University of Alberta

Characterization of the response of melanoma cell lines to inhibition of anti-apoptotic Bcl-2 proteins

by

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ABSTRACT

Malignant melanoma is resistant to almost all conventional forms of chemotherapy. Recent evidence suggests that anti-apoptotic proteins of the Bcl-2 family are overexpressed in melanoma and may contribute to melanoma's striking resistance to apoptosis. ABT-737, a small-molecule inhibitor of Bcl-2, Bcl-xl and Bcl-w, has demonstrated efficacy in several forms of cancer. However, overexpression of Mcl-1, a frequent observance in melanoma, is known to confer ABT-737 resistance.

My results demonstrate that the combination of ABT-737 and RNA silencing of Mcl-1 induces significant cell death in six different melanoma cell lines, representing a potential new therapeutic strategy. I show that the apoptotic response to the combination treatment involves both the intrinsic pathway and a death receptor-independent role for extrinsic pathway proteins. The combination treatment also induces a number of gene expression changes as assessed by cDNA microarray and follow-up analyses. Based on the results of the array, I investigated the effects of inhibition of MAPK proteins combined with ABT-737 and/or Mcl-1 knockdown. I found that the combination of a p38 MAPK inhibitor and ABT-737 strongly and synergistically induces apoptosis in melanoma cell lines, thus suggesting a second novel treatment combination with potential for melanoma therapy. Finally, I provide novel evidence that Bcl-2 family member PUMA is cleaved in a caspase-dependent fashion during apoptosis and may play a role in treatment response.

Currently, there are no effective treatments for metastatic melanoma. My findings describe two potential combination therapies for melanoma as well as provide novel evidence as to the mechanisms involved in treatment response.

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LIST OF ABBREVIATIONS, SYMBOLS AND NOMENCLATURE

ABT-263	Orally available analog of ABT-737 (Abbott)
ABT-737	Bcl-2, Bcl-xL and Bcl-w inhibitor (Abbott)
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
AP-1	Activator protein 1
Bcl-2	B-cell leukemia 2
BH	Bcl-2 Homology
BSA	Bovine Serum Albumin
Bp	Base pair
CASVM	Server for SVM prediction of caspase cleavage sites
cDNA	complementary DNA
CDK	Cyclin-Dependent Kinase
CLL	Chronic Lymphocytic Leukemia
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DsiRNA	Dicer substrate siRNA
DTIC	Dacarbazine
ER	Endoplasmic Reticulum
ERK	Extracellular signal-related Kinase
HEM	Human Epidermal Melanocytes
IAP	Inhibitor of Apoptosis Protein
IL-2	Interleukin-2
JNK	Jun N-terminal Kinase
kDa	kilo Dalton
LDH	Lactate Dehydrogenase
MAPK	Mitogen-Activated Protein Kinase
Mcl-1	Myeloid cell leukemia 1
MEF	Mouse Embryonic Fibroblast
MEK	MAP ERK kinase
MG132	Proteasome inhibitor
MOM	Mitochondrial Outer Membrane
mRNA	messenger RNA
mTOR	mammalian Target Of Rapamycin
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide
SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly ADP-Ribose Polymerase
PCR	Polymerase Chain Reaction
PD98059	MEK inhibitor
PI	Propidium Iodide
PI3K	Phosphoinositide 3-Kinase

PUMA	p53 upregulated modulator of apoptosis
RNA	Ribonucleic acid
RNAi	RNA interference
RGP	Radial Growth Phase
RISC	RNA-Induced Silencing Complex
RT-PCR	Reverse Transcription PCR
SB202190	p38 inhibitor
SB203580	p38 inhibitor
SP600125	JNK inhibitor
SEM	Standard Error of the Mean
SCLC	Small Cell Lung Cancer
siRNA	small interfering RNA
tBid	truncated Bid
TBST	Tris-Buffered Saline Tween-20
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TUNEL	Terminal deoxynucleotidyl dUTP Nick End Labeling
TMZ	Temozolomide
UVR	Ultraviolet Radiation
VGP	Vertical Growth Phase
Z-AEVD-FMK	Caspase-10 inhibitor
Z-IETD-FMK	Caspase-8 inhibitor
Z-LEHD-FMK	Caspsae-9 inhibitor
Z-VAD-FMK	General/Pan caspase inhibitor

~Chapter 1~

Literature Review

Introduction

Malignant melanoma is the most aggressive form of cutaneous cancer and shows little response to conventional therapeutics. As a result, survival times for metastatic melanoma are exceedingly low. New treatments have been developed, but as yet, none have significantly prolonged survival time. The majority of anti-cancer agents act through the induction of apoptosis, specifically via the intrinsic apoptotic pathway. Melanoma cells are highly resistant to apoptosis due both to the inherent survival characteristics of parental melanocytes as well as genetic alterations acquired during tumorigenesis. Ultimately, control of the intrinsic pathway of apoptosis is largely dependent on the balance between pro- and anti-apoptotic members of the Bcl-2 family of proteins. Multiple Bcl-2 family proteins are dysregulated in melanoma, significantly contributing to melanoma's apoptotic resistance. In the past few years, a variety of new therapeutic agents have been developed to target Bcl-2 proteins and related upstream pathways. Recent evidence suggests that simultaneous targeting of multiple anti-apoptotic Bcl-2 proteins may be the most promising treatment strategy. Metastatic melanoma is essentially incurable and new therapies are urgently required. Targeted treatment strategies to restore melanoma's apoptotic function are thus of great significance and make an important contribution to cancer research.

Malignant Melanoma

Melanoma incidence and risk factors

The three major forms of skin cancer are basal cell carcinoma, squamous cell carcinoma and malignant melanoma. Of these, melanoma is the least common; however, it is responsible for 65% of skin cancer-associated deaths (Cummins et al., 2006). The incidence of malignant melanoma is rising in Canada, due both to increased leisure time spent in the sun as well as improvements in detection and diagnosis (Canadian Cancer Society website). In 2009, there are estimated to be 5000 new cases of melanoma in Canada, making it the seventh most common form of cancer. Furthermore, melanoma is the third most common cancer in young women age 15 to 29.

The major environmental risk factor for melanoma development is exposure to ultraviolet radiation (UVR). In contrast to other forms of skin cancer, melanoma is primarily associated with intense and intermittent sun exposure, such as severe sunburns, rather than cumulative sun exposure (Chudnovsky et al., 2005). People with fair complexions, blonde or red hair and an inability to tan display a greater incidence of melanoma than the general population (Tsao et al., 2004a). Individuals with a large number of nevi (moles), atypical or dysplastic nevi, or a tendency to freckle also have an increased susceptibility (Tsao et al., 2004a). Furthermore, personal or family history of melanoma and/or non-melanoma skin cancer is a strong predictive factor.

Familial melanoma

Familial melanoma accounts for approximately 10% of melanoma cases (reviewed in Sekulic et al., 2008). Through linkage analysis and follow-up studies, two high-risk loci have been identified. The first is the *CDKN2A* gene on chromosome 9p21(Hussussian et al., 1994; Kamb et al., 1994). *CDKN2A* is an unusual locus that, via alternative reading frames, encodes two unrelated proteins, $p16^{INK4A}$ and $p14^{ARF}$. Both proteins are key tumor suppressors that regulate cell cycle and apoptosis, respectively. A second high-susceptibility locus is the *CDK4* gene on chromosome 12q14 (Zuo et al., 1996). *CDK4* mutations block the interaction between CDK4 and $p16^{INK4A}$, resulting in a loss of cell cycle control. Mutations in *CDKN2A* and *CDK4* account for less than 50% of families with hereditary cutaneous melanoma; however, as yet, no other high-susceptibility genes have been identified (Tucker, 2009). Furthermore, mutations in either gene are infrequent in more common, sporadic melanomas.

Melanoma biology

Cutaneous melanoma arises from the pigment-producing melanocyte cells of the skin epidermis. Melanocytes are derived from the embryonic neural crest and subsequently migrate to their final location in the skin (reviewed in Uong and Zon, 2010). There are four major types of cutaneous melanoma: superficial spreading melanoma, nodular melanoma, lentigo maligna and acral lentiginous melanoma (Gray-Schopfer et al., 2007). There are also non-cutaneous types including uveal melanoma, which arises from pigmented cells of the eye.

Superficial spreading melanoma (SSM) is by far the most common form, accounting for approximately 70% of all melanoma cases (Cummins et al., 2006).

Melanoma can develop from benign or dysplastic nevi or it can arise *de novo* without evidence of a previous benign lesion (Chudnovsky et al., 2005). Initially, there is a radial growth phase (RGP) in which the cancer spreads laterally in the epidermis (pagetoid spread). The RGP is followed by a vertical growth phase (VGP) characterized by invasion of the dermis. Melanoma cells in VGP also gain the ability to metastasize. Initial metastasis is commonly to nearby lymph nodes. Further sites of metastases include distant lymph nodes, skin and subcutaneous tissue, brain, liver, lung, gastrointestinal tract and bone. The developmental genetic program responsible for migration of the parental melanocytes from the neural crest to the skin epidermis is thought to contribute to melanoma's strong aggessiveness and high metastatic rate (Gupta et al., 2005).

Survival rates for non-disseminated melanoma are high: 5-year survival is ~98% for RGP and 84% for VGP melanoma (Payette et al., 2009). However, outcome for metastatic melanoma remains grim with estimated 5-year survival rates of 14% (skin, lymph node or GI metastasis), 4% (lung) and 3% (liver, brain or bone) (Barth et al., 1995). Median survival time for metastatic melanoma is generally less than one year after diagnosis.

Treatment of malignant melanoma

If melanoma is diagnosed early, it can be cured by surgical removal of the primary tumor (Testori et al., 2009). For cases with a high risk of recurrence,

adjuvant therapy may also be recommended. Currently, the only adjuvant therapy that has shown a reproducible increase in relapse-free survival in clinical trials is interferon alpha-2b (Tsao et al., 2004a). However, because interferon treatment is associated with considerable toxic side effects and its benefit to overall survival is questionable, it is not widely used in clinical practice.

There are currently no effective treatment options for metastatic melanoma. Radiotherapy has limited efficacy in metastatic melanoma and its use is largely palliative (Testori et al., 2009). The only FDA-approved treatments for stage-IV melanoma are dacarbazine (DTIC) and high-dose bolus interleukin-2. Alkylating agent DTIC is considered the standard for melanoma therapy; however, response rates are estimated at only 13.4% and there is little improvement to survival time (Yang and Chapman, 2009). Although not FDA-approved, there is off-license use of DTIC analogue, temozolomide (TMZ) (Lorigan et al., 2008). TMZ displays greater oral bioavailability and central nervous system penetration than DTIC; however, an overall survival benefit has yet to be shown. Interleukin-2 (IL-2) is a cytokine that stimulates expansion of activated T-cells to improve host immune response against melanoma cells (Stein and Brownell, 2008). High doses of IL-2 have been shown to produce complete responses in a small percent of patients (~7%); however, use of IL-2 is limited by its severe toxic side effects including myocarditis and transient renal insufficiency.

Chemoresistance in melanoma

In addition to displaying a poor response to DTIC and TMZ, melanoma is resistant to essentially all major classes of chemotherapeutic agents including nitrosoureas, platinating agents, topoisomerase inhibitors, taxanes and vinca alkaloids. None of these agents have been shown to provide a durable survival advantage to melanoma patients in randomized clinical trials (Soengas and Lowe, 2003). Melanoma thus displays broad resistance to cytotoxic drugs regardless of their mechanism of action.

Nitrosoureas, including carmustine (BCNU), lomustine (CCNU) and fotemustine (FTMU), are alkylating agents that mediate their cytotoxic effects through formation of DNA interstrand crosslinks (Huitema et al., 2000). Response rates for nitrosoureas in melanoma range from around 13 to 18% and there is little improvement to overall survival time (Mouawad et al., 2009). Platinum analogs cisplatin and carboplatin are also DNA damaging agents that act by intercalating into the DNA. Response rates range between 10 and 20% in phase II trials, with survival times remaining under 6 months (Yang and Chapman, 2009). Melanoma is also highly resistant to topoisomerase inhibitors such as etoposide that interfere with DNA replication, producing DNA strand breaks.

Taxanes (docetaxel, paclitaxel, etc.) and vinca alkaloids (vincristine and vinblastine) mediate their cytotoxic effects by disrupting microtubule depolymerization or preventing microtubule assembly, respectively (Soengas and Lowe, 2003). None of these agents displays significant activity in melanoma and, as a result, taxanes and vinca alkaloids are primarily used as components of

combination therapies rather than as single agents. Importantly, although some chemotherapeutic agents and combination treatments have shown promising results in phase II clinical trials, currently no treatments have produced a significant increase in overall survival in phase III trials of metastatic melanoma (Mouawad et al., 2009).

A number of mechanisms have been proposed to explain the chemoresistance phenotype in melanoma. Melanoma cells express multiple members of the ABC family of transporter proteins, including MDR1 (Multi-Drug Resistance 1), that can function as drug efflux pumps (Chen et al., 2009a). There is also some evidence that altered DNA repair function, including increased O^6-methylguanine DNA methyltransferase activity, contributes to resistance to DNA damaging agents (Bradbury and Middleton, 2004). In addition, down-regulation or mutation of topoisomerase II has been suggested to account for melanoma's resistance to topoisomerase inhibitors such as etoposide (Grossman and Altieri, 2001). However, none of these mechanisms can fully account for melanoma's widespread resistance to all forms of treatment.

Ultimately, the majority of chemotherapeutic agents act through the induction of apoptosis (Hocker et al., 2008). Therefore, dysregulation of apoptotic pathways has been suggested as the major mechanism of chemoresistance in melanoma. As discussed below, melanoma cells display significant defects in key apoptotic regulatory networks as well as upstream survival signaling pathways.

Apoptosis overview

Apoptosis is a form of programmed cell death characterized by cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (reviewed in Hacker, 2000). Apoptotic cells break apart into small apoptotic bodies, which are then phagocytosed by macrophages. Unlike necrotic death, cellular constituents are not released from the dying cells; therefore, there is no inflammatory response.

Apoptosis occurs through two major pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (See Elmore, 2007 for a comprehensive review) (Figure 1-1). Despite these designations, however, apoptosis is not a linear process and there is significant overlap between pathways. The central executioners of both pathways are the caspases (cysteine-aspartic proteases), which are divided into initiator caspases (caspase-2, -8, -9, -10) and effector caspases (caspase-3, -6, -7). The effector caspases cleave key cytoplasmic, cytoskeletal and nuclear proteins to produce the characteristic morphology of apoptotic cells.

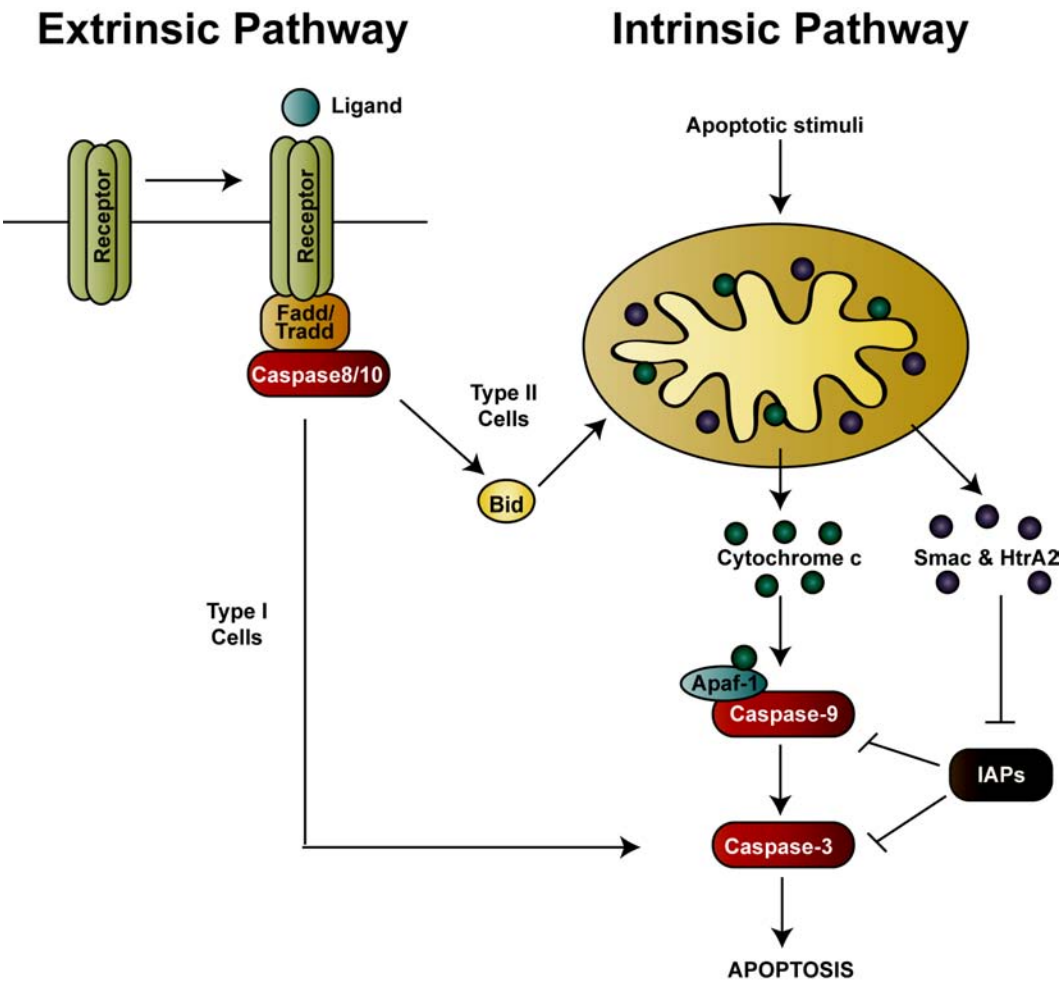


Figure 1-1: Schematic of the extrinsic and intrinsic pathways of apoptosis.

The extrinsic pathway of apoptosis transduces signals from the death receptors to the apoptotic machinery either directly (by activation of caspase-3) or indirectly (by activation of Bid). The intrinsic pathway is activated in response to a variety of apoptotic stimuli including DNA damage and growth factor withdrawal. The key event in the intrinsic pathway is the release of apoptogenic factors from the mitochondria, which activates the caspase cascade.

Extrinsic pathway

The extrinsic pathway is initiated through stimulation of death receptors on the cell surface (Figure 1-1). Receptors and corresponding ligands are members of the tumor necrosis factor (TNF) superfamily (Locksley et al., 2001). The most characterized ligand/receptor pairings are FasL/Fas, TNF-α/TNF-R1 and TRAIL/TRAIL-R1 and TRAIL-R2. Receptors of this family are transmembrane proteins with a N-terminal extracellular domain responsible for ligand binding and a C-terminal cytoplasmic domain responsible for transducing signals to the apoptotic machinery. Extracellular ligand binding induces a conformational change in the receptors, resulting in recruitment of cytoplasmic adapter proteins (e.g. FADD, TRADD) (See Guicciardi and Gores, 2009 for a review of death receptor signaling). The adapter proteins then recruit initiator procaspases 8 and/or 10. The resulting complex is referred to as the death-inducing signaling complex or DISC. DISC formation induces autocatalytic activation of caspase-8 and -10. In type I cells, active caspase-8/-10 directly activate downstream effector caspase-3 and induce apoptosis independently of the mitochondria (Mahalingam et al., 2009). In type-II cells, levels of active caspase-8 and -10 are insufficient to activate caspase-3; instead, caspase-8 cleaves Bcl-2 family member Bid, which in turn activates the intrinsic pathway of apoptosis.

Intrinsic pathway

The intrinsic pathway of apoptosis is activated in response to a variety of stimuli, including growth factor deprivation, hypoxia and DNA damage (Soengas

and Lowe, 2003). The majority of cytotoxic drugs are thought to act through this pathway. A key event in the induction of intrinsic apoptosis is the permeabilization of the outer membrane of the mitochondria, allowing release of apoptogenic factors from the intermembrane space (Jourdain and Martinou, 2009) (Figure 1-1). One of these factors is cytochrome c, which forms a complex with Apaf-1 and procaspase-9 in the cytoplasm. This complex, referred to as the apoptosome, provides a platform for activation of caspase-9 (Hill et al., 2004). Caspase-9 then activates effector caspase-3. Smac/Diablo and HtrA2/Omi are also released from the mitochondria during apoptosis. Both proteins function to inhibit IAP (inhibitor of apoptosis protein) activity, thus facilitating activation of caspase-9 and caspase-3 (Wei et al., 2008). The mitochondria can also release AIF and EndoG, which are involved in caspase-independent cell death (Cregan et al., 2004a). The release of pro-apoptotic factors from the mitochondria is largely controlled by the Bcl-2 family of proteins.

Bcl-2 Family

The Bcl-2 family is comprised of pro- and anti-apoptotic regulatory proteins defined by the presence of conserved Bcl-2 homology (BH) domains (Thomadaki and Scorilas, 2006) (Figure 1-2). Anti-apoptotic Bcl-2 proteins possess three or four BH domains (BH1-4) and include Bcl-2, Bcl-xl, Bcl-w, Mcl-1 and A1. In anti-apoptotic family members, the BH1, BH2 and BH3 domains form a hydrophobic cleft, which may be further stabilized by the N-terminal BH4 domain (Muchmore et al., 1996). Pro-apoptotic Bcl-2 proteins are divided into

multi-domain (BH1-3) proteins, namely Bax and Bak, as well as BH3-only proteins including Bad, Bim, Bid, Bik, Bmf, Hrk, PUMA and Noxa (Lomonosova and Chinnadurai, 2008). The α-helical BH3 domain of pro-apoptotic proteins interacts with the hydrophobic cleft of anti-apoptotic binding partners. The interactions between pro- and anti-apoptotic Bcl-2 proteins regulate the release of apoptogenic factors from the mitochondria.

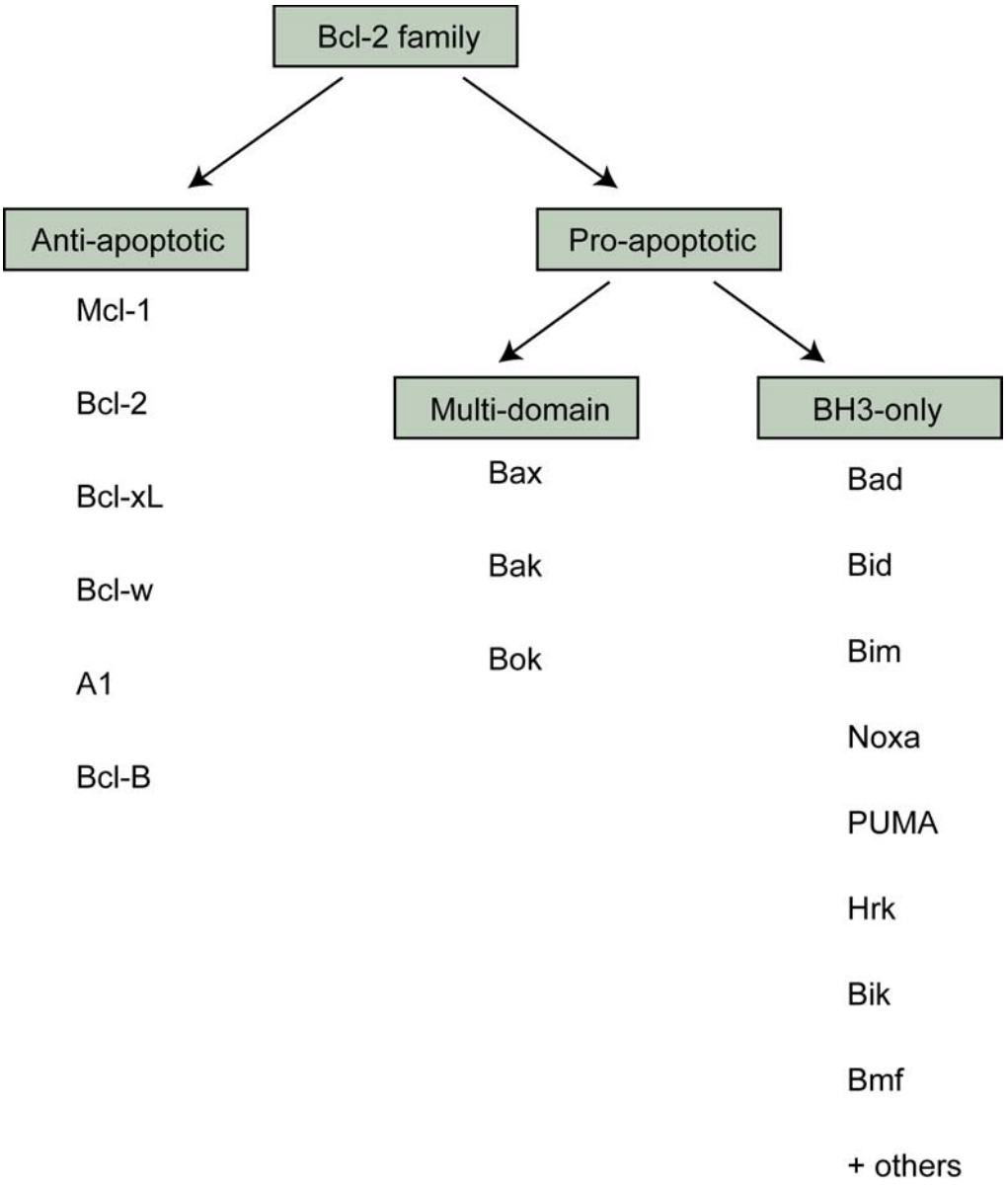


Figure 1-2: Major members of the Bcl-2 family

The Bcl-2 family is divided into pro- and anti-apoptotic proteins, with the pro-apoptotic proteins further divided into multi-domain and BH3-only. Adapted from www.researchapoptosis.com.

Multi-domain pro-apoptotic proteins

The multi-domain pro-apoptotic proteins Bax and Bak are directly involved in mitochondrial outer membrane permeabilization during apoptosis (Reed, 2006; reviewed in Fletcher and Huang, 2008). In healthy cells, monomeric Bax protein is primarily localized to the cytosol. In response to apoptotic stimuli, Bax translocates to the mitochondria and oligomerizes in the outer membrane. In contrast, Bak is constitutively localized to the mitochondrial outer membrane in an inactive state. Upon induction of apoptosis, Bak undergoes a conformational change and, like Bax, forms high molecular weight oligomers. Oligomerization of Bax and Bak is associated with release of cytochrome c and other factors from the mitochondria; however, there is debate as to whether Bax and Bak form functional protein channels *in vivo* (Martinez-Caballero et al., 2009) or induce a more general disruption of the mitochondrial membrane (Kuwana et al., 2002).

BH3-only proteins

BH3-only proteins act upstream of Bax and Bak, functioning as sensors of apoptotic signals and facilitating Bax and Bak activation (reviewed in Lomonosova and Chinnadurai, 2008; Youle and Strasser, 2008). Activity of BH3 proteins can be regulated by a variety of mechanisms. For example, transcription of *PUMA* and *NOXA* is induced by p53 in response to DNA damage (Oda et al., 2000; Nakano and Vousden, 2001; Villunger et al., 2003). In contrast, activity of the Bad protein is largely controlled by phosphorylation. Dephosphorylation of Bad in response to growth factor withdrawal results in its release from the 14-3-3

protein and translocation to the mitochondria (Zha et al., 1996). In healthy cells, Bim is sequestered in a microtubule-associated dynein complex (Puthalakath et al., 1999). Exposure to apoptotic stimuli induces release of Bim and translocation to the mitochondria. As discussed above, Bid is activated by caspase-8 cleavage to produce its functional form, tBid (truncated Bid) (Li et al., 1998; Luo et al., 1998). There is now evidence that Bid can also be cleaved by caspase-10, granzyme B and lysosomal proteases (reviewed in Yin, 2006).

BH3-only proteins also differ in their ability to bind to anti-apoptotic Bcl-2 proteins (Chen et al., 2005). Bim and PUMA potently engage all major anti-apoptotic Bcl-2 proteins: Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1. The BH3 domains of Bid, Bik, and Hrk bind all pro-survival proteins except Mcl-1; although there is evidence that tBid interacts with Mcl-1 *in vivo* (Weng et al., 2005; Clohessy et al., 2006). In contrast, Noxa only binds Mcl-1 and A1, and Bad engages only Bcl-2, Bcl-xL and Bcl-w. Several BH3-only proteins have also been shown to directly interact with Bax and/or Bak, namely Bim, Bid and, in some studies, PUMA (Wei et al., 2000; Letai et al., 2002; Walensky et al., 2006; Gavathiotis et al., 2008; Gallenne et al., 2009). The unique regulatory mechanisms and protein interactions for BH3-only proteins suggests that they act as sensors for distinct stress signals and apoptotic stimuli (Danial, 2007).

Anti-apoptotic Bcl-2 proteins

The activity of Bax/Bak and the BH3-only proteins is opposed by anti-apoptotic Bcl-2 family members, the best characterized of which are Bcl-2, Bcl-

xL and Mcl-1. *BCL-2* was originally cloned from the breakpoint of the 14;18 translocation associated with follicular lymphoma (Pegoraro et al., 1984). *BCL-2* was the first proto-oncogene identified that did not promote proliferation but, instead, inhibited cell death. The Bcl-2 protein contains all four conserved BH domains (BH1-4) and localizes to the mitochondria, endoplasmic reticulum and nuclear membrane (Krajewski et al., 1993). Expression of *BCL-2* is regulated by a variety of transcription factors, including up-regulation by MITF and transcriptional repression by p53 (Wu et al., 2001; McGill et al., 2002). Bcl-2 activity can also be regulated by phosphorylation by multiple kinases including ERK, JNK, ASK and PKC (Ruvolo et al., 2001). In addition to interacting with multiple BH3-only proteins, Bcl-2 has also been shown to heterodimerize with Bax (Otter et al., 1998).

The *BCL-X* locus encodes several alternatively spliced transcripts, of which *BCL-xL* is the major isoform expressed in post-natal tissues (Boise et al., 1993; Gonzalez-Garcia et al., 1994). The Bcl-xL protein localizes primarily to the mitochondria and, to a lesser extent, the perinuclear envelope. Bcl-xL possesses all four BH domains. A smaller variant, Bcl-xS, lacks the BH1 and BH2 domains and functions similar to other BH3-only pro-apoptotic proteins. Expression of Bcl-xL is controlled by a variety of transcription factors including Sp1, AP-1, Nf-κB, as well as members of the STAT family (Grad et al., 2000). In addition to interacting with several BH3-only proteins, Bcl-xL directly binds and inhibits Bak (Willis et al., 2005).

Mcl-1 differs from Bcl-2 and Bcl-xL in that it does not possess a BH4 domain, but contains two N-terminal PEST domains, characteristic of proteins with rapid turnover (reviewed in Michels et al., 2005). Indeed, Mcl-1 displays a short half-life, ranging from approximately 30 minutes to a few hours. During apoptosis induced by DNA damaging agents (including UV) or anoikis, Mcl-1 is rapidly degraded via the proteasome (Nijhawan et al., 2003; Willis et al., 2005; Woods et al., 2007). Furthermore, Mcl-1 can undergo caspase-dependent cleavage during apoptosis to produce a pro-apoptotic protein product (Weng et al., 2005). Expression of *MCL-1* is induced by multiple transcription factors including STAT3, STAT5, CREB and HIF-1 (Akgul, 2009). Similar to Bcl-xS, a BH3-only splice variant, Mcl-1_S can also be expressed from the *MCL-1* locus (Bingle et al., 2000). Mcl-1 primarily localizes to the outer mitochondrial membrane where it directly binds and inhibits Bak (Willis et al., 2005).

Several other anti-apoptotic Bcl-2 family members have been identified including Bcl-w, A1/Bfl1 and Bcl-B. Bcl-w bears all four BH domains and displays similar binding kinetics to Bcl-xL (Chen et al., 2005). Bcl-B also contains all four domains and has been suggested to interact with Bax but not Bak, Bid or Bad (Zhai et al., 2003; Zhai et al., 2008). Like Mcl-1, A1 lacks the conserved BH4 domain and selectively antagonizes Bak and tBid (Simmons et al., 2008). Thus, the induction of apoptosis requires neutralization of multiple pro-survival Bcl-2 family members. Two major models have been proposed to describe the interaction between pro- and anti-apoptotic Bcl-2 family members and the activation of Bax and Bak (Figure 1-3).

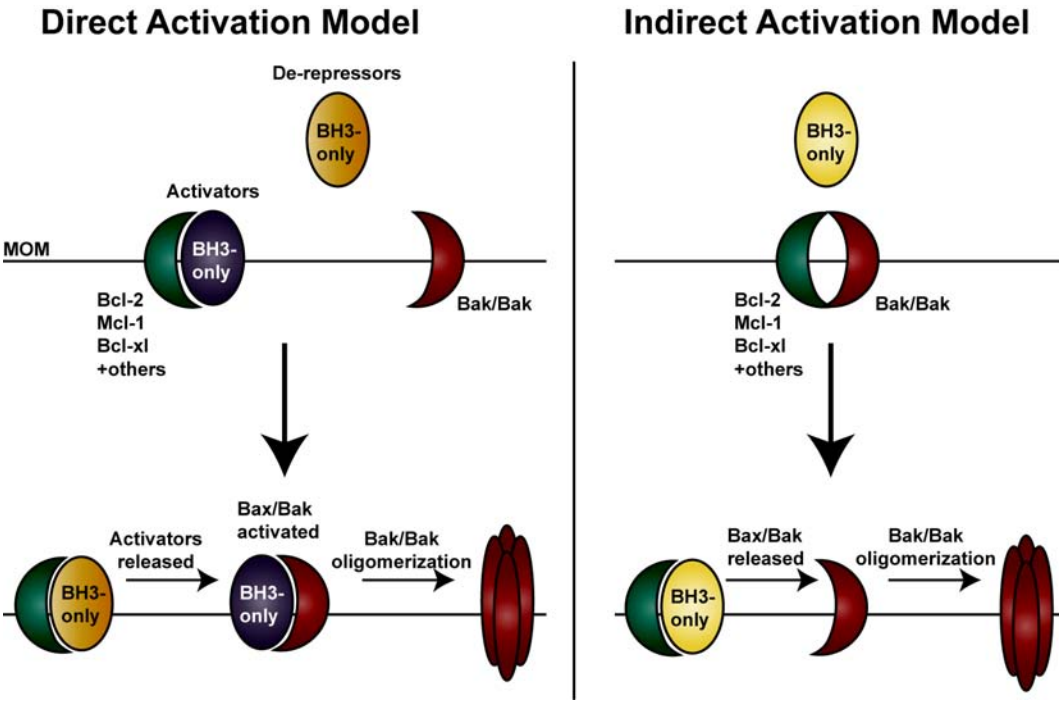


Figure 1-3: Models of Bax/Bak activation.

The direct activation model posits that a subset of BH3-only proteins termed "activators" (Bim, Bid, PUMA) directly activate Bax and Bak, but are held in check by anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Mcl-1, etc.). In response to apoptotic stimuli, "de-repressor" BH3-only proteins (e.g. Bad, Noxa, Bmf, etc.) bind to the anti-apoptotic proteins, releasing the activators to bind with Bax and Bak. In contrast, the indirect activation model suggests that BH3-only proteins function solely to release Bax and Bak from anti-apoptotic family members. MOM = mitochondrial outer membrane. Adapted from (Danial, 2007).

Direct activation model

The direct activation or hierarchical model (Letai et al., 2002; Kuwana et al., 2005) postulates that there are two distinct sets of BH3-only proteins termed "activators" (Bid, Bim and possibly PUMA) and "de-repressors" or "sensitizers" (Bad, Bmf, Noxa, Bik, etc). Activators directly interact with Bax and Bak, inducing mitochondrial translocation (Bax), conformational change and oligomerization. The function of the de-repressors is therefore to bind and inhibit anti-apoptotic Bcl-2 family members, allowing release of the activators. This model is supported by studies demonstrating direct binding between the BH3 domains of Bid, Bim and PUMA with Bax and/or Bak (Letai et al., 2002; Walensky et al., 2006; Gallenne et al., 2009). Furthermore, recently, a Bim SAHB (stabilized α-helix of Bcl-2 domains) was shown to bind Bax at a novel interaction site and directly activate Bax function in multiple *in vitro* and *in vivo* assays (Gavathiotis et al., 2008).

Indirect activation model

The indirect activation or classical model (Chen et al., 2005; Uren et al., 2007; Fletcher et al., 2008) asserts that BH3-only proteins do not directly activate Bax and Bak, but primarily function to release Bax and Bak from anti-apoptotic Bcl-2 proteins. In support of this model, Mcl-1 and Bcl-xL have been shown to directly bind and inhibit Bak (Willis et al., 2005). Neutralization of both Mcl-1 and Bcl-xL (by Noxa plus Bad) induces Bak-mediated apoptosis. One limitation of this model has been in explaining the activation of Bax, which exists mainly as

a cytosolic monomer in healthy cells (discussed in Lomonosova and Chinnadurai, 2008). Thus, a complementary model has been proposed suggesting that a small proportion of Bax constitutively resides in the mitochondrial membrane, held in check by anti-apoptotic Bcl-2 proteins (Fletcher et al., 2008). Up-regulation of BH3-only proteins releases mitochondrial Bax, which then recruits cytosolic Bax to allow for oligomerization and membrane permeabilization (Tan et al., 2006). Ultimately, the *in vivo* mechanism of Bax and Bak activation may be a combination of the direct and indirect models, dependent on cell type and apoptotic stimulus.

Dysregulation of apoptosis in melanoma

Bcl-2 family

Parental melanocytes themselves are resistant to apoptosis. Melanocytes have a photoprotective role in the skin, producing melanin in response to UVR, which is then transferred to keratinocytes (reviewed in Gilchrest et al., 1999). After exposure to damaging levels of UVR, keratinocytes rapidly undergo apoptosis. "Sunburn cells" refer to apoptotic keratinocytes. In contrast, apoptosis in rarely observed for melanocytes. One possible explanation for this resistance to apoptosis is the high expression of Bcl-2 observed in melanocytes (Plettenberg et al., 1995; Selzer et al., 1998; Bowen et al., 2003).

Because melanoma is strongly resistant to almost all forms of chemotherapy, it has been suggested that the chemoresistance phenotype is not due to mutations selected in response to treatment, but rather due to the inherent

apoptotic resistance of the melanoma cells (Soengas and Lowe, 2003). Although Bcl-2 plays a strong role in melanocyte survival, its role in melanoma is unclear, with some studies suggesting that levels increase with tumor progression and others demonstrating decreased Bcl-2 at advanced tumor stages and metastasis (reviewed in Bush and Li, 2003).

In contrast to Bcl-2, our group and others have found that anti-apoptotic proteins Mcl-1 and Bcl-xL are consistently overexpressed in melanoma compared to benign nevi (Tang et al., 1998; Leiter et al., 2000; Zhuang et al., 2007; Wong et al., 2008). Expression tends to increase with tumor grade and stage, with metastatic cells displaying highest levels of Mcl-1 and Bcl-xL. Overexpression of Mcl-1 has particularly been linked to chemoresistance in melanoma (Hocker et al., 2008). Indeed, down-regulation of Mcl-1 levels sensitizes melanoma cells to DTIC (Thallinger et al., 2003).

Furthermore, pro-apoptotic Bcl-2 family members can also be dysregulated in melanoma. Low levels of pro-apoptotic Bax and Bak are frequently observed in primary melanoma and correlate with increased risk of metastasis and an overall poor prognosis (Fecker et al., 2006; Tchernev and Orfanos, 2007). BH3-only proteins Bim and PUMA have also been shown to be down-regulated in melanoma and levels are inversely correlated with 5-year survival (Karst et al., 2005; Dai et al., 2008).

Other components of the intrinsic pathway

The p53 protein is a critical regulator of the apoptotic response to DNA damages and, as such, the *TP53* gene is the most commonly mutated gene in human cancer. However, only around 13% of melanomas are associated with *TP53* mutations (Hocker et al., 2008). One possible explanation for this observation is that the selective pressure to mutate *TP53* may be alleviated by the loss of downstream apoptotic function (Soengas and Lowe, 2003). In addition to the Bcl-2 family, multiple components of the intrinsic pathway are dysregulated in melanoma. Several studies have demonstrated that expression of apoptotic effector Apaf-1 is down-regulated in melanoma (Dai et al., 2004; Mustika et al., 2005).

There is also evidence that expression of IAPs, including survivin and Livin (ML-IAP) and XIAP, increases with melanoma progression and predicts poor disease outcome (Gong et al., 2005; Takeuchi et al., 2005; Kluger et al., 2007; Chen et al., 2009b). High Livin expression is associated with resistance to etoposide *in vitro* and poor responses to chemotherapy *in vivo* (Nachmias et al., 2003*)*. Knockdown of Livin by siRNA induces apoptosis, cell cycle arrest and/or decreased proliferation in melanoma cell lines (Wang et al., 2007a). Inhibition of survivin via a dominant-negative construct also induces apoptosis in melanoma (Liu et al., 2004). Down-regulation of XIAP sensitizes melanoma cells to apoptosis induction by TRAIL (Thayaparasingham et al., 2009).

Extrinsic pathway

Melanoma cells, particularly those derived from melanoma metastases, are often resistant to the induction of apoptosis by death ligands (reviewed in Eberle et al., 2007). Down-regulation of death receptors Fas, TRAIL-R1 and TRAIL-R2 has been noted in melanoma (Bullani et al., 2002; Zhuang et al., 2006). Melanoma also displays overexpression of c-FLIP (cellular FLICE inhibitory protein), which inhibits activity of caspase-8 and -10 (Bullani et al., 2001). Recently, *CASP8* and *CASP10* polymorphisms have been shown to contribute to melanoma susceptibility; however, the functional effects of these variants on induction of apoptosis remains to be elucidated (Li et al., 2008a).

Dysregulation of survival pathways in melanoma

MAPK pathway

Resistance to apoptosis can also be conferred by overactivity of key survival pathways, many of which themselves regulate Bcl-2 family expression and function. Of particular importance in melanoma is the mitogen-activated protein kinase (MAPK) network, namely the RAS/RAF/MEK/ERK pathway (Sekulic et al., 2008; Russo et al., 2009). Growth factor stimulation of receptor tyrosine kinases induces activation of G protein RAS, of which there are three isoforms: HRAS, KRAS and NRAS. RAS then induces activation of serine-threonine kinase RAF (ARAF, BRAF, CRAF). RAF phosphorylates MEK (MEK1 and MEK2), which in turn phosphorylates and activates ERK (ERK1 and ERK2). Active ERK translocates to the nucleus and phosphorylates transcription

factors promoting cell survival and proliferation. ERK activity promotes the expression of anti-apoptotic Bcl-2, Bcl-xL and Mcl-1 (Boucher et al., 2000). In addition, ERK also phosphorylates BH3-only protein Bim, inhibiting its pro-apoptotic activity (Harada et al., 2004).

Up to 90% of melanomas display constitutive activation of ERK (Cohen et al., 2002). ERK hyperactivity is due to the high frequency of *NRAS* (15-30%) and *BRAF* (50-70%) mutations in melanoma (Gray-Schopfer et al., 2007). *NRAS* and *BRAF* mutations are largely mutually exclusive. The most common mutation of *NRAS* is a leucine to glutamine substitution at position 61 (Q61L), resulting in constitutive activity of the protein. The most common BRAF mutation in melanoma is the glutamic acid for valine substitution (V600E), which results in constitutive activation and enhanced kinase activity (Davies et al., 2002). As discussed below, inhibition of RAS, RAF and MEK signaling is being investigated as a potential therapeutic strategy for melanoma.

PI3K/Akt pathway

The PI3K/Akt pathway also plays a role in survival and apoptotic resistance in melanoma (reviewed in Hocker et al., 2008; Lomas et al., 2008; Sekulic et al., 2008). Phosphoinositide 3-kinase (PI3K) is activated downstream of receptor tyrosine kinases, G-protein coupled receptors or RAS proteins. PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), leading to activation of the major effector of this pathway, Akt. Akt activity promotes Bcl-2 expression and inhibits

activity of Bad. Other downstream effectors of Akt, including mTOR and FOXO1, also contribute to cell proliferation and survival. Negative regulation of the PI3K/Akt pathway comes from PTEN, which functions to dephosphorylate PIP3, preventing Akt activation.

High levels of phosphorylated (active) Akt have been detected in melanoma, with highest expression levels in melanoma metastases (Dai et al., 2005). Loss of PTEN function is also commonly reported for late-stage melanomas, due to point mutations, promoter hypermethylation or loss of heterozygosity of chromosome 10q (Gray-Schopfer et al., 2007; Lomas et al., 2008). Interestingly, *PTEN* mutations co-occur with *BRAF* mutations in about 20% of cases; however, *PTEN* and *NRAS* mutations appear to be mutually exclusive (Tsao et al., 2004b).

Multiple other survival regulatory proteins, including MITF, NF-κB, β-catenin and c-KIT are also reported to be dysregulated in some cases of melanoma. For excellent comprehensive reviews of melanoma genetics see (Hocker et al., 2008) and (Sekulic et al., 2008).

Targeted therapies in melanoma

Targeting the MAPK pathway

A number of novel targeted therapeutic strategies have entered clinical trials for treatment of advanced stage melanoma. Based on the high frequency of *BRAF* and *NRAS* mutations in melanoma, several treatment strategies have been investigated to inhibit RAS, RAF or MEK. As specific RAS inhibitors have

proven difficult to develop, treatments to target post-translational modification of the RAS protein have been investigated (reviewed in Fecher et al., 2009). Farnesylation of RAS is required for proper membrane localization; thus, farnesyltranferase inhibitors (FTIs) have been investigated for melanoma therapy. However, a phase II clinical trial of FTI R115777 did not produce an objective clinical response and the trial was subsequently terminated. A major limitation of RAS-targeted therapies is the higher frequency of *BRAF* mutations compared to *NRAS* mutations in melanoma.

Sorafenib (BAY43-9006) is an oral, multi-targeted kinase inhibitor, shown to inhibit RAF as well as other kinases including VEGFR, PDGFR, Flt-3, c-KIT and FGFR-1 (Wilhelm et al., 2004). In clinical trials, sorafenib displayed little single agent activity in melanoma (Eisen et al., 2006). Combination treatments of sorafenib with DTIC or carboplatin and paclitaxel produced improved response rates; however, overall survival was largely unchanged (Lorigan et al., 2008). One proposed explanation for the lack of sorafenib efficacy in melanoma is that the drug more potently targets CRAF than BRAF (Wilhelm et al., 2004). As a result, specific BRAF inhibitors are currently entering clinical trials (Russo et al., 2009). Furthermore, a specific inhibitor of the V600E mutant BRAF has been developed (PLX4720) with promising anti-tumor effects in *in vivo* mouse models (Tsai et al., 2008).

In addition, MEK inhibitors have come to light as a potential therapeutic strategy for melanoma. MEK inhibitors have shown promising preclinical data, effectively inducing regression of melanoma xenograft tumors (Collisson et al.,

2003). However, *BRAF* mutant cells are much more sensitive to MEK inhibition than *NRAS* mutants (Solit et al., 2006). As a result, a phase II trial of MEK inhibitor AZD6244 demonstrated objective responses in only a small subset of *BRAF* mutant patients and not in patients with *NRAS* mutations (Fecher et al., 2009). Furthermore, there is evidence that melanoma can become resistant to MEK inhibition, as well as BRAF inhibition, via mutations in *MEK1* (Emery et al., 2009). In addition, based on evidence in lung cancer, high Akt activity may also confer resistance to MEK inhibitors (Meng et al., 2009).

Targeting other signaling pathways

Several drugs have been developed to target mTOR, one of the major downstream effectors of Akt. However, mTOR inhibitors CCI-779 and RAD001 have shown little single agent activity in metastatic melanoma in phase II trials (Margolin et al., 2005; Rao et al., 2006). *In vitro* studies have suggested that simultaneous inhibition of the PI3K/Akt/mTOR pathway (by rapamycin) and the RAS/RAF/MEK/ERK pathway (by sorafenib) synergistically kills melanoma cells (Molhoek et al., 2005; Lasithiotakis et al., 2008). Thus, clinical trials of this combination treatment strategy are in progress.

More general inhibitors of cell signaling are also being investigated for treatment of melanoma. These include proteasome inhibitors, such as bortezomib, which is currently licensed for use in multiple myeloma (Lorigan et al., 2008). However, phase-II trials in metastatic melanoma have not been encouraging (Markovic et al., 2005). Results of *in vitro* studies have shown that bortezomib increases levels of pro-apoptotic Noxa; however, anti-apoptotic Mcl-1 levels are

also increased and inhibition of Mcl-1 may be needed to improve treatment efficacy (Qin et al., 2005; Qin et al., 2006). Both histone deacetylase inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMT) are also in clinical trial for melanoma (Lorigan et al., 2008). In a phase I trial, DNMT decitabine has shown some promise in combination with IL-2 and further studies are ongoing to fully assess the efficacy of the combination treatment (Gollob et al., 2006).

Targeting Bcl-2

As therapies targeting survival pathways have proven disappointing, novel treatments that directly target the apoptotic machinery are being investigated. Currently, one Bcl-2 family-targeted therapy has been assessed in clinical trials for melanoma. Oblimersen (Genasense) is an antisense oligonucleotide targeting expression of Bcl-2. The anti-cancer activity of oblimersen is due to both specific down-regulation of Bcl-2 as well as non-specific effects such as production of reactive oxygen species and immunostimulation (Kim et al., 2007). In a phase-III trial comparing oblimersen plus DTIC with DTIC alone, there was a significant increase in response rate and progression free survival (Bedikian et al., 2006). However, overall survival improved only in a subset of patients without elevated serum LDH. As aforementioned, in many melanomas, Bcl-2 levels appear to decrease with advancing stage; thus, the use of Bcl-2 as a therapeutic target is questionable. Targeting of other anti-apoptotic proteins has therefore been proposed. In addition, as both Mcl-1 and Bcl-xL are consistently overexpressed in melanoma, simultaneous target of multiple anti-apoptotic Bcl-2 proteins may provide a stronger therapeutic benefit.

New strategies to target anti-apoptotic Bcl-2 proteins

Several drugs that target anti-apoptotic Bcl-2 proteins have been developed including HA-14 (Bcl-2), Antimycin A (Bcl-2 and Bcl-xL), BH3I-1 and BH3I-2 (Bcl-xL), gossypol (Bcl-2, Bcl-xl, Bcl-w, Mcl-1), obatoclax (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, Bcl-B) and ABT-737 (Bcl-2, Bcl-xL, Bcl-w) (Kang and Reynolds, 2009). Here I will focus on the agents that inhibit multiple Bcl-2 family members, namely gossypol, obatoclax and ABT-737.

Gossypol

Gossypol is a natural product derived from the cotton plant that has been used in a variety of therapeutic applications (reviewed in Lessene et al., 2008). In terms of anti-cancer activity, the (-) enantiomer of gossypol displays greater cytotoxicity than the (+) enantiomer, due to the ability of (-)-gossypol to bind Bcl-2, Bcl-xL, Bcl-w and Mcl-1. Ascenta is currently developing (-)-gossypol as AT-101. In pre-clinical studies, AT-101 induced apoptosis in multiple myeloma, chronic lymphocytic leukemia (CLL) and B-cell lymphoma cells and displayed synergism with conventional chemotherapeutics (Balakrishnan et al., 2008; Kline et al., 2008; Paoluzzi et al., 2008). In melanoma cell lines, (-)-gossypol improved response to proteasome inhibitor bortezomib (Wolter et al., 2007). In a phase I clinical trial of prostate cancer, AT-101 was well-tolerated and displayed encouraging single-agent clinical activity (Liu et al., 2009). A semi-synthetic derivative, apogossypol, has also been developed, which displays improved efficacy and reduced toxicity *in vivo* (Kitada et al., 2008).

Obatoclax

Obatoclax (GX15-070) is a synthetic small molecule that inhibits the interaction of anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and Bcl-B) with the BH3 domains of pro-apoptotic proteins (Zhai et al., 2006). Obatoclax has been shown to disrupt the association between Mcl-1 and Bak (Nguyen et al., 2007). Pre-clinical studies of obatoclax have demonstrated anti-cancer activity in multiple cancers including multiple myeloma and non-small cell lung cancer (Trudel et al., 2007a; Li et al., 2008b). Obatoclax alone has little effect on melanoma cells; however, it enhances apoptosis when combined with ER stress inducers thapsigargin or tunicamycin (Jiang et al., 2009). A phase-I clinical trial of obatoclax in hematological malignancies demonstrated that the treatment was generally well tolerated and produced one complete response in a patient with acute myeloid leukemia (Schimmer et al., 2008).

ABT-737

ABT-737 is a BH3-mimetic initially discovered by Abbott laboratories in NMR-based screening for small molecules that bind to the hydrophobic groove of Bcl-xL (Oltersdorf et al., 2005). ABT-737 binds with high affinity to Bcl-2, Bcl-xL and Bcl-w ($K_i \leq 1$ nM) but not to Mcl-1, A1 or Bcl-B. Thus, ABT-737 acts in a similar fashion to BH3-only protein Bad. In accordance with the indirect activation model, ABT-737 does not directly interact with Bax, but induces apoptosis through inactivation of anti-apoptotic Bcl-2 family members (van Delft et al., 2006). This drug displays single-agent activity in multiple solid tumors and

hematological malignancies including glioblastoma, small cell lung cancer, multiple myeloma and CLL (Oltersdorf et al., 2005; Del Gaizo Moore et al., 2007; Kline et al., 2007; Tagscherer et al., 2008). ABT-737 also synergistically enhances the effects of other cytotoxic agents such as TRAIL, dexamethasone and melphalan (Trudel et al., 2007b; Huang and Sinicrope, 2008). When my studies were initiated, there were no published reports of ABT-737 in melanoma. An orally available analog, ABT-263, has also been developed and is currently in clinical trials (Tse et al., 2008).

Importantly, recent studies have suggested that ABT-737 is the most specific inhibitor of anti-apoptotic Bcl-2 proteins out of the drugs mentioned above. Inhibition of Bcl-2 proteins is expected to induce the intrinsic pathway of apoptosis via activation of Bax and Bak. However, several Bcl-2 inhibitors including gossypol, apogossypol, obatoclax, HA14-1, and Antimycin A display similar toxicities in Bax/Bak double knockout cells as in wild type cells (van Delft et al., 2006; Vogler et al., 2009). In contrast, double knockout cells are almost completely resistant to ABT-737 treatment. Thus, Vogler et al. (2009) proposed that ABT-737 is a true BH3-mimetic whereas most other Bcl-2 inhibitors have non-specific targets that largely mediate their cytotoxic effects.

The major factor that confers resistance to ABT-737 in cancer cells is expression of Mcl-1 (Konopleva et al., 2006; van Delft et al., 2006; Lin et al., 2007). Reduction of Mcl-1 levels by RNA interference (RNAi; discussed in Chapter 3) therefore improves ABT-737 efficacy. In addition, drugs such as roscovitine, which indirectly lower Mcl-1 levels, act synergistically with ABT-

737 to induce apoptosis (Chen et al., 2007). Thus, ABT-737, in combination with an agent to reduce activity of Mcl-1, represents a promising strategy to specifically target the apoptotic machinery of cancer cells.

Hypothesis and Summary of Studies

Malignant melanoma is highly resistant to apoptosis and, as a result, does not respond well to conventional chemotherapy and radiation. Novel therapies targeting survival pathways have only modest effects in melanoma. Treatments that directly target apoptotic regulatory proteins may hold greater promise. Anti-apoptotic proteins Mcl-1, Bcl-xL and, to a lesser extent, Bcl-2, are frequently overexpressed in melanoma. BH3-mimetic ABT-737 is a potent and specific inhibitor of Bcl-2, Bcl-xL and Bcl-w. ABT-737 does not inhibit Mcl-1 and thus down-regulation of Mcl-1 often improves ABT-737 efficacy in cancer cells. I therefore hypothesized that the combination of Mcl-1 knockdown and ABT-737 would overcome the anti-apoptotic effects of Mcl-1 and Bcl-xL in melanoma cells, resulting in the induction of apoptosis.

I investigated the effects of Mcl-1 knockdown and ABT-737 treatment, alone and in combination, on cell viability and apoptosis in six well-known melanoma cell lines. I also investigated the mechanisms involved treatment response by assaying levels of Bcl-2 family proteins and examining global gene expression changes by microarray analyses. My results led me to elucidate the role of the extrinsic pathway in treatment response as well as develop a novel treatment strategy combining ABT-737 with inhibition of p38. My findings therefore describe promising, new treatment strategies for melanoma and provide novel evidence as to the mechanisms involved in treatment response. This research is important as there are currently no effective therapies for melanoma and targeted treatments to induce apoptosis are of great clinical value.

~Chapter 2~

Materials and Methods

Drugs and other reagents

ABT-737 and its enantiomer were generously supplied by Abbott Laboratories (Abbott Park, IL). Both compounds were dissolved in DMSO to final concentrations of 20 mM and 5 mM (working stock) and stored at -20^{o}C. Caspase inhibitors Z-IETD-FMK, Z-LEHD-FMK, and Z-VAD-FMK were obtained from Calbiochem, EMD Chemicals (San Diego, CA). Caspase-10 inhibitor Z-AEVD-FMK and recombinant death receptor chimera (Fas/Fc, TNF-R1/Fc, TRAIL-R1/Fc) were from R&D Systems (Minneapolis, MN). The caspase inhibitors were dissolved in DMSO and the recombinant chimera were dissolved in 0.1% BSA in PBS. p38 inhibitors SB202190 and SB203580, JNK inhibitor SP600125 and MEK inhibitor PD98059 were purchased from Sigma (St. Louis, MO) and dissolved in DMSO. Ammonium chloride (NH_4Cl) and chloroquine were also from Sigma. Proteasome inhibitors MG-132 and clasto-Lactacystin β-lactone were from Calbiochem.

Cell culture

Human melanoma cell lines were maintained in RPMI-1640 (Hyclone, Logan, UT) supplemented with 10% FBS (Hyclone). Human epidermal melanocytes were plated in Melanocyte Medium with melanocyte growth supplement, 0.5% FBS and 1% penicillin/streptomycin (all from ScienCell, Carlsbad, CA). After the second passage, melanocytes were maintained in RPMI-1640 with 10% FBS for at least 48 hours prior to harvest. All cells were grown in 5% CO_2 at 37^{o}C.

siRNA transfection

Cell lines were transfected with Dicer-substrate siRNA (DsiRNA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's directions. Mcl-1 DsiRNA sequence: Sense: CCC GCC GAA UUC AUU AAU UUA CUG T, Antisense: ACA GUA AAU UAA UGA AUU CGG CGG GUA (HSC.RNAI.N021960.2.3, Integrated DNA Technologies, Coralville, IA). Caspase-8 DsiRNA sequence: Sense: CUA CCA GAA AGG UAU ACC UGU UGA G, Antisense: CUC AAC AGG UAU ACC UUU CUG GUA GUU (ACC NM_001228.4_3, IDT). Scrambled Control sequence: Sense: CUU CCU CUC UUU CUC UCC CUU GUG A, Antisense: UCA CAA GGG AGA GAA AGA GAG GAA GGA (DS Scrambled Neg, IDT).

Cotransfection of Mcl-1 and caspase-8 DsiRNA

To assess the effect of caspase-8 knockdown on survival, cells were first transfected with 40 nM caspase-8 or Scrambled DsiRNA in 60 mm plates, then split into 96 well plates 24 hours post-transfection. 24 hours later, cells were transfected with Mcl-1 or Scrambled DsiRNA and/or treated with ABT-737 as appropriate.

Immunoblot analysis

Standard SDS-PAGE

Adherent and suspension cells were harvested and lysed (50 mM Tris pH 7.5, 10 nM MgCl2, 1% SDS) in the presence of protease inhibitors (Complete

protease inhibitor cocktail, Roche, Laval, PQ). Samples were sonicated to fragment the DNA. After addition of loading dye (2.5% SDS, 5% β-mercatoethanol, 12.5% glycerol, 6.25 mM Tris pH 6.8 + Bromophenol blue), samples were boiled for 10 minutes and run on SDS polyacrylamide gels. Western transfer to 0.45 μM PVDF membrane was performed using standard protocols.

Bis-Tris electrophoresis

After standard whole cell lysis, 20-50 μg protein was denatured at 70^{o}C with 2.5 μL NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) and 1 μL NuPAGE reducing agent (Invitrogen) in a total of 10 μL. Samples were then run on NuPAGE Novex 4-12% Bis-Tris gradient gels in MES SDS Running Buffer (Invitrogen) according to manufacturer's instructions.

Immunoblotting

Immunoblots were blocked in 5% dry milk in TBST. Primary antibodies were incubated in 5% BSA/TBST or 5% milk/TBST according to manufacturer's recommendations, with the exception of the blots in Chapter 7, Figures 7-2 to 7-5, which were performed using HIKARI western enhancing solution (Nacalai USA, San Diego, CA). Antibody binding was visualized using the appropriate HRP-conjugated secondary antibody and Amersham ECL (Amersham Biosciences, now GE Healthcare, Piscataway, NJ). Densitometry was performed using Quantity One software (Bio-Rad, Mississauga, ON, Canada). Loading was

assessed by γ-tubulin levels or by the amido black stain of the immunoblot. All antibodies used are listed in Table 2-1.

Table 2-1: Antibodies used in western immunoblotting experiments

Antibody	Source	Catalog #
A1	Cell Signaling	4647
Bad	Cell Signaling	9292
Bak (active)	Calbiochem	AM04
Bax (active)	BD	556467
Bcl-2	BD	610538
Bcl-w	Cell Signaling	2724
Bcl-xL	BD	610211
Bid	Cell Signaling	2003
Bik	Cell Signaling	4592
Bim	Cell Signaling	4582
Bmf	Cell Signaling	4692
Caspase 3 (cleaved)	Cell Signaling	9664
Caspase 8 (total)	Cell Signaling	9746
Caspase 8 (cleaved)	Cell Signaling	9496
Caspase 9 (total)	Cell Signaling	9502
Caspase 9 (cleaved)	Cell Signaling	9505
Caspase 10	Cell Signaling	9752
Gamma tubulin	Sigma	T6557
Mcl-1	Sigma	M8434
NOXA	Calbiochem	OP180
p21	BD	556431
p53	BD	554293
PARP (cleaved)	Cell Signaling	9541
PUMA	Cell Signaling	4976
PUMA (2)	Genetex	29643

MTT assay for viability

Overall viability was assessed colourimetrically using MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma). Cells were plated to ~2000-3000 cells/well in 96 well plates the day prior to treatment. Twenty-four hours after treatment, 25 μl MTT solution (2 mg/mL in PBS) was added to each well and plates were incubated at 37^{o}C for 6 hours. Following incubation, the formazan precipitate was solubilized by adding 100 μL 24:1 isopropanol:HCl and mixing by pipet 50 times. Plates were then incubated an additional hour at 37^{o}C. Absorbance was measured at 570 nm on a plate reader (BioTek uQuant spectrophotometer, Fisher Scientific, Pittsburgh, PA).

TUNEL

Adherent and suspension cells were collected, fixed in 1% paraformaldehyde and stored at -20^{o}C until analysis. Terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) was performed using the Apo-BrdU *In Situ* DNA Fragmentation assay kit (Biovision, Mountain View, CA) according to manufacturer's instructions. Flow cytometry was performed on a FACSCalibur using CellQuest-Pro software (BD BioSciences, San Jose, CA).

Propidium iodide staining for cell cycle analysis

Adherent and suspension cells were collected, pelleted and resuspended in 500 μL Vindelov propidium iodide solution (10 mM Tris pH 8.0, 10 mM NaCl,

700 U RNase A, 0.075 mM propidium iodide, 1 mM NP40). Flow cytometry was performed on a FACSCalibur using CellQuest-Pro software (BD BioSciences).

Crystal violet

Crystal violet assay for viability/cell density was performed by removing media from plates, washing in PBS, then staining for 10 minutes with crystal violet (0.5% crystal violet, 20% methanol). Plates were then washed with water and dried. The remaining dye was redissolved in methanol and absorbance read at 600 nm.

PCR and sequencing of *CASP8* polymorphisms

DNA was extracted using the QIAquick DNA extraction columns (Qiagen, Valencia, CA). Previously described primers and PCR conditions were used to amplify the -652 6N del polymorphism (Sun et al., 2007), and the D302H polymorphism (Li et al., 2008a). Bands were purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were run on 3130x1 or 3100-Avant Applied Biosystems genetic analyzers.

RNA extraction and purification

Adherent and suspension cells were harvested and RNA extracted using Trizol reagent (Invitrogen) according to manufacturer's instructions. To ensure a high level of purity, samples were purified and concentrated using the RNeasy

MinElute Cleanup Kit (Qiagen). To test RNA quality, 1 μg of each sample was run on a MOPS formaldehyde gel.

cDNA microarray protocols

Custom cDNA microarrays were manufactured by Agilent Technologies (La Jolla, CA). Each slide contained 8 identical arrays for a total of 16 arrays. Array design is discussed in greater detail in Chapter 5. Array procedures were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) Protocol by Xiao (Nick) Zhang at the Gene Microarray Facility of Queen's University, Kingston, ON. Labeling was performed using the Quick Amp Labeling Kit, One Color (Agilent). Briefly, total RNA is first reverse transcribed to cDNA. The cDNA is transcribed with T7 RNA polymerase in the presence of Cy3-dCTP to produced fluorescently-labeled "cRNA". The cRNA is then column-purified and quantified. Samples are then prepared for hybridization using the Gene Expression Hybridization Kit (Agilent) and incubated on the arrays for 17 hours at 65^{o}C. Arrays are then washed in GE Wash Buffers 1 and 2 and scanned using an Agilent DNA Microarray Scanner and DNA Microarray Scan Control Software v7. Data were extracted using Agilent Feature Extraction software. Data normalization and analysis was performed using Genespring GX software (Silicon Genetics, Redwood City, CA).

RT-PCR (Reverse Transcription - PCR)

RNA samples were diluted to equal concentrations. Reverse transcription was performed on 400 ng RNA using the AccuScript High Fidelity 1st Stand cDNA Synthesis Kit (Stratagene, Agilent Technologies, La Jolla, CA). PCR amplifications were carried out in 25 μl reactions containing 1 μl cDNA, 2.5 μl 10X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM forward primer, 0.4 μM reverse primer, and 0.5 U *Taq* polymerase (Recombinant, Invitrogen). Amplification conditions were 95°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The first PCR amplification reaction was performed using primers to GAPDH (Kihara et al., 2001) to confirm equal loading between samples. If there were any differences, the amount of cDNA in each PCR reaction was adjusted accordingly. Remaining primers are listed in Table 2-2.

Table 2-2: Primer sets for RT-PCR experiments

Primer	Accession	Sequence[1]	Product (bp)
BAX Forward	NM_138763	TGG GCT GGA CAT TGG ACT TC	173
BAX Reverse		GCC TCA GCC CAT CTT CTT CC	
BCLB Forward	NM_020396	GCC CAA CTG TGA CCA ACT AAA	177
BCLB Reverse		GAG CCA TTT GCA TTC TTG TC	
CASP4 Forward	NM_033306	GCG GAA CTG TGC ATG ATG AG	274
CASP4 Reverse		GGC GTT GAA GAG CAG AAA GC	
CASP8 Forward	NM_033355	GCC CAA ACT TCA CAG CAT TAG	528
CASP8 Reverse		TGG CAA AGT GAC TGG ATG TAC	
CCND2 Forward	NM_001759	GCC AAA GGA AGG AGG TCA G	441
CCND2 Reverse		GGC CAG GAA CAT GCA GAC A	
FOS Forward	NM_005252	CGG GCT TCA ACG CAG ACT AC	346
FOS Reverse		CGG CCT CCT GTC ATG GTC TT	
HSPA5 Forward	NM_005347	GGG TCC CAC AGA TTG AAG TC	442
HSPA5 Reverse		GGG CCT GCA CTT CCA TAG A	
JUN Forward	NM_002228	CGC GCG AGT CGA CAA GTA AGA	568
JUN Reverse		GCC CTC CTG CTC ATC TGT CAC	
MAPK11 Forward	NM_002751	CCG GGA AGC GAC TAC ATT GAC	635
MAPK11 Reverse		TGC CCA GAC TCC TAC ACA TG	
MCL1 Forward	NM_021960	CGG GGA ATC TGG TAA TAA CAC	757
MCL1 Reverse		GCC ATA ATC CTC TTG CCA CTT	
MYC Forward	NM_002467	CCG GAC GAC GAG ACC TTC AT	579
MYC Reverse		CGC TGC GTA GTT GTG CTG AT	
NOXA Forward	NM_021127	GGT GCA CGT TTC ATC AAT TTG	586
NOXA Reverse		CGC CCA ACA GGA ACA CAT T	
TNF Forward	NM_000594	GCC CAG GCA GTC AGA TCA TC	254
TNF Reverse		CGG CTG ATG GTG TGG GTG A	

[1] All sequences are shown 5′ to 3′

Statistics

All statistical analyses were performed on GraphPad Prism version 5 for Macintosh (GraphPad Software, San Diego, CA). Unless otherwise stated, P-values were determined by two-tailed, unpaired *t* tests. To assess the synergistic effect of the combination treatment, we used two-way ANOVA as previously described (Slinker, 1998). For the Mcl-1 DsiRNA and ABT-737 combination, overall viability by MTT assay (i.e. absorbance at 570 nm without normalization) of cells treated with the combination treatment (Mcl-1 DsiRNA 10 nM and ABT-737 10 μM) were compared against cells treated with either single treatment or cells treated with only a Scrambled DsiRNA control. The same data is shown in its normalized form in Figure 3-5. For the SB202190/ABT-737 combination, surviving fraction of cells treated with the combination of SB202190 (25 μM) and ABT-737 (10 μM) was compared against cells treated with either single treatment (+DMSO) or cells treated with only the enantiomer control. The same data is shown in the left panel of Figure 6-4. In all statistical analyses, P-values less than 0.05 were considered statistically significant.

~Chapter 3~
Comparison of the response of six melanoma cell lines to Mcl-1 knockdown and ABT-737, alone and in combination

Data presented in this chapter have been published.

Angela M. Keuling, Kathleen E. A. Felton, Arabesque A. Parker, Majid Akbari, Susan E. Andrew and Victor A. Tron. (2009) RNA silencing of Mcl-1 enhances ABT-737-mediated apoptosis in melanoma: Role for a caspase-8-dependent pathway. *PLoS ONE* 17 (8): e6651.

All experimental design, data analysis and experimentation were performed by Angela Keuling, with the exception of some of the time courses in Figure 3-2 and some of the MTT assays in Figure 3-5, which were performed with assistance from Lindsey Schultz and Arabesque Parker, respectively. Initial knockdown studies were performed by Kathleen Felton.

Introduction

Anti-apoptotic Bcl-2 family members Mcl-1, Bcl-xL and, to a lesser extent, Bcl-2, are frequently overexpressed in melanoma (Tang et al., 1998). The BH3-mimetic ABT-737 targets Bcl-2, Bcl-xL and Bcl-w but does not affect activity of Mcl-1 (Oltersdorf et al., 2005). Tahir *et al.* demonstrated an inverse relationship between expression of Mcl-1 and sensitivity to ABT-737 in cancer cell lines (Tahir et al., 2007). In addition, ABT-737-resistant cell lines, generated by continuous drug exposure over several months, develop striking Mcl-1 overexpression. We therefore proposed to combine ABT-737 with genetic down-regulation of Mcl-1 to simultaneously target multiple anti-apoptotic Bcl-2 family members in melanoma.

RNA interference (RNAi) is a gene silencing mechanism originally described by Andrew Fire and Craig Mello in 1998 (Fire et al., 1998). In brief, long double-stranded RNA (dsRNA) molecules are cleaved by the RNase III enzyme Dicer into small interfering RNAs (siRNAs) of approximately 21-23 nucleotides in length. The siRNA duplex is then incorporated into the RNA-induced silencing complex (RISC), which is the effector complex of gene silencing. RISC uses one strand of the siRNA (the "guide" strand) as a template to bind and degrade complementary mRNA transcripts (reviewed in Dorsett and Tuschl, 2004).

RNAi has been utilized both as a research tool and as a therapeutic application to block expression of genes responsible for human disease (Kim and Rossi, 2007). RNAi can be induced in mammalian cells either by expression of

short-hairpin RNAs from specialized vectors or by use of chemically synthesized siRNA duplexes. Most synthetic siRNAs are approximately 21 nucleotides in length and therefore are directly incorporated into endogenous RISC without prior Dicer processing. However, there is evidence that longer siRNAs (27-mers) that serve as Dicer substrates are more efficiently incorporated into RISC and thereby display an up to 100-fold increase in potency compared to conventional 21-mers (Kim et al., 2005). Therefore, to knockdown expression of Mcl-1 in melanoma cell lines, I utilized a Mcl-1-specific Dicer-substrate siRNA (DsiRNA).

To examine the effects of Mcl-1 knockdown and ABT-737 on melanoma *in vitro*, I used six different malignant melanoma cell lines: Lox IMVI, Malme-3M, MeWo, SK-MEL-2, SK-MEL-5 and SK-MEL-28. With the exception of SK-MEL-28, which was derived from primary tumor tissue, all cell lines were derived from melanoma metastases. In addition, all lines, with the exception of MeWo, are part of the National Cancer Institute's NCI-60 panel of cancer cell lines. These cell lines represent some of the genetic diversity in melanoma. Mutation status at key melanomagenesis genes *CDKN2A*, *NRAS*, *BRAF*, and *TP53* is shown in Table 3-1. Three cell lines with wild type *TP53* were chosen as well as three cell lines with known *TP53* mutations. Of note, although *CDKN2A* mutations are associated with familial melanoma more than sporadic; mutations affecting the $p16^{INK4A}$ transcript are commonly acquired during culture of melanoma cell lines (Fujimoto et al., 1999).

Table 3-1: Melanoma cell line characteristics

Cell Line	**Primary site**	**Metastatic site**[1]	**Mutations**[2]				
			CDKN2A	*BRAF*	*NRAS*	*TP53*	Other
Lox IMVI	skin	lymph node	M1 *157del	V600E			
Malme-3M	skin	lung	M1 *157del	V600E			
MeWo	skin	lymph node	R80*			Q317*, E258K	
SK-MEL-2	skin	skin of thigh			Q61R	G245S	
SK-MEL-5	skin	axillery node	M1 *157del	V600E			*STK11*
SK-MEL-28	skin	none		V600E		L145R	*EGFR*

1. With the exception of SK-MEL-28, all cell lines are derived from metastatic site
2. Mutation data obtained from the COSMIC (Catalogue of Somatic Mutations In Cancer) database at www.sanger.ac.uk/genetics/CGP/cosmic

I began my studies by optimizing knockdown of Mcl-1 by Dicer-substrate siRNA in the six melanoma cell lines. I then investigated the effects of Mcl-1 DsiRNA and ABT-737 treatment, alone and in combination, on overall viability and the induction of apoptosis. I found that while neither single agent significantly induces cell death in all cell lines, the combination treatment is consistently effective in both reducing overall viability and increasing the induction of apoptosis. Since my work began in 2005, several studies have shown that genetic silencing of Mcl-1 synergistically increases ABT-737-mediated cell death (See Discussion). Thus, the combination therapy represents a promising new treatment strategy for malignant melanoma.

Results

Optimization of Mcl-1 knockdown

Prior to the initiation of my studies, Kathleen Felton in our laboratory tested three Mcl-1-specific DsiRNAs. I used the most effective of the three to reduce levels of Mcl-1 in the six melanoma cell lines used in this study (DsiRNA sequence provided on page 37). I observed noticeably reduced levels of Mcl-1 with as little as 0.01 nM DsiRNA (Figure 3-1). In all cell lines, maximum knockdown is achieved by a dose of 10 nM DsiRNA, resulting in 80-90% reduction in Mcl-1 levels (Figure 3-1). When optimizing knockdown, I also examined levels of cleaved PARP (Poly ADP-ribose polymerase) as a marker for apoptosis induction. PARP is a nuclear protein cleaved by caspase-3 during apoptosis (Boulares et al., 1999). Overall, the effect of Mcl-1 knockdown on PARP cleavage was minor. However, in cell lines that displayed increased PARP cleavage, maximum cleavage was achieved by the 10 nM dose or lower. I therefore utilized 10 nM of Mcl-1 DsiRNA as a standard dose in subsequent assays.

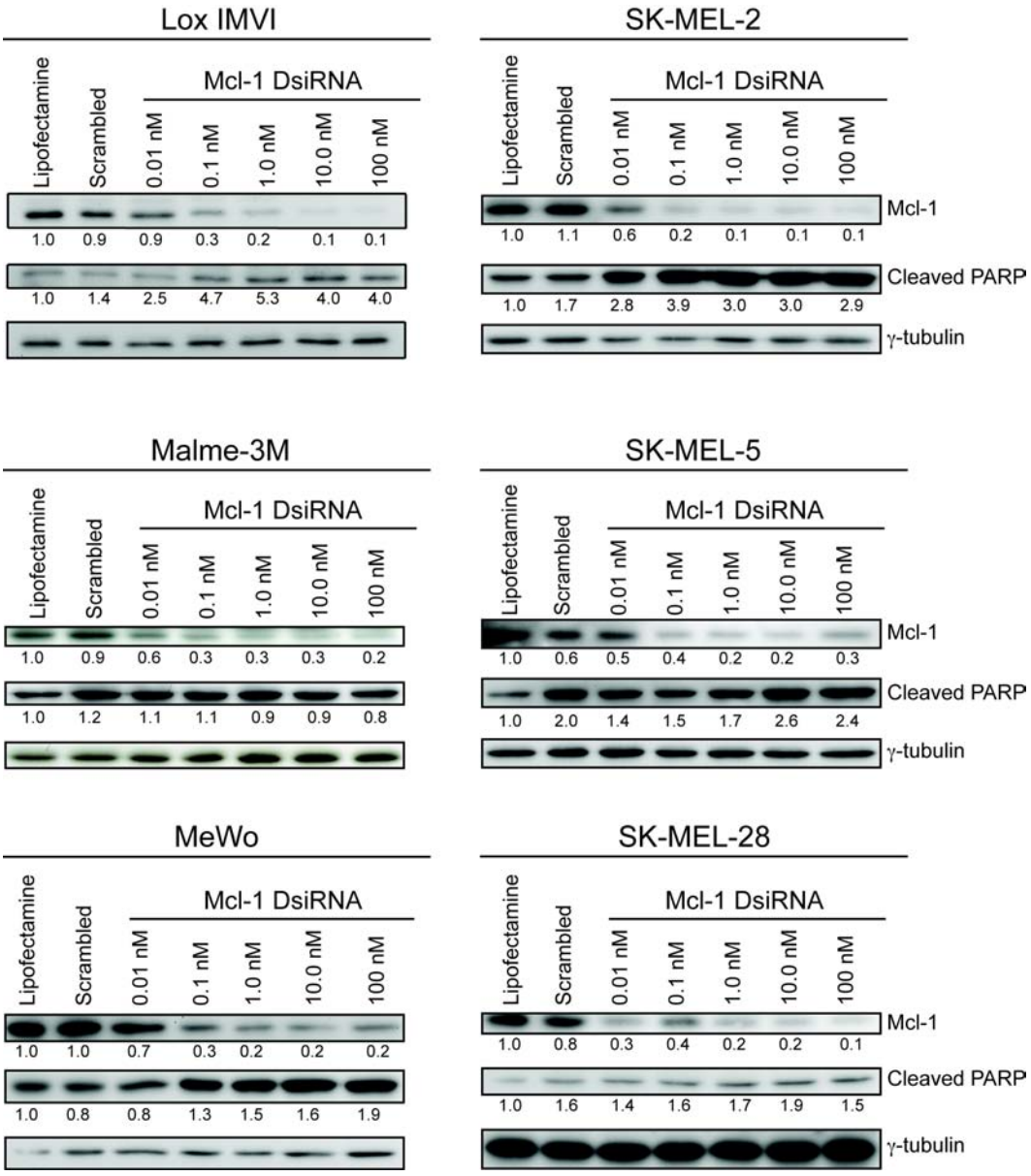
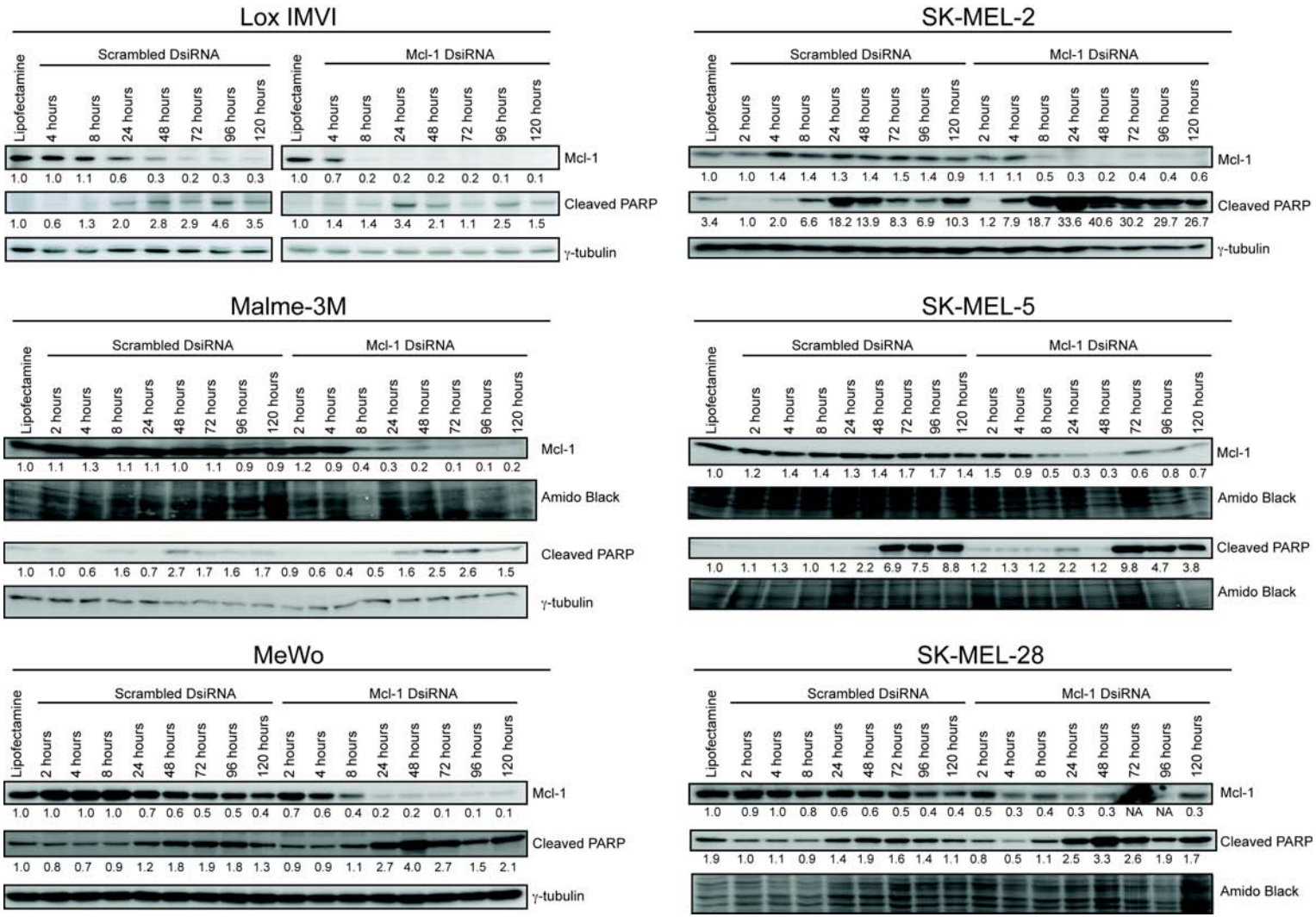


Figure 3-1: Knockdown of Mcl-1 with Dicer substrate siRNA

Western blot analysis demonstrating dose-dependent knockdown by Mcl-1 DsiRNA. Cells were treated with Lipofectamine-only, 100 nM Scrambled DsiRNA or increasing doses of Mcl-1 DsiRNA for 24 hours. Values represent quantification of bands using γ-tubulin to correct for loading and normalization to the Lipofectamine-only band.

Optimization of time point for Mcl-1 knockdown

Using the 10 nM dose of Mcl-1 DsiRNA, I then examined the length of the knockdown effect. In all cell lines, knockdown is detectable by four to eight hours post-transfection and maximum knockdown is achieved at 24 hours (Figure 3-2). Increased PARP cleavage is apparent as early as four hours. Both knockdown and PARP cleavage are maintained for up to five days (120 hours). Surprisingly, increased PARP cleavage is also observed in the Scrambled control-treated cells at later time points, indicative of non-specific cell death. This is likely due to the rapid growth of the melanoma cell lines, leading to overgrowth and nutrient deprivation at later time points. Confirming this hypothesis, in SK-MEL-2, I changed the media after the 48-hour time point, and observed a noticeable decrease in PARP cleavage in the control cells. Therefore, to minimize non-specific effects, subsequent assays were performed at 24 hours: the earliest time point at which maximum knockdown is achieved.


Lox IMVI
Scrambled DsiRNA
Mcl-1 DsiRNA
Lipofectamine
4 hours
8 hours
24 hours
48 hours
72 hours
96 hours
120 hours
Mcl-1
1.0 1.0 1.1 0.6 0.3 0.2 0.3 0.3 1.0 0.7 0.2 0.2 0.2 0.2 0.1 0.1
Cleaved PARP
1.0 0.6 1.3 2.0 2.8 2.9 4.6 3.5 1.0 1.4 1.4 3.4 2.1 1.1 2.5 1.5
γ-tubulin
SK-MEL-2
Scrambled DsiRNA
Mcl-1 DsiRNA
Lipofectamine
2 hours
4 hours
8 hours
24 hours
48 hours
72 hours
96 hours
120 hours
Mcl-1
1.0 1.0 1.4 1.4 1.3 1.4 1.5 1.4 0.9 1.1 1.1 0.5 0.3 0.2 0.4 0.4 0.6
Cleaved PARP
3.4 1.0 2.0 6.6 18.2 13.9 8.3 6.9 10.3 1.2 7.9 18.7 33.6 40.6 30.2 29.7 26.7
γ-tubulin
Malme-3M
Scrambled DsiRNA
Mcl-1 DsiRNA
Lipofectamine
2 hours
4 hours
8 hours
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48 hours
72 hours
96 hours
120 hours
Mcl-1
1.0 1.1 1.3 1.1 1.1 1.0 1.1 0.9 0.9 1.2 0.9 0.4 0.3 0.2 0.1 0.1 0.2
Amido Black
Cleaved PARP
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γ-tubulin
SK-MEL-5
Scrambled DsiRNA
Mcl-1 DsiRNA
Lipofectamine
2 hours
4 hours
8 hours
24 hours
48 hours
72 hours
96 hours
120 hours
Mcl-1
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Amido Black
Cleaved PARP
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Amido Black
MeWo
Scrambled DsiRNA
Mcl-1 DsiRNA
Lipofectamine
2 hours
4 hours
8 hours
24 hours
48 hours
72 hours
96 hours
120 hours
Mcl-1
1.0 1.0 1.0 1.0 0.7 0.6 0.5 0.5 0.4 0.7 0.6 0.4 0.2 0.2 0.1 0.1 0.1
Cleaved PARP
1.0 0.8 0.7 0.9 1.2 1.8 1.9 1.8 1.3 0.9 0.9 1.1 2.7 4.0 2.7 1.5 2.1
γ-tubulin
SK-MEL-28
Scrambled DsiRNA
Mcl-1 DsiRNA
Lipofectamine
2 hours
4 hours
8 hours
24 hours
48 hours
72 hours
96 hours
120 hours
Mcl-1
1.0 0.9 1.0 0.8 0.6 0.6 0.5 0.4 0.4 0.5 0.3 0.4 0.3 0.3 NA NA 0.3
Cleaved PARP
1.9 1.0 1.1 0.9 1.4 1.9 1.6 1.4 1.1 0.8 0.5 1.1 2.5 3.3 2.6 1.9 1.7
Amido Black

Figure 3-2: Time course of Mcl-1 knockdown.

Cells were treated with Lipofectamine-only for 24 hours or with 10 nM Scrambled DsiRNA or Mcl-1 DsiRNA for the indicated time points. Values represent quantification of bands using γ-tubulin or amido black to correct for loading and normalization to the Lipofectamine-only band (except for SK-MEL-2 and SK-MEL-28, which were normalized to the 2 hour Scrambled DsiRNA bands). Note: In the SK-MEL-2 cell line, media was changed after the 48-hour time point.

Effects of Mcl-1 knockdown on cell viability

I next investigated whether knockdown of Mcl-1 alone could reduce viability of melanoma cell lines. I compared the effect of Mcl-1 DsiRNA to that of a Scrambled DsiRNA control at a dose range of 0.01 to 100 nM. Overall, Mcl-1 DsiRNA had minor effects on melanoma cell viability (Figure 3-3). At our standard 10 nM dose, Mcl-1 DsiRNA significantly reduced viability in only two cell lines, SK-MEL-2 and Lox IMVI ($P<0.05$, compared to equal dose of Scrambled DsiRNA). Mcl-1 knockdown had the greatest effect on SK-MEL-2, reducing viability by up to 20%. In all other cell lines, the difference between Mcl-1 and Scrambled DsiRNA was less than 10%, even at the highest doses. Thus, targeting Mcl-1 alone is insufficient to reduce melanoma viability to an extent that would provide a therapeutic benefit.

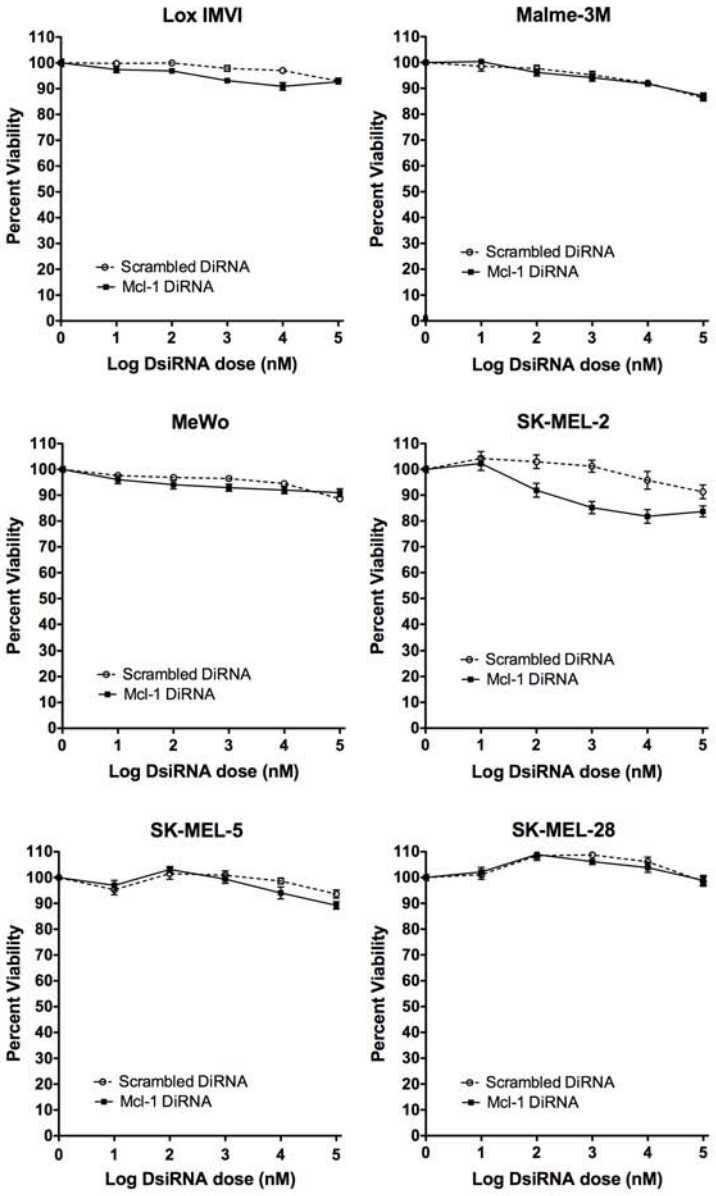

Figure 3-3: Effect of Mcl-1 DsiRNA compared to Scrambled DsiRNA on cell viability in melanoma cell lines.

Cells were transfected with 0, 0.01, 0.1, 1, 10, or 100 nM Mcl-1 DsiRNA or Scrambled DsiRNA. Each dose was increased by three orders of magnitude before the logarithm was taken to allow us to graph all doses as positive values. Viability was assessed 24 hours post-transfection via MTT assay. Curves were normalized to the 0 nM treatment. Points represent mean (n=4); error bars represent SEM (on all graphs).

Effects of ABT-737 on cell viability

As knockdown of Mcl-1 alone was not effective in the induction of cell death in all cell lines, I tested the effects of ABT-737, which inhibits Bcl-2, Bcl-xl and Bcl-w. The effects of ABT-737 were compared against its enantiomer control at a dose range of 0.1 to 50 μM (Figure 3-4). At doses higher than 50 μM, non-specific DMSO toxicity had a strong effect, thus 50 μM was used as the maximum dose. ABT-737 single treatment reduced viability in three cell lines (SK-MEL-5, Lox IMVI and SK-MEL-2) compared to the control. However, even in SK-MEL-5, which displays the strongest response, viability remains over 50% at the highest dose. Furthermore, Malme-3M, MeWo and SK-MEL-28 show little to no response. Thus, ABT-737 has little single agent activity in melanoma cell lines. I therefore examined the combined effect of Mcl-1 knockdown and ABT-737.

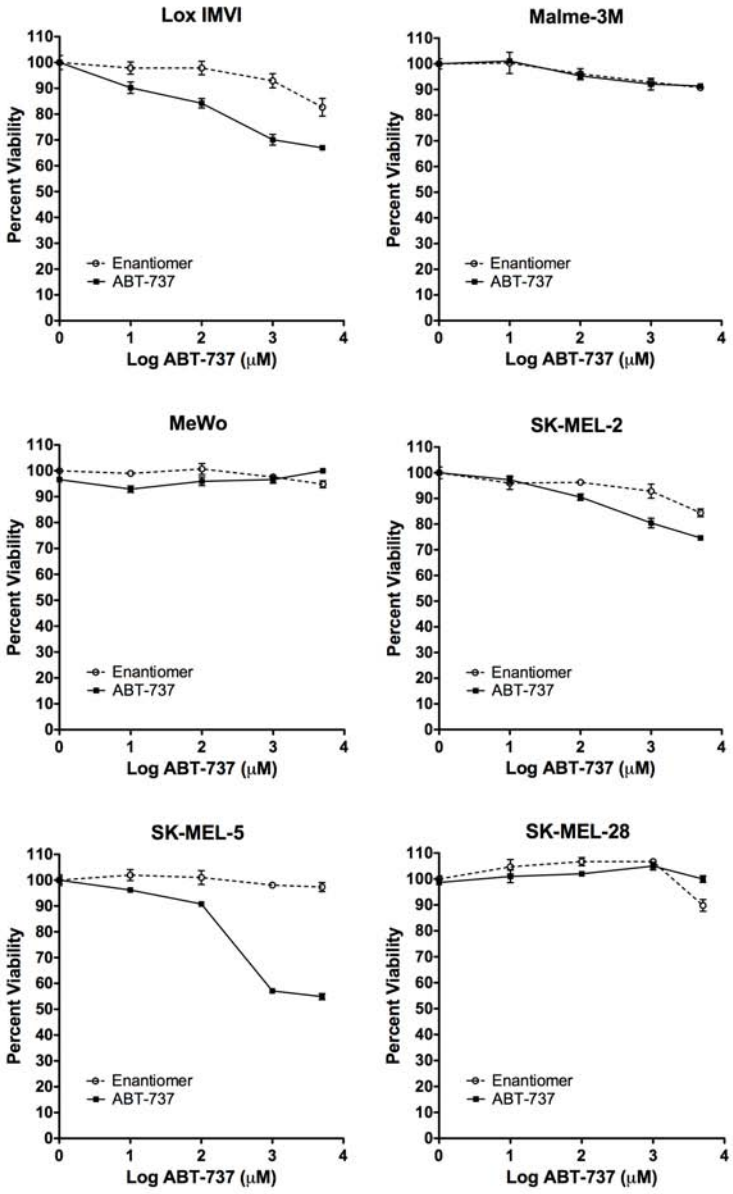

Figure 3-4: Effect of ABT-737 compared to drug enantiomer on cell viability of melanoma cell lines.

Cells were treated with 0, 0.1, 1, 10, or 50 μM of ABT-737. Each dose was increased by two orders of magnitude before the logarithm was taken to allow us to graph all doses as positive values. Viability was assessed 24 hours post-transfection via MTT assay. Curves were normalized to the 0 μM treatment. Points represent mean (n=4); error bars represent SEM (on all graphs).

Combination of Mcl-1 DsiRNA and ABT-737 synergistically reduces viability in multiple melanoma cell lines

In contrast to the single treatments, the combination of Mcl-1 DsiRNA and ABT-737 strongly decreased viability in all cell lines compared to the Scrambled DsiRNA and ABT-737 control (Figure 3-5). SK-MEL-2 was the most responsive cell line, followed by Lox IMVI. In both cell lines, viability was reduced to under 50% by the 10 μM dose of ABT-737. MeWo was the most resistant cell line, although viability was still reduced to around 70%.

To assess the synergistic interaction between Mcl-1 knockdown and ABT-737, I used two-way ANOVA calculations as previously described (Slinker, 1998). In all cell lines there was a statistically significant interaction between the two treatments, with cell lines varying in the extent of the synergism. SK-MEL-5 displays the weakest effect of the interaction (i.e. interaction accounts for 2% of total variation, $P=0.001$) whereas Malme-3M shows the greatest synergistic effect (interaction accounts for 25% of variation, $P<0.0001$). The Malme-3M cell line shows only minor effects of the single treatments but displays a strong viability decrease with the combination treatment, illustrating the strength of the synergistic effect of the combination therapy.

Viability data is summarized in Table 3-2A, ordering the cell lines from most responsive to least responsive, in terms of overall toxicity, for each of the single treatments and the combination. A summary of the ANOVA data, comparing the difference in synergism between the cell lines, is shown in Table 3-2B.

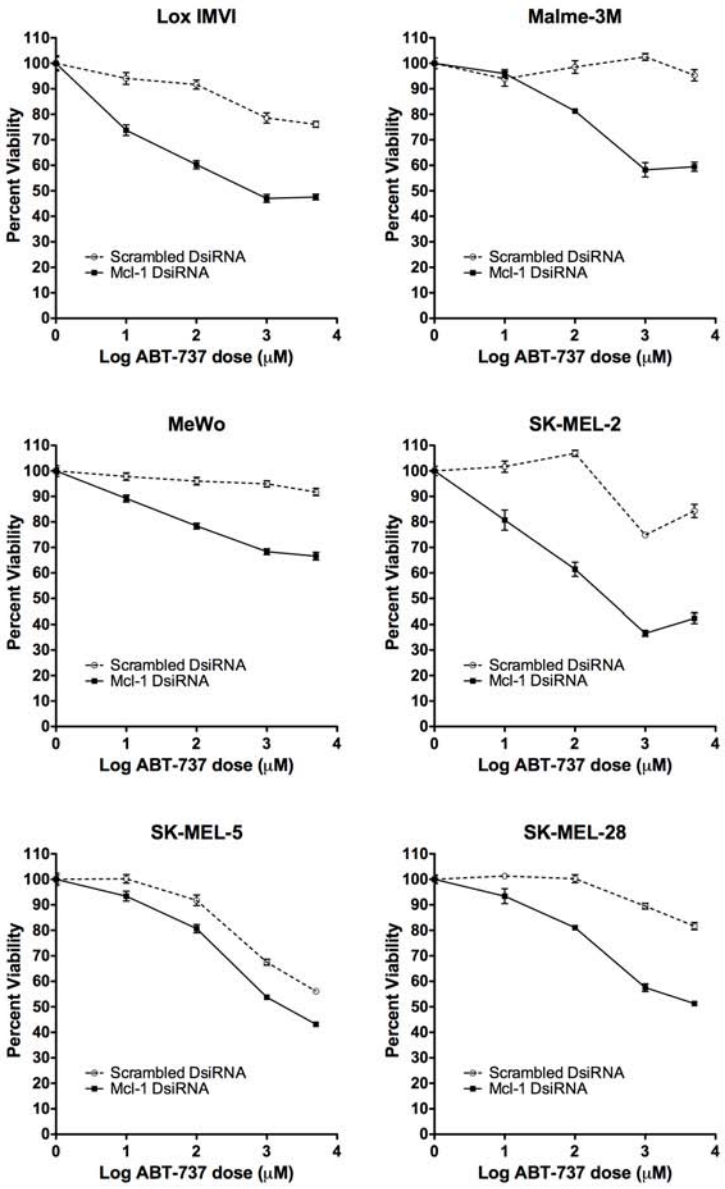

Figure 3-5: Synergistic effect of Mcl-1 knockdown and ABT-737 on cell viability in melanoma cell lines.

Cells were transfected with either 10 nM Mcl-1 or Scrambled DsiRNA and treated with 0, 0.1, 1, 10, or 50 μM of ABT-737. Each dose was increased by two orders of magnitude before the logarithm was taken to allow us to graph all doses as positive values. Viability was assessed 24 hours post-transfection via MTT assay. Each curve was normalized back to the 0 μM ABT-737 treatment: the Scrambled DsiRNA curve was normalized to cells treated with only Scrambled DsiRNA (10 nM) and the Mcl-1 DsiRNA curve was normalized to cells treated with only Mcl-1 DsiRNA (10 nM). Points represent mean (n=4); error bars represent SEM (on all graphs).

Table 3-2A: Summary of viability data: Overall toxicity

Mcl-1 DsiRNA	ABT-737	Combination
SK-MEL-2	SK-MEL-5	SK-MEL-2
Lox IMVI	Lox IMVI	Lox IMVI
SK-MEL-5	SK-MEL-2	SK-MEL-5
MeWo	SK-MEL-28	SK-MEL-28
SK-MEL-28	MeWo	Malme-3M
Malme-3M	Malme-3M	MeWo

Most responsive

Least responsive

Note: Cell lines with little to no response are shown in gray

Table 3-2B: Summary of viability data: Synergism

Cell Line	Interaction	P-value
Malme-3M	25%	P<0.0001
SK-MEL-28	23%	P=0.0018
MeWo	17%	P<0.0001
Lox IMVI	13%	P<0.0001
SK-MEL-2	6%	P<0.0001
SK-MEL-5	2%	P=0.001

Strongest Synergism

Weakest Synergism

Note: Interaction and P-values were determined by two-way ANOVA

Mcl-1 knockdown and ABT-737 induce apoptotic cell death

I then confirmed that the observed decreases in viability were due to corresponding increases in apoptosis. To quantify apoptosis, I measured DNA fragmentation using the TUNEL (Terminal dUTP Nick End Labeling) assay. In all cell lines, decreases in viability, in response to either single treatments or the

combination treatment, were accompanied by significant increases in DNA fragmentation (Figure 3-6). The marked increase in apoptosis with the combination treatment (Mcl-1 DsiRNA and ABT-737) confirms the synergistic effect on viability observed in Figure 3-5.

In agreement with the viability data, SK-MEL-2 displays the largest fold induction of apoptosis with the combination treatment, followed by Lox IMVI. In Malme-3M and SK-MEL-28, the combination treatment induced similar, lower levels of apoptosis, again supporting the MTT assay results. Interestingly, SK-MEL-5, which displays strong ABT-737-mediated viability effects, shows only low levels of DNA fragmentation with ABT-737 alone or the combination treatment. Furthermore, MeWo, which is largely resistant to the combination treatment by viability assay, displays an unexpectedly high TUNEL-positive signal. Of note, crystal violet assays for cell density support the MTT viability results (data not shown).

TUNEL measures DNA fragmentation, a key biochemical event during apoptosis. There is evidence that different cell lines (of the same cell type) can differ in both rate and extent of DNA fragmentation following exposure to the same cytotoxic agent (Ploski and Aplan, 2001). Thus, while an increase in DNA fragmentation within a cell line is indicative of apoptosis, comparisons between cell lines should be made with caution. Therefore, for comparison of treatment sensitivity between cell lines (Chapter 4), I used overall viability data rather than induction of apoptosis. Furthermore, apoptosis in each cell line was independently confirmed by examining PARP cleavage (see next section).

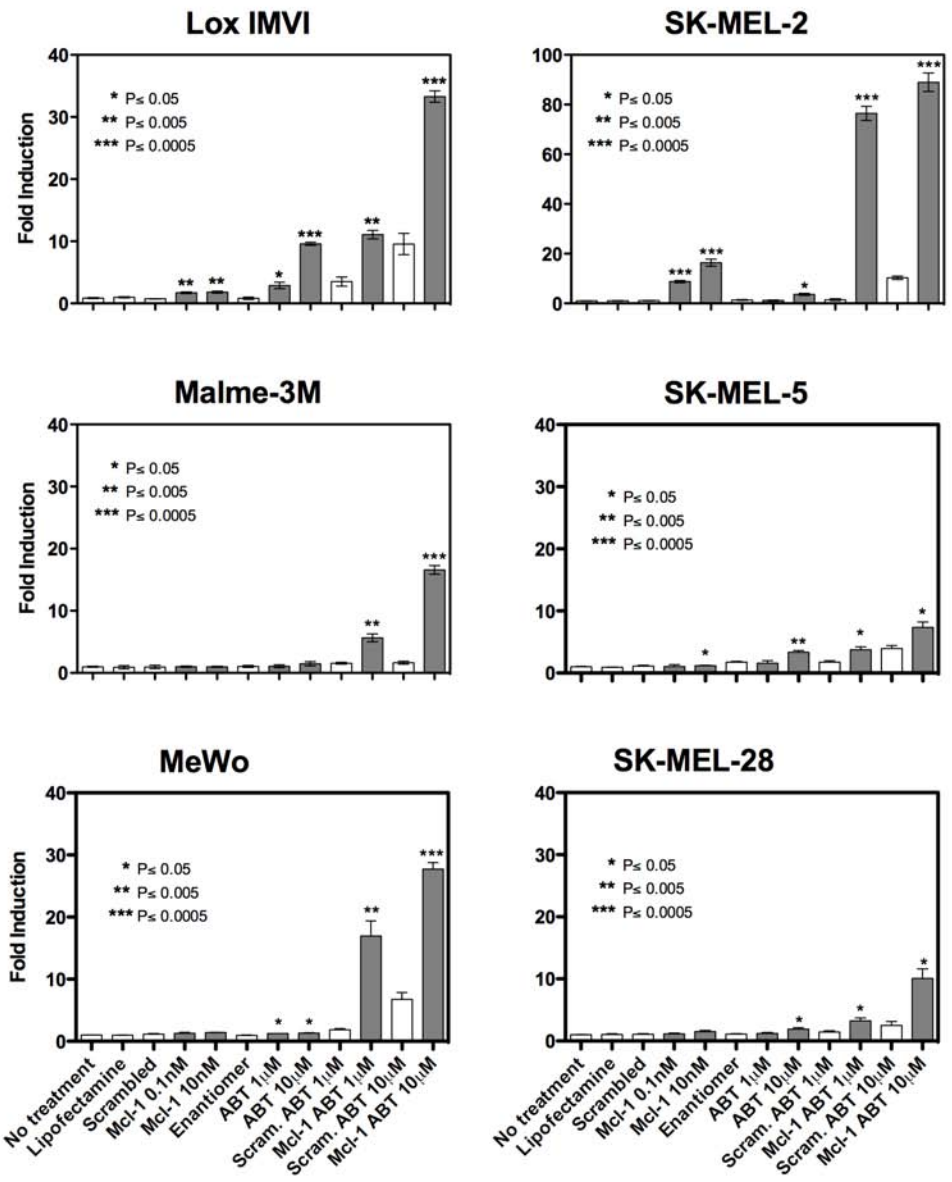

Figure 3-6: Mcl-1 knockdown and ABT-737 induce apoptotic cell death.

Assessment of apoptosis via TUNEL assay. Cells were treated with No treatment, Lipofectamine-only, Scrambled DsiRNA (10 nM), Mcl-1 DsiRNA (0.1 and 10 nM), Enantiomer (10 μM), ABT-737 (1 and 10 μM), Scrambled DsiRNA + ABT-737 (10 nM DsiRNA, 1 and 10 μM ABT-737) and Mcl-1 DsiRNA + ABT-737 (10 nM DsiRNA, 1 and 10 μM ABT-737). Cells were harvested 24 hours post-transfection. Apoptosis is expressed as a fold induction over No Treatment. Data represent mean ± SEM (n=3). Statistical significance was determined using two-tailed, unpaired *t* tests (Mcl-1 DsiRNA compared to Scrambled, ABT-737 compared to Enantiomer, Mcl-1 +ABT-737 compared to Scrambled + ABT-737). Note the different scale on the SK-MEL-2 graph.

Mcl-1 knockdown and ABT-737 induce PARP cleavage

As aforementioned, PARP is cleaved by caspase-3 during apoptosis and can therefore be used as a marker for apoptotic cell death. Both Mcl-1 DsiRNA and ABT-737 induce PARP cleavage in cell lines that respond to the single treatments (as measured by MTT assay and TUNEL). Furthermore, the combination treatment strongly increased levels of cleaved PARP in all cell lines. In some cell lines, namely Lox IMVI, MeWo and SK-MEL-28, the Scrambled DsiRNA plus ABT-737 treatment induces more PARP cleavage than ABT-737 alone, suggesting a small non-specific effect of the Scrambled control. However, levels of cleaved PARP are much higher in the Mcl-1 DsiRNA and ABT-737 treatment, confirming the specific toxicity of the combination treatment. Taken together, the TUNEL and PARP cleavage results confirm that Mcl-1 DsiRNA and ABT-737 induce apoptotic cell death.

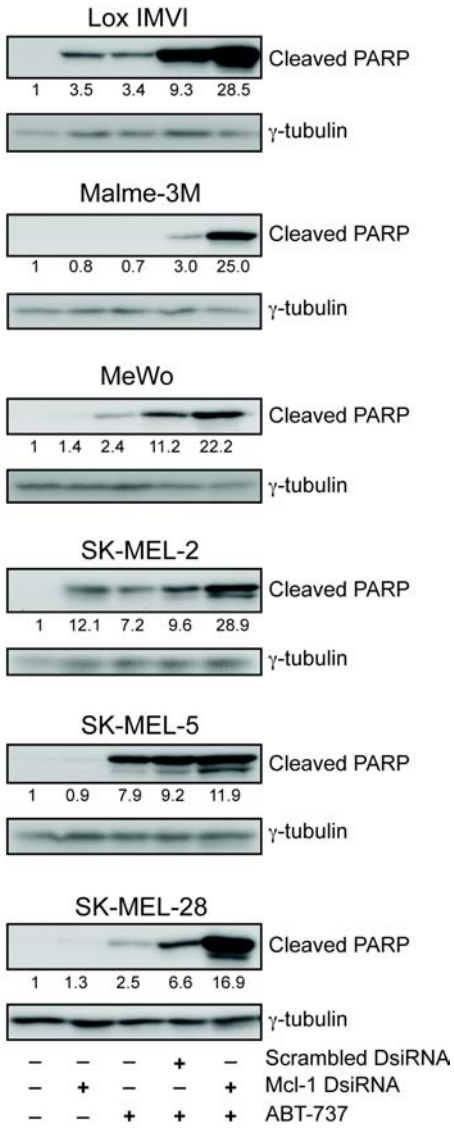

Figure 3-7: Mcl-1 DsiRNA and ABT-737 induce PARP cleavage

Assessment of apoptosis via PARP cleavage. Cells were treated with 10 nM Scrambled DsiRNA, 10 nM Mcl-1 DsiRNA and/or 10 μM ABT-737 in the combinations indicated for 24 hours. Values represent quantification of bands using γ-tubulin to correct for loading and normalization to the no treatment band (first lane).

Discussion

Malignant melanoma is highly resistant to chemotherapeutic treatments largely due to an intrinsic resistance of the neoplastic melanocytes to undergo apoptosis. Our group was one of the first to describe increased expression of the anti-apoptotic proteins Mcl-1 and Bcl-xL in melanoma (Tang et al., 1998). I specifically targeted Mcl-1 in melanoma cell lines using RNAi technology. Mcl-1 is an excellent candidate for gene silencing as it has a relatively short half-life and thus, by blocking production of new protein, Mcl-1 levels should rapidly decrease. A relatively low dose of DsiRNA (10 nM) was shown to strikingly knockdown Mcl-1 levels by up to 90% (Figure 3-1). One of the potential non-specific effects of siRNAs is the induction of an interferon response (Sledz et al., 2003). Although longer RNA molecules are more likely to induce an immune reaction, the lower concentrations of DsiRNA needed to reduce gene expression are thought to circumvent this effect (Amarzguioui et al., 2006; Kim and Rossi, 2007).

In time course experiments, knockdown of Mcl-1 by DsiRNA was detectable at early time points post-transfection (four or eight hours) and the knockdown effect was maintained up to five days (Figure 3-2). Therefore, a single DsiRNA transfection is sufficient to rapidly and consistently lowers Mcl-1 levels. A limitation to *in vitro* assays appears to be the non-specific death of melanoma cells at late time points, likely due to nutrient deprivation and over-confluency. Thus, earlier time points (such as 24 hours post-transfection) should be used to assess specific Mcl-1 knockdown-mediated cellular effects.

Although DsiRNA strongly reduces Mcl-1 levels in melanoma cells, I found that targeting Mcl-1 alone is insufficient to reduce cell viability in the majority of cell lines examined (Figure 3-3). Only one cell line, SK-MEL-2, displayed a substantial response to Mcl-1 single treatment. I then examined the effects of ABT-737, which targets Bcl-2, Bcl-xL and Bcl-w (Figure 3-4). ABT-737 had a modest effect on viability in three of the six lines. However, the remaining three cell lines are almost completely resistant to ABT-737 treatment. I therefore combined Mcl-1 knockdown with ABT-737 to simultaneously inhibit multiple anti-apoptotic Bcl-2 family members. Mcl-1 DsiRNA enhances the effect of ABT-737 in all cell lines examined, resulting in significant decreases in overall viability (Figure 3-5). The synergistic effect of the combination treatment is statistically significant by two-way ANOVA.

I then confirmed that Mcl-1 DsiRNA and ABT-737 mediate their effects on overall viability through induction of apoptosis. I assessed apoptosis induction by examining DNA fragmentation (TUNEL; Figure 3-6) and PARP cleavage (Figure 3-7). In agreement with the viability data, neither single treatment significantly induces apoptosis in all cell lines. However, the combination significantly increases DNA fragmentation and PARP cleavage in all cell lines. Thus, the combination therapy represents a promising new treatment strategy for malignant melanoma that calls for further investigation *in vivo*.

In initial mouse model studies, ABT-737 has been well tolerated, with little effect on overall animal weight and no observed caspase activation in normal tissues such as liver, heart and intestine (Oltersdorf et al., 2005). ABT-737 does

appear to reduce platelet levels; however, the effect is reversible and platelet counts are rapidly restored post-treatment (Zhang et al., 2007). Future studies using mouse melanoma models will be required to assess both the anti-tumor activity and toxicity of the Mcl-1 DsiRNA and ABT-737 combination therapy *in vivo*.

Our results confirm studies utilizing Mcl-1 knockdown and ABT-737 in other forms of cancer (van Delft et al., 2006; Chen et al., 2007; Lin et al., 2007). At the start of my thesis, there were no published reports showing the effect of ABT-737, alone or in combination, in melanoma. More recently, Miller et al. reported that, similar to my findings, ABT-737 has little effect as a single agent in melanoma cell lines (Miller et al., 2008). However, they found that ABT-737 synergizes with proteasome inhibitor MG-132. A second study by Weber et al. also found that while ABT-737 is largely ineffective in melanoma, but enhances the effects of chemotherapeutic agents DTIC, fotemustine and imiquimod (Weber et al., 2009). The authors of both studies also confirmed that down-regulation of Mcl-1 sensitizes melanoma cells to ABT-737 treatment.

There is currently no effective treatment for metastatic melanoma. Here we present evidence that the combination of ABT-737 and Mcl-1 knockdown by DsiRNA efficiently induces cell death in multiple melanoma cell lines well over the effect seen with the individual treatments. The combination treatment is therefore a promising, new treatment strategy for malignant melanoma.

~Chapter 4~
Role of extrinsic pathway proteins in response to Mcl-1 DsiRNA and ABT-737

Data presented in this chapter have been published.

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All experimental design, experimentation and data analysis were performed by Angela Keuling.

Introduction

Elucidation of the genetic factors involved in the apoptotic response to treatment paves the way for therapies tailored to individual tumor characteristics. The two major pathways of apoptosis are the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. Inhibition of anti-apoptotic Bcl-2 family members is known to activate the intrinsic pathway of apoptosis. ABT-737 has been shown to displace Bak and Bax from Bcl-2 and Bcl-xl, resulting in their conformational change (Konopleva et al., 2006; Huang and Sinicrope, 2008). In addition, ABT-737 disrupts the association of "activator" BH3-only protein Bim with Bcl-2 and Bcl-xL. As a result, ABT-737 treatment rapidly induces Bax and Bak oligomerization in the mitochondrial outer membrane of treatment-sensitive cell lines (Del Gaizo Moore et al., 2007), activating apoptosis.

Double knockout *Bax -/-; Bak-/-* MEFs (mouse embryonic fibroblasts) are highly resistant to ABT-737-mediated apoptosis (van Delft et al., 2006; Vogler et al., 2009). Double knockout cells are also resistant to combination treatments of ABT-737 plus CDK inhibitor roscovitine (which down-regulates Mcl-1 levels) and ABT-737 plus Noxa (which inhibits Mcl-1 and A1) (van Delft et al., 2006; Chen et al., 2007). The requirement of Bim for ABT-737-mediated cell death is more controversial, with one study reporting down-regulation of Bim reduces ABT-737 sensitivity in CLL cells (Del Gaizo Moore et al., 2007) and another reporting that Bim knockdown has no effect on treatment response in AML (acute myeloid leukemia) cells (Konopleva et al., 2006). These conflicting reports may

reflect the different requirements of different cell types for direct activation of Bax and Bak by BH3-only proteins.

Subsequent to oligomerization of Bax and Bak in the mitochondrial membrane, ABT-737-treated cells release cytochrome c from the mitochondria and activate intrinsic initiator caspase-9 and effector caspase-3 (Konopleva et al., 2006; Chauhan et al., 2007; Kline et al., 2007). These studies also report caspase-8 activation following ABT-737 treatment; however, the role of the extrinsic pathway in treatment response remains to be elucidated.

Regardless of the model for Bax and Bak activation (direct or indirect), the balance between pro- and anti-apoptotic Bcl-2 family members have a large effect on the cellular decision to undergo apoptosis. As ABT-737 specifically targets Bcl-2, Bcl-xL and Bcl-w, cells with high levels of Bcl-2 and Bcl-xL are more sensitive to ABT-737 treatment (Tahir et al., 2007; Witham et al., 2007; Tagscherer et al., 2008). In contrast, high expression or activity of Mcl-1 is associated with ABT-737 resistance in multiple cancer cell types including AML, SCLC and glioblastoma (Konopleva et al., 2006; Tahir et al., 2007; Tagscherer et al., 2008). There is also evidence that BH3-only protein expression can influence treatment response in that ABT-737-sensitive SCLC cell lines express relatively high levels of Bim and Noxa compared to resistant lines (Tahir et al., 2007). High levels of BH3-only proteins may facilitate activation of Bax and Bak upon neutralization of anti-apoptotic Bcl-2 family members. The effects of Bcl-2 family protein expression on treatment response in melanoma have not yet been assessed.

To identify factors responsible for sensitivity to ABT-737 and Mcl-1 knockdown in melanoma, I examined basal expression levels of pro- and anti-apoptotic Bcl-2 family proteins in the six melanoma cell lines. I found that levels of BH3-only protein Bid, as well as its upstream activator caspase-8, correlate with sensitivity to the combination treatment. These results led me to investigate the role of the extrinsic pathway of apoptosis in treatment response. I demonstrate death receptor-independent activation of extrinsic pathway proteins in response to combination treatment. Activation of this pathway is required for full treatment response. These data provide novel insight into the mechanism of apoptosis induction in treatments targeting Bcl-2 family members as well as provide potential markers for treatment sensitivity.

Results

Differences in expression of Bcl-2 family proteins between cell lines

As shown in Figure 3-5, the combination of Mcl-1 DsiRNA and ABT-737 reduces viability in all cell lines examined. However, there are differences between the cell lines in terms of treatment sensitivity. To identify factors affecting treatment response, I initially examined basal expression of both pro- and anti-apoptotic Bcl-2 family proteins in the six cell lines used in this study (Figure 4-1).

In contrast to previous studies in SCLC, AML, glioblastoma and ovarian cancer cells (Konopleva et al., 2006; Tahir et al., 2007; Witham et al., 2007; Tagscherer et al., 2008), expression of anti-apoptotic Bcl-2 proteins did not appear to relate to treatment sensitivity in melanoma cell lines. See Table 3-2A (Chapter 3) for a comparison of treatment response between the cell lines. I found that Bcl-2 levels are highest in MeWo (resistant to ABT-737 single treatment and displays the smallest response to the combination treatment) followed by SK-MEL-2 (responds to ABT-737 and displays the largest response to the combination treatment). Furthermore, there is little difference in Bcl-xL and Bcl-w levels between cell lines. Interestingly, high Mcl-1 expression did not predict resistance to ABT-737 or the combination treatment. Indeed, the three cell lines that display detectable responses to ABT-737 single treatment (SK-MEL-5, Lox IMVI and SK-MEL-2) display higher Mcl-1 expression than the three resistant cell lines. Of note, levels of anti-apoptotic A1 are lowest in the most responsive cell lines, SK-MEL-2 and Lox IMVI, which may indicate a role for A1 in

resistance to the combination treatment. However, as A1 was exceedingly difficult to detect by western blot, it was not pursued further.

In terms of pro-apoptotic Bcl-2 proteins, basal levels of Bid most closely correlated with how the cell lines respond to the combination treatment. Bid levels were lowest in the most responsive cell lines (SK-MEL-2 and Lox IMVI) and highest in the most resistant cell line (MeWo). Cell lines with intermediate responses had intermediate levels of Bid. Interestingly, this relationship is opposite to what one would expect, as Bid is pro-apoptotic. Levels of Bim are also lower in SK-MEL-2 and Lox IMVI than in more resistant cell lines. In addition, SK-MEL-2 and Lox IMVI display slightly higher levels of PUMA-α. However, as Bid displayed the strongest relationship between protein level and combination treatment response, Bid was chosen for follow-up analyses. For further studies on the role of PUMA in treatment response, see Chapter 7.

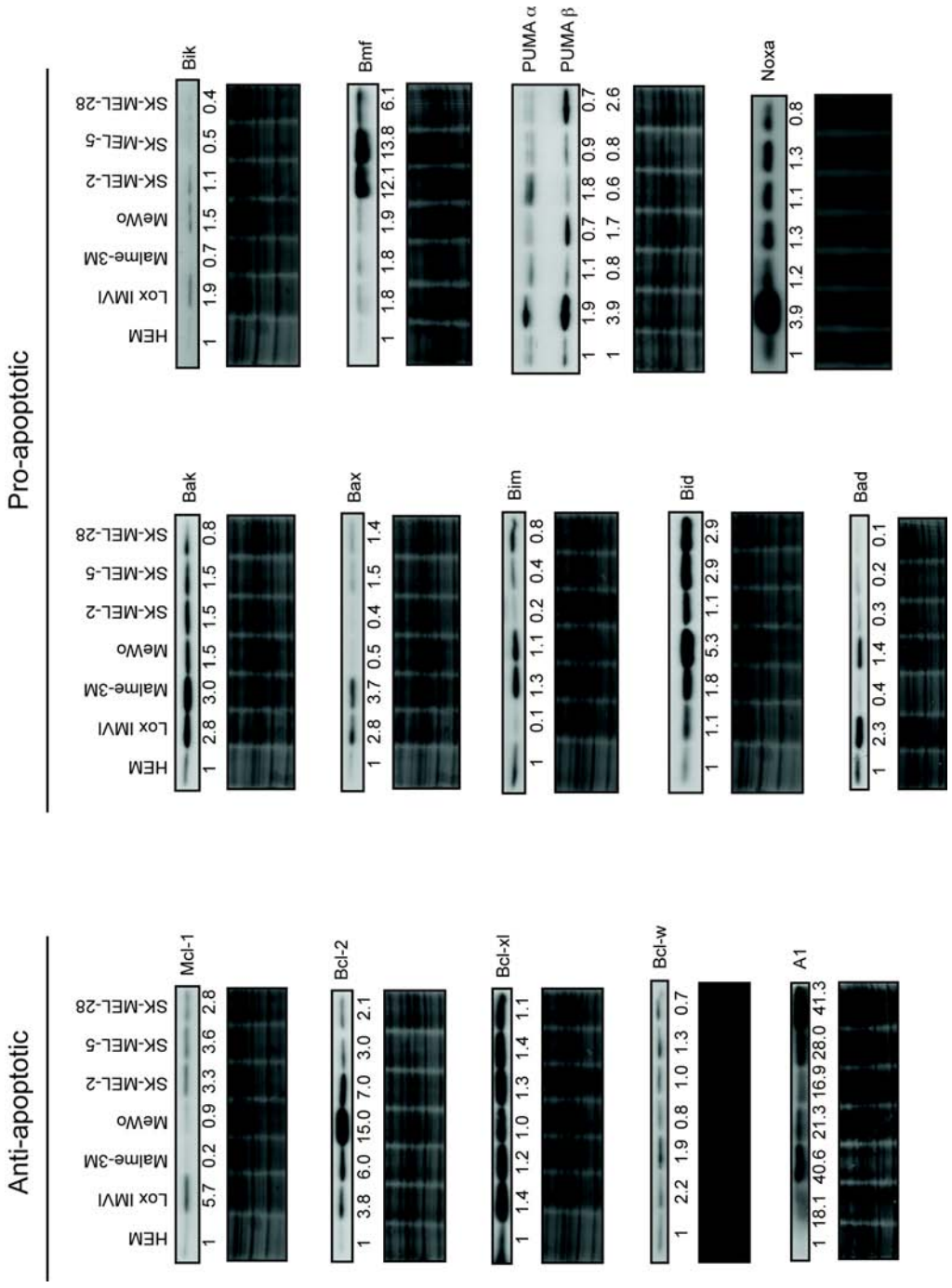

Figure 4-1: Basal expression levels of Bcl-2 family proteins

Western immunoblots to the indicated anti- and pro-apoptotic Bcl-2 proteins in six melanoma cell lines compared to normal human epidermal melanocytes (HEM). Cells were harvested for protein at approximately equal confluency one day after splitting. Values represent quantification of bands using the amido black stain to correct for loading and normalization to the HEM band. Note: SK-MEL-2 and SK-MEL-5 are reversed on the Bad blot.

Levels of Bid correlate with combination treatment response

To expand on the results of Figure 4-1, I repeated the western immunoblots to Bid and plotted average Bid levels against survival in response to the combination treatment (Figure 4-2A). As I was unable to calculate EC_{50} values for all the cell lines (owing to the fact that some of the survival curves level off before reaching 50%) I calculated the surviving fraction of cells treated with 10 μM ABT-737, combined with our standard dose of 10 nM Mcl-1 DsiRNA. At this dose of the combination treatment, there is an almost two-fold difference in viability between the most responsive cell line (SK-MEL-2) and the least responsive (MeWo) ($P<0.0001$). I found that there is a statistically significant correlation between the surviving fraction and basal levels of Bid, with the most responsive cell lines displaying lower levels of full-length Bid (Figure 4-2A).

Levels of cleaved caspase-8 correlate with combination treatment response

The pro-apoptotic function of Bid is primarily carried out by its caspase-cleaved form, tBid (Li et al., 1998). Bid is cleaved to tBid by caspases 8 and 10, the major initiator caspases of the extrinsic pathway of apoptosis (Fischer et al., 2006). I therefore examined levels of caspase-8 and -10 in the six cell lines and plotted average levels against surviving fraction. I found that there was no correlation between caspase-10 levels and treatment response (data not shown). However, I observed that cell lines with a strong response to treatment have slightly higher levels of full-length procaspase-8 (Figure 4-2B). While I could not

consistently detect tBid in untreated cells, I was able to detect and quantify the intermediate cleavage products of caspase-8 (p41/p43 isoforms). I demonstrated a strong, direct correlation between steady-state levels of cleaved caspase-8 and the surviving fraction (Figure 4-2C). As cleaved caspase-8 levels increase, there is an increase in treatment response.

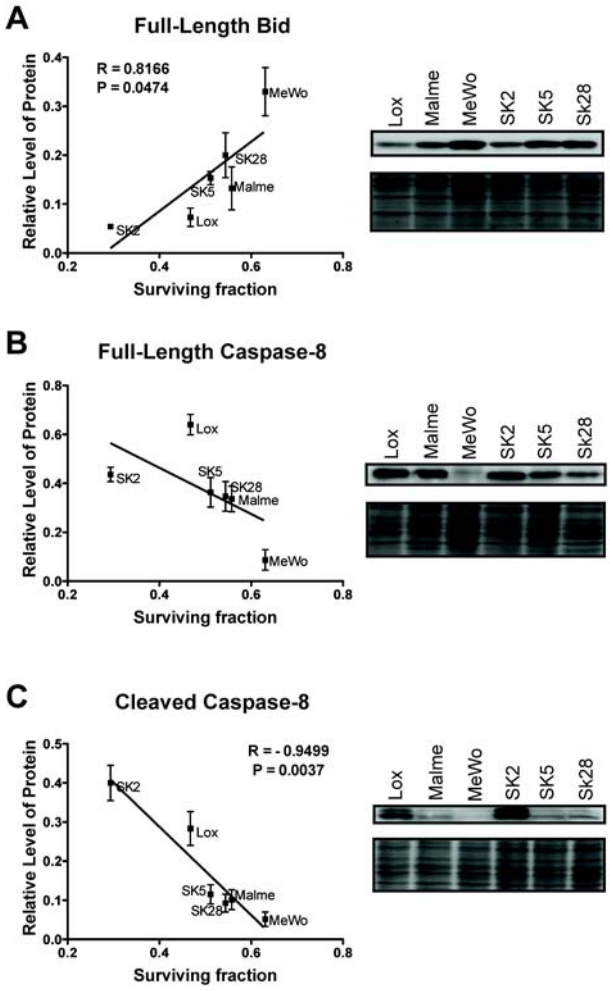

Figure 4-2: Expression of Bid and caspase-8 compared to combination treatment response

Right, western immunoblot of basal levels of full-length Bid (A), full-length caspase-8 (B) and cleaved caspase-8 (C). Cells were harvested for protein at approximately equal confluency one day after splitting. *Left*, relative expression levels were quantified by densitometry, normalized to the amido black loading control, then plotted against the surviving fraction at 10 nM DsiRNA, 10 μM ABT-737 (expressed as a fraction of cells treated with Scrambled DsiRNA only). Correlation was assessed by Pearson correlation analysis: *R* (correlation coefficient) and *P* values are indicated on graph. *Lox* (Lox IMVI), *Malme* (Malme-3M), *SK2* (SK-MEL-2), *SK5* (SK-MEL-5), *SK28* (SK-MEL-28).

Cell line differences are not due to known *CASP8* polymorphisms

In a recent report by Li *et al*., two putatively functional polymorphisms of *CASP8* were shown to contribute to melanoma susceptibility (Li et al., 2008a). I therefore sequenced both the D302H (rs1045485:G>C) and –652 6N ins/del (rs3834129: –/CTTACT) polymorphisms in the six melanoma cell lines used in this study (Table 4-1). None of the cell lines has the G to C transition to produce the D302H amino acid change. With regard to the –652 6N ins/del polymorphism: Malme-3M, MeWo, SK-MEL-2 and SK-MEL-5 are homozygous for the insertion allele, SK-MEL-28 is homozygous for the deletion and Lox IMVI is a heterozygote. The –652 6N deletion is thought to reduce expression of *CASP8* by eliminating an SP1 promoter binding site (Sun et al., 2007). However, given that I observe highest levels of full-length caspase-8 in Lox IMVI (a heterozygote) and lowest levels in MeWo (an insertion homozygote), genotype at this locus likely does not play a major role in determining caspase-8 expression levels in the cell lines in this study.

Table 4-1: *CASP8* polymorphisms in melanoma cell lines

Cell line	rs1045485:G>C	rs3834129 6N ins/del
Lox IMVI	GG	ins/del
Malme-3M	GG	ins/ins
MeWo	GG	ins/ins
SK-MEL-2	GG	ins/ins
SK-MEL-5	GG	ins/ins
SK-MEL-28	GG	del/del

Mcl-1 DsiRNA and ABT-737 treatment induces cleavage of caspase-9 as well as caspase-8, caspase-10 and Bid

The combination treatment strategy of Mcl-1 DsiRNA and ABT-737 specifically targets mitochondrial apoptotic regulators, thus activating the intrinsic pathway of apoptosis. However, Bid and caspase-8 function primarily as part of the extrinsic pathway. I therefore examined the activation of the intrinsic and extrinsic pathways in response to Mcl-1 DsiRNA, ABT-737 and the combination treatment. I used the SK-MEL-2 and Malme-3M cell lines for these experiments. SK-MEL-2 shows some response to the single treatments and a strong synergistic response to the combination. Malme-3M demonstrates little to no response to the single treatments but responds to the combination.

I first confirmed that intrinsic initiator caspase-9 is cleaved in response to Mcl-1 DsiRNA and ABT-737 single treatments (SK-MEL-2) and activation is increased in response to the combination treatment (both cell lines) (Figure 4-3). Interestingly, I also observed cleavage of caspase-8 following Mcl-1 knockdown and ABT-737 treatment, and found that caspase-8 cleavage dramatically increases with the combination treatment (Figure 4-3). Increased levels of cleaved caspase-8 are accompanied by increased levels of tBid (Figure 4-3). Furthermore, the Mcl-1 DsiRNA and ABT-737 combination treatment significantly decreases levels of procaspase-10 (Figure 4-3). Decreased procaspase-10 has previously been shown to correspond to caspase-10 cleavage and activation (Milhas et al., 2005; Sutheesophon et al., 2005; Filomenko et al., 2006).

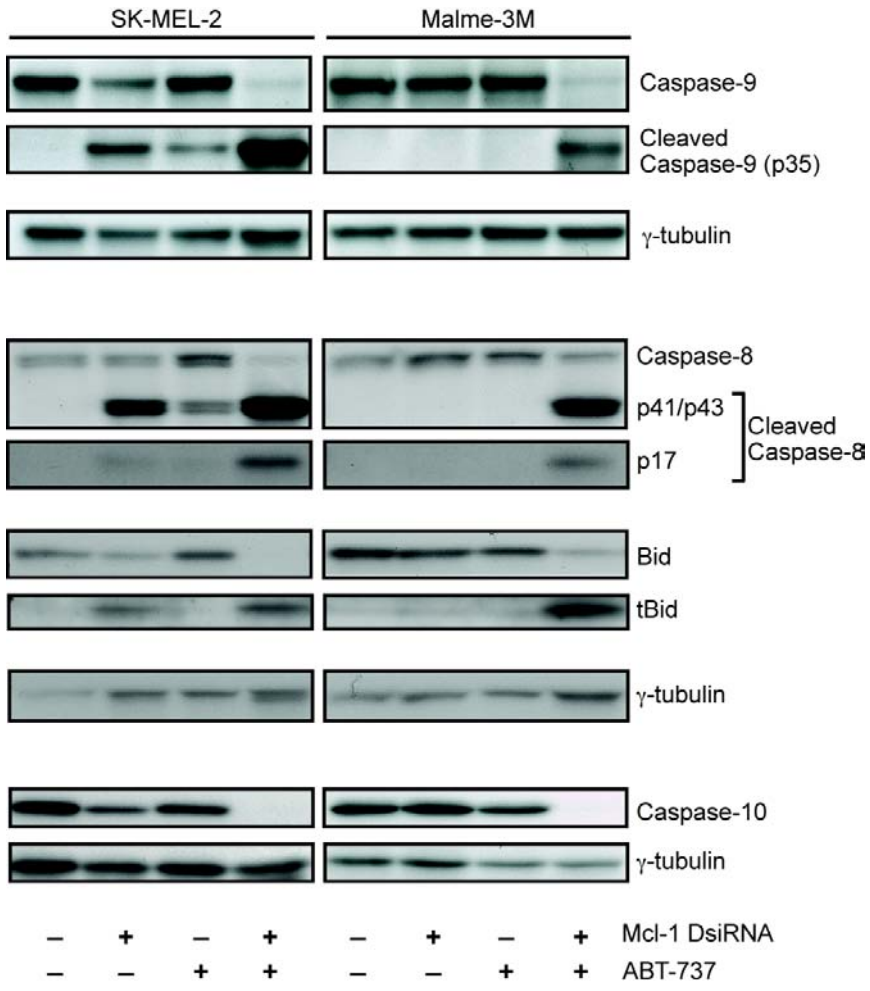

Figure 4-3: Mcl-1 knockdown and ABT-737 induces cleavage of caspase-8, caspase-9, caspase-10 and Bid

Western blots to caspase-8, -9, -10 and Bid, full-length and cleaved isoforms. SK-MEL-2 and Malme-3M were treated with 10 nM Mcl-1 DsiRNA and 10 μM ABT-737, alone and in combination, for 24 hours.

Caspase-8/-10 cleavage is dependent on caspase-9 activity

Caspase-8 and caspase-10 are initiator caspases of the death receptor pathway; however, there is some evidence that they can also be cleaved downstream of the mitochondria subsequent to caspase-9 activation (Slee et al., 1999). I therefore assessed whether the caspase-8/-10 cleavage observed following combination treatment is dependent on caspase-9 activity. I demonstrate that inhibition of caspase-9 decreases cleavage of caspase-8 in a dose-dependent fashion (Figure 4-4). At the 50 μM dose of the caspase-9 inhibitor, caspase-8 cleavage is almost completely abolished. The caspase-9 inhibitor also completely restores full-length caspase-10 (Figure 4-4).

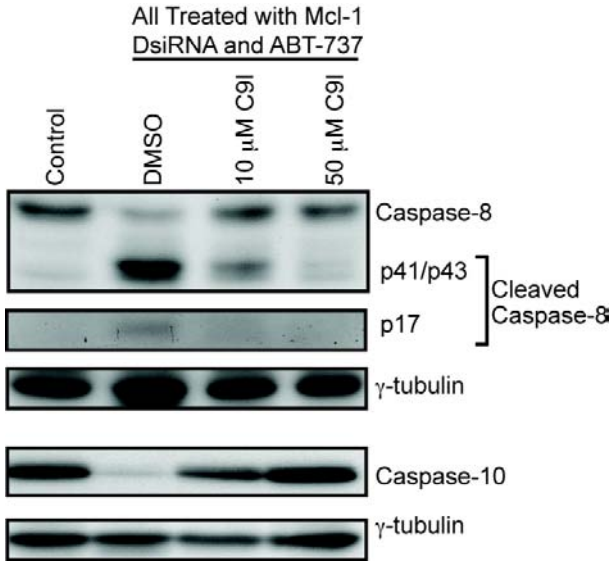

Figure 4-4: Caspase-8/-10 cleavage is dependent on caspase-9

Western blots showing reduced caspase-8/-10 cleavage with inhibition of caspase-9. Lox IMVI cells were pretreated with 50 μM DMSO or the indicated doses of Z-LEHD-FMK (caspase-9 inhibitor, C9I). Cells were then treated with the combination of 10 nM Mcl-1 DsiRNA and 10 μM ABT-737. Control cells were pretreated with DMSO and transfected with Scrambled DsiRNA.

Caspase-8/-10 cleavage is independent of death receptor activity

To assess the contribution of death receptor signaling to caspase-8/-10 activation, I used death receptor recombinant chimera (Fas/Fc, TNF-R1/Fc, and TRAIL-R1/Fc). These soluble receptors bind all corresponding ligand (both soluble and membrane-bound), thus preventing ligand from interacting with endogenous receptors and preventing death receptor-mediated apoptosis (Keogh et al., 2007; Luce et al., 2009). I found that neither the individual recombinant chimera nor the combination of all three had any effect on caspase-8/-10 activation in response to Mcl-1 DsiRNA/ABT-737 treatment (Figure 4-5).

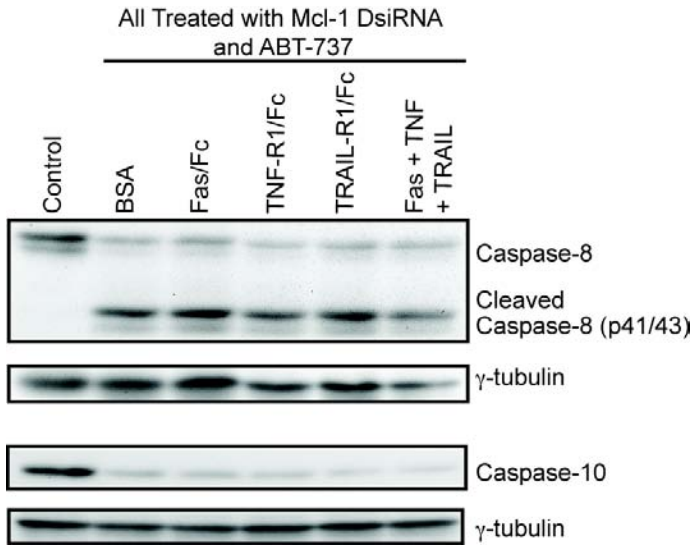

Figure 4-5: Caspase-8/-10 cleavage is not dependent on death receptors

Western blots showing that caspase-8/-10 cleavage is not affected by inhibition of Fas, TNF or TRAIL signaling. Lox IMVI cells were pretreated with 1 μg/mL Fas/Fc, TNF-R1/Fc, TRAIL-R1/Fc or the combination of all three (all at 1 μg/mL) prior to transfection. Cells were then treated with 10 nM Mcl-1 DsiRNA and 10 μM ABT-737 for 24 hours.

Response to combination treatment requires caspase-8, -9 and -10

To elucidate the pathways required for response to the combination treatment (Mcl-1 DsiRNA and ABT-737) I examined the effects of inhibitors of caspase-8 (Z-IETD-FMK, C8I), -9 (Z-LEHD-FMK, C9I) and -10 (Z-AEVD-FMK, C10I). The pan-caspase inhibitor Z-VAD-FMK (PCI) is also shown for reference. All inhibitors significantly increase viability (and thus decrease treatment response) in a dose dependent fashion in combination-treated cells (Figure 4-6).

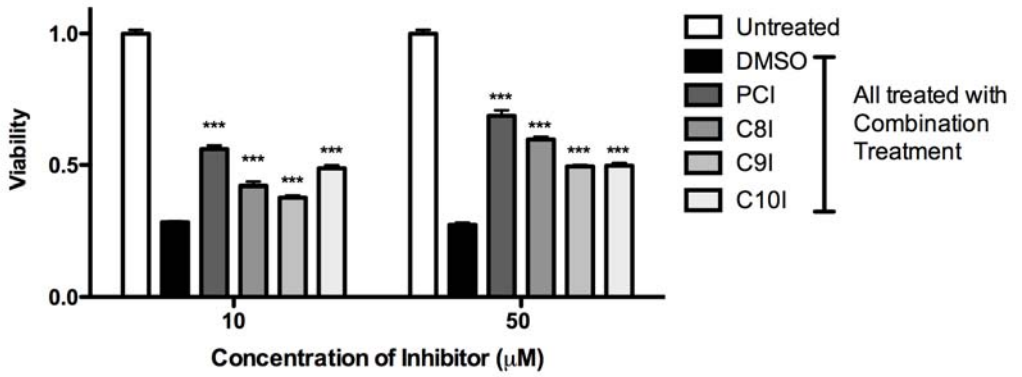

Figure 4-6: Inhibition of caspase-8, -9 and -10 reduces treatment efficacy

Effects of Z-VAD-FMK (pan-caspase inhibitor, PCI), Z-IETD-FMK (caspase-8 inhibitor, C8I), Z-LEHD-FMK (caspase-9 inhibitor, C9I) and Z-AEVD-FMK (caspase-10 inhibitor, C10I) on efficacy of the Mcl-1 DsiRNA and ABT-737 combination treatment. Lox IMVI cells were pretreated with DMSO or inhibitor 1 hour prior to transfection. Viability was assessed by MTT assay. Data represent mean ± SEM (n=4). Statistically significant differences between the DMSO treatment and the inhibitor treatments were determined using two-tailed, unpaired t tests. ***P ≤ 0.0005.

Confirmation of role of caspase-8 in treatment response

To further confirm that caspase-8 plays a role in treatment response I also knocked down caspase-8 expression. Using DsiRNA, I was able to knockdown caspase-8 protein levels by approximately 50% without interfering with Mcl-1 knockdown (Figure 4-7A). Pre-treatment with caspase-8 DsiRNA significantly increases viability in cells treated with the Mcl-1 DsiRNA/ABT-737 combination ($P<0.0001$; Figure 4-7B). Knockdown of caspase-8 was less effective in reducing treatment efficacy compared to the caspase-8 inhibitor, which may be due to incomplete knockdown. However, while the effect was not as strong as the peptide inhibitor, caspase-8 knockdown confirms that down-regulation of caspase-8 activity decreases efficacy of the combination treatment.

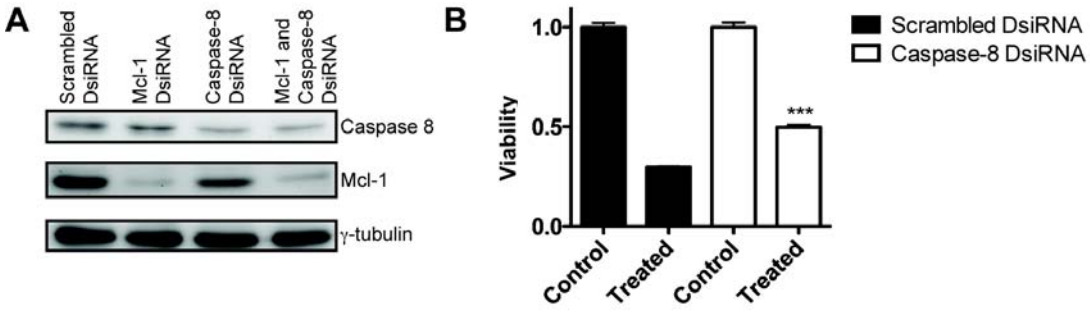

Figure 4-7: Knockdown of caspase-8 impairs treatment response

(A) Western immunoblot demonstrating knockdown of caspase-8. Lox IMVI cells were treated with Scrambled (40 nM), Mcl-1 (10 nM), caspase-8 (40 nM) or both Mcl-1 and caspase-8 DsiRNA for 24 hours. Immunoblotting was performed against both caspase-8 and Mcl-1 to demonstrate simultaneous knockdown. (B) Effects of caspase-8 knockdown on efficacy of the Mcl-1 DsiRNA and ABT-737 combination treatment. Cotransfection procedure as described in Materials and Methods. Viability was assessed by MTT assay. Data represent mean ± SEM (n=4). P value determined by two-tailed, unpaired t test. ***P ≤ 0.0005. Representative of three independent experiments.

Response to combination treatment does not require death receptor activity

To assess the requirement for death receptor signaling, I examined the effects of the recombinant chimera Fas/Fc, TNF-R1/Fc and TRAIL-R1/Fc on treatment response. Neither the individual chimera, nor the combination of all three, had any effect on viability of cells treated with the Mcl-1 DsiRNA and ABT-737 combination treatment (Figure 4-8). Thus, my results demonstrate that caspase-8/-10 function in treatment response is death receptor-independent.

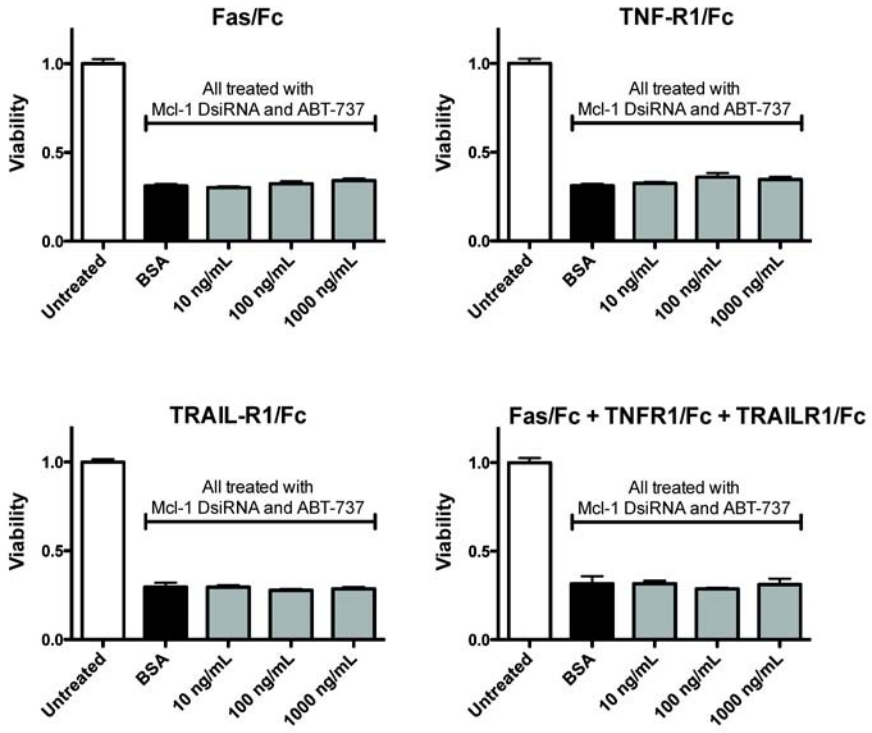

Figure 4-8: Inhibition of death receptor signaling does not affect treatment efficacy.

Lox IMVI cells were pretreated with Fas/Fc, TNF-R1/Fc, TRAIL-R1/Fc or a BSA control (1 μg/mL) at the indicated doses prior to treatment with 10 nM DsiRNA, 10 μM ABT-737 for 24 hours. For the triple combination of inhibitors, the indicated dose represents the dose of each individual inhibitor. Viability was assessed by MTT assay. Data represent mean ± SEM (n=4).

Discussion

By identifying the genetic factors involved in the induction of cell death following treatment, the long-term goal is to tailor therapy to individual tumor characteristics. I therefore sought to identify factors that modify sensitivity of melanoma cells to the combination treatment of Mcl-1 DsiRNA and ABT-737. Previous studies have shown high Mcl-1 levels to be predictive of ABT-737 resistance in SCLC, glioblastoma and AML cell lines (Konopleva et al., 2006; Tahir et al., 2007; Tagscherer et al., 2008). In contrast, I found that high basal levels of Mcl-1 were not predictive of resistance to ABT-737 or the combination treatment in the melanoma cell lines used in this study. Indeed, I found a trend towards higher Mcl-1 levels in the more sensitive cell lines (Figure 4-1). Furthermore, there was no apparent relationship between treatment sensitivity and levels of other anti-apoptotic Bcl-2 proteins, including Bcl-2, Bcl-xL and Bcl-w. Levels of A1 were higher in more resistant cell lines, suggesting a potential role for A1 in treatment resistance; however, due to technical limitations in detection of A1 by western blot, it was not pursued further. Thus, my results suggest that expression of anti-apoptotic Bcl-2 proteins is not the sole determinant of treatment response in melanoma cell lines. Furthermore, my results raise the possibility that the synergistic effect of the combination treatment may be due more to the relative *decrease* in Mcl-1 levels rather than the final level of Mcl-1 achieved.

I demonstrate a significant inverse correlation between levels of Bid and treatment response (Figure 4-2A). While this appears paradoxical, as Bid is pro-

apoptotic, our data is concordant with a report on cervical cancer demonstrating a correlation between high Bid expression and poor radiotherapy outcome (Green et al., 2005). I propose that low levels of full-length Bid correspond to higher levels of cleaved tBid, although the cleavage product is difficult to detect owing to its short half-life. There are only slightly higher levels of full-length procaspase-8 in highly responsive cell lines; however, there is a strong, linear relationship between cleaved caspase-8 (p43/p41) and treatment response (Figure 4-2B, C). Presumably, high levels of the intermediate cleavage products of caspase-8 correlate with high levels of the active protein (p17). Given that a caspase-8 dependent pathway appears to play an important role in treatment response (discussed below), cell lines with high levels of active caspase-8 and Bid may therefore be better "primed" to die upon treatment with Mcl-1 DsiRNA/ABT-737. Thus, high levels of cleaved caspase-8 and, to a lesser extent, low levels of full-length Bid could potentially be used as predictors of response to ABT-737 and Mcl-1 knockdown.

A number of chemotherapeutic treatments have been shown to activate the death receptor pathway, resulting in cleavage of extrinsic initiator caspases 8 or 10 (Debatin and Krammer, 2004). However, death receptor-independent caspase-8/-10 activation has been reported in response to several anti-cancer agents including camptothecin, paclitaxel (Taxol) and etoposide (von Haefen et al., 2003; Filomenko et al., 2006; de Vries et al., 2007). In such studies, loss or inhibition of caspase-8 or -10 significantly reduces treatment efficacy. Caspase-8 and -10 can be cleaved by caspase-3 or caspase-6 downstream of the

mitochondria (Slee et al., 1999; Tang et al., 2000). In such instances, the active caspase-8 or -10 are thought to function as part of a feedback loop to enhance the caspase cascade and amplify apoptotic signals. Cleavage of Bid to tBid further enhances the release of cytochrome c from the mitochondria via activation of Bax and Bak. Thus, death receptor-independent activation of caspase-8/-10 appears to be an important apoptotic mechanism for certain chemotherapeutic agents.

My results demonstrate cleavage of caspase-8 and -10 as well as downstream target Bid following treatment with Mcl-1 DsiRNA and ABT-737 (Figures 4-3). Caspase-8/-10 cleavage is dependent on caspase-9 activity but not on death receptor activation (Figure 4-4 and 4-5). Inhibition of either caspase-8 or caspase-10 (as well as caspase-8 knockdown) significantly reduces treatment efficacy suggesting that this downstream pathway may play an important role in the response of melanoma cells to the combination treatment (Figures 4-6 and 4-7). In contrast, inhibition of the major death receptor pathways has no effect on treatment response (Figure 4-8). While it is possible that a death receptor activated by a ligand other than TNF, TRAIL or FasL is involved in the induction of cell death, my results strongly suggest a death-receptor independent function of caspase-8, caspase-10 and Bid in response to Bcl-2 family inhibition. A proposed model is presented in Figure 4-9.

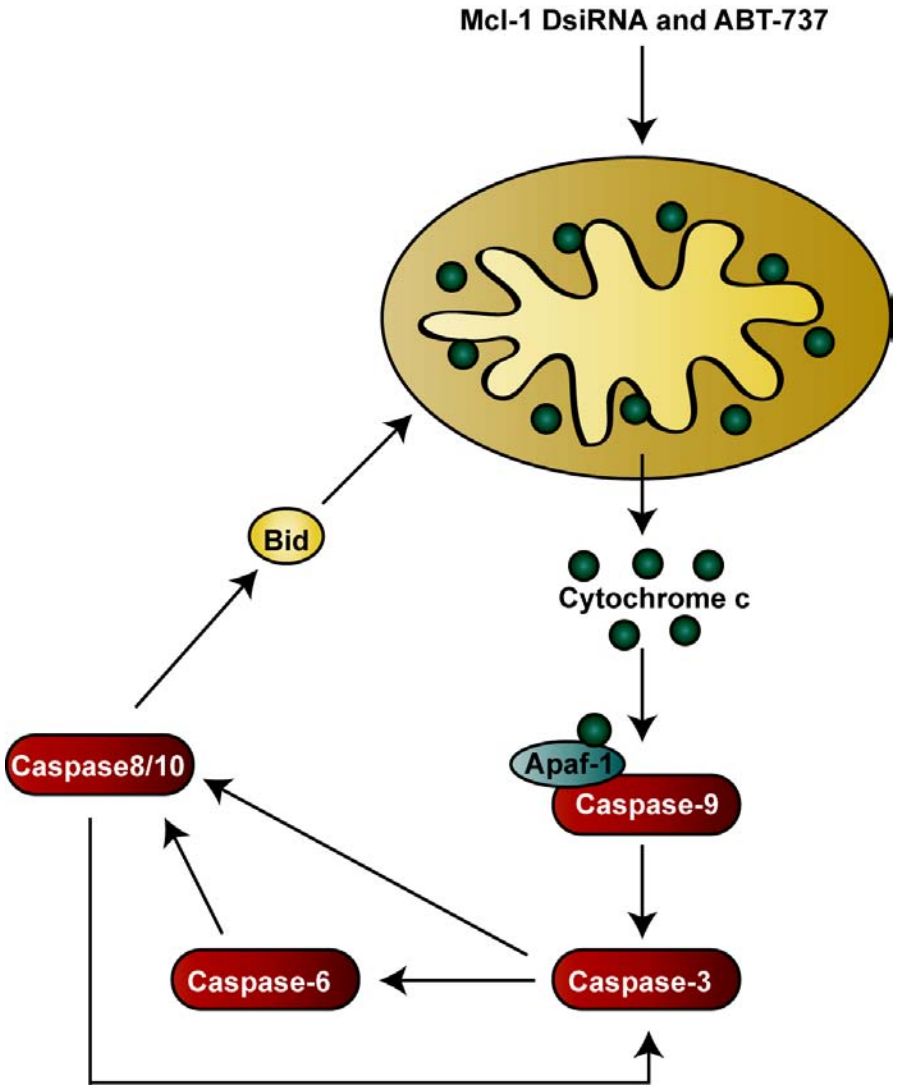

Figure 4-9: Putative model of caspase-8/-10 role in treatment response

In response to the combination treatment of Mcl-1 DsiRNA and ABT-737, the intrinsic pathway of apoptosis is activated as evidenced by cleavage of caspase-9. Downstream of caspase-9 activation, caspase-8 and -10 are cleaved by either caspase-3 or caspase-6. Active caspase-8/-10 then acts as part of a feedback loop by cleaving caspase-3 and Bid. Active tBid translocates to the mitochondria to promote Bax and Bak activation and facilitate cytochrome c release.

Interestingly, although the individual caspase inhibitors and the pan-caspase inhibitor significantly increase viability of combination treated cells (Figure 4-6), viability is not fully restored to that of untreated cells. Thus, the effect of the combination treatment is not abolished. One possible explanation is simply that the doses of inhibitor weren't high enough to completely block caspase activity. I used the highest dose (50 μM) that did not result in non-specific DMSO toxicity in the melanoma cell lines; however, a higher dose may have more strongly blocked caspase function. A second explanation is that a proportion of the cell death induced by the combination treatment is caspase-independent. In addition to cytochrome c, Smac/DIABLO and HtrA2, the mitochondria can release AIF and EndoG, which are involved in caspase-independent cell death (Cregan et al., 2004a). As Bcl-2 family members regulate mitochondrial membrane permeabilization, inhibition of anti-apoptotic Bcl-2 proteins may also induce release of caspase-independent factors.

Overall, my results demonstrate that a complex apoptotic pathway is induced upon inhibition of anti-apoptotic Bcl-2 family members, involving components of both the intrinsic and extrinsic networks. My findings also describe two potential markers of treatment sensitivity, Bid and caspase-8, which may be used to identify patients likely to respond to our proposed treatment strategy.

~Chapter 5~
Microarray analysis of gene expression changes associated with the combination treatment of Mcl-1 DsiRNA and ABT-737

Experimental design, data analysis and experimentation were performed by Angela Keuling, with the exception of the array preparation and hybridization, which was performed by Xiao (Nick) Zhang.

Introduction

My results have shown that the induction of cell death via Mcl-1 DsiRNA and ABT-737 involves a complex interplay between components of the intrinsic and extrinsic apoptotic pathways (Chapter 4). Other groups have also demonstrated diverse cellular responses to Bcl-2 inhibitory treatment. ABT-737 has been shown to induce mitochondrial autophagy (mitophagy) by disrupting the interaction between Bcl-2/Bcl-xL and Beclin-1, a key autophagic regulator (Maiuri et al., 2007). Indeed, the authors describe a "BH3-like" domain in Beclin-1 that mediates its interaction with anti-apoptotic Bcl-2 family members. Interestingly, ABT-737-mediated autophagy was observed in viable, adherent cells and was not associated with the induction of cell death. Furthermore, a recent study demonstrated that ABT-737 treatment induces oxidative stress as evidenced by increased levels of reactive oxygen species hydrogen peroxide and superoxide (Howard et al., 2009). Anti-oxidant treatment reduces ABT-737-mediated apoptosis, suggesting that oxidative stress plays a role in treatment response.

ABT-737 has also been shown to up-regulate transcription of the gene encoding death receptor 5 (DR5, TRAIL-R2), which mediates the synergistic interaction between ABT-737 and TRAIL treatment (Song et al., 2008). This effect was cell line-specific as it was observed in the LNCaP prostate cancer line but not in DU145 prostate cancer cells. In multiple myeloma cell lines, ABT-737 treatment decreases levels of Mcl-1, Bcl-2, Bcl-xL and Bcl-w as well as down-

regulating phospho-Akt and phospho-STAT3 (Kline et al., 2007). Thus, response to ABT-737 treatment involves multiple, diverse pathways.

Furthermore, Bcl-2 family members have also been shown to function in other cellular processes including regulation of the cell cycle and DNA damage response. Bcl-2 and Bcl-xL are anti-apoptotic but they can also display an anti-proliferative function by delaying cell cycle entry out of G0 (reviewed in Zinkel et al., 2006). In addition, Mcl-1 directly interacts with PCNA (proliferating cell nuclear antigen) and Mcl-1 overexpression inhibits cell cycle progression from S phase to G2 (Fujise et al., 2000). Mcl-1 is also involved in phosphorylation of Chk1 in response to DNA damage, possibly by acting as an adaptor molecule (Jamil et al., 2008). BH3-only protein Bid is phosphorylated by ATM following DNA damage and functions in the S-phase checkpoint (Kamer et al., 2005). Finally, Bad is involved in multiple cellular processes including G0/G1 cell cycle arrest (Chattopadhyay et al., 2001), glycolysis (Danial et al., 2003) as well as insulin secretion in pancreatic beta cells (Danial et al., 2008).

We therefore hypothesized that the combination treatment of Mcl-1 knockdown and ABT-737 could induce a wide array of signaling pathways in melanoma cells. To get a broader idea of the pathways activated with treatment, we used cDNA microarray analysis. We examined gene expression in cells treated with the combination treatment of Mcl-1 DsiRNA and ABT-737 compared to a control treatment. By analyzing gene expression changes, we aimed to identify pathways that enhance treatment response (that could be pharmacologically stimulated) or pathways that limit treatment response (that could be

pharmacologically inhibited). Based on the array results and follow-up analyses, we focused on the immediate early response, and thus the MAPK pathway, as a potential target for increasing treatment response.

Results

Design of custom cDNA microarray

To investigate the pathways involved in the induction of cell death in response to the Mcl-1 DsiRNA and ABT-737 combination treatment, I designed a custom cDNA microarray, manufactured by Agilent. At the initiation of this project there were two commercial apoptosis-specific arrays available: SA Biosciences' Oligo GEArray Human Apoptosis Microarray and Miltenyi Biotec's PIQOR Cell Death Microarray. I used the gene lists from these arrays as the starting point for my design. However, as discussed in the introduction, Bcl-2 family members are involved in diverse processes; therefore, I expanded my array design to include a variety of key genes involved in cell cycle, growth and proliferation signaling, DNA damage and stress response, autophagy, etc. The array contained probes to a total of 435 genes as summarized in Table 5-1. As the aim of these experiments was to identify viable therapeutic targets, the array was not designed to be comprehensive but rather to focus on well-characterized genes with available associated inhibitors, siRNAs, antibodies, etc.

For each of six melanoma cell lines (Lox IMVI, Malme-3M, MeWo, SK-MEL-2, SK-MEL-5, SK-MEL-28), I extracted RNA from cells treated with either the Mcl-1 DsiRNA and ABT-737 combination treatment, or a control treatment of Scrambled DsiRNA and the drug enantiomer. RNA samples were fluorescently labeled using Agilent's One-Color Labeling protocol, then each hybridized to one of the custom-designed microarrays (for more details, see Chapter 2, Materials

and Methods). Expression levels, that is, the intensity of the fluorescent signals, were then compared using Genespring microarray analysis software.

Table 5-1: Composition of custom cDNA microarray

Category	Genes	Examples
Bcl-2 family and related genes	39	*BCL2, MCL1, BAX, BID, HRK, BAG1, BNIP1, BCLG*
Death receptor pathway	49	*FASLG, TNF, TRAIL, FAS, TNFR1, TRAILR1, FADD, TRADD, TRAF1, RIPK1*
Apoptotic and mitochondrial	89	*AIF, BIRC5, CARD10, CASP9, DIABLO, MFN1, ROCK-1, VDAC1*
Oncogenes	23	*BRAF, JUN, FOS, KIT, NRAS, MYC*
Cell cycle	54	*CCNA1, CDC2, CDK4, CDKN2A, PCNA, WEE1*
DNA damage and stress response	34	*ATM, BRCA1, CHK1, HSPA5, HSP90AA1, RB1, TP53, TP73*
Transcription factors	25	*CEBPA, CREB1, E2F1, FOXO1, PAX3, SP1, STAT3*
Growth factors and receptors	22	*EGF, EGFR, IGF1, IGF1R, PDGFA, VEGFA, VEGFR1*
Melanoma-associated	25	*MC1R, MLANA, MITF, POMC, TYR, TYRP1*
Autophagy	7	*BECN1, RAPTOR, RICTOR*
Signaling pathways	58	*FAK, MAPK3, NFKB1, PI3KCA, PRKCA, PTEN*
Housekeeping	10	*GAPDH, ACTB, TUBG1*

Summary of array results

For each cell line, I compared expression levels between cells treated with the combination treatment (Mcl-1 DsiRNA and ABT-737) versus the control (Scrambled DsiRNA and the enantiomer). I focused on genes with at least a two-fold expression difference between the combination treatment and the control. This cut-off eliminated all of the housekeeping genes with the exception of *HIST4H4,* which is consistently up-regulated in multiple cell lines. To narrow down my list of genes further, I focused on genes with a two-fold or greater expression difference in at least three of the six cell lines (Table 5-2).

On this list, multiple diverse signaling pathways are represented. There are several genes of the Bcl-2 family: *BAX, BCL2L10* (BCL-B), *NOXA* and BH3-like gene *BLID*. There are also several other apoptotic effectors including *CASP4, CASP8,* and death receptor family genes *TNF, TNFRSF9,* and *TNFSF11. JUN* and *MYC* are part of the immediate early response, which also involves *MAPK11* (p38 beta). There is a cell cycle gene (*CCND2* - CyclinD2), a marker of endoplasmic reticulum stress (*HSPA5* - BiP) and a regulator of p53 (*MDM2*). There are also several growth factors and receptors: *IGF2, IGFBP5, KDR* (VEGF receptor 2) and *VEGFA*. Interestingly, almost all expression changes were treatment-dependent increases in expression levels rather than transcriptional decreases. Thus, the combination treatment of Mcl-1 DsiRNA and ABT-737 induces multiple gene expression changes in diverse pathways.

Table 5-2: Genes with at least a two-fold expression difference between treated and control in at least three of six cell lines

	Cell Line					
Gene	**Lox**	**Malme**	**MeWo**	**SK-2**	**SK-5**	**SK-28**
BAX		27.9 up	2.0 down	8.8 up		6.2 up
BCL2L10	2.7 up	2.3 up		2.4 up		
BLID		2.2 up	2.8 up			3.0 up
CARD11	2.5 up	7.6 up				6.2 up
CASP4		22.2 up		7.1 up		3.6 up
CASP8		137.3 up		40.5 up	3.3 up	4.2 up
CCND2		2.6 up		3.1 up	8.1 up	
CEBPB	2.4 up	2.0 up			2.1 up	
CXCL1	2.3 up		2.2 up			2.0 up
HIST4H4	8.4 up	10.7 up		3.4 up		9.7 up
HSPA5				2.4 up	3.3 up	2.4 up
IGF2	5.4 up	2.7 up				2.5 up
IGFBP5	2.3 up			2.8 up		3.7 up
JUN	3.2 up	11.2 up		2.7 up	3.9 up	6.2 up
KDR			2.4 up	2.1 up		2.1 up
MAPK11		63.4 up	4.0 down	24.0 up		4.4 up
MDM2	2.9 up	7.0 up				3.8 up
MYC	4.4 up	6.2 up			4.1 up	2.6 up
NOXA	11.6 up	3.1 up			2.4 up	
TNF	2.7 up	2.0 up		2.0 up		
TNFRSF9	5.4 up	2.1 up		2.7 up	2.0 down	
TNFSF11	2.3 up	3.3 up	2.0 up	2.3 up		2.1 up
VEGFA	2.8 up				2.9 up	2.6 up

Confirmation of array results by semi-quantitative RT-PCR

I chose several of the expression differences in Table 5-2, representative of multiple pathways, to confirm by semi-quantitative RT-PCR (Table 5-3). Confirmed expression changes are shown in Figure 5-1. As a control for the sensitivity of the assay, I also examined levels of *MCL-1*, which is specifically knocked down in the combination treated samples but not in the controls. Of the array genes that I examined, *JUN* displayed the most consistent expression change, with four of the six cell lines showing increased levels of *JUN* with treatment. The JUN protein forms a heterodimer with FOS, forming the AP-1 transcription factor, and thus expression of *JUN* is closely regulated with the *FOS* gene (Thomson et al., 1999). By the array data, *FOS* is upregulated in two cell lines (Lox IMVI and MeWo), which I also confirmed by RT-PCR (Figure 5-1).

The only other gene to display consistent treatment-dependent up-regulation in multiple cell lines was *BCL-B*. The three cell lines most resistant to ABT-737 and the combination treatment (Malme-3M, MeWo and SK-MEL-28) displayed increased expression of *BCL-B* with treatment. Interestingly, on the array, Lox IMVI and SK-MEL-2 displayed increased expression of *BCL-B* with treatment, which was not confirmed by RT-PCR. These results suggest that the up-regulation of *BCL-B* in these cell lines is minimal and may require a more sensitive technique to detect.

Furthermore, the expression differences observed for *CCND2, NOXA*, and *TNF* by microarray were only confirmed in one cell line each by RT-PCR. In each case, only the cell line that displayed the greatest fold change on the array

displayed a noticeable expression difference by RT-PCR. Again, other cell lines may display more minor expression differences requiring a more sensitive technique. However, as we were more interested in larger expression differences than more subtle changes, I did not investigate these genes further.

Table 5-3: Confirmation of microarray results by RT-PCR

Gene	Confirmed in
BAX	-
BCLB	Malme-3M, MeWo, SK-MEL-28
CASP4	-
CASP8	-
CCND2	SK-MEL-5
HSPA5	-
JUN	Malme-3M, MeWo, SK-MEL-5, SK-MEL-28
MAPK11	-
MYC	-
NOXA	Lox IMVI
TNF	Lox IMVI

Note: Dash represents expression changes not confirmed

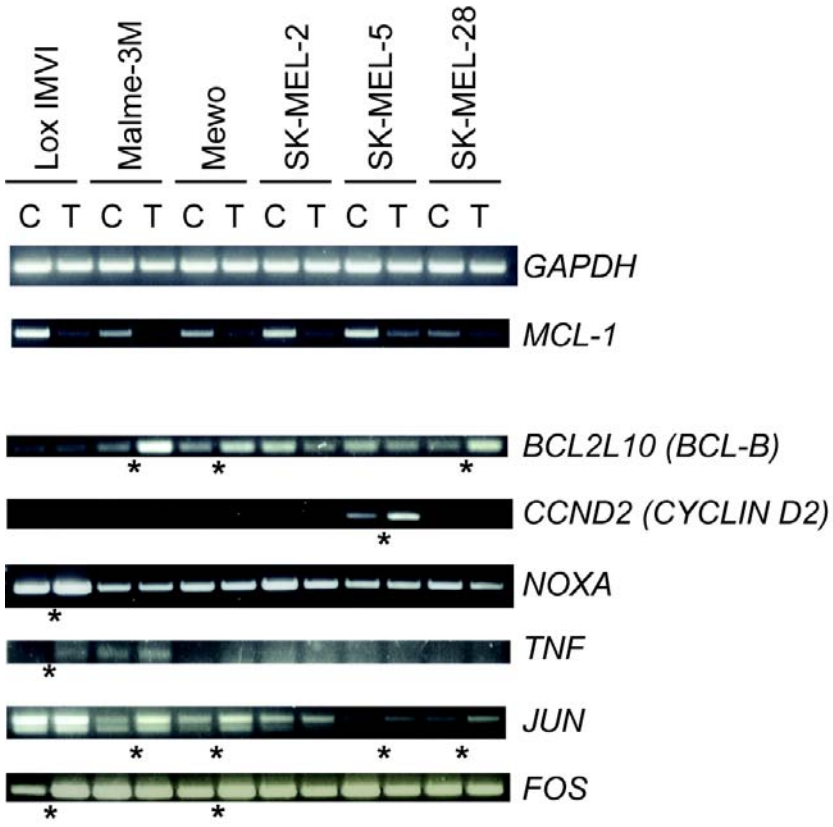

Figure 5-1: RT-PCR confirmation of microarray results

RT-PCR results confirming expression differences observed by cDNA microarray. Only confirmed differences are shown. *GAPDH* is shown as a control for equal amounts of cDNA. *MCL1* is also shown as a positive control for a known expression difference. C = Control (Scrambled DsiRNA and enantiomer). T = Treated (Mcl-1 DsiRNA and ABT-737). * = expression difference between Control and Treated. Representative of at least two independent experiments.

Discussion

Induction of cell death via inhibition of Bcl-2 family members may involve the action of multiple interconnected pathways. A better understanding of the pathways involved will allow us to better tailor treatment to the unique signaling milieu in malignant melanoma cells. To investigate relevant genes and pathways induced by the combination treatment of Mcl-1 DsiRNA and ABT-737, I designed a custom cDNA microarray. Gene expression differences were assessed in all six melanoma cell lines used in my previous studies. I focused on expression changes observed in at least three of the six cell lines, indicative of a general response to the combination treatment, rather than cell line-unique expression changes, which may be dependent on specific genetic alterations in the individual tumor line. Using this criteria, I identified 23 genes with a greater than two-fold expression changes between treated and the control in at least three cell lines. The genes represented in this list encode products involved in multiple functions including apoptosis (intrinsic and extrinsic), cell cycle, endoplasmic reticulum stress as well as the immediate early response. Thus, the inhibition of anti-apoptotic Bcl-2 family members induces a complex response in melanoma cell lines, involving diverse cellular processes.

I confirmed several of the expression differences observed on the array by RT-PCR. Interestingly, on the array, several genes of the TNF superfamily were up-regulated by the combination treatment. One, *TNF*, was confirmed in the Lox IMVI cell line by RT-PCR. This cell line was also used in the previous chapter to confirm that death receptor function was *not* required for the induction of cell

death with treatment. Thus, the up-regulation of TNF family genes may be a consequence of treatment that is not essential for the apoptotic response. Alternatively, since my studies focus on a relatively early time point (24 hours, see Figure 3-2), up-regulation of TNF may play a role in a later death response as has been observed for γ-radiation in breast cancer cells (Luce et al., 2009).

The gene encoding Bcl-B was found to be up-regulated in Malme-3M, MeWo and SK-MEL-28: the three most resistant cell lines to the combination treatment. Bcl-B is a recently identified anti-apoptotic Bcl-2 protein shown to specifically interact with Bax but not Bak (Zhai et al., 2008). ABT-737 binds Bcl-B with low affinity, similar to its effects on Mcl-1 and A1 (Oltersdorf et al., 2005). The up-regulation of Bcl-B in response to ABT-737 and Mcl-1 knockdown may be a cellular feedback response to the loss of Bcl-2, Bcl-xL, Bcl-w and Mcl-1 activity. The observation that Bcl-B expression is induced in more resistant cell lines suggests that Bcl-B may limit the induction of apoptosis in those lines. However, to my knowledge, no drugs specifically targeting Bcl-B are currently commercially available. Furthermore, I was unable to detect Bcl-B by western blot using available antibodies, which would thus make optimization of Bcl-B knockdown difficult. Therefore, although Bcl-B is a promising therapeutic target, it was not studied further in this thesis.

The most consistently up-regulated gene, by both the array and RT-PCR results, was the proto-oncogene *JUN*. *JUN* is considered to be an immediate early gene, along with *FOS*, *MYC* and others (Thomson et al., 1999). Expression of *FOS* is also induced in response to the combination treatment in two cell lines;

however, I could not confirm the up-regulation of *MYC* (observed on the array) by RT-PCR. Nor could I confirm the up-regulation of *MAPK11* (p38 beta, an upstream regulator of the immediate early response) also observed on the array. I therefore focused on *JUN* and, to a lesser extent, *FOS*.

As indicated by their classification as immediate-early genes, transcription of *JUN* and *FOS* is rapidly and transiently induced in response to extracellular stimuli including growth factors, cytokines, UV radiation and tumor promoting agents (e.g. TPA) (reviewed in Thomson et al., 1999; Malemud, 2007). These stimuli activate one or more of the three major MAPK cascades, the central kinases of which are ERK (extracellular signal related kinase), JNK (JUN N-terminal kinase) or p38. The three kinases in turn phosphorylate and activate transcription factors for up-regulation of *JUN* and *FOS*.

JUN and FOS, along with related proteins, form the transcription factor complex AP-1, which is involved in regulating cell proliferation, survival and differentiation (reviewed in Eferl and Wagner, 2003). The *JUN* gene itself is an AP-1 target gene, thus promoting its own transcription. JUN is a positive regulator of proliferation in transformed cells. The proliferative activity of JUN requires phosphorylation by JNK (Derijard et al., 1994; Behrens et al., 1999). Studies have also suggested that ERK and p38 can phosphorylate JUN in some cell types (Pulverer et al., 1991; Yamagishi et al., 2001). Interestingly, JUN can either be pro- or anti-apoptotic depending on cell type (Hess et al., 2004). JUN promotes apoptosis in neuronal cells but is anti-apoptotic in keratinocytes and hepatocytes. A recent study using mouse B16 melanoma cells found that the

combining knockdown of JunB (a related Jun protein) with inhibition of Jun by a JNK inhibitor induced cell cycle arrest and apoptosis (Gurzov et al., 2008).

I therefore hypothesized that induction of JUN (and FOS) may promote survival and proliferation of melanoma cells, thus limiting the effect of the combination treatment of Mcl-1 DsiRNA and ABT-737. As, to my knowledge, no specific inhibitors of JUN or FOS are commercially available, I focused my follow-up studies on inhibition of the upstream MAPK cascades. As aforementioned, ERK, JNK and p38 contribute to both JUN expression and function. Furthermore, in melanoma, unique mechanisms closely link the activities of the three kinases, promoting cell proliferation and survival (see Introduction Chapter 6). Thus, I examined the effects of inhibitors of ERK, JNK and p38 on treatment response (Chapter 6).

A number of studies have utilized microarrays to identify prognostic factors for melanoma, by examining expression difference between tumor and non-tumor tissue (Mandruzzato et al., 2006; Winnepenninckx et al., 2006; Jensen et al., 2007). However, few melanoma studies have investigated the gene expression changes induced by cytotoxic treatment. My results have shown that the combination treatment of Mcl-1 DsiRNA and ABT-737 induces the expression of multiple genes of diverse function in melanoma cell lines. Based on both the array data and confirmatory RT-PCR, expression of *JUN* was found to be consistently up-regulated in multiple cell lines. I therefore focused my next experiments on inhibition of the upstream regulatory pathways controlling JUN expression and activity.

~Chapter 6~
Effects of MAPK inhibitors on Mcl-1 DsiRNA, ABT-737 and combination treatment response: Synergism between p38 inhibition and ABT-737

Data presented in this chapter have been submitted for publication.

Angela M. Keuling, Susan E. Andrew and Victor A. Tron. Inhibition of p38 MAPK enhances ABT-737-induced cell death in melanoma cell lines: Novel regulation of PUMA. Submitted to *Pigment Cell & Melanoma Research*.

All experimental design, experimentation and data analysis were performed by Angela Keuling.

Introduction

The three major MAPK proteins, ERK, JNK and p38, play critical roles in regulating cell survival, proliferation, differentiation and stress responses (Thomson et al., 1999). The MAPK network features prominently in melanoma as up to 90% of cases have activating mutations in either *BRAF* or *NRAS*, resulting in constitutive activation of the MEK-ERK pathway (Hocker et al., 2008). ERK1 and ERK2 phosphorylate a variety of substrates including transcription factors, cytoskeletal proteins, and regulators of apoptosis (Yoon and Seger, 2006). Phosphorylation of FOS and JUN by ERK promotes proliferation. Furthermore, ERK phosphorylates and inhibits Bad, Bim and caspase-9, preventing the induction of cell death. Few specific ERK inhibitors exist; however, both BRAF and MEK inhibitors have been investigated as potential therapeutic agents for melanoma (reviewed in Dhomen and Marais, 2009).

The three major isoforms of JNK are JNK1, JNK2 and JNK3 (Bode and Dong, 2007). JNK1 and 2 are ubiquitously expressed, whereas JNK3 is largely restricted to the brain. JNKs display both oncogenic and tumor suppressor functions, dependent on cell type, stimulus and interactions with other pathways (Wagner and Nebreda, 2009). JNK can promote cell survival and proliferation, primarily though activation of JUN and AP-1. However, JNK can also function in the induction of apoptosis, in part due to inhibition of anti-apoptotic Bcl-2 proteins. In melanoma cells, hyperactivity of ERK results in increased JNK activity via a positive feedback loop involving JUN and RACK1 (Lopez-Bergami et al., 2007). Inhibition of JNK induces cell cycle arrest in some melanoma cell

lines and apoptosis in others (Alexaki et al., 2008). Thus, like ERK, JNK plays a role in cell survival and proliferation in melanoma.

The four major isoforms of p38 are p38-α, p38-β, p38-δ, and p38-γ (Thornton and Rincon, 2009; Wagner and Nebreda, 2009). p38-α and p38-β are ubiquitously expressed and are thought to have primarily overlapping functions. Most published literature on p38 is on the alpha isoform. p38 is traditionally considered to be a tumor suppressor and plays roles in promoting differentiation, cell cycle arrest and the induction of apoptosis. In most cancers, there is an inverse relationship between ERK (proliferative) and p38 (growth arresting and apoptotic) activity due to a negative feedback loop between the two pathways (Aguirre-Ghiso et al., 2003). However, in melanoma, this negative feedback loop is lost and instead there is positive feedback between ERK and p38, resulting in high levels of active p38 (Estrada et al., 2009). Inhibition or knockdown of p38-alpha reduces melanoma cell proliferation and migration (Estrada et al., 2009). Thus, in melanoma, p38 appears to promote tumor growth and metastasis.

Based on my observations of increased *JUN* expression in melanoma cells treated with Mcl-1 DsiRNA and ABT-737, I hypothesized that inhibition of upstream MAPK pathways could improve treatment efficacy (see Discussion Chapter 5). Given that ERK, JNK and p38 display interrelated functions in melanoma, I examined the effects of inhibitors of each of the three kinases. I combined the MAPK inhibitors with Mcl-1 DsiRNA and ABT-737 single treatments as well as the combination therapy. I found that inhibition of p38 strongly improves the efficacy of ABT-737 or the combination treatment.

Furthermore, I demonstrate that while p38 inhibition alone induces G1 cell cycle arrest, combined with ABT-737 or Mcl-1 DsiRNA/ABT-737, apoptosis is induced. Providing evidence as to the mechanism of this synergistic interaction, I show that inhibition of p38 increases expression of pro-apoptotic Bcl-2 protein PUMA. My results thus describe the potential for a novel therapeutic strategy for melanoma through simultaneous targeting of the p38 MAPK pathway and anti-apoptotic Bcl-2 proteins.

Results

Inhibition of MAPK pathways: Initial screen for combinational therapies

I examined the effects of combining MAPK inhibitors with Mcl-1 DsiRNA, ABT-737 or the combination treatment on overall survival. For my initial experiments, I used the Malme-3M and Lox IMVI cell lines. Malme-3M, which displays a treatment-dependent increase in *JUN* expression (Figure 5-1), shows little to no response to Mcl-1 DsiRNA or ABT-737 alone, and a modest response to the combination treatment. Lox IMVI, which displays increased *FOS* levels with the combination treatment (Figure 5-1), shows small responses to the single treatments and a relatively strong response to the combination. For simplicity, only the normalized data are shown in Figures 6-1 to 6-3.

Inhibition of JNK

To inhibit JNK, I used inhibitor SP600125, which has been shown to reduce viability of chemotherapy-resistant cancer cell lines (Kim et al., 2009). SP600125 inhibits JNK1, JNK2 and JNK3. I found that SP600125 reduces viability of Lox IMVI and Malme-3M (Figure 6-1). Addition of ABT-737 slightly reduces viability compared to the JNK inhibitor alone; however, there is no effect of combining SP600125 with Mcl-1 DsiRNA alone or the combination treatment (Figure 6-1).

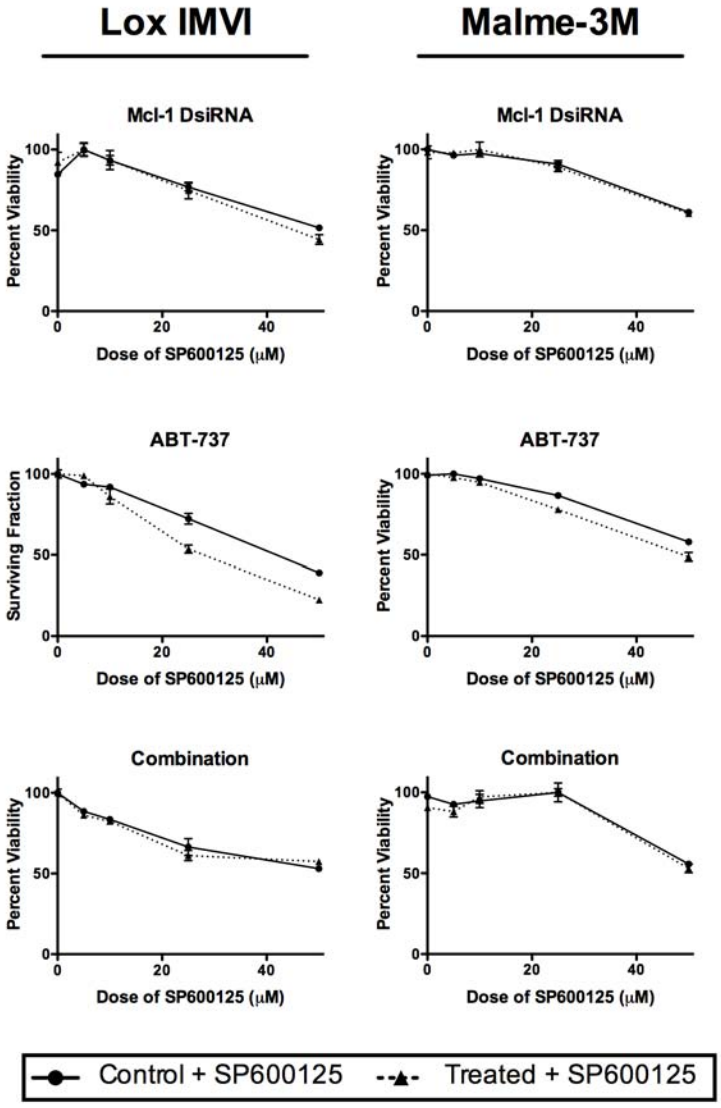

Figure 6-1: Effects of JNK inhibitor combined with Mcl-1 DsiRNA, ABT-737, alone and in combination

Lox IMVI and Malme-3M cells were pre-treated with JNK inhibitor SP600125 then treated with either 10 nM Mcl-1 DsiRNA, 10 μM ABT-737 or the combination of 10 nM Mcl-1 DsiRNA and 10 μM ABT-737. Control treatments were 10 nM Scrambled DsiRNA, 10 μM enantiomer or Scrambled DsiRNA and enantiomer. Viability was assessed at 24 hours by MTT assay. Normalized data is shown: Control curves were normalized to Control + 0 μM SP600125 and Treated curves were normalized to Treated + 0 μM SP600125. Points represent mean (n=4); error bars represent SEM (on all graphs).

Inhibition of MEK/ERK

As there are no specific ERK inhibitors commercially available, to inhibit ERK activity, I used PD98059, an inhibitor of upstream kinase MEK (MEK1 and MEK2). As aforementioned, MEK inhibitors have previously been investigated for use in melanoma therapy. I therefore examined the effects of PD98059 combined with Mcl-1 DsiRNA, ABT-737 or the combination treatment (Figure 6-2). MEK inhibition alone reduces viability of Malme-3M cells but had little effect on Lox IMVI cells. Mcl-1 DsiRNA single treatment has no effect on this response. In contrast, ABT-737 and the combination treatment both enhance the effect of MEK inhibition, resulting in a stronger reduction in viability in both cell lines (Figure 6-2).

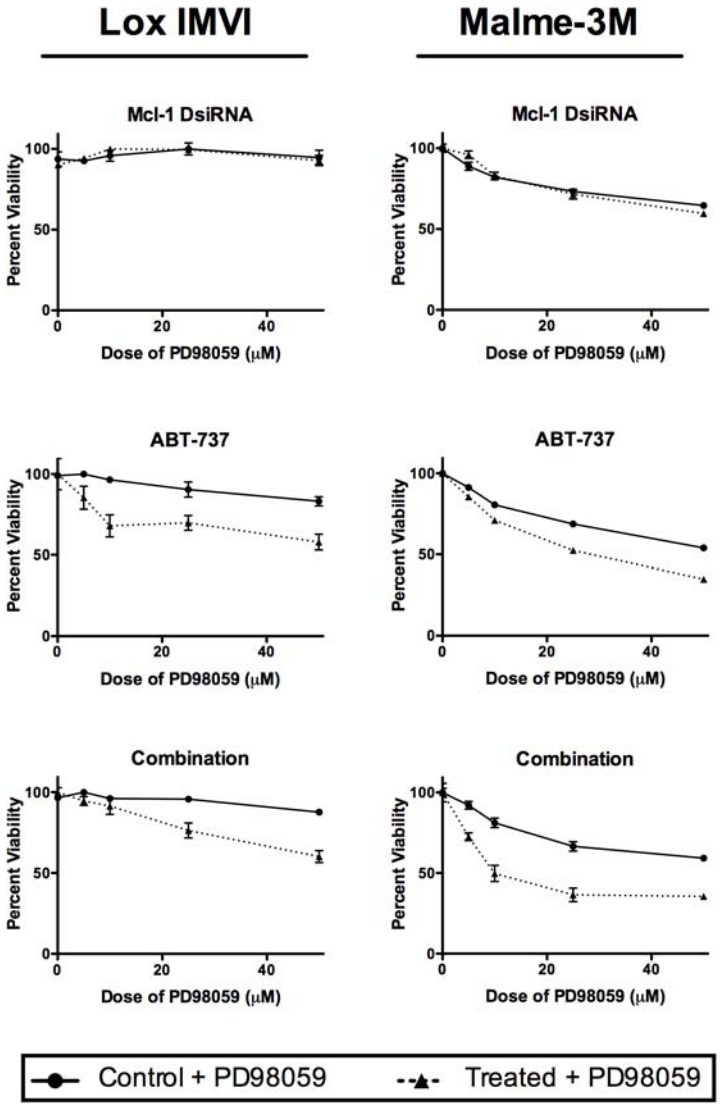

Figure 6-2: Effects of MEK inhibitor combined with Mcl-1 DsiRNA, ABT-737, alone and in combination

Lox IMVI and Malme-3M cells were pre-treated with MEK inhibitor PD98059 then treated with either 10 nM Mcl-1 DsiRNA, 10 μM ABT-737 or the combination of 10 nM Mcl-1 DsiRNA and 10 μM ABT-737. Control treatments were 10 nM Scrambled DsiRNA, 10 μM enantiomer or Scrambled DsiRNA and enantiomer. Viability was assessed at 24 hours by MTT assay. Normalized data are shown: Control curves were normalized to Control + 0 μM PD98059 and Treated curves were normalized to Treated + 0 μM PD98059. Points represent mean (n=4); error bars represent SEM (on all graphs).

Inhibition of p38

Similar to the JNK and MEK inhibitors, I found that SB202190, which inhibits p38-α and p38-β, produces a modest viability reduction in Lox IMVI and Malme-3M cells (Figure 6-3). Also in agreement with the other MAPK inhibitors, Mcl-1 DsiRNA does not improve the efficacy of p38 inhibitor treatment. However, when SB202190 is combined with ABT-737 or the combination treatment, there is a strong synergistic effect, particularly in the Malme-3M cell line (Figure 6-3). Inhibition of p38 had a stronger effect on treatment efficacy than either JNK or MEK inhibition. Furthermore, as the SB202190/ABT-737 treatment displays similar efficacy to the "triple combination" (SB202190/ABT-737/Mcl-1 DsiRNA), and is a much simpler treatment strategy, I focused on this combination in further studies.

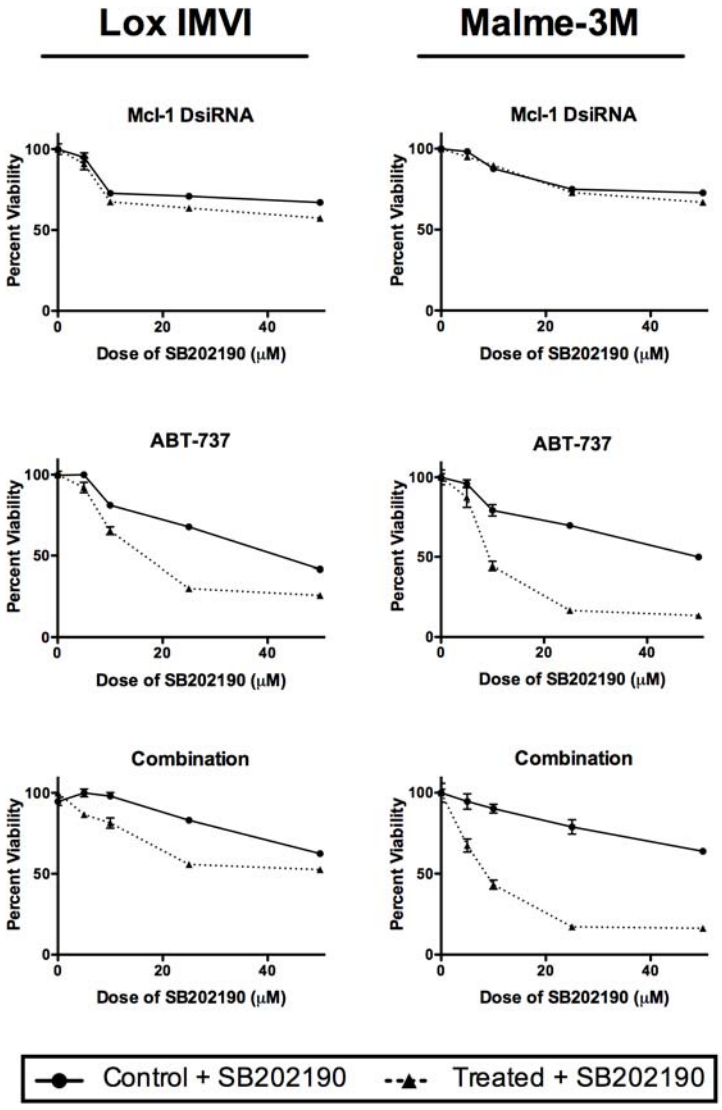

Figure 6-3: Effects of p38 inhibitor combined with Mcl-1 DsiRNA, ABT-737, alone and in combination

Lox IMVI and Malme-3M cells were pre-treated with p38 inhibitor SB202190 then treated with either 10 nM Mcl-1 DsiRNA, 10 μM ABT-737 or the combination of 10 nM Mcl-1 DsiRNA and 10 μM ABT-737. Control treatments were 10 nM Scrambled DsiRNA, 10 μM enantiomer or Scrambled DsiRNA and enantiomer. Viability was assessed at 24 hours by MTT assay. Normalized data are shown: Control curves were normalized to Control + 0 μM SB202190 and Treated curves were normalized to Treated + 0 μM SB202190. Points represent mean (n=4); error bars represent SEM (on all graphs).

Combination of p38 inhibitor and ABT-737 synergistically reduces cell viability in multiple melanoma cell lines

To confirm the combinational effect of ABT-737 and inhibition of p38 (Figure 6-3), I examined two additional cell lines, SK-MEL-2 and SK-MEL-28 (Figure 6-4). Both Lox IMVI and Malme-3M are p53 wild-type and BRAF mutant. I therefore examined SK-MEL-2 and SK-MEL-28, both of which are mutant for p53. SK-MEL-2 is also the only NRAS mutant of the six cell lines used in this study. Lox IMVI and Malme-3M are shown again in Figure 6-4 for reference. In Figure 6-4 and 6-5, both raw and normalized data are shown to illustrate the overall effect of treatment on survival and the synergism of the treatments, respectively.

I combined ABT-737 (or its enantiomer control) with p38 inhibitor SB202190 (Figure 6-4). A DMSO control treatment had little effect on ABT-737 or the enantiomer, ruling out non-specific toxicity effects. The p38 inhibitor single treatment (i.e. combined only with the enantiomer) decreases viability in all four cell lines in a dose-dependent fashion; however, even at the highest dose of 50 μM, a substantial proportion of cells remain viable.

In contrast, the combination of ABT-737 and SB202190 strongly reduces viability such that, at the 50 μM dose, viability is under 20% in all cell lines examined. The synergistic interaction between the two treatments was statistically significant in all cell lines using two-way ANOVA. The Malme-3M displayed the greatest synergistic effect (interaction accounts for 25% of variation, $P<0.0001$) and Lox IMVI displayed the weakest (2% interaction, $P=0.0017$).

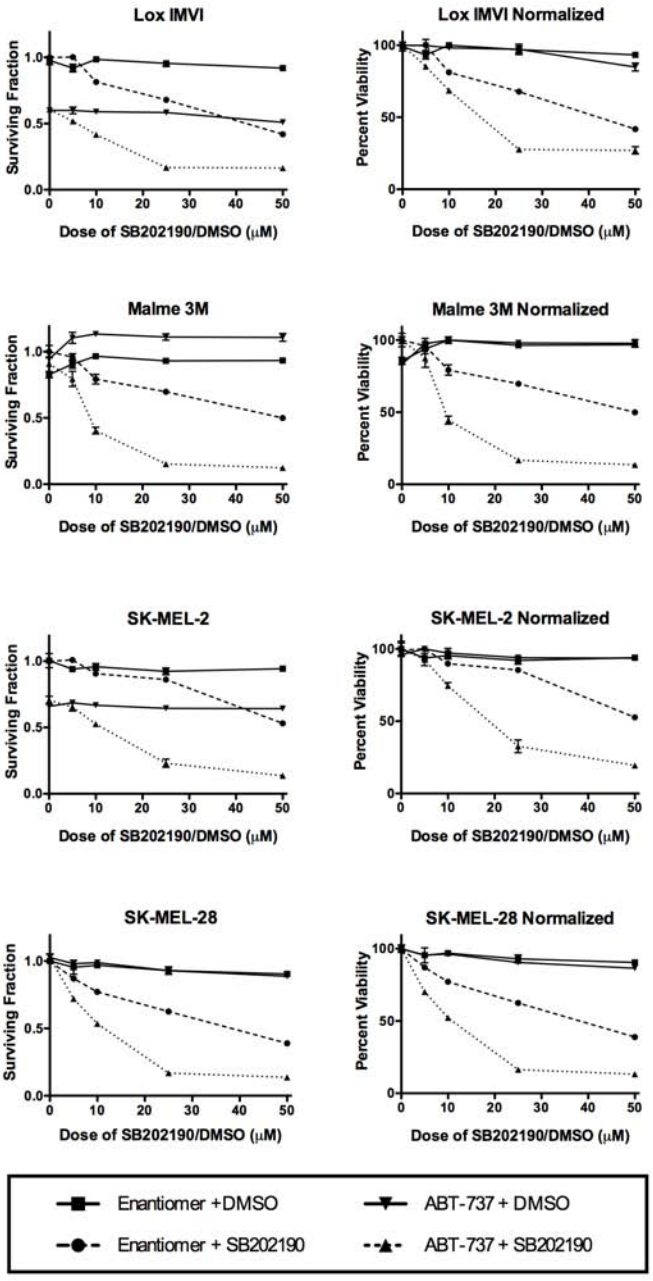

Figure 6-4: Combination of ABT-737 and SB202190 in multiple cell lines

Cells were treated with either 10 μM ABT-737 or enantiomer, combined with increasing doses of DMSO or SB202190. Viability was assessed at 24 hours by MTT assay. *Left*, Viability is expressed as the surviving fraction compared to the Enantiomer-only control (0 μM DMSO/SB202190). *Right*, Enantiomer curves were normalized to Enantiomer-only; ABT-737 curves were normalized to ABT-737-only. Points represent mean (n=4); error bars represent SEM (on all graphs).

Confirmation with second p38 inhibitor

The combinational effect of p38 inhibition and ABT-737 was further confirmed with an independent p38 inhibitor, SB203580, which inhibits p38-α and p38-β (Figure 6-5). SB203580 is an older generation p38 inhibitor and, as such, was not as effective as SB202190. However, I still observed a statistically significant synergistic interaction between the inhibitor and ABT-737 in both Malme-3M (p53+) and SK-MEL-28 (p53-) (interaction accounts for 6% and 5%, respectively; $P<0.0001$). I also attempted to confirm this interaction by knocking down p38; however, despite using three independent DsiRNAs for each of p38-α and p38-β, I was not able to achieve significant knockdown of either protein.

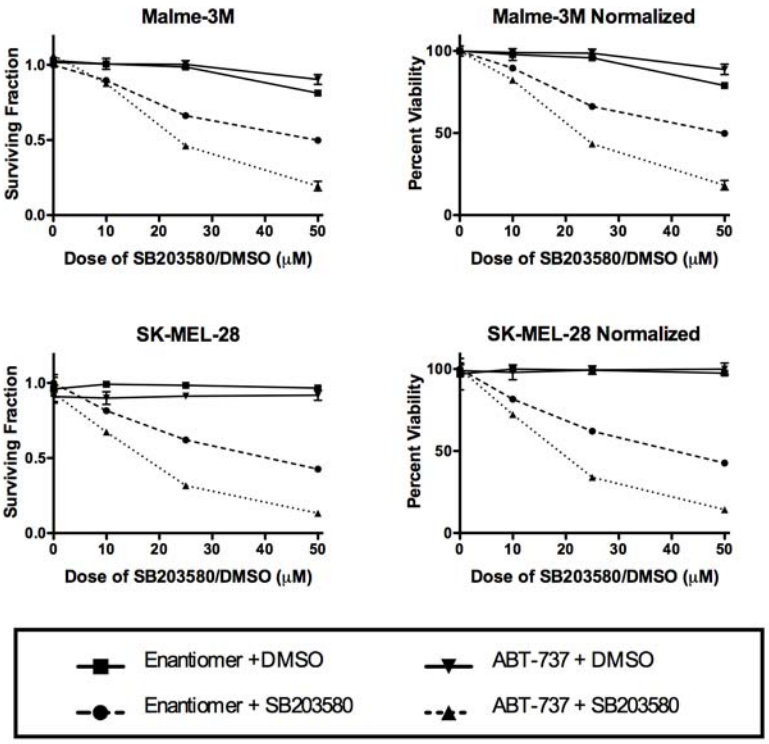

Figure 6-5: Combination treatment of ABT-737 and SB203580

Cells were treated with either 10 μM ABT-737 or enantiomer, combined with increasing doses of DMSO or SB203580. Viability was assessed at 24 hours by MTT assay. *Left*, Viability is expressed as the surviving fraction compared to the Enantiomer-only control (0 μM DMSO/SB203580). *Right*, Enantiomer curves were normalized to Enantiomer-only; ABT-737 curves were normalized to ABT-737-only. Points represent mean (n=4); error bars represent SEM (on all graphs).

Triple combination treatment with Mcl-1 knockdown

Based on my initial experiments with the p38 inhibitor (Figure 6-3), the triple combination of Mcl-1 DsiRNA/SB202190/ABT-737 did not appear to be substantially more effective than the SB202190/ABT-737 treatment. To investigate this further, I directly compared the two treatments (Figure 6-6). I used the Malme-3M (p53+) and SK-MEL-28 (p53-) cell lines, which show little to no response to ABT-737 or Mcl-1 DsiRNA alone.

I examined the effects of adding SB202190 to the Mcl-1 DsiRNA and ABT-737 treatment compared to Scrambled DsiRNA and ABT-737 treatment. As previously demonstrated, combining ABT-737 with Mcl-1 DsiRNA decreases viability compared to a Scrambled DsiRNA control. Addition of SB202190 to both treatments drives viability down further. However, as the dose of SB202190 increases, the difference between the SB202190/ABT-737/Scrambled DsiRNA and SB202190/ABT-737/Mcl-1 DsiRNA treatments decreases. Thus, at higher doses of the p38 inhibitor, knockdown of Mcl-1 provides only a minor improvement to efficacy of the SB202190 and ABT-737 combination treatment.

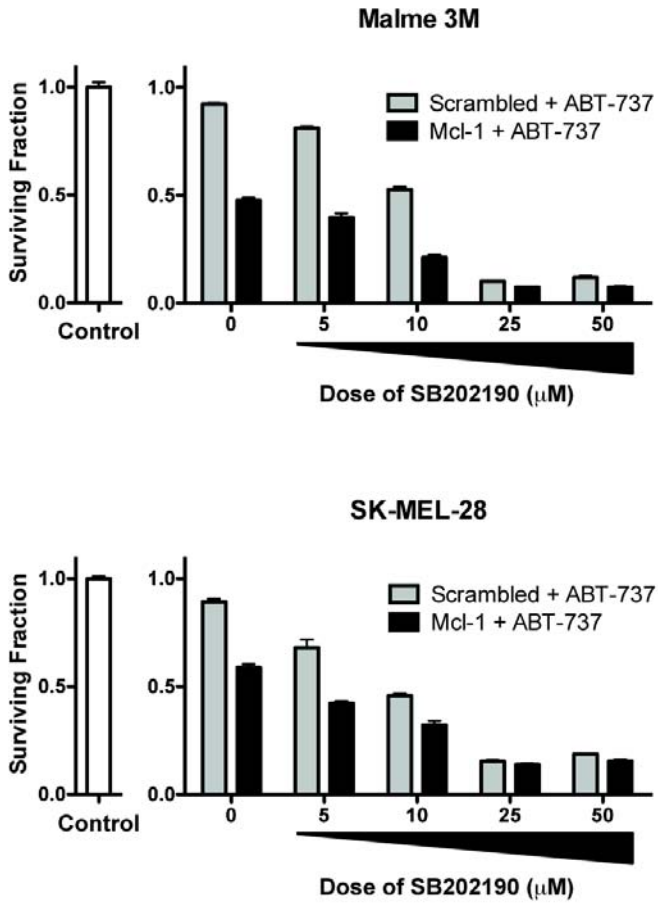

Figure 6-6: Triple combination of Mcl-1 DsiRNA, ABT-737 and SB202190

Malme-3M and SK-MEL-28 cells were treated with 10 nM Scrambled DsiRNA + 10 μM ABT-737 (grey bars) or 10 nM Mcl-1 DsiRNA + 10 μM ABT-737 (black bars), with increasing doses of SB202190. Control cells (white bar) were treated 10 nM Scrambled DsiRNA only. Viability was assessed at 24 hours by MTT assay. Surviving fraction was calculated in comparison to the Scrambled DsiRNA-only control. Data represent mean ± SEM (n=4).

Combination of p38 inhibition and ABT-737 induces apoptosis

I then tested my hypothesis that the combination of SB202190 and ABT-737 decreases viability through induction of apoptotic cell death. I also included the Mcl-1 DsiRNA/ABT-737 combination and the triple combination (SB202190/ABT-737/Mcl-1 DsiRNA) for comparison. Apoptosis and cell cycle changes were assessed by measuring DNA content by propidium iodide staining (PI). ABT-737 alone does not induce apoptosis in either Malme-3M or SK-MEL-28 (Figure 6-7), consistent with the viability data. Interestingly, although the p38 inhibitor single treatment decreases overall viability by MTT assay, there is no increase in apoptosis in Malme-3M and only a small apoptotic effect in SK-MEL-28 (Figure 6-7). Rather, p38 inhibition appears to primarily induce G1 arrest as evidenced by a loss of cells from the G2/M and S fractions.

In contrast, the combination of the p38 inhibitor and ABT-737 results in an almost complete loss of the G2/M and S fractions combined with a significant increase in apoptosis (Figure 6-7; $P<0.05$ compared to either single treatment, both cell lines). The apoptotic effect of the SB202190/ABT-737 treatment is significantly greater than that of the Mcl-1 DsiRNA and ABT-737 combination ($P<0.05$, both cell lines). Compared to the SB202190/ABT-737 treatment, the triple combination induces a shift of cells from the G1 fraction to sub-G1 (Figure 6-7). This effect is greater in Malme-3M than SK-MEL-28; however, the difference in apoptosis is not statistically significant in either cell line.

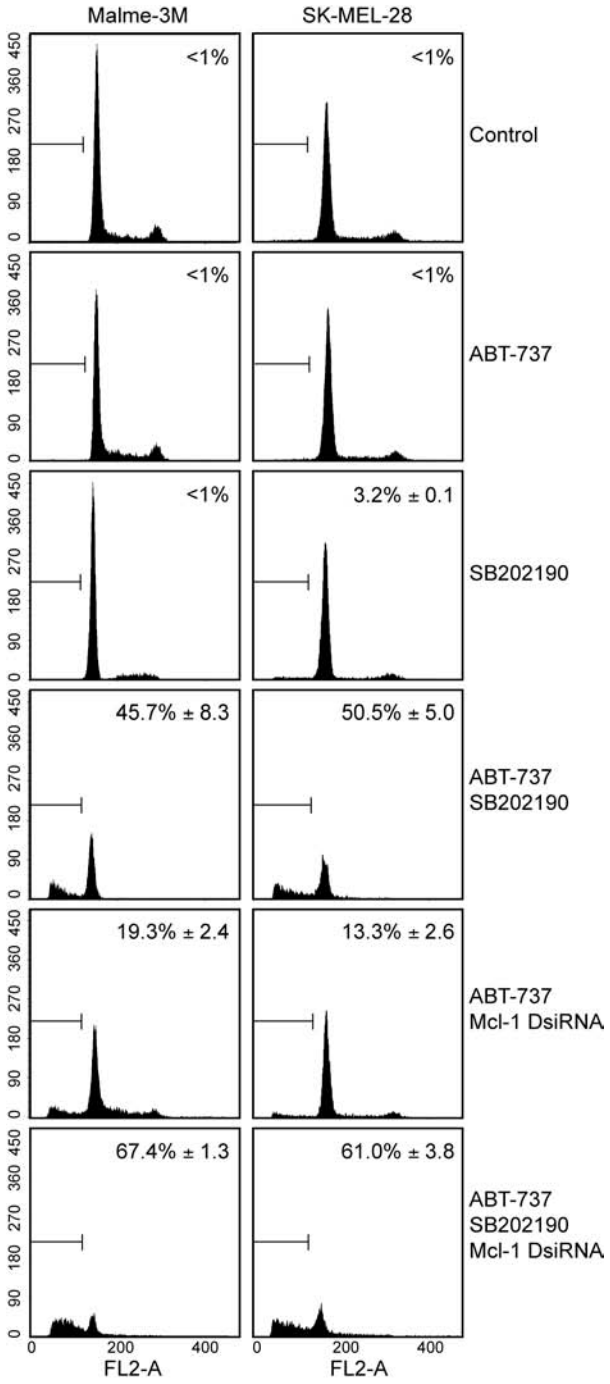

Figure 6-7: Combination of ABT-737 and SB202190 induces apoptosis

Representative histograms showing DNA content in Malme-3M and SK-MEL-28 cells treated with the indicated treatments and combinations for 24 hours. Doses: ABT-737 (10 μM), SB202190 (25 μM), Mcl-1 DsiRNA (10 nM). Apoptotic cells are identified as cells with sub-G1 DNA content. Mean percent apoptosis ± SEM (n=3) is indicated on each plot.

Confirmation of cytostatic effect of p38 inhibition

To confirm that the effect of p38 inhibitor single treatment is primarily cytostatic, I used crystal violet staining to assess fold change in cell density over 24 hours (Figure 6-8). I found that while control-treated plates displayed an increase in cell density over 24 hours, density of SB202190-treated plates was largely unchanged (fold change 0.99±0.02 Malme-3M, 0.97±0.1 SK-MEL-28) suggesting a failure to proliferate. Thus, apoptosis is only induced when the p38 inhibitor is combined with ABT-737 (or Mcl-1 DsiRNA and ABT-737).

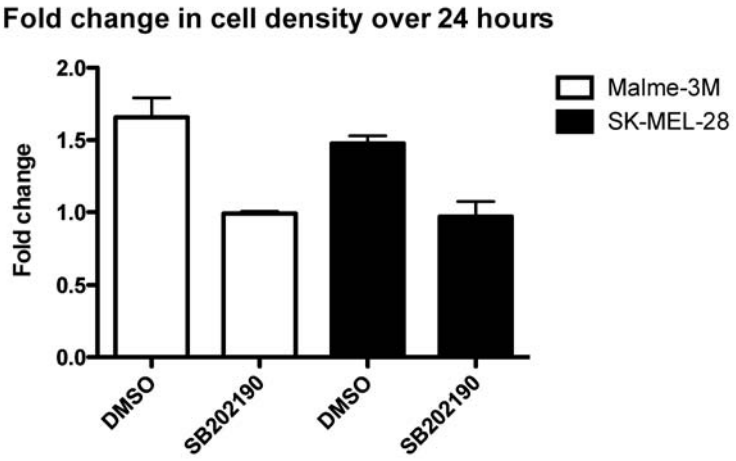

Figure 6-8: Cytostatic effect of SB202190 on melanoma cell proliferation

The day before treatment, Malme-3M and SK-MEL-28 cells were evenly plated on 60 mm diameter plates for ~50% next-day confluency. On the day of treatment, one plate of each cell line was stained by crystal violet to set the baseline for cell density. Remaining plates were treated with either 25 μM DMSO or 25 μM SB202190 for 24 hours. After 24 hours, density was again assessed by crystal violet and fold increase over the baseline was calculated. Data represent mean ±SEM of three independent experiments.

Combination of p38 inhibition and ABT-737 induces caspase activation

To further confirm that the combination of SB202190 and ABT-737 induces apoptosis, I examined the activation of the caspase cascade in Malme-3M and SK-MEL-28 (Figure 6-9). I assessed caspase activation in the SB202190/ABT-737 combination treatment as well as the Mcl-1 DsiRNA/ABT-737 treatment and the triple combination. I found activation of initiator caspases 9 and 8 as well as effector caspase-3 in all three combination treatments (Figure 6-9). Consistent with my apoptosis data, neither ABT-737 nor SB202190 alone induce a detectable increase in caspase cleavage.

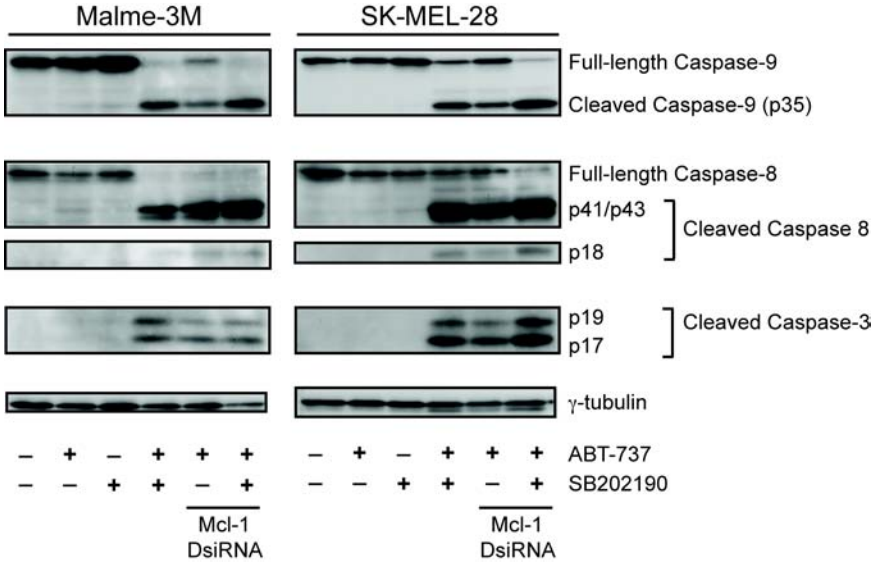

Figure 6-9: Combination of ABT-737 and SB202190 induces caspase cleavage

Western immunoblots showing caspase-9, caspase-8 and cleaved caspase-3 levels with treatment. Malme-3M and SK-MEL-28 cells were treated with 10 μM ABT-737 and 25 μM SB202190, alone and in combination. Also shown are the Mcl-1 DsiRNA (10 nM) + ABT-737 (10 μM) combination and the triple combination (10 nM Mcl-1 DsiRNA, 10 μM ABT-737 and 25 μM SB202190).

Inhibition of p38 has no effect on Mcl-1

As Mcl-1 is a major factor in ABT-737 resistance, many ABT-737-based combination therapies inhibit Mcl-1 either directly or indirectly through up-regulation of pro-apoptotic Bcl-2 family proteins (Chen et al., 2007; Lin et al., 2007; Cragg et al., 2008; Miller et al., 2008). In Figure 6-6, I showed that as the dose of SB202190 increases, the effect of Mcl-1 knockdown on SB202190/ABT-737 efficacy decreases. I therefore hypothesized that the p38 inhibitor may substitute for Mcl-1 knockdown. However, I observed no effect of SB202190 on Mcl-1 levels, suggesting that p38 inhibition does not directly affect Mcl-1 (Figure 6-10).

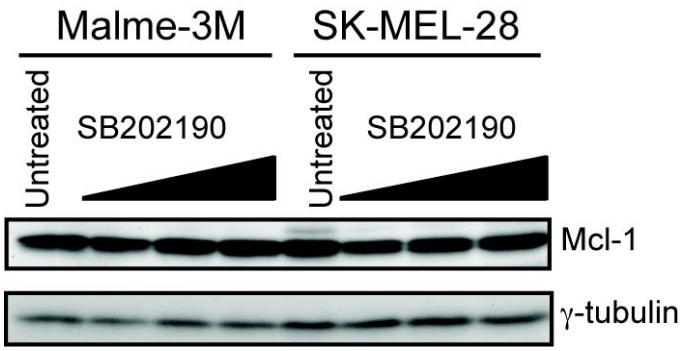

Figure 6-10: Effect of p38 inhibition on Mcl-1 levels

Western immunoblot showing levels of Mcl-1 in Malme-3M and SK-MEL-28 cells treated with increasing doses of SB202190 (0, 5, 10 and 25 μM) for 24 hours.

Inhibition of p38 up-regulates PUMA-α and the combination treatments decrease both PUMA-α and PUMA-β

As discussed in the next chapter, I observed unusual regulation of pro-apoptotic Bcl-2 protein PUMA in response to Mcl-1 DsiRNA and ABT-737 treatment. Therefore, I assessed the effect of the p38 inhibitor on levels of PUMA. I observed substantial up-regulation of pro-apoptotic Bcl-2 family member PUMA with inhibition of p38 (Figure 6-11). Specifically, SB202190 treatment increases expression of PUMA-α but not PUMA-β. This effect is p53-independent as it is observed in both p53 wild-type (Malme-3M) and p53 mutant (SK-MEL-28) cell lines. Preliminary experiments on other Bcl-2 family members, including Bax and Bim, did not reveal p38 inhibitor-dependent changes (data not shown).

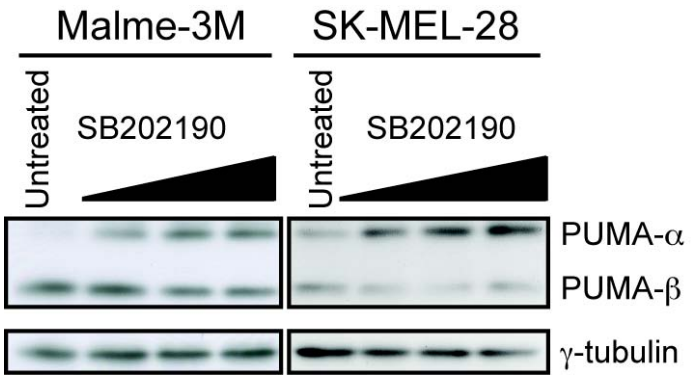

Figure 6-11: Effect of p38 inhibition on PUMA levels

Western immunoblot showing levels of PUMA in Malme-3M and SK-MEL-28 cells treated with increasing doses of SB202190 (0, 5, 10 and 25 μM) for 24 hours.

I then examined PUMA levels in cells treated with the combination treatments of SB202190 and ABT-737, compared to the Mcl-1 DsiRNA/ABT-737 combination and the triple combination. I found that while the p38 inhibitor alone increases PUMA-α, both PUMA-α and PUMA-β levels decrease when the inhibitor is combined with ABT-737 (Figure 6-12). PUMA-β decreases below untreated levels, and PUMA-α returns approximately to baseline. Furthermore, the Mcl-1 DsiRNA/ABT-737 combination and the triple combination treatment drastically reduce levels of both PUMA isoforms below untreated levels.

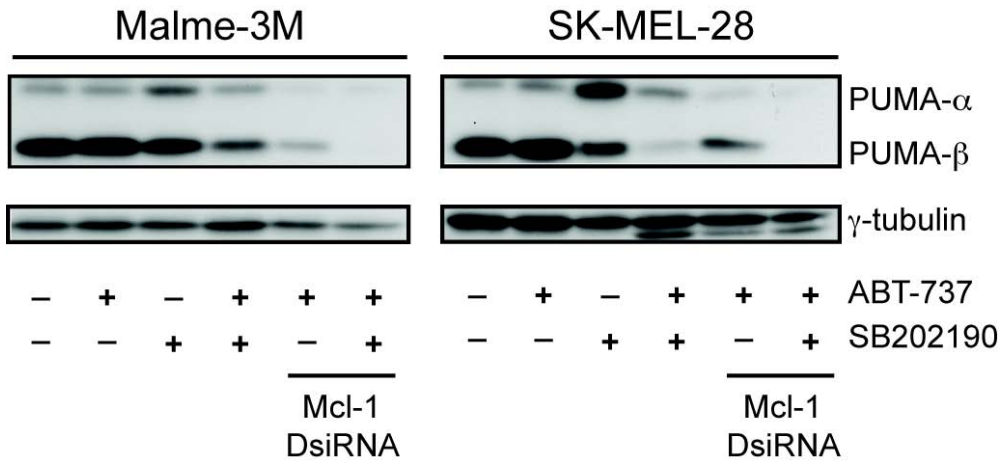

Figure 6-12: Effect of p38 inhibition and ABT-737 on PUMA levels

Western immunoblots showing PUMA levels in cells treated with 10 μM ABT-737 and 25 μM SB202190, alone and in combination. Also shown are the Mcl-1 DsiRNA (10 nM) + ABT-737 (10 μM) combination and the triple combination (10 nM DsiRNA, 10 μM ABT-737 and 25 μM SB202190).

Discussion

The three major arms of the MAPK network, namely the ERK, JNK and p38 pathways, play critical roles in regulating survival and proliferation in melanoma. My results also suggest that the MAPK target JUN may play a role in treatment response to inhibition of anti-apoptotic Bcl-2 proteins (Chapter 5). As no specific JUN inhibitors were available, I examined the effects of MAPK inhibitors in the response of melanoma cell lines to Mcl-1 DsiRNA, ABT-737 and the combination treatment.

I found that inhibition of JNK alone reduced melanoma cell viability (Figure 6-1). This observation is concordant with previously published results demonstrating that inhibition of JNK in melanoma induces cell cycle arrest and apoptosis (Alexaki et al., 2008). However, I observed no substantial synergistic effect between the JNK inhibitor and Mcl-1 DsiRNA and ABT-737, alone or in combination (Figure 6-1). Thus, although a JNK-targeted therapy may hold promise in melanoma, there is likely little benefit to a combination therapy co-targeting anti-apoptotic Bcl-2 proteins.

Inhibition of the ERK pathway, via a specific MEK inhibitor, reduced viability of Malme-3M but not Lox IMVI (Figure 6-2). Addition of ABT-737 or the combination treatment (Mcl-1 DsiRNA and ABT-737) synergistically reduced viability in both cell lines (Figure 6-2). Several papers have shown that MEK inhibition induces either G1 arrest or apoptosis in melanoma cells (Wang et al., 2007b; Cragg et al., 2008; VanBrocklin et al., 2009). G1 arrest is often the first response, followed by apoptosis at later time points. Cragg et al. (2008)

demonstrated a synergistic response to the combination of MEK inhibition and ABT-737 in BRAF mutant tumor lines, including two melanoma lines. Conversely, vanBrocklin et al. (2009) suggest a more additive relationship between the two treatments. Of note, the van Brocklin (2009) study examined cell death at 72 hours post-treatment, a time point that I've found to be associated with non-specific growth effects (See Figure 3-2). My results (at 24 hours) are thus more concordant with Cragg et al. (2008), which assessed apoptosis at an earlier time point (48 hours). Therefore, the evidence points more strongly in favor of a synergistic interaction between ABT-737 and inhibition of MEK.

Although traditionally thought of as a tumor suppressor, evidence is beginning to emerge suggesting that p38 may be oncogenic in some cancers (Wagner and Nebreda, 2009). Thus, p38 inhibitory treatments are currently being investigated for cancer therapy. In multiple myeloma, p38 inhibition has been shown to substantially increase the effects of anti-cancer agents including proteasome inhibitors bortezomib and MG-132 (Hideshima et al., 2004; Navas et al., 2006). In colorectal cancer, p38 inhibition synergizes with anti-inflammatory agents sulindac and exisulind (Sun and Sinicrope, 2005; Lim et al., 2006).

I found that inhibition of p38 alone had a modest growth effect in melanoma cell lines (Figure 6-3, 6-4, 6-5). As aforementioned, inhibition of p38 was previously shown to reduce proliferation of melanoma cells (Estrada et al., 2009). My data expand on this finding to show that p38 inhibition induces G1 cell cycle arrest, without inducing a substantial apoptotic response (Figure 6-7, 6-8, 6-9). Due to the highly aggressive nature of malignant melanoma, growth inhibition

alone may not be enough to provide a long-term therapeutic benefit. In contrast, the combination of the p38 inhibitor and ABT-737 strongly and synergistically induces apoptotic cell death in melanoma cell lines (Figure 6-7, 6-9). As a result, viability is reduced by over 80% at the highest doses of SB202190 (Figure 6-4). The synergistic interaction between the two treatments is statistically significant by two-way ANOVA. Addition of Mcl-1 DsiRNA reduces viability further; however, at high doses of the p38 inhibitor, the improvement to treatment efficacy is minor (Figure 6-5). I therefore propose that the combination of p38 inhibition and ABT-737 is a highly effective *in vitro* treatment that calls for further investigation *in vivo.*

Recently, orally-available p38 inhibitors (e.g. SD0006 (Burnette et al., 2009)) and an orally-available analog of ABT-737 (ABT-263 (Tse et al., 2008)) have been developed, thus our proposed treatment strategy may be readily translated into *in vivo* therapy. Furthermore, several p38 inhibitors are in clinical trials as anti-inflammatories for rheumatoid arthritis and other inflammatory disorders (www.clinicaltrials.gov), suggesting that such compounds have relatively good safety profiles.

Many treatments that synergize with ABT-737 directly or indirectly inhibit Mcl-1. The CDK inhibitor roscovitine reduces levels of Mcl-1 protein (Chen et al., 2007), proteasome inhibitor MG-132 increases levels of NOXA (Miller et al., 2008), and MEK inhibitors increase levels of Bim and PUMA (Wang et al., 2007b). I did not observe any effect of p38 inhibition on Mcl-1 itself; however, I did observe a substantial increase in levels of pro-apoptotic Bcl-

2 protein, PUMA-α. As discussed in Chapter 1, PUMA binds and inhibits anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1 (Yu and Zhang, 2008). As I found that p38 inhibitor treatment alone does not induce apoptosis, PUMA levels may not be high enough to overcome all of the anti-apoptotic Bcl-2 proteins. However, combined with ABT-737, which potently inhibits Bcl-2, Bcl-xL and Bcl-w, the increased amount of PUMA protein may be sufficient to inhibit Mcl-1 (and A1), thus removing the remaining block to apoptosis induction. The subsequent decrease in PUMA following addition of ABT-737 is discussed in the next chapter. Following completion of this thesis, I performed PUMA-α knockdown experiments suggesting that PUMA-α is required for the full induction of cell death with the combination treatment (Appendix I). Thus, PUMA plays a unique and important role in treatment response, although the full mechanism of apoptotic induction remains to be elucidated.

Currently, metastatic melanoma is considered incurable and new treatment strategies are urgently required. Multiple pathways controlling cell survival, proliferation and apoptosis are dysregulated in melanoma; therefore, combination therapies may hold the key to effective treatment. Here I report that the combination of a p38 MAPK inhibitor and a Bcl-2 family inhibitor effectively induces cell death in melanoma cell lines. Furthermore, I provide evidence that pro-apoptotic protein PUMA may play a role in response to this combination treatment. My results thus describe a promising and novel treatment strategy for malignant melanoma.

~Chapter 7~
Novel regulation of PUMA in treatment response

Data presented in this chapter have been submitted for publication.

Angela M. Keuling, Susan E. Andrew and Victor A. Tron. Inhibition of p38 MAPK enhances ABT-737-induced cell death in melanoma cell lines: Novel regulation of PUMA. Submitted to *Pigment Cell & Melanoma Research*.

All experimental design, experimentation and data analysis were performed by Angela Keuling, with the exception of the mass spectrometry, which was performed by Fang Wu and Peng Wang.

Introduction

PUMA (p53 up-regulated modulator of apoptosis) is a pro-apoptotic BH3-only member of the Bcl-2 family. The *PUMA* locus undergoes alternative splicing to produce multiple protein isoforms, namely PUMA-α, -β, -δ and -γ (Nakano and Vousden, 2001). Via the BH3 domain of the α and β isoforms, PUMA binds and inhibits anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1 (Chen et al., 2005). In addition, there is some evidence that the BH3 domain of PUMA can directly bind and activate Bax, although the role of PUMA as a direct activator is controversial (Gallenne et al., 2009). In melanoma, expression of PUMA is greatly reduced, correlating with poor overall and disease-free survival (Karst et al., 2005). Exogenous expression of PUMA-α significantly induces apoptosis in melanoma cell lines.

Activity of PUMA is primarily regulated at the level of transcription (reviewed in Yu and Zhang, 2008). The best-characterized regulator of PUMA is p53, which induces *PUMA* transcription in response to genotoxic stress (Nakano and Vousden, 2001). Indeed, PUMA is thought to account for the majority of the proapoptotic activity of p53 as *Puma* knockout mice recapitulate almost all the apoptotic defects of p53 null mice (Jeffers et al., 2003; Yu and Zhang, 2003). In addition to PUMA, p53 also induces expression of fellow BH3-only protein Noxa (Oda et al., 2000). However, the apoptotic defects of the *Noxa*-/- mouse are not as pronounced as the *Puma* knockout (Villunger et al., 2003). Furthermore, overexpression of PUMA more potently induces cell death than Noxa in some cell lines (Cregan et al., 2004b). The strong proapoptotic activity of PUMA is likely

due to its ability to engage all anti-apoptotic Bcl-2 proteins, whereas Noxa binds only Mcl-1 and A1 (Chen et al., 2005).

In addition to p53, a variety of other transcription factors including p73, FoxO3a, CHOP, E2F1, MYC, CREB, JUN and Sp1, can also activate expression of *PUMA* (Yu and Zhang, 2008). Consequently, PUMA is up-regulated during apoptosis induced by diverse stimuli such as oncogenic stress, growth factor and cytokine withdrawal, ER stress, hypoxia and oxidative stress, and ischemia. Inhibition of MEK has been shown to up-regulate PUMA (Wang et al., 2007b) and, as discussed in Chapter 6, I have found that inhibition of p38 also induces PUMA in a p53-independent fashion. Thus, PUMA is a critical regulator of apoptosis in a variety of cellular stress responses.

While assessing the effect of Bcl-2 family members on sensitivity to the combination treatment (Chapter 4), I briefly examined the effect of treatment on a number of Bcl-2 proteins, including PUMA. Unexpectedly, I observed a strong down-regulation of PUMA in response to the combination treatment. The observed decrease in PUMA levels in cells undergoing apoptosis appears paradoxical as PUMA is pro-apoptotic. I therefore investigated the down-regulation of PUMA further. Here I report that the down-regulation of PUMA in response to treatment is caspase-dependent and independent of proteasomal or lysosomal degradation. Furthermore, I provide evidence of a putative PUMA cleavage product. My results therefore represent the first evidence of endogenous PUMA cleavage.

Results

PUMA levels decrease with combination treatment

In examining the effect of the combination treatment on pro-apoptotic PUMA, I observed, unexpectedly, that levels of PUMA decrease in melanoma cells treated with Mcl-1 DsiRNA and ABT-737 (Figure 7-1). PUMA-α and PUMA-β levels drastically decrease with combination treatment in all six melanoma cell lines used in this study. I therefore investigated this phenomenon further. For further analyses, I focused on the Lox IMVI cell line, which displays the highest PUMA expression out of the six cell lines.

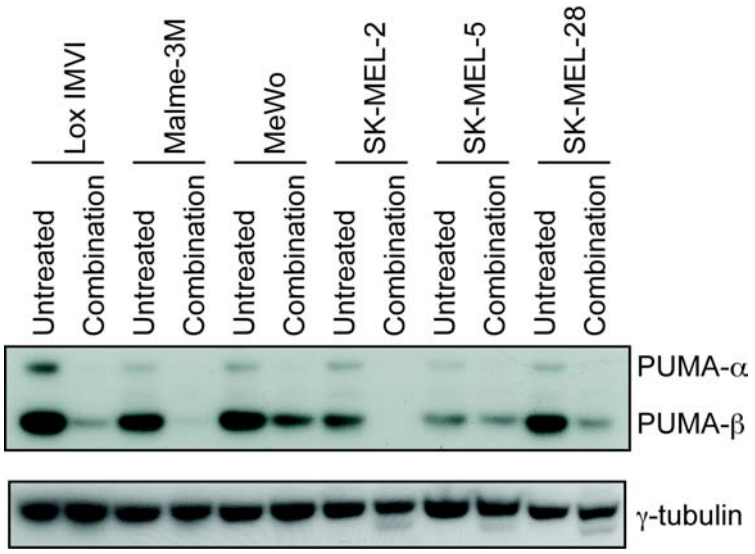

Figure 7-1: Mcl-1 DsiRNA and ABT-737 combination treatment decreases levels of PUMA

Western immunoblot showing levels of PUMA-α and PUMA-β in cells treated with 10 nM Mcl-1 DsiRNA and 10 μM ABT-737 (for 24 hours) compared to an untreated control.

Detection of novel PUMA isoform

I analyzed combination-treated Lox IMVI cell lysates on Bis-Tris gradient gels instead of conventional SDS-PAGE gels, as the former are thought to provide greater stability and sharper resolution to a wide range of proteins. Using the Bis-Tris electrophoresis system, I observed a novel, low molecular weight product (~16-17 kDa) on PUMA immunoblots, running just under PUMA-β (18 kDa) (Figure 7-2). This product is present in cells undergoing apoptosis as it appears in conjunction with cleaved caspase-3. Lox IMVI responds to ABT-737, both alone and in combination with Mcl-1 DsiRNA, and correspondingly the low molecular weight product is detected in both treatments. Also in agreement, Lox IMVI displays a small response to Mcl-1 DsiRNA alone (viability reduced by ~10%); and the small PUMA band is only faintly visible with this treatment.

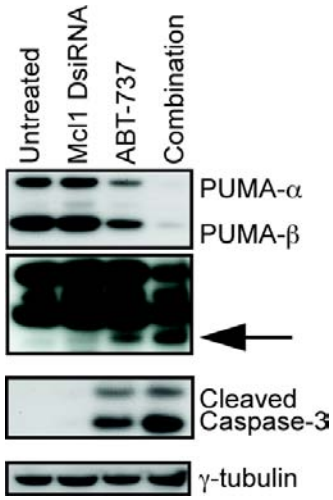

Figure 7-2: Detection of novel PUMA isoform

Lox IMVI cells were treated for 24 hours with Mcl-1 DsiRNA (10 nM) and ABT-737 (10 μM), alone and in combination. Samples were analyzed by Bis-Tris electrophoresis as described in the Materials and Methods. Both a low and high exposure of the PUMA blot are shown. Arrow points to novel PUMA isoform. Also shown is cleaved caspase-3 as a marker of apoptosis.

Confirmation with second antibody

I confirmed detection of the novel PUMA band using two independent antibodies to PUMA (one from Cell Signaling, one from Genetex), both of which are directed to the shared C-terminal region of PUMA-α and PUMA-β (Figure 7-3). The cleavage band was also detected in other cell lines, including Malme-3M and SK-MEL-2; although, the band was very faint compared to the Lox IMVI blots (data not shown).

Of note, using the Bis-Tris gel system, I sometimes observe a middle band running between PUMA-α and PUMA-β. This band is detected with both PUMA antibodies. This observation is consistent with other studies in which PUMA sometimes appears to run as a doublet or triplet (Wong et al., 2005; Mahmoudi et al., 2009); however, as this isoform does not consistently follow the same expression pattern as PUMA-α and PUMA-β, I did not investigate it further.

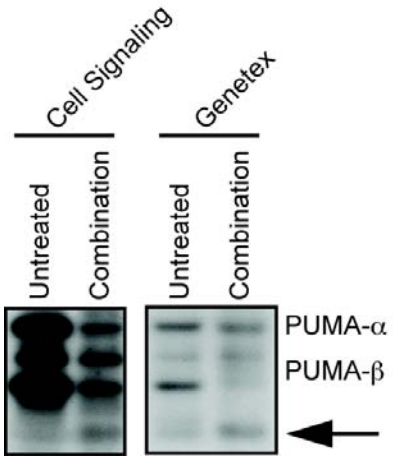

Figure 7-3: Detection of PUMA isoform with second antibody

PUMA immunoblots using two antibodies: Cell Signaling antibody 4976 (same antibody as in all other figures in this chapter) and Genetex antibody GTX29643. Lox IMVI cells were treated with either no treatment of the combination of Mcl-1 DsiRNA and ABT-737 for 24 hours. Arrow points to novel PUMA isoform.

Caspase-dependent cleavage of PUMA

A number of pro-apoptotic Bcl-2 family proteins are reported to be cleaved during apoptosis, including Bid, Bad, Bim, and Bax (Gross et al., 1999; Gao and Dou, 2000; Condorelli et al., 2001; Chen and Zhou, 2004). I therefore hypothesized that the low molecular weight band on the PUMA immunoblots may represent a novel PUMA cleavage product. Thus, the observed decrease in full-length PUMA-α and -β could be due to caspase-dependent cleavage. In support of this hypothesis, the general caspase inhibitor Z-VAD-FMK, both eliminates the small cleavage band and fully restores full-length PUMA levels (Figure 7-4).

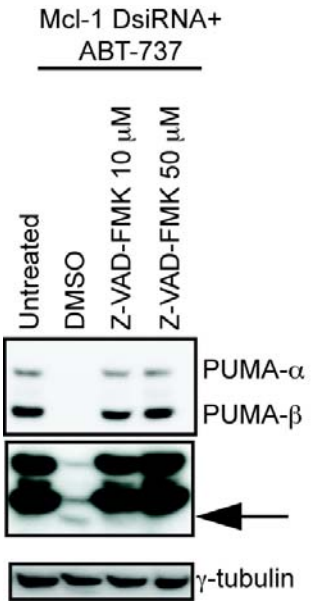

Figure 7-4: Caspase-dependent cleavage of PUMA

Lox IMVI cells were pre-treated with either DMSO or Z-VAD-FMK at the indicated doses, then treated with the combination of Mcl-1 DsiRNA and ABT-737 for 24 hours. Both low and high exposure of PUMA are shown. Arrow points to novel (cleaved) PUMA isoform.

Down-regulation of PUMA is not due to protein degradation

To confirm that the observed treatment-dependent down-regulation of PUMA is not due to degradation, I examined the effects of two independent proteasome inhibitors (MG-132 or Lactacystin) and lysosome inhibitors (ammonium chloride or chloroquine) on PUMA levels. Neither proteasome nor lysosome inhibition fully restore full-length PUMA, confirming that PUMA is not being degraded (Figure 7-5).

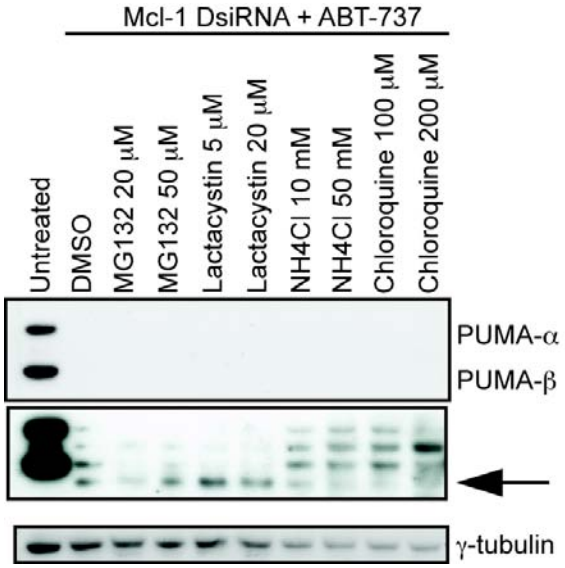

Figure 7-5: Effects of proteasome and lysosome inhibition

Lox IMVI cells were pre-treated proteasome inhibitors MG132 and clasto-Lactacystin β-lactone or weak bases (lysosome inhibitors) ammonium chloride (NH_4Cl) and chloroquine prior to treatment with Mcl-1 DsiRNA and ABT-737 for 24 hours. Both a low and high exposure of PUMA are shown. Arrow points to novel (cleaved) isoform.

Mass spectrometry

My results strongly suggest that PUMA is cleaved during apoptosis; however, confirmation of the identity of the cleavage product by mass spectrometry has proven a challenge. I was unable to immunoprecipitate PUMA using the antibodies that can detect the low molecular weight cleavage band (neither of which are optimized for immunoprecipitation). I then sought to identify the band in whole cell lysate. Running whole cell lysate (of combination-treated cells) on a gradient gel, I cut an approximately 1-2 mm section around the site of the cleavage band and sent this for mass spectrometry. Over 600 proteins were detected, 92 of which were represented by multiple peptides. No peptides representing PUMA were detected. However, mass spectrometry also failed to detect several other proteins of similar size to the PUMA band, including cleavage products of Bid (15 kDa), caspase-3 (17 kDa), and caspase-9 (17 kDa). Thus, the results of the mass spectrometry were difficult to interpret and the identity of the PUMA cleavage product has yet to be confirmed.

Potential PUMA cleavage sites

Caspases cleave after specific four amino-acid motifs, typically with an aspartate residue at position 4 (Fischer et al., 2003). In a small number of caspase substrates, cleavage occurs following a glutamate residue. Using the CASVM server for prediction of caspase cleavage sites (Wee et al., 2006), four potential sites are predicted in PUMA-α (Figure 7-6). Only one predicted site is a classical motif with an aspartate residue in position 4 (LARD - aa20). The remaining three

sites are non-canonical motifs ending with glutamate. As I observe cleavage of both PUMA-α and PUMA-β with treatment, the PUMA cleavage site is likely within the region of shared sequence between the two isoforms. Furthermore, the antibodies that detect the PUMA cleavage product are targeted to the C-terminal shared region. Two predicted cleavage sites are within the shared region. One, PEME (191) is only two amino acids away from the C-terminus of either protein, and therefore could not produce the ~16-17 kDa cleavage product I observed. On the other hand, cleavage at the upstream SLAE (101) site could potentially produce a product running slightly faster than PUMA-β, as observed on my PUMA immunoblots. However, there are several other aspartate and glutamate residues in the two proteins, thus cleavage may occur at a site other than those predicted by CASVM and will have to be confirmed experimentally.

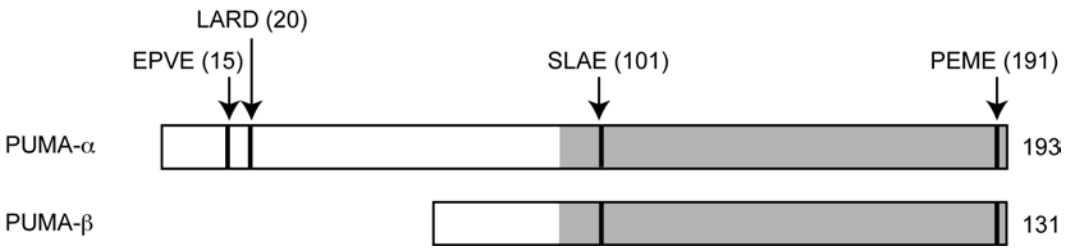

Figure 7-6: Predicted cleavage sites in PUMA

Results of CASVM cleavage site prediction server for PUMA-α and PUMA-β. Predicted tetrapeptide motifs are indicated along with their position in the PUMA-α protein. Region of shared sequence between the two isoforms is shown in grey. Total amino acid number is shown to the right of each schematic.

Discussion

BH3-only members of the Bcl-2 family are regulated by a variety of mechanisms including transcriptional activation, phosphorylation, localization and caspase-dependent cleavage. Induction of BH3-only activity is associated with induction of apoptosis. Thus, my observation that levels of BH3-only protein PUMA decrease with Mcl-1 DsiRNA and ABT-737 treatment appeared paradoxical (Figure 7-1). However, I found that down-regulation of PUMA was accompanied by detection of a novel, low molecular weight PUMA isoform (Figure 7-2 and 7-3). Caspase inhibition both restores full-length PUMA and eliminates the small protein product (Figure 7-4), suggesting that PUMA is cleaved during apoptosis.

Here I present the first report of endogenous PUMA cleavage. BH3-only proteins Bad and Bim are also reported to be cleaved downstream of the caspase cascade (Condorelli et al., 2001; Chen and Zhou, 2004). The truncated proteins have increased apoptotic activity and are thought to function as part of a feedback loop to amplify apoptotic signals. My results raise the possibility that PUMA acts by a similar mechanism. Of note, knockdown of PUMA-α (Appendix I) did not affect response to Mcl-1 DsiRNA/ABT-737; however, further work is needed to examine the role of PUMA-β in treatment response. Future studies will also elucidate the structure and function of the PUMA cleavage product.

Although generally reported to be up-regulated during apoptosis, there are a small number of reports in which PUMA levels decrease by an unknown mechanism (Fandy et al., 2007; Zantl et al., 2007; Ngan et al., 2008).

Interestingly, although MEK inhibitors are reported to increase PUMA at early time points (Wang et al., 2007b), at later time points, PUMA appears to decrease (VanBrocklin et al., 2009). Furthermore, a recent study expressing a PUMA construct in BaF_3 cells found that recombinant PUMA levels decreased more rapidly than a GFP control, an effect that can be prevented by caspase inhibition (Callus et al., 2008). My results suggest an endogenous cleavage mechanism that may serve to explain some of these unexpected decreases in PUMA protein levels; however, further experimentation is required.

In explanation as to why the PUMA cleavage product had not been detected previously, few papers include high enough exposures to visualize the cleavage product (based on my experience). Furthermore, as I was only able to detect the cleavage band with antibodies directed against the C-terminus of PUMA-α and PUMA-β, studies using more N-terminal-directed antibodies would be unlikely to detect the cleaved isoform. Finally, my use of Bis-Tris gradient gels allowed for sharper resolution and separation than conventional SDS-PAGE, which may be necessary to separate cleaved PUMA from PUMA-β.

As discussed in Chapter 6, although p38 inhibition up-regulates PUMA-α, addition of ABT-737 to this treatment results in a decrease of both PUMA-α and PUMA-β levels. These results suggest that the SB202190/ABT-737 combination therapy induces cleavage of PUMA, similar to the Mcl-1 DsiRNA and ABT-737 treatment. Thus, PUMA cleavage may occur during apoptosis in response to a variety of stimuli in melanoma cells.

Using a server for prediction of caspase cleavage sites, four potential cleavage sites are predicted in PUMA-α, two of which are shared with PUMA-β (Figure 7-6). Neither site is a canonical aspartate motif, providing further evidence as to why PUMA had not previously been identified as a caspase substrate. The *in vivo* PUMA cleavage site will need to be determined experimentally.

To date, little is known about the post-transcriptional regulation of PUMA activity. My results provide the first evidence that endogenous PUMA can be cleaved in a caspase-dependent mechanism during apoptosis. Thus, the role of PUMA in apoptosis induction is more complex than originally surmised. Future studies will elucidate the complex regulation of PUMA function.

~Chapter 8~

General Discussion

The combination of Mcl-1 DsiRNA and ABT-737 induces apoptosis in multiple melanoma cell lines

Melanoma is resistant to conventional chemotherapy and radiation due to the intrinsic ability of melanoma cells to elude apoptotic stimuli. Our previous results and others have shown that anti-apoptotic Bcl-2 family members Mcl-1, Bcl-xL and, in some cases, Bcl-2 are highly overexpressed in melanoma (Tang et al., 1998; Bush and Li, 2003; Zhuang et al., 2007). ABT-737 is a potent and specific small-molecule inhibitor of Bcl-2, Bcl-xL and Bcl-w, with demonstrated single agent activity in a variety of cancers (Oltersdorf et al., 2005). Resistance to ABT-737 is largely conferred by expression and activity of Mcl-1 (Tahir et al., 2007). I found that neither ABT-737 nor Mcl-1 knockdown alone induce substantial cell death in the melanoma cell lines used in this study. However, the combination of the two treatments significantly induces apoptosis and reduces overall viability in all cell lines examined.

The next step in this project is to assess the efficacy of the combination treatment in an *in vivo* model of melanoma. First, knockdown of Mcl-1 *in vivo* must be optimized. There are a variety of delivery methods for siRNA molecules, with liposomes being a promising option in terms of high delivery and relatively low toxicity (Tran et al., 2009). Ideally, the Mcl-1 DsiRNA-carrying liposome would be tagged to target it specifically to melanoma cells. These experiments are currently underway in the Tron laboratory. Once optimal knockdown of Mcl-1 is achieved, dose and delivery of ABT-737 will be optimized to produce the maximal apoptotic effect in melanoma cells while limiting toxicity to normal

tissues. Initial *in vivo* studies of mice bearing SCLC, ovarian cancer or myeloma xenograft tumors demonstrated that ABT-737 is generally well tolerated (Oltersdorf et al., 2005; Trudel et al., 2007b; Witham et al., 2007). Furthermore, an orally available analog of ABT-737 has been developed (ABT-263) (Tse et al., 2008), allowing for simpler treatment schedules. Thus, my results describe a promising, new treatment strategy that calls for further investigation *in vivo*.

Induction of apoptosis by Mcl-1 DsiRNA and ABT-737 requires death receptor-independent activity of extrinsic pathway proteins

My results demonstrate a role for extrinsic pathway proteins in response to Mcl-1 DsiRNA and ABT-737 treatment. I found that, in addition to intrinsic initiator caspase-9, extrinsic caspases 8 and 10 as well as Bid are activated in response to the combination treatment. Caspase-8/-10 activation is dependent on caspase-9 activity but is independent of death receptor function. I also demonstrate that caspase-8 (and -10) are required for the full induction of cell death following treatment. Furthermore, my results suggest that Bid and cleaved caspase-8 can act as markers for sensitivity to the combination treatment.

Further studies are needed to elucidate the pathway between caspase-9 and caspase-8/-10. Previous studies have shown that caspase-8 can be cleaved by either caspase-3 or caspase-6, downstream of the mitochondria (Slee et al., 1999; Tang et al., 2000). Furthermore, future studies are needed to determine whether Bid, acting downstream of caspase-8/-10, is required for combination treatment response. Thus, my results suggest that extrinsic pathway proteins play an

important role in treatment response to Mcl-1 DsiRNA and ABT-737. A putative model is presented in Figure 4-9. Future studies will further elucidate the complex feedback mechanism that ultimately results in the induction of apoptosis.

Mcl-1 and ABT-737 induce multiple gene expression changes

Analysis of gene expression patterns by cDNA microarrays in the six melanoma cell lines revealed multiple expression changes between cells treated with Mcl-1 DsiRNA and ABT-737 compared to a control treatment. A variety of pathways were thus represented including in apoptosis, cell cycle, ER stress and immediate early response. A small number of these expression differences were confirmed by semi-quantitative RT-PCR. Future studies utilizing real-time quantitative RT-PCR could help to confirm more subtle differences in transcript levels.

Interestingly, the array and follow-up RT-PCR demonstated up-regulation of the gene encoding anti-apoptotic Bcl-2 protein Bcl-B. *BCLB* up-regulation was observed specifically in the cell lines most resistant to the combination treatment. These data suggest that Bcl-B activity may contribute to treatment resistance in some cell lines. Combined with my results from Chapter 4, demonstrating high levels of A1 in treatment-resistant cell lines, I propose that expression and activity of anti-apoptotic Bcl-2 family members not targeted by ABT-737 or Mcl-1 DsiRNA substantially affects treatment response. However, due to technical limitations in detecting the Bcl-B and A1 proteins by western blot, this hypothesis could not be pursued further during my thesis work. Upon optimization of Bcl-B

and A1 detection, I foresee future work in combining knockdown of either protein with ABT-737 and/or Mcl-1 DsiRNA.

In several cell lines, by both the array and RT-PCR, I found that *JUN* expression is up-regulated in response to the combination treatment. Furthermore, I also observed up-regulation of its binding partner *FOS* in two of the six cell lines. I therefore focused my studies on inhibition of the upstream MAPK pathways. However, the mechanism by which inhibition of Bcl-2 proteins leads to induction of immediate early genes remains to be elucidated. There is evidence that ABT-737 treatment induces increased levels of reactive oxygen species (Howard et al., 2009). Oxidative stress is known to activate ERK, JNK and p38, which in turn can induce expression of *JUN* and *FOS* (McCubrey et al., 2006). Thus, the pathway by which *JUN/FOS* are transactivated in responses to treatment represents an interesting new area of investigation.

ABT-737 and inhibition of p38 synergistically induce cell death in melanoma cell lines

The combination of ABT-737 and an inhibitor of p38 MAPK is a novel treatment strategy that effectively induces apoptosis in multiple melanoma cell lines. In the majority of cancers, p38 is thought to act as a tumor suppressor. However, in melanoma, p38 acts in conjunction with ERK to promote tumor growth and metastasis (Estrada et al., 2009). Thus, it will be interesting to see if the apoptotic effects of the p38 inhibitor/ABT-737 treatment are unique to

melanoma. To date, no other reports on this treatment combination have been published.

The next step in developing this treatment strategy will be to optimize treatment *in vivo*. As aforementioned, a variety of orally available p38 inhibitors are in clinical trials for use as anti-inflammatories (www.clinicaltrials.gov). Thus, if approved, these treatments are likely to be well tolerated. Dose and delivery schedule of ABT-737 (or ABT-263) and a relevant p38 inhibitor will be determined experimentally in melanoma xenograft models.

Many treatments that synergize with ABT-737 directly or indirectly inhibit Mcl-1 (Chen et al., 2007; Lin et al., 2007; Cragg et al., 2008). I observed no effect of p38 inhibition on levels of Mcl-1; however, I observed substantially increased expression of the alpha isoform of PUMA. Knockdown experiments suggest that PUMA-α is required for the full induction of cell death with treatment. Given the complexity of targeting both the p38 pathway and the Bcl-2 family, up-regulation of PUMA is likely just one of the mechanisms involved in the synergistic response to treatment. Future studies are thus needed to fully eluciduate the mechanisms required for the induction of cell death with treatment. My results therefore describe both a novel, potential treatment strategy for melanoma as well as describe a novel connection between p38 and PUMA.

PUMA is cleaved during apoptosis

My results provide the first indication that endogenous PUMA is cleaved by a caspase-dependent mechanism during apoptosis. Future studies are needed to

confirm the identity and amino acid sequence of the PUMA cleavage product. In addition, the cleavage site must be confirmed experimentally. I attempted to express a PUMA construct in melanoma cell lines; however, I could not detect expression of recombinant PUMA regardless of transfection condition, cell line or antibody used. If a tagged recombinant PUMA could be expressed in melanoma cells, it could facilitate the detection of the cleavage product as well as immunoprecipitation of the protein for mass spectrometry. Furthermore, aspartate and glutamate residues could be mutated to reveal the cleavage site.

Although cleavage of endogenous PUMA has not been previously reported, there are published studies in which PUMA levels decrease during apoptosis by an unknown mechanism (Fandy et al., 2007; Zantl et al., 2007; Ngan et al., 2008). These results suggest that cleavage of PUMA may not be restricted to melanoma cells. The specific combination of stimuli and cell types that result in PUMA cleavage will be an interesting area of investigation. Thus, my results open the door to future studies on the complex role of PUMA in apoptosis.

As a final note, my studies were carried out in six melanoma cell lines reflective of the genetic diversity in melanoma. Further work is required to expand these results to a broader number of melanomas and determine the overall efficacy of the proposed treatment strategies as well as the extent of the apoptotic mechanisms described here.

Conclusions and Perspectives

There are currently no effective treatments for metastatic melanoma and survival rates are exceedingly low. Recent focus has been on development of drugs to target key proliferative and survival pathways. Based on the high frequency of *BRAF* and *NRAS* mutations in melanoma, inhibition of this pathway has been in the spotlight. However, clinical trials of general kinase inhibitors, such as sorafenib, have not been promising (Eisen et al., 2006). Furthermore, use of newer generation BRAF and MEK inhibitors may be hindered by treatment resistance conferred by *MEK1* mutations (Emery et al., 2009). Thus, new therapeutic strategies are needed.

Given the extensive and complex dysregulation of proliferative and survival pathways in melanoma, I believe that combination therapies, hitting multiple key targets, are the future of melanoma therapy. Here I provide evidence of two such combination treatments: ABT-737 combined with either Mcl-1 DsiRNA or an inhibitor of p38. Of these two, the p38 inhibitor/ABT-737 combination stands out as the simplest and most effective treatment strategy. In particular, given the recent development of oral analogs of both components, this drug combination should be readily adapted for *in vivo* treatment.

Personalized medicine, in my view, is a critical component to development of effective cancer therapies. Protein markers, genotypes at key loci and genomic profiles could all be used to subdivide patients into those most and least likely to respond to a given treatment. Combination therapies could then be devised to target the specific combination of dysregulated pathways in the

individual cancer. Thus efficacy could be maximized and patients would not endure potentially harsh treatments to which they are unlikely to respond.

The crux of personalized cancer therapies comes out of understanding the mechanisms involved in treatment response and resistance. My data provide novel evidence as to the mechanisms involved in response to the Mcl-1 DsiRNA/ABT-737 and p38 inhibitor/ABT-737 treatment strategies. I also describe two potential markers of treatment response, namely Bid and cleaved caspase-8.

The results of my thesis provide exciting new directions to take melanoma therapy. Further elucidation of the pathways and mechanisms involved in treatment response will aid in effective implementation of my proposed treatment strategies in a clinical setting.

References

Aguirre-Ghiso JA, Estrada Y, Liu D, Ossowski L (2003) ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). Cancer Res 63:1684-1695.

Akgul C (2009) Mcl-1 is a potential therapeutic target in multiple types of cancer. Cell Mol Life Sci 66:1326-1336.

Alexaki VI, Javelaud D, Mauviel A (2008) JNK supports survival in melanoma cells by controlling cell cycle arrest and apoptosis. Pigment Cell Melanoma Res 21:429-438.

Amarzguioui M, Lundberg P, Cantin E, Hagstrom J, Behlke MA, Rossi JJ (2006) Rational design and in vitro and in vivo delivery of Dicer substrate siRNA. Nat Protoc 1:508-517.

Balakrishnan K, Wierda WG, Keating MJ, Gandhi V (2008) Gossypol, a BH3 mimetic, induces apoptosis in chronic lymphocytic leukemia cells. Blood 112:1971-1980.

Barth A, Wanek LA, Morton DL (1995) Prognostic factors in 1,521 melanoma patients with distant metastases. J Am Coll Surg 181:193-201.

Bedikian AY, Millward M, Pehamberger H, Conry R, Gore M, Trefzer U, Pavlick AC, DeConti R, Hersh EM, Hersey P, Kirkwood JM, Haluska FG (2006) Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. J Clin Oncol 24:4738-4745.

Behrens A, Sibilia M, Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. Nat Genet 21:326-329.

Bingle CD, Craig RW, Swales BM, Singleton V, Zhou P, Whyte MK (2000) Exon skipping in Mcl-1 results in a bcl-2 homology domain 3 only gene product that promotes cell death. J Biol Chem 275:22136-22146.

Bode AM, Dong Z (2007) The functional contrariety of JNK. Mol Carcinog 46:591-598.

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597-608.

Boucher MJ, Morisset J, Vachon PH, Reed JC, Laine J, Rivard N (2000) MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1 and promotes survival of human pancreatic cancer cells. J Cell Biochem 79:355-369.

Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S, Smulson M (1999) Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem 274:22932-22940.

Bowen AR, Hanks AN, Allen SM, Alexander A, Diedrich MJ, Grossman D (2003) Apoptosis regulators and responses in human melanocytic and keratinocytic cells. J Invest Dermatol 120:48-55.

Bradbury PA, Middleton MR (2004) DNA repair pathways in drug resistance in melanoma. Anticancer Drugs 15:421-426.

Bullani RR, Huard B, Viard-Leveugle I, Byers HR, Irmler M, Saurat JH, Tschopp J, French LE (2001) Selective expression of FLIP in malignant melanocytic skin lesions. J Invest Dermatol 117:360-364.

Bullani RR, Wehrli P, Viard-Leveugle I, Rimoldi D, Cerottini JC, Saurat JH, Tschopp J, French LE (2002) Frequent downregulation of Fas (CD95) expression and function in melanoma. Melanoma Res 12:263-270.

Burnette BL, Selness S, Devraj R, Jungbluth G, Kurumbail R, Stillwell L, Anderson G, Mnich S, Hirsch J, Compton R, De Ciechi P, Hope H, Hepperle M, Keith RH, Naing W, Shieh H, Portanova J, Zhang Y, Zhang J, Leimgruber RM, Monahan J (2009) SD0006: a potent, selective and orally available inhibitor of p38 kinase. Pharmacology 84:42-60.

Bush JA, Li G (2003) The role of Bcl-2 family members in the progression of cutaneous melanoma. Clin Exp Metastasis 20:531-539.

Callus BA, Moujallad DM, Silke J, Gerl R, Jabbour AM, Ekert PG, Vaux DL (2008) Triggering of apoptosis by Puma is determined by the threshold set by prosurvival Bcl-2 family proteins. J Mol Biol 384:313-323.

Chattopadhyay A, Chiang CW, Yang E (2001) BAD/BCL-[X(L)] heterodimerization leads to bypass of G0/G1 arrest. Oncogene 20:4507-4518.

Chauhan D, Velankar M, Brahmandam M, Hideshima T, Podar K, Richardson P, Schlossman R, Ghobrial I, Raje N, Munshi N, Anderson KC (2007) A

novel Bcl-2/Bcl-X(L)/Bcl-w inhibitor ABT-737 as therapy in multiple myeloma. Oncogene 26:2374-2380.

Chen D, Zhou Q (2004) Caspase cleavage of BimEL triggers a positive feedback amplification of apoptotic signaling. Proc Natl Acad Sci U S A 101:1235-1240.

Chen KG, Valencia JC, Gillet JP, Hearing VJ, Gottesman MM (2009a) Involvement of ABC transporters in melanogenesis and the development of multidrug resistance of melanoma. Pigment Cell Melanoma Res.

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, 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 N, Gong J, Chen X, Meng W, Huang Y, Zhao F, Wang L, Zhou Q (2009b) Caspases and inhibitor of apoptosis proteins in cutaneous and mucosal melanoma: expression profile and clinicopathologic significance. Hum Pathol 40:950-956.

Chen S, Dai Y, Harada H, Dent P, Grant S (2007) Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. Cancer Res 67:782-791.

Chudnovsky Y, Khavari PA, Adams AE (2005) Melanoma genetics and the development of rational therapeutics. J Clin Invest 115:813-824.

Clohessy JG, Zhuang J, de Boer J, Gil-Gomez G, Brady HJ (2006) Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c

release and its role in receptor-mediated apoptosis. J Biol Chem 281:5750-5759.

Cohen C, Zavala-Pompa A, Sequeira JH, Shoji M, Sexton DG, Cotsonis G, Cerimele F, Govindarajan B, Macaron N, Arbiser JL (2002) Mitogen-actived protein kinase activation is an early event in melanoma progression. Clin Cancer Res 8:3728-3733.

Collisson EA, De A, Suzuki H, Gambhir SS, Kolodney MS (2003) Treatment of metastatic melanoma with an orally available inhibitor of the Ras-Raf-MAPK cascade. Cancer Res 63:5669-5673.

Condorelli F, Salomoni P, Cotteret S, Cesi V, Srinivasula SM, Alnemri ES, Calabretta B (2001) Caspase cleavage enhances the apoptosis-inducing effects of BAD. Mol Cell Biol 21:3025-3036.

Cragg MS, Jansen ES, Cook M, Harris C, Strasser A, Scott CL (2008) Treatment of B-RAF mutant human tumor cells with a MEK inhibitor requires Bim and is enhanced by a BH3 mimetic. J Clin Invest 118:3651-3659.

Cregan SP, Dawson VL, Slack RS (2004a) Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 23:2785-2796.

Cregan SP, Arbour NA, Maclaurin JG, Callaghan SM, Fortin A, Cheung EC, Guberman DS, Park DS, Slack RS (2004b) p53 activation domain 1 is essential for PUMA upregulation and p53-mediated neuronal cell death. J Neurosci 24:10003-10012.

Cummins DL, Cummins JM, Pantle H, Silverman MA, Leonard AL, Chanmugam A (2006) Cutaneous malignant melanoma. Mayo Clin Proc 81:500-507.

Dai DL, Martinka M, Li G (2005) Prognostic significance of activated Akt expression in melanoma: a clinicopathologic study of 292 cases. J Clin Oncol 23:1473-1482.

Dai DL, Martinka M, Bush JA, Li G (2004) Reduced Apaf-1 expression in human cutaneous melanomas. Br J Cancer 91:1089-1095.

Dai DL, Wang Y, Liu M, Martinka M, Li G (2008) Bim expression is reduced in human cutaneous melanomas. J Invest Dermatol 128:403-407.

Danial NN (2007) BCL-2 family proteins: critical checkpoints of apoptotic cell death. Clin Cancer Res 13:7254-7263.

Danial NN, Gramm CF, Scorrano L, Zhang CY, Krauss S, Ranger AM, Datta SR, Greenberg ME, Licklider LJ, Lowell BB, Gygi SP, Korsmeyer SJ (2003) BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. Nature 424:952-956.

Danial NN, Walensky LD, Zhang CY, Choi CS, Fisher JK, Molina AJ, Datta SR, Pitter KL, Bird GH, Wikstrom JD, Deeney JT, Robertson K, Morash J, Kulkarni A, Neschen S, Kim S, Greenberg ME, Corkey BE, Shirihai OS, Shulman GI, Lowell BB, Korsmeyer SJ (2008) Dual role of proapoptotic BAD in insulin secretion and beta cell survival. Nat Med 14:144-153.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K,

Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA (2002) Mutations of the BRAF gene in human cancer. Nature 417:949-954.

de Vries JF, Wammes LJ, Jedema I, van Dreunen L, Nijmeijer BA, Heemskerk MH, Willemze R, Falkenburg JH, Barge RM (2007) Involvement of caspase-8 in chemotherapy-induced apoptosis of patient derived leukemia cell lines independent of the death receptor pathway and downstream from mitochondria. Apoptosis 12:181-193.

Debatin KM, Krammer PH (2004) Death receptors in chemotherapy and cancer. Oncogene 23:2950-2966.

Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A (2007) Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. J Clin Invest 117:112-121.

Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 76:1025-1037.

Dhomen N, Marais R (2009) BRAF signaling and targeted therapies in melanoma. Hematol Oncol Clin North Am 23:529-545, ix.

Dorsett Y, Tuschl T (2004) siRNAs: applications in functional genomics and potential as therapeutics. Nat Rev Drug Discov 3:318-329.

Eberle J, Kurbanov BM, Hossini AM, Trefzer U, Fecker LF (2007) Overcoming apoptosis deficiency of melanoma-hope for new therapeutic approaches. Drug Resist Updat 10:218-234.

Eferl R, Wagner EF (2003) AP-1: a double-edged sword in tumorigenesis. Nat Rev Cancer 3:859-868.

Eisen T, Ahmad T, Flaherty KT, Gore M, Kaye S, Marais R, Gibbens I, Hackett S, James M, Schuchter LM, Nathanson KL, Xia C, Simantov R, Schwartz B, Poulin-Costello M, O'Dwyer PJ, Ratain MJ (2006) Sorafenib in advanced melanoma: a Phase II randomised discontinuation trial analysis. Br J Cancer 95:581-586.

Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495-516.

Emery CM, Vijayendran KG, Zipser MC, Sawyer AM, Niu L, Kim JJ, Hatton C, Chopra R, Oberholzer PA, Karpova MB, MacConaill LE, Zhang J, Gray NS, Sellers WR, Dummer R, Garraway LA (2009) MEK1 mutations confer resistance to MEK and B-RAF inhibition. Proc Natl Acad Sci U S A 106:20411-20416.

Estrada Y, Dong J, Ossowski L (2009) Positive crosstalk between ERK and p38 in melanoma stimulates migration and in vivo proliferation. Pigment Cell Melanoma Res 22:66-76.

Fandy TE, Ross DD, Gore SD, Srivastava RK (2007) Flavopiridol synergizes TRAIL cytotoxicity by downregulation of FLIPL. Cancer Chemother Pharmacol 60:313-319.

Fecher LA, Amaravadi RK, Schuchter LM, Flaherty KT (2009) Drug targeting of oncogenic pathways in melanoma. Hematol Oncol Clin North Am 23:599-618, x.

Fecker LF, Geilen CC, Tchernev G, Trefzer U, Assaf C, Kurbanov BM, Schwarz C, Daniel PT, Eberle J (2006) Loss of proapoptotic Bcl-2-related multidomain proteins in primary melanomas is associated with poor prognosis. J Invest Dermatol 126:1366-1371.

Filomenko R, Prevotat L, Rebe C, Cortier M, Jeannin JF, Solary E, Bettaieb A (2006) Caspase-10 involvement in cytotoxic drug-induced apoptosis of tumor cells. Oncogene 25:7635-7645.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806-811.

Fischer U, Janicke RU, Schulze-Osthoff K (2003) Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 10:76-100.

Fischer U, Stroh C, Schulze-Osthoff K (2006) Unique and overlapping substrate specificities of caspase-8 and caspase-10. Oncogene 25:152-159.

Fletcher JI, Huang DC (2008) Controlling the cell death mediators Bax and Bak: puzzles and conundrums. Cell Cycle 7:39-44.

Fletcher JI, Meusburger S, Hawkins CJ, Riglar DT, Lee EF, Fairlie WD, Huang DC, Adams JM (2008) Apoptosis is triggered when prosurvival Bcl-2 proteins cannot restrain Bax. Proc Natl Acad Sci U S A 105:18081-18087.

Fujimoto A, Morita R, Hatta N, Takehara K, Takata M (1999) p16INK4a inactivation is not frequent in uncultured sporadic primary cutaneous melanoma. Oncogene 18:2527-2532.

Fujise K, Zhang D, Liu J, Yeh ET (2000) Regulation of apoptosis and cell cycle progression by MCL1. Differential role of proliferating cell nuclear antigen. J Biol Chem 275:39458-39465.

Gallenne T, Gautier F, Oliver L, Hervouet E, Noel B, Hickman JA, Geneste O, Cartron PF, Vallette FM, Manon S, Juin P (2009) Bax activation by the BH3-only protein Puma promotes cell dependence on antiapoptotic Bcl-2 family members. J Cell Biol 185:279-290.

Gao G, Dou QP (2000) N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. J Cell Biochem 80:53-72.

Gavathiotis E, Suzuki M, Davis ML, Pitter K, Bird GH, Katz SG, Tu HC, Kim H, Cheng EH, Tjandra N, Walensky LD (2008) BAX activation is initiated at a novel interaction site. Nature 455:1076-1081.

Gilchrest BA, Eller MS, Geller AC, Yaar M (1999) The pathogenesis of melanoma induced by ultraviolet radiation. N Engl J Med 340:1341-1348.

Gollob JA, Sciambi CJ, Peterson BL, Richmond T, Thoreson M, Moran K, Dressman HK, Jelinek J, Issa JP (2006) Phase I trial of sequential low-dose 5-aza-2'-deoxycytidine plus high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma. Clin Cancer Res 12:4619-4627.

Gong J, Chen N, Zhou Q, Yang B, Wang Y, Wang X (2005) Melanoma inhibitor of apoptosis protein is expressed differentially in melanoma and melanocytic naevus, but similarly in primary and metastatic melanomas. J Clin Pathol 58:1081-1085.

Gonzalez-Garcia M, Perez-Ballestero R, Ding L, Duan L, Boise LH, Thompson CB, Nunez G (1994) bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. Development 120:3033-3042.

Grad JM, Zeng XR, Boise LH (2000) Regulation of Bcl-xL: a little bit of this and a little bit of STAT. Curr Opin Oncol 12:543-549.

Gray-Schopfer V, Wellbrock C, Marais R (2007) Melanoma biology and new targeted therapy. Nature 445:851-857.

Green MM, Hutchison GJ, Valentine HR, Fitzmaurice RJ, Davidson SE, Hunter RD, Dive C, West CM, Stratford IJ (2005) Expression of the proapoptotic protein Bid is an adverse prognostic factor for radiotherapy outcome in carcinoma of the cervix. Br J Cancer 92:449-458.

Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 274:1156-1163.

Grossman D, Altieri DC (2001) Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. Cancer Metastasis Rev 20:3-11.

Guicciardi ME, Gores GJ (2009) Life and death by death receptors. Faseb J 23:1625-1637.

Gupta PB, Kuperwasser C, Brunet JP, Ramaswamy S, Kuo WL, Gray JW, Naber SP, Weinberg RA (2005) The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. Nat Genet 37:1047-1054.

Gurzov EN, Bakiri L, Alfaro JM, Wagner EF, Izquierdo M (2008) Targeting c-Jun and JunB proteins as potential anticancer cell therapy. Oncogene 27:641-652.

Hacker G (2000) The morphology of apoptosis. Cell Tissue Res 301:5-17.

Harada H, Quearry B, Ruiz-Vela A, Korsmeyer SJ (2004) Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. Proc Natl Acad Sci U S A 101:15313-15317.

Hess J, Angel P, Schorpp-Kistner M (2004) AP-1 subunits: quarrel and harmony among siblings. J Cell Sci 117:5965-5973.

Hideshima T, Podar K, Chauhan D, Ishitsuka K, Mitsiades C, Tai YT, Hamasaki M, Raje N, Hideshima H, Schreiner G, Nguyen AN, Navas T, Munshi NC, Richardson PG, Higgins LS, Anderson KC (2004) p38 MAPK inhibition

enhances PS-341 (bortezomib)-induced cytotoxicity against multiple myeloma cells. Oncogene 23:8766-8776.

Hill MM, Adrain C, Duriez PJ, Creagh EM, Martin SJ (2004) Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. Embo J 23:2134-2145.

Hocker TL, Singh MK, Tsao H (2008) Melanoma genetics and therapeutic approaches in the 21st century: moving from the benchside to the bedside. J Invest Dermatol 128:2575-2595.

Howard AN, Bridges KA, Meyn RE, Chandra J (2009) ABT-737, a BH3 mimetic, induces glutathione depletion and oxidative stress. Cancer Chemother Pharmacol 65:41-54.

Huang S, Sinicrope FA (2008) BH3 mimetic ABT-737 potentiates TRAIL-mediated apoptotic signaling by unsequestering Bim and Bak in human pancreatic cancer cells. Cancer Res 68:2944-2951.

Huitema AD, Smits KD, Mathot RA, Schellens JH, Rodenhuis S, Beijnen JH (2000) The clinical pharmacology of alkylating agents in high-dose chemotherapy. Anticancer Drugs 11:515-533.

Hussussian CJ, Struewing JP, Goldstein AM, Higgins PA, Ally DS, Sheahan MD, Clark WH, Jr., Tucker MA, Dracopoli NC (1994) Germline p16 mutations in familial melanoma. Nat Genet 8:15-21.

Jamil S, Mojtabavi S, Hojabrpour P, Cheah S, Duronio V (2008) An essential role for MCL-1 in ATR-mediated CHK1 phosphorylation. Mol Biol Cell 19:3212-3220.

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

Jensen EH, Lewis JM, McLoughlin JM, Alvarado MD, Daud A, Messina J, Enkemann S, Yeatman TJ, Sondak VK, Riker AI (2007) Down-regulation of pro-apoptotic genes is an early event in the progression of malignant melanoma. Ann Surg Oncol 14:1416-1423.

Jiang CC, Wroblewski D, Yang F, Hersey P, Zhang XD (2009) Human melanoma cells under endoplasmic reticulum stress are more susceptible to apoptosis induced by the BH3 mimetic obatoclax. Neoplasia 11:945-955.

Jourdain A, Martinou JC (2009) Mitochondrial outer-membrane permeabilization and remodelling in apoptosis. Int J Biochem Cell Biol 41:1884-1889.

Kamb A, Shattuck-Eidens D, Eeles R, Liu Q, Gruis NA, Ding W, Hussey C, Tran T, Miki Y, Weaver-Feldhaus J, et al. (1994) Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. Nat Genet 8:23-26.

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

Kang MH, Reynolds CP (2009) Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. Clin Cancer Res 15:1126-1132.

Karst AM, Dai DL, Martinka M, Li G (2005) PUMA expression is significantly reduced in human cutaneous melanomas. Oncogene 24:1111-1116.

Keogh RJ, Harris LK, Freeman A, Baker PN, Aplin JD, Whitley GS, Cartwright JE (2007) Fetal-derived trophoblast use the apoptotic cytokine tumor necrosis factor-alpha-related apoptosis-inducing ligand to induce smooth muscle cell death. Circ Res 100:834-841.

Kihara C, Tsunoda T, Tanaka T, Yamana H, Furukawa Y, Ono K, Kitahara O, Zembutsu H, Yanagawa R, Hirata K, Takagi T, Nakamura Y (2001) Prediction of sensitivity of esophageal tumors to adjuvant chemotherapy by cDNA microarray analysis of gene-expression profiles. Cancer Res 61:6474-6479.

Kim DH, Rossi JJ (2007) Strategies for silencing human disease using RNA interference. Nat Rev Genet 8:173-184.

Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ (2005) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol 23:222-226.

Kim JH, Kim TH, Kang HS, Ro J, Kim HS, Yoon S (2009) SP600125, an inhibitor of Jnk pathway, reduces viability of relatively resistant cancer cells to doxorubicin. Biochem Biophys Res Commun 387:450-455.

Kim R, Emi M, Matsuura K, Tanabe K (2007) Antisense and nonantisense effects of antisense Bcl-2 on multiple roles of Bcl-2 as a chemosensitizer in cancer therapy. Cancer Gene Ther 14:1-11.

Kitada S, Kress CL, Krajewska M, Jia L, Pellecchia M, Reed JC (2008) Bcl-2 antagonist apogossypol (NSC736630) displays single-agent activity in Bcl-2-transgenic mice and has superior efficacy with less toxicity compared with gossypol (NSC19048). Blood 111:3211-3219.

Kline MP, Rajkumar SV, Timm MM, Kimlinger TK, Haug JL, Lust JA, Greipp PR, Kumar S (2007) ABT-737, an inhibitor of Bcl-2 family proteins, is a potent inducer of apoptosis in multiple myeloma cells. Leukemia 21:1549-1560.

Kline MP, Rajkumar SV, Timm MM, Kimlinger TK, Haug JL, Lust JA, Greipp PR, Kumar S (2008) R-(-)-gossypol (AT-101) activates programmed cell death in multiple myeloma cells. Exp Hematol 36:568-576.

Kluger HM, McCarthy MM, Alvero AB, Sznol M, Ariyan S, Camp RL, Rimm DL, Mor G (2007) The X-linked inhibitor of apoptosis protein (XIAP) is up-regulated in metastatic melanoma, and XIAP cleavage by Phenoxodiol is associated with Carboplatin sensitization. J Transl Med 5:6.

Konopleva M, Contractor R, Tsao T, Samudio I, Ruvolo PP, Kitada S, Deng X, Zhai D, Shi YX, Sneed T, Verhaegen M, Soengas M, Ruvolo VR, McQueen T, Schober WD, Watt JC, Jiffar T, Ling X, Marini FC, Harris D, Dietrich M, Estrov Z, McCubrey J, May WS, Reed JC, Andreeff M (2006) Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. Cancer Cell 10:375-388.

Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC (1993) Investigation of the subcellular distribution of the bcl-2 oncoprotein:

residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res 53:4701-4714.

Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD (2005) BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol Cell 17:525-535.

Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 111:331-342.

Lasithiotakis KG, Sinnberg TW, Schittek B, Flaherty KT, Kulms D, Maczey E, Garbe C, Meier FE (2008) Combined inhibition of MAPK and mTOR signaling inhibits growth, induces cell death, and abrogates invasive growth of melanoma cells. J Invest Dermatol 128:2013-2023.

Leiter U, Schmid RM, Kaskel P, Peter RU, Krahn G (2000) Antiapoptotic bcl-2 and bcl-xL in advanced malignant melanoma. Arch Dermatol Res 292:225-232.

Lessene G, Czabotar PE, Colman PM (2008) BCL-2 family antagonists for cancer therapy. Nat Rev Drug Discov 7:989-1000.

Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2:183-192.

Li C, Zhao H, Hu Z, Liu Z, Wang LE, Gershenwald JE, Prieto VG, Lee JE, Duvic M, Grimm EA, Wei Q (2008a) Genetic variants and haplotypes of the caspase-8 and caspase-10 genes contribute to susceptibility to cutaneous melanoma. Hum Mutat.

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

Li J, Viallet J, Haura EB (2008b) A small molecule pan-Bcl-2 family inhibitor, GX15-070, induces apoptosis and enhances cisplatin-induced apoptosis in non-small cell lung cancer cells. Cancer Chemother Pharmacol 61:525-534.

Lim SJ, Lee YJ, Lee E (2006) p38MAPK inhibitor SB203580 sensitizes human SNU-C4 colon cancer cells to exisulind-induced apoptosis. Oncol Rep 16:1131-1135.

Lin X, Morgan-Lappe S, Huang X, Li L, Zakula DM, Vernetti LA, Fesik SW, Shen Y (2007) 'Seed' analysis of off-target siRNAs reveals an essential role of Mcl-1 in resistance to the small-molecule Bcl-2/Bcl-XL inhibitor ABT-737. Oncogene 26:3972-3979.

Liu G, Kelly WK, Wilding G, Leopold L, Brill K, Somer B (2009) An open-label, multicenter, phase I/II study of single-agent AT-101 in men with castrate-resistant prostate cancer. Clin Cancer Res 15:3172-3176.

Liu T, Brouha B, Grossman D (2004) Rapid induction of mitochondrial events and caspase-independent apoptosis in Survivin-targeted melanoma cells. Oncogene 23:39-48.

Locksley RM, Killeen N, Lenardo MJ (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104:487-501.

Lomas J, Martin-Duque P, Pons M, Quintanilla M (2008) The genetics of malignant melanoma. Front Biosci 13:5071-5093.

Lomonosova E, Chinnadurai G (2008) BH3-only proteins in apoptosis and beyond: an overview. Oncogene 27 Suppl 1:S2-19.

Lopez-Bergami P, Huang C, Goydos JS, Yip D, Bar-Eli M, Herlyn M, Smalley KS, Mahale A, Eroshkin A, Aaronson S, Ronai Z (2007) Rewired ERK-JNK signaling pathways in melanoma. Cancer Cell 11:447-460.

Lorigan P, Eisen T, Hauschild A (2008) Systemic therapy for metastatic malignant melanoma--from deeply disappointing to bright future? Exp Dermatol 17:383-394.

Luce A, Courtin A, Levalois C, Altmeyer-Morel S, Romeo PH, Chevillard S, Lebeau J (2009) Death receptor pathways mediate targeted and non-targeted effects of ionizing radiations in breast cancer cells. Carcinogenesis 30:432-439.

Luo X, Budihardjo I, Zou H, Slaughter C, 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.

Mahalingam D, Szegezdi E, Keane M, Jong S, Samali A (2009) TRAIL receptor signalling and modulation: Are we on the right TRAIL? Cancer Treat Rev 35:280-288.

Mahmoudi S, Henriksson S, Corcoran M, Mendez-Vidal C, Wiman KG, Farnebo M (2009) Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage. Mol Cell 33:462-471.

Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, Juin P, Tasdemir E, Pierron G, Troulinaki K, Tavernarakis N, Hickman JA, Geneste O, Kroemer G (2007) Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. Embo J 26:2527-2539.

Malemud CJ (2007) Inhibitors of stress-activated protein/mitogen-activated protein kinase pathways. Curr Opin Pharmacol 7:339-343.

Mandruzzato S, Callegaro A, Turcatel G, Francescato S, Montesco MC, Chiarion-Sileni V, Mocellin S, Rossi CR, Bicciato S, Wang E, Marincola FM, Zanovello P (2006) A gene expression signature associated with survival in metastatic melanoma. J Transl Med 4:50.

Margolin K, Longmate J, Baratta T, Synold T, Christensen S, Weber J, Gajewski T, Quirt I, Doroshow JH (2005) CCI-779 in metastatic melanoma: a phase II trial of the California Cancer Consortium. Cancer 104:1045-1048.

Markovic SN, Geyer SM, Dawkins F, Sharfman W, Albertini M, Maples W, Fracasso PM, Fitch T, Lorusso P, Adjei AA, Erlichman C (2005) A phase II study of bortezomib in the treatment of metastatic malignant melanoma. Cancer 103:2584-2589.

Martinez-Caballero S, Dejean LM, Kinnally MS, Oh KJ, Mannella CA, Kinnally KW (2009) Assembly of the mitochondrial apoptosis-induced channel, MAC. J Biol Chem 284:12235-12245.

McCubrey JA, Lahair MM, Franklin RA (2006) Reactive oxygen species-induced activation of the MAP kinase signaling pathways. Antioxid Redox Signal 8:1775-1789.

McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK, Lin YL, Ramaswamy S, Avery W, Ding HF, Jordan SA, Jackson IJ, Korsmeyer SJ, Golub TR, Fisher DE (2002) Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell 109:707-718.

Meng J, Peng H, Dai B, Guo W, Wang L, Ji L, Minna JD, Chresta CM, Smith PD, Fang B, Roth JA (2009) High level of AKT activity is associated with resistance to MEK inhibitor AZD6244 (ARRY-142886). Cancer Biol Ther 8:2073-2080.

Michels J, Johnson PW, Packham G (2005) Mcl-1. Int J Biochem Cell Biol 37:267-271.

Milhas D, Cuvillier O, Therville N, Clave P, Thomsen M, Levade T, Benoist H, Segui B (2005) Caspase-10 triggers Bid cleavage and caspase cascade activation in FasL-induced apoptosis. J Biol Chem 280:19836-19842.

Miller LA, Goldstein NB, Johannes WU, Walton CH, Fujita M, Norris DA, Shellman YG (2008) BH3 Mimetic ABT-737 and a Proteasome Inhibitor Synergistically Kill Melanomas through Noxa-Dependent Apoptosis. J Invest Dermatol.

Molhoek KR, Brautigan DL, Slingluff CL, Jr. (2005) Synergistic inhibition of human melanoma proliferation by combination treatment with B-Raf

inhibitor BAY43-9006 and mTOR inhibitor Rapamycin. J Transl Med 3:39.

Mouawad R, Sebert M, Michels J, Bloch J, Spano JP, Khayat D (2009) Treatment for metastatic malignant melanoma: Old drugs and new strategies. Crit Rev Oncol Hematol.

Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, Ng SL, Fesik SW (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature 381:335-341.

Mustika R, Budiyanto A, Nishigori C, Ichihashi M, Ueda M (2005) Decreased expression of Apaf-1 with progression of melanoma. Pigment Cell Res 18:59-62.

Nachmias B, Ashhab Y, Bucholtz V, Drize O, Kadouri L, Lotem M, Peretz T, Mandelboim O, Ben-Yehuda D (2003) Caspase-mediated cleavage converts Livin from an antiapoptotic to a proapoptotic factor: implications for drug-resistant melanoma. Cancer Res 63:6340-6349.

Nakano K, Vousden KH (2001) PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 7:683-694.

Navas TA, Nguyen AN, Hideshima T, Reddy M, Ma JY, Haghnazari E, Henson M, Stebbins EG, Kerr I, O'Young G, Kapoun AM, Chakravarty S, Mavunkel B, Perumattam J, Luedtke G, Dugar S, Medicherla S, Protter AA, Schreiner GF, Anderson KC, Higgins LS (2006) Inhibition of p38alpha MAPK enhances proteasome inhibitor-induced apoptosis of

myeloma cells by modulating Hsp27, Bcl-X(L), Mcl-1 and p53 levels in vitro and inhibits tumor growth in vivo. Leukemia 20:1017-1027.

Ngan CY, Yamamoto H, Takagi A, Fujie Y, Takemasa I, Ikeda M, Takahashi-Yanaga F, Sasaguri T, Sekimoto M, Matsuura N, Monden M (2008) Oxaliplatin induces mitotic catastrophe and apoptosis in esophageal cancer cells. Cancer Sci 99:129-139.

Nguyen M, Marcellus RC, Roulston A, Watson M, Serfass L, Murthy Madiraju SR, Goulet D, Viallet J, Belec L, Billot X, Acoca S, Purisima E, Wiegmans A, Cluse L, Johnstone RW, Beauparlant P, Shore GC (2007) Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. Proc Natl Acad Sci U S A 104:19512-19517.

Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F, Wang X (2003) Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev 17:1475-1486.

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 288:1053-1058.

Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen

W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 435:677-681.

Otter I, Conus S, Ravn U, Rager M, Olivier R, Monney L, Fabbro D, Borner C (1998) The binding properties and biological activities of Bcl-2 and Bax in cells exposed to apoptotic stimuli. J Biol Chem 273:6110-6120.

Paoluzzi L, Gonen M, Gardner JR, Mastrella J, Yang D, Holmlund J, Sorensen M, Leopold L, Manova K, Marcucci G, Heaney ML, O'Connor OA (2008) Targeting Bcl-2 family members with the BH3 mimetic AT-101 markedly enhances the therapeutic effects of chemotherapeutic agents in in vitro and in vivo models of B-cell lymphoma. Blood 111:5350-5358.

Payette MJ, Katz M, 3rd, Grant-Kels JM (2009) Melanoma prognostic factors found in the dermatopathology report. Clin Dermatol 27:53-74.

Pegoraro L, Palumbo A, Erikson J, Falda M, Giovanazzo B, Emanuel BS, Rovera G, Nowell PC, Croce CM (1984) A 14;18 and an 8;14 chromosome translocation in a cell line derived from an acute B-cell leukemia. Proc Natl Acad Sci U S A 81:7166-7170.

Plettenberg A, Ballaun C, Pammer J, Mildner M, Strunk D, Weninger W, Tschachler E (1995) Human melanocytes and melanoma cells constitutively express the Bcl-2 proto-oncogene in situ and in cell culture. Am J Pathol 146:651-659.

Ploski JE, Aplan PD (2001) Characterization of DNA fragmentation events caused by genotoxic and non-genotoxic agents. Mutat Res 473:169-180.

Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR (1991) Phosphorylation of c-jun mediated by MAP kinases. Nature 353:670-674.

Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell 3:287-296.

Qin JZ, Xin H, Sitailo LA, Denning MF, Nickoloff BJ (2006) Enhanced killing of melanoma cells by simultaneously targeting Mcl-1 and NOXA. Cancer Res 66:9636-9645.

Qin JZ, Ziffra J, Stennett L, Bodner B, Bonish BK, Chaturvedi V, Bennett F, Pollock PM, Trent JM, Hendrix MJ, Rizzo P, Miele L, Nickoloff BJ (2005) Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. Cancer Res 65:6282-6293.

Rao RD, Windschitl HE, Allred JB, Lowe VJ, Maples WJ, Gornet MK (2006) Phase II trial of mTOR inhibitor everolimus (RAD001) in metastatic melanoma. Journal of Clinical Oncology 24.

Reed JC (2006) Proapoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, and therapeutic opportunities. Cell Death Differ 13:1378-1386.

Russo AE, Torrisi E, Bevelacqua Y, Perrotta R, Libra M, McCubrey JA, Spandidos DA, Stivala F, Malaponte G (2009) Melanoma: molecular pathogenesis and emerging target therapies (Review). Int J Oncol 34:1481-1489.

Ruvolo PP, Deng X, May WS (2001) Phosphorylation of Bcl2 and regulation of apoptosis. Leukemia 15:515-522.

Schimmer AD, O'Brien S, Kantarjian H, Brandwein J, Cheson BD, Minden MD, Yee K, Ravandi F, Giles F, Schuh A, Gupta V, Andreeff M, Koller C, Chang H, Kamel-Reid S, Berger M, Viallet J, Borthakur G (2008) A phase I study of the pan bcl-2 family inhibitor obatoclax mesylate in patients with advanced hematologic malignancies. Clin Cancer Res 14:8295-8301.

Sekulic A, Haluska P, Jr., Miller AJ, Genebriera De Lamo J, Ejadi S, Pulido JS, Salomao DR, Thorland EC, Vile RG, Swanson DL, Pockaj BA, Laman SD, Pittelkow MR, Markovic SN (2008) Malignant melanoma in the 21st century: the emerging molecular landscape. Mayo Clin Proc 83:825-846.

Selzer E, Schlagbauer-Wadl H, Okamoto I, Pehamberger H, Potter R, Jansen B (1998) Expression of Bcl-2 family members in human melanocytes, in melanoma metastases and in melanoma cell lines. Melanoma Res 8:197-203.

Simmons MJ, Fan G, Zong WX, Degenhardt K, White E, Gelinas C (2008) Bfl-1/A1 functions, similar to Mcl-1, as a selective tBid and Bak antagonist. Oncogene 27:1421-1428.

Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR (2003) Activation of the interferon system by short-interfering RNAs. Nat Cell Biol 5:834-839.

Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, Martin SJ (1999)

Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol 144:281-292.

Slinker BK (1998) The statistics of synergism. J Mol Cell Cardiol 30:723-731.

Soengas MS, Lowe SW (2003) Apoptosis and melanoma chemoresistance. Oncogene 22:3138-3151.

Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A, Ye Q, Lobo JM, She Y, Osman I, Golub TR, Sebolt-Leopold J, Sellers WR, Rosen N (2006) BRAF mutation predicts sensitivity to MEK inhibition. Nature 439:358-362.

Song JH, Kandasamy K, Kraft AS (2008) ABT-737 induces expression of the death receptor 5 and sensitizes human cancer cells to TRAIL-induced apoptosis. J Biol Chem 283:25003-25013.

Stein JA, Brownell I (2008) Treatment approaches for advanced cutaneous melanoma. J Drugs Dermatol 7:175-179.

Sun T, Gao Y, Tan W, Ma S, Shi Y, Yao J, Guo Y, Yang M, Zhang X, Zhang Q, Zeng C, Lin D (2007) A six-nucleotide insertion-deletion polymorphism in the CASP8 promoter is associated with susceptibility to multiple cancers. Nat Genet 39:605-613.

Sun Y, Sinicrope FA (2005) Selective inhibitors of MEK1/ERK44/42 and p38 mitogen-activated protein kinases potentiate apoptosis induction by sulindac sulfide in human colon carcinoma cells. Mol Cancer Ther 4:51-59.

Sutheesophon K, Nishimura N, Kobayashi Y, Furukawa Y, Kawano M, Itoh K, Kano Y, Ishii H, Furukawa Y (2005) Involvement of the tumor necrosis factor (TNF)/TNF receptor system in leukemic cell apoptosis induced by histone deacetylase inhibitor depsipeptide (FK228). J Cell Physiol 203:387-397.

Tagscherer KE, Fassl A, Campos B, Farhadi M, Kraemer A, Bock BC, Macher-Goeppinger S, Radlwimmer B, Wiestler OD, Herold-Mende C, Roth W (2008) Apoptosis-based treatment of glioblastomas with ABT-737, a novel small molecule inhibitor of Bcl-2 family proteins. Oncogene.

Tahir SK, Yang X, Anderson MG, Morgan-Lappe SE, Sarthy AV, Chen J, Warner RB, Ng SC, Fesik SW, Elmore SW, Rosenberg SH, Tse C (2007) Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. Cancer Res 67:1176-1183.

Takeuchi H, Morton DL, Elashoff D, Hoon DS (2005) Survivin expression by metastatic melanoma predicts poor disease outcome in patients receiving adjuvant polyvalent vaccine. Int J Cancer 117:1032-1038.

Tan C, Dlugosz PJ, Peng J, Zhang Z, Lapolla SM, Plafker SM, Andrews DW, Lin J (2006) Auto-activation of the apoptosis protein Bax increases mitochondrial membrane permeability and is inhibited by Bcl-2. J Biol Chem 281:14764-14775.

Tang D, Lahti JM, Kidd VJ (2000) Caspase-8 activation and bid cleavage contribute to MCF7 cellular execution in a caspase-3-dependent manner during staurosporine-mediated apoptosis. J Biol Chem 275:9303-9307.

Tang L, Tron VA, Reed JC, Mah KJ, Krajewska M, Li G, Zhou X, Ho VC, Trotter MJ (1998) Expression of apoptosis regulators in cutaneous malignant melanoma. Clin Cancer Res 4:1865-1871.

Tchernev G, Orfanos CE (2007) Downregulation of cell cycle modulators p21, p27, p53, Rb and proapoptotic Bcl-2-related proteins Bax and Bak in cutaneous melanoma is associated with worse patient prognosis: preliminary findings. J Cutan Pathol 34:247-256.

Testori A, Rutkowski P, Marsden J, Bastholt L, Chiarion-Sileni V, Hauschild A, Eggermont AM (2009) Surgery and radiotherapy in the treatment of cutaneous melanoma. Ann Oncol 20 Suppl 6:vi22-29.

Thallinger C, Wolschek MF, Wacheck V, Maierhofer H, Gunsberg P, Polterauer P, Pehamberger H, Monia BP, Selzer E, Wolff K, Jansen B (2003) Mcl-1 antisense therapy chemosensitizes human melanoma in a SCID mouse xenotransplantation model. J Invest Dermatol 120:1081-1086.

Thayaparasingham B, Kunz A, Peters N, Kulms D (2009) Sensitization of melanoma cells to TRAIL by UVB-induced and NF-kappaB-mediated downregulation of xIAP. Oncogene 28:345-362.

Thomadaki H, Scorilas A (2006) BCL2 family of apoptosis-related genes: functions and clinical implications in cancer. Crit Rev Clin Lab Sci 43:1-67.

Thomson S, Mahadevan LC, Clayton AL (1999) MAP kinase-mediated signalling to nucleosomes and immediate-early gene induction. Semin Cell Dev Biol 10:205-214.

Thornton TM, Rincon M (2009) Non-classical p38 map kinase functions: cell cycle checkpoints and survival. Int J Biol Sci 5:44-51.

Tran MA, Watts RJ, Robertson GP (2009) Use of liposomes as drug delivery vehicles for treatment of melanoma. Pigment Cell Melanoma Res 22:388-399.

Trudel S, Li ZH, Rauw J, Tiedemann RE, Wen XY, Stewart AK (2007a) Preclinical studies of the pan-Bcl inhibitor obatoclax (GX015-070) in multiple myeloma. Blood 109:5430-5438.

Trudel S, Stewart AK, Li Z, Shu Y, Liang SB, Trieu Y, Reece D, Paterson J, Wang D, Wen XY (2007b) The Bcl-2 family protein inhibitor, ABT-737, has substantial antimyeloma activity and shows synergistic effect with dexamethasone and melphalan. Clin Cancer Res 13:621-629.

Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, Bremer R, Gillette S, Kong J, Haass NK, Sproesser K, Li L, Smalley KS, Fong D, Zhu YL, Marimuthu A, Nguyen H, Lam B, Liu J, Cheung I, Rice J, Suzuki Y, Luu C, Settachatgul C, Shellooe R, Cantwell J, Kim SH, Schlessinger J, Zhang KY, West BL, Powell B, Habets G, Zhang C, Ibrahim PN, Hirth P, Artis DR, Herlyn M, Bollag G (2008) Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. Proc Natl Acad Sci U S A 105:3041-3046.

Tsao H, Atkins MB, Sober AJ (2004a) Management of cutaneous melanoma. N Engl J Med 351:998-1012.

Tsao H, Goel V, Wu H, Yang G, Haluska FG (2004b) Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma. J Invest Dermatol 122:337-341.

Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, Johnson EF, Marsh KC, Mitten MJ, Nimmer P, Roberts L, Tahir SK, Xiao Y, Yang X, Zhang H, Fesik S, Rosenberg SH, Elmore SW (2008) ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer Res 68:3421-3428.

Tucker MA (2009) Melanoma epidemiology. Hematol Oncol Clin North Am 23:383-395, vii.

Uong A, Zon LI Melanocytes in development and cancer. J Cell Physiol 222:38-41.

Uren RT, Dewson G, Chen L, Coyne SC, Huang DC, Adams JM, Kluck RM (2007) Mitochondrial permeabilization relies on BH3 ligands engaging multiple prosurvival Bcl-2 relatives, not Bak. J Cell Biol 177:277-287.

van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, Willis SN, Scott CL, Day CL, Cory S, Adams JM, Roberts AW, Huang DC (2006) The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 10:389-399.

VanBrocklin MW, Verhaegen M, Soengas MS, Holmen SL (2009) Mitogen-activated protein kinase inhibition induces translocation of Bmf to promote apoptosis in melanoma. Cancer Res 69:1985-1994.

Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, Adams JM, Strasser A (2003) p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science 302:1036-1038.

Vogler M, Weber K, Dinsdale D, Schmitz I, Schulze-Osthoff K, Dyer MJ, Cohen GM (2009) Different forms of cell death induced by putative BCL2 inhibitors. Cell Death Differ 16:1030-1039.

von Haefen C, Wieder T, Essmann F, Schulze-Osthoff K, Dorken B, Daniel PT (2003) Paclitaxel-induced apoptosis in BJAB cells proceeds via a death receptor-independent, caspases-3/-8-driven mitochondrial amplification loop. Oncogene 22:2236-2247.

Wagner EF, Nebreda AR (2009) Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer 9:537-549.

Walensky LD, Pitter K, Morash J, Oh KJ, Barbuto S, Fisher J, Smith E, Verdine GL, Korsmeyer SJ (2006) A stapled BID BH3 helix directly binds and activates BAX. Mol Cell 24:199-210.

Wang H, Tan SS, Wang XY, Liu DH, Yu CS, Bai ZL, He DL, Zhao J (2007a) Silencing livin gene by siRNA leads to apoptosis induction, cell cycle arrest, and proliferation inhibition in malignant melanoma LiBr cells. Acta Pharmacol Sin 28:1968-1974.

Wang YF, Jiang CC, Kiejda KA, Gillespie S, Zhang XD, Hersey P (2007b) Apoptosis induction in human melanoma cells by inhibition of MEK is caspase-independent and mediated by the Bcl-2 family members PUMA, Bim, and Mcl-1. Clin Cancer Res 13:4934-4942.

Weber A, Kirejczyk Z, Potthoff S, Ploner C, Hacker G (2009) Endogenous Noxa Determines the Strong Proapoptotic Synergism of the BH3-Mimetic ABT-737 with Chemotherapeutic Agents in Human Melanoma Cells. Transl Oncol 2:73-83.

Wee LJ, Tan TW, Ranganathan S (2006) SVM-based prediction of caspase substrate cleavage sites. BMC Bioinformatics 7 Suppl 5:S14.

Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev 14:2060-2071.

Wei Y, Fan T, Yu M (2008) Inhibitor of apoptosis proteins and apoptosis. Acta Biochim Biophys Sin (Shanghai) 40:278-288.

Weng C, Li Y, Xu D, Shi Y, Tang H (2005) Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. J Biol Chem 280:10491-10500.

Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujath J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnane L, Lynch M, Auclair D, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post LE, Bollag G, Trail PA (2004) BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 64:7099-7109.

Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM, Huang DC (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 19:1294-1305.

Winnepenninckx V, Lazar V, Michiels S, Dessen P, Stas M, Alonso SR, Avril MF, Ortiz Romero PL, Robert T, Balacescu O, Eggermont AM, Lenoir G, Sarasin A, Tursz T, van den Oord JJ, Spatz A (2006) Gene expression profiling of primary cutaneous melanoma and clinical outcome. J Natl Cancer Inst 98:472-482.

Witham J, Valenti MR, De-Haven-Brandon AK, Vidot S, Eccles SA, Kaye SB, Richardson A (2007) The Bcl-2/Bcl-XL family inhibitor ABT-737 sensitizes ovarian cancer cells to carboplatin. Clin Cancer Res 13:7191-7198.

Wolter KG, Verhaegen M, Fernandez Y, Nikolovska-Coleska Z, Riblett M, de la Vega CM, Wang S, Soengas MS (2007) Therapeutic window for melanoma treatment provided by selective effects of the proteasome on Bcl-2 proteins. Cell Death Differ 14:1605-1616.

Wong HK, Fricker M, Wyttenbach A, Villunger A, Michalak EM, Strasser A, Tolkovsky AM (2005) Mutually exclusive subsets of BH3-only proteins are activated by the p53 and c-Jun N-terminal kinase/c-Jun signaling pathways during cortical neuron apoptosis induced by arsenite. Mol Cell Biol 25:8732-8747.

Wong RP, Khosravi S, Martinka M, Li G (2008) Myeloid leukemia-1 expression in benign and malignant melanocytic lesions. Oncol Rep 19:933-937.

Woods NT, Yamaguchi H, Lee FY, Bhalla KN, Wang HG (2007) Anoikis, initiated by Mcl-1 degradation and Bim induction, is deregulated during oncogenesis. Cancer Res 67:10744-10752.

Wu Y, Mehew JW, Heckman CA, Arcinas M, Boxer LM (2001) Negative regulation of bcl-2 expression by p53 in hematopoietic cells. Oncogene 20:240-251.

Yamagishi S, Yamada M, Ishikawa Y, Matsumoto T, Ikeuchi T, Hatanaka H (2001) p38 mitogen-activated protein kinase regulates low potassium-induced c-Jun phosphorylation and apoptosis in cultured cerebellar granule neurons. J Biol Chem 276:5129-5133.

Yang AS, Chapman PB (2009) The history and future of chemotherapy for melanoma. Hematol Oncol Clin North Am 23:583-597, x.

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

Yoon S, Seger R (2006) The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors 24:21-44.

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

Yu J, Zhang L (2003) No PUMA, no death: implications for p53-dependent apoptosis. Cancer Cell 4:248-249.

Yu J, Zhang L (2008) PUMA, a potent killer with or without p53. Oncogene 27 Suppl 1:S71-83.

Zantl N, Weirich G, Zall H, Seiffert BM, Fischer SF, Kirschnek S, Hartmann C, Fritsch RM, Gillissen B, Daniel PT, Hacker G (2007) Frequent loss of expression of the pro-apoptotic protein Bim in renal cell carcinoma: evidence for contribution to apoptosis resistance. Oncogene 26:7038-7048.

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 87:619-628.

Zhai D, Jin C, Satterthwait AC, Reed JC (2006) Comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins. Cell Death Differ 13:1419-1421.

Zhai D, Jin C, Huang Z, Satterthwait AC, Reed JC (2008) Differential regulation of Bax and Bak by anti-apoptotic Bcl-2 family proteins Bcl-B and Mcl-1. J Biol Chem 283:9580-9586.

Zhai D, Ke N, Zhang H, Ladror U, Joseph M, Eichinger A, Godzik A, Ng SC, Reed JC (2003) Characterization of the anti-apoptotic mechanism of Bcl-B. Biochem J 376:229-236.

Zhang H, Nimmer PM, Tahir SK, Chen J, Fryer RM, Hahn KR, Iciek LA, Morgan SJ, Nasarre MC, Nelson R, Preusser LC, Reinhart GA, Smith ML, Rosenberg SH, Elmore SW, Tse C (2007) Bcl-2 family proteins are essential for platelet survival. Cell Death Differ 14:943-951.

Zhuang L, Lee CS, Scolyer RA, McCarthy SW, Zhang XD, Thompson JF, Hersey P (2007) Mcl-1, Bcl-XL and Stat3 expression are associated with

progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma. Mod Pathol 20:416-426.

Zhuang L, Lee CS, Scolyer RA, McCarthy SW, Zhang XD, Thompson JF, Screaton G, Hersey P (2006) Progression in melanoma is associated with decreased expression of death receptors for tumor necrosis factor-related apoptosis-inducing ligand. Hum Pathol 37:1286-1294.

Zinkel S, Gross A, Yang E (2006) BCL2 family in DNA damage and cell cycle control. Cell Death Differ 13:1351-1359.

Zuo L, Weger J, Yang Q, Goldstein AM, Tucker MA, Walker GJ, Hayward N, Dracopoli NC (1996) Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. Nat Genet 12:97-99.

Appendix I

Data obtained after thesis defense

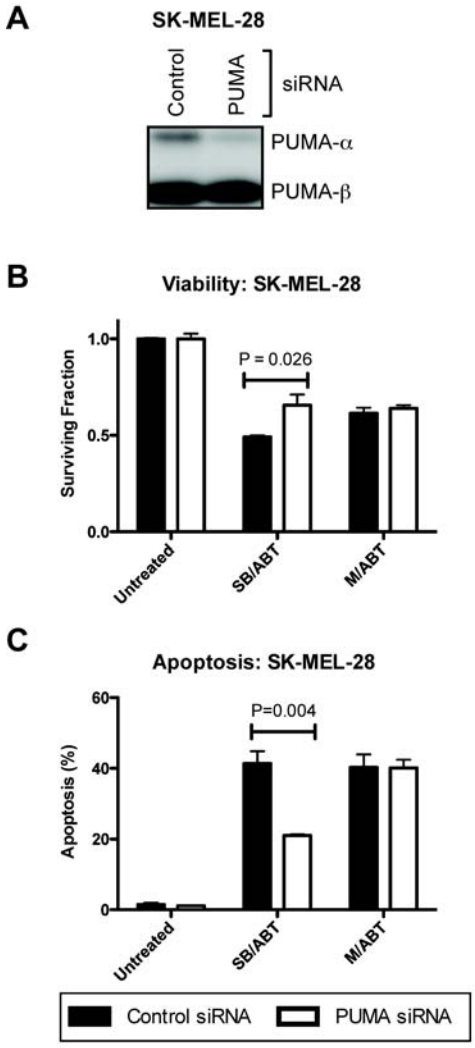

Figure A-1: Effects of knockdown of PUMA-α on treatment response. (A) Western immunoblot demonstrating knockdown of PUMA-α. SK-MEL-28 cells were treated with Scrambled control siRNA (10 nM) or PUMA siRNA (10 nM) for 48 hours. (B) Effects of PUMA knockdown on viability. SK-MEL-28 cells were treated with Scrambled siRNA (10 nM) or PUMA siRNA (10 nM) for 24 hours, then treated with SB/ABT: (SB202190 (10 μM) + ABT-737 (10 μM)) or M/ABT: (Mcl-1 DsiRNA (10 nM) + ABT-737 (10 μM)) for an additional 24 hours (total treatment time = 48 hours). Viability was assessed by MTT assay (n=4). (C) Effects of PUMA knockdown on apoptosis. Treatments as in (B). Apoptosis was assessed by PI staining (n=3). *P* values determined by two-tailed, unpaired *t* tests.