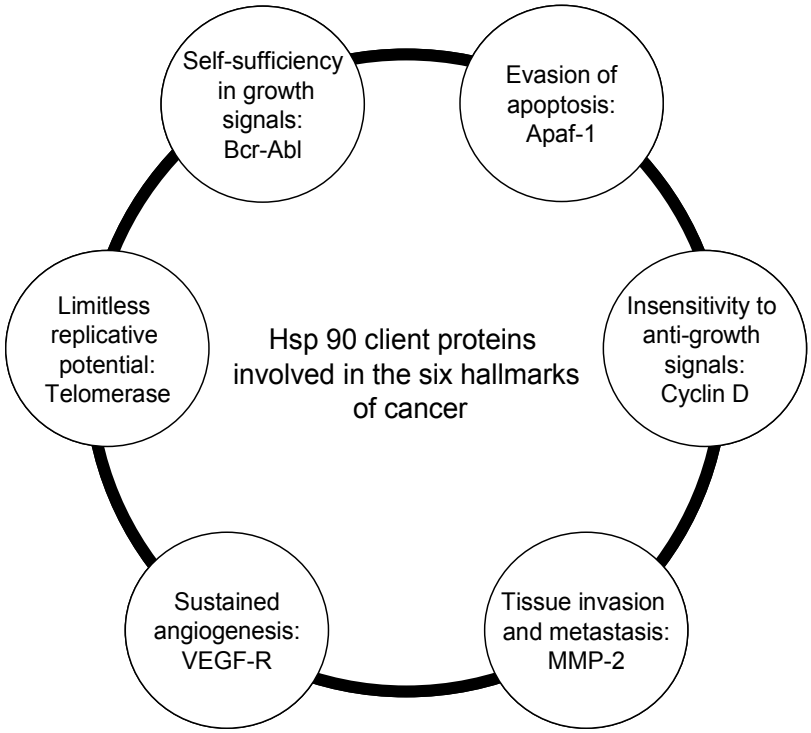


Figure 2: Example of Hsp90 client proteins involved in the six hallmarks of cancer. *Bcr-Abl,* translocation gene product resulting from the fusion of a portion of the *Breakpoint Cluster Region* gene fused with the *Abl1* gene; *Apaf-1*, *apoptotic protease activating factor-1; MMP-2, matrix metalloproteinase-2; VEGF-R, vascular endothelial growth factor receptor.*

2). It has been reported that Hsp90 is often seen as a homo-dimer in healthy cells whereas it is often seen in a multimeric protein complex in cancer cells. In addition, Hsp90 has also has been shown to have an increased amount of ATPase activity in cancer cells compared to healthy cells (Kamal et al., 2003).

Significantly, whereas many Hsp90 client proteins are being pursued individually as targets for anti-cancer drug development, inhibition of Hsp90 in cancer cells would be expected to prevent the maturation and stabilization of numerous Hsp90 client proteins, leading to their ultimate degradation within the ubiquitin-proteasome pathway (Whitesell et al., 1994; Schulte et al., 1995). In this regard, Hsp90 has emerged as a target for cancer chemotherapeutic drug design because inhibition of its chaperoning function simultaneously leads to the destabilization and degradation of multiple oncogenic proteins.

The earliest Hsp90 inhibitors were the natural products geldanamycin (GA), an analogue of GA, i.e.,17-AAG (17-allylaminogeldanamycin), and radicicol (Whitesell et al., 1994; Sharma et al., 1998). These compounds were shown to inhibit Hsp90 by binding to its N-terminal ATP-binding site, which, in turn, induces destabilization of Hsp90-dependent client proteins (Roe et al. 1999). In the case of GA and 17-AAG, these compounds were shown to exert antineoplastic effects in numerous preclinical experimental models. However, the likelihood of

these compounds making it into the clinic seems limited, in part, because of concerns about their adverse hepatotoxic effects (Egorin et al., 1998) and poor solubility (Chiosis et al., 2003). A more significant limitation is that the cytotoxic potential of these agents is limited because they frequently induce a strong stress response leading to increased *de novo* expression of the antiapoptotic proteins Hsp70 and Hsp90 (Whitesell et al., 2003; Schmitt et al., 2007; Workman and Powers, 2007).

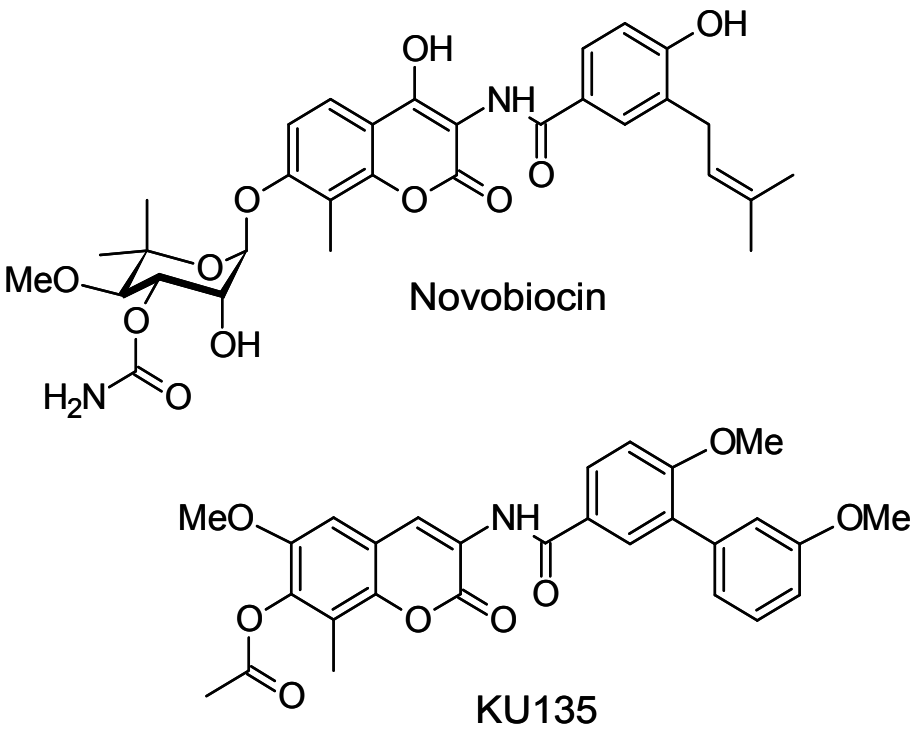


Figure 3: Novobiocin and KU135 chemical structures. KU135 is one of the novobiocin-derived analogues that inhibit Hsp90.

More recently, it has been shown that the antibiotic novobiocin can inhibit Hsp90 chaperone function of client proteins by binding to a previously unrecognized C-terminal ATP-binding domain in Hsp90 (Marcu et al., 2000a; Marcu et al., 2000b). However, the binding affinity of novobiocin for the C-terminal ATP-binding site was shown to be poor, manifesting an IC_{50} of ~ 700 μM (Marcu et al., 2000a). Currently, more than 300 analogues of novobiocin, designated "KU" compounds, have been developed in the medicinal chemistry laboratory of Brian Blagg on the KU-Lawrence campus, many of which exhibit significantly increased potency (Yu et al., 2005; Burlison et al., 2008a). Among these compounds is the novobiocin analogue KU135, which we have found binds to Hsp90 and is a potent inducer of apoptosis in human leukemia cells (Figure 3).

1.5 Overview of Heat-Induced Apoptosis

There has recently been an increasing interest in the potential use of heat-induced apoptosis as novel treatment of neoplasms; furthermore, it has also gained interest as a way of inducing apoptosis by damaging the mitochondria directly which would be useful as mutations within the apoptotic pathway are usually upstream of the mitochondria (Johnstone et al., 2002; Milleron and Bratton, 2007a). As mentioned in the previous

section, sub-lethal doses of heat exposure induce adaptive responses such as the expression of heat shock proteins (Hsp27, 70, and 90), which protects cells from subsequent stress, such as higher temperatures and chemotherapeutic agents (Jolly and Morimoto, 2000). However, when cells are exposed initially to a lethal temperature, these adaptive responses do not prevent cell death. It is currently thought that temperatures above 42°C induce apoptosis; however, as the temperature increases, it has been reported that the amount of apoptosis decreases with an increase in necrosis (Milleron and Bratton, 2007a).

Currently, there is research being conducted on the therapeutic potential of heat shock using nanoparticles targeted to cancer cells, which are then heated to induce cell death (Huang et al., ; Pissuwan et al., 2006). Heat-induced cell death is also being explored as a putative component of adjuvant therapy (Kouloulias et al., 2005). Although heat shock is known to induce apoptosis, the mechanism is poorly understood and currently controversial.

1.6 Overview of Apoptosis and Key Protein Families

Apoptosis or programmed cell death is a process ultimately leading to a cell's demise in a tightly regulated and precise fashion. It is distinct

from the necrotic form of cell death in several ways. Key features of a cell undergoing apoptosis include cellular shrinkage, chromatid condensation,

Table 1: Comparison of Apoptosis vs. Necrosis

Apoptosis	**Necrosis**
Physiological/ Pathological	Accidental
Tightly Regulated	Unregulated or Poorly Regulated
Membranes Intact Late	Membrane Integrity Lost Early
No Leakage of Cell Content	Leakage of Cell Content
Oligonucleosomal DNA-Fragmentation	Random DNA Degradation
Cell Shrinkage	Cytoplasmic Swelling
No Mitochondrial Swelling	Mitochondrial Swelling
ATP-dependent	ATP Not Required
No Inflammatory Response	Inflammatory Response

and the formation of apoptotic bodies (Kerr et al., 1972; Wyllie et al., 1980). These apoptotic bodies are engulfed by phagocytes to avoid the initiation of an inflammatory response (Danial and Korsmeyer, 2004). Typical features of necrosis, on the other hand, are cellular swelling and rupture, random DNA degradation, and generation of an inflammatory response (Table 1). Apoptosis plays a necessary role during development

and in maintaining tissue homeostasis (Danial and Korsmeyer, 2004). Several diseases can arise when this process is aberrant resulting in too much cell accumulation or cell attrition. For example, cancers arise when cells accumulate rather than undergo apoptosis, and Parkinson's disease has been associated with increased or accelerated cell death (Thompson, 1995).

Nobel Laureate H. Robert Horvitz is credited with identifying the regulatory genes responsible for the process of apoptosis in *Caenorhabditis elegans* (*C. elegans*) and helping to identify mammalian homologues of those genes (Figure 4) (Metzstein et al., 1998). His work was largely based on the 131 cells that are pre-determined to die during normal development of *C. elegans* as determined by mapping the fate of all 1090 cells in the worm (Metzstein et al., 1998). The genetic basis of apoptosis is now considered well-defined and consists of highly conserved core proteins. In general, there are three primary protein categories that function to govern apoptosis: regulator, adaptor, and effector proteins. As illustrated at the bottom of Figure 4, humans have also evolved an additional level of complexity with respect to apoptosis signaling pathways. Specifically, human cells can be triggered to undergo a receptor-mediated form of apoptosis. In addition, there is an important difference between *C. elegans* and humans. Whereas Ced-9 can inhibit death by binding to and inhibiting Ced-4, the mammalian homologue of

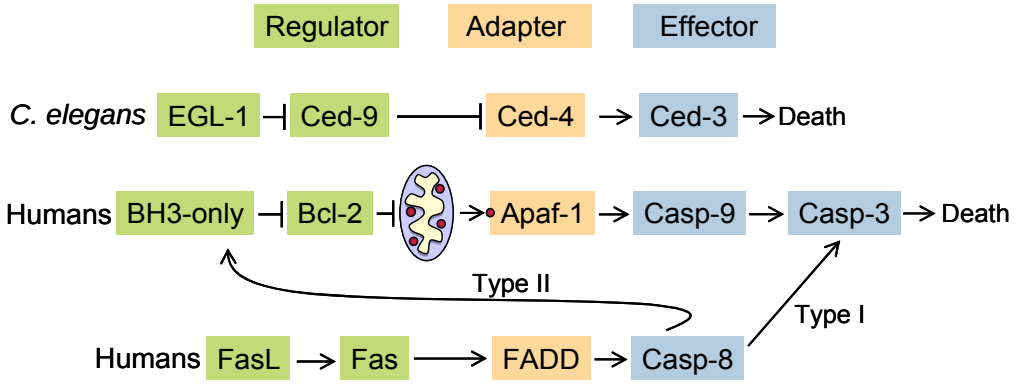


Figure 3: Conservation of Core Cell Death Genes Between *C. elegans* and Humans. *C. elegans* have one apoptotic pathway where Egl-1 inhibits Ced-9 which, in turn, alleviates the inhibition of Ced-9 on Ced-4, thereby facilitating Ced-4 activation. Ced-4 then activates Ced-3 which results in death. Two important differences have evolved in humans with the first being that the human homologue of Ced-9, Bcl-2, does not directly bind and inhibit Apaf-1. Bcl-2 exerts its inhibition at the level of mitochondria by preventing the release of cytochrome *c* which is necessary for the activation of Apaf-1. The other important difference is that humans have evolved an additional pathway leading to cell death known as the receptor mediated pathway which is executed differently depending on whether a cell is characterized as either type I or type II (also see Figure 8). *Ced, cell death abnormal; Egl, egg laying defective; BH3, Bcl-2 homology domain 3; Bcl-2, B-cell lymphoma 2; Apaf-1, Apoptotic protease activating factor 1; FADD, fas associated death domain.*

Ced-9, Bcl-2, does not bind to, and directly inhibit, Apaf-1(Apoptotic protease activating factor-1), but rather exerts its inhibitory effect by localizing to the outer mitochondrial membrane where it presumably prevents the release of cytochrome *c* (Figure 4) (Danial and Korsmeyer, 2004). Cytochrome *c* is required for activation of Apaf-1 in mammalian systems (Li et al., 1997) .

1.6.1 Caspases

Caspases (cysteine proteases that cleave their substrates after aspartate residues) are the biochemical foundation of apoptosis and are responsible for the execution of cell death. These enzymes are present constitutively in the cell as proforms and are activated in response to pro-apoptotic stress. Caspases are classified as either initiator caspases (e.g., caspase-8, -9, -10, possibly -2) or effector caspases (e.g., caspase-6, -7, -3) according to their timing of activation within the caspase cascade, substrate specificity, and/or unique structural features (Fuentes-Prior and Salvesen, 2004). The cysteine within the conserved penta-peptide consensus sequence QACxG is considered to be the active site of caspases, and as mentioned above, active caspases cleave substrates following an aspartate amino acid residue (Cohen, 1997). Initiator

caspases are activated early during apoptosis, whereas effector caspases are activated later by active initiator caspases (Nunez et al., 1998) (Figure 5).

Caspases have three distinct protein domains including a prodomain, a large subunit (~20 kDa), and a small subunit (~10 kDa) – with an aspartate residue being present at the interface of the different domains (Nunez et al., 1998). The key structural feature unique to initiator caspases is the long prodomain that interacts with an adaptor molecule (e.g., Apaf-1, FADD, and possibly RIP-associated ICH-1/CED-3 homologous protein with a death domain (RAIDD)). This interaction facilitates their activation by bringing initiator procaspases of a single type into close proximity which facilitates their activation through dimerization and/or a conformational change, auto-activation, and subsequent proteolytic cleavage. Active initiator caspases cleave downstream effector procaspases, which then dimerize to form an active effector caspase. Effector caspases destroy the cell by cleaving over 1000 known substrates and are responsible for the typical biochemical and morphological features observed during apoptosis, for example, chromatin condensation and apoptotic body formation.

Currently, controversy surrounds the precise mechanism(s) required for initiator caspase activation. There are two paradigms, namely the induced proximity and induced conformation models, attempting to

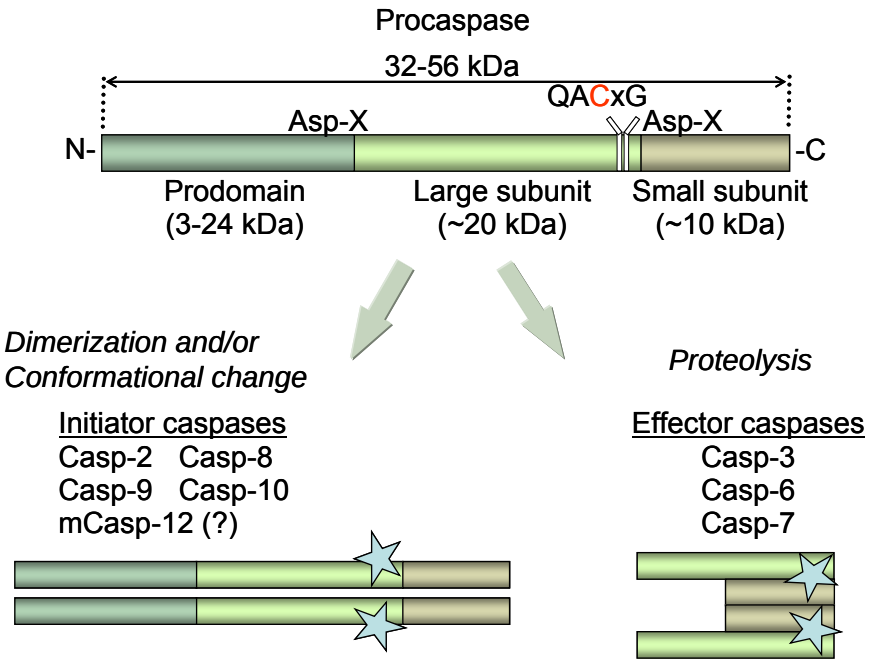


Figure 5: Caspase classification and activation. Caspases are cysteine proteases that cleave target substrates following an aspartate amino acid residue. Procaspases have three domains with an aspartate amino acid and a non-specific amino acid (X) present at the interface of the different subunits. The pentapeptide sequence, QACxG, within the large subunit, contains the active site where x represents any amino acid. Initiator caspases are activated through dimerization and/or a conformational change, whereas effector caspases are activated through cleavage by an active initiator caspase, which cleaves the effecotor caspases between their different domains. Activated effector caspases cleave over 1000 known substrates which results in cellular death. *Casp, caspase; mCasp-12, mouse caspase-12; Asp, Aspartate; Q, glutamine; A, alanine; C, cysteine; x, non-specific; G, glycine.*

describe the process of initiator caspase activation where it is possible that both perspectives are correct and not mutually exclusive. The induced proximity model suggests that the adaptor molecules required for initiator caspase activation function to bring these caspases into close proximity to one another which results in their homo-dimerization and subsequent activation. In this model the activating event is dimerization (Boatright and Salvesen, 2003). The issue surrounding this perspective is that although engineered homo-dimerized caspase-9 does exhibit increased activity compared to monomeric caspase-9, the activity is much less than the activity of caspase-9 when it is activated within the apoptosome complex (Chao et al., 2005). An alternative model is known as the induced conformation model and suggests that caspase-9 undergoes a conformational change upon binding to the apoptosome which results in its activation (Bao and Shi, 2007). It is worth mentioning that the experimental methods used to describe these models primarily focused on caspase-9, and it seems plausible that different initiator caspases utilize different mechanisms for their activation.

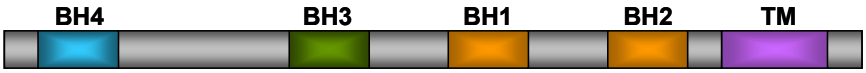
1.6.2 Bcl-2 family of proteins

The Bcl-2 family of proteins serve as key regulators of caspase activation. In particular, the Bcl-2 family of proteins contains both pro- and

Figure 6. Well-characterized Bcl-2-family proteins and how they regulate mitochondrial outer membrane permeabilization.

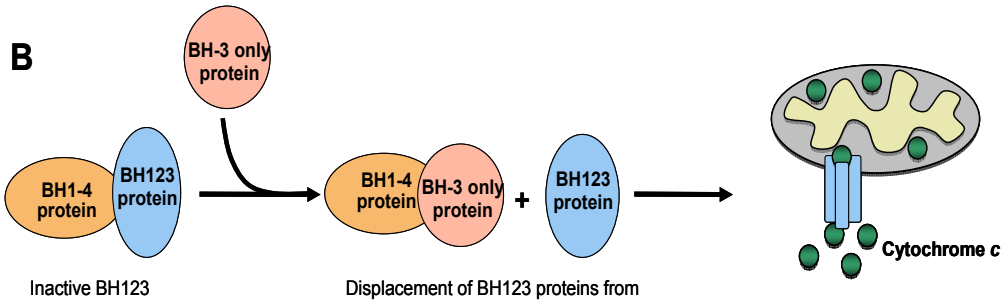
A, Well-characterized Bcl-2 faimly members include both pro- and anti-apoptotic family members. Anti-apoptotic family members contain four Bcl-2 homology (BH) domains, whereas pro-apoptotic members contain BH domains 1-3 or only the BH3 domain. *B*, Activation of a pro-apoptotic BH123 protein occurs by displacing it from an anti-apoptotic family member (BH1-4 protein). This displacement is mediated by a BH3-only family member. *C*, Activation of a pro-apoptotic BH123 family member is mediated by an "activator" BH3-only family member. The "activator" protein is maintained in an inactive state by an anti-apoptotic Bcl-2 family member (BH1-4 protein). An "inactivator" BH3-only family member displaces the "activator" BH3-only family member from the anti-apoptotic Bcl-2 family member (BH1-4 protein). This displacement allows the "activator" protein to activate the BH123 famly member.

A



- **Anti-apoptotic**
  - *BH1-4-domains*
    - Bcl-2
    - Bcl-x_L
    - Mcl-1
- **Pro-apoptotic**
  - *BH123-domain*
    - Bax
    - Bak
  - *BH3-domain only*
    - Bid, Bim, Puma — *"activator"*
    - Bad, Noxa — *"inactivator"*

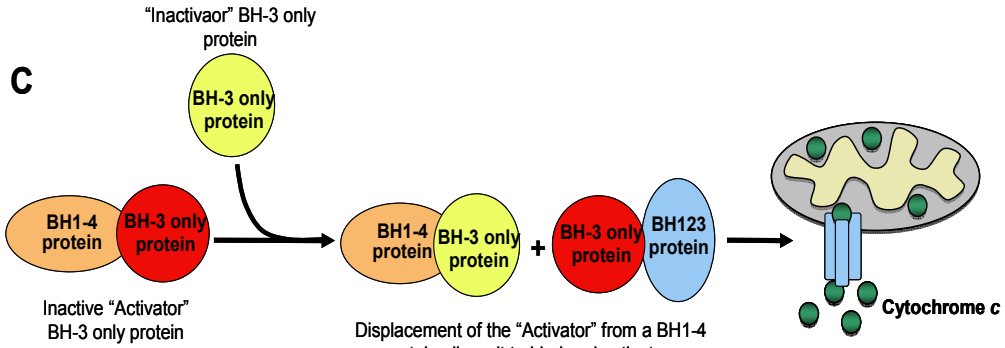
B



Inactive BH123 protein

Displacement of BH123 proteins from BH1-4 proteins results in activation of the BH123 protein.

C



Inactive "Activator" BH-3 only protein

Displacement of the "Activator" from a BH1-4 protein allows it to bind and activate a BH123 protein.

anti-apoptotic members that are thought to regulate apoptosis at the level of mitochondria by either promoting or inhibiting the release of cytochrome *c* and other intermembrane space proteins into the cytosol (Breckenridge and Xue, 2004). The release of cytochrome *c* into the cytosol is significant insofar as it activates Apaf-1, which, in turn, activates procaspase-9 to initiate a caspase cascade (Li et al., 1997).

In 1986, Bcl-2 was first identified as an oncogene in a B-cell lymphoma resistant cancer where this protein was overexpressed and contributed to drug resistance (Cleary et al., 1986). Following the identification of Bcl-2, other family members have been identified and include both pro- and anti-apoptotic proteins. The anti-apoptotic proteins contain four Bcl-2 homology domains (BH1-4), while the pro-apoptotic family members are further characterized as containing “BH123” domains (e.g. Bak and Bax) or “BH3-only” domains (e.g. Bim, Puma, and Bid) (Figure 6A). The anti-apoptotic family members exert their protective effects in a similar fashion; however, the pro-apoptotic family members have different pro-death mechanisms (Breckenridge and Xue, 2004).

Anti-apoptotic Bcl-2 family members are thought to exert their inhibitory effects primarily by localizing to the outer mitochondrial membrane (OMM), where they inhibit the release of cytochrome *c* by one of several proposed mechanisms. One model involves the direct binding of an anti-apoptotic Bcl-2 protein to a BH123 protein to prevent its

activation (Figure 6B), while a second model suggests they bind to BH3-only proteins and inhibit the ability of these proteins to activate a BH123 protein. The latter model supports a scheme in which specific BH3-only family members are classified as either inactivators (e.g. Bad and Noxa) or activator proteins (e.g. Bid, Bim, and Puma). In this scheme, Bcl-2/xL normally bind and sequester the activators. In response to an apoptotic stimulus, this inhibition is relieved by inactivator proteins, which displace the activators bound to Bcl-2/xL. This displacement then allows the activators to activate the BH123 family members to induce MOMP (mitochondrial outer membrane permeabilization) (Figure 6C) (Kim et al., 2006a).

In response to intracellular damage, the pro-apoptotic Bcl-2 family members are thought to "sense" the damage and relay the signal to activate the mitochondrial apoptotic pathway, resulting in extrusion of intermembrane space proteins into the cytosol. It is widely believed that the BH123 proteins homo-oligomerize forming pores that span the outer mitochondrial membrane in response to a pro-apoptotic signal, often involving the prior activation of a BH3-only protein. The BH123 proteins, Bak and Bax, double knockout (DKO) phenotype is highly resistant to most, if not all, cytotoxic stressors including DNA-damaging agents, endoplasmic reticulum (ER) stressors, and hormone withdrawal-induced cell death (Wei et al., 2001). Because they show this DKO phenotype but

have no observed phenotype when knocked out individually, it is accepted throughout the literature that these proteins are redundant in function with the primary function being pore formation spanning the OMM.

Pore formation is necessary to facilitate MOMP in that it allows the release of cytochrome *c* and other intermembrane space proteins into the cytosol. Cytochrome *c* will then interact with and activate Apaf-1 causing activation of the intrinsic caspase cascade, commencing with procaspase-9. The release of cytochrome *c* is considered to be the "point of no return" for the cell by some investigators because it is thought that all mitochondria release all of their cytochrome *c* within a very short period of time following MOMP (Goldstein et al., 2005). However, there is also evidence to suggest that this is not the case, but rather that the amount of cytochrome *c* initially released is only a "trickle" and increases because of a feed-forward mechanism. The first view favors a linear progression of apoptosis, while the second view favors a higher degree of complexity to the process. The two perspectives also differ in the dependence of caspase activation for cytochrome *c* release; the former is caspase-independent, while the latter appears to depend on caspase activation (Lakhani et al., 2006; Franklin and Robertson, 2007; Shawgo et al., 2008b).

1.6.3 Additional Modulators of Caspase Activation

Inhibitors of apoptosis (IAPs) make up an eight-membered family of proteins which include: c-IAP-1, c-IAP-2, XIAP, NAIP, Bruce, Survivin, ML-IAP, and ILP2 (Eckelman et al., 2006). The hallmark feature of all members within this family is that they contain at least one baculoviral IAP repeat (BIR) domain. In addition, some also contain a really interesting new gene (RING) domain which is thought to be an E3 ubiquitin ligase domain (Eckelman et al., 2006). Although they were initially discovered based on their ability to inhibit apoptosis in insect cells, it is now known that they play a functional role in a variety of cellular processes including cell cycle, cell signaling, and apoptosis(Clem et al., 1991; Schimmer, 2004; Srinivasula and Ashwell, 2008) .

Of the eight IAP proteins, X-linked inhibitor of apoptosis (XIAP) has been the best characterized related to its inhibitory effects on caspase activation. It is unique in that it is the only known IAP able to inhibit active caspases directly through binding via its BIR domains (Eckelman et al., 2006). XIAP binds caspase-9 through an interaction with the BIR3 domain, whereas caspases-3 and -7 are inhibited through an interaction with the BIR2 domain (Figure 7). Although it has been shown that other IAP family members are able to bind to caspases (e.g., c-IAP1 and c-IAP2), the inhibitory effects of this binding have yet to be determined;

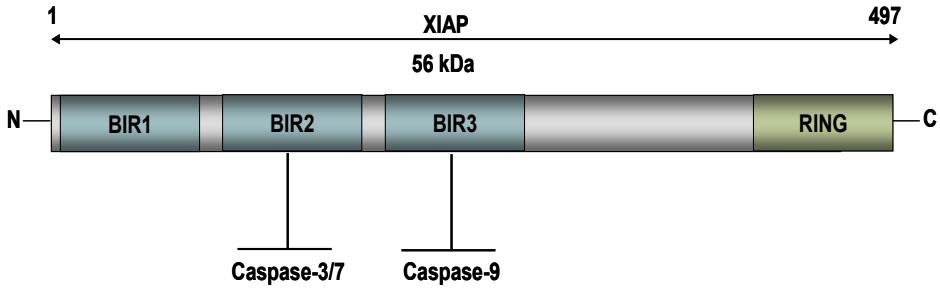


Figure 7: XIAP (X-linked inhibitor of apoptosis) protein and its inhibition on caspase-3,-7, and -9. XIAP has three baculoviral inhibitor of apoptosis repeat (BIR) domains. The BIR2 domain is required for inhibition of activated caspase-3 and caspase-7. The BIR3 domain is rerquired for binding and inhibition of caspase-9.

however, there is ongoing research focused on the possibility of caspase inhibition mediated by the RING domain resulting in ubiquitination and degradation (Choi et al., 2009).

Some resident proteins of the mitochondrial intermembrane space are released into the cytosol following MOMP where they function to facilitate caspase activation. As mentioned, the role of cytochrome *c* in the cytosol is required for apoptosome formation and activation of caspase-9. It is worth noting that the function of cytochrome *c* during apoptosis is unrelated to its role during oxidative phosphorylation, where it shuttles electrons between complex III and IV of the electron transport

chain. It has been shown experimentally *in vitro* and *in vivo* that it is possible to disrupt the pro-apoptotic function of cytochrome *c*, while maintaining its function during cellular respiration (Hao et al., 2005; Vempati et al., 2007) .

In addition to cytochrome *c*, there are two additional proteins that are released following MOMP that can facilitate caspase activation, reportedly by antagonizing XIAP. Although both of these proteins function as XIAP antagonists, they do so by distinct mechanisms. The first is second mitochondrial activator of caspases/direct IAP-binding protein with low pI (SMAC/DIABLO)(Du et al., 2000; Verhagen et al., 2000). The second is High temperature requirement A2 (Htr2 /Omi) (Vande Walle et al., 2008). Both proteins are synthesized in the nucleus with a mitochondrial localization signal on their amino terminus, which is removed upon successful translocation to the intermembrane space of the mitochondria. The removal of the localization signal exposes a tetrapeptide sequence that is required for binding to XIAP (Srinivasula and Ashwell, 2008). SMAC binds both BIR2 and BIR3 domains of XIAP, where it is thought to relieve the caspase inhibitory properties of XIAP (Shiozaki and Shi, 2004) . Omi/HtrA2 is a serine protease that not only binds to the BIR3 domain of XIAP but irreversibly degrades the protein through its proteolytic activity (Yang et al., 2003).

1.7 Overview of Apoptosis: The Intrinsic and Extrinsic Pathways

Apoptosis can be induced through either the extrinsic (death receptor-mediated) or intrinsic (mitochondria-mediated) pathway (Figure 8) (Green, 2000). Current cytotoxic therapeutic approaches seem to utilize primarily the intrinsic pathway by causing intracellular damage (Johnstone et al., 2002). The extrinsic pathway is activated extracellularly upon binding of a death ligand to its cognate receptor, for example, Fas ligand binding to the Fas receptor, whereas the intrinsic pathway is activated intracellularly in response to internal stress, such as DNA damage (Green, 2000). However, activation of the extrinsic pathway will, in turn, activate the intrinsic pathway in some cell types. These unique cell types are termed "type II cells" and include hepatocytes (Yin et al., 1999; Samraj et al., 2006).

As mentioned above, the extrinsic pathway is activated upon binding of a ligand to its cognate receptor. The activated receptor will then cluster and recruit a corresponding adaptor molecule. For example, the binding of Fas ligand to the Fas receptor results in trimerization of the receptor and recruitment of the adaptor protein FADD (Fas-Associated Death Domain). FADD, in turn, recruits initiator procaspase-8 molecules to the cytoplasmic side of the receptor (Rensing-Ehl et al., 1995). These protein-protein interactions are facilitated through complementary protein-

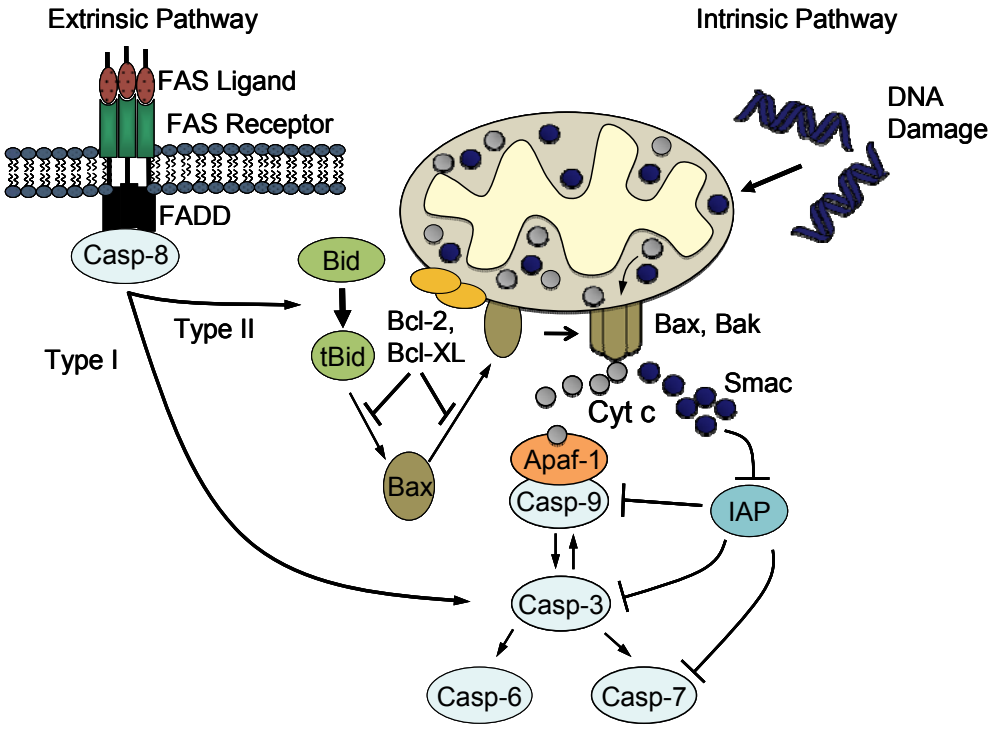


Figure 8: Overview of the extrinsic and intrinsic apoptosis pathways. The extrinsic pathway is activated upon ligand binding of a death receptor, such as Fas ligand binding to Fas. In this case, apoptosis occurs either by caspase-8-mediated direct activation of caspase-3 (type I cell) or by caspase-8-mediated cleavage of Bid to tBid, which then engages mitochondria (type II cell). The intrinsic pathway is activated by an internal stress leading to cytochrome *c* release, which then interacts with Apaf-1 and facilitates the activation of caspase-9. Active caspase-9 then activates caspase-3 resulting in the activation of other effector caspases and cell death. *Bid, BH3 interacting domain; tBid, truncated Bid; Bak, Bcl-2 antagonist/killer 1; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma x-long.*

interacting domains, such as the death domain (DD) shared by the Fas receptor and FADD, and the death effector domain (DED) shared by FADD and procaspase-8. The Fas-FADD-procaspase-8 protein complex is known as the death-inducing signaling complex (DISC), which promotes the activation of procaspase-8 by bringing two caspase-8 molecules into close proximity allowing their dimerization and/or conformational change, auto-activation, and subsequent proteolytic cleavage (Bao and Shi, 2007). In a type I cell, active initiator caspase-8 will directly cleave and activate effector procaspase-3, which will then cleave and activate other effector caspases, as well as target intracellular protein substrates resulting in dismantling of the cell.

In a type II cell, activated caspase-8 does not directly cleave and activate caspase-3. Instead, it is thought that the amount of activated caspase-8 in a type II cell is insufficient to cleave and activate enough caspase-3 to execute cell death (Schmitz et al., 1999). Active caspase-8 does, however, cleave the BH3-only Bcl-2 family member Bid to truncated Bid (tBid) (Li et al., 1998). The cleavage of Bid to tBid leads to the engagement of the mitochondria-mediated pathway, presumably through tBid-mediated activation of the BH123 proteins Bak and/or Bax, which then homo-oligomerize to induce MOMP (Kim et al., 2006a). The ensuing release of cytochrome *c* and other pro-apoptotic proteins leads to the activation of caspase-9 and caspase-3.

The type I and type II model of receptor-mediated cell death was originally described in 1998 when Jurkat T-lymphocytes were shown to be type II cells due to the fact that Bcl-2 or Bcl-X_L overexpression effectively inhibited Fas-induced apoptosis (Scaffidi et al., 1998). Controversy has surrounded the exact role that MOMP plays in mediating type II cell death. It has been reported that engagement of mitochondria is necessary for cytochrome *c* release and apoptosome formation; however, it has also been suggested that MOMP is necessary to neutralize XIAP-mediated inhibition of caspase-3 presumably through the release of SMAC (Sun et al., 2002; Wilkinson et al., 2004; Samraj et al., 2006). Our laboratory has recently shown that Jurkat T-lymphocytes lacking the ability to form an apoptosome and therefore unable to activate caspase-9, depend on MOMP upon activation of the receptor-mediated pathway; however, it is SMAC that is needed to facilitate caspase activation and cell death rather than activation of caspase-9. In other words, these cells are not dependent on activation of the intrinsic pathway per se, but rely on the presence of SMAC presumably to relieve XIAP-mediated inhibition of caspase-3 (Shawgo et al., 2009).

As mentioned above, the mitochondria-mediated pathway is activated in response to intracellular stress; however, the precise mechanism of how the damage is integrated to produce a death signal is unclear. Upon its release from the mitochondria, cytochrome *c* will bind to Apaf-1, which

serves as the adaptor protein for procaspase-9 (Li et al., 1997). The binding of cytochrome *c* to Apaf-1 causes Apaf-1 to undergo a conformational change that unmasks its caspase recruitment domain (CARD). The CARD of procaspase-9 will then bind the CARD of Apaf-1 causing the dimerization and/or conformational change, auto-activation, and subsequent cleavage of initiator procaspase-9. This protein complex, consisting of Apaf-1, caspase-9, cytochrome *c*, and dATP/ATP, is termed the apoptosome and serves as the initiating platform for procaspase-9 (Bao and Shi, 2007). Activated caspase-9 will then activate downstream effector procaspases through cleavage. As mentioned above, the effector caspases will then dismantle the cell through the cleavage of target protein substrates.

1.8 Overview of the "BH3-Domain" Only Protein Bid

Bid is a pro-apoptotic member of the Bcl-2 family of proteins containing only the BH3 domain. The BH3 domain is required for the pro-apoptotic effect(s) of this and other proapoptotic members of this protein family. Full-length Bid is a 22-kDa protein that contains ~195 amino acids. During apoptosis, Bid often undergoes proteolysis to tBid. Caspase-8-mediated cleavage of Bid occurs after aspartate residue 60 where it is the C-terminal cleavage fragment that promotes apoptosis (Figure 9). It has

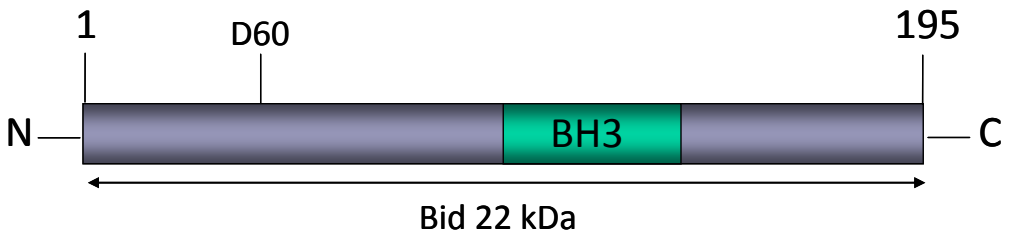


Figure 9: Full Length Bid (BH3-Interacting Domain Death Agonist) Protein. Apartate amino acid residue 60 (D60) represents the caspase-8 cleavage site. Upon cleavage, the C-terminal portion of the protein is the "active" form know as truncated Bid (tBid).

been suggested that tBid may insert into the OMM because it contains typical structural features associated with transmembrane proteins. One possibility is that the pro-apoptotic function of Bid is not limited to its activation of the BH123 proteins but that it also has pro-apoptotic function through protein-membrane lipid interactions. In this scenario, tBid interacts with cardiolipin at mitochondrial contact sites causing cristae reorganization, which also promotes cytochrome *c* release (Yin, 2006).

Bid is a unique BH3-only protein in that it requires post-translational modification for activation, most notably involving caspase-8-mediated cleavage to tBid (Wang et al., 1996; Li et al., 1998; Luo et al., 1998). Bid normally resides in the cytosol and possibly the nucleus (Zinkel et al., 2005). Upon being cleaved, tBid is myristolyated at its N-terminus, translocates to the OMM, and/or activates a BH123 protein. The best characterized setting for tBid involvement is in the engagement of the

mitochondrial apoptotic pathway in certain cell types, such as hepatocytes, upon activation of the extrinsic pathway (Yin et al., 1999). The role of Bid/tBid in response to other stressors that activate the intrinsic pathway remains poorly understood and controversial. More specifically, there has been controversy surrounding the role of Bid/tBid in response to DNA damage- and heat-induced apoptosis.

1.9 Concluding Remarks

The majority of chemotherapeutic agents lack the specificity to directly target neoplastic cells, but often target cells actively participating in the cell cycle or those undergoing cell division. Cytotoxic chemotherapeutics more often than not are thought to induce cell death through the initiation of mitochondria-mediated apoptosis. Over the past decade, a better understanding of the molecular pathway(s) and the key players leading to the activation and execution of apoptosis has evolved. However, the precise details of how these pathways are regulated in response to anti-cancer therapies remain only partially understood. New information regarding the details of these pathways would be expected to contribute to the development of more specific drug design and therapeutic approaches.

Chapter 2: Statement of Purpose

2.1 Significance

Given the modest curative rates for cancer and given the considerable toxic adverse effects of many chemotherapeutic agents, a more detailed understanding of the mechanism of action of many anticancer therapeutics is warranted. In general, cytotoxic anticancer drugs kill cells by causing the cell to undergo mitochondria-mediated or intrinsic apoptosis (Lowe and Lin, 2000). Furthermore, defects in the apoptotic machinery, which contribute to tumorigenesis and therapeutic resistance, are considered to be one of the six hallmarks of cancer (Hanahan and Weinberg, 2000). Although cytotoxic anticancer drugs are known to engage the intrinsic pathway of apoptosis, the precise mechanistic details remain elusive and, in some instances, controversial. It is also the case that the precise molecular requirements necessary for the induction of apoptosis in response to a given cytotoxic agent may be, at least in part, unique to that stressor. For my dissertation research, I have investigated the underlying mechanisms responsible for mitochondria-mediated apoptosis induced by a DNA topoisomerase II inhibitor, a novel heat shock protein 90 (Hsp90) inhibitor, and elevated temperature. The results of my studies are expected to contribute new

information toward an understanding of mitochondria-mediated apoptosis induced by cytotoxic stress.

Topoisomerase II inhibitors (e.g. etoposide) are DNA-damaging anti-cancer drugs that are often administered to fight a plethora of human malignancies. Although these drugs are known to induce mitochondria-mediated apoptosis, the exact details of how this process occurs are only partially understood. Over the last several years, there has been an interest to develop new drugs that disrupt the function of Hsp90 by targeting the N-terminal ATP-binding site of the protein. Two such agents, 17-allylamino-demethoxygeldanamycin (17-AAG) and17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), have entered phase II clinical trials. However, the potential clinical utility of these drugs as anticancer agents has been dampened significantly due to concerns about their adverse effects and tendency to induce new expression of cytoprotective Hsp90 and Hsp70 proteins (Whitesell et al., 2003; Schmitt et al., 2007; Workman and Powers, 2007). Finally, there is therapeutic interest in using methods to deliver heat to tumor masses to induce cell death. Although this option is becoming more of a realistic approach for cancer therapy, the mechanisms responsible for heat-induced apoptosis remain virtually unknown; furthermore, controversy surrounds the limited amount of mechanistic details that have been proposed for this form of cell death (Milleron and Bratton, 2006).

2.2 Overall Hypothesis

The primary objective of my dissertation is to determine how cytotoxic stress leads to the initiation and execution of cell death pathways, more specifically, the mitochondria-mediated apoptotic cell death pathway. The primary focus is to better understand the underlying molecular mechanistic details of apoptosis induced by DNA damage, inhibition of Hsp90, and heat shock. The *central hypothesis* is that the molecular requirements necessary for irreversible engagement of the mitochondria-mediated apoptotic pathway vary depending upon the nature of the stimulus. This central hypothesis will be tested by pursuing three Specific Aims. Following the completion of these studies, an enhanced understanding of the molecular mechanisms linking stress-induced apoptosis to the activation, regulation, and execution of the mitochondria-mediated pathway in cancer cells is expected.

2.3 Specific Aims

2.3.1 Specific Aim #1: Investigate the initiation and execution of mitochondria-mediated apoptosis in response to DNA damage induced by topoisomerase II inhibition.

- Specific Aim 1a. Determine the molecular requirements necessary for mitochondrial outer membrane permeabilization (MOMP) in response to the DNA-damaging agent etoposide.
- Specific Aim 1b. Investigate the role of the BH3-only Bcl-2 family member, Bid, in response to the DNA-damaging agent etoposide.

2.3.2 Specific Aim #2: To investigate the initiation and execution of mitochondria-mediated apoptosis in response to a novel novobiocin-derived C-terminal Hsp90 inhibitor, designated KU135.

- Specific Aim 2a. Compare the antiproliferative effects of KU135 to the N-terminal Hsp90 inhibitor, 17-AAG.
- Specific Aim 2b. Determine if KU135 can directly bind to Hsp90, more specifically, the C-terminus portion of Hsp90.
- Specific Aim 2c. Evaluate and characterize the ability of KU135 to induce mitochondria-mediated apoptosis.

2.3.3 Specific Aim #3: Investigate the initiation and execution of apoptosis in response to heat stress at 44°C.

- Specific Aim 3a. Identify and characterize the initiator caspase responsible for the onset of heat-induced apoptosis.
- Specific Aim 3b. Evaluate the role of MOMP in heat-induced apoptosis.

- Specific Aim 3c. Investigate the role of the BH-3 only Bcl-2 family member, Bid, during heat shock-induced apoptosis.

Chapter 3: Materials and Methods

3.1 Cell Culture

Wild-type (clones E6.1 and A3), caspase-8-deficient (clone I 9.2), and FADD-deficient (clone I 2.1) Jurkat T-lymphocytes (ATCC, Manasses, VA) were cultured in RPMI 1640 complete medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), 2% (w/v) glutamine (Invitrogen), 100 U/mL penicillin (Invitrogen) and 100 µg/mL streptomycin (Invitrogen) at 37°C in a humidified 5% CO_2 incubator. Cells were maintained at their optimal growth density of 10^5 - 10^6 cells/mL for a maximum time period of 3 months. For transfected Jurkat cells (pSFFV-Bcl-2, pSFFV-Bcl-xL, pSFFV-neo, pSUPER-Apaf-1, pSUPER-Bid, pSUPER-Caspase-2, pSUPER-RAIDD, pSUPER-neo, pcDNA3-XIAP, pcDNA3-XIAP-BIR1/BIR2, or pcDNA3-neo), 1 mg/mL Geneticin (Invitrogen) was substituted for penicillin and streptomycin. All cells were re-plated in fresh complete nonselective medium prior to all experiments. Cells (5×10^6) were retained in liquid nitrogen for long-term storage in 1 mL of cryostorage medium (5% DMSO and RPMI 1640 with appropriate antibiotics). Prior to submersion in liquid nitrogen, cells were placed at

-80°C in a Mr. Frosty (4 h – 1 week). Cryopreserved cells were thawed at 37°C and diluted with 10 mL of appropriate growth medium. Cells were then incubated at room temperature (22°C) for 5 min before centrifugation at 300 x g for 5 min. Cell pellets were resuspended in 10 mL of appropriate medium and allowed to recover for 1 week before experiments were performed. Cells were treated with etoposide (2.4 nM – 47 μM) (Sigma), anti-Fas antibody (100 ng/mL) (clone CH-11, MBL International, Woburn, MA), KU135 (2.5 nM – 50 μM), 17-AAG (0.5 nM – 10 μM) (InvivoGen, San Diego, CA), DMSO (≤ 0.5% final concentration), or heat (44°C for 1 h). The caspase inhibitor quinoline-Val-Asp-CH_2-difluorophenoxy (qVD-OPh) (MP Biomedicals, Solon, OH) was used at a final concentration of 20 μM.

3.2 Measurement of Caspase Activity

Cells (5 x 10^5) were pelleted and washed once with ice-cold phosphate-buffered saline (PBS). Cells were resuspended in 25 μL of PBS, added to a microtiter plate, and combined with DEVD-aminomethylcoumarin (AMC) (Peptide Institute, Osaka, Japan) dissolved in a standard reaction buffer (100 mM Hepes, pH 7.25, 10% sucrose, 10 mM dithiothreitol, 0.1% CHAPS). Cleavage of DEVD-AMC was monitored by AMC production in a FLx800 Multi-detection Microplate

Reader (BioTek Instruments, Winooski, Vermont) using 355 nm excitation and 460 nm emission wavelengths.

3.3 RNAi (RNA interference)

Initially, transient transfections were performed to validate the ability of an individual siRNA (short interfering RNA) oligonucleotide to knockdown the target gene of interest. The target siRNA molecules were 21 nucleotides in length. For transient transfections, 1.5 x 10^6 cells were pelleted and resuspended in 75 µL of electroporation buffer (Ambion, Austin, TX). 1.5 µg of the target siRNA was added to cells. Next, the solution mixture was added to a 0.1-cm cuvette and electroporated using a Bio-Rad Gene Pulser Xcell system at 200V. The cells were then incubated for 48 h in complete growth medium at 37°C followed by processing for Western blot analysis. The appropriate primary antibody was then used to determine the protein expression level of the gene of interest.

The vector-based pSUPER (pSuper.neo) RNAi system (Oligoengine, Seattle, WA) was used to stably suppress the protein expression of the gene of interest by generating stable gene knockdown cell lines. The gene-specific targeting insert specifies a 19-nucleotide sequence downstream of the transcription start site followed by a 9

nucleotide non-complementary spacer sequence, followed by the reverse complement of the same 19-nucleotide sequence that is at the beginning of the target. The sequence was ligated into *BglII* and *XhoI* sites of the pSUPER vector, which was then transformed into TOP10 competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA) using the heat-pulse method. Several clones were screened for the correct insert size by restriction enzyme digest and agarose gel eletrophoresis to separate the DNA fragments followed by sequence analysis to verify the sequence.

3.4 Transfections

Jurkat T-lymphocytes (10^7 cells) were washed with PBS and resuspended in 800 µL of RPMI. Cells were then transfected with 20 µg of plasmid DNA (pSFFV-Bcl-2, pSFFV-Bcl-xL, pSFFV-neo, pSUPER-Apaf-1, pSUPER-Bid, pSUPER-Caspase-2, pSUPER-RAIDD, pSUPER-neo, pcDNA3-XIAP, pcDNA3-XIAP-BIR1/BIR2, or pcDNA3-neo) by electroporation using a Bio-Rad Gene Pulser Xcell system (0.4-cm cuvette, 300V, and 950 µF). Cells were then allowed to recover in complete medium for 48 h at 37°C in a humidified CO_2 incubator. Selection of transfected cells was done in the presence of 1 mg/mL Geneticin for one month followed by serial dilutions to obtain single-cell

clones with silenced or overexpressed genes. The extent of protein overexpression or knockdown was determined by Western blot analysis.

3.5 Flow Cytometry Measurements for Cell Death, Mitochondrial Membrane Potential (ΔΨ), Bak Activation, and Cell Cycle Analysis

Phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane was detected by staining with Annexin V-FITC (BD PharMingen), whereas disruption of the plasma membrane was detected by staining DNA with the non-cell permeable dye propidium iodide (PI). In brief, 10^6 cells were pelleted by centrifugation at 300 x g for 5 min following treatment. Cells were then washed with PBS and centrifuged at 300 x g for 5 min. Pellets were resuspended in 500 µL of binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 1.5 µg annexin V-FITC and 5 µL PI. Flow cytometric analysis was then performed using the BD LSRII and BD FACS Diva v6.1.2 software.

Dissipation of the ΔΨ was detected using the stain $DiIC_1(5)$ (Invitrogen), which is a cell-permeable dye that accumulates in mitochondria with active membrane potentials. The intensity of the fluorescence decreases as ΔΨ diminishes. Briefly, cells (10^6) were pelleted by centrifugation at 300 x g for 5 min at the end of the experiment. Cells were then washed once in PBS, and resuspended in 1 mL of warm

PBS. Next, 5 µL of $DiIC_1(5)$ (10 µM) was added to the cells and incubated in a humidified 5% CO2 incubator at 37°C for 15 min. Cells were then pelleted by centrifugation at 300 x g for 5 min and resuspended in 500 µL of cold PBS. Flow cytometric analysis was then performed using the BD LSRII and BD FACS Diva v6.1.2 software.

For detection of activated Bak by flow cytometry, cells (2.5×10^6) were washed in PBS, fixed in 400 µL of 0.25% paraformaldehyde in PBS for 5 min, then washed with 1% fetal bovine serum in PBS. Following centrifugation, the cells were resuspended in 50 µL of staining buffer (1% fetal bovine serum and 100 µg/mL digitonin in PBS) containing a conformation-specific mouse monoclonal antibody against the active conformation of Bak (1:30; AM03, Calbiochem) for 30 min at room temperature (22°C). Cells were then washed and resuspended in 50 µL of staining buffer containing 0.25 µg of Alexa Fluor 488-labeled chicken anti-mouse antibody for 30 min in the dark. Cells were washed and resuspended in PBS. Flow cytometric analysis was then performed using the BD LSRII and histogram overlays were performed using FlowJo software (Tree Star, Ashland, OR).

For cell cycle analysis, 10^6 cells were pelleted following treatment and washed with cold PBS. Cells were fixed in 1 mL of ice-cold 70% ethanol and incubated at room temperature (22°C) for 30 min. Next, the fixed cells were pelleted, resupended in 500 µL of PI/RNase Staining

Buffer (BD PharMingen), incubated at room temperature (22°C), and analyzed by flow cytometry using the BD LSRII and BD FACS Diva v6.1.2 software.

3.6 Western Blot Analysis

Cells (5×10^6) were centrifuged at 300 x g for 5 min. Pelleted cells were resuspended and lysed in 200 µL of ice-cold lysis buffer (10 mM 50 Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% Nonidet P-40) supplemented with a mixture of protease inhibitors (Complete Mini EDTA-Free, Roche Applied Science). Protein concentrations were determined using the bicinchoninic acid assay (Pierce). BSA standards (0 µg, 1 µg, 2 µg, 4 µg, 8 µg, 16 µg, 32 µg, 64 µg) were used to determine sample protein concentrations. The protein was then mixed with Laemmli's buffer at a final concentration of 1X (50mM Tris HCl pH 6.8, 2% SDS, 6% glycerol, 1% β-mercaptoethanol, 0.004 % bromophenol blue) before being incubated for 5 min at 100°C. Protein (30 – 40 µg) was loaded on a polyacrylamide gel (8-15%). Polyacrylamide gels were allowed to run for ~ 45 min at 195 V in a Mini-PROTEAN® 3 Cell (BioRad, Hercules, CA) in electrode buffer (25 mM Tris, 192 mM glycine, 1% SDS). The proteins were then transferred to a nitrocellulose membrane in a BioRad transfer apparatus for 1 h at 100 V using the

Trans-Blot® Electrophoretic Transfer Cell (BioRad) with transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). Nitrocellulose membranes were incubated in 5% non-fat dry milk in PBS for 1 h at 22°C with gentle shaking. Nitrocellulose membranes were then washed in PBS before incubation with primary antibody overnight. The primary antibodies were diluted in 1% BSA in PBS. Nitrocellulose membranes were incubated with primary antibody overnight at 4°C with gentle shaking. Antibodies and dilutions used were mouse 1:1,000 rabbit anti-Akt (pan) (clone C67E7, Cell Signaling Technology, Danvers, MA), 1:500 anti-Apaf-1 (clone 94408, R&D Systems, Minneapolis, MN), 1:1,000 rabbit anti-Bax (Cell Signaling Technology, Danvers, MA), 1:1,000 rabbit anti-Bak NT (Millipore), 1:500 mouse anti-Bcl-2 (DakoCytomation, Glostrup, Denmark), 1:1,000 rabbit anti-Bcl-xL (clone 54H6, Cell Signaling), 1:5,000 mouse anti-β-actin (clone AC-15, Sigma), 1:1,000 rabbit anti-Bid (Cell Signaling), 1: 1,000 mouse anti-caspase-2 (clone 35, BD Pharmingen, San Jose, CA), 1:1,000 rabbit anti-caspase-3 (clone 8G10, Cell Signaling), 1:1,000 rabbit anti-caspase-6 (Cell Signaling), 1:1,000 rabbit anti-caspase-7 (Cell Signaling), 1:1,000 mouse anti-caspase-8 (clone 1C12, Cell Signaling), 1:1,000 rabbit anti-caspase-9 (Cell Signaling), 1:1,000 mouse anti-cdc2 p34 (sc-54, Santa Cruz Biotechnology),1:1,000 mouse anti-cytochrome *c* (clone 7H8.2C12, Pharmingen), 1:5,000 rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (Trevigen, Gaithersburg, MD), 1: 2,000 rat anti-

GRP94 (clone 9G10, Assay Designs, Ann Arbor, MI), 1:1,000 rabbit anti-Hif-1α (Novus Biologicals, Littleton, CO), 1:1,000 mouse anti-Hsp70 (Hsp72) (clone C92F3A-5, Assay Designs), 1:250 rat anti-Hsp90α (clone 9D2, Assay Designs), 1:2,000 mouse anti-Hsp90β (clone K3705, Assay Designs), 1:500 mouse anti-Myc (clone 9E10, Santa Cruz Biotechnology), 1:1,000 rabbit anti-phospho-Akt (Ser473) (clone 193H12, Cell Signaling), 1:1,000 mouse anti-Smac/DIABLO (Cell Signaling), 1:2,000 mouse anti-TRAP (Affinity BioReagents, Rockford, IL), and 1:1,000 mouse anti-XIAP (clone 28, BD Transduction Laboratories). Nitrocellulose membranes were washed with 1X PBS for 10 min, 1X PBST for 10 min, and 1X PBS for 10 min at room temperature (22°C) with gentle shaking to remove excess primary antibody. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature (22°C) with gentle shaking. Secondary antibodies were diluted at 1:10,000 in 2.5% non-fat dry milk in PBS. Secondary antibodies used include goat anti-mouse IgG, (Thermo Scientific, Pierce Biotechnology), goat anti-rabbit IgG (Thermo Scientific, Pierce Biotechnology), and goat anti-rat (Pierce Biotechnology). The nitrocellulose membranes were then washed with 1X PBS for 10 min, 1X PBST for 10 min, and 1X PBS for 10 min at room temperature (22°C) with gentle shaking. The membrane was then blotted to remove excess buffer and incubated with 2 mL each of ECL Detection Reagent 1 and ECL

Detection Reagent 2 (Amersham, Buckinghamshire, UK) for 1 min before developing using classic autoradiography film BX (MidSci, St. Louis, Missouri).

3.7 Subcellular Fractionation

Subcellular fractionation was used to detect the release of cytochrome *c* from the intermembrane space of the mitochondria into the cytosol. Cells (2.5×10^6) were centrifuged at 300 x g for 5 min at room temperature (22°C) and washed in cold PBS. Cells were then centrifuged at 300 x g for 5 min at room temperature (22°C). Cells were resuspended in 125 µL of buffer (140 mM mannitol, 46 mM sucrose, 50 mM KCl, 1 mM KH_2PO4, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM Tris, pH 7.4) supplemented with a mixture of protease inhibitors (Complete Mini EDTA-Free), and permeabilized with 7.5 µg of digitonin (Sigma) on ice for 10 min with occasional vortexing. Plasma membrane permeabilization was monitored by trypan blue staining. Cell suspensions were centrifuged at 12,000 x g for 10 min at 4°C to separate the cytosol (supernatant) from the mitochondria (pellet). Supernatant and pellet fractions were subjected to Western blot analysis.

3.8 Determination of Bak Oligomerization

Bak oligomerization can be visualized by Western Blot analysis after

incubation with a cross-linking agent (Wei et al., 2000). For detection of Bak oligomerization, cells (2.5 x 10^6) were harvested after treatment by centrifugation at 300 x g for 5 min at room temperature (22°C). Cells were washed in cold PBS and centrifuged for 5 min at 300 x g at room temperature (22°C). Cells were then resuspended in 200 µL of 100 mM EDTA/PBS and incubated with the cross-linking agent bismaleimidohexane (BMH) at a final concentration of 1 mM. After the addition of BMH, tubes were mixed by inverting 2-3X followed by a 30 min incubation at room temperature (22°C) while rocking. The reaction was quenched by the addition of 100 mM DTT followed by a 15 min incubation at room temperature (22°C) while rocking. The cell suspension was then centrifuged at 500 x g for 5 min and processed for Western blot analysis.

3.9 Digitonin-Permeabilized Cells

Jurkat cells (2.5 x 10^6) were washed in PBS, resuspended in 125 µL of buffer (140 mM mannitol, 46 mM sucrose, 50 mM KCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM succinate, 1 mM EGTA, 5 mM Tris, pH 7.4) supplemented with a mixture of protease inhibitors (Complete Mini EDTA-Free). Recombinant purified protein, truncated Bid or full-length Bid (R & D systems, Minneapolis, MN), was added at a final concentration ranging from 5 nM to 250 nM. The cells were then permeabilized with 7.5 µg of

digitonin (Sigma) and incubated at room temperature (22ºC) for 15 min followed by centrifugation at 12,000 x *g* for 10 min at 4°C. Supernatant and pellet fractions were subjected to Western blot analysis.

3.10 Cell proliferation assay

Cellular metabolic activity/viability was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. This approach uses the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) that is bioreduced by metabolically active/viable cells into a colored formazan product that is soluble in tissue culture medium. In brief, 3×10^4 cells/well were cultured in 96-well plates in 100 µL of complete growth medium for 24 or 48 h. At the end of incubation, 20 µL of MTS solution was added to each well and incubated for 1-4 h at 37°C. The amount of soluble formazan from cellular reduction of MTS was assessed by measurement of absorbance at 490 nm VICTOR^{3}V Multilabel Reader (PerkinElmer). Data were analyzed using non-linear regression and sigmoidal dose-response curves (GraphPad Prism) from which IC_{50} values were calculated.

*These experiments were done by Shawna B. Matthews

3.11 Novobiocin affinity column chromatography

Novobiocin-sepharose was prepared as described previously (Marcu et al., 2000b). In brief, three grams of epoxy-activated sepharose 6B (Sigma) were washed and swollen in 100 mL of distilled water for 1 h at room temperature (25°C). The resin was washed further with coupling buffer (300 mM sodium carbonate, pH 9.5). The gel was mixed with 400 mg of novobiocin (sodium salt, Sigma) in 10 mL of coupling buffer and incubated at 37°C with gentle rotation for 20 h. Excess ligand was washed away with coupling buffer, and the remaining epoxy-active groups were blocked with 1 M ethanolamine in coupling buffer for 12 h at 30°C with gentle shaking. The gel was washed sequentially with coupling buffer, 500 mM NaCl in coupling buffer, distilled water, 500 mM NaCl in 100 mM sodium acetate (pH 4.0), and again in distilled water. The resin was subsequently equilibrated in 25 mM Hepes (pH 8.0) containing 1 mM EDTA, 10% ethylene glycol, and 200 mM KCl and stored in the dark at 4°C.

Rabbit reticulocyte lysate was incubated in the presence of an ATP-regenerating system (10 mM creatine phosphate and 20 units/mL creatine phosphokinase) for 5 min at 37°C, after which, the reticulocyte lysate was applied to the novobiocin column that had been pre-equilibrated with 10

mM Tris-HCl (pH 7.4) buffer. The novobiocin column was then washed with 20 volumes of 10 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl. Subsequently, bound proteins were eluted with increasing concentrations of KU135 or 10 mM novobiocin. All experiments were carried out under dark conditions at 4°C. The eluents were separated via SDS-PAGE and analyzed with Hsp90 antibodies.

*These experiments were done in the laboratory of Brian Blagg.

3.12 Proteolytic fingerprinting of Hsp90.

Proteolytic fingerprinting was performed using a modified method described previously (Yun et al., 2004). In brief, 50% (v/v) rabbit reticulocyte lysate, an ATP-regenerating system (10 mM creatine phosphate and 20 units/mL creatine phosphokinase), and 75 mM KCl were incubated in the presence of drug or vehicle for 5 min at 37 °C. Following the incubation period, the reaction mixtures were chilled on ice and digested with 125 µg/mL TPCK-treated trypsin (Worthington Biomedical, Lakewood, NJ) for 6 min (Hartson et al., 1999). Reactions were terminated by immediate boiling in Laemmli loading buffer. Subsequently, the samples were separated by SDS-PAGE and Western blotted using antibodies specific to the N-terminus of Hsp90 (PA3-013,

Affinity BioReagents) or the C-terminus of Hsp90 (clone AC88, Assay Designs).

*These experiments were done in the laboratory of Brian Blagg.

3.13 Surface plasmon resonance (SPR) analysis of KU135 binding to Hsp90

Insect Sf9 cells overexpressing Hsp90β were cultured and harvested by the Baculovirus/Monoclonal Antibody Core Facility at Baylor College of Medicine. Hsp90β was extracted and purified (>98% pure) as described previously (Grenert et al., 1997; Owen et al., 2002) but without the initial DEAE-cellulose chromatography step. The surface of a SSOO COOH1 SPR sensor chip mounted in a SensiQ SPR instrument (ICX Nomadics) was activated by treatment with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide for preferential cross-linking of the protein's N-terminus to the surface. For Hsp90 immobilization, 250 µL of Hsp90 (7 mg/ml) in 20 mM sodium bicarbonate buffer (pH 8) containing 150 mM NaCl was injected at a flow rate of 10 µL/min, giving 2800 response units of protein stably immobilized to the surface of the flow cell, representing ~0.08 picomoles of Hsp90. Unreactive groups were then quenched with 1 M ethanolamine (pH 8), and the surface washed with buffer containing 10 mM Pipes (pH 7.4), 300

mM NaCl, 2% DMSO and 0.01% Igepal CA-630 followed by equilibration in assay buffer. KU135 or novobiocin was diluted in assay buffer containing 10 mM Pipes, pH 7.4, 300 mM NaCl, 2% DMSO and 0.00075% Igepal CA-630 and injected over the surface of the derivatized chip at a flow rate of 25 µL/min at 25 °C at the indicated concentrations. All measurements were done in triplicate. SPR binding curves were analyzed using QDAT software (ICX Nomadics). To calculate the K_d, binding constants were fitted using the equations: $R_{eq} = K_A (A) R_{max} / (K_A(A) +1)$ and re-graphed using Origin software for Scatchard analysis and to demonstrate saturation of binding. Novobiocin's binding isotherm was globally fit for total and nonspecific binding using the equation $Y= (B_{max}(X))/(K_D +X)) +NS(X)$ to fit total binding and the equation $Y=NX(X)$ to fit nonspecific binding.

*These experiments were done in the laboratory of Robert L. Matts.

3.14 b-VAD-fmk affinity labeling of initiator caspases

Cells (2.5×10^6)/mL were incubated with 120 µM of the biotinylated pan-caspase inhbitor biotin-Val-Ala-Asp(OMe)-CH_2F (b-VAD-fmk) (Kamiya Biomedical Company, Seattle, Washington) for 3 hours. The cells were then incubated at 44°C for 1 h followed by a 6 h recovery period at 37°C . Following the six-hour recovery time period post-heat shock, cells were washed with PBS and lysed with 500 µL of lysis buffer (50mM Tris/HCl pH

7.4, 150 mM NaCl, 1% NP-40, 1 mM DTT) supplemented with a mixture of protease inhibitors (Complete Mini EDTA-Free, Roche Applied Science). The lysate was then incubated on ice for 30 min, followed by centrifugation at 12,000 x *g* for 10 min at 4°C. The supernatants were then incubated with streptavidin-sepharose beads (GE Healthcare, Piscataway, NJ) that had been pre-equilibrated with lysis buffer followed by gentle rocking overnight. The beads were then washed four times with wash buffer (50mM Tris/HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM and subsequently incubated with 2X Laemmli buffer and boiled followed by Western blot analysis.

Chapter 4: Cleavage of Bid by Executioner Caspases Mediates Feed Forward Amplification of Mitochondrial Outer Membrane Permeabilization During Genotoxic Stress-Induced Apoptosis in Jurkat Cells

4.1 Abstact

The extent to which the BH3-only protein Bid is important for intrinsic (mitochondria-mediated) apoptotic cell death induced by genotoxic stress remains controversial. In the present study, we examine this issue using a panel of gene-manipulated Bax-deficient Jurkat T-lymphocytes. Cells stably depleted of Bid were far less sensitive than control-transfected cells to etoposide-induced apoptosis. In particular, drug-induced Bak activation, cytochrome *c* release, loss of mitochondrial membrane potential, and caspase activation were all decreased in cells lacking Bid. Reconstitution experiments using recombinant proteins and permeabilized Bid-deficient cells demonstrated that truncated Bid (tBid), but not full-length Bid, potently induced Bak activation and the release of cytochrome *c*. Further, caspase-8-deficient Jurkat cells efficiently cleaved Bid and were sensitive to drug-induced apoptosis. By comparison, Apaf-1-deficient cells, as well as cells overexpressing full-length XIAP or the

BIR1/BIR2 domains of XIAP, failed to cleave Bid in response to genotoxic stress. These data suggest that tBid plays an important regulatory role in the execution of DNA damage-induced cytochrome *c* release and apoptosis. However, the fact that cleavage of Bid to tBid is mediated by executioner caspases suggests that a self-amplifying feed forward loop involving caspases, Bid, and mitochondria may help determine irreversible commitment to apoptosis.

4.2 Introduction

Apoptosis is an active form of cell death that plays an essential role during normal embryonic development and in the maintenance of tissue homeostasis in the adult organism (Danial and Korsmeyer, 2004). Consequently, dysregulation of apoptosis has been implicated as a contributing factor to the onset of different pathological conditions, including cancer. In addition, it is now generally accepted that many genotoxic anticancer drugs are effective against tumor cells for their ability to induce mitochondria-mediated apoptosis (Lowe and Lin, 2000). Similarly, mutations or the altered expression of pro- and anti-apoptotic proteins can contribute to the development of drug resistance.

Execution of apoptosis is mediated by a family of cysteine-dependent aspartate-specific proteases (caspases). During true

mitochondria-mediated apoptosis, members of the Bcl-2 family of proteins are the primary regulators of caspase activation for their role in controlling mitochondrial outer membrane permeabilization (MOMP) (Adams and Cory, 1998). The process of MOMP results in the release of cytochrome *c*, second mitochondria-derived activator of caspase (Smac, also known as DIABLO), and Omi (also known as HtrA2) into the cytosol where they converge to promote the activation of caspase-9 within the apoptotic protease-activating factor-1 (Apaf-1) apoptosome complex. The Bcl-2 family contains proteins with opposing functions, and it is generally thought that the induction of MOMP requires the activation of either Bak or Bax triggered by a Bcl-2 homology 3 (BH3)-only protein (Chipuk et al., 2006; Ow et al., 2008; Youle and Strasser, 2008). Indeed, evidence in the literature indicates that cells lacking either Bak or Bax exhibit only subtle defects in MOMP, whereas doubly-deficient cells are often found to be highly resistant to mitochondria-mediated apoptosis (Lindsten et al., 2000; Wei et al., 2001).

At present, there are two models for the activation of Bax or Bak by BH3-only proteins. One model argues that BH3-only proteins function as either "sensitizer" (*e.g.* Bad and Noxa) or "activator" proteins (*e.g.* truncated Bid (tBid), Bim, and perhaps Puma) (Kim et al., 2006b). In this scenario, a sensitizer protein is needed to displace an activator protein from a prosurvival protein (*e.g.* Bcl-2, Bcl-x_L, or Mcl-1) in order to activate

Bak or Bax. The second model argues that BH3-only proteins bind and inhibit the function of prosurvival Bcl-2 proteins, which normally bind to and inhibit Bak and Bax (Chen et al., 2005; Willis et al., 2007). Of the seven or so known BH3-only proteins (Youle and Strasser, 2008), Bid is unique in that it requires post-translational modification for activation, most notably involving caspase-8-mediated cleavage to tBid (Wang et al., 1996; Li et al., 1998; Luo et al., 1998). Bid normally resides in the cytosol and possibly the nucleus (Zinkel et al., 2005). Upon being cleaved, the C-terminal fragment (tBid) is myristoylated at its newly exposed N-terminus, translocates to the outer mitochondrial membrane (OMM), and/or activates Bak or Bax protein (Zha et al., 2000). Recently, it was shown that the N-terminal cleavage fragment of Bid is quickly ubiquitinated for degradation and that this degradation is necessary for the pro-apoptotic function of tBid (Tait et al., 2007). The same study also concluded that, although full-length Bid is capable of translocating to the OMM, it is not able to induce MOMP on its own (Tait et al., 2007). A well-characterized example of tBid involvement during apoptosis is in the engagement of the mitochondrial apoptotic pathway in so-called type II cells upon activation of the extrinsic pathway (Yin et al., 1999).

Here, we have investigated whether Bid plays a functional role in the induction of MOMP during apoptosis in response to the genotoxic anticancer drug etoposide. To that end, we used Bax-deficient Jurkat

cells that are stably depleted of Bid and evaluated the extent to which these cells underwent drug-induced MOMP. In addition, Jurkat clones in which the intrinsic pathway had been inhibited due to the stable knockdown of Apaf-1 or the overexpression of full-length XIAP or the baculoviral IAP repeat 1 and 2 (BIR1/BIR2) of XIAP were used to gain insight into the molecular requirements necessary for cleavage of Bid to tBid during drug-induced apoptosis. Strikingly, the data showed that etoposide-induced apoptosis was decreased in Bid-deficient Jurkat cells. In particular, cells lacking Bid expression exhibited decreased Bak activation, cytochrome *c* release, loss of mitochondrial membrane potential ($\Delta\psi$), and caspase activation. Further, incubation of permeabilized Bid-deficient cells with recombinant tBid, but not full-length Bid, induced Bak dimerization and cytochrome *c* release. Significantly, we also found that cleavage of Bid to tBid occurred strictly downstream of Apaf-1 by a mechanism that required active executioner caspases.

4.3 Results and Discussion

4.3.1 Bid Is Cleaved in Response to the DNA-damaging Chemotherapeutic Agent Etoposide

Although it has been reported that overexpression of full-length Bid causes cell death (Wang et al., 1996) and that full-length Bid can translocate to the OMM in response to an apoptotic stimulus (Tafani et al., 2002; Sarig et al., 2003; Valentijn and Gilmore, 2004), it is generally accepted that tBid is far more potent than full-length Bid at activating Bak or Bax to induce MOMP (Li et al., 1998; Luo et al., 1998; Yin et al., 1999). The mechanism responsible for cleavage of Bid to tBid is at least partially understood. Caspase-8-mediated proteolysis of Bid to tBid during receptor-mediated cell killing in type II cells is probably the best characterized example of Bid's involvement during apoptotic cell death (Li et al., 1998; Luo et al., 1998). It is also known that cathepsins, calpains, and Granzyme B can cleave Bid, although in most instances the cleavage sites are different from those targeted by caspase-8 (Yin, 2006). It has also been suggested that caspase-3, and possibly caspase-2, can cleave Bid *in vitro*, although other evidence indicates that Bid is a relatively poor substrate for caspase-2 (Guo et al., 2002; Bonzon et al., 2006). Irrespective of the mechanism responsible for the cleavage of Bid, it is the C-terminal fragment that promotes apoptosis. Further, caspase-8-mediated cleavage of Bid to tBid exposes a glycine residue at the newly formed N-terminus that, in turn, undergoes myristoylation to facilitate translocation of tBid to mitochondria where it promotes MOMP induction (Zha et al., 2000).

Although tBid is a potent inducer of Bak/Bax-controlled MOMP during receptor-mediated cell killing, whether tBid is an important upstream regulator of MOMP during true intrinsic apoptotic cell death is controversial (Kamer et al., 2005; Zinkel et al., 2005; Kaufmann et al., 2007; Zinkel et al., 2007). Thus, we set out to determine the extent to which Bid or tBid is functionally important for MOMP induction and apoptosis in response to DNA damage. As illustrated in Fig. 10*A*, Western blot analysis of Jurkat whole-cell lysates obtained at 6 h following incubation with etoposide (10 μM) revealed that Bid had been cleaved to tBid. Drug-induced cleavage of Bid to tBid was accompanied by an increase in the percentage of cells undergoing apoptosis (28%) as determined by annexin V-FITC and propidium iodide co-staining (Fig. 10*B*). Further, in agreement with our previous findings, incubation of cells with 10 μM etoposide for 6 h resulted in the proteolytic cleavage of caspase-9, -3, and -7, and a concomitant increase in caspase (DEVDase) activity (Fig. 10*C* and *D*).

4.3.2 Stable Knockdown of Bid Desensitizes Jurkat Cells to Genotoxic Drug-induced Apoptosis

Figure 10. Etoposide-induced Bid cleavage and apoptosis in wild-type Jurkat T cells. *A*, wild-type cells (10^6/mL) were cultured with DMSO or 10 μM etoposide for 6 h, harvested, and lysed for Western blotting. *B-D*, duplicate aliquots of cells in *A* were harvested and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining (*B*), Western blotting of caspases (*C*), or caspase (DEVDase) activity measurements (*D*). In *B*, quadrants are defined as live (*lower left*), early apoptotic (*lower right*), late apoptotic (*upper right*), and necrotic (*upper left*). *Numbers* refer to the percentage of cells in each quadrant. *Casp*, caspase; *RFU*, relative fluorescence units.

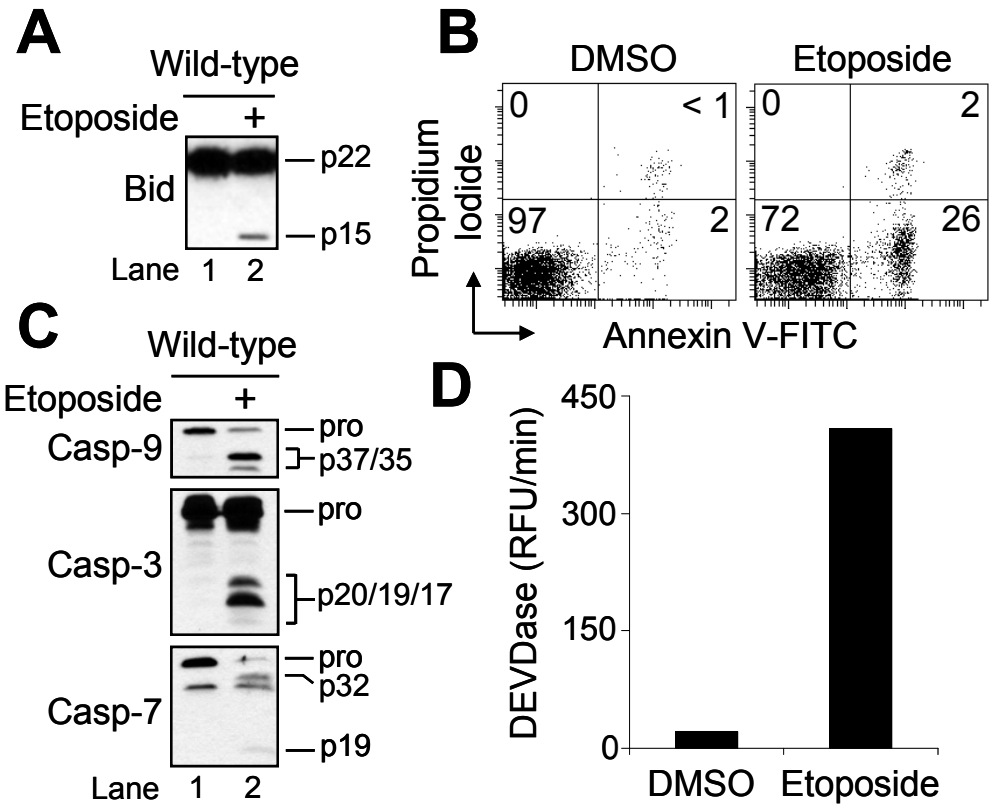

A
Wild-type
Etoposide
+
Bid
p22
p15
Lane 1 2
B
DMSO
Etoposide
Propidium Iodide
0
< 1
97
2
0
2
72
26
Annexin V-FITC
C
Wild-type
Etoposide
+
Casp-9
pro
p37/35
Casp-3
pro
p20/19/17
Casp-7
pro
p32
p19
Lane 1 2
D
DEVDase (RFU/min)
450
300
150
0
DMSO
Etoposide

Figure 10.

Having shown that tBid is generated in wild-type Jurkat cells undergoing DNA damage-induced apoptosis, we next used a vector-based system that directs the synthesis of short hairpin RNA (shRNA) to produce stable silencing of Bid. We hypothesized that such cells would shed light on whether Bid was important for genotoxic stress-induced apoptosis or if cleavage of Bid to tBid (Fig. 10*A*) was largely a bystander event in this setting with little biological significance.

Two single-cell Bid-deficient Jurkat clones (#7 and #13) were used for these studies, and the extent to which Bid was suppressed was confirmed by Western blot analysis (Fig. 11*A*). As mentioned previously, the requirement for Bid cleavage to tBid upon activation of the extrinsic pathway in type II cells has been well characterized and is widely accepted (Li et al., 1998; Luo et al., 1998; Scaffidi et al., 1998). Because Jurkat cells are considered to be of type II origin, we first tested the sensitivity of these cells to apoptosis induced by agonistic anti-Fas antibody (100 ng/mL). As anticipated, Bid-deficient cells were highly resistant to anti-Fas-induced apoptosis with clone #13 exhibiting the greatest degree of resistance as assessed by annexin V-FITC and propidium iodide co-staining (Fig. 11*B*) and proteolytic processing of caspases (Fig. 11*C*, *lanes 6* and *9 versus 3*). Next, having demonstrated that Bid was strictly required for receptor-mediated apoptosis in Jurkat cells, we incubated the cells for 6 h in the presence of etoposide (10 μM)

Figure 11. Bid-deficient Jurkat cells are less susceptible to etoposide-induced apoptosis. *A*, vector control or two single-cell Bid-deficient Jurkat clones (#7 and #13) were harvested and lysed for Western blotting. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. *B*, cells (10^6/mL) were cultured in the presence or absence of DMSO, 10 μM etoposide, or 100 ng/mL agonistic anti-Fas antibody for 6 h, harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as live (*lower left*), early apoptotic (*lower right*), late apoptotic (*upper right*), and necrotic (*upper left*). *Numbers* refer to the percentage of cells in each quadrant. *C*, duplicate aliquots of cells in *B* were harvested and lysed for Western blotting. *shRNA*, short hairpin RNA; *Casp*, caspase.

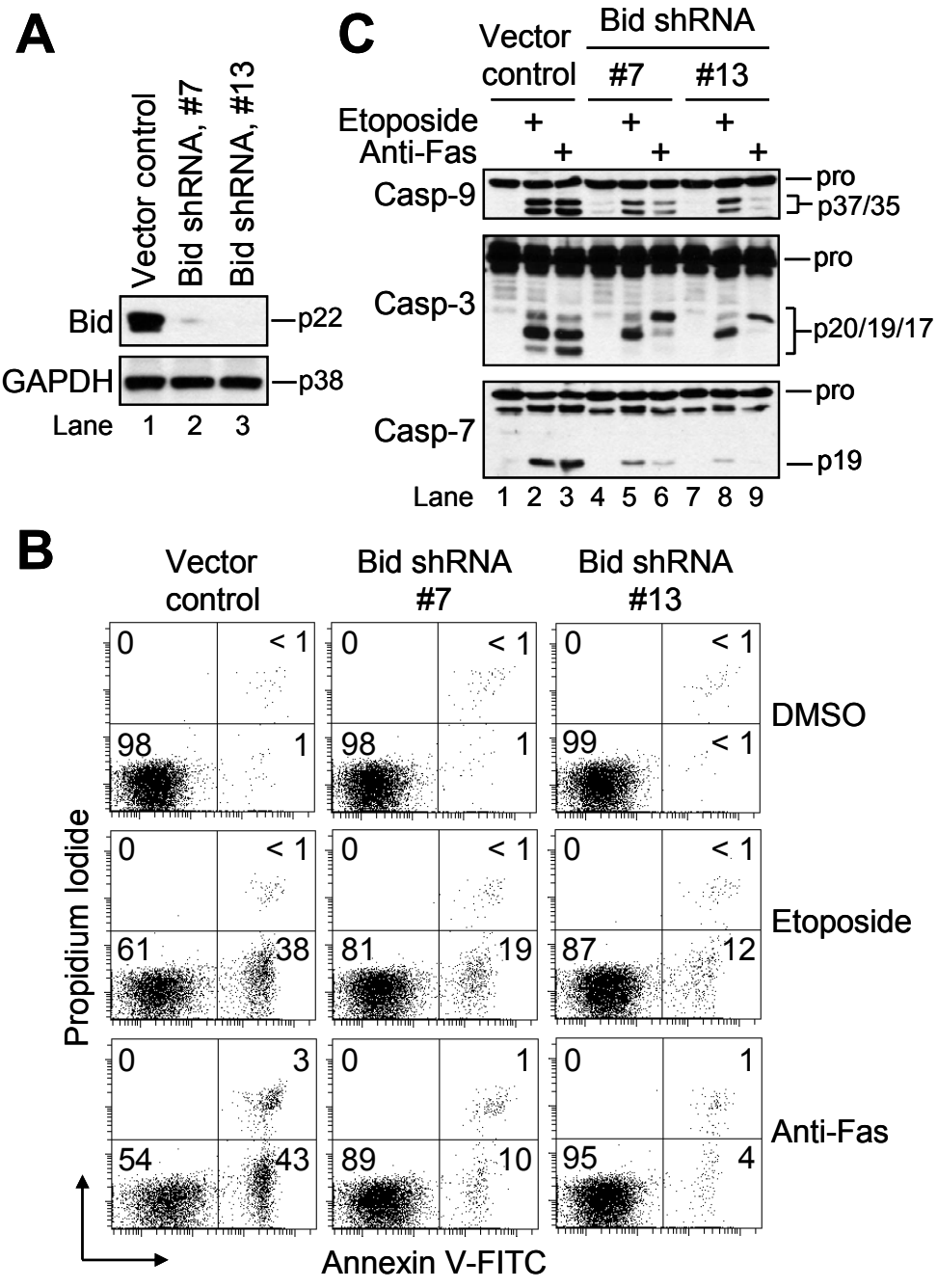

A
Vector control
Bid shRNA, #7
Bid shRNA, #13
Bid
p22
GAPDH
p38
Lane 1 2 3
C
Vector control
Bid shRNA
#7
#13
Etoposide
Anti-Fas
Casp-9
pro
p37/35
Casp-3
pro
p20/19/17
Casp-7
pro
p19
Lane 1 2 3 4 5 6 7 8 9
B
Vector control
Bid shRNA #7
Bid shRNA #13
DMSO
Etoposide
Anti-Fas
Propidium Iodide
Annexin V-FITC
0
< 1
98
1
99
61
38
81
19
87
12
3
54
43
89
10
95
4

Figure 11.

and evaluated the different clones for their sensitivity to apoptosis induction. The results indicated that both Bid-deficient clones were less sensitive than control-transfected cells to etoposide-induced apoptosis with the degree of resistance paralleling the extent to which Bid expression had been suppressed (Fig. 11*B*). In agreement with these findings, Western blot analysis of cell lysates obtained at 6 h post-etoposide treatment showed that proteolytic processing of caspase-9, -3, and -7 was decreased in Bid-deficient cells (Fig. 11*C*, *lanes 5* and *8 versus 2*). Overall, these data suggest that Bid plays an important function during genotoxic drug-induced apoptosis in Jurkat cells.

4.3.3 DNA Damage-induced Mitochondrial Events Are Attenuated in Bid-deficient Jurkat Cells

Studies from numerous laboratories have shown that MOMP is tightly regulated by pro- and anti-apoptotic proteins of the Bcl-2 family (Youle and Strasser, 2008). In particular, MOMP is widely accepted to require the activation of a multidomain protein, notably Bak or Bax. The presence and activation of either Bak or Bax is thought to be sufficient to induce MOMP. In fact, because the deletion of either gene alone produces only subtle defects in apoptosis, whereas doubly-deficient cells exhibit severe apoptosis deficiencies (Lindsten et al., 2000), Bak and Bax

are generally thought to be redundant in function. The activation of Bak and Bax coincides with their homo-oligomerization and in most instances is thought to depend on the presence and prior activation of a BH3-only Bcl-2 family member. However, the mechanism by which BH3-only proteins activate Bak or Bax is controversial (Chipuk and Green, 2008). As mentioned previously, one model suggests that BH3-only proteins activate Bak and Bax directly, whereas the second model indicates BH3-only proteins activate Bak and Bax indirectly by neutralizing the anti-apoptotic function of pro-survival Bcl-2 family proteins.

Because we had observed that etoposide-induced apoptosis was decreased in Bid-deficient Jurkat cells, we next evaluated mitochondrial events that characteristically define MOMP, such as the dimerization and activation of Bak or Bax, the release of intermembrane space proteins, and the loss of $\Delta\Psi$. In a recent paper, we showed that Jurkat (E6.1) cells express Bak, but not Bax, protein (Shawgo et al., 2008a). Those findings were confirmed in Fig. 12*A*, and we also determined that silencing Bid had no effect on the expression level of Bak protein (*lane 4 versus 3* and *2*). Therefore, we next examined the extent to which Bak had undergone oligomerization in response to etoposide, using the cross-linking agent bismaleimidohexane. As illustrated in Fig. 12*B*, cross-linked complexes consistent with Bak dimers were detected in control-transfected cells following treatment with 10 μM etoposide, whereas the extent of drug-

Figure 12. Inhibition of etoposide-induced mitochondrial apoptotic changes in cells lacking Bid. *A*, whole-cell lysates of Jurkat (E6.1) and MCF-7 (positive control for Bax expression) cells were subjected to SDS-PAGE and Western blotted. *B* and *C*, cells (10^6/mL) were cultured with DMSO or 10 μM etoposide for 6 h and processed for determination of Bak oligomerization by Western blotting (*B*) or Bak activation by flow cytometric analysis (*C*). *Numbers* in *C* refer to the percentage increase in Bak-associated fluorescence between DMSO- and etoposide-treated samples. *D*, duplicate aliquots of cells in *B* and *C* were harvested and processed for subcellular fractionation. Supernatant (*s*) and pellet (*p*) fractions were analyzed by Western blotting. *E*, duplicate aliquots of cells in *B* and *C* were harvested and processed for mitochondrial membrane potential ($\Delta\Psi$) determination by flow cytometry. Reduced $DiIC_1(5)$ fluorescence is indicative of a loss of $\Delta\Psi$, and *numbers* refer to the percentage of cells that underwent a dissipation of $\Delta\Psi$. *shRNA*, short hairpin RNA; *BMH*, bismaleimidohexane; *Cyt c*, cytochrome *c*.

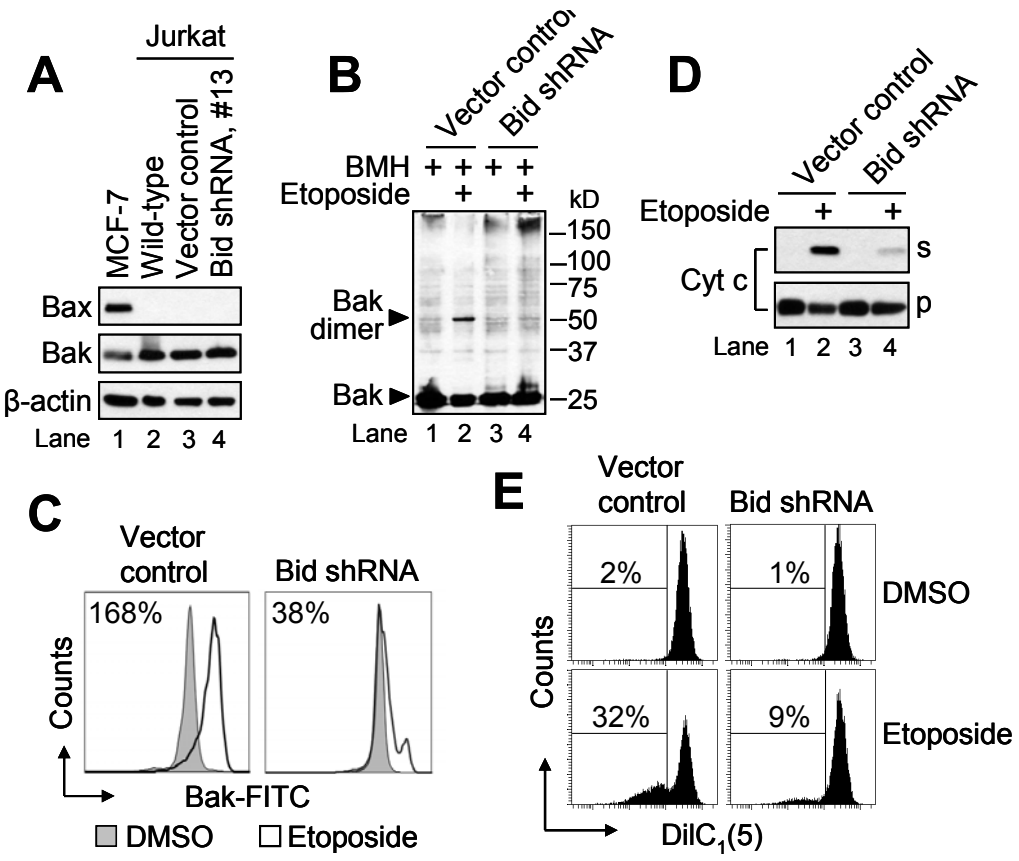

A
Jurkat
MCF-7
Wild-type
Vector control
Bid shRNA, #13
Bax
Bak
β-actin
Lane 1 2 3 4
B
Vector control
Bid shRNA
BMH + + + +
Etoposide + +
kD
150
100
75
50
37
25
Bak dimer
Bak
Lane 1 2 3 4
D
Vector control
Bid shRNA
Etoposide + +
Cyt c
s
p
Lane 1 2 3 4
C
Vector control
Bid shRNA
168%
38%
Counts
Bak-FITC
DMSO
Etoposide
E
Vector control
Bid shRNA
2%
1%
DMSO
32%
9%
Etoposide
Counts
DilC1(5)

Figure 12.

induced dimerization was markedly decreased in the Bid-deficient cells as illustrated in lane 4 of Fig. 12*B*. These findings were in line with results obtained using an active conformation-specific monoclonal Bak antibody and flow cytometric analysis where activation causes a shift to the right of the resulting histogram (Fig. 12*C*).

Next, consistent with the inhibition of Bak activation in cells lacking Bid, the release of cytochrome *c* in response to etoposide was also markedly inhibited (Fig. 12*D*). Finally, as illustrated in Fig. 12*E*, the loss of $\Delta\Psi$ in response to etoposide was also decreased in the Bid-deficient cell line (Fig. 12*E*). Combined, these data strongly suggest that Bid functions to promote Bak-controlled MOMP during DNA damage-induced apoptosis in Jurkat cells.

4.3.4 Cleaved Bid (tBid) Mediates MOMP and Is Generated Independently of Caspase-8 in Response to DNA damage

A previous study reported that the expression of sublethal levels of wild-type or uncleavable Bid in *bid*$^{-/-}$ mouse embryonic fibroblasts resulted in a similar sensitization of cells to DNA damage-induced apoptosis that was accompanied by an enhanced localization of Bid to mitochondria and release of cytochrome *c* (Sarig et al., 2003). In this regard, because we had demonstrated that cells lacking Bid were resistant to drug-induced

apoptosis, we next sought to determine if the phenotype we observed was due to the absence of full-length Bid or tBid. To address this issue, we made several attempts to rescue the phenotype of our Bid-deficient cells by reintroducing wild-type human Bid and mutants of Bid that were uncleavable or unable to be myristoylated. Although it had been reported previously that overexpression of Bid is lethal to Jurkat cells (Wang et al., 1996), we reasoned that reintroduction of wild-type or mutant Bid into cells that were deficient in this protein would yield viable cell clones. Surprisingly, however, all attempts to express wild-type or mutant Bid into Bid-silenced cells were unsuccessful because we found that cells simply could not be transfected with any form of Bid and remain viable. Since our efforts to rescue the phenotype in whole cells failed, we next performed experiments in which we tried to reconstitute the phenotype using permeabilized cells. Specifically, recombinant full-length and caspase-8-cleaved Bid were used to treat permeabilized Bid-deficient Jurkat cells to evaluate more precisely whether Bid, tBid, or both could promote the engagement of the mitochondrial pathway. As shown in Fig. 13*A*, when Bid-deficient Jurkat cells were permeabilized with digitonin and incubated with 5, 10, 25, 50, 100, or 250 nM recombinant tBid for 15 min at 22 ºC, cytochrome *c* was released into the cytosol. By comparison, full-length Bid failed to stimulate the release of any cytochrome *c*, even at the highest concentration of 250 nM. Consistent with the cytochrome *c* release data,

Figure 13. tBid, but not Bid, mediates MOMPand is generated independently of caspase-8 in response to etoposide. *A*, Bid-deficient (clone #13) Jurkat cells (2.5 x 10^6) were permeabilized with 7.5 µg of digitonin and incubated with recombinant tBid (*top panels*) or Bid (*bottom panels*) protein (5-250 nM) at 22 ºC for 15 min. Subsequently, supernatant (*s*) and pellet (*p*) fractions were obtained, subjected to SDS-PAGE, and Western blotted. *B*, digitonin-permeabilized Bid-deficient cells as in *A* were incubated in the presence or absence of either 25 nM tBid or Bid and processed for determination of Bak oligomerization by Western blotting. *C*, wild-type cells (10^6/mL) were pre-incubated in the presence or absence of 20 µM qVD-OPh for 1 h and subsequently incubated in the presence or absence of DMSO or 10 µM etoposide for an additional 6 h at which time cells were harvested and processed for Western blot analysis. *D*, whole-cell lysates of wild-type (A3) and caspase-8-deficient (I 9.2) Jurkat cells were subjected to SDS-PAGE and Western blotted. *E* and *F*, cells (10^6/mL) were incubated in the absence or presence of DMSO, 10 µM etoposide, or 100 ng/mL agonistic anti-Fas antibody for 6 h, harvested, and lysed for Western blotting (*E*) or processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining (*F*). Quadrants in *F* are defined as live (*lower left*), early apoptotic (*lower right*), late apoptotic (*upper right*), and necrotic (*upper left*). *Numbers* refer to the percentage of cells in each quadrant.

shRNA, short hairpin RNA; *Cyt c*, cytochrome *c*; *Conc*, concentration; BMH, bismaleimidohexane; *Casp*, caspase.

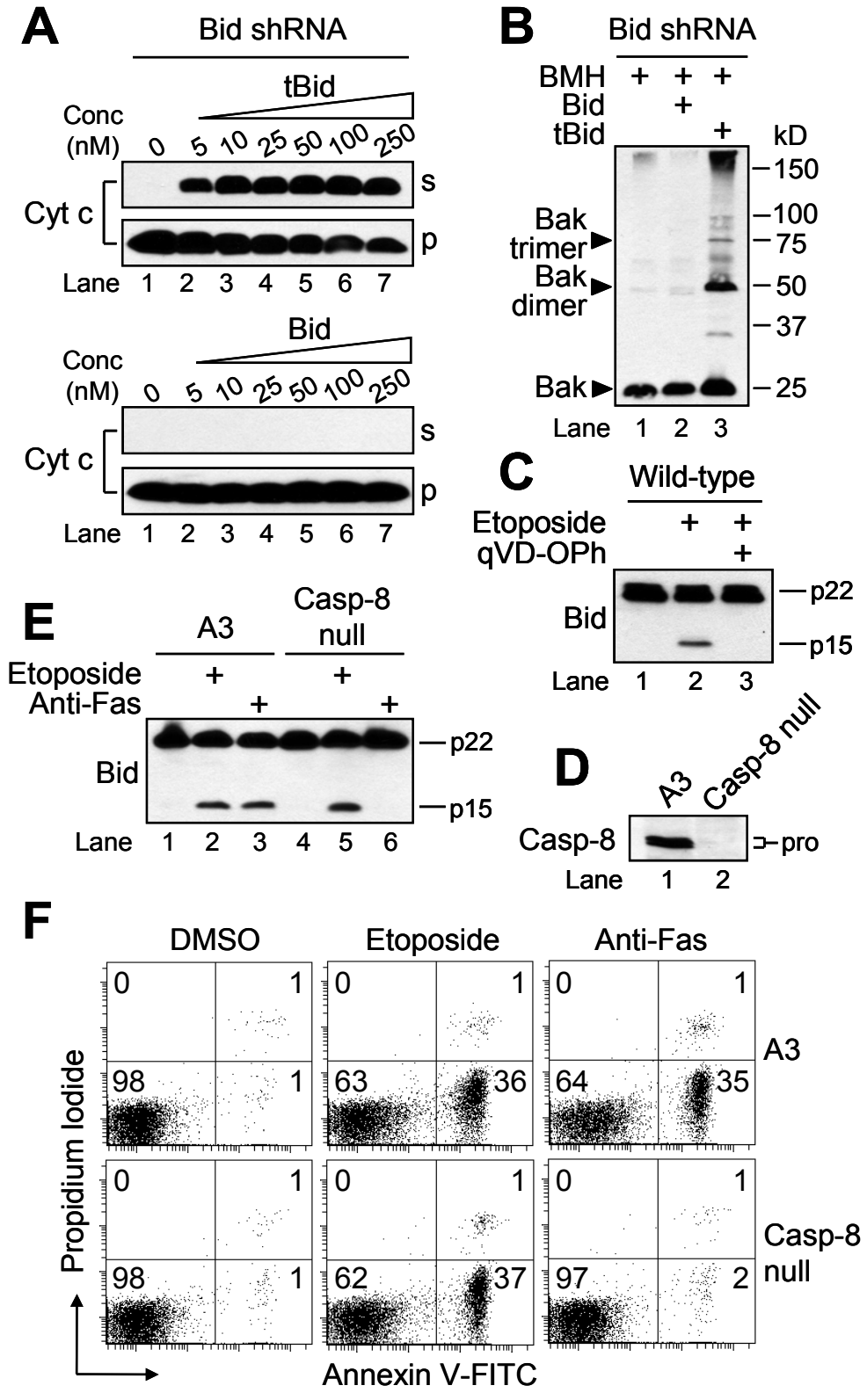

A
Bid shRNA
tBid
Conc
(nM)
0 5 10 25 50 100 250
Cyt c
s
p
Lane 1 2 3 4 5 6 7
Bid
Conc
(nM)
0 5 10 25 50 100 250
Cyt c
s
p
Lane 1 2 3 4 5 6 7
B
Bid shRNA
BMH + + +
Bid +
tBid +
kD
150
100
75
50
37
25
Bak trimer
Bak dimer
Bak
Lane 1 2 3
C
Wild-type
Etoposide + +
qVD-OPh +
Bid
p22
p15
Lane 1 2 3
E
A3
Casp-8 null
Etoposide + +
Anti-Fas + +
Bid
p22
p15
Lane 1 2 3 4 5 6
D
A3
Casp-8 null
Casp-8
pro
Lane 1 2
F
DMSO
Etoposide
Anti-Fas
0 1
98 1
63 36
64 35
A3
0 1
98 1
62 37
97 2
Casp-8 null
Propidium Iodide
Annexin V-FITC

Figure 13.

only tBid (25 nM) induced the oligomerization of Bak when added to digitonin-permeabilized Bid-deficient cells (Fig. 13*B*). These findings indicate that tBid, and not full-length Bid, is most probably responsible for drug-induced activation of Bak and the induction of MOMP in response to genotoxic stress.

As mentioned previously, it is widely accepted that caspase-8-mediated cleavage of Bid is required for execution of extrinsic apoptotic cell death in type II cells. It has also been suggested that other caspases may have the ability to cleave Bid, such as caspase-2 (Guo et al., 2002; Bonzon et al., 2006) and caspase-3 (Bossy-Wetzel and Green, 1999; Slee et al., 2000). Additional lines of evidence have shown that non-caspase proteases, such as calpains (Chen et al., 2001; Mandic et al., 2002) and cathepsins (Stoka et al., 2001; Droga-Mazovec et al., 2008), are also capable of cleaving full-length Bid. Further, a different study reported that tBid is important during DNA damage-induced apoptosis and that the cleavage of Bid to tBid in this context was not caspase-dependent. Instead, cleavage was suggested to occur by an unidentified aspartate protease (Werner et al., 2004). The same study concluded that the importance of tBid for genotoxic stress-induced apoptosis was that it functioned to promote MOMP upstream of caspase activation.

To determine if the cleavage of Bid to tBid was a caspase-mediated event in our experimental system, we incubated wild-type Jurkat cells with

the caspase inhibitor quinoline-Val-Asp-CH_2-difluorophenoxy (qVD-OPh, 20 μM) for 1 h prior to the addition of etoposide (10 μM). As illustrated in Fig. 13*C*, pretreatment of cells with qVD-OPh completely prevented drug-induced generation of tBid. Next, because of early reports indicating that caspase-8 is important during DNA damage-induced apoptosis (Friesen et al., 1996; Kasibhatla et al., 1998; Petak et al., 2000) and because caspase-8 is the best characterized protease responsible for the cleavage of Bid, we used caspase-8-deficient Jurkat cells (Fig. 13*D*) to determine whether caspase-8 is necessary for mitochondria-mediated apoptosis and/or Bid cleavage. As expected, these cells were highly resistant to receptor-mediated apoptosis induced by anti-Fas (Fig. 13*F*). By comparison, caspase-8-deficient cells underwent etoposide-induced apoptosis to the same extent as A3 control cells (Fig. 13*F*), suggesting that caspase-8 is dispensable for etoposide-induced apoptosis. Further, cleavage of Bid to tBid was prevented in caspase-8 null cells incubated in the presence of anti-Fas, whereas Bid was cleaved to a similar extent in caspase-8-deficient and A3 control cells treated with etoposide (Fig. 13*E*). Combined, these data suggest that caspase-8 is dispensable for etoposide-induced cleavage of Bid to tBid.

4.3.5 Executioner Caspases Mediate Bid Cleavage During Etoposide-induced Apoptosis

To extend these findings and to determine whether drug-induced Bid cleavage was mediated by a caspase other than caspase-8, we first used Apaf-1-deficient Jurkat cells described previously that are totally resistant to etoposide-induced apoptosis (Franklin and Robertson, 2007). As illustrated in Fig. 14*A*, cleavage of Bid to tBid did not occur in Apaf-1-deficient cells incubated in the presence of 10 μM etoposide for 6 h. Because Bid cleavage appeared to require apoptosome-dependent caspase activation, we were next interested to determine the extent to which this was mediated by an initiator or executioner caspase protease. To distinguish between these two possibilities, we used Jurkat cells described recently that overexpress either full-length XIAP or the BIR1/BIR2 domains of XIAP (Shawgo et al., 2008a). Full-length XIAP inhibits apoptosis by preventing caspase-9, -3, and -7 activity, whereas the BIR1/BIR2 domains of XIAP can inhibit caspase-3 and -7, but not caspase-9 (Shiozaki et al., 2003; Scott et al., 2005). During either mitochondria-mediated apoptosis or receptor-mediated apoptosis in type II cells, the prosurvival activity of XIAP is neutralized by Smac and Omi, which are released into the cytosol from the intermembrane space of mitochondria as a consequence of MOMP. In agreement with our previous findings (Shawgo et al., 2008a), Jurkat cells that overexpressed either full-length XIAP or the BIR1/BIR2 domains of XIAP were impaired in their ability to activate caspases and undergo apoptosis, exhibiting 7%

Figure 14. Cleavage of Bid in response to etoposide is mediated by executioner caspases. *A*, wild-type, control-transfected, and Apaf-1-deficient Jurkat cells (10^6/mL) were incubated in the presence or absence of DMSO or 10 μM etoposide for 6 h, harvested, and lysed for Western blotting. *B*, control-transfected, XIAP-overexpressing, and Myc-BIR1/BIR2-expressing Jurkat cells (10^6/mL) were incubated in the presence or absence of DMSO or 10 μM etoposide for 6 h, harvested, and lysed for Western blotting. *C*, duplicate aliquots of cells in *B* were processed for determination of Bak activation by flow cytometric analysis. *Numbers* in *C* refer to the percentage increase in Bak-associated fluorescence between DMSO- and etoposide-treated samples. *shRNA*, short hairpin RNA.

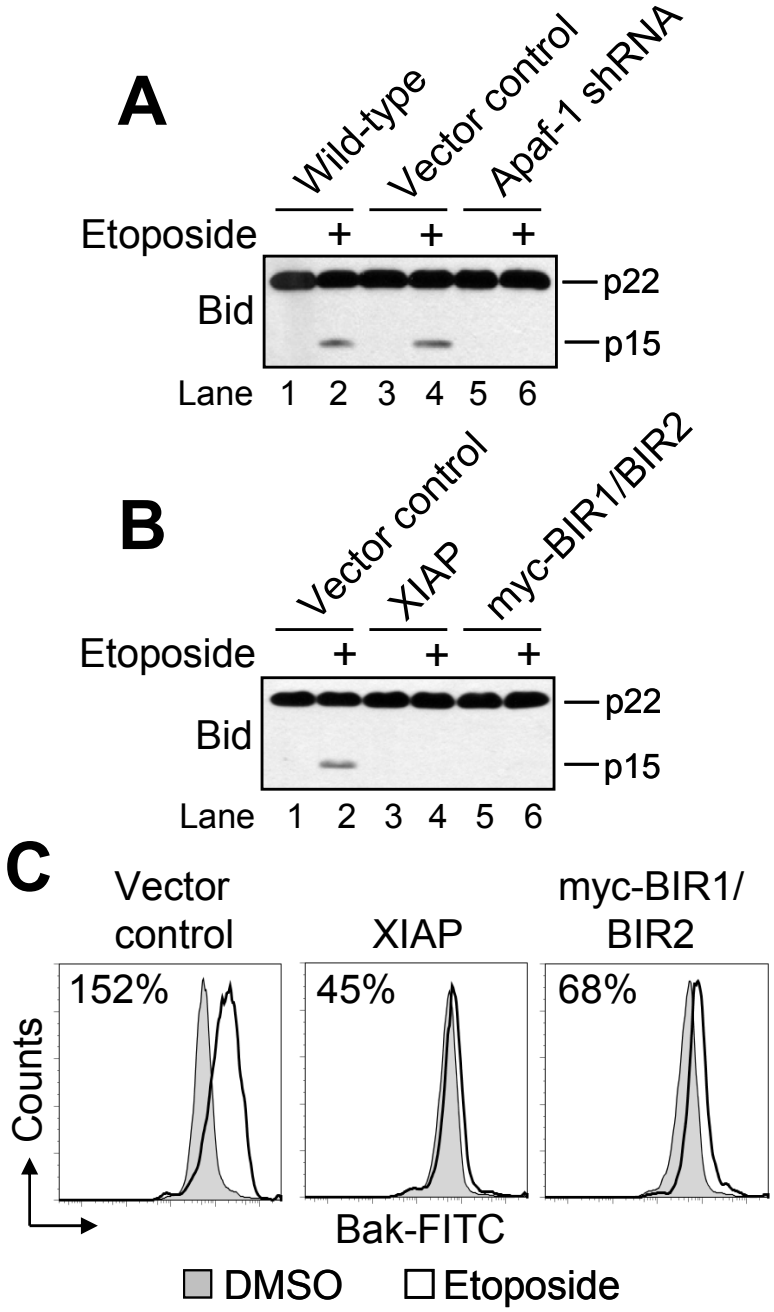

A
Wild-type
Vector control
Apaf-1 shRNA
Etoposide + + +
Bid
p22
p15
Lane 1 2 3 4 5 6
B
Vector control
XIAP
myc-BIR1/BIR2
Etoposide + + +
Bid
p22
p15
Lane 1 2 3 4 5 6
C
Vector control
XIAP
myc-BIR1/ BIR2
152%
45%
68%
Counts
Bak-FITC
DMSO
Etoposide

Figure 14.

and 12% apoptotic cells, respectively, in response to etoposide (data not shown). Western blot analysis of duplicate cell aliquots revealed that control-transfected, but not XIAP- or BIR1/BIR2-transfected, cells had cleaved Bid in response to etoposide treatment (Fig. 14*B*). Importantly, the absence of etoposide-induced Bid cleavage in cells overexpressing either XIAP or the BIR1/BIR2 domains of XIAP was accompanied by a marked decrease in Bak activation in these cells incubated under the same conditions (Fig. 14*C*). Taken together, these data suggest that etoposide-induced cleavage of Bid to tBid and tBid-mediated activation of Bak occur by a mechanism that requires the prior activation of executioner caspases.

4.4 Concluding Remarks

A key step during true intrinsic apoptosis is the induction of MOMP mediated by an active multidomain pro-apoptotic protein Bak or Bax (Chipuk and Green, 2008). In fact, it is generally accepted that the presence and activation of either Bak or Bax is strictly required for MOMP. BH3-only proteins are most often responsible for the activation of Bak and Bax, which involves their homo-oligomerization and insertion into the OMM to form pores through which intermembrane space proteins, such as cytochrome *c* and Smac, are released into the cytosol to activate

caspases. As mentioned previously, the best characterized example of a BH3-only protein being responsible for the activation of a multidomain protein is during receptor-mediated apoptosis in type II cells where tBid activates Bak/Bax to induce MOMP. A similar emergent role for a BH3-only protein in the activation of Bak or Bax during true intrinsic apoptotic cell death is currently lacking.

Some evidence in the literature indicates that the activation of Bak or Bax following DNA damage can be mediated by p53-dependent upregulation of the BH3-only protein Puma (Jeffers et al., 2003; Villunger et al., 2003). However, Jurkat cells, which are highly susceptible to DNA damage-induced apoptosis, possess mutant p53 (Cheng and Haas, 1990). In addition, cycling T lymphoma cells and mitogenically activated T lymphocytes from *p53*$^{-/-}$ mice were shown to be sensitive to genotoxic stress-induced apoptosis (Strasser et al., 1994). In this regard, p53-induced upregulation of Puma is unlikely to be the sole mediator of Bak/Bax-dependent apoptosis in response to DNA damage. In fact, accumulating evidence in the literature suggests that the activation of Bak or Bax during true intrinsic apoptosis requires the presence of either Bid or Bim, irrespective of p53 status (Letai, 2008).

Recent studies investigating a potential role for Bid during DNA damage-induced apoptosis have produced conflicting results (Kamer et al., 2005; Zinkel et al., 2005; Kaufmann et al., 2007). On one hand,

embryonic fibroblasts and myeloid progenitor cells from Bid-deficient mice were shown to be less susceptible to etoposide-induced apoptosis (Kamer et al., 2005; Zinkel et al., 2005), whereas another study reported that Bid plays no role in DNA damage-induced apoptosis (Kaufmann et al., 2007). The fact that each of these studies used cells that were cultivated from *bid*$^{-/-}$ mice on a C57BL/6 background makes it difficult to reconcile the different findings in the absence of additional experimentation.

As mentioned previously, a separate study using Jurkat T cells suggested that tBid was required for apoptosis signaling during DNA damage-induced apoptosis (Werner et al., 2004). The authors also concluded that Bid cleavage occurred independently of caspase activity. Rather, it was speculated that an unidentified aspartate protease was responsible for the cleavage of Bid to tBid, which, in turn, was critical for MOMP and the activation of all caspases.

Our findings using a panel of gene-manipulated Jurkat cells also support a role for cleavage of Bid to tBid in eliciting cytochrome *c* release and apoptosis in response to etoposide. However, unlike Werner *et al.* (38), our data suggest that most, if not all, Bid cleavage occurs downstream of MOMP by a mechanism that depends on active executioner caspases (Fig. 15). In this regard, our current data support and extend a model of the intrinsic pathway in which downstream caspases and mitochondria forge a circuit of positive amplification to

Figure. 15. Hypothetical scheme of etoposide-induced apoptosis. Genotoxic stress in response to etoposide induces initial MOMP by generating a still poorly defined death signal that activates Bax/Bak. As a consequence of early MOMP, intermembrane space proteins, including cytochrome *c* and Smac, are released into the cytosol. The initial release of these proteins into the cytosol *per se* is not an irreversible commitment point to apoptotic cell death. Instead, the point of commitment occurs when the accumulation of Smac is sufficient to inhibit XIAP-mediated inhibition of caspase-9, -3, and -7 and/or the accumulation of cytochrome *c* surpasses a threshold needed for the conversion of sufficient numbers of monomeric Apaf-1 molecules to heptameric apoptosome signaling platforms necessary for initial caspase-9 activation. The ensuing caspase-9 activity would need to be sufficiently strong to activate enough executioner caspase-3 or -7 molecules to kill a cell outright and/or to elicit feed forward amplification of MOMP by cleaving Bid to tBid. *BH3*, Bcl-2 homology 3; *Cyt c*, cytochrome *c*.

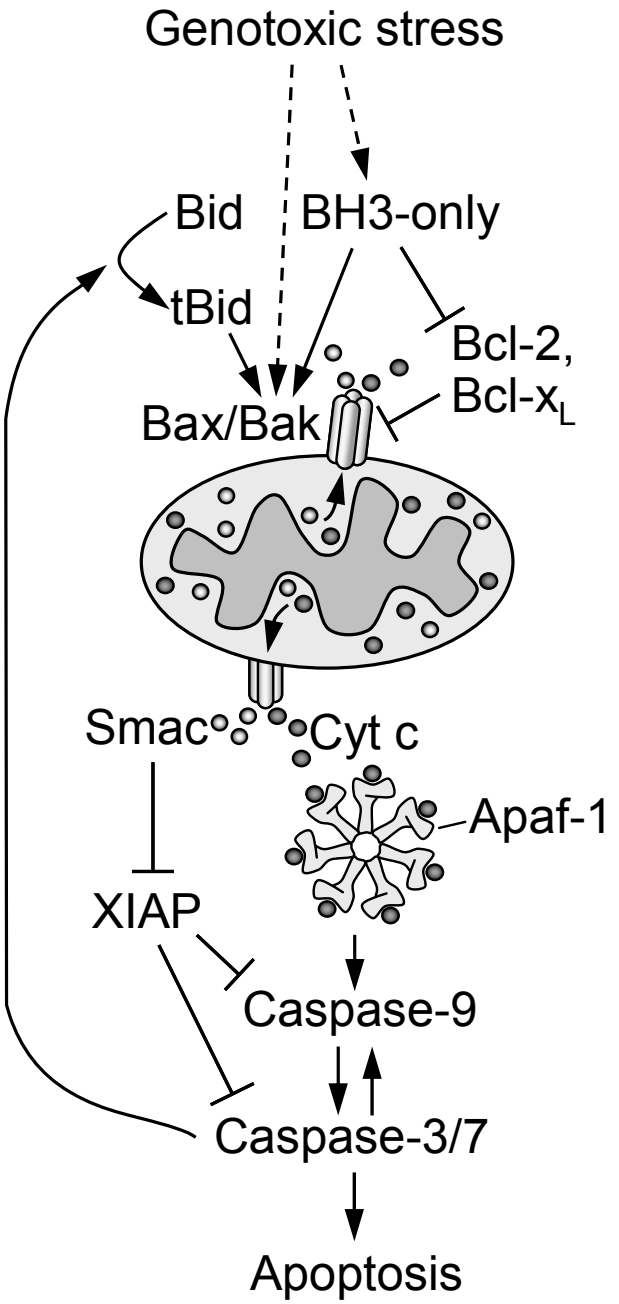

Genotoxic stress
Bid
BH3-only
tBid
Bcl-2,
Bcl-x$_L$
Bax/Bak
Smac
Cyt c
Apaf-1
XIAP
Caspase-9
Caspase-3/7
Apoptosis

Figure 15.

ensure irreversible commitment to apoptosis. Indeed, it is tempting to speculate that some cell types may be spared death if feed forward amplification of early mitochondrial apoptotic events is inhibited.

We envision a scenario where genotoxic stress triggers initial MOMP and the release of intermembrane space proteins by activating Bak or Bax by an as yet poorly understood mechanism that may or may not involve a BH3-only protein (Fig. 15). The initial induction of MOMP would not necessarily mark the “point of no return” for an injured cell unless, or until, the release of intermembrane space proteins, including cytochrome *c*, exceeded a threshold necessary to trigger apoptosome formation resulting in caspase-9 activation and the subsequent activation of caspase-3/7. In turn, active caspase-3 (or -7) would mediate feed forward amplification of initial MOMP by cleaving Bid to tBid to increase the activation of Bak or Bax.

Chapter 5: KU135, a Novel Novobiocin-derived C-terminal Inhibitor of Hsp90, Exerts Potent Antiproliferative Effects in Human Leukemic Cells

5.1 Abstact

Heat shock protein 90 (Hsp90) assists in the proper folding of numerous mutated or overexpressed signal transduction proteins that are involved in cancer. Consequently, there is considerable interest in developing chemotherapeutic drugs that specifically disrupt the function of Hsp90. Here, we investigated the extent to which a novel novobiocin-derived C-terminal Hsp90 inhibitor, designated KU135, induced antiproliferative effects in Jurkat T-lymphocytes. The results indicated that KU135 bound directly to Hsp90, caused the degradation of known Hsp90 client proteins, and induced more potent antiproliferative effects than the established N-terminal Hsp90 inhibitor 17-AAG. Closer examination of the cellular response to KU135 and 17-AAG revealed that only 17-AAG induced a strong upregulation of Hsp70 and Hsp90. Also, KU135 caused wild-type cells to undergo G_2/M arrest, whereas cells treated with 17-AAG accumulated in G_1. Further, KU135, but not 17-AAG, was found to be a potent inducer of mitochondria-mediated apoptosis as evidenced, in part,

by the fact that cell death was inhibited to a similar extent by Bcl-2/Bcl-x_L overexpression or the depletion of Apaf-1. Combined, these data suggest that KU135 inhibits cell proliferation by regulating signaling pathways that are mechanistically different from those targeted by 17-AAG and as such represents a novel opportunity for Hsp90 inhibition.

5.2 Introduction

Members of the heat shock protein (Hsp) 90 protein family are commonly overexpressed in cancer cells and play critical roles in promoting survival by chaperoning client proteins associated with all six of the acquired cancer capabilities (Hanahan and Weinberg, 2000; Isaacs et al., 2003; Blagg and Kerr, 2006). A growing number of natural product, synthetic, and semi-synthetic Hsp90 inhibitors are being developed that largely target the N-terminal ATP-binding pocket and have been shown to cause potent antiproliferative effects (Roe et al., 1999; Whitesell and Lindquist, 2005; Avila et al., 2006a; Avila et al., 2006b). However, the potential clinical utility of several of the N-terminal inhibitors as anticancer drugs has been dampened significantly due to concerns about their adverse hepatotoxic effects (Egorin et al., 1998) and tendency to induce expression of cytoprotective Hsp90 and Hsp70 proteins (Chiosis et al., 2003; Whitesell et al., 2003; Powers and Workman, 2007; Schmitt et al.,

2007). More recently, the observation was made that Hsp90 contains a previously unrecognized C-terminal ATP-binding domain (Marcu et al., 2000a; Marcu et al., 2000b), which has led several groups to pursue the development of specific C-terminal Hsp90 inhibitors as potential anticancer drug modalities (Burlison et al., 2006; Burlison et al., 2008b; Donnelly et al., 2008). Both N-terminal and C-terminal Hsp90 inhibitors can exert an antiproliferative response, in some instances, by stimulating apoptosis (Isaacs et al., 2003; Georgakis and Younes, 2005; Whitesell and Lindquist, 2005), although the underlying mechanisms are not well understood.

Apoptotic cell death is mediated by a family of cysteine proteases that cleave after aspartate residues (caspases). In general, the activation of caspases can occur by two distinct signaling pathways. Within the extrinsic (receptor-mediated) pathway, ligand (e.g., FasL and TNFα) binding to a corresponding death receptor (e.g., Fas and TNF-R1) leads to recruitment of FADD and procaspase-8 molecules to the cytosolic side of the cell membrane to form the death-inducing signaling complex (DISC) (Kischkel et al., 1995). Activation of procaspase-8 occurs at the DISC, and active caspase-8, in turn, can activate caspase-3 directly or by first cleaving and activating the BH3-only protein Bid to truncated Bid (tBid), which, in turn, can engage the intrinsic or mitochondria-mediated apoptotic pathway (Li et al., 1998; Luo et al., 1998). The intrinsic (mitochondria-

mediated) pathway, however, is most often initiated by cytotoxic stress, including growth factor withdrawal, DNA damage, γ-radiation, and heat. In response to these types of stimuli, mitochondrial outer membrane permeabilization (MOMP) generally occurs, resulting in the release of cytochrome *c*, Smac (also known as DIABLO), and Omi into the cytosol, where they work together to activate procaspase-9 within the apoptotic protease-activating factor-1 (Apaf-1) apoptosome complex (Li et al., 1997). Once activated, caspase-9 can cleave and thereby activate caspase-3, which, in turn, is responsible for proteolytic dismantling of the cell.

The aim of the current study was to investigate the extent to which a novobiocin-derived C-terminal Hsp90 inhibitor, KU135, could induce an antiproliferative response and how such an effect compared to that exhibited by the established N-terminal inhibitor 17-allylamino-demethoxygeldanamycin (17-AAG). The results indicated that KU135 binds directly to Hsp90 and exhibits more potent antiproliferative effects than 17-AAG. Both 17-AAG and KU135 stimulated the degradation of known Hsp90 client proteins, whereas only 17-AAG triggered a pronounced increased expression of Hsp70 and Hsp90 proteins. KU135 was found to induce both G_2/M cell cycle arrest and mitochondria-mediated apoptosis, whereas 17-AAG predominantly induced G_1 cell cycle arrest.

5.3 Results

5.3.1 A novobiocin-derived C-terminal Hsp90 inhibitor, KU135, induces a potent antiproliferative response in Jurkat T-lymphocytes.

As mentioned previously, a new possibility for Hsp90 inhibition was raised when the antibiotic novobiocin was found to bind a previously unidentified C-terminal ATP-binding site (Fig. 16A), albeit with poor affinity (IC_{50}, ~700 μM). Through systematic investigations, new novobiocin analogues have been produced that exhibit significantly better efficacy. We began our studies by evaluating the extent to which wild-type Jurkat T-lymphoblastoid leukemia cells (clone E6.1) were sensitive to the novel novobiocin analogue KU135 (Fig. 16B). KU135 is part of a larger library of structurally related compounds that will be discussed in detail in a forthcoming manuscript under preparation by Blagg and coworkers (unpublished information). The potency of KU135 was compared to that of novobiocin, 17-AAG, and the anticancer drug etoposide, which damages DNA by inhibiting topoisomerase II and is a well-established inducer of mitochondria-mediated apoptosis. Wild-type Jurkat cells were incubated with increasing concentrations of KU135 (2.5 nM – 50 μM), novobiocin (25 nM – 500 μM), 17-AAG (0.5 nM – 10 μM), and etoposide (2.4 nM – 47 μM) or DMSO (0.5% final concentration) for 24 or 48 h and

Figure 16. KU135, a novel analogue of novobiocin, causes antiproliferation. A, Hsp90 contains four functional domains and the numbering (1-732) reflects the approximate number of amino acids that comprise each region. NCR refers to the negatively-charged region that links the N-terminal and middle domains. The regions where nucleotides, small molecule inhibitors, and/or cisplatin bind Hsp90 are shown. B, the structures of novobiocin and KU135. C, cells (3 x 10^4/well) were cultured in a 96-well plate in the absence or presence of DMSO (●), KU135 (2.5 nM – 50 μM) (▲), 17-AAG (0.5 nM – 10 μM) (□), etoposide (2.4 nM – 47 μM) (✱), or novobiocin (25 nM – 500 μM) (◆) for 24 or 48 h and processed for cell proliferation determination by the MTS assay.

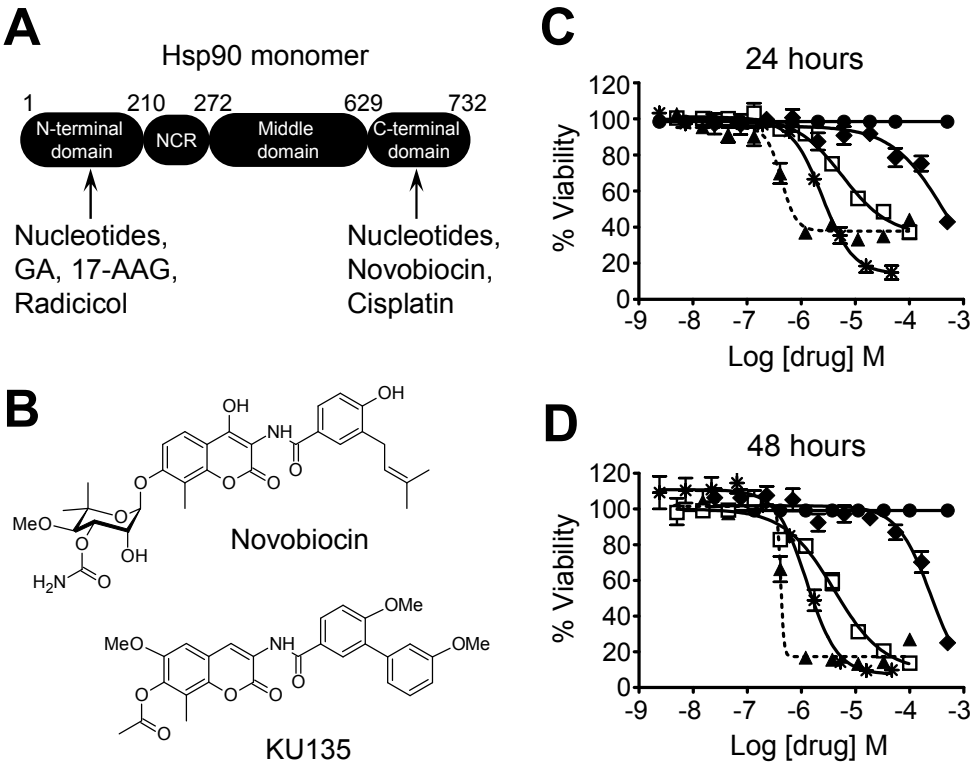

A
Hsp90 monomer
1
210
272
629
732
N-terminal domain
NCR
Middle domain
C-terminal domain
Nucleotides, GA, 17-AAG, Radicicol
Nucleotides, Novobiocin, Cisplatin
B
Novobiocin
KU135
C
24 hours
% Viability
Log [drug] M
D
48 hours
% Viability
Log [drug] M

Figure 16.

cell viability was determined by the MTS proliferation assay. As illustrated in Fig. 16C, all four compounds (i.e., KU135, novobiocin, 17-AAG, and etoposide) inhibited cell proliferation in a concentration- and time-dependent manner. In particular, the IC_{50} values for KU135, etoposide, 17-AAG, and novobiocin at 48 h post-treatment were found to be 416 nM, 1.3 μM, 4 μM, and 252 μM, respectively. Thus, while all four drug treatments inhibited Jurkat cell proliferation, KU135 was ~3, ~10, and ~600 times more potent than etoposide, 17-AAG, and novobiocin, respectively.

5.3.2 KU135 inhibits cell division and induces apoptotic cell death in wild-type Jurkat cells.

Next, we were interested in determining whether the decrease in viability observed in response to KU135 administration using the MTS assay was due to an induction of apoptosis. Indeed, as shown in Fig. 16A, wild-type Jurkat cells underwent apoptosis in a concentration-dependent manner when incubated with KU135 for 24 h. In particular, we found that treatment with 1 μM and 2.5 μM KU135 induced 38% and 47% apoptosis, respectively (Fig. 17A). The level of KU135-induced apoptosis did not increase further when cells were incubated with concentrations of KU135 greater than 2.5 μM (data not shown). By comparison, and in

Figure 17. KU135 and 17-AAG exert different effects on apoptosis and cell cycle distribution. A, cells (5 x 10^5/mL) were cultured with DMSO or the indicated concentrations of 17-AAG or KU135 for 24 h and processed for apoptotic cell death determination by flow cytometric analysis of annexin V-FITC staining in a buffer containing propidium iodide. Increased annexin V-FITC fluorescence reflects phosphatidylserine externalization on the plasma membrane, and numbers refer to the percentage of cells undergoing apoptosis. B, cells (5 x 10^5/mL) were cultured with DMSO, 1 μM KU135, or 10 μM 17-AAG for 24 h, fixed in 70% ethanol, and analyzed for cell cycle status using PI/RNase Staining Buffer and flow cytometry. C, quantification of cell cycle distribution of viable/euploid cells as obtained in B. D, quantification of the apoptotic hypodiploid (sub-G_1) cells as obtained in B. AU, arbitrary units.

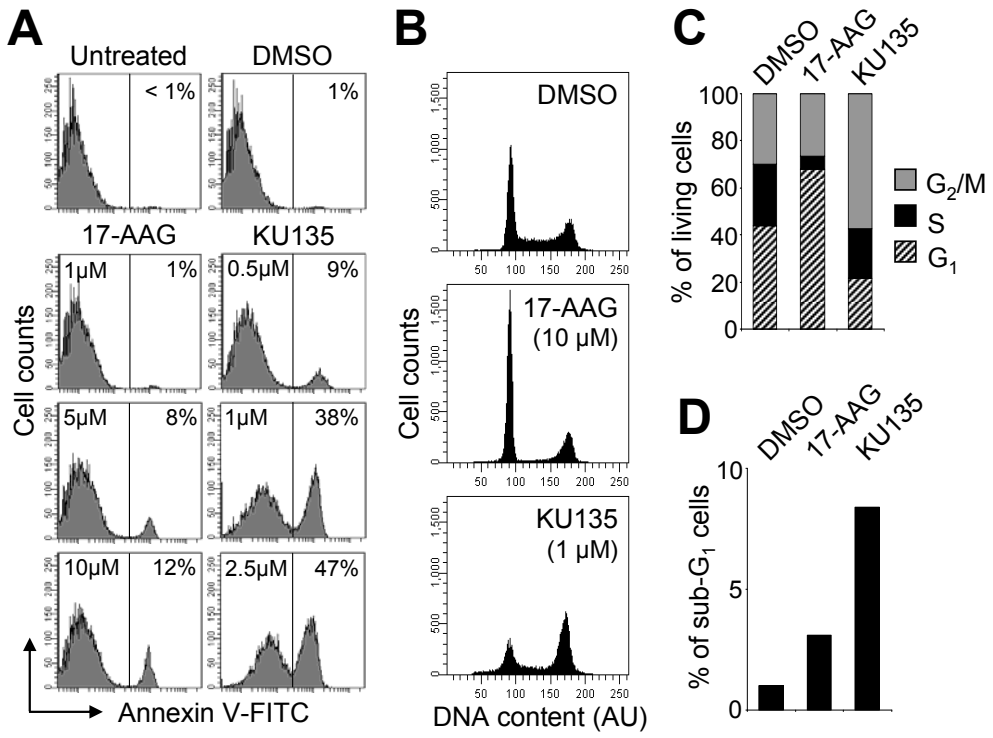

A
Untreated
DMSO
< 1%
1%
17-AAG
KU135
1μM
1%
0.5μM
9%
5μM
8%
1μM
38%
10μM
12%
2.5μM
47%
Cell counts
Annexin V-FITC
B
DMSO
17-AAG
(10 μM)
KU135
(1 μM)
Cell counts
DNA content (AU)
C
DMSO
17-AAG
KU135
% of living cells
G2/M
S
G1
D
DMSO
17-AAG
KU135
% of sub-G1 cells

Figure 17.

agreement with previous findings (Rahmani et al., 2003), 17-AAG exhibited a much milder apoptotic effect in Jurkat cells, even at the highest concentration of 10 μM, where only 12% of cells had externalized PS. Combined, these data suggest that the more potent antiproliferative effect of KU135 as compared to 17-AAG (Fig. 17) is due, at least in part, to the ability of KU135 to induce apoptotic cell death.

Because 17-AAG induced significantly less apoptosis than KU135, and yet was found to have an IC_{50} value relatively similar to that of etoposide (Fig. 16), we speculated that the antiproliferative activity of 17-AAG might involve an effect on cell cycle status. As illustrated in Fig. 17B and C, the distribution of non-apoptotic cells differed between KU135- and 17-AAG-treated cells at 24 h. Specifically, 66% and 26% of cells incubated with 17-AAG had accumulated in G_1 and G_2/M phases of the cell cycle, respectively. This was compared to KU135-treated cells in which 20% and 53% of cells had accumulated in G_1 and G_2/M phases of the cell cycle, respectively (Fig. 17B and C). In agreement with the low level of 17-AAG-induced apoptosis that was observed compared to KU135 as assessed by annexin V positivity (Fig. 17A), cell cycle distribution analysis revealed a considerably smaller fraction of sub-G_1 cells in response to 17-AAG than KU135 (Fig. 17B and D). The fact that the percentage of KU135-treated cells containing hypodiploid DNA content was low as compared to the amount of apoptosis that was observed using

annexin V-FITC and propidium iodide staining as an endpoint can likely be explained by the fact that DNA fragmentation is a late apoptotic event relative to PS externalization. Overall, these data indicate that the antiproliferative effect of KU135 is mechanistically different to that exhibited by 17-AAG.

5.3.3 Binding of KU135 to Hsp90 and the effect of KU135 and 17-AAG on the levels of Hsp90 isoforms and associated client proteins.

Next, using three complementary approaches, we tested the extent to which KU135 bound Hsp90. Initially, we performed novobiocin-binding experiments in which rabbit reticulocyte lysate was loaded onto a novobiocin-sepharose column in order to bind endogenous Hsp90. Thereafter, the column was washed extensively with 10 mM Tris-HCl (pH 7.4) buffer, containing 150 mM NaCl, and bound proteins were eluted with increasing concentrations of KU135 or 10 mM novobiocin. As shown in Fig. 18A, Hsp90 was eluted from the column with KU135 in a concentration-dependent manner (lanes 3-7). In contrast to novobiocin, which binds the C-terminus of Hsp90 (lane 2), the N-terminal Hsp90 inhibitor geldanamycin was unable to elute Hsp90 from the novobiocin-sepharose column (data not shown), which is consistent with previous studies by Neckers and coworkers (Marcu et al., 2000b). Next, proteolytic

Figure 18. KU135 binds Hsp90 and exhibits different effects than 17-AAG on the expression levels of Hsp90 isoforms and known client proteins. A, rabbit reticulocyte lysate (50 µl) was applied to a novobiocin-sepharose column after ATP regeneration and eluted with the indicated concentrations of KU135 and Western blotted. DMSO and 10 mM novobiocin were used as controls. B, rabbit reticulocyte lysate (50 µl) was incubated at 30 °C for 10 min with DMSO, 20 mM geldanamycin, novobiocin and molybdate (lanes 1–4, respectively) or 0, 1, 5, 10 and 20 mM KU135 (lanes 5–9). Subsequently, the reactions were chilled on ice and incubated for 6 min with 125 µg/mL trypsin and Western blotted. C – F, representative curves of surface plasmon resonance (SPR) analysis of KU135 binding to Hsp90β injected at the indicated concentrations (C), hyperbolic replot of the amount of KU135 bound versus concentration of KU135 (D), a scatchard plot of the binding of KU135 to Hsp90 (E), and hyperbolic replot of the amount of novobiocin bound versus concentration of novobiocin, corrected for nonspecific binding as described under "Materials and Methods" (F). G and H, cells (5 x 10^5/mL) were cultured with DMSO, 1 µM KU135, or 10 µM 17-AAG for 24 h, harvested, and lysed for Western blotting. β-actin was used as a loading control. RU, relative units.

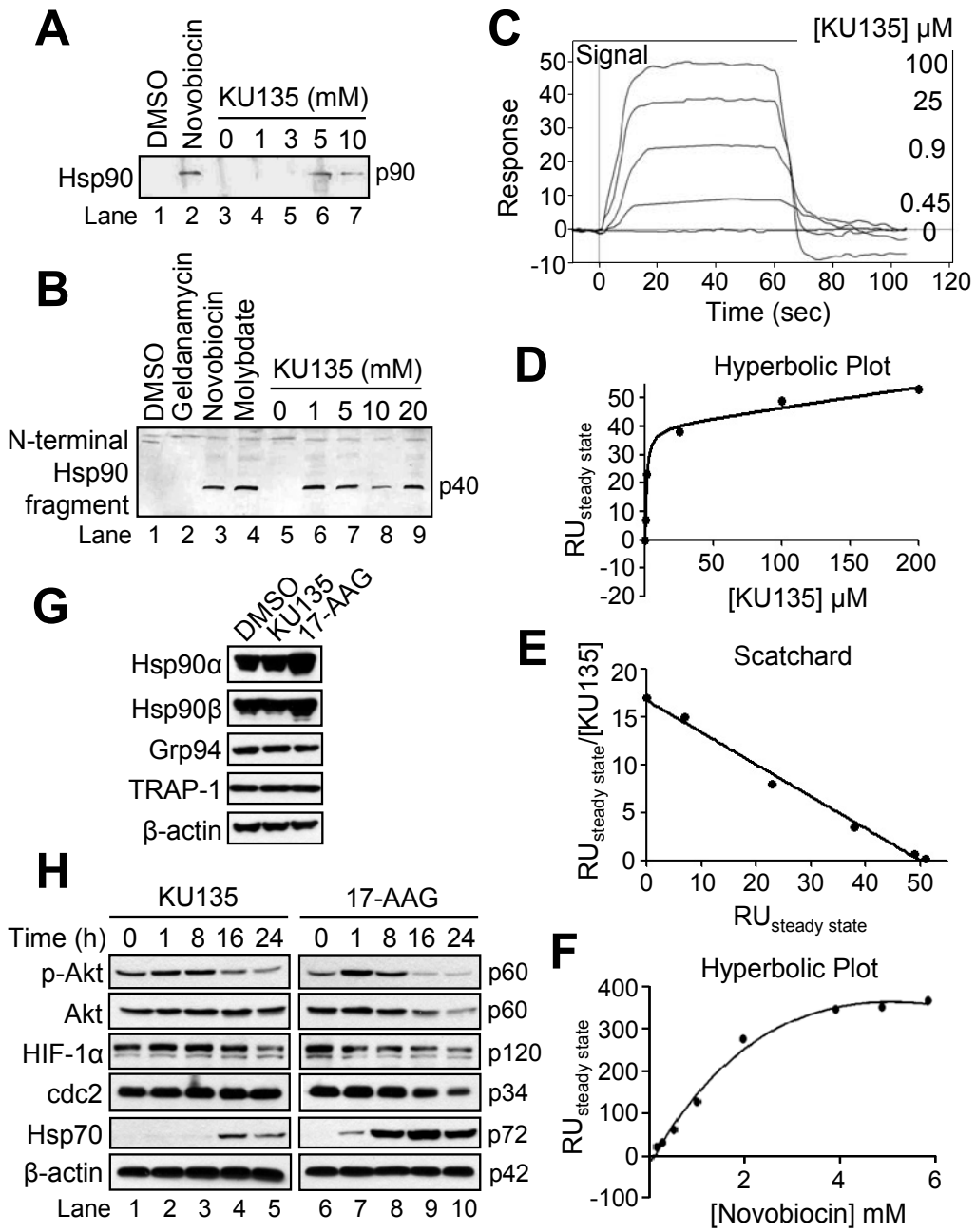

A
DMSO
Novobiocin
KU135 (mM)
0 1 3 5 10
Hsp90
p90
Lane 1 2 3 4 5 6 7
B
DMSO
Geldanamycin
Novobiocin
Molybdate
KU135 (mM)
0 1 5 10 20
N-terminal Hsp90 fragment
p40
Lane 1 2 3 4 5 6 7 8 9
C
[KU135] μM
Signal
100
25
0.9
0.45
0
Response
Time (sec)
D
Hyperbolic Plot
RU steady state
[KU135] μM
E
Scatchard
RU steady state/[KU135]
RU steady state
F
Hyperbolic Plot
RU steady state
[Novobiocin] mM
G
DMSO
KU135
17-AAG
Hsp90α
Hsp90β
Grp94
TRAP-1
β-actin
H
KU135
17-AAG
Time (h) 0 1 8 16 24 0 1 8 16 24
p-Akt
Akt
HIF-1α
cdc2
Hsp70
β-actin
p60
p60
p120
p34
p72
p42
Lane 1 2 3 4 5 6 7 8 9 10

Figure 18.

fingerprinting of Hsp90 in rabbit reticulocyte lysate was performed to determine whether binding of a C-terminal inhibitor, such as novobiocin, molybdate, and perhaps KU135, caused Hsp90 to adopt a conformation that differs from the tertiary structure it assumes upon binding of the N-terminal inhibitor geldanamycin (Fig. 18B) (Yun et al., 2004). Briefly, rabbit reticulocyte lysate was incubated at 37 °C for 5 min with DMSO, 20 mM geldanamycin, novobiocin, or molybdate (lanes 1–4, respectively), or 0, 1, 5, 10 and 20 mM KU135 (lanes 5–9), partially digested with trypsin, subjected to SDS-PAGE, and Western blotted with an Hsp90 antibody against the N-terminus of the protein. As shown in Fig. 18B, reticulocyte lysate incubated with KU135 produced a similar proteolytic fingerprint pattern as previously reported for novobiocin and molybdate (Yun et al., 2004). In contrast, incubation of reticulocyte lysate with geldanamycin (lane 2) resulted in no proteolytic fingerprinting. Combined, these data strongly suggest that KU135 binding of the C-terminus of Hsp90 in reticulocyte lysate exposes a cleavage site necessary for tryptic digestion and production of a 40-kDa N-terminal fragment of Hsp90.

To further support that Hsp90 specifically binds KU135, the interaction of KU135 with immobilized Hsp90β was measured by surface plasmon resonance spectroscopy. Fig. 18C shows representative curves for the binding of KU135 to Hsp90β. As illustrated in Fig. 18D and E, the binding of KU135 to Hsp90 was saturable with a calculated K_d of 1-2 μM

and a stoichiometry of 1 mole per mole of Hsp90β monomer. Thus, the high affinity binding of KU135 correlates directly with its potency in whole-cell assays (Fig. 16 and 17). By comparison, the K_d for novobiocin binding of Hsp90 was mathematically determined to be 3 ± 1 mM, and the half-maximal saturation concentration of novobiocin determined by extrapolation from the hyperbolic fitted curve was just below 1 mM (Fig. 18F). Taken together, these results demonstrate that Hsp90 exhibits a binding affinity for KU135 that is more than 500 times greater than its affinity for novobiocin. Significantly, Hsp90 binding affinity for KU135 is comparable to that reported for geldanamycin and 17-AAG (K_d approx. 1 μM) (Marcu et al., 2000b).

Currently, approximately 100-200 Hsp90-dependent client proteins have been identified. Many of these clients are oncogenic proteins (e.g., Akt, p-Akt, HIF-1α, cdc2, and Hsp70) that are involved in regulating signal transduction, cell growth, and apoptosis (Isaacs et al., 2003). In many instances, the molecular profile of Hsp90 inhibition includes depletion of numerous client proteins and induction of Hsp70 and/or Hsp90 in a concentration- and/or time-dependent manner (Banerji et al., 2005a; Banerji et al., 2005b). Because KU135 was found to be more potent than 17-AAG at inhibiting cell proliferation, we sought to investigate the extent to which this might correspond to differences in the expression profiles of the four different Hsp90 proteins (*i.e.*, Hsp90α, Hsp90β, Grp94, and

TRAP1), the depletion of associated client proteins, and/or the induction of Hsp70.

As illustrated in Fig. 18G, Jurkat cells constitutively express Hsp90α, Hsp90β, Grp94, and TRAP-1, and incubation with 17-AAG, but not KU135, for 24 h led to a considerable increase in the expression levels of Hsp90α and Hsp90β. In addition, both KU135 and 17-AAG caused significant alterations in the level of known Hsp90 client proteins (Fig. 18H). Specifically, treatment with 17-AAG or KU135 caused a time-dependent decrease in the level of p-Akt. We also found that incubation with KU135 or 17-AAG caused a decrease in the level of Hif-1α over 24 h, whereas only 17-AAG was found to affect the expression level of the cell cycle regulator cdc2. Significantly, Fig. 18H also illustrates that Hsp70 expression was induced to a far greater extent in cells treated with 17-AAG than cells treated with KU135. Collectively, these findings demonstrate that both 17-AAG and KU135 effectively inhibit the chaperoning function of Hsp90. Furthermore, the fact that the expression of Hsp90α, Hsp90β, and Hsp70 were markedly increased only in response to 17-AAG might partially explain why KU135 was found to exert more potent cytotoxic effects than 17-AAG (Fig. 17).

5.3.4 KU135-induced apoptosis occurs independently of the extrinsic pathway.

In light of the finding that KU135 was a potent inducer of apoptosis, we next investigated the extent to which this effect involved the extrinsic (receptor-mediated) pathway. To test this possibility, we used a clone of Jurkat cells lacking caspase-8 (I 9.2). The absence of this protein was confirmed previously by Western blot analysis, and this clone was found to be completely resistant to Fas-induced apoptosis (Shelton et al., 2009). As illustrated in Fig. 19A, cells lacking caspase-8 were as sensitive as control A3 cells to KU135-induced apoptosis at 24 h, as determined by annexin V-FITC and propidium iodide fluorescence. In addition, Western blot analysis of cell lysates obtained at 24 h post-treatment revealed that KU135 (1 μM) caused robust processing of procaspase-9, -3, and -2 (Fig. 19B). Further, caspase-3-like (DEVDase) activity was stimulated to the same extent in caspase-8-null cells as control A3 cells when incubated in the presence of KU135 for 24 h (data not shown). Although caspase-9 underwent proteolytic cleavage in response to 17-AAG (Fig. 19B, lanes 3 and 6), only the p37 inactive form of this caspase was observed (Srinivasula et al., 1998; Denault et al., 2007). Combined, these findings confirm that 17-AAG is a relatively poor inducer of apoptosis (*cf.* Fig. 17) and demonstrate that KU135-induced apoptosis occurs independently of the extrinsic (receptor-mediated) pathway.

5.3.5 KU135-induced apoptosis relies heavily on the intrinsic pathway.

Figure 19. KU135-induced apoptosis occurs independently of the receptor-mediated apoptotic pathway. A, cells (5 x 10^5/mL) were cultured in the presence or absence of DMSO, 1 μM KU135, or 10 μM 17-AAG, harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as live (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic (upper left). Numbers refer to the percentage of cells in each quadrant. B, duplicate aliquots of cells in *A* were harvested and lysed for Western blotting. Casp, caspase.

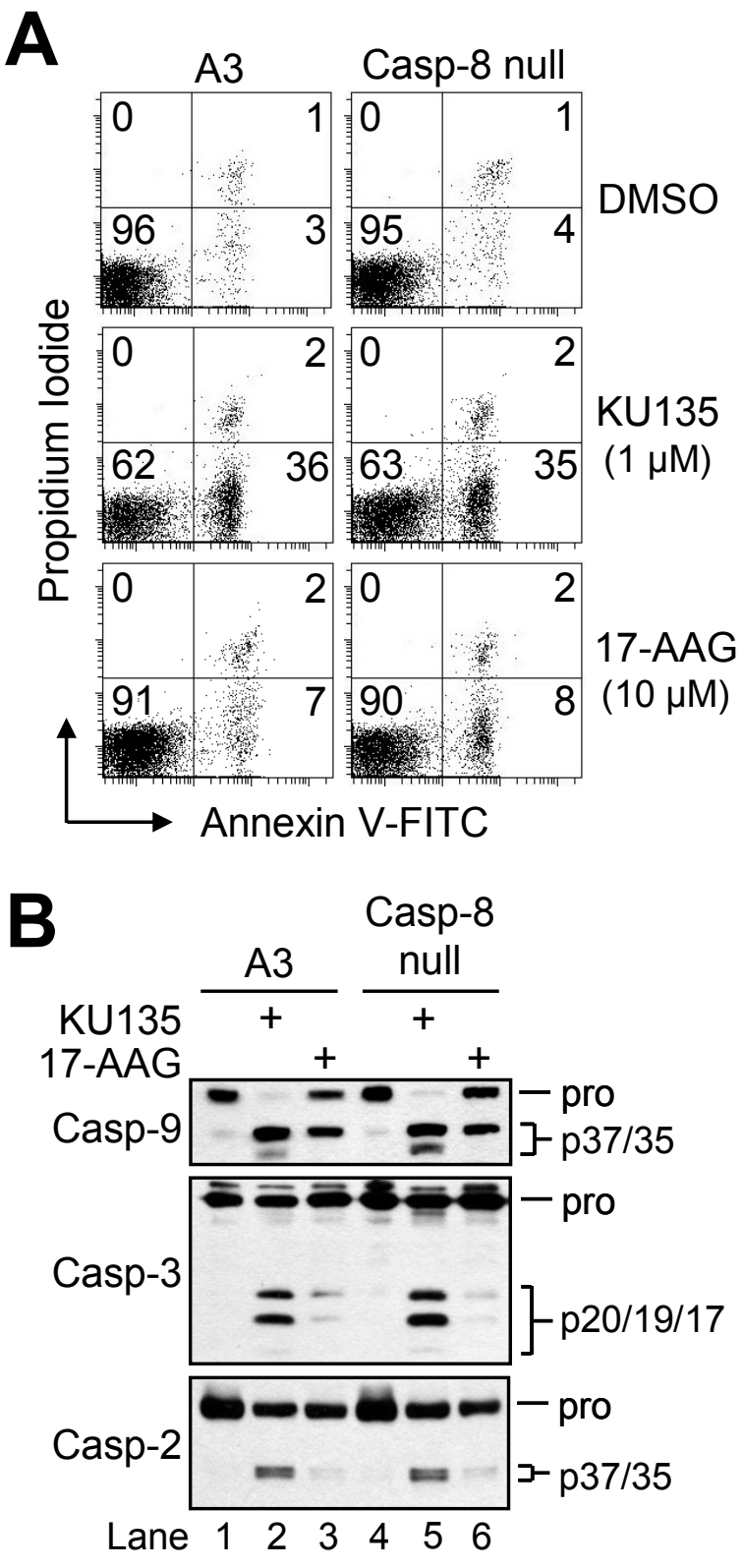

A
A3
Casp-8 null
0
1
96
3
95
4
DMSO
0
2
62
36
63
35
KU135
(1 μM)
0
2
91
7
90
8
17-AAG
(10 μM)
Propidium Iodide
Annexin V-FITC
B
A3
Casp-8
null
KU135
17-AAG
+
+
+
+
Casp-9
pro
p37/35
Casp-3
pro
p20/19/17
Casp-2
pro
p37/35
Lane 1 2 3 4 5 6

Figure 19.

We next investigated whether the intrinsic (mitochondria-mediated) pathway was responsible for KU135-induced cell killing. For these experiments, we took advantage of Apaf-1-deficient Jurkat cells (Franklin and Robertson, 2007), as well as cells in which we had overexpressed Bcl-2 or Bcl-x_L (Shawgo et al., 2008b). Apaf-1 is strictly required for apoptosome-mediated activation of initiator caspase-9 within the intrinsic pathway, whereas Bcl-2 and Bcl-x_L are well characterized anti-apoptotic proteins whose overexpression is known to inhibit intrinsic apoptosis by preventing MOMP and the release of intermembrane space pro-apoptotic proteins (e.g., cytochrome *c* and Smac) into the cytosol.

As illustrated in Fig. 20A, cells lacking Apaf-1 or overexpressing Bcl-2/Bcl-x_L were resistant to KU135-induced apoptosis, having undergone only 3-6% apoptosis after incubation with KU135 (1 μM) for 24 h, whereas control-transfected cells had undergone 40% apoptosis at the same time-point. In agreement with these findings, Western blot analysis of cell lysates obtained at 24 h post-KU135 treatment revealed that extensive proteolytic processing of procaspase-9, -3, and -2 occurred in wild-type and control-transfected cells (Fig. 20B). Whereas proteolytic processing of these three caspases did not occur in Bcl-2- or Bcl-x_L-overexpressing cells, some processing was observed in Apaf-1-deficient cells (Fig. 20B), despite their being resistant to KU135-induced apoptosis. However, the cleavage of caspase-9 in response to KU135 in the wild-

Figure 20. KU135-induced apoptosis relies on the mitochondria-mediated apoptotic pathway. A, cells (5 x 10^5/mL) were cultured in the presence or absence of DMSO or 1 μM KU135 for 24 h, harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as live (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic (upper left). Numbers refer to the percentage of cells in each quadrant. B, duplicate aliquots of cells in *A* were harvested and lysed for Western blotting. C, cells (5 x 10^5/mL) were cultured in the presence or absence of DMSO or 1 μM KU135 for 24 h, harvested, and processed for determination of Bak oligomerization by Western blotting. D and E, duplicate aliquots of cells in C were processed for subcellular fractionation into supernatant (s) and pellet (p) fractions (D) or for mitochondrial membrane potential (ΔΨ) determination by flow cytometry (E). Reduced $DiIC_1(5)$ fluorescence is indicative of a loss of ΔΨ, and numbers refer to the percentage of cells that underwent a dissipation of ΔΨ. BMH, bismaleimidohexane; Cyt c, cytochrome *c*; Casp, caspase.

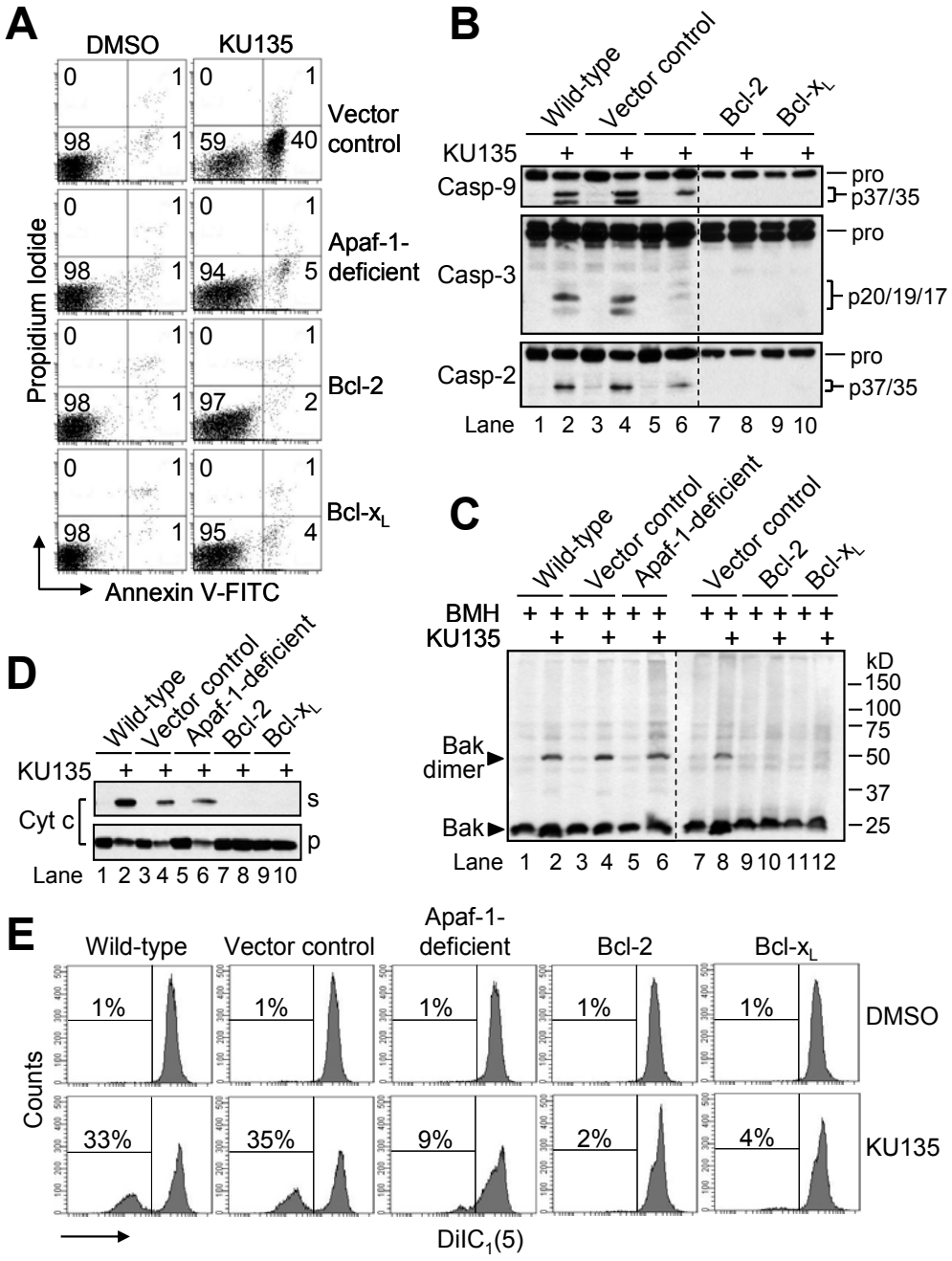

A
DMSO
KU135
Vector control
Apaf-1-deficient
Bcl-2
Bcl-xL
Propidium Iodide
Annexin V-FITC
B
Wild-type
Vector control
Bcl-2
Bcl-xL
KU135
Casp-9
Casp-3
Casp-2
pro
p37/35
p20/19/17
Lane 1 2 3 4 5 6 7 8 9 10
C
Wild-type
Vector control
Apaf-1-deficient
Vector control
Bcl-2
Bcl-xL
BMH
KU135
kD
150
100
75
50
37
25
Bak dimer
Bak
Lane 1 2 3 4 5 6 7 8 9 10 11 12
D
Wild-type
Vector control
Apaf-1-deficient
Bcl-2
Bcl-xL
KU135
Cyt c
s
p
Lane 1 2 3 4 5 6 7 8 9 10
E
Wild-type
Vector control
Apaf-1-deficient
Bcl-2
Bcl-xL
DMSO
KU135
Counts
1%
33%
35%
9%
2%
4%
DiIC1(5)

Figure 20.

type and control-transfected cells produced two fragments (p37/p35), whereas only one caspase-9 cleavage fragment (p37) was detected in the Apaf-1-deficient cells (Fig. 20B). This finding and the observation made with 17-AAG (Fig. 19B) are significant because apoptosome-dependent activation of caspase-9 yields the p35 fragment, whereas the p37 form of caspase-9 is generally thought to be produced by caspase-3-mediated cleavage and to be catalytically inactive (Srinivasula et al., 1998; Denault et al., 2007). However, only a trace amount of active caspase-3 was detected in both instances (Fig. 20B and 19B), suggesting that the generation of the p37 fragment of caspase-9 may be caspase-3-independent. Finally, caspase-2 also underwent some proteolytic cleavage in KU135-treated Apaf-1-deficient cells (Fig. 20B). However, whether proteolysis of caspase-2 in this context reflects its activation or whether caspase-2 is being cleaved as an "innocent bystander" remains to be determined. Notwithstanding, these data demonstrate that the antiproliferative effect of KU135 involves an ability to induce intrinsic (mitochondria-mediated) apoptosis.

We next evaluated the extent to which mitochondrial endpoints typically associated with MOMP, including Bak/Bax activation, the release of mitochondrial intermembrane space proteins (e.g., cytochrome *c*), and the loss of mitochondrial membrane potential ($\Delta\Psi$), were inhibited in KU135-resistant cells. As illustrated in Fig. 20C, wild-type, control-

transfected, and Apaf-1-deficient cells treated with 1 μM KU135 for 24 h activated Bak (Jurkat E6.1 cells do not express Bax) as evidenced by the presence of Bak dimers in these cells using the cross-linking agent bismaleidmidohexane. In agreement with these data, KU135 induced cytochrome *c* release in wild-type, control-transfected, and Apaf-1-deficient cells (Fig. 20D). Both Bak oligomerization and cytochrome *c* release were completely inhibited in Bcl-2- or Bcl-x_L-overexpressing cells incubated under the same conditions (Fig. 20C and D). Finally, we evaluated the different clones for KU135-induced changes in ΔΨ at 24 h (Fig. 20E). Wild-type and control-transfected cells incubated in the presence of 1 μM KU135 showed that 35% of cells had lost ΔΨ, whereas cells overexpressing Bcl-2 or Bcl-x_L had retained ΔΨ. By comparison, Apaf-1-deficient cells, which were found to be resistant to KU135, despite activating Bak, releasing cytochrome *c*, and cleaving certain caspases, largely retained ΔΨ (Fig. 20E). Overall, these findings offer support of a model of KU135-induced apoptosis that requires the involvement of the mitochondrial pathway.

5.4 Discussion

Hsp90 represents a family of ATPase-containing molecular chaperones that comprise as much as 1-2% of total cellular protein under

unstressed conditions. In response to various stressors, this amount can increase to 4-6% (Whitesell and Lindquist, 2005). Hsp90 is often present at elevated levels in cancer cells and functions to stabilize oncogenic proteins involved in signal transduction, growth, and apoptosis regulation (Isaacs et al., 2003). A possible explanation for Hsp90 overexpression in cancer cells is that malignant cells are in a persistent state of proteotoxic stress due, in part, to acidosis and/or the accumulation of mutated signaling molecules that would otherwise result in cell lethality. In this way, the presence of Hsp90 (as well as other heat shock proteins) is thought to compensate for the hostility of many cancer cell microenvironments (Whitesell and Lindquist, 2005; Solit and Rosen, 2006). As mentioned previously, there are currently between one and two hundred reported cytosolic and nuclear client proteins for Hsp90, including protein kinases (e.g., Akt and Her2), transcription factors (e.g., mutant p53 and HIF-1α), chimeric signaling proteins (e.g., Bcr-Abl), and several proteins involved in apoptosis (e.g., Bid, Apaf-1, and RIP) (for an updated list see http://www.picard.ch/downloads/downloads.htm). Significantly, whereas many of the aforementioned Hsp90 client proteins are being pursued *individually* as targets for anti-cancer drug development, inhibition of Hsp90 would prevent the maturation and stabilization of numerous Hsp90 client proteins simultaneously, leading to their ultimate degradation within the ubiquitin-proteasome pathway (Whitesell et al., 1994; Schulte et

al., 1995). In this regard, Hsp90 has emerged as an exciting novel target for cancer chemotherapeutic drug design because inhibition of its chaperoning function simultaneously leads to the destabilization and degradation of multiple oncogenic proteins.

Although one of the earliest Hsp90 inhibitors, 17-AAG, which targets the N-terminal ATP-binding pocket of Hsp90, has entered clinical trials for refractory malignancies, it has shown little clinical effect as monotherapy (Goetz et al., 2005; Nowakowski et al., 2006). Further, even though 17-AAG is less toxic than its parent compound geldanamycin, its widespread use is still hindered by concerns regarding its hepatotoxicity (Egorin et al., 1998; Solit et al., 2007). A new approach to targeting Hsp90 began with the observation that the antibiotic novobiocin binds with low affinity to a C-terminal ATP-binding pocket (Marcu et al., 2000a; Marcu et al., 2000b). More potent analogues of novobiocin have been developed, and we report here that one such lead candidate inhibitor, designated KU135, binds directly to Hsp90 and suppresses cell proliferation by engaging signaling pathways that are mechanistically different from those affected by 17-AAG.

Indeed, KU135 was found to exert more potent antiproliferative effects than 17-AAG. Whereas 17-AAG predominantly induces cell cycle arrest, KU135 causes both cell cycle arrest and an induction of apoptosis. This is significant since an Hsp90 inhibitor exhibiting combined cytostatic

and cytotoxic effects would be expected to eradicate tumor cells and thereby lower tumor cell burden, while a purely cytostatic inhibitor would only inhibit tumor cell growth/division. A possible explanation for why 17-AAG is not able to induce a similar apoptotic response as KU135 is that differences may exist in the number and types of client proteins that are affected by an N-terminal (17-AAG) versus a C-terminal (KU135) Hsp90 inhibitor. Another possibility is that the relative absence of cytotoxic activity with 17-AAG is due to the robust upregulation of Hsp90α, Hsp90β, and Hsp70 that is observed compared to KU135.

Overall, the data presented here provide compelling evidence for the continued development of novobiocin-based C-terminal Hsp90 inhibitors as promising alternatives to N-terminal inhibitors, such as 17-AAG, for the treatment of human malignancies. KU135 is one such lead candidate novobiocin-derived compound. Experiments are currently ongoing in the laboratory to characterize the specific molecular ordering of the caspase activation cascade induced by KU135, as well as the molecular requirements necessary for MOMP to occur in response to this compound.

Chapter 6: Caspase-9 activation is Essential for Heat-induced Apoptosis in Jurkat Cells

6.1 Abstract

Exposure of cells to heat is known to induce apoptosis, although the underlying mechanisms remain poorly understood. In the present study, we examined the molecular requirements necessary for heat-induced apoptosis using a panel of genetically modified Jurkat T-lymphocytes. Wild-type Jurkat cells cultured at 44°C for 1 h underwent apoptosis as determined by caspase activation and phosphatidylserine externalization. By comparison, cells stably overexpressing Bcl-2/Bcl-X_L or stably depleted of Apaf-1 were completely resistant to heat-induced apoptosis, implicating the involvement of the mitochondria-mediated pathway in this form of cell death. Pretreatment of wild-type cells with a biotinylated general caspase inhibitor (b-VAD-fmk) both inhibited heat-induced apoptosis and trapped activated initiator caspase-2, 8,and 9. In spite of this finding, however, cells deficient in either caspase-8 or the caspase-2 adaptor RAIDD were highly susceptible to heat-induced apoptosis. Furthermore, b-VAD-fmk pretreatment failed to label any activated caspase in Apaf-1-deficient cells exposed to heat. Additionally, cells stably lacking Apaf-1 or the BH3-only protein Bid displayed less heat-induced Bak activation, cytochrome *c* release, and loss of $\Delta\Psi$, even

though cleavage of Bid to tBid occurred downstream of caspase-9 activation. Combined, these data suggest that caspase-9 is the critical apical caspase activated during heat-induced apoptosis and that tBid may function to promote cytochrome *c* release during this process as part of a feed forward amplification loop.

6.2 Introduction

Exposure to elevated temperatures is known to induce an evolutionarily conserved adaptive response known as the heat shock response. A key feature of this response includes transcriptional upregulation of several heat shock proteins (Hsp), which is thought to protect the organism from proteotoxic stress and lethality (Jolly and Morimoto, 2000). In addition, induction of the heat shock response is known to offer protection against subsequent exposure to otherwise lethal cellular stressors, including chemotherapeutic agents (Parsell et al., 1993). Due to the harsh microenvironment of many tumors, induction of the heat shock response is often seen in tumorigenic cells where it is thought to play a key role in oncogenesis and therapeutic resistance (Jolly and Morimoto, 2000). However, initial exposure to more severe or prolonged elevated temperatures, is known to bypass this protective heat shock response and induce an apoptotic cellular death (Milleron and

Bratton, 2007b). The ability of hyperthermia to induce cell death has led to its successful use in the treatment of a variety of cancers (Wust et al., 2002; Kouloulias et al., 2005), and interest has been gaining in developing ways to directly target tumorigenic cells with heat (Ito et al., 2006; Pissuwan et al., 2006).

There are two distinct apoptotic pathways, namely, the mitochondria-mediated (i.e. intrinsic) pathway and the receptor-mediated (i.e. extrinsic) pathway. The extrinsic pathway is activated upon binding of death ligand to its cognate receptor (e.g. Fas binding to the Fas receptor), which causes the receptors to move within close proximity to one another and recruit an adaptor protein, such as FADD, followed by recruitment of an initiator procaspase, such as caspase-8 (Kischkel et al., 1995; Boatright and Salvesen, 2003). This protein complex is termed the death-inducing signaling-complex (DISC) and serves as the activating platform for initator caspases 8 and 10 during receptor-mediated apoptosis. In so-called type I cells, there is sufficient activation of caspase-8 at the DISC to directly cleave and activate effector caspase-3 resulting in execution of apoptosis. However, in so-called type II cells, activated caspase-8 does not directly cleave and activate a sufficient amount of effector caspase-3 to execute apoptosis. In this cell type, activated caspase-8 cleaves the pro-apoptotic Bcl-2 family member, Bid, to truncated-Bid (tBid) which then engages the mitochondria-mediated pathway (Barnhart et al., 2003).

Mitochondria-mediated apoptosis is activated following exposure to cytotoxic stressors, including DNA damage, growth factor withdrawal, and γ-radiation. During intrinsic apoptosis, caspase activation is largely regulated by the Bcl-2 family of proteins. The Bcl-2 family of proteins contains both pro- and anti-apoptotic members and largely function to either promote or inhibit mitochondrial outer membrane permeabilization (MOMP). MOMP allows the release of cytochrome *c* from the intermembrane space of mitochondria into the cytosol where it is required for caspase activation (Jiang and Wang, 2004). Cytosolic cytochrome *c* interacts with the adaptor protein, apoptotic protease activating factor-1 (Apaf-1), which then recruits initiator caspase-9 to this protein complex, termed the apoptosome (Jiang and Wang, 2004). This protein complex serves as the activating platform for initiator caspase-9. Activated initiator caspase-9 cleaves and activates downstream effector casapses which then cleave various target substrates resulting in the biochemical and morphological characteristics associated with apoptotic cell death.

Although adaptive cellular responses to heat have been studied for decades, heat-induced apoptosis has been studied to a much lesser extent and conflicting results have emerged from these studies (Milleron and Bratton, 2006; Tu et al., 2006; Milleron and Bratton, 2007b). In this regard, the aim of the current study was to help determine the molecular requirements necessary for heat-induced apoptosis. Because several of

the more recent studies have used Jurkat T-lymphocytes as a model system to investigate apoptosis induced by elevated temperatures, we also used a large panel of genetically modified Jurkat cells in which key steps in the intrinsic or extrinsic pathway were inhibited. In agreement with previous studies, our results indicated that heat-induced apoptosis relies heavily on the mitochondria-mediated apoptotic pathway. Significantly, although caspases 2, 8, and 9 were affinity labeled as apical proteases, subsequent experiments revealed that only activated caspase-9 was strictly required for heat-induced apoptosis. Bid was observed to play a role in this form of apoptosis, although its cleavage to tBid occurred downstream of Apaf-1.

6.3 Results and Discussion

6.3.1 Heat induces Jurkat cells to undergo apoptosis in a Bcl-2- and Bcl-X_L-inhibitable manner

Previous studies have suggested several different mechanisms for the initiation of heat-induced apoptosis that, in many cases, are difficult to reconcile. For instance, it has been suggested that caspase-2 is the most apical caspase activated during heat-induced apoptosis (Tu et al., 2006; Bouchier-Hayes et al., 2009), and that caspase-2 functions by cleaving

Bid to tBid (Bonzon et al., 2006), which then induces MOMP. However, a different study suggested that the apical caspase activated in response to heat is a z-VAD-inhibitable protease that functions, at least in part, by directly cleaving and activating caspase-3, implying that caspase-9 may not always be necessary (Milleron and Bratton, 2006; Milleron and Bratton, 2007b). In addition, a very recent study found that Mcl-1 degradation is important in response to heat where its ubiquitination disrupts the binding between Mcl-1 and Noxa which, in turn, facilitates Bax activation (Stankiewicz et al., 2009); however, this is in contrast to a different study indicating that Bax and/or Bak can be activated directly by heat (Pagliari et al., 2005).

Because of the conflicting and, in some cases, contradictory nature of the reported data, we set out to better characterize and resolve some of the confusion relating to the mechanistic details of heat-induced apoptosis. As illustrated in Fig. 21A, exposure of wild-type Jurkat cells to1 h of heat at 44°C, followed by a 6 h recovery time period at 37°C caused 32% of the cells to undergo apoptosis as determined by annexin V-FITC and propidium iodide co-staining followed by flow cytometric analysis. Heat-induced apoptosis was accompanied by proteolytic cleavage of caspases 9, 3, and 2, as well as an increase in caspase-3-like (DEVDase) activity (Fig. 21, B and C). In addition, incubation of the pan-caspase inhibitor, qVD-OPH (20 uM), for 1 h prior to heat exposure completely

prevented cell death (data not shown). Next, the requirement of MOMP in response to heat-induced apoptosis was evaluated in clones of Jurkat cells described previously (Shawgo et al., 2008b) that do not undergo MOMP due to the overexpression of Bcl-2 or Bcl-X_L. As illustrated in Fig. 21D, Jurkat cells overexpressing either Bcl-2 or Bcl-X_L were completely resistant to heat-induced apoptosis. Taken together, these data suggest that heat-induced cell death is a caspase-mediated apoptotic event that requires MOMP.

6.3.2 Caspase-2, -8, and -9 are activated apically during heat-induced apoptosis

Execution of apoptosis requires the activation of the caspase cascade, where active initiator caspases cleave and thereby activate downstream effector caspases that, in turn, dismantle the cell. Whereas proteolytic cleavage of an effector caspase is indicative of its activation, the same is not necessarily true with initiator caspases. There are currently two prevailing models that describe initiator caspase activation. The induced proximity model suggests that the adaptor molecules required for initiator caspase activation function to bring caspases into close proximity leading to caspase homo-dimerization and subsequent activation (Boatright and Salvesen, 2003). By comparison, the induced

Figure 21. Heat induces Jurkat cells to undergo apoptosis in a Bcl-2- and Bcl-X_L-inhibitable manner. *A*, wild-type cells (10^6/mL) were heat shocked for 1 h at 44°C followed by a 6 h recovery period at 37°C, cells were then harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and PI staining. Quadrants are defined as: live (lower left); early apoptotic (lower right); late apoptotic (upper right); and necrotic (upper left). Numbers refer to the percentage of cells in each quadrant. *B-C*, duplicate aliquots of cells were harvested and lysed for *(B,)* Western blotting of caspases *(C)*, or caspase (DEVDase) activity measurements. *D*, control-transfected or Bcl-x_L- and Bcl-2-overexpressing, Jurkat cells (10^6/mL) were heat shocked for 1 h at 44°C followed by a 6 h recovery period at 37°C, harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and PI staining. Quadrants are defined as in *A.*

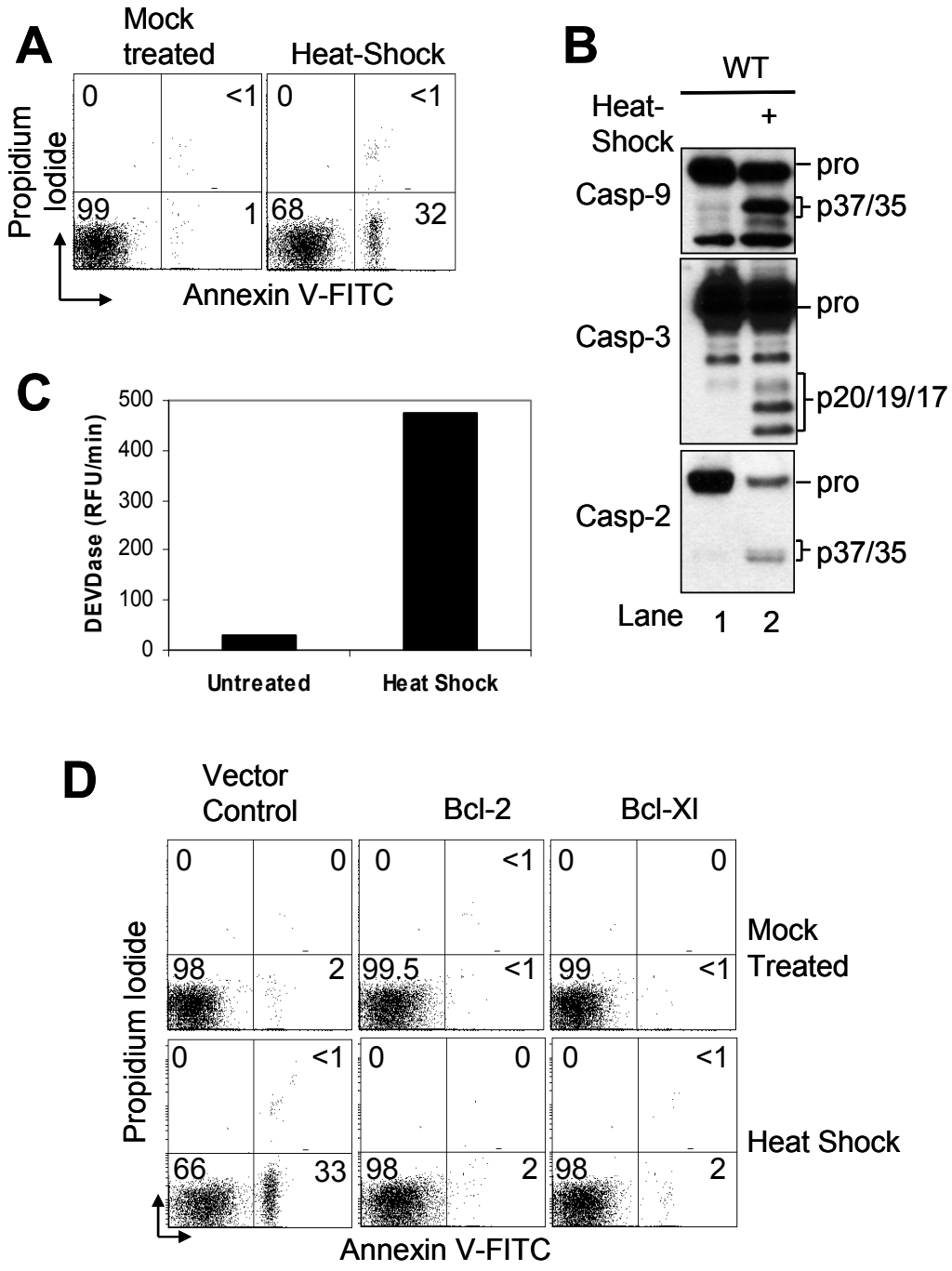

A
Mock treated
Heat-Shock
Propidium Iodide
0
<1
0
<1
99
1
68
32
Annexin V-FITC
B
WT
Heat-Shock
+
Casp-9
pro
p37/35
Casp-3
pro
p20/19/17
Casp-2
pro
p37/35
Lane 1 2
C
DEVDase (RFU/min)
500
400
300
200
100
0
Untreated
Heat Shock
D
Vector Control
Bcl-2
Bcl-Xl
Propidium Iodide
0
0
0
<1
0
0
98
2
99.5
<1
99
<1
Mock Treated
0
<1
0
0
0
<1
66
33
98
2
98
2
Heat Shock
Annexin V-FITC

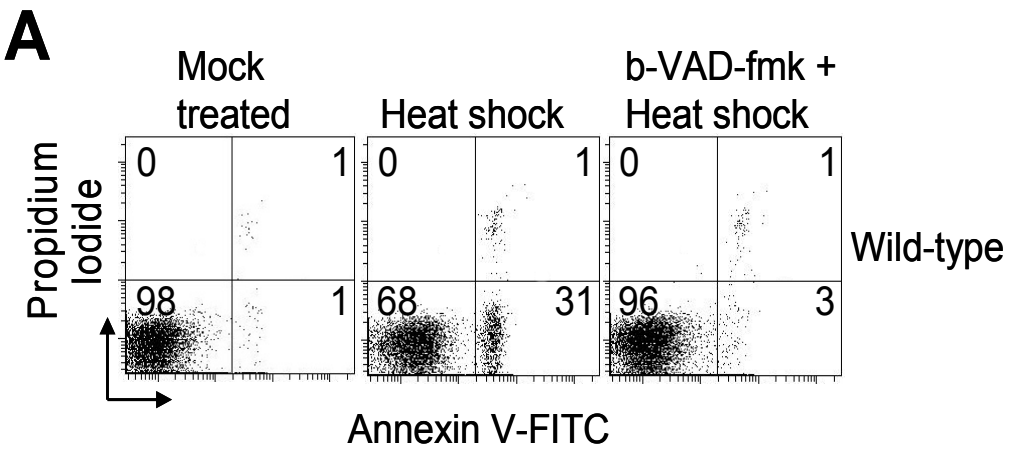
Figure 21.

conformation model suggests that initiator caspases undergo a conformational change upon binding to an adaptor protein resulting in activation (Bao and Shi, 2007). Neither model suggests that cleavage is an activating event, although it was recently shown that dimerization and cleavage are necessary for caspase-8 activation (Oberst *et al.*).

Because proteolytic cleavage of initiator caspases does not necessarily reflect their activation, and because so-called specific peptide substrates are not specific (McStay et al., 2008), it has been technically challenging to unambiguously identify the most apical caspase activated in response to a given insult. A recent study, however, used an innovative approach to affinity label or "trap" initiator caspases as they become activated inside cells (Tu et al., 2006). The method relies on a biotinylated form of the general caspase inhibitor z-VAD-fmk and immobilized streptavidin (Boatright et al., 2003).

Using this approach to identify the most apical initiator caspase(s) activated in response to heat, we pretreated wild-type Jurkat cells with b-VAD-fmk (120 μM) for 3 h at 37°C prior to a 1 h exposure to 44°C and a 6 h recovery at 37°C. As illustrated in Fig. 22A, b-VAD-fmk completely inhibited heat-induced apoptosis as determined by annexin V-FITC and propidium iodide co-staining. Next, having demonstrated that b-VAD-fmk inhibited cell death, we lysed the cells and incubated them in the presence of immobilized streptavidin to pull down any activated caspases. As

Figure 22. Caspase-2, -8, and -9 are activated apically during heat-induced apoptosis. *A*, wild-type cells (10^6/mL) were preincubated in the presence or abasece of 120 μM b-VAD-fmk for 3 h then heat shocked for 1 h at 44°C followed by a 6 h recovery period at 37°C. Cells were then harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and PI staining. Quadrants are defined as: live (lower left); early apoptotic (lower right); late apoptotic (upper right); and necrotic (upper left). Numbers refer to the percentage of cells in each quadrant. *B*, duplicate aliquots of cells were harvested, lysed, and incubated with strepavidin beads followed by washing and boiling of beads with sample buffer followed by Western blot analysis.


A
Mock treated
Heat shock
b-VAD-fmk + Heat shock
Propidium Iodide
0
1
98
1
0
1
68
31
0
1
96
3
Wild-type
Annexin V-FITC

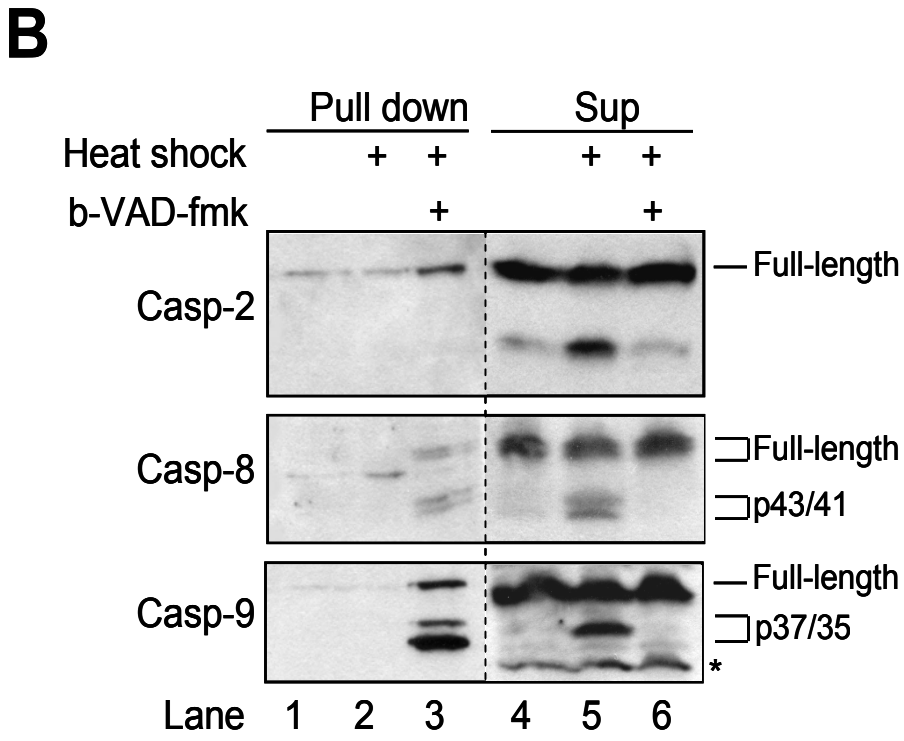

B
Pull down
Sup
Heat shock
+ + + +
b-VAD-fmk
+ +
Casp-2
Full-length
Casp-8
Full-length
p43/41
Casp-9
Full-length
p37/35
*
Lane 1 2 3 4 5 6

Figure 22.

illustrated in Fig. 22B, initiator caspases 2, 8, and 9 were affinity labeled, although the intensity of the bands detected were different with caspase-9 showing the greatest intensity. Taken together, these data indicate that b-VAD-fmk both effectively inhibits apoptosis and labels initiator caspases 2, 8, and 9 as the apical caspases activated in response to heat.

6.3.3 RAIDD is dispensable during heat-induced apoptosis

Because we had observed, in agreement with a recent study (Tu et al., 2006), that caspase-2 was activated apically in response to heat, we next sought to determine the significance of its activation. To do so, we used the pSUPER vector-based system that directs the synthesis of siRNAs and a recently validated target (Shi et al., 2009) to silence caspase-2. Two single-cell caspase-2-deficient Jurkat clones (#8 and #14) were used for these studies, and the extent to which caspase-2 was suppressed was confirmed by Western blot (Fig. 23A). To test the extent to which caspase-2 activation was important for heat-induced apoptosis, we incubated the individual clones for 1 h at 44°C and evaluated them for differences in annexin V-FITC fluorescence after a 6-h recovery at 37°C. Unexpectedly, despite a very similar level of caspase-2 knockdown between the two clones (#8 and #14), the percentage of cells undergoing heat-induced apoptosis was markedly dissimilar at 37% and 12%,

Figure 23. RAIDD is dispensable during heat-induced apoptosis. *A*, vector control or two single-cell Caspase-2-deficient Jurkat clones (#8 and #14) were harvested and lysed for Western blotting. Actin was used as a loading control. *B*, cells (10^6/mL) were cultured in the presence or absence of 1 h at 44°C followed by a 6 h recovery period,then harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as live (*lower left*), early apoptotic (*lower right*), late apoptotic (*upper right*), and necrotic (*upper left*). *Numbers* refer to the percentage of cells in each quadrant. *C*,. vector control or two single-cell RAIDD-deficient Jurkat clones (2C3 and 1C7) were harvested and lysed for Western blotting. Actin was used as a loading control. *B*, cells (10^6/mL) were cultured in the presence or absence of 1 h at 44°C followed by a 6 h recovery period, then harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as in *B.*

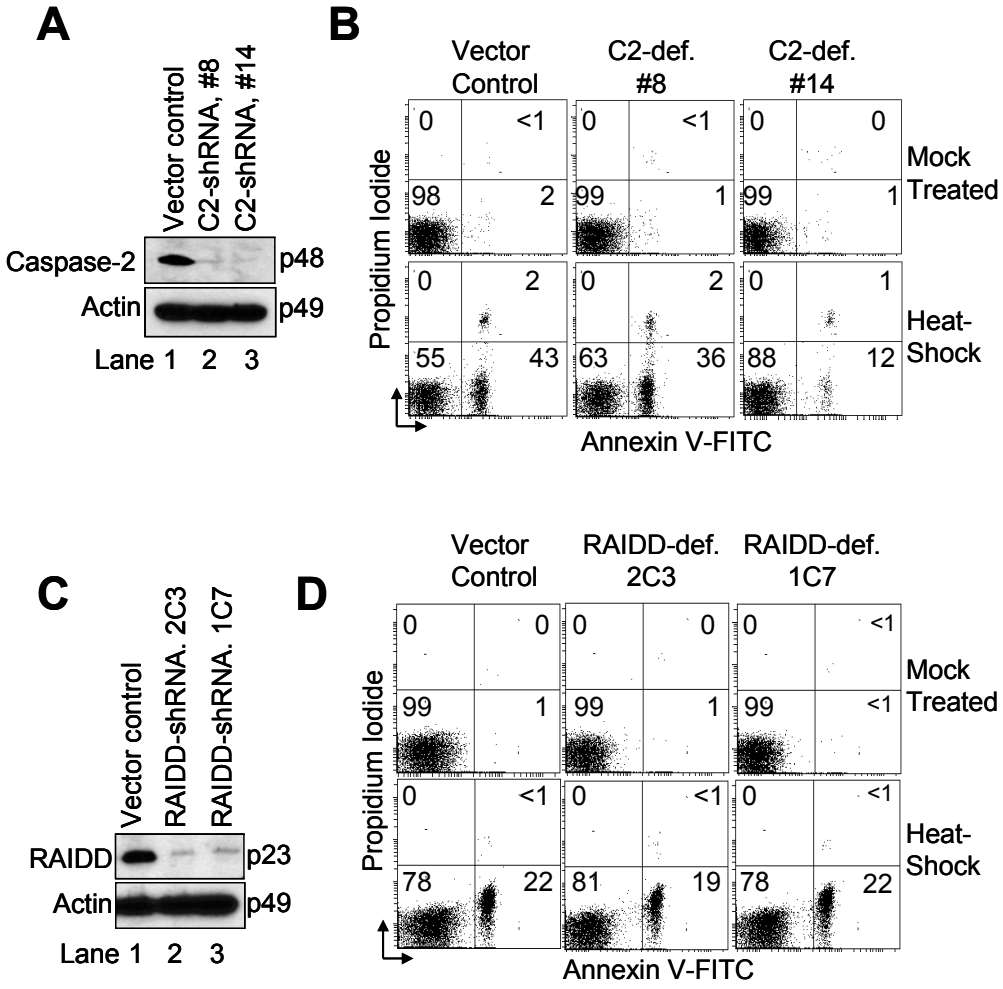

A
Vector control
C2-shRNA, #8
C2-shRNA, #14
Caspase-2
p48
Actin
p49
Lane 1 2 3
B
Vector Control
C2-def. #8
C2-def. #14
Propidium Iodide
Mock Treated
Heat-Shock
Annexin V-FITC
C
Vector control
RAIDD-shRNA. 2C3
RAIDD-shRNA. 1C7
RAIDD
p23
Actin
p49
Lane 1 2 3
D
Vector Control
RAIDD-def. 2C3
RAIDD-def. 1C7
Propidium Iodide
Mock Treated
Heat-Shock
Annexin V-FITC

Figure 23.

respectively (Fig. 23B). The inconsistency in our findings with these cells is similar to a previous report in which the authors had used a different siRNA target to silence caspase-2 (Lassus et al., 2002).

Because our attempt to directly evaluate the requirement of caspase-2 in response to heat was inconclusive, we next used shRNA to generate RAIDD (RIP associated Ich-1/CED homologous protein with death domain)-deficient Jurkat cells. We hypothesized that because RAIDD is the adaptor protein responsible for activation of caspase-2 (Duan and Dixit, 1997; Tinel and Tschopp, 2004; Park et al., 2007), such cells would offer important insight into the requirement of caspase-2 in response to heat. Additionally, a previous study reported that RAIDD is important for caspase-2 activation during heat-induced cell death (Tu et al., 2006). To that end, two single-cell RAIDD-deficient Jurkat clones (2C3 and 1C7) were generated and the extent to which RAIDD was suppressed was confirmed by Western blot analysis (Fig. 23C). As illustrated in Fig. 22D, both RAIDD-deficient cell lines were as sensitive to heat-induced apoptosis as control-transfected cells. Combined, although b-VAD-fmk labeled active caspase-2 as an apical caspase following heat shock, our findings do not support an essential role for RAIDD-mediated activation of caspase-2 during heat-induced apoptosis.

6.3.4 Apaf-1 is required for heat-induced apoptosis, whereas caspase-8 is dispensable

In addition to caspase-2, caspase-8 and -9 were pulled down as apical caspases using the b-VAD-fmk approach (Fig. 22B). Furthermore, it has been reported that caspase-8 plays a role in heat-induced apoptosis due to an increased expression of Fas ligand, which acts in a paracrine manner (Cippitelli et al., 2005; Tu et al., 2006). To determine the significance of caspase-8 activation in our hands, caspase-8-deficient Jurkat cells that have been described previously and are completely resistant to Fas-induced apoptosis (Shelton et al., 2009) were evaluated for their sensitivity to heat-induced apoptosis. As illustrated in Fig. 24A and B, the results indicated that caspase-8-deficient cells underwent heat-induced apoptosis and processed caspases-9, -3, and -2 to the same extent as A3 control cells (Fig. 24A and B) suggesting caspase-8 is dispensable in this setting.

Having shown that neither caspase-2 nor caspase-8 was necessary for heat-induced apoptosis, we were interested to determine the extent to which caspase-9 was involved. To do so, we used Apaf-1-deficient Jurkat cells that were shown previously to not activate caspase-9 (Shawgo et al., 2009) and to be completely resistant to mitochondria-mediated apoptosis induced by etoposide (Franklin and Robertson, 2007). This is significant

Figure 24. Apaf-1 is required for heat-induced apoptosis, whereas caspase-8 is dispensable. *A*, Caspse-8-deficient cells (10^6/mL) were heat-shocked for 1 h at 44°C followed by a 6 h recovery period at 37°C, cells were then harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and PI staining. Quadrants are defined as: live (lower left); early apoptotic (lower right); late apoptotic (upper right); and necrotic (upper left). Numbers refer to the percentage of cells in each quadrant. *B*, duplicate aliquots of cells were harvested and lysed for Western blotting of caspases. *C*, Apaf-1-deficient cells (10^6/mL) were heat-shocked for 1 h at 44°C followed by a 6 h recovery period at 37°C, cells were then harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and PI staining. Quadrants are defined as: live (lower left); early apoptotic (lower right); late apoptotic (upper right); and necrotic (upper left). Numbers refer to the percentage of cells in each quadrant. *D*, duplicate aliquots of cells were harvested and lysed for Western blotting of caspases.

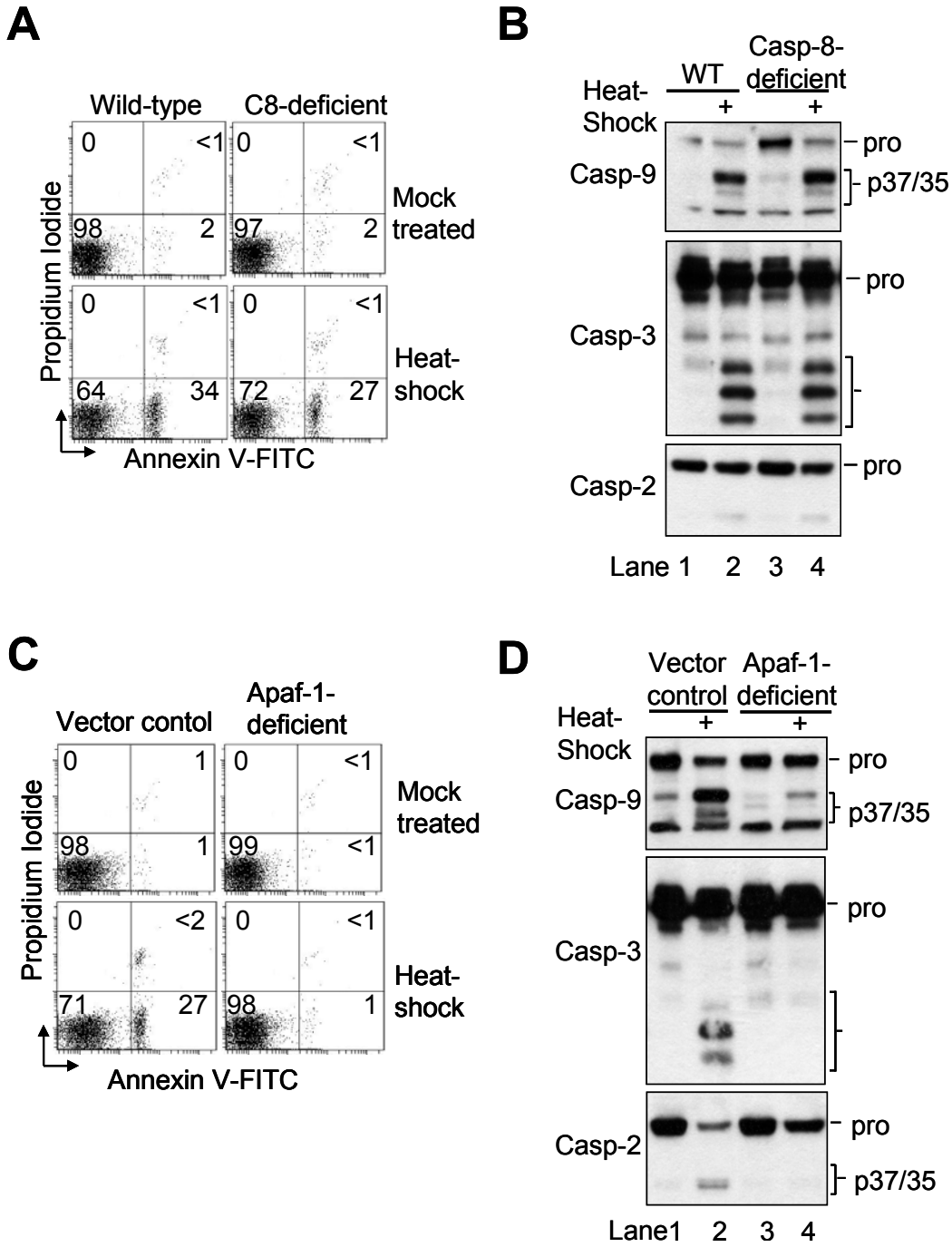

A
Wild-type
C8-deficient
Propidium Iodide
0
<1
98
2
97
Mock treated
64
34
72
27
Heat-shock
Annexin V-FITC
B
Casp-8-deficient
WT
Heat-Shock
+
Casp-9
pro
p37/35
Casp-3
Casp-2
Lane 1 2 3 4
C
Vector contol
Apaf-1-deficient
99
71
D
Vector control
Lane1 2 3 4

Figure 24.

because Apaf-1 is strictly required for caspase-9 activation within the apoptosome complex (Malladi et al., 2009). In stark contrast to caspase-8-deficient cells, Apaf-1-deficient Jurkat cells were completely resistant to heat-induced apoptosis (Fig. 24C). In agreement with these findings, Western blot analysis of cell lysates showed that proteolytic processing of caspase-2, -3, and-9 was inhibited in Apaf-1-deficient cells following heat exposure. Taken together, these data suggest that caspase-8 is dispensable during heat-induced apoptosis, whereas caspase-9 is required for this form of cell death.

6.3.5 b-VAD-fmk does not associate with any initiator caspase in Apaf-1-deficient cells exposed to heat

Because up to this point caspase-9 appeared to be the only initiator caspase that was essential for heat-induced apoptosis, we sought to extend these findings by testing whether caspase-2 or -8 could be pulled down by b-VAD-fmk in the Apaf-1-deficient cells following heat exposure. As illustrated in Fig. 25A, b-VAD-fmk failed to label caspase-2, caspase-8, or caspase-9 in the Apaf-1 knockdown cells providing additional evidence that caspase-9 is the most apical caspase during heat-induced apoptosis.

As mentioned previously, stimulation of the Fas receptor with Fas ligand or agonistic anti-Fas antibody results in DISC formation and

Figure 25. b-VAD-fmk does not associate with any initiator caspase in Apaf-1-deficient cells exposed to heat. *A*, Apaf-1-deficient cells (10^6/mL) were preincubated in the presence or abasece of 120 μM b-VAD-fmk for 3 h, then heat shocked for 1 h at 44°C followed by a 6 h recovery period at 37°C. Cells were then harvested, lysed, and incubated with strepavidin beads followed by washing and boiling of beads with sample buffer followed by Western blot analysis. *B*, Apaf-1-deficient cells (10^6/mL) were preincubated in the presence or abasece of 120 μM b-VAD-fmk for 3 h, then incubated for 6 h with anti-Fas antibody (100ng/mL). Cells were then harvested, lysed, and incubated with strepavidin beads followed by washing and boiling of beads with sample buffer followed by Western blot analysis.

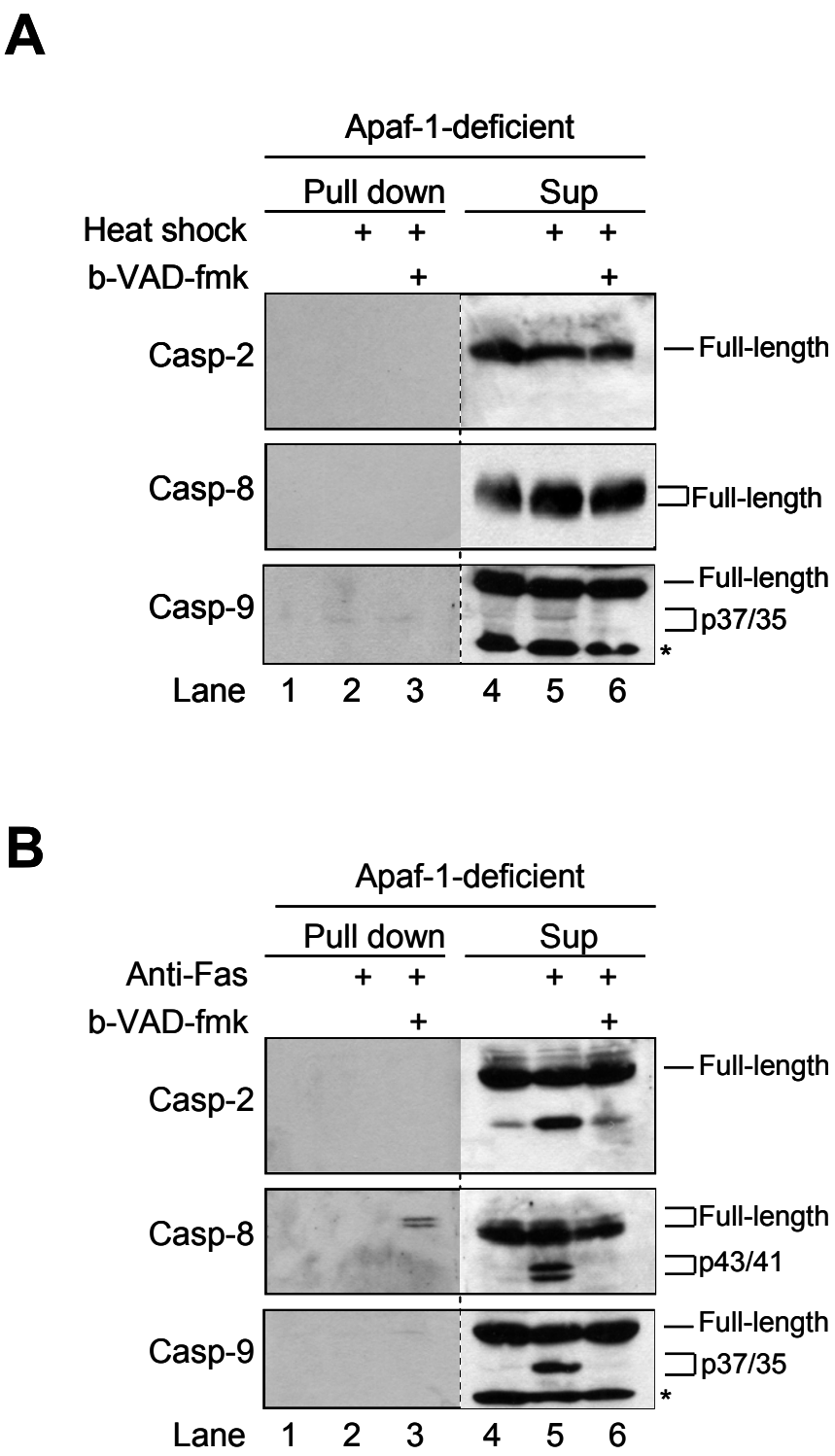

A
Apaf-1-deficient
Pull down
Sup
Heat shock
+ + + +
b-VAD-fmk
+ +
Casp-2
Full-length
Casp-8
Full-length
Casp-9
Full-length
p37/35
*
Lane 1 2 3 4 5 6
B
Apaf-1-deficient
Pull down
Sup
Anti-Fas
+ + + +
b-VAD-fmk
+ +
Casp-2
Full-length
Casp-8
Full-length
p43/41
Casp-9
Full-length
p37/35
*
Lane 1 2 3 4 5 6

Figure 25.

activation of caspase-8 (Boatright and Salvesen, 2003). To ensure that the b-VAD-fmk labeling method was effectively working in the Apaf-1-deficient cells, we pretreated the cells with b-VAD-fmk for 3 h and then incubated in the presence of anti-Fas agonistic antibody (100 ng/mL) for 6 h. Consistent with our previous results, Apaf-1-deficient Jurkat cells were partially sensitive to apoptosis induced by anti-Fas (Shawgo et al., 2009). Further, as expected, caspase-8 was the only initiator caspase that was labeled by b-VAD-fmk (Fig. 25B). Overall, these data strongly suggest that caspase-9 is the only required apically-activated caspase during heat-induced apoptosis.

6.3.6 Heat-induced mitochondrial events are attenuated in both Bid-deficient and Apaf-1-deficient Jurkat cells

Several studies have concluded that MOMP is tightly regulated by pro- and anti-apoptotic members of the Bcl-2 family of proteins (Youle and Strasser, 2008). It is widely accepted that Bax and/or Bak are required to facilitate MOMP (Lindsten et al., 2000), where their activation results in homo-oligomerization and pore formation spanning the outer mitochondrial membrane. It is through this pore that cytochrome *c* and other pro-apoptotic proteins are released from the intermembrane space of the mitochondria into the cytosol. Although it is widely accepted that

the activation of Bak and Bax depends on the prior activation of a BH3-only Bcl-2 family member, the precise mechanism of Bax and Bak activation during mitochondria-mediated apoptosis remains poorly understood (Chipuk and Green, 2008).

Because it has been reported that caspase-2-mediated Bid cleavage to tBid is required for MOMP during heat-induced apoptosis, Bid-deficient Jurkat cells, which were described previously and shown to be completely resistant to apoptosis receptor-mediated apoptosis (Shelton et al., 2009), were evaluated for their ability to undergo heat-induced apoptosis. As illustrated in Fig. 26A, flow cytometric analysis of Bid-deficient cells showed that these cells were partially resistant to heat-induced apoptosis with 25% of the population undergoing apoptosis as compared to 47% in the control-transfected cells suggesting a role for Bid in this process.

Given that Bid-deficient Jurkat cells were partially resistant and Apaf-1-deficient Jurkat cells were completely resistant to heat-induced apoptosis, mitochondrial events that characteristically define MOMP such as Bak and/or Bax activation, release of intermembrane space proteins, and loss of $\Delta\Psi$ were evaluated. Previously, we have reported that Jurkat (E6.1) cells express Bak, but do not express Bax (Shawgo et al., 2008b; Shelton et al., 2009). Therefore, Bak activation was evaluated using an active conformation-specific monoclonal Bak antibody and flow cytometric

Figure 26. Heat-induced mitochondrial events are attenuated in both Bid-deficient and Apaf-1-deficient Jurkat cells. *A*, vector control or Bid-deficient Jurkat cells (10^6/mL) were heat-shocked for 1 h at 44°C followed by a 6 h recovery time period, then harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as live (*lower left*), early apoptotic (*lower right*), late apoptotic (*upper right*), and necrotic (*upper left*). *Numbers* refer to the percentage of cells in each quadrant. *B*, cells (10^6/mL) were heat-shocked for 1 h at 44°C followed by 6 h of recovery, and processed for determination of Bak activation by flow cytometric analysis. *Numbers* in *B* refer to the percentage increase in Bak-associated fluorescence between untreated and heat-shocked samples. *C*, duplicate aliquots of cells in *B* were harvested and processed for subcellular fractionation. Supernatant (*s*) and pellet (*p*) fractions were analyzed by Western blotting. *D*, duplicate aliquots of cells in *B* were harvested and processed for mitochondrial membrane potential ($\Delta\Psi$) determination by flow cytometry. Reduced $DiIC_1(5)$ fluorescence is indicative of a loss of $\Delta\Psi$, and *numbers* refer to the percentage of cells that underwent a dissipation of $\Delta\Psi$.

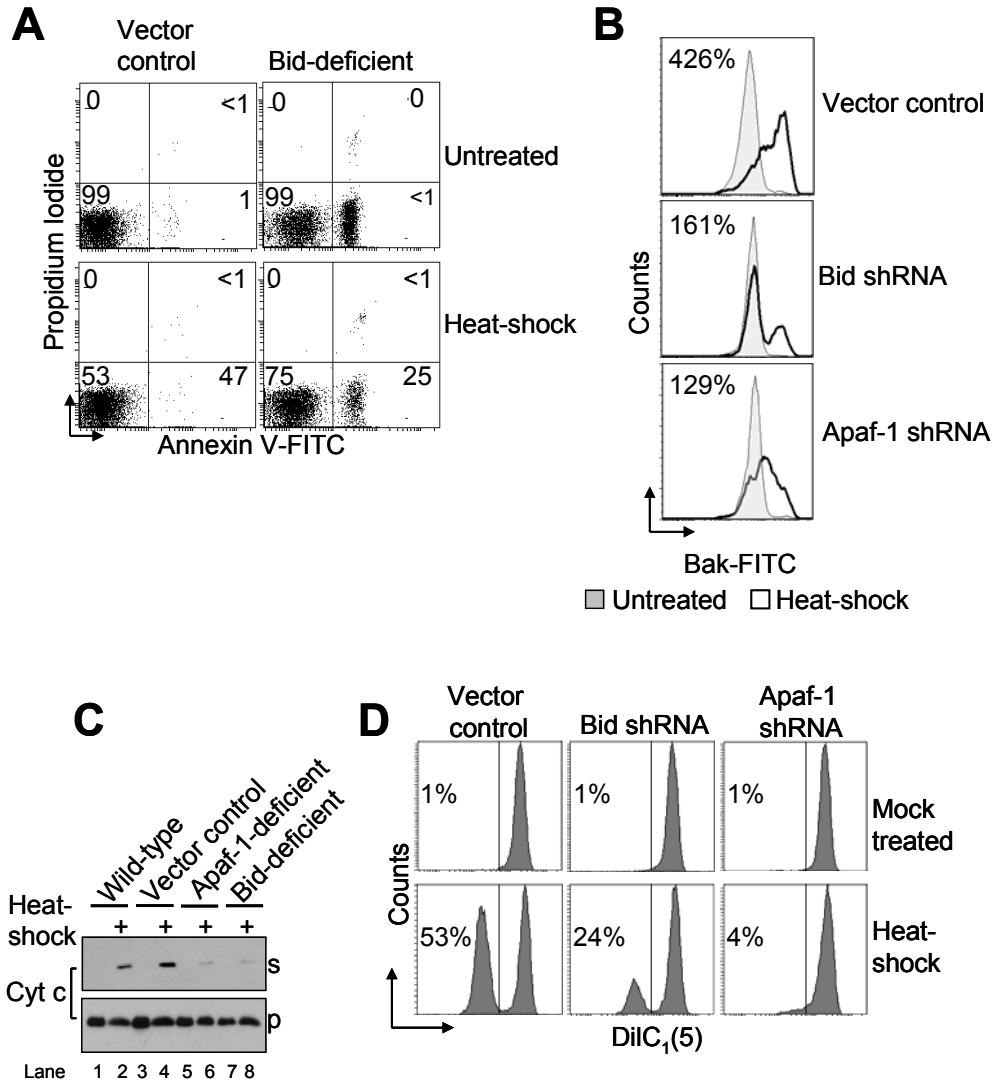

A
Vector control
Bid-deficient
Propidium Iodide
Annexin V-FITC
Untreated
Heat-shock
0
<1
99
1
53
47
75
25
B
426%
161%
129%
Vector control
Bid shRNA
Apaf-1 shRNA
Counts
Bak-FITC
Untreated
Heat-shock
C
Wild-type
Vector control
Apaf-1-deficient
Bid-deficient
Heat-shock
Cyt c
s
p
Lane 1 2 3 4 5 6 7 8
D
Vector control
Bid shRNA
Apaf-1 shRNA
1%
53%
24%
4%
Mock treated
Heat-shock
Counts
DiIC1(5)

Figure 26.

analysis where activation causes a shift to the right of the resulting histogram. As illustrated in Fig. 26B, Bak activation was markedly decreased in both the Apaf-1-deficient and Bid-deficient cell lines.

Next, consistent with the inhibition of Bak activation in cells lacking either Bid or Apaf-1, the release of cytochrome *c* in response to heat-shock was also markedly decreased (Fig. 26C). Finally, as illustrated in Fig. 26D, the loss of $\Delta\Psi$ was also decreased in the Apaf-1-deficient and Bid-deficient cell lines. Combined, these data suggest that Bid functions to promote Bak-mediated MOMP and that MOMP largely depends on events downstream of Apaf-1 during heat-induced apoptosis.

6.3.7 Cleavage of Bid is a caspase-mediated event that occurs downstream of Apaf-1

Previously, we reported that effector caspases cleaved Bid to tBid during etoposide-induced apoptosis, and tBid functioned as part of a feed forward amplification loop to induce Bak oligomerization and cytochrome *c* release (Shelton et al., 2009). In light of those observations, we next set out to determine if cleavage of Bid was a caspase-mediated event in response to heat. To do so, wild-type Jurkat cells were incubated with qVD-OPH (20 uM) for 1 h prior to heat exposure at 44°C for 1 h followed by a 6-h recovery at 37°C. As illustrated in Fig. 27A, the inclusion of qVD-

Figure 27. Cleavage of Bid is a caspase-mediated event that occurs downstream of Apaf-1. *A*, wild-type cells (10^6/mL) were pre-incubated in the presence or absence of 20 μM qVD-OPh for 1 h and subsequently incubated at 44°C for 1 h followed by a 6 h recovery period at which time cells were harvested and processed for Western blot analysis. *B*, control-transfected, and Apaf-1-deficient Jurkat cells (10^6/mL) were heat-shocked for 1 h followed by a recovery of 6 h, harvested, and lysed for Western blotting.

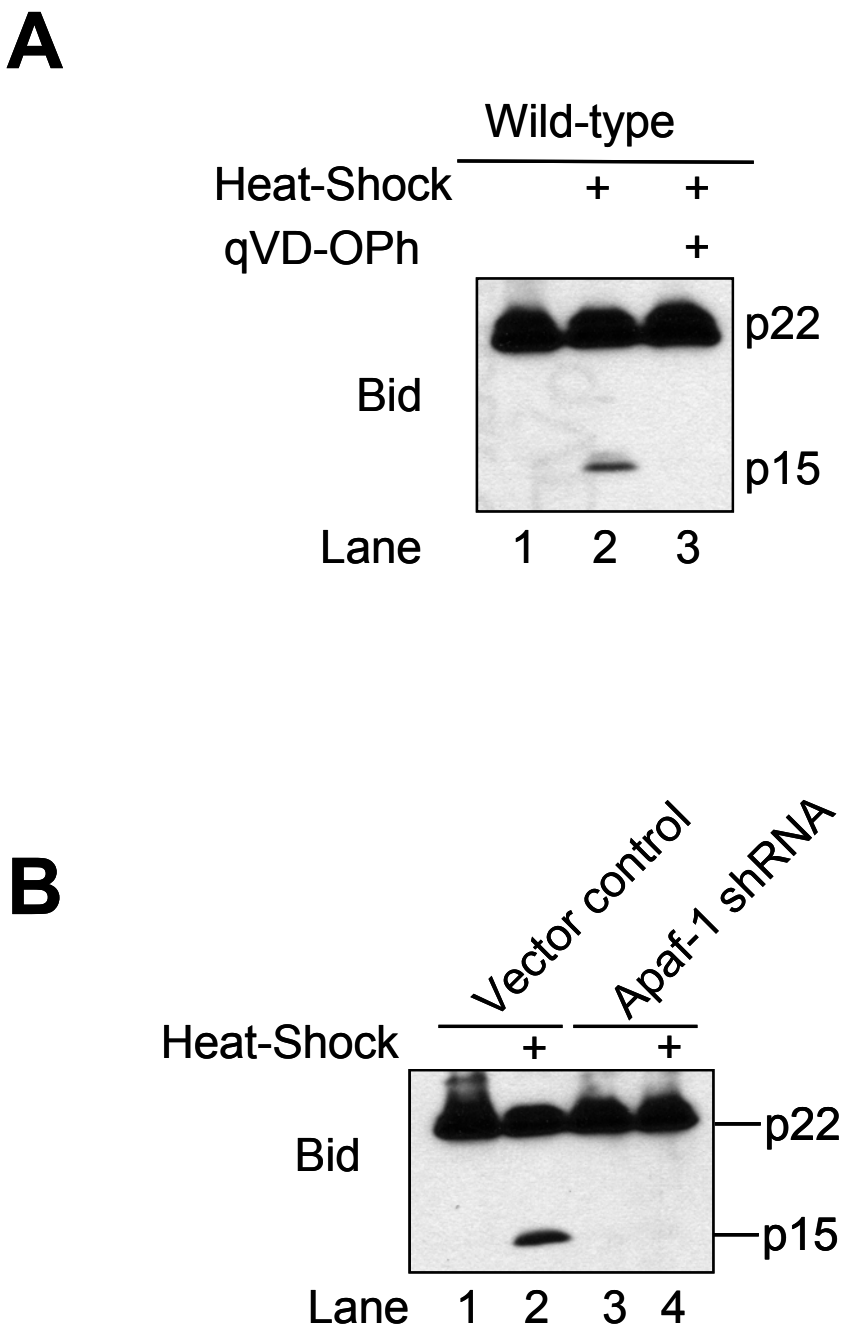

A
Wild-type
Heat-Shock
+
+
qVD-OPh
+
p22
Bid
p15
Lane
1
2
3
B
Vector control
Apaf-1 shRNA
Heat-Shock
+
+
Bid
p22
p15
Lane
1
2
3
4

Figure 27.

OPH completely inhibited the heat-induced generation of tBid. To extend these findings and to determine if Bid was cleaved upstream or downstream of apoptosome formation, we evaluated the extent of Bid cleavage in the Apaf-1-deficient cell line in response to heat. As illustrated in Fig. 27B, cleavage of Bid to tBid did not occur in the Apaf-1-deficient cell line when exposed to 44°C for 1 h followed by a recovery at 37°C for 6 h. These data suggest that the cleavage of Bid to tBid is a caspase-mediated event that occurs downstream of apoptosome-dependent caspase activation during heat-induced apoptosis.

6.4 Concluding remarks

Previous studies characterizing heat-induced apoptosis have suggested several different possible mechanisms generating conflicting results (Pagliari et al., 2005; Bonzon et al., 2006; Milleron and Bratton, 2006; Tu et al., 2006). Caspase-2 was identified as the apical caspase in response to heat through the innovative b-VAD-fmk affinity labeling approach (Tu et al., 2006). Intriguingly, caspase-2 was labeled even in Bcl-2 and Bcl-x_L-overexpressing cells leading the authors to conclude that it must be activated upstream of MOMP. In addition, heat-induced apoptosis was markedly reduced but not eliminated in caspase-2-deficient splenocytes and MEFs. Further, b-VAD-fmk failed to label caspase-2 in

RAIDD-deficient splenocytes and these cells were partially resistant to heat-shock leading to the conclusion that RAIDD was important for caspase-2 activation (Tu et al., 2006). However, a different study that was published around the same time reported that caspase-2-deficient MEFs were sensitive to heat-induced apoptosis and suggested that an unknown protease able to activate caspase-3 was the apical caspase (Milleron and Bratton, 2006). Although the authors also reported that Apaf-1 and caspase-9 were both dispensable for heat-induced apoptosis, the case was made that MOMP may still be necessary to antagonize IAPs via Smac release (Milleron and Bratton, 2006).

Although it is known that MOMP is required during intrinsic apoptosis and that prior activation of Bak or Bax is necessary to facilitate permeabilization of the outer mitochondrial membrane, the mechanistic details of Bak and Bax activation are only partially understood (Chipuk and Green, 2008). During extrinsic apoptosis in type II cells, caspase-8-mediated cleavage of the BH3-only protein Bid to its active form, tBid, is required for the activation of Bax and Bak (Li et al., 1998; Luo et al., 1998). A similar emergent role for any one of the BH3-only family members during true intrinsic apoptotic signaling has not been established. Nevertheless, the prevailing view is that BH3-only proteins are responsible for Bax and Bak activation during mitochondria-mediate apoptosis. A possible exception to the rule is during heat-induced

apoptosis where it was reported that Bax and Bak can be activated by heat *in vitro* (Pagliari et al., 2005). However, because of the fact that Bcl-X_L could inhibit heat-induced Bax activation, the point was made that activation of Bax and Bak in intact cells may still require a BH3-only protein to antagonize the anti-apoptotic Bcl-2 family members (Pagliari et al., 2005) .

As mentioned previously, it has also been reported that caspase-2-mediated Bid cleavage is required for MOMP during heat-induced apoptosis (Bonzon et al., 2006). This study also reported that Bid-deficient MEFs were largely resistant to heat-induced apoptosis. Although there was a percentage of the population of cells that remained sensitive, this population was also sensitive in the presence of qVD-OPh, leading the authors to conclude that the population of sensitive cells died by a caspase-independent cell death (Bonzon et al., 2006).

A more recent study has suggested a model where the depletion of Mcl-1 is important for Bax activation in response to heat (Stankiewicz et al., 2009). The authors suggest that the BH3-only protein, Noxa, plays an important role in regulating the expression levels of Mcl-1 where heat-shock results in displacement of Noxa from Mcl-1 which then allows the ubiquitin ligase, Mule, to bind Mcl-1 leading to its proteosomal degradation. In addition, they found that Hsp70 overexpression prevented Mcl-1 degradation and Bax activation (Stankiewicz et al., 2009).

Our findings using a large panel of gene-manipulated Jurkat cells suggest that caspase-9 is activated apically during heat-induced apoptosis. The activation of caspase-9 is strictly dependent on formation of the apoptosome, which requires cytochrome *c*. Significantly, however, there is very little cytochrome *c* released upstream of apoptosome formation. In fact, our data showed that the majority of MOMP occurred downstream of initial caspase-9 activation and was mediated largely by tBid. Although these data support a role for Bid during heat-induced apoptosis, most, if not all, cleavage of Bid occurred downstream of MOMP. Combined, these data support an emerging model of mitochondria-mediated apoptosis where the activation of caspases downstream of caspase-9 is necessary to feed forward and amplify the initial apoptotic signal to ensure cell demise. In such a scenario, the earliest release of cytochrome *c* into the cytosol would remain sublethal unless or until it accumulated to an extent that is sufficient to promote sustained apoptosome-mediated activation of caspase-9.

Chapter 7: Conclusions and Future Directions

7.1 General Discussion

The implication that apoptosis plays an important role in human disease has driven the desire to better understand and characterize the apoptotic pathway in hopes that cell survival and cell death can be manipulated for therapeutic purposes. It is now known that deregulated apoptosis is a contributing factor in several diseases characterized by either excessive or insufficient apoptosis (Thompson, 1995). Parkinson's disease is an example of a disease associated with excessive apoptosis, whereas the lack of apoptosis is considered to be a hallmark of cancer (Thompson, 1995; Hanahan and Weinberg, 2000). In addition, the therapeutic end-point of most cytotoxic anti-cancer drugs is to induce apoptosis, more specifically, mitocondria-mediated apoptosis (Lowe and Lin, 2000).

Initial observations of what we now know to be apoptotic cell death date back to the late 1800s (Lockshin and Zakeri, 2001), but the field progressed slowly until the mid-1980s after which time apoptosis-focused research expanded rapidly (Hacker and Vaux, 1997). It was during the late 1980s through the 1990s that the key regulators and mediators of

apoptosis were identified and characterized in humans (Hacker and Vaux, 1997) . An apoptotic signaling model emerged from insights gained during this time period, and this model is considered to be the conventional paradigm today.

The current perspective of mitochondria-mediated apoptosis assumes a linear apoptotic signaling cascade where an initial cytotoxic stress results in the permeabilization of the outer mitochondrial membrane followed by caspase activation and cell death (Goldstein et al., 2000; Goldstein et al., 2005). However, there is some evidence that is at odds with a strictly linear view of the pathway (Hao et al., 2005; Lakhani et al., 2006; Shawgo et al., 2008b; Shelton et al., 2009). At issue is that the conventional linear paradigm has argued that cytochrome *c* release is a caspase-independent event, and that MOMP leading to cytochrome *c* release is an all-or-none event that marks the "point of no return" (Goldstein et al., 2000; Goldstein et al., 2005). There have been some studies, however, that have shown there are differences in engagement and execution of the mitochondria-mediated apoptotic pathway in response to different stressors. However, several of these studies have produced conflicting results and thus led to considerable confusion within the field. One example is caspase-2-mediated apoptosis where several studies have reported novel functions for caspse-2 upstream of MOMP in

response to different stimuli (Bouchier-Hayes), although none of these studies has fostered an emergent understanding of caspase-2's function.

It seems reasonable to suspect that different cytotoxic agents induce apoptosis through similar as well as distinct mechanisms. The results of the studies in my dissertation contribute to the understanding of apoptotic cell death pathways in response to three different cytotoxic therapeutic strategies including a topoisomerase II inhibitor, an Hsp90 inhibitor, and elevated temperature.

7.2 Overall Conclusions

7.2.1 The role of Bid in DNA-damage induced apoptosis

The best characterized setting of Bid's involvement in apoptotic cell death is during receptor-mediated apoptosis in so-called type II cells, where it functions to activate Bak and/or Bax leading to MOMP (Li et al., 1998; Luo et al., 1998). By comparison, there have also been reports in the literature that have produced conflicting results regarding the role of Bid during DNA-damage induced apoptosis (Werner et al., 2004; Zinkel et al., 2005; Kaufmann et al., 2007). My data support a role for Bid in

mediating MOMP downstream of effector caspase activation during apoptosis induced by etoposide (Shelton et al., 2009).

As mentioned, the prevailing view of mitochondria-mediated apoptosis is a linear view beginning with the induction of MOMP. The data generated under Specific Aim 1 support more complex signaling events leading to the irreversible commitment of a cell to die. In response to the DNA-damaging drug, etoposide, my data suggest that MOMP and caspases forge an amplification loop to execute cell death in Jurkat T-lymphocytes. In this scenario, the initial amount of cytochrome *c* released may not always be sufficient to irreversibly activate caspases and execute cell death. In other words, cytochrome *c* release does not occur in an all-or-none fashion and is not the "point of no return."

Instead, the initial amount of released cytochrome *c* remains sublethal until some poorly defined threshold is exceeded that caspase-9 and subsequently caspase-3/7 are activated. Active caspase-3/7, in turn, cleaves the BH3-only protein, Bid, to tBid, which functions to induce more MOMP to seal the cell's fate. The fact that tBid induces MOMP is in agreement with its known function; however, the fact that it appears to be important during DNA-damage induced apoptosis where its cleavage is mediated by caspases-3 and/or -7 was unexpected. These data offer new insights into mechanism(s) responsible for DNA damage-induced apoptosis.

7.2.2 Targeting Hsp90 via its C-terminal domain

Hsp90 functions as a molecular chaperone where it is required for the conformational stability and function of numerous client proteins. Although Hsp90 is present constitutively, it is also induced in response to cellular stress. Hsp90 expression is often elevated in human malignancies where it is thought to be a compensatory mechanism to the harsh microenvironment often found in tumors (Whitesell and Lindquist, 2005). There are currently over 200 known Hsp90 client proteins and many are known oncogenic proteins involved in all six hallmarks of cancer (Whitesell et al., 2003; Bagatell and Whitesell, 2004; Whitesell and Lindquist, 2005).

Hsp90 is currently being pursued as an anti-cancer drug target where its inhibition would presumably lead to the destabilization and degradation of numerous client proteins. Whereas several Hsp90 client proteins are being pursued individually as drug targets, inhibition of Hsp90 would attack cancer cells on multiple levels by simultaneously destabilizing several oncogenic client proteins. N-terminal Hsp90 inhibitors (e.g. 17-AAG) have been developed and entered clinical trials (Whitesell and Lindquist, 2005); however, their clinical use has been

limited due to adverse effects and the compensatory induction of cytoprotective Hsp90 and Hsp70 (Solit et al., 2003; Banerji et al., 2005b).

Data generated under Specific Aim 2 investigated a novel C-terminal Hsp90 inhibitor, KU135, and compared the antiproliferative effects of this compound to the N-terminal inhibitor, 17-AAG. KU135 was found to have more potent antiproliferative effects than 17-AAG. In addition, 17-AAG was found to be primarily cytostatic, whereas KU135 was both cytostatic and cytotoxic. The cytotoxicity of KU135 was found to be due to induction of the mitochondria-mediated apoptotic pathway, where activation of caspase-9 was required for cell death but not MOMP. In other words, MOMP occurred upstream and independently of caspse-9 activation.

Although the first Hsp90 inhibitor entered clinical trials more than 10 years ago, there are no drugs within this class approved for anti-cancer therapy (Solit and Chiosis, 2008). My data show that KU135 and 17-AAG have different antiproliferative mechanisms, and that KU135 does not induce a robust cytoprotective response. These data provide evidence and support for the continued development of C-terminal Hsp90 inhibitors where these compounds could be a viable alternative to the N-terminal Hsp90 inhibitors in the clinic.

7.2.3 Mechanisms of heat-induced apoptosis

Exposure of cells to stress is known to induce the heat shock response which includes the induction of heat shock proteins. However, acute exposure to elevated temperatures can induce an apoptotic response (Milleron and Bratton, 2007b) . Therapeutic approaches to directly target tumor cells with heat are currently underway (Huang et al., ; Ito et al., 2006; Pissuwan et al., 2006), but the mechanistic details surrounding heat-induced apoptosis remain poorly defined. Currently, the existing literature on heat-induced apoptosis is somewhat controversial.

Of the few studies that have been performed investigating heat-induced apoptosis, differences regarding the mechanistic details have been reported surrounding key points including the apically activated caspase and whether MOMP is necessary (Pagliari et al., 2005; Bonzon et al., 2006; Milleron and Bratton, 2006; Tu et al., 2006). It has been reported that caspase-2 is the apically activated caspase and induces MOMP by cleaving Bid to tBid (Bonzon et al., 2006); however, it has also been reported that the apical caspase is an unidentified caspase that is capable of directly cleaving and activating caspase-3 (Milleron and Bratton, 2006). Whereas one study suggested that tBid is needed to induce MOMP by activating Bak and/or Bax (Bonzon et al., 2006), another study has suggested that Bax and Bak are activated directly by heat

(Pagliari et al., 2005). The data generated under Specific Aim 3 resolve some of these issues using Jurkat-T-lymphocytes, which were also used as a model system in most of the studies mentioned above.

The data generated in response to heat-induced apoptosis clearly show that this stressor activates the mitochondria-mediated pathway and that MOMP is required. Caspase-9 was identified as the apical caspase, and Bid was found to play a role in heat-induced apoptosis by inducing MOMP. As in specific aim 1, tBid was generated downstream of caspase-9 activation leading to the conclusion that it facilitates an amplification of the initial apoptotic signal where the initial amount of cytochrome *c* that is released to activate caspase-9 is mediated by an unknown mechanism and may not commit the cell to death. The data generated under this specific aim offer some resolution to the conflicting results in the literature and provide insight into the mechanistic details of heat-induced apoptosis.

7.3 Concluding Remarks

Combined, the results presented in my dissertation support the idea that different cytotoxic stressors utilize similar as well as distinct mechanisms to induce mitochondria-mediated apoptosis. The results of specific aims 1 and 3 support a scenario of an initial amount of MOMP that does not irreversibly commit a cell to die. In this scenario, executioner

caspases and mitochondria are required to mediate amplification of the initial signal and irreversibly commit a cell to death. On the other hand, the results of specific aim 2 support an initial signal that is sufficient to induce enough MOMP to irreversibly commit the cell to death by a mechanism that is independent of caspase activation.

Chapter 8: Literature Cited

Adams JM and Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. *Science* 281:1322-1326.

Avila C, Hadden MK, Ma Z, Kornilayev BA, Ye QZ and Blagg BS (2006a) High-throughput screening for Hsp90 ATPase inhibitors. *Bioorg Med Chem Lett* 16:3005-3008.

Avila C, Kornilayev BA and Blagg BS (2006b) Development and optimization of a useful assay for determining Hsp90's inherent ATPase activity. *Bioorg Med Chem* 14:1134-1142.

Bagatell R and Whitesell L (2004) Altered Hsp90 function in cancer: a unique therapeutic opportunity. *Mol Cancer Ther* 3:1021-1030.

Banerji U, O'Donnell A, Scurr M, Pacey S, Stapleton S, Asad Y, Simmons L, Maloney A, Raynaud F, Campbell M, Walton M, Lakhani S, Kaye S, Workman P and Judson I (2005a) Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. *J Clin Oncol* 23:4152-4161.

Banerji U, Walton M, Raynaud F, Grimshaw R, Kelland L, Valenti M, Judson I and Workman P (2005b) Pharmacokinetic-pharmacodynamic relationships for the heat shock protein 90

molecular chaperone inhibitor 17-allylamino, 17-demethoxygeldanamycin in human ovarian cancer xenograft models. *Clin Cancer Res* 11:7023-7032.

Bao Q and Shi Y (2007) Apoptosome: a platform for the activation of initiator caspases. *Cell Death Differ* 14:56-65.

Barnhart BC, Alappat EC and Peter ME (2003) The CD95 type I/type II model. *Semin Immunol* 15:185-193.

Blagg BS and Kerr TD (2006) Hsp90 inhibitors: small molecules that transform the Hsp90 protein folding machinery into a catalyst for protein degradation. *Med Res Rev* 26:310-338.

Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR and Salvesen GS (2003) A unified model for apical caspase activation. *Mol Cell* 11:529-541.

Boatright KM and Salvesen GS (2003) Mechanisms of caspase activation. *Curr Opin Cell Biol* 15:725-731.

Bonte FJ, Storaasli JP and Weisberger AS (1956) Comparative evaluation of radioactive colloidal gold and nitrogen mustard in the treatment of serous effusions of neoplastic origin. *Radiology* 67:63-66.

Bonzon C, Bouchier-Hayes L, Pagliari LJ, Green DR and Newmeyer DD (2006) Caspase-2-induced apoptosis requires bid cleavage: a

physiological role for bid in heat shock-induced death. *Mol Biol Cell* 17:2150-2157.

Bossy-Wetzel E and Green DR (1999) Caspases induce cytochrome c release from mitochondria by activating cytosolic factors. *J Biol Chem* 274:17484-17490.

Bouchier-Hayes L The role of caspase-2 in stress-induced apoptosis. *J Cell Mol Med*.

Bouchier-Hayes L, Oberst A, McStay GP, Connell S, Tait SW, Dillon CP, Flanagan JM, Beere HM and Green DR (2009) Characterization of cytoplasmic caspase-2 activation by induced proximity. *Mol Cell* 35:830-840.

Bouck N, Stellmach V and Hsu SC (1996) How tumors become angiogenic. *Adv Cancer Res* 69:135-174.

Breckenridge DG and Xue D (2004) Regulation of mitochondrial membrane permeabilization by BCL-2 family proteins and caspases. *Curr Opin Cell Biol* 16:647-652.

Brown JM and Wouters BG (1999) Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res* 59:1391-1399.

Bryan TM and Cech TR (1999) Telomerase and the maintenance of chromosome ends. *Curr Opin Cell Biol* 11:318-324.

Burlison JA, Avila C, Vielhauer G, Lubbers DJ, Holzbeierlein J and Blagg BS (2008a) Development of novobiocin analogues that manifest

anti-proliferative activity against several cancer cell lines. *J Org Chem* 73:2130-2137.

Burlison JA, Avila C, Vielhauer G, Lubbers DJ, Holzbeierlein J and Blagg BS (2008b) Development of Novobiocin Analogues That Manifest Anti-proliferative Activity against Several Cancer Cell Lines. *J Org Chem* 73:2130-2137.

Burlison JA, Neckers L, Smith AB, Maxwell A and Blagg BS (2006) Novobiocin: redesigning a DNA gyrase inhibitor for selective inhibition of hsp90. *J Am Chem Soc* 128:15529-15536.

Chao Y, Shiozaki EN, Srinivasula SM, Rigotti DJ, Fairman R and Shi Y (2005) Engineering a dimeric caspase-9: a re-evaluation of the induced proximity model for caspase activation. *PLoS Biol* 3:e183.

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM and Huang DC (2005) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 17:393-403.

Chen M, He H, Zhan S, Krajewski S, Reed JC and Gottlieb RA (2001) Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion. *J Biol Chem* 276:30724-30728.

Cheng J and Haas M (1990) Frequent mutations in the p53 tumor suppressor gene in human leukemia T-cell lines. *Mol Cell Biol* 10:5502-5509.

Chiosis G, Huezo H, Rosen N, Mimnaugh E, Whitesell L and Neckers L (2003) 17AAG: low target binding affinity and potent cell activity--finding an explanation. *Mol Cancer Ther* 2:123-129.

Chipuk JE, Bouchier-Hayes L and Green DR (2006) Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ* 13:1396-1402.

Chipuk JE and Green DR (2008) How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* 18:157-164.

Choi YE, Butterworth M, Malladi S, Duckett CS, Cohen GM and Bratton SB (2009) The E3 ubiquitin ligase cIAP1 binds and ubiquitinates caspase-3 and -7 via unique mechanisms at distinct steps in their processing. *J Biol Chem* 284:12772-12782.

Christofori G and Semb H (1999) The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci* 24:73-76.

Cippitelli M, Fionda C, Di Bona D, Piccoli M, Frati L and Santoni A (2005) Hyperthermia enhances CD95-ligand gene expression in T lymphocytes. *J Immunol* 174:223-232.

Cleary ML, Smith SD and Sklar J (1986) Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* 47:19-28.

Clem RJ, Fechheimer M and Miller LK (1991) Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science* 254:1388-1390.

Cohen GM (1997) Caspases: the executioners of apoptosis. *Biochem J* 326 (Pt 1):1-16.

Danial NN and Korsmeyer SJ (2004) Cell death: critical control points. *Cell* 116:205-219.

Denault JB, Eckelman BP, Shin H, Pop C and Salvesen GS (2007) Caspase 3 attenuates XIAP (X-linked inhibitor of apoptosis protein)-mediated inhibition of caspase 9. *Biochem J* 405:11-19.

Donnelly AC, Mays JR, Burlison JA, Nelson JT, Vielhauer G, Holzbeierlein J and Blagg BS (2008) The design, synthesis, and evaluation of coumarin ring derivatives of the novobiocin scaffold that exhibit antiproliferative activity. *J Org Chem* 73:8901-8920.

Downward J (2006) Signal transduction. Prelude to an anniversary for the RAS oncogene. *Science* 314:433-434.

Droga-Mazovec G, Bojic L, Petelin A, Ivanova S, Romih R, Repnik U, Salvesen GS, Stoka V, Turk V and Turk B (2008) Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues. *J Biol Chem* 283:19140-19150.

Du C, Fang M, Li Y, Li L and Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102:33-42.

Duan H and Dixit VM (1997) RAIDD is a new 'death' adaptor molecule. *Nature* 385:86-89.

Eckelman BP, Salvesen GS and Scott FL (2006) Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep* 7:988-994.

Egorin MJ, Rosen DM, Wolff JH, Callery PS, Musser SM and Eiseman JL (1998) Metabolism of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) by murine and human hepatic preparations. *Cancer Res* 58:2385-2396.

Evan G and Littlewood T (1998) A matter of life and cell death. *Science* 281:1317-1322.

Felix CA, Kolaris CP and Osheroff N (2006) Topoisomerase II and the etiology of chromosomal translocations. *DNA Repair (Amst)* 5:1093-1108.

Folkman J (1997) Addressing tumor blood vessels. *Nat Biotechnol* 15:510.

Franklin EE and Robertson JD (2007) Requirement of Apaf-1 for mitochondrial events and the cleavage or activation of all procaspases during genotoxic stress-induced apoptosis. *Biochem J* 405:115-122.

Friesen C, Herr I, Krammer PH and Debatin KM (1996) Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nat Med* 2:574-577.

Fuentes-Prior P and Salvesen GS (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J* 384:201-232.

Georgakis GV and Younes A (2005) Heat-shock protein 90 inhibitors in cancer therapy: 17AAG and beyond. *Future Oncol* 1:273-281.

Goetz MP, Toft D, Reid J, Ames M, Stensgard B, Safgren S, Adjei AA, Sloan J, Atherton P, Vasile V, Salazaar S, Adjei A, Croghan G and Erlichman C (2005) Phase I trial of 17-allylamino-17-demethoxygeldanamycin in patients with advanced cancer. *J Clin Oncol* 23:1078-1087.

Goldstein JC, Munoz-Pinedo C, Ricci JE, Adams SR, Kelekar A, Schuler M, Tsien RY and Green DR (2005) Cytochrome c is released in a single step during apoptosis. *Cell Death Differ* 12:453-462.

Goldstein JC, Waterhouse NJ, Juin P, Evan GI and Green DR (2000) The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat Cell Biol* 2:156-162.

Green DR (2000) Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 102:1-4.

Grenert JP, Sullivan WP, Fadden P, Haystead TA, Clark J, Mimnaugh E, Krutzsch H, Ochel HJ, Schulte TW, Sausville E, Neckers LM and Toft DO (1997) The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J Biol Chem* 272:23843-23850.

Guo Y, Srinivasula SM, Druilhe A, Fernandes-Alnemri T and Alnemri ES (2002) Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. *J Biol Chem* 277:13430-13437.

Hacker G and Vaux DL (1997) A chronology of cell death. *Apoptosis* 2:247-256.

Hahn JS (2009) The Hsp90 chaperone machinery: from structure to drug development. *BMB Rep* 42:623-630.

Hanahan D and Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86:353-364.

Hanahan D and Weinberg RA (2000) The hallmarks of cancer. *Cell* 100:57-70.

Hande KR (1998) Etoposide: four decades of development of a topoisomerase II inhibitor. *Eur J Cancer* 34:1514-1521.

Hao Z, Duncan GS, Chang CC, Elia A, Fang M, Wakeham A, Okada H, Calzascia T, Jang Y, You-Ten A, Yeh WC, Ohashi P, Wang X and Mak TW (2005) Specific ablation of the apoptotic functions of

cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. *Cell* 121:579-591.

Harris CC (1996a) p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. *Carcinogenesis* 17:1187-1198.

Harris CC (1996b) Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. *J Natl Cancer Inst* 88:1442-1455.

Hartson SD, Thulasiraman V, Huang W, Whitesell L and Matts RL (1999) Molybdate inhibits hsp90, induces structural changes in its C-terminal domain, and alters its interactions with substrates. *Biochemistry* 38:3837-3849.

Hayflick L (1997) Mortality and immortality at the cellular level. A review. *Biochemistry (Mosc)* 62:1180-1190.

Huang HC, Rege K and Heys JJ Spatiotemporal Temperature Distribution and Cancer Cell Death in Response to Extracellular Hyperthermia Induced by Gold Nanorods. *ACS Nano*.

Isaacs JS, Xu W and Neckers L (2003) Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell* 3:213-217.

Ito A, Honda H and Kobayashi T (2006) Cancer immunotherapy based on intracellular hyperthermia using magnetite nanoparticles: a novel

concept of "heat-controlled necrosis" with heat shock protein expression. *Cancer Immunol Immunother* 55:320-328.

Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, MacLean KH, Han J, Chittenden T, Ihle JN, McKinnon PJ, Cleveland JL and Zambetti GP (2003) Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* 4:321-328.

Jiang X and Wang X (2004) Cytochrome C-mediated apoptosis. *Annu Rev Biochem* 73:87-106.

Johnstone RW, Ruefli AA and Lowe SW (2002) Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108:153-164.

Jolly C and Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J Natl Cancer Inst* 92:1564-1572.

Kamal A, Thao L, Sensintaffar J, Zhang L, Boehm MF, Fritz LC and Burrows FJ (2003) A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature* 425:407-410.

Kamer I, Sarig R, Zaltsman Y, Niv H, Oberkovitz G, Regev L, Haimovich G, Lerenthal Y, Marcellus RC and Gross A (2005) Proapoptotic BID is an ATM effector in the DNA-damage response. *Cell* 122:593-603.

Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A and Green DR (1998) DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. *Mol Cell* 1:543-551.

Kaufmann T, Tai L, Ekert PG, Huang DC, Norris F, Lindemann RK, Johnstone RW, Dixit VM and Strasser A (2007) The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest. *Cell* 129:423-433.

Kerr JF, Wyllie AH and Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26:239-257.

Kim H, Rafiuddin-Shah M, Tu HC, Jeffers JR, Zambetti GP, Hsieh JJ and Cheng EH (2006a) Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol* 8:1348-1358.

Kim H, Rafiuddin-Shah M, Tu HC, Jeffers JR, Zambetti GP, Hsieh JJ and Cheng EH (2006b) Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol* 8:1348-1358.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH and Peter ME (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-

associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* 14:5579-5588.

Knudson AG, Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 68:820-823.

Kouloulias V, Plataniotis G, Kouvaris J, Dardoufas C, Gennatas C, Uzunoglu N, Papavasiliou C and Vlahos L (2005) Chemoradiotherapy combined with intracavitary hyperthermia for anal cancer: feasibility and long-term results from a phase II randomized trial. *Am J Clin Oncol* 28:91-99.

Kregel KC (2002) Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol* 92:2177-2186.

Lakhani SA, Masud A, Kuida K, Porter GA, Jr., Booth CJ, Mehal WZ, Inayat I and Flavell RA (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* 311:847-851.

Lassus P, Opitz-Araya X and Lazebnik Y (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 297:1352-1354.

Letai AG (2008) Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer* 8:121-132.

Li H, Zhu H, Xu CJ and Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94:491-501.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479-489.

Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K, Chen Y, Wei M, Eng VM, Adelman DM, Simon MC, Ma A, Golden JA, Evan G, Korsmeyer SJ, MacGregor GR and Thompson CB (2000) The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* 6:1389-1399.

Lockshin RA and Zakeri Z (2001) Programmed cell death and apoptosis: origins of the theory. *Nat Rev Mol Cell Biol* 2:545-550.

Lowe SW and Lin AW (2000) Apoptosis in cancer. *Carcinogenesis* 21:485-495.

Luo X, Budihardjo I, Zou H, Slaughter C and Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94:481-490.

Mandic A, Viktorsson K, Strandberg L, Heiden T, Hansson J, Linder S and Shoshan MC (2002) Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis. *Mol Cell Biol* 22:3003-3013.

Marcu MG, Chadli A, Bouhouche I, Catelli M and Neckers LM (2000a) The heat shock protein 90 antagonist novobiocin interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone. *J Biol Chem* 275:37181-37186.

Marcu MG, Schulte TW and Neckers L (2000b) Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins. *J Natl Cancer Inst* 92:242-248.

Martincic D and Hande KR (2005) Topoisomerase II inhibitors. *Cancer Chemother Biol Response Modif* 22:101-121.

McStay GP, Salvesen GS and Green DR (2008) Overlapping cleavage motif selectivity of caspases: implications for analysis of apoptotic pathways. *Cell Death Differ* 15:322-331.

Metzstein MM, Stanfield GM and Horvitz HR (1998) Genetics of programmed cell death in C. elegans: past, present and future. *Trends Genet* 14:410-416.

Milleron RS and Bratton SB (2006) Heat shock induces apoptosis independently of any known initiator caspase-activating complex. *J Biol Chem* 281:16991-17000.

Milleron RS and Bratton SB (2007a) 'Heated' Debates in Apoptosis. *Cell Mol Life Sci*.

Milleron RS and Bratton SB (2007b) 'Heated' debates in apoptosis. *Cell Mol Life Sci* 64:2329-2333.

Morimoto RI and Santoro MG (1998) Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nat Biotechnol* 16:833-838.

Nitiss JL (2009) Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer* 9:338-350.

Nitiss JL and Beck WT (1996) Antitopoisomerase drug action and resistance. *Eur J Cancer* 32A:958-966.

Nowakowski GS, McCollum AK, Ames MM, Mandrekar SJ, Reid JM, Adjei AA, Toft DO, Safgren SL and Erlichman C (2006) A phase I trial of twice-weekly 17-allylamino-demethoxy-geldanamycin in patients with advanced cancer. *Clin Cancer Res* 12:6087-6093.

Nunez G, Benedict MA, Hu Y and Inohara N (1998) Caspases: the proteases of the apoptotic pathway. *Oncogene* 17:3237-3245.

Oberst A, Pop C, Tremblay AG, Blais V, Denault JB, Salvesen GS and Green DR Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. *J Biol Chem*.

Ow YL, Green DR, Hao Z and Mak TW (2008) Cytochrome c: functions beyond respiration. *Nat Rev Mol Cell Biol* 9:532-542.

Owen BA, Sullivan WP, Felts SJ and Toft DO (2002) Regulation of heat shock protein 90 ATPase activity by sequences in the carboxyl terminus. *J Biol Chem* 277:7086-7091.

Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tu S, Beere HM and Green DR (2005) The multidomain proapoptotic molecules Bax and Bak are directly activated by heat. *Proc Natl Acad Sci U S A* 102:17975-17980.

Park HH, Logette E, Raunser S, Cuenin S, Walz T, Tschopp J and Wu H (2007) Death domain assembly mechanism revealed by crystal structure of the oligomeric PIDDosome core complex. *Cell* 128:533-546.

Parsell DA, Taulien J and Lindquist S (1993) The role of heat-shock proteins in thermotolerance. *Philos Trans R Soc Lond B Biol Sci* 339:279-285; discussion 285-276.

Petak I, Tillman DM, Harwood FG, Mihalik R and Houghton JA (2000) Fas-dependent and -independent mechanisms of cell death following DNA damage in human colon carcinoma cells. *Cancer Res* 60:2643-2650.

Pissuwan D, Valenzuela SM and Cortie MB (2006) Therapeutic possibilities of plasmonically heated gold nanoparticles. *Trends Biotechnol* 24:62-67.

Powers MV and Workman P (2007) Inhibitors of the heat shock response: biology and pharmacology. *FEBS Lett* 581:3758-3769.

Rahmani M, Yu C, Dai Y, Reese E, Ahmed W, Dent P and Grant S (2003) Coadministration of the heat shock protein 90 antagonist 17-allylamino- 17-demethoxygeldanamycin with suberoylanilide hydroxamic acid or sodium butyrate synergistically induces apoptosis in human leukemia cells. *Cancer Res* 63:8420-8427.

Rensing-Ehl A, Frei K, Flury R, Matiba B, Mariani SM, Weller M, Aebischer P, Krammer PH and Fontana A (1995) Local Fas/APO-1 (CD95) ligand-mediated tumor cell killing in vivo. *Eur J Immunol* 25:2253-2258.

Ritossa F (1996) Discovery of the heat shock response. *Cell Stress Chaperones* 1:97-98.

Roe SM, Prodromou C, O'Brien R, Ladbury JE, Piper PW and Pearl LH (1999) Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J Med Chem* 42:260-266.

Samraj AK, Keil E, Ueffing N, Schulze-Osthoff K and Schmitz I (2006) Loss of caspase-9 provides genetic evidence for the type I/II

concept of CD95-mediated apoptosis. *J Biol Chem* 281:29652-29659.

Sarig R, Zaltsman Y, Marcellus RC, Flavell R, Mak TW and Gross A (2003) BID-D59A is a potent inducer of apoptosis in primary embryonic fibroblasts. *J Biol Chem* 278:10707-10715.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH and Peter ME (1998) Two CD95 (APO-1/Fas) signaling pathways. *Embo J* 17:1675-1687.

Schimmer AD (2004) Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer Res* 64:7183-7190.

Schmitt E, Gehrmann M, Brunet M, Multhoff G and Garrido C (2007) Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. *J Leukoc Biol* 81:15-27.

Schmitz I, Walczak H, Krammer PH and Peter ME (1999) Differences between CD95 type I and II cells detected with the CD95 ligand. *Cell Death Differ* 6:821-822.

Schulte TW, Blagosklonny MV, Ingui C and Neckers L (1995) Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. *J Biol Chem* 270:24585-24588.

Scott FL, Denault JB, Riedl SJ, Shin H, Renatus M and Salvesen GS (2005) XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. *EMBO J* 24:645-655.

Sharma SV, Agatsuma T and Nakano H (1998) Targeting of the protein chaperone, HSP90, by the transformation suppressing agent, radicicol. *Oncogene* 16:2639-2645.

Shawgo ME, Shelton SN and Robertson JD (2008a) Caspase-mediated Bak Activation and Cytochrome c Release during Intrinsic Apoptotic Cell Death in Jurkat Cells. *J Biol Chem* 283:35532-35538.

Shawgo ME, Shelton SN and Robertson JD (2008b) Caspase-mediated Bak activation and cytochrome c release during intrinsic apoptotic cell death in Jurkat cells. *J Biol Chem* 283:35532-35538.

Shawgo ME, Shelton SN and Robertson JD (2009) Caspase-9 activation by the apoptosome is not required for fas-mediated apoptosis in type II Jurkat cells. *J Biol Chem* 284:33447-33455.

Shay JW and Bacchetti S (1997) A survey of telomerase activity in human cancer. *Eur J Cancer* 33:787-791.

Shelton SN, Shawgo ME and Robertson JD (2009) Cleavage of Bid by executioner caspases mediates feed forward amplification of mitochondrial outer membrane permeabilization during genotoxic stress-induced apoptosis in Jurkat cells. *J Biol Chem* 284:11247-11255.

Shi M, Vivian CJ, Lee KJ, Ge C, Morotomi-Yano K, Manzl C, Bock F, Sato S, Tomomori-Sato C, Zhu R, Haug JS, Swanson SK, Washburn MP, Chen DJ, Chen BP, Villunger A, Florens L and Du C (2009) DNA-PKcs-PIDDosome: a nuclear caspase-2-activating complex with role in G2/M checkpoint maintenance. *Cell* 136:508-520.

Shiozaki EN, Chai J, Rigotti DJ, Riedl SJ, Li P, Srinivasula SM, Alnemri ES, Fairman R and Shi Y (2003) Mechanism of XIAP-mediated inhibition of caspase-9. *Mol Cell* 11:519-527.

Shiozaki EN and Shi Y (2004) Caspases, IAPs and Smac/DIABLO: mechanisms from structural biology. *Trends Biochem Sci* 29:486-494.

Slee EA, Keogh SA and Martin SJ (2000) Cleavage of BID during cytotoxic drug and UV radiation-induced apoptosis occurs downstream of the point of Bcl-2 action and is catalysed by caspase-3: a potential feedback loop for amplification of apoptosis-associated mitochondrial cytochrome c release. *Cell Death Differ* 7:556-565.

Solit DB and Chiosis G (2008) Development and application of Hsp90 inhibitors. *Drug Discov Today* 13:38-43.

Solit DB, Ivy SP, Kopil C, Sikorski R, Morris MJ, Slovin SF, Kelly WK, DeLaCruz A, Curley T, Heller G, Larson S, Schwartz L, Egorin MJ, Rosen N and Scher HI (2007) Phase I trial of 17-allylamino-17-

demethoxygeldanamycin in patients with advanced cancer. *Clin Cancer Res* 13:1775-1782.

Solit DB and Rosen N (2006) Hsp90: a novel target for cancer therapy. *Curr Top Med Chem* 6:1205-1214.

Solit DB, Scher HI and Rosen N (2003) Hsp90 as a therapeutic target in prostate cancer. *Semin Oncol* 30:709-716.

Sporn MB (1996) The war on cancer. *Lancet* 347:1377-1381.

Srinivasula SM, Ahmad M, Fernandes-Alnemri T and Alnemri ES (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* 1:949-957.

Srinivasula SM and Ashwell JD (2008) IAPs: what's in a name? *Mol Cell* 30:123-135.

Stankiewicz AR, Livingstone AM, Mohseni N and Mosser DD (2009) Regulation of heat-induced apoptosis by Mcl-1 degradation and its inhibition by Hsp70. *Cell Death Differ* 16:638-647.

Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ, Ellerby LM, Bredesen D, Freeze H, Abrahamson M, Bromme D, Krajewski S, Reed JC, Yin XM, Turk V and Salvesen GS (2001) Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *J Biol Chem* 276:3149-3157.

Strasser A, Harris AW, Jacks T and Cory S (1994) DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell* 79:329-339.

Sun XM, Bratton SB, Butterworth M, MacFarlane M and Cohen GM (2002) Bcl-2 and Bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of Smac/DIABLO and subsequent inactivation of X-linked inhibitor-of-apoptosis protein. *J Biol Chem* 277:11345-11351.

Tafani M, Karpinich NO, Hurster KA, Pastorino JG, Schneider T, Russo MA and Farber JL (2002) Cytochrome c release upon Fas receptor activation depends on translocation of full-length bid and the induction of the mitochondrial permeability transition. *J Biol Chem* 277:10073-10082.

Tait SW, de Vries E, Maas C, Keller AM, D'Santos CS and Borst J (2007) Apoptosis induction by Bid requires unconventional ubiquitination and degradation of its N-terminal fragment. *J Cell Biol* 179:1453-1466.

Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* 267:1456-1462.

Tinel A and Tschopp J (2004) The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science* 304:843-846.

Tu S, McStay GP, Boucher LM, Mak T, Beere HM and Green DR (2006) In situ trapping of activated initiator caspases reveals a role for caspase-2 in heat shock-induced apoptosis. *Nat Cell Biol* 8:72-77.

Valentijn AJ and Gilmore AP (2004) Translocation of full-length Bid to mitochondria during anoikis. *J Biol Chem* 279:32848-32857.

Vande Walle L, Lamkanfi M and Vandenabeele P (2008) The mitochondrial serine protease HtrA2/Omi: an overview. *Cell Death Differ* 15:453-460.

Veikkola T and Alitalo K (1999) VEGFs, receptors and angiogenesis. *Semin Cancer Biol* 9:211-220.

Vempati UD, Diaz F, Barrientos A, Narisawa S, Mian AM, Millan JL, Boise LH and Moraes CT (2007) Role of cytochrome C in apoptosis: increased sensitivity to tumor necrosis factor alpha is associated with respiratory defects but not with lack of cytochrome C release. *Mol Cell Biol* 27:1771-1783.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ and Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102:43-53.

Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, Adams JM and Strasser A (2003) p53- and drug-induced

apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 302:1036-1038.

Volpert OV, Dameron KM and Bouck N (1997) Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. *Oncogene* 14:1495-1502.

Wang JC (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* 3:430-440.

Wang K, Yin XM, Chao DT, Milliman CL and Korsmeyer SJ (1996) BID: a novel BH3 domain-only death agonist. *Genes Dev* 10:2859-2869.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB and Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 292:727-730.

Weinberg RA (1995) The retinoblastoma protein and cell cycle control. *Cell* 81:323-330.

Werner AB, Tait SW, de Vries E, Eldering E and Borst J (2004) Requirement for aspartate-cleaved bid in apoptosis signaling by DNA-damaging anti-cancer regimens. *J Biol Chem* 279:28771-28780.

Whitesell L, Bagatell R and Falsey R (2003) The stress response: implications for the clinical development of hsp90 inhibitors. *Curr Cancer Drug Targets* 3:349-358.

Whitesell L and Lindquist SL (2005) HSP90 and the chaperoning of cancer. *Nat Rev Cancer* 5:761-772.

Whitesell L, Mimnaugh EG, De Costa B, Myers CE and Neckers LM (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc Natl Acad Sci U S A* 91:8324-8328.

Wilkinson JC, Cepero E, Boise LH and Duckett CS (2004) Upstream regulatory role for XIAP in receptor-mediated apoptosis. *Mol Cell Biol* 24:7003-7014.

Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, Strasser A, Kluck RM, Adams JM and Huang DC (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* 315:856-859.

Workman P and Powers MV (2007) Chaperoning cell death: a critical dual role for Hsp90 in small-cell lung cancer. *Nat Chem Biol* 3:455-457.

Wust P, Hildebrandt B, Sreenivasa G, Rau B, Gellermann J, Riess H, Felix R and Schlag PM (2002) Hyperthermia in combined treatment of cancer. *Lancet Oncol* 3:487-497.

Wyllie AH, Kerr JF and Currie AR (1980) Cell death: the significance of apoptosis. *Int Rev Cytol* 68:251-306.

Yang QH, Church-Hajduk R, Ren J, Newton ML and Du C (2003) Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. *Genes Dev* 17:1487-1496.

Yang X, Li W, Prescott ED, Burden SJ and Wang JC (2000) DNA topoisomerase IIbeta and neural development. *Science* 287:131-134.

Yin XM (2006) Bid, a BH3-only multi-functional molecule, is at the cross road of life and death. *Gene* 369:7-19.

Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA and Korsmeyer SJ (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 400:886-891.

Youle RJ and Strasser A (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 9:47-59.

Yu XM, Shen G, Neckers L, Blake H, Holzbeierlein J, Cronk B and Blagg BS (2005) Hsp90 inhibitors identified from a library of novobiocin analogues. *J Am Chem Soc* 127:12778-12779.

Yun BG, Huang W, Leach N, Hartson SD and Matts RL (2004) Novobiocin induces a distinct conformation of Hsp90 and alters Hsp90-cochaperone-client interactions. *Biochemistry* 43:8217-8229.

Zha J, Weiler S, Oh KJ, Wei MC and Korsmeyer SJ (2000) Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science* 290:1761-1765.

Zinkel SS, Hurov KE and Gross A (2007) Bid plays a role in the DNA damage response. *Cell* 130:9-10; author reply 10-11.

Zinkel SS, Hurov KE, Ong C, Abtahi FM, Gross A and Korsmeyer SJ (2005) A role for proapoptotic BID in the DNA-damage response. *Cell* 122:579-591.

Zou J, Guo Y, Guettouche T, Smith DF and Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 94:471-480.