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The Role of BCL-2 Family in Clinical Response of Chronic Lymphocytic Leukemia

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THE ROLE OF BCL-2 FAMILY IN CLINICAL RESPONSE OF CHRONIC LYMPHOCYTIC LEUKEMIA

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DEDICATION

This dissertation is dedicated to my parents, for whom this journey would never have begun, To my wife Haifa who has patiently stood by me and I could not have continued without her help and support and to Dr. Alex Almasan, who without whose guidance, I could not have completed it. Thank you all.

THE ROLE OF BCL-2 FAMILY IN CLINICAL RESPONSE OF CHRONIC LYMPHOCYTIC LEUKEMIA

SAYER ALHARBI

ABSTRACT

The anti-apoptotic Bcl-2 proteins regulate lymphocyte survival and are over-expressed in lymphoid malignancies, including chronic lymphocytic leukemia (CLL). The small molecule inhibitor ABT-737 binds with high affinity to Bcl-2, Bcl-xl, and Bcl-w but with low affinity to Mcl-1, Bfl-1, and Bcl-b. The active analog of ABT-737, navitoclax, has shown a high therapeutic index in lymphoid malignancies; developing a predictive marker for it would be clinically valuable for patient selection or choice of drug combinations. We compared expression of anti-apoptotic Bcl-2 genes that are known to be targeted by ABT-737. Our findings reveal that the relative ratio of Mcl-1 and Bfl-1 to Bcl-2 expression provides a highly significant linear correlation with ABT-737 sensitivity ($r = 0.6$, $P < .001$). The (Mcl-1 + Bfl-1)/Bcl-2 ratio was validated in a panel of leukemic cell lines subjected to genetic and pharmacologic manipulations. We also tested whether anti-apoptotic Bcl-2 proteins can be used as prognostic marker in CLL, since the current prognostic markers, such as CD38 and ZAP70 fail to predict the clinical outcome in a substantial number of CLL patients. Our findings indicate that only high Bcl-xl expression is strongly correlated ($P=0.002$) with short

treatment-free survival. Strikingly, Bcl-xl was able to identify patients with high risk in the ZAP70 negative and CD38 negative groups as well as in patients with normal/unknown p53 deletion, thus providing a very powerful prognostic value for CLL. Finally, we investigated the potential mechanism responsible for ABT-737 resistance in leukemic cell lines. Compared with parental cells, cells that have developed acquired resistance to ABT-737 showed increased expression of Mcl-1 due to increased protein stability. Interestingly, increased Mcl-1 levels sequester the BH3-only protein Bim which mediates resistance to ABT-737. These data therefore reveal novel insights into the role of anti-apoptotic Bcl-2 proteins in clinical response, acquired resistance, and tumor progression in CLL patients.

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LIST OF ABBREVIATIONS

ABT-R	ABT-737-resistant
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATM	Ataxia-telangiectasia mutated
APAF-1	Apoptotic protease activating factor-1
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 associated killer
Bax	Bcl-2 associated x protein
Bcl-2	B- cell lymphoma 2
Bcl-xl	B- cell lymphoma extra large
Bcl-w	B- cell lymphoma-w
BECN1	Beclin 1
BH3-only	Bcl-2 Homology domain 3 only
Bid	Bcl-2 homology interacting domain death
Bim	Bcl-2 interacting mediator of cell death
PBS	Phosphate buffered saline
C	Contol
Caspase	Cysteine aspartic acid
cDNA	Copy deoxyribonucleic acid
CHX	Cyclohexiamide

CLL	Chronic lymphocytic leukemia
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamminetetraacetate
ERK	Extracellular signal-regulated kinases
FADD	Fas-associated death domain
FBS	Fetal bovin serum
FCR	Fludarabine combined with cyclophosphamide and rituximab
FOXO3a	Forkead box transcription factor-3A
FISH	Fluorescence in situ hybridization
GSK-3	Glycogen synthase kinase-3
IRB	Institutional review board
IMS	Intermembrane space
Mcl-1	Myeloid cell leukemia 1
miRNA	microRNAs
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic acid
MULE	Mcl-1 ubiquitin Ligase E3
NMR	Nuclear magnetic resonance
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nur77	Nuclear receptor

OMM	The outer mitochondrial membrane
PARP1	Poly-(ADP-ribose) polymerase 1
P53	Protein 53
PS	Phosphatidylserine
PI	Propidium iodide
PUMA	P53 upregulated modulator of apoptosis
RT-PCR	Quantitative real-time poly chain reaction
SDS	Sodium dodecyl sulphate
ShRNA	Small hairpin RNA
STAT3	Signal transducer and activator of transcription 3
tBid	Truncated Bid
TM	Transmembrane domain
TNF	Tumor necrosis factor
TNFR1	TNF receptor-1
Ubqln1	Ubiquilin1
WBC	White blood cells

CHAPTER I

INTRODUCTION

1.1 Apoptosis

Apoptosis, or programmed cell death, is a cellular mechanism used to eliminate cells that are injured, infected, or have reached the end of their life span (Kerr, Wyllie et al. 1972). This process is tightly regulated by a family of proteases called caspases, which are normally found in healthy cells as inactive precursors, but which become activated during apoptosis (Autret and Martin 2009). Apoptosis ensures removal of cellular bodies before these cells can rupture and release their contents. This process is critical, as cell rupture caused by necrosis can provoke inflammatory immune reactions that can augment promote damage and lead to further cell death (Chen, Kono et al. 2007). Disturbed regulation of apoptosis can lead to many diseases, including cancer, autoimmunity, and degenerative disorders (Cory and Adams 2002). Apoptotic cell death is characterized by a series of morphological and biochemical features, such as plasma membrane blebbing, chromatin condensation, DNA cleavage, and exposure of phosphatidylserine on the extracellular side of the plasma membrane (Bouillet and Strasser 2002).

1.2 Apoptosis pathways

There are two predominant pathways that cells utilize to trigger apoptosis. The two pathways of apoptosis can be distinguished by whether the Bcl-2 family proteins are involved and which caspases are critical for mediating the cell death (Youle and Strasser 2008). The intrinsic pathway or mitochondrial pathway is mediated by the Bcl-2 family and primarily leads to the activation of caspase-9 (Hakem, Hakem et al. 1998). The Bcl-2 family consists of anti and pro-apoptotic members that mediate opposing functions to control life or cell death in the intrinsic pathway by controlling the integrity of the outer mitochondrial membrane (OMM).

Bcl-2 family members are classified into anti-apoptotic and pro-apoptotic (Chipuk, Moldoveanu et al. 2010). Anti-apoptotic members include Bcl-2-related gene (Bcl-2), Bcl-2-related gene A1 (A1), long isoform (Bcl-xl), Bcl-2-like 2 (Bcl-w), Bcl-b and myeloid cell leukemia 1 (Mcl-1). The anti-apoptotic members work predominantly to inhibit apoptosis by sequestering pro-apoptotic proteins and blocking their ability to oligomerize and advance the outer mitochondrial membrane permeabilization (OMMP). The pro-apoptotic Bcl-2 members are divided into the effector proteins and the BH3-only proteins. The effector proteins Bcl-2 antagonist killer 1 (Bak) and Bcl-2-associated x protein (Bax) can initiate apoptosis by causing pores within the OMM to promote MOMP. The BH3-only proteins Bad, Bid, Bim, Noxa, and Puma have a conserved BH3 domain that can bind to the anti-apoptotic Bcl-2 proteins and thus allow the effector proteins to be released and then promote apoptosis.

The intrinsic pathway can be triggered by various intracellular stresses, including cytokine deprivation and genotoxic damage (Zha, Harada et al. 1996; Oda, Ohki et al. 2000) (Figure 1.1). Once the intrinsic pathway is triggered, this promotes MOMP, which allows cytochrome c, located in the mitochondrial intermembrane space (IMS), to be released into the cytosol. Cytochrome c engages apoptotic protease activating factor-1 (APAF-1) to oligomerize into a caspase activation complex called the apoptosome. This binds and promotes the activation of initiator caspase-9, which then activates executioner caspases-3 and -7 (Li, Nijhawan et al. 1997).

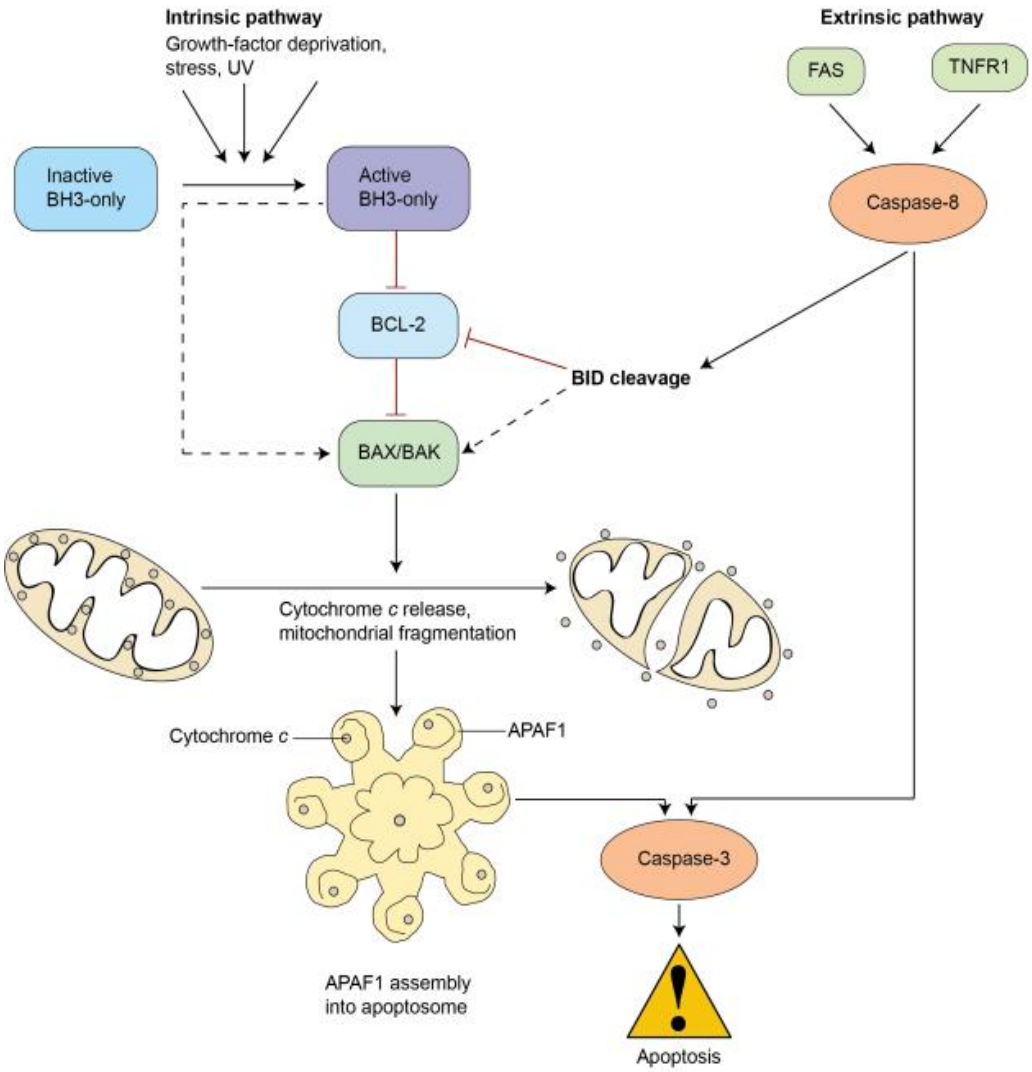

Figure 1.1: Scheme showing intrinsic and extrinsic pathways of apoptosis. Apoptosis can be induced by intrinsic or extrinsic pathways. The intrinsic pathway is activated either by BH3-only protein induction or by post-translational modification which resulted in inhibition of the anti-apoptotic Bcl-2 proteins. This releases BAK or BAX, which in turn release cytochrome c and promote apoptosis by activating APAF1 and caspases. The extrinsic pathway can be activated directly by caspase-8, which leads to caspase-3 activation and cell death. The intrinsic pathway can be linked to the extrinsic pathway through cleaved BID. Modified from (Youle and Strasser 2008).

The extrinsic pathway is activated by ligation of death receptors at the cell surface independently of the Bcl-2 family. The tumor necrosis factor (TNF) receptor family members, such as Fas or TNF receptor-1 (TNFR1), contain an intracellular death domain that can recruit and activate caspase-8 through the adaptor protein Fas-associated death domain (FADD). This recruitment leads to activation of downstream caspases, such as caspase-3, -6, or -7 (Ashkenazi 2002; Almasan and Ashkenazi 2003). Interestingly, the extrinsic pathway can be linked with the intrinsic pathway through caspase-8 cleavage-mediated activation of the pro-apoptotic BH3-only protein Bid (Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998). The cleaved Bid (tBid) can translocate to the mitochondria and further activate caspase through the intrinsic pathway (Figure 1.1).

1.3 Bcl-2 family structure

The Bcl-2 family shares at least one conserved homology domain, called the Bcl-2 homology (BH) domain (Youle and Strasser 2008) (Figure 1.2). Anti-apoptotic Bcl-2 proteins contain four Bcl-2 homology domains (BH1–4) and are mostly localized within the OMM. The carboxy terminal hydrophobic transmembrane domain (TM) of the anti-apoptotic Bcl-2 proteins mediates its targeting to the outer mitochondrial membrane, where the BH1-BH3 domains and the N-terminal BH4 domain stabilizes the structure (Tzifi, Economopoulou et al. 2012). BH1-BH3 domains also form a hydrophobic groove on the surface of anti-

apoptotic proteins that can be targeted by the BH3 domain of their pro-apoptotic ligands (Sattler, Liang et al. 1997).

The three-dimensional structure is conserved between Bcl-xl and Bcl-2 (Petros, Medek et al. 2001). However, there are similarities and differences between the Bcl-2 cores of anti-apoptotic proteins and the BH3 peptide binding (Chipuk, Moldoveanu et al. 2010), thus creating a preferential binding between anti-apoptotic and BH3-only Bcl-2 family proteins. For example, Bim and Puma can bind to all anti-apoptotic proteins, where Noxa binds only to Mcl-1 and Bfl-1 (Figure 1.3). These variations in the Bcl-2 cores are critical to cancer drug discovery, as Mcl-1 and Bfl-1 are not targeted by the most promising BH3 mimetic, ABT-737, which only inhibits Bcl-2, Bcl-w, and Bcl-xl (Oltersdorf, Elmore et al. 2005).

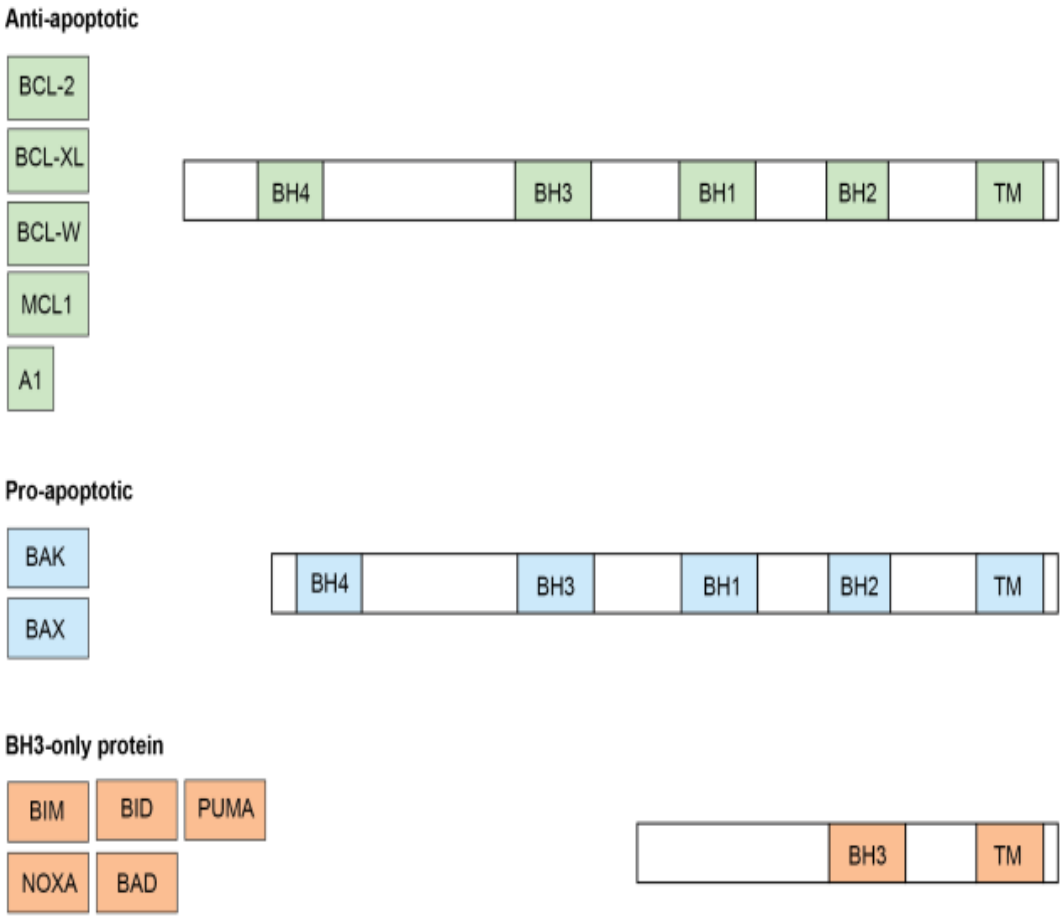

Figure 1.2: The Bcl-2 family related proteins. This family comprises anti-apoptotic (highlighted in green boxes), pro-apoptotic (highlighted in blue) and BH3-only proteins (highlighted in orange). The anti-apoptotic and pro-apoptotic proteins share four Bcl-2 homology domains BH1-4, where BH3-only proteins display only one BH3 domain. Modified from (Lessene, Czabotar et al. 2008).

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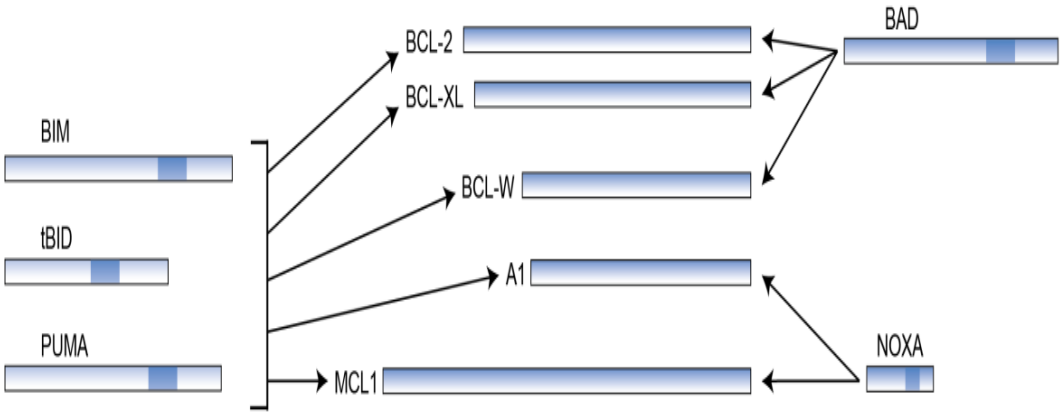

Figure 1.3. The binding specificity of BH3-only proteins for anti-apoptotic Bcl-2 family proteins. Bim, Bid and Puma can bind to all anti-apoptotic Bcl-2 family proteins. Noxa binds only to Mcl-1 and Bfl-1 (A1), where as Bad binds to Bcl-1, Bcl-xl, and Bcl-w. Modified from (Youle and Strasser 2008).

The effector proteins Bax and Bak were first described as containing only BH1-3 domains, however it was later discovered that they also contain a BH4 domain (Kvansakul, Yang et al. 2008). The BH3-only proteins contain a BH3 domain that is essential for binding to the anti-apoptotic members and mediating apoptosis (Huang and Strasser 2000). The BH3 peptide is an amphipathic α-helix of around 15 amino acids long that binds to the hydrophobic groove of the anti-apoptotic Bcl-2-family proteins (Zha, Aime-Sempe et al. 1996). The BH3-only proteins are divided into direct activator (Bim and Bid) and sensitizers/de-repressor (Bad, Noxa, and Puma) proteins.

1.4 Bax and Bak activation models

Initiation of apoptosis requires not only pro-apoptotic family members, such as Bax and Bak, but also the BH3-only proteins. However, the BH3-only proteins alone fail to induce apoptosis in the absence of Bax and Bak (Zong, Lindsten et al. 2001). During apoptosis, Bax and Bak oligomerize into the mitochondrial outer membrane, disrupt its integrity and release cytochrome c, which allows activation of caspases (Wei, Zong et al. 2001). The BH3-only proteins Bid and Bim can directly trigger Bax and Bak activation whereas the other members were suggested to activate Bax and Bak indirectly (Wei, Lindsten et al. 2000; Letai, Bassik et al. 2002). In fact, there are three models for activating Bax and Bak: sensitizer, de-repressor, and neutralization models (Chipuk, Moldoveanu et al. 2010) (Figure 1.4). According to the sensitizer model, an anti-apoptotic protein

is sequestered with a BH3-only protein and this interaction blocks the inhibition of direct activators (Letai, Bassik et al. 2002). For example, if Bcl-2 is in complex with Puma, any further induction of Bim will not be inhibited, since Bcl-2 is already sequestered by Puma and in this case MOMP will proceed (Figure 1.4A). According to the derepression model, a direct activator is inhibited by an anti-apoptotic Bcl-2 protein, and any cellular stress induces a BH3-only protein, which then releases the direct activator from Bcl-2 and promotes MOMP (Certo, Del Gaizo Moore et al. 2006; Del Gaizo Moore, Brown et al. 2007) (Figure 1.4B). In the neutralization model, the effecter protein Bax or Bak is inhibited (Uren, Dewson et al. 2007). For example, activated Bak is sequestered by the anti-apoptotic Bcl-2, and following any cellular stress, a BH3-only protein will be induced to neutralize the anti-apoptotic protein, which frees Bak, and then promotes apoptosis (Figure 1.4C).

Activation of Bax appears to involve sub-cellular translocation and oligomerization. In healthy cells, Bax is localized as an inactive form in the cytosol where an α9 helix in the C-terminal occupies its BH3 binding hydrophobic groove, which inhibits both its mitochondrial localization and pro-apoptotic activity (Suzuki, Youle et al. 2000). During apoptosis, Bax undergoes conformational change, which allows to translocate to the mitochondria by inserting its C-terminal into the OMM and thus, allowing its BH3 domain exposure, which mediates Bax oligomerization. Unlike Bax, Bak is localized on the mitochondria, where it is sequestered by anti-apoptotic Mcl-1 and Bcl-xl to

prevent its oligomerization (Willis, Chen et al. 2005). Following cytotoxic signals that activate BH3-only proteins, the interaction can be disrupted by the BH3-only proteins where Bak can be released from the anti-apoptotic proteins and promote apoptosis.

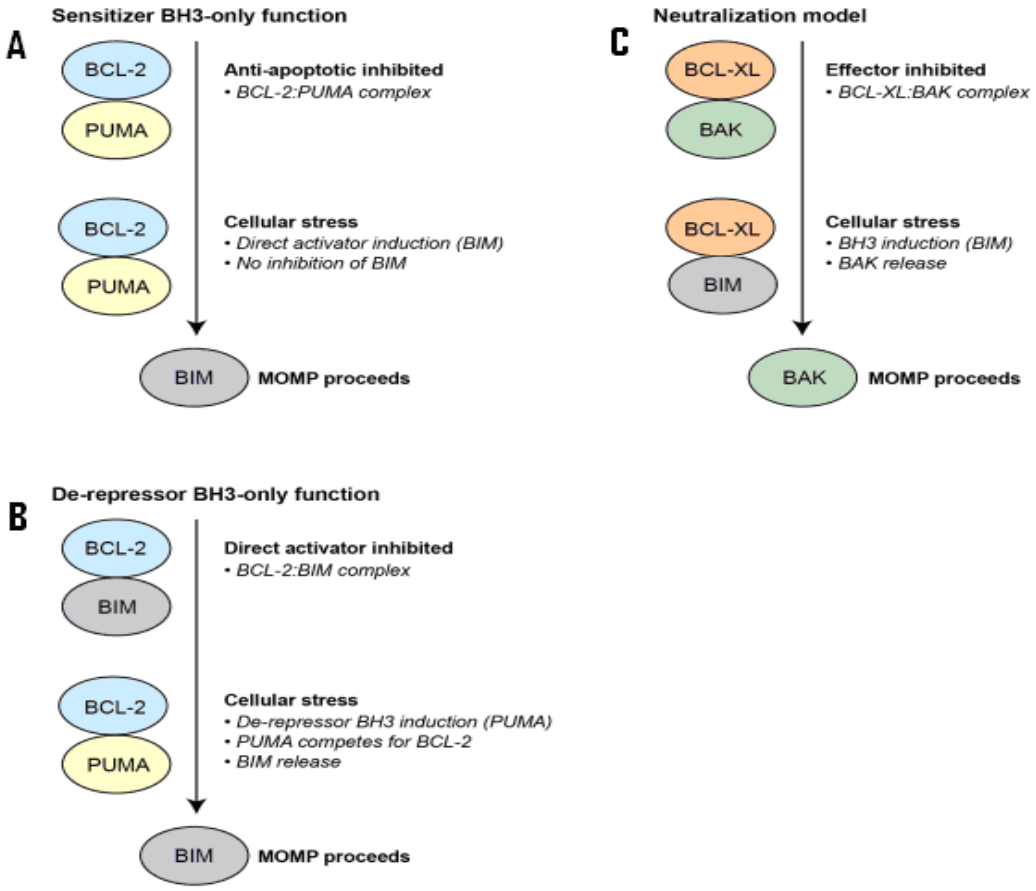

Figure 1.4: Three models for activating Bax and Bak by BH3-only proteins. (A) Sensitizer model: BH3-only protein inhibits the anti-apoptotic Bcl-2 protein. Upon activation following a cellular stress, a direct activator can not be inhibited and will go directly to promote MOMP. (B) De-repressor model. A direct activator (BIM) is sequestered by an anti-apoptotic Bcl-2 protein. Following cellular stress, a de-repressor BH3-only protein is induced and releases the direct activator from the anti-apoptotic protein and then MOMP proceeds. (C) The neutralization model: The effector BAK is inhibited by the anti-apoptotic protein. Following cellular stress, BH3-only protein is induced and then binds to the anti-apoptotic protein which displaces the effector and then promotes MOMP. Modified from (Chipuk, Moldoveanu et al. 2010).

1.5 Bcl-2 family regulation

BH3-only proteins are the major sensors for any cellular stress. BH3-only proteins can be activated in a variety of ways, such as transcriptional upregulation, proteolysis, or post-translational modifications (Figure 1.5). Noxa and Puma can be induced transcriptionally by the tumor suppressor p53 in response to DNA damage (Oda, Ohki et al. 2000; Nakano and Vousden 2001).

Bim is regulated transcriptionally by the class O forkhead box transcription factor-3A (FOXO3A) in response to growth factor deprivation (Dijkers, Medema et al. 2000). Bim can be also regulated at transcriptional level, where bim mRNA is negatively regulated by the miRNA cluster miRNA-17-92 (Xiao, Srinivasan et al. 2008). In healthy cells, Bim is sequestered away from the anti-apoptotic proteins by binding to LC8 dynein light chain in the microtubule-associated dynein motor complex (Puthalakath, Huang et al. 1999). Following a death signal, Bim dissociates from the motor complex and translocates to neutralize Bcl-2 anti-apoptotic activity. Moreover, phosphorylation of pro-apoptotic Bim by ERK1/2 on serine 69 promotes its degradation by the ubiquitin-proteasome pathway (Luciano, Jacquel et al. 2003).

The BH3-only protein Bad is regulated by post-translational modification on two phosphorylation sites, ser-112 and ser-136. The phosphorylated Bad is sequestered in the cytosol by a 14-3-3 molecule that prevents interaction with anti-apoptotic Bcl-xl (Zha, Harada et al. 1996). The survival signaling molecule Akt has been shown to lead to phosphorylated Bad at ser-136 and inactivate its

function, however, dephosphorylated Bad can neutralize Bcl-xl to promote apoptosis following IL-3 deprivation (Datta, Dudek et al. 1997).

Interestingly, unlike other pro-apoptotic members, Bid undergoes a cleavage mediated by caspase-8 (Li, Zhu et al. 1998) and this cleavage allows the buried BH3 domain to be exposed. The cleaved Bid (tBid) can then translocate to the mitochondria to promote apoptosis (Figure 1.5).

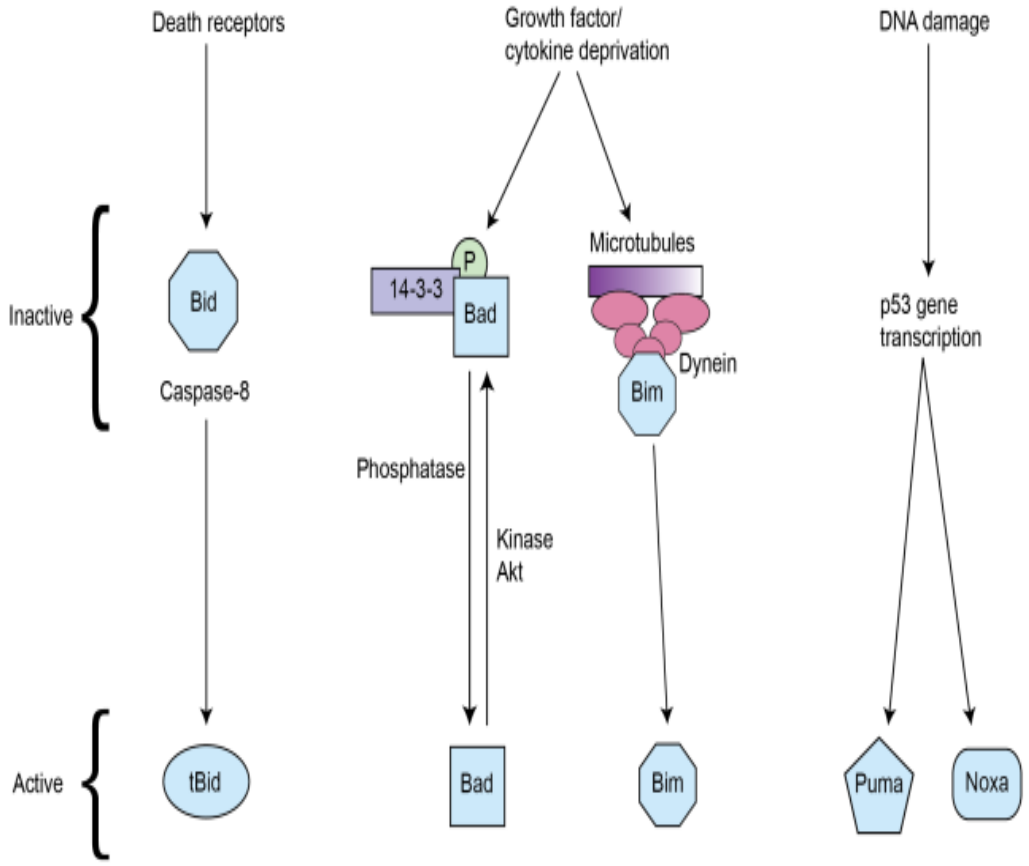

Figure 1.5: Activation of BH3-only proteins. BH3-only proteins are activated in a variety of ways, including proteolysis, post-translational modifications, or transcriptional up regulation. Bid is activated by cleavage, whereas Bad is kept in an inactive phosphorylated form bound to 14-3-3 in the cytoplasm. Following growth factor/cytokine deprivation, Bad is activated by de-phosphorylation. Bim is bound to the microtubules and released after cytokine deprivation. Puma and Noxa are transcriptionally up regulated by p53 following DNA damage. The activated BH3-only proteins can then translocate to the mitochondria where they promote apoptosis by activating the effector proteins or by neutralizing the anti-apoptotic proteins. Modified from (Gardner 2004).

The anti-apoptotic protein Mcl-1 also undergoes several post-translational modifications since it has a large N terminal that contains two sequences rich in proline (P), glutamic acid (E), serine (S), and threonine (T), which mediate rapid degradation or promote stability of the proteins (Gores and Kaufmann 2012). These modifications are critical for determining whether Mcl-1 promotes or prevents apoptosis. Mcl-1 protein has a unique feature that differentiates it from other anti-apoptotic Bcl-2 family members: it has a short half-life estimated at less than 1 hour (Stewart, Koss et al. 2010). The short half-life of Mcl-1 is due to constitutive polyubiquitination and degradation by the proteasome. It has been shown that following IL-3 withdrawal, Mcl-1 is phosphorylated on serine159 by glycogen synthase kinase-3 (GSK-3) and thus accelerating ubiquitinylation and degradation of Mcl-1 (Maurer, Charvet et al. 2006). Mcl-1 is ubiquitinylated by MULE (Mcl-1 Ubiquitin Ligase E3) at five lysines of its N terminus (Zhong, Gao et al. 2005). MULE contains a BH3 domain that binds to Mcl-1 and promotes its degradation. FBW7 is another E3 ubiquitin ligase that has been recently identified to promote Mcl-1 degradation (Inuzuka, Shaik et al. 2011). It has been reported that removing ubiquitin groups conjugated to Mcl-1 by the deubiquitinase USP9X was found to increase Mcl-1 stability and thus, promote cell survival (Schwickart, Huang et al. 2010). USP9X binds Mcl-1 and removes the polyubiquitin chains that flags Mcl-1 for proteasomal degradation, which results in high Mcl-1 levels. Interestingly, Mcl-1 activity can be changed from anti-apoptotic to pro-apoptotic activity during apoptosis following cleavage by caspase-3 at Asp127 and/or Asp157 (Michels, O'Neill et al. 2004).

Ubiquilin-1 (Ubqln1) has been identified to interact specifically with Bcl-b and not with other anti-apoptotic proteins. Ubqln1 promotes Bcl-b protein stabilization and re-localization from the mitochondria to the cytosol which leads to more potent anti-apoptotic activity (Beverly, Lockwood et al. 2012).

The anti-apoptotic protein Bcl-2 can also be regulated by post-translational modification; caspase-3 cleaves the carboxyl terminal at Asp34, which converts the Bcl-2 phenotype from anti-apoptotic to pro-apoptotic (Cheng, Kirsch et al. 1997; Chen, Gong et al. 2000). This conformational change reveals the pro-apoptotic activity of Bcl-2. Moreover, binding the orphan nuclear receptor Nur77 to Bcl-2 causes a conformational change that leads to expose the buried BH3 domain of Bcl-2 (Lin, Kolluri et al. 2004). On the other hand, anti-apoptotic Bcl-xl and Bfl-1 genes are directly regulated by the transcription factors signal transducer and activator of transcription (STAT3) and NF-kB (Grumont, Rourke et al. 1999; Zong, Edelstein et al. 1999; Grad, Zeng et al. 2000).

1.6 Bcl-2 family and its physiological roles

Knockout mice studies have revealed the essential role of the Bcl-2 family in embryogenesis and tissue homeostasis. Bcl-xl was found to be essential for embryogenesis, neuronal cells, and hematopoietic cell survival (Motoyama, Wang et al. 1995). It has been recently shown that Bcl-xl is also critical for lymphoma development (Kelly, Grabow et al. 2011). Bcl-2-deficient mice show complete embryonic development, however, they have defects in lymphocyte

differentiation and severe polycystic kidney disease (Veis, Sorenson et al. 1993). Mcl-1 deletion was reported to be lethal due to the failure of embryonic implantation (Rinkenberger, Horning et al. 2000), Mcl-1 was also found to be critical for the development and survival of acute myeloid leukemia (AML) cells (Glaser, Lee et al. 2012).

Bax knockout mice exhibit B-cells hyperplasia (Knudson, Tung et al. 1995), whereas Bak-deficient mice have been reported to be developmentally normal. Interestingly, Bak and Bax double knockout mice showed a huge accumulation of hematopoietic cells due to lack of apoptosis (Lindsten, Ross et al. 2000).

Gene targeting studies have defined the essential functions of different BH3-only members in mediating apoptosis. Bid has been reported to be a critical factor for cell death initiated by death receptor Fas, as Bid-deficient mice were highly resistant to Fas-induced apoptosis (Yin, Wang et al. 1999). In addition, deleted Bim causes resistance to apoptosis induced by growth factor deprivation in a broad range of cell types (Bouillet, Metcalf et al. 1999), whereas loss of Puma and Noxa mediate resistance to DNA damage agents (Villunger, Michalak et al. 2003).

1.7 Bcl-2 family regulation in cancer

Blocking apoptosis is considered one of the hallmarks of cancer as impaired apoptosis maintains cancer survival and progression (Hanahan and Weinberg 2011). Cancer cells block apoptosis either by promoting overexpression of the

anti-apoptotic proteins or by downregulating the pro-apoptotic proteins. Both increased levels of anti-apoptotic proteins or the loss of pro-apoptotic proteins are associated with genetic alterations in many tumor malignancies. Chromosomal translocations, deletion, additional copy numbers, post-translational modifications, or epigenetic alterations have been reported to modulate Bcl-2 family expression that can promote survival of cancer cells.

In follicular B-cell lymphoma, a high Bcl-2 expression level is associated with (14;18) chromosomal translocations, which place the Bcl-2 gene on chromosome 18 under the control of an immunoglobulin heavy chain enhancer on chromosome 14, resulting in excessive Bcl-2 production though transcriptional regulation (Tsujimoto, Cossman et al. 1985). High Bcl-2 levels have also been reported in diffuse large B-cell lymphoma and chronic lymphocytic leukemia due to Bcl-2 gene amplification or hypomethylation of the Bcl-2 promoter (Hanada, Delia et al. 1993; Monni, Joensuu et al. 1997). In addition, the single nucleotide polymorphisms 938 C >A in the Bcl-2 promoter enhanced its binding activity and resulted in high Bcl-2 levels in chronic lymphocytic leukemia cells (Nuckel, Frey et al. 2007).

Preventing Bcl-2 pro-apoptotic activity of anti-apoptotic proteins can be a strategy for blocking apoptosis in cancer cells. Mutation of the aspartic acid residue required for caspase cleavage that changes Bcl-2 function from anti-apoptotic to pro-apoptotic has been identified in human lymphoma (Cory, Huang et al. 2003).

Interestingly, the loss of endogenous microRNAs that normally repress anti-apoptotic Bcl-2 protein expression can be a mechanism for elevated levels of these anti-apoptotic proteins. MicroRNAs are short, noncoding RNAs of about 19–24 nt that bind to 3′ UTRs of target mRNAs and promote their degradation. MiR-15a and miR-16-1 are located in a cluster at 13q14.3, which are frequently deleted in CLL (Calin, Ferracin et al. 2005). Deletions of miR-15a and miR-16-1 result in high Bcl-2 expression.

In cancer cells, Mcl-1 undergoes post-translational modification to enhance cell survival by increasing the rate of protein stability. Extracellular signal-regulated kinases (ERK) activation promotes Mcl-1 phosphorylation at Thr163 and stabilization of the Mcl-1 protein (Domina, Vrana et al. 2004). The E3 ubiquitin ligase that targets phosphorylated Mcl-1 for degradation has been recently identified as FBW7 (Inuzuka, Shaik et al. 2011). FBW7 is a E3 ubiquitin ligase which controls Mcl-1 stability. FBW7 has tumor-suppressor activity, as mutation or deletion of this gene resulted in a significant increase in Mcl-1 levels in human ALL cell lines.

In a large study to analyze gene copy number in many cancer specimens, the Mcl-1 genomic region has been found to be amplified in lung and breast cancers (Beroukhim, Mermel et al. 2010). MiR-29 was found to negatively regulate Mcl-1 expression and miR-29 loss is consequently associated with high Mcl-1 levels (Mott, Kobayashi et al. 2007). Like Mcl-1, Bfl-1 protein has a short half-life, which can be markedly increased by the mutation of C-terminal lysines 151,163, and 172 to arginines (Fan, Simmons et al. 2010). These ubiquitination-resistant

mutants show that Bfl-1 deubiquitination is critical for its tumor promotion mechanism.

It has recently been reported that chromosomal translocation of the transcription factor BACH2 gene on 6q15 to the second exon of Bcl-xl on 20q11 resulted in strong expression of Bcl-xl in the Burkitt lymphoma cell line BLUE-1 (Turkmen, Riehn et al. 2011).

Genetic alterations in pro-apoptotic genes have also been reported in many cancers. These alterations can result in loss of the tumor suppressor function of pro-apoptotic genes. Interestingly, p53 is the transcription factor for Bax, Bid, Puma and Noxa; therefore, any deletion or mutation in the p53 gene will directly affect the transcriptional levels of these genes (Miyashita and Reed 1995; Oda, Ohki et al. 2000; Nakano and Vousden 2001). Bim function is reported to be lost in hematological malignancies; for example, the 2q13 locus where the BIM gene is located has been reported to be deleted in mantle cell lymphoma (Tagawa, Karnan et al. 2005). Bim expression can also be regulated transcriptionally by microRNA as gene amplification of miR-17 that negatively regulates Bim level is observed in several human cancers (Ventura, Young et al. 2008). Mutations that cause inactive Bax have been reported in colon cancer (Rampino, Yamamoto et al. 1997). AKT pathway is known to be highly activated in many cancers. Upon activation, AKT phosphorylates the pro-apoptotic protein Bad, which results in its sequestration by 14-3-3 (Zha, Harada et al. 1996).

1.8 Bcl-2 family and clinical response in cancer

Apoptosis is often impaired in cancer and can significantly limit the response to conventional therapy. Resistance of tumor cells to radiation or chemotherapy can be due to a defect in the apoptosis machinery. Increased anti-apoptotic protein levels have been reported in many human malignancies and generally correlate with aggressive tumors and chemotherapeutic resistance (Letai 2008). A high Mcl-1 level has been correlated with chemoresistance in various haematopoietic and lymphoid cancers, CLL (Kitada, Andersen et al. 1998) and multiple myeloma (Derenne, Monia et al. 2002). Interestingly, resistance to 122 standard chemotherapy agents was correlated with high Bcl-xl levels in 60 NCI cell lines (Amundson, Myers et al. 2000). The resistance to a group of compounds that repress Mcl-1 expression was correlated with high mRNA expression of Bcl-xl, indicating that the patients-selection strategy for development of any Mcl-1 inhibitor in breast and non-small cells lung carcinoma (NSCLC) tumors should be based on patients with low Bcl-xl expression (Wei, Margolin et al. 2012). High Bfl-1 levels were also found to contribute to fludarabine resistance in CLL cells (Olsson, Norberg et al. 2007).

Several studies have attempted to correlate Bcl-2 family expression and response to treatments, which highlight the potential application of the Bcl-2 family as a marker for predicting clinical outcome. We have shown that the relative expression of Mcl-1 and Bfl-1 to Bcl-2 predicts ABT-737 response in hematological and solid malignancies (Al-Harbi, Hill et al. 2011). High levels of Bcl-2 and low levels of Bax were associated with resistance to chlorambucil in

CLL (Thomas, Pepper et al. 2000). Interestingly, It was found that patients with highly primed cancer cells or a high apoptotic threshold exhibited superior clinical response to chemotherapy (Ni Chonghaile, Sarosiek et al. 2011).

1.9 Targeting anti-apoptotic proteins by small molecule inhibitors

Most conventional cytotoxic therapies act primarily through p53 activation to induce apoptosis, and this indirect effect may explain the poor response in patients with p53 deletion. Thus, it can be concluded that there is a need to develop a new generation of targeted therapies that mediate response through direct activation of the apoptotic machinery. This could be a very promising approach since most cancer cells are addicted to high expression of anti-apoptotic proteins in order to maintain their survival and progression.

Obatoclax: A screen of a natural products library for Bcl-2 inhibitors identified obatoclax as a molecular antagonist that binds to Bcl-2, Bcl-xl, Bcl-w, and Mcl-1 with low micromolar affinity (Perez-Galan, Roue et al. 2007). Obatoclax has shown high anti-tumor activity through disruption of the Mcl-1:Bak interactions (Li, Viallet et al. 2008). In contrast, another report showed that obatoclax has the same anti-tumor activity in Bak/Bax double knockout mice as wild type cells, and the anti tumor activity was similar in cells with both high and low anti-apoptotic proteins levels (Lessene, Czabotar et al. 2008). This leads to the conclusion that the cell death induced by the drug is not mediated by apoptosis and sensitivity to the drug was not correlated to expression levels of anti-apoptotic Bcl-2 proteins.

Gossypol: Another small molecule antagonist that targets anti-apoptotic proteins is gossypol. It is a drug derived from the cotton plant and has shown high anti-tumor activity in several hematological malignancies, including lymphoma and multiple myeloma cell lines, as well as in *vivo* models (Paoluzzi, Gonen et al. 2008); however, one major drawback of the drug is that its anti tumor activity requires a high concentration (≈20μM), which it is difficult to achieve clinically.

ABT-737: Crystal structural studies have shown that the BH3 peptide is 16 to 20 amino acids in length and binds a hydrophobic pocket formed by the BH1, BH2 and BH3 domains on the surface of anti-apoptotic proteins (Muchmore, Sattler et al. 1996; Sattler, Liang et al. 1997). This provides the platform for a new drug discovery based on mimicking BH3 peptides that can be rational antagonists of the anti-apoptotic proteins. Nuclear magnetic resonance (NMR)-based screening and structure-based design structure methods were applied rationally to develop a small molecule inhibitor that binds with high affinity to Bcl-2, Bcl-xl, and Bcl-w, and with low affinity to Mcl-1 and Bfl-1 (Oltersdorf, Elmore et al. 2005) (Figure 1.6). ABT-737 has maintained its high activity even in the presence of 10% human serum with a minimum effect on normal primary cells derived from healthy donors. As compared to seven anti-apoptotic protein inhibitors, ABT-737 was the only one to mediate cell death in a Bak/Bax-dependent manner (van Delft, Wei et al. 2006).

We have recently shown that ABT-737 activity is based on relative expression of Mcl-1 and Bfl-1 to Bcl-2 in hematological malignancies and small cell lung carcinoma (Al-Harbi, Hill et al. 2011). This indicates that ABT-737 is a highly

selective target. ABT-737 and its oral derivative compound navitoclax had a marked cytotoxic effect as a single agent in a variety of tumor types, including lymphoma and lung cancer when used within nanomolar range (Tse, Shoemaker et al. 2008). Tumor volume was significantly reduced in small cell lung cancer xenografts and haematological xenografts when treated with navitoclax (Tse, Shoemaker et al. 2008). Recent clinical trials show that the drug is highly effective with minimum off target-effects (Wilson, O'Connor et al. 2010; Gandhi, Camidge et al. 2011; Roberts, Seymour et al. 2012).

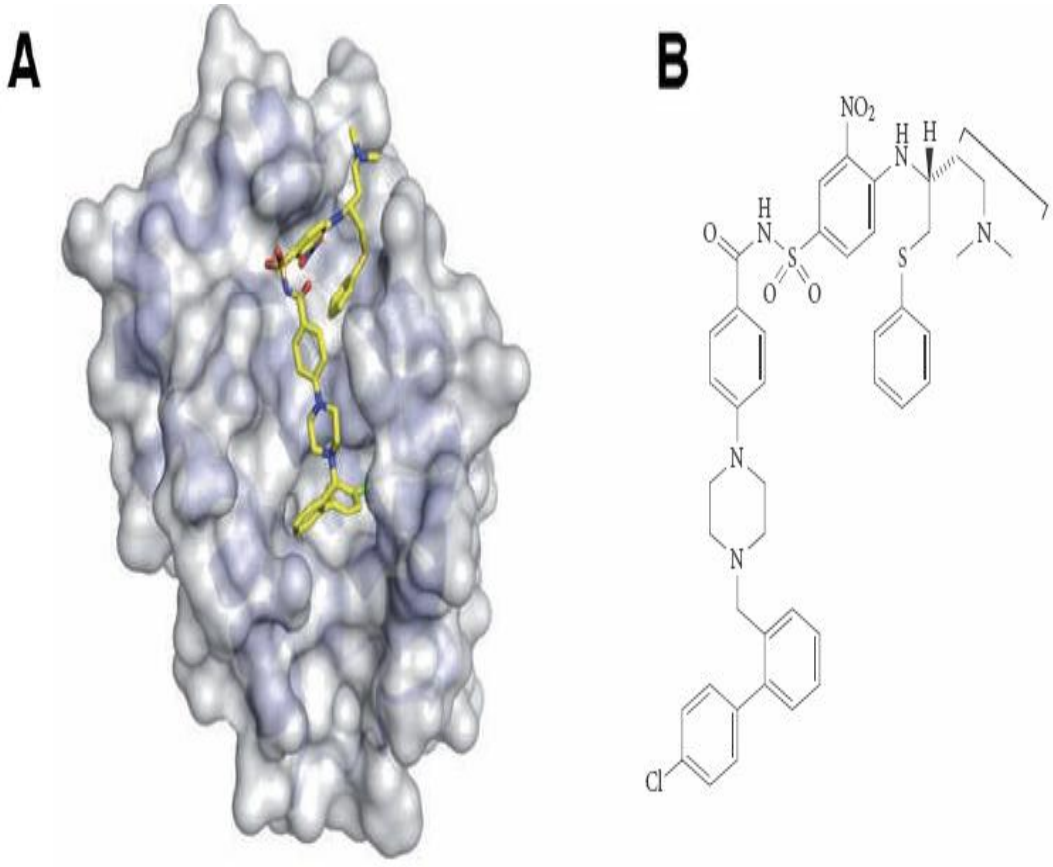

Figure 1.6: Interaction between anti-apoptotic protein Bcl-xl and ABT-737. (A) Structure of ABT-737 binding to the hydrophobic pocket on the surface of anti-apoptotic Bcl-xl protein. (B) ABT-737 chemical structure. Modified from (Lessene, Czabotar et al. 2008).

1.10 Bcl-2 family and Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world, and accounts for approximately 30% of leukemia cases in the United States (Lee, Dixon et al. 1987). CLL is characterized by an excessive accumulation of morphologically mature B lymphocytes in the blood, marrow, and lymphatic tissues. Blocking apoptosis by high levels of anti-apoptotic proteins is considered the hallmark of CLL. Interestingly, it has been reported that high levels of Bcl-2 correlate with high white blood cell (WBC) counts in CLL patients (Del Gaizo Moore, Brown et al. 2007). This leads to the conclusion that high B-cell counts are more likely to be a result of decreased apoptosis rather than of an increased in proliferation rate.

Clinical staging (Rai/Binet) in CLL is based on classification into three stages (Bazargan, Tam et al. 2012): low stage (0) is characterized by lymphocytosis located in the blood or bone marrow, intermediate stage (I, II) is a patient with lymphocytosis and enlarged lymph nodes, liver, or spleen. High stage (III, IV): is characterized by lymphocytosis and anemia (Hgb <11 g/dl), or lymphocytosis and thrombocytopenia (platelet count <100,000/μl). This clinical staging system is essential to estimate the tumor burden, however it is not useful in predicting which patients in an early stage are likely to have an aggressive form of the disease and a short survival rate. P53 deletion, ATM deletion or mutation have been described to influence CLL prognosis and clinical response to DNA-damaging agents (Pleyer, Egle et al. 2009).

1.11 Bcl-2 family interaction with non Bcl-2 family proteins

The anti-apoptotic Bcl-2 proteins have been reported to regulate another aspect of cell death. Bcl-2 and Bcl-xl have been linked to autophagy. Autophagy is a nonapoptotic cell death that is found to be suppressed by Bcl-2 and Bcl-xl (Shimizu, Kanaseki et al. 2004). These proteins can bind to the tumor suppressor beclin 1 (BECN1), which contains a BH3 domain, to inhibit autophagosome formation (Oberstein, Jeffrey et al. 2007). ATG12, which is an autophagic protein, was recently reported to have a unique BH3-like motif that mediates binding and inactivates the anti-apoptotic activity of Bcl-2 and Mcl-1 (Rubinstein, Eisenstein et al. 2011).

The Bcl-2 proteins usually interact through the BH3 domain; however, proteins without a distinct BH3 domain can interact with the Bcl-2 family. This suggest that a BH3 domain may not be required to engage the Bcl-2 family interaction where molecules containing a BH3-like domain may also mediate this function (Chipuk, Moldoveanu et al. 2010). For example, cytosolic p53 has been reported to interact with anti-apoptotic Bcl-2 protein and/or Bak and Bax. Following genotoxic stress, p53 can promote apoptosis by localizing into the mitochondria (Marchenko, Zaika et al. 2000). It has been shown that p53 is sequestered by Bcl-xl in the cytosol, and this interaction can be disrupted by Puma following genotoxic stress (Chipuk, Bouchier-Hayes et al. 2005). Recently, three conserved hydrophobic residues (F19, W23 and L26) of p53 (SN15) peptide have been identified to anchor into three hydrophobic pockets of Bcl-xl in a similar manner as the BH3 peptide (Bharatham, Chi et al. 2011).

CHAPTER II

AN ANTI-APOPTOTIC BCL-2 FAMILY EXPRESSION INDEX PREDICTS THE RESPONSE OF CHRONIC LYMPHOCYTIC LEUKEMIA TO ABT-737

Sayer Al-harbi et al, *BLOOD,* 29 SEPTEMBER 2011, VOLUME 118,

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2.1 Abstract

The anti-apoptotic Bcl-2 proteins regulate lymphocyte survival and are overexpressed in lymphoid malignancies, including CLL. The small molecule inhibitor ABT-737 binds with high affinity to Bcl-2, Bcl-xl, and Bcl-w but with low affinity to Mcl-1, Bfl-1, and Bcl-b. The active analog of ABT-737, navitoclax has shown a high therapeutic index in lymphoid malignancies; developing a predictive marker for it would be clinically valuable for patient selection or choice of drug combinations. Here we used RT-PCR as a highly sensitive and quantitative assay to compare expression of anti-apoptotic Bcl-2 genes that are known to be targeted by ABT-737. Our findings reveal that the relative ratio of Mcl-1 and Bfl-1 to Bcl-2 expression provides a highly significant linear correlation with ABT-737 sensitivity (r=0.6, P<0.001). In contrast, anti-apoptotic transcript levels, used individually or in combination for high or low affinity

ABT-737-binding proteins could not predict ABT-737 sensitivity. The (Mcl-1 + Bfl-1)/Bcl-2 ratio was validated in a panel of leukemic cell lines subjected to genetic and pharmacologic manipulations. Changes following ABT-737 treatment included increased expression of Bfl-1 and Bcl-b that may contribute to treatment resistance. This study defines a highly significant Bcl-2 expression index for predicting the response of CLL to ABT-737.

2.2 Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western World (Lee, Dixon et al. 1987). It is characterized by an expansion of small mature B cells in the peripheral blood, lymph nodes, and bone marrow. The standard of care for medically-fit patients involves sequential cycles of chemo-immunotherapy with the purine nucleoside analog fludarabine combined with cyclophosphamide and the anti-CD20 monoclonal antibody rituximab (FCR) (Hallek, Fischer et al. 2010; Robak, Dmoszynska et al. 2010).

The Bcl-2 family proteins regulate apoptosis primarily at the mitochondria through the intrinsic apoptotic pathway (Del Gaizo Moore, Brown et al. 2007; Letai 2008). These proteins are divided into three classes based on sequence homology (Bcl-2 homology domains BH1 to BH4) and function (Adams and Cory 2007; Deng, Carlson et al. 2007). The anti-apoptotic proteins that display sequence homology in all BH1-BH4 domains are Bcl-2, Bcl-xl, Bcl-w, Mcl-1, Bfl-1, and Bcl-b. The pro-apoptotic Bcl-2 family members are divided into multidomain effectors (Bax and Bak containing BH1-BH3 domains) and the BH3-only proteins (Bim, Puma, and Noxa). Increased expression of the anti-apoptotic proteins represents the main factor that accounts for blocking the intrinsic apoptosis pathway. This inhibition of apoptosis is accomplished by sequestering pro-apoptotic proteins and thus preventing mitochondrial outer membrane permeabilization (MOMP) (Tait and Green 2010), (Cheng, Wei et al. 2001; Willis, Chen et al. 2005; Certo, Del Gaizo Moore et al. 2006).

Elevated levels of Bcl-2, Mcl-1, and Bcl-xl mRNA and protein are found in many solid tumors and hematologic malignancies, including CLL (Gottardi, Alfarano et al. 1996; Kitada, Andersen et al. 1998; Pepper, Hoy et al. 1998; Rust, Harms et al. 2005; Stam, Den Boer et al. 2010). Importantly, Bcl-2 and Mcl-1 protein expression parallels their mRNA levels in diffuse large B-cell lymphoma (DLBCL) and CLL (Shen, Iqbal et al. 2004; Veronese, Tournilhac et al. 2008). In most cases of follicular lymphoma, high Bcl-2 levels could be caused by chromosomal translocation t(14;18), which places the Bcl-2 gene under the control of the IgH enhancer, leading to overproduction of the Bcl-2 transcript and protein (Tsujimoto, Finger et al. 1984). In CLL, loss of microRNAs (miR) is involved in regulation of the Bcl-2 family expression levels. 13q14 is the most commonly detected chromosomal deletion in CLL that leads to loss of miR-15a and miR-16, which causes increased Bcl-2 levels (Cimmino, Calin et al. 2005). In addition, Mcl-1 can also be regulated by miRs, as miR-29 is known to negatively regulate Mcl-1 levels (Calin, Ferracin et al. 2005). Several reports suggest that Bcl-2 family proteins can be used as a predictive marker for the CLL response to chemotherapy. The Mcl-1/Bax ratio was reported to correlate with chemo-resistance to fludarabine and rituximab (Bannerji, Kitada et al. 2003; Pepper, Lin et al. 2008). The Bcl-2/Bax ratio was also associated with resistance to chlorambucil (Williamson, Kelly et al. 1998). In addition, Bcl-xl levels were found to correlate with resistance to many standard chemotherapeutic agents in the 60-tumor cell line NCI panel (Amundson, Myers et al. 2000).

ABT-737 was developed as a rationally-designed, small-molecule inhibitor that binds with high affinity to Bcl-2, Bcl-xl, and Bcl-w but with much lower affinity to Mcl-1, Bfl-1, and Bcl-b (Oltersdorf, Elmore et al. 2005). The orally available Bcl-2-family inhibitor navitoclax (ABT-263) is now in clinical development for lymphoid malignancies, including CLL, both as a single agent and in combination with chemotherapy and/or monoclonal antibodies (Oltersdorf, Elmore et al. 2005). Navitoclax showed a high therapeutic index in drug-resistant lymphoid malignancies with low incidence of off-target toxic effects in a phase 1 dose-escalation study (Wilson, O'Connor et al. 2010). Moreover, preliminary efficacy data from a phase I study with navitoclax (ABT-263) in patients with small cell lung carcinoma (SCLC) and other solid tumors are encouraging as they show that the drug is safe and well-tollerated (Gandhi, Camidge et al. 2011). ABT-737 induces apoptosis in primary follicular lymphoma, CLL, acute myeloid leukemia, and solid tumor cells (Oltersdorf, Elmore et al. 2005; Konopleva, Contractor et al. 2006; Del Gaizo Moore, Brown et al. 2007). Interestingly, tumor cell lines tested for ABT-737 response in cell culture have shown similar activity in xenografts derived from the same cell lines *in vivo* (Oltersdorf, Elmore et al. 2005; Hann, Daniel et al. 2008).

Sensitivity to ABT-737 is thought to depend on a Bcl-2 complex enriched for Bim (Del Gaizo Moore, Brown et al. 2007; Deng, Carlson et al. 2007). High levels of Mcl-1 were also suggested to account for ABT-737 resistance mostly based on cell line studies (Konopleva, Contractor et al. 2006; van Delft, Wei et al. 2006; Chen, Dai et al. 2007). In contrast, other studies report that there is no

correlation between Mcl-1 expression and the response to ABT-737 in primary CLL and in acute lymphoblastic leukemia xenografts (Del Gaizo Moore, Brown et al. 2007; Mason, Khaw et al. 2009; Vogler, Butterworth et al. 2009; High, Szymanska et al. 2010). Moreover, the ratios of Bcl-2/Bim, Bcl-2/Bax, Mcl-1/Bim, or Mcl-1/Bax could not predict sensitivity to ABT-737 in primary CLL (Mason, Khaw et al. 2009). Interestingly, long-term exposure to ABT-737 resulted in increased levels of both RNA and protein levels of Mcl-1 and Bfl-1 and development of resistance in lymphoma cell lines that were initially sensitive (Yecies, Carlson et al. 2010).

There are currently no satisfactory predictive markers for the wide range of response to ABT-737 in primary lymphoid cells. Developing a predictive index for ABT-737 response may help in patient selection or choice of drug combinations. In this study we examined whether quantifying expression of all anti-apoptotic Bcl-2 family transcripts whose encoded proteins are known to be targeted by ABT-737 could be used to identify a gene expression index that predicts ABT-737 sensitivity. Here, we used a highly sensitive and quantitative real-time (RT-PCR) assay to examine expression of six anti-apoptotic Bcl-2 family genes. We defined the relative expression of (Mcl-1 + Bfl-1)/Bcl-2 as the most significant predictive marker for the response of primary CLL and leukemic cell lines to ABT-737 treatment.

2.3 Methods

2.3.1 Cell culture

Peripheral blood samples from 57 patients with CLL and six healthy donors were obtained with the patients' informed consent according to protocols approved by the institutional review board (IRB) according to the Declaration of Helsinki. All primary CLL cells were freshly processed without freezing. Lymphocytes were purified by Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ) gradient centrifugation. Cells were washed twice in media and cultured at a density of 1 x 10^6 cells/ml in RPMI-1640 medium supplemented with 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), L-glutamine (Invitrogen, Carlsbad, CA), and 100 units/ml penicillin-streptomycin (Gibco BRL, Gaithersburg, MD). Primary CLL cells were treated for 18 hours with ABT-737 at 50 nM, a concentration previously shown to be effective in most primary CLL patients tested (Mason, Khaw et al. 2009). Nine leukemic cell lines were used in the study: (i) three CLL (Mec-1, Mec-2, and PTA-3920), (ii) two Pre-B (Nalm-6 and Reh), two B-cell-derived (NCI-H929 and IM-9), and two T-cell-derived (Jurkat and Molt-4). These cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA), with the exception of Mec-1 and Mec-2, which were a gift from Dr. Y. Sauntharajah (Cleveland Clinic). The cell lines, CLL primary cells, and human lymphocytes obtained from healthy donors were all cultured under similar conditions. ABT-737 was obtained from Abbott Laboratories (Abbott Park, IL).

2.3.2 Purification of lymphocytes from healthy donors

Highly purified lymphocytes were obtained using the Gambro Elutra^{TM} Cell Separation System. Following the apheresis procedure, cells were washed in a saline/tricitrasol solution prior to elutriation. Cell composition was verified on Cell Dyn 3700 (Abbott Diagnostics, Abbott Park, IL) prior to elutriation. Cells were separated using a continuous countercurrent buffer flow of Hank's Balanced Salt Solution (Lonza BioWhittaker, Switzerland), with simultaneous centrifugation, using the Caridian Elutra Cell Separation System (CaridianBCT, Lakewood, CO). Fractions were centrifuged at 2500 rpm for 5 min at 4C° and pooled to concentrate the product. Purity of the final combined product as determined on the Cell Dyn 3700 was >97%.

2.3.3 Flow cytometry

Cell death was assessed by phosphatidylserine (PS) externalization as we have previously described (Chen, Gong et al. 2001). After 18 hours of culture, primary CLL cells and established cell lines were stained with fluorescein-conjugated Annexin V (BD Biosciences, San Jose, CA) and propidium iodide (PI) and analyzed on a BD FACSCalibur flow cytometer. Raw data were analyzed using the CellQuest software. Results were normalized to survival of untreated cells. Spontaneous cell death in freshly derived CLL cells was less than 30%.

Flow cytometric immunophenotyping was performed at Cleveland Clinic as part of the diagnostic evaluation on a FACSCanto instrument (BD Biosciences, San

Jose, CA). Each of these flow cytometric evaluations was performed using fluorescently labeled monoclonal antibodies against CD38 and ZAP70. Staining protocols were standard lyses/washing protocols, as previously described (Chen, Gong et al. 2001).

2.3.4 FISH

A dual-color probe for CLL PANEL 1 (ATM/p53) and a tri-color probe for CLL PANEL 2 (D13S319/13q34/CEP12), (Abbott Molecular, Inc., Des Plaines, IL) were used. Fluorescence in situ hybridization (FISH) was performed according to the manufacturer's instructions. The p53 probe corresponds to chromosomal region 17p13.1. The ATM probe corresponds to chromosome region 11q22.3. Chromosomal region 13q34 was detected by the D13S319 probe. Trisomy of chromosome 12 was detected by the CEP12 probe. Abnormalities of 17p present in ≥ 15% of nucleated cells were considered positive. Abnormalities of other probes (13q34, CEP12, and ATM) present in ≥ 10% of nucleated cells were considered positive.

2.3.5 Pharmacologic and genetic manipulation of Mcl-1 and Bfl-1

Flavopiridol was obtained from Sanofi-Aventis though the NCI Cancer Therapy Evaluation Program. All the ABT-737-resistant cell lines: Mec-1, Mec-2, PTA-3920, NCI-H929, and Nalm-6 were treated with flavopiridol for 4 hours

with a 300 nM concentration, which was reported to be effective in decreasing Mcl-1 levels (Yecies, Carlson et al. 2010). After the drug was removed, cells were then treated with 100 nM of ABT-737 (EC_{50} of sensitive cell lines) for 18 hours.

Mcl-1 and Bfl-1 knockdown in Mec-1 cells was achieved by shRNAs delivered with the use of lentiviral-mediated transduction. Cell populations with stable expression of shMcl-1, shBfl-1, and as control a scrambled shRNA (shControl) were selected by puromycin (Sigma). Lentiviral particles targeting three different regions of Mcl-1 (sc-35877-V), and Bfl-1 (sc-37285-V), and the scrambled shRNA (sc-108080) were obtained from Santa Cruz, CA.

2.3.6 RNA isolation and quantitative RT-PCR

Total RNA was isolated using the Trizol method (Invitrogen). Two µg of RNA were reverse transcribed using the TaqMan reverse transcription kit and amplified using the SYBR Green Master Mix (Applied Biosystems, N8080234 and 4309155) and examined on a 7500 Real-Time PCR system (Applied Biosystems). Quantitative, real-time reverse transcription polymerase chain reaction (RT-PCR) was performed as we have described previously (Crosby, Jacobberger et al. 2007) using the following specific primers: Bcl-B: 5'-GCTGGGATGGCTTTTGTCA-3' (forward) and 5'- GCCTGGACCAGCTGTTTTCTC-3' (reverse); Bcl-w: 5'-ACCCCAGGCTCAGCCCAACA-3' (forward) and 5'-CAGCACACAGTGCAGCCCCA-3' (reverse); Bcl-2 (Stecca, Mas et al. 2007): 5'-GGAGGATTGTGGCCTTCTTT-3' (forward) and 5'-

GGAGGATTGTGGCCTTCTTT-3’ (reverse); Bcl-xl (Aerbajinai, Giattina et al. 2003): 5’-GGATGGCCACTTACCTGA-3’ (forward) and 5’-GCCGTACAGTTCCACAAAGG-3’ (reverse); Bfl-1 (Yecies, Carlson et al. 2010): 5’-TTACAGGCTGGCTCAGGACT-3’ (forward) and 5’-AGCACTCTGGACGTTTTGCT-3’ (reverse); Mcl-1 (Yecies, Carlson et al. 2010): ATGCTTCGGAAACTGGACAT-3’ (forward) and 5’-TCCTGATGCCACCTTCTAGG-3’ (reverse); Bim (Nordigarden, Kraft et al. 2009): 5’-TGGCAAAGCAACCTTCTGATG-3’ (forward) and 5’-GCAGGCTGCAATTGTCTACCT-3’ (reverse); Noxa (Jiang, Lucas et al. 2008): 5’-TGGAAGTCGAGTGTGCTACTCAA-3’ (forward) and

5’-CAGAAGAGTTTGGATATCAGATTCAGA-3’ (reverse); Puma (Jiang, Lucas et al. 2008): 5’-GCATGCCTGCCTCACCTT-3’ (forward) and 5’-TCACACGTCGCTCTCTCTAAACC-3’ (reverse); Actin (Yecies, Carlson et al. 2010): 5’-AGAAAATCTGGCACCACACC-3’ (forward) and 5’-AGAGGCGTACAGGGATAGCA-3’ (reverse). Each experiment was repeated twice. The Bcl-b and Bcl-w primers were designed using the Primer Express (Applied Biosystems), and Primer 3 (LIST sequence) software, respectively. A lymphocyte sample set was used to establish a baseline comparison between mRNA levels in CLL and healthy donors. The extent of knockdown of Mcl-1 and Bfl-1 levels was determined by quantitative RT-PCR using the above primers as well as those suggested by the manufacturer (Santa Cruz, CA) for Mcl-1 (sc-35877-PR) and Bfl-1 (sc-37285-PR).

2.3.7 Statistical analysis

Baseline characteristics were grouped by *in vitro* culture sensitivity to ABT-737 treatment and compared between the resulting two groups using Fisher's exact test or *t*-test. Spearman correlation was used to assess the strength and direction of association between post-treatment cell viability and each Bcl-2 family member individually or in combination. The Cohen interpretation of correlation (r) indicates that when $r<0.3$ it represents weak correlation, 0.3-0.5 is moderate, and >0.5 is strong. Three groups were defined based on cell viability. Expression of Bcl-2 family members, individually or in combination were compared among the three groups using the Kruskal-Wallis test. Pairwise comparisons between groups were performed using the Wilcoxon rank sum test. A Bonferroni correction was used for pairwise comparisons. Pre- to post-treatment changes in Bcl-2 family transcripts were assessed using the Wilcoxon signed rank test. All statistical tests were two-sided. For pairwise comparisons, $p<0.017$ was used to indicate statistical significance. For all other comparisons, $p<0.05$ was used to indicate statistical significance.

2.4 Results

2.4.1 Primary CLL cells display differential sensitivity to ABT-737

To determine the sensitivity of CLL to ABT-737, freshly isolated primary CLL cells derived from the peripheral blood of patients were examined by flow cytometry for Annexin V staining as a cell death assay (Chen, Gong et al. 2001). The majority of primary CLL samples underwent apoptosis after treatment with ABT-737; however, there was a wide range of sensitivity, in agreement with earlier reports (Figure 2.1A) (Oltersdorf, Elmore et al. 2005; Mason, Khaw et al. 2009). Based on their response, primary CLL samples from 57 patients could be classified into three groups: (i) A sensitive group (33/57 cases, 57.8%) with cell viability less than 40%, (ii) An intermediate group (15/57, 26.3%) with viability of 40-65%, and (iii) A resistant group (9/57, 15.7%) with viability > 65%. Increasing the time exposures of ABT-737 from 18 to 36 hours did not change the sensitivity groups for the CLL samples (data not shown).

2.4.2 Baseline characteristics of patients with ABT-737 sensitive and resistant CLL are similar

Baseline characteristics of CLL patients in this study (Table 2.1, Figure 2.2A-B) were grouped by *in vitro* culture sensitivity to ABT-737 treatment. The median age was similar for both groups. The majority of patients in both the ABT-737 sensitive and resistant groups had early stage (Rai 0, I, or II) disease and had not been treated for their CLL prior to ABT-737 testing. The proportion of cases

positive for ZAP70 and CD38 by flow cytometry and commonly detected chromosomal abnormalities by fluorescence *in situ* hybridization (FISH) testing that are indicated do not differ significantly between the ABT-737 sensitive and resistant groups. ABT-737 sensitivity was similar for patients with early versus advanced stage of the disease (P = 0.7) (Figure 2.2A). It was also similar for common prognostic markers for CLL, such as ZAP70 (P = 0.72) or CD38 (P = 0.54) expression (Del Poeta, Maurillo et al. 2001; Rassenti, Huynh et al. 2004) (Figure 2.2B), prior treatment, 17p deletion (p53) (Dohner, Stilgenbauer et al. 2000; Seiler, Dohner et al. 2006), or 13q14 deletion (Table 2.1), consistent with previous findings (Del Gaizo Moore, Brown et al. 2007; Mason, Khaw et al. 2009; Vogler, Butterworth et al. 2009).

Table 2.1: Patients characteristics

Characteristic	Sensitive to ABT-737 (N = 33)		Resistant/intermediate to ABT-737 (N = 24)		P
	No. (%)	Value (range)	No. (%)	Value (range)	
Median age, y (range)		66.5 (48-87)		61.0 (50-77)	.53
	26 (78.8)	–	19 (79.1)	–	1.0
Sex, male/female	7 (21.2)	–	5 (20.8)	–	
Treatment for CLL before ABT-737 testing	10 (30.3)	–	8 (33.3)	–	1.0
FISH					
17p	2 of 14 (14.3)	–	2 of 11 (18.2)	–	1.0
11q	4 of 14 (28.6)	–	2 of 11 (18.2)	–	.66
13q14	8 of 14 (57.1)	–	7 of 11 (63.6)	–	1.0
Trisomy 12	2 of 13 (15.4)	–	1 of 10 (10)	–	1.0
13q34	1 of 13 (7.7)	–	3 of 11 (27.3)	–	.30

P values are indicated based on using Fisher exact test (categorical variables) or the Student *t* test (continuous variables).
– indicates not applicable; and FISH, fluorescence in situ hybridization.

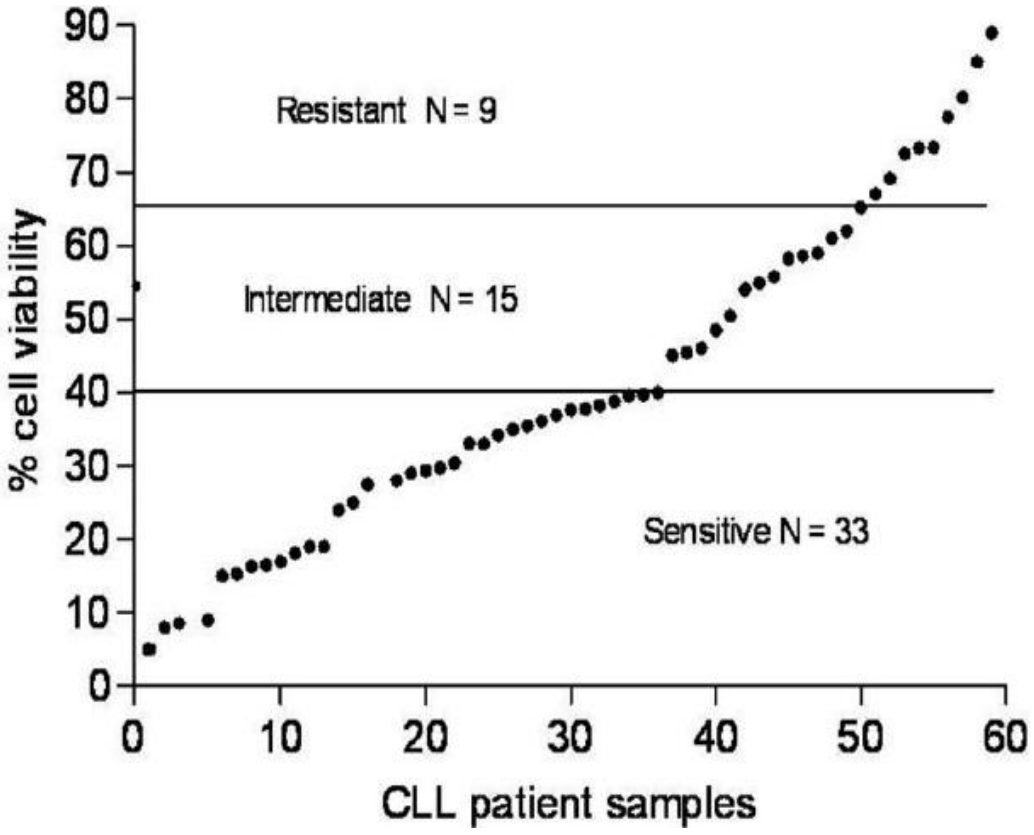


Figure 2.1. A wide-range of responses to ABT-737 in primary CLL. Primary CLL cells derived from 57 patients were incubated with ABT-737 (50 nM) for 18 hours. Cell survival was assessed by Annexin V/FITC binding, as determined by flow cytometric analysis. Values are normalized to survival of untreated cells and each data point represents one CLL patient. Based on cell viability data, the response to ABT-737 was classified into: resistant (> 65%), intermediate (40% to 65%), and sensitive (< 40%).

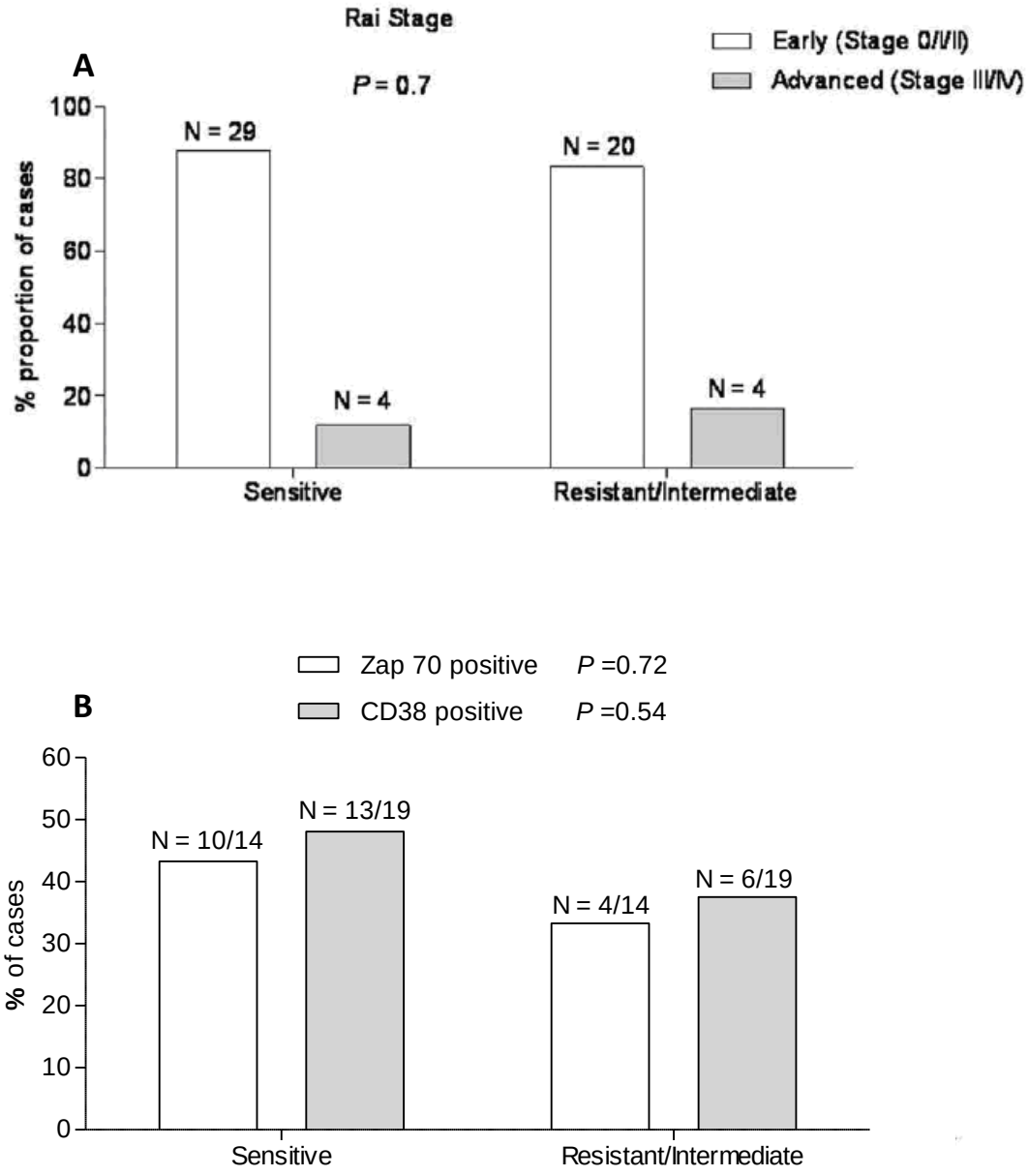


Figure 2.2. Responses to ABT-737 in primary CLL is independent of known prognostic markers. The proportion of CLL patients in the resistant/intermediate and sensitive groups respectively was examined with regard to the common CLL prognostic markers: (A) Rai stage and (B) ZAP70, CD38 expression. *P*-values are shown for comparison of groups.

2.4.3 Lack of correlation between expression of Bcl-2 family members, individually or combined, and their response to ABT-737

To investigate the possible contribution of Bcl-2 family members to ABT-737 response, we determined their expression levels. Of the nine Bcl-2 family members that we have examined, we focused first on the six anti-apoptotic members since ABT-737 was designed to target anti-apoptotic proteins and we reasoned that their differential expression could define ABT-737 sensitivity. Expression of each individual anti-apoptotic transcript or their combinations in untreated CLL was plotted against cell viability following ABT-737 treatment (Figure 2.3). No linear correlation was found between expression levels of any single anti-apoptotic Bcl-2 family member ($r < 0.3$, $P > 0.05$) or their combination ($r < 0.3$, $P > 0.05$), considering those with either high affinity (Bcl-2, Bcl-w, and Bcl-xl) or those with low affinity (Mcl-1, Bfl-1, and Mc-1) to ABT-737 (Figure 2.3). Next, we examined the expression of pro-apoptotic BH3-only Bcl-2 family members Noxa, Bim, and Puma. Their mRNA levels were relatively high in most of the tested CLL (data not shown). Nevertheless, no significant correlation was found between expression levels of any of these pro-apoptotic Bcl-2 family members, taken individually ($r < 0.3$, $P > 0.05$) or in combination ($r < 0.3$, $P > 0.05$) and ABT-737 sensitivity.

Moreover, the six mRNAs were expressed at relatively similar levels in all the healthy donors when they normalized to each other (Figure 2.4A). However, normalizing all anti-apoptotic Bcl-2 family to β-actin revealed that Mcl-1 levels were more abundant in normal lymphocytes compared to Bfl-1, Bcl-b, and Bcl-w,

which were expressed at very low levels ((Figure 2.4b), note logarithmic scale used to accommodate these large differences). In contrast, Bcl-b, Bfl-1, and Bcl-w were highly upregulated compared to Mcl-1 in primary CLL.

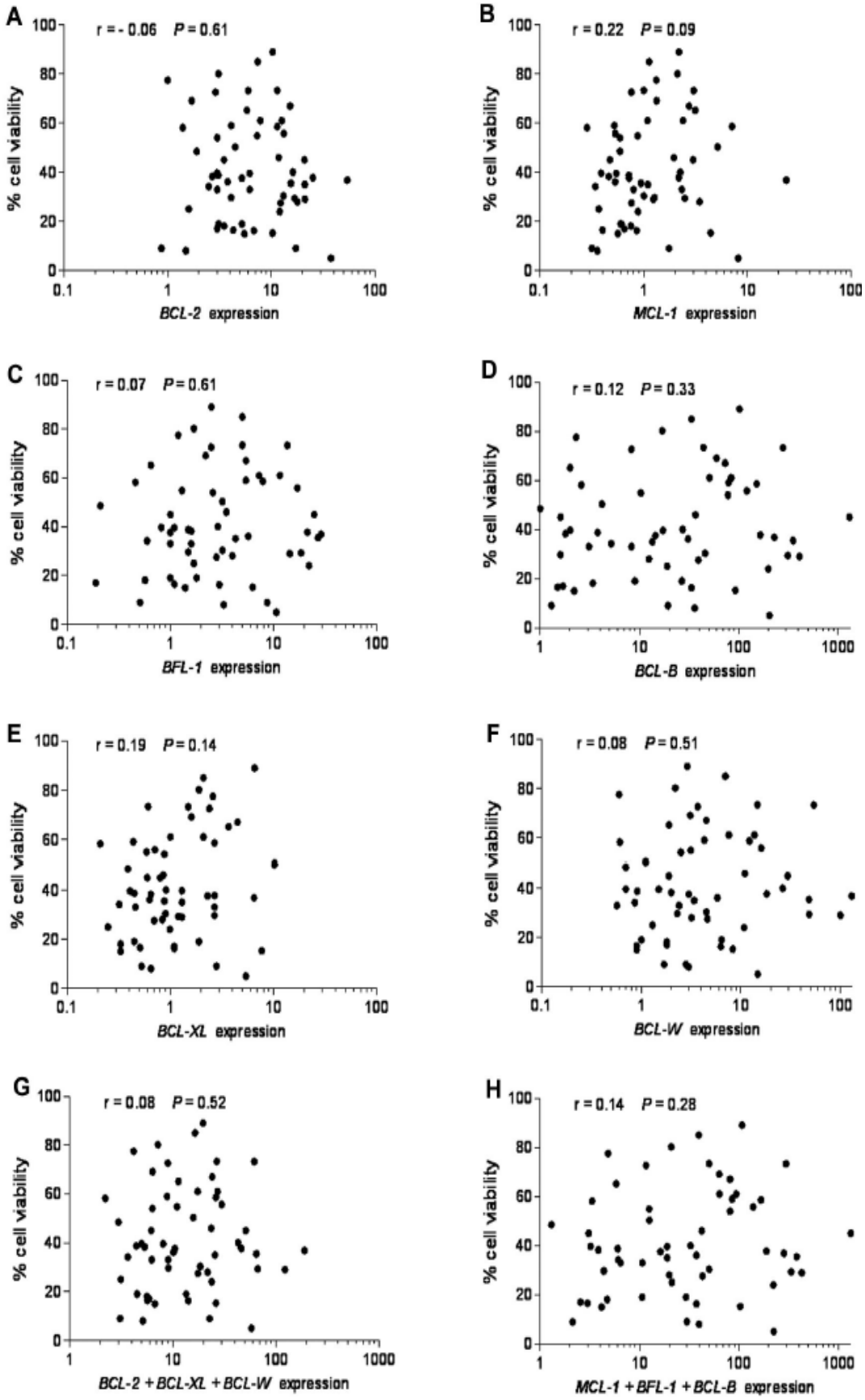

A
r = - 0.06 P = 0.61
% cell viability
BCL-2 expression
B
r = 0.22 P = 0.09
% cell viability
MCL-1 expression
C
r = 0.07 P = 0.61
% cell viability
BFL-1 expression
D
r = 0.12 P = 0.33
% cell viability
BCL-B expression
E
r = 0.19 P = 0.14
% cell viability
BCL-XL expression
F
r = 0.08 P = 0.51
% cell viability
BCL-W expression
G
r = 0.08 P = 0.52
% cell viability
BCL-2 + BCL-XL + BCL-W expression
H
r = 0.14 P = 0.28
% cell viability
MCL-1 + BFL-1 + BCL-B expression

Figure 2.3. Lack of correlation between mRNA expressions of anti-apoptotic Bcl-2 family members, individually or combined, and response to ABT-737. Quantitative RT-PCR was used to study anti-apoptotic Bcl-2 family mRNAs. Expression values of each anti-apoptotic transcript or their combinations were plotted against cell viability following ABT-737 treatment. RNA expression for each transcript was normalized to that obtained in lymphocytes isolated from six healthy donors. (A) Bcl-2, (B) Mcl-1, (C) Bfl-1, (D) Bcl-b, (E) Bcl-xl, (F) Bcl-w, (G) Bcl-2 + Bcl-xl + Bcl-w, and (H) Mcl-1 + Bfl-1 + Bcl-b expression. Spearman correlation (*r*) and *P*-values are shown.

2.4.4 An anti-apoptotic Bcl-2 family index predicts ABT-737 sensitivity

Because each anti-apoptotic transcript was expressed at different basal levels in the lymphocytes from healthy donors (Figure 2.4B), we next standardized the expression of the six mRNAs to one basal level to provide a better method of comparison in CLL cells. Normalization to β-actin revealed the most abundant anti-apoptotic determinants in untreated primary CLL (Figure 2.5). This approach guided us to identify the critical determinants of the ABT-737 sensitivity. The expression levels of Mcl-1, Bcl-2, and Bfl-1 were much higher compared to those of Bcl-xl and Bcl-w. The sensitive group had the highest levels of Bcl-2 as compared to expression of Mcl-1 and Bfl-1. The index mean was 0.45 for Bcl-2, 0.40 for Mcl-1, and 0.21 for Bfl-1**.** In contrast, compared to Bcl-2, the intermediate group had relatively higher levels of Mcl-1 and Bfl-1, while the resistant group had much higher levels of Mcl-1 and Bfl-1. In the intermediate group, the index mean was 0.25 for Bcl-2, 0.37 for Mcl-1, and 0.21 for Bfl-1. In the resistant group, the index mean was 0.21 for Bcl-2, 0.48 for Mcl-1, and 0.19 for Bfl-1. Next, we examined the expression of pro-apoptotic BH3-only Bcl-2 family members Noxa, Bim, and Puma. Noxa levels were the highest compared to those of Bim and Puma in the CLL samples examined (data not shown). Thus Noxa levels were 3.5 and 6 fold higher than those of Bim and Puma, respectively (data not shown).

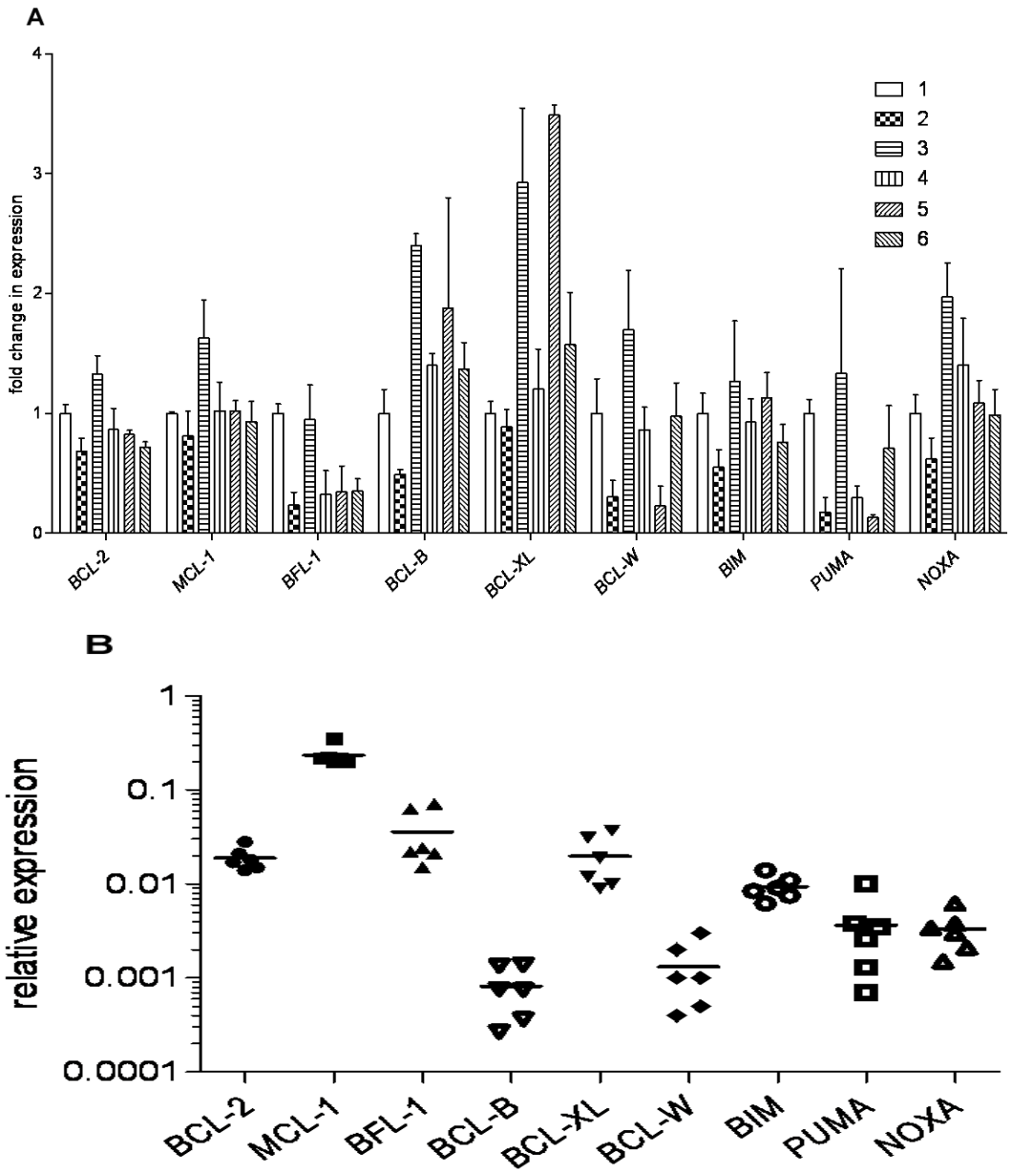
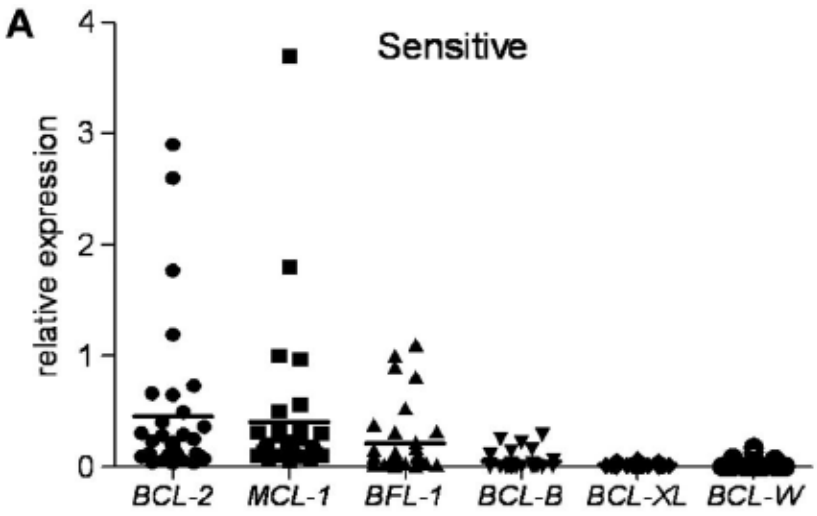
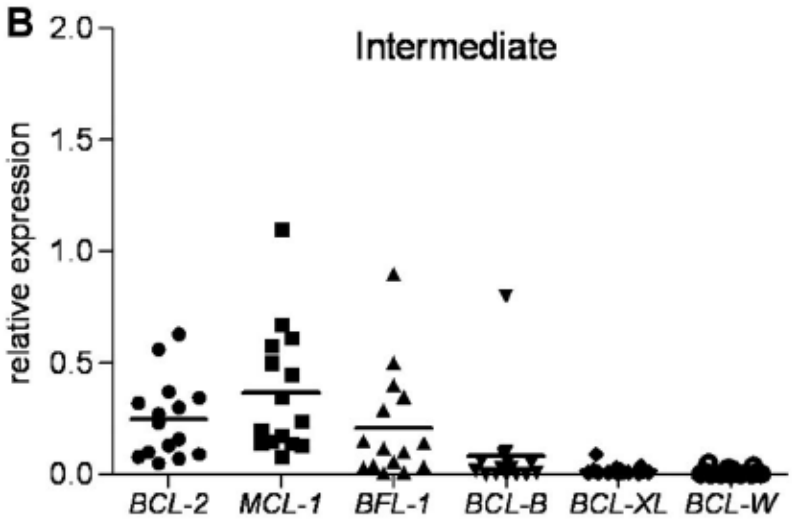


Figure 2.4. Bcl-2 family mRNA expression in lymphocytes isolated from healthy donors. (A) mRNA levels of Bcl-2 family members determined by quantitative RT-PCR normalized to those of the first healthy donor show similar expression for each Bcl-2 family transcript. (B) Bcl-2 family members have different basal levels normalized to β-actin. Note the logarithmic scale used to reveal the large differences in the expression levels.


A
Sensitive
relative expression
4
3
2
1
0
BCL-2
MCL-1
BFL-1
BCL-B
BCL-XL
BCL-W


B
Intermediate
relative expression
2.0
1.5
1.0
0.5
0.0
BCL-2
MCL-1
BFL-1
BCL-B
BCL-XL
BCL-W

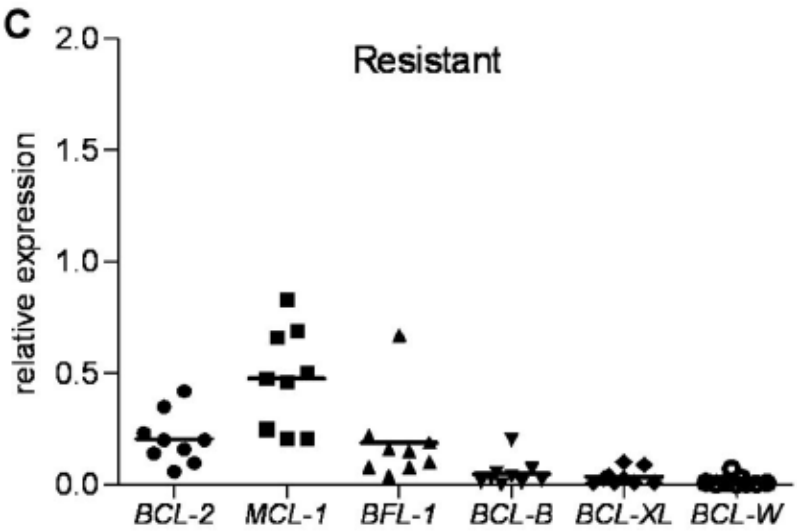

C
Resistant
relative expression
2.0
1.5
1.0
0.5
0.0
BCL-2
MCL-1
BFL-1
BCL-B
BCL-XL
BCL-W

Figure 2.5. Anti-apoptotic Bcl-2 family mRNA levels in ABT-737 sensitive, intermediate, and resistant CLL groups. RNA expression of anti-apoptotic Bcl-2 family members, as determined by quantitative RT-PCR, was normalized to β-actin for each CLL patient sample. Bcl-2, Mcl-1, and Bfl-1 levels were more abundant than those of Bcl-b, Bcl-xl, and Bcl-w in primary CLL. (A) The sensitive group. (B) The intermediate group. (C) The resistant group. Mean values are shown.

In order to define an anti-apoptotic gene expression index predictive of ABT-737 sensitivity, we plotted several combinations of genes relative to cell viability. The relative ratio of Mcl-1 and Bfl-1 to Bcl-2 expression provided the best linear correlation with ABT-737 sensitivity (r = 0.6, $P < 0.001$) (Figure 2.6A). By comparison, the relative ratios of Mcl-1/Bcl-2 (Figure 2.6B) or Bfl-1/Bcl-2 (Figure 2.6C) showed a less robust linear correlation: (r = 0.45, $P < 0.001$), (r = 0.28, $P = 0.030$), respectively. The relative ratio of Bcl-b/Bcl-2 (Figure 2.6D) showed no significant correlation (r = 0.16, $P = 0.22$). Including Bcl-b, Bcl-xl, (Figure 2.6E-F), or Bcl-w (data not shown) into the (Mcl-1 + Bfl-1)/Bcl-2 model had no significant effect on this correlation. The different ratios of anti-apoptotic Bcl-2 family mRNAs were determined in the resistant, intermediate, and sensitive groups (Figure 2.7). Of the different anti-apoptotic Bcl-2 family combinations evaluated, (Mcl-1 + Bfl-1)/Bcl-2 provided the highest predictive value for the ABT-737 response in CLL (Figure 2.7A), with $P < 0.001$ among all the three groups. In the sensitive class (average cell viability 26.2%), the average ratio of (Mcl-1 + Bfl-1)/Bcl-2 was 1.67. For the intermediate class (average cell viability 53.5%), the average ratio was 2.3. In the resistant class (average cell viability, 76.3), the average ratio was 3.3 (Table 2.2).

Table 2.2: The (Mcl-1/Bfl-1)/Bcl-2 index in CLL and SCLC cell lines

	Resistant	Intermediate	Sensitive
CLL	3.3 (9/57); 15.7%	2.3 (15/57); 26.3%	1.67 (33/57); 57.9%
SCLC	2.8 (4/22); 18.1%	2.1 (7/22); 31.8%	1.7 (11/22); 50%

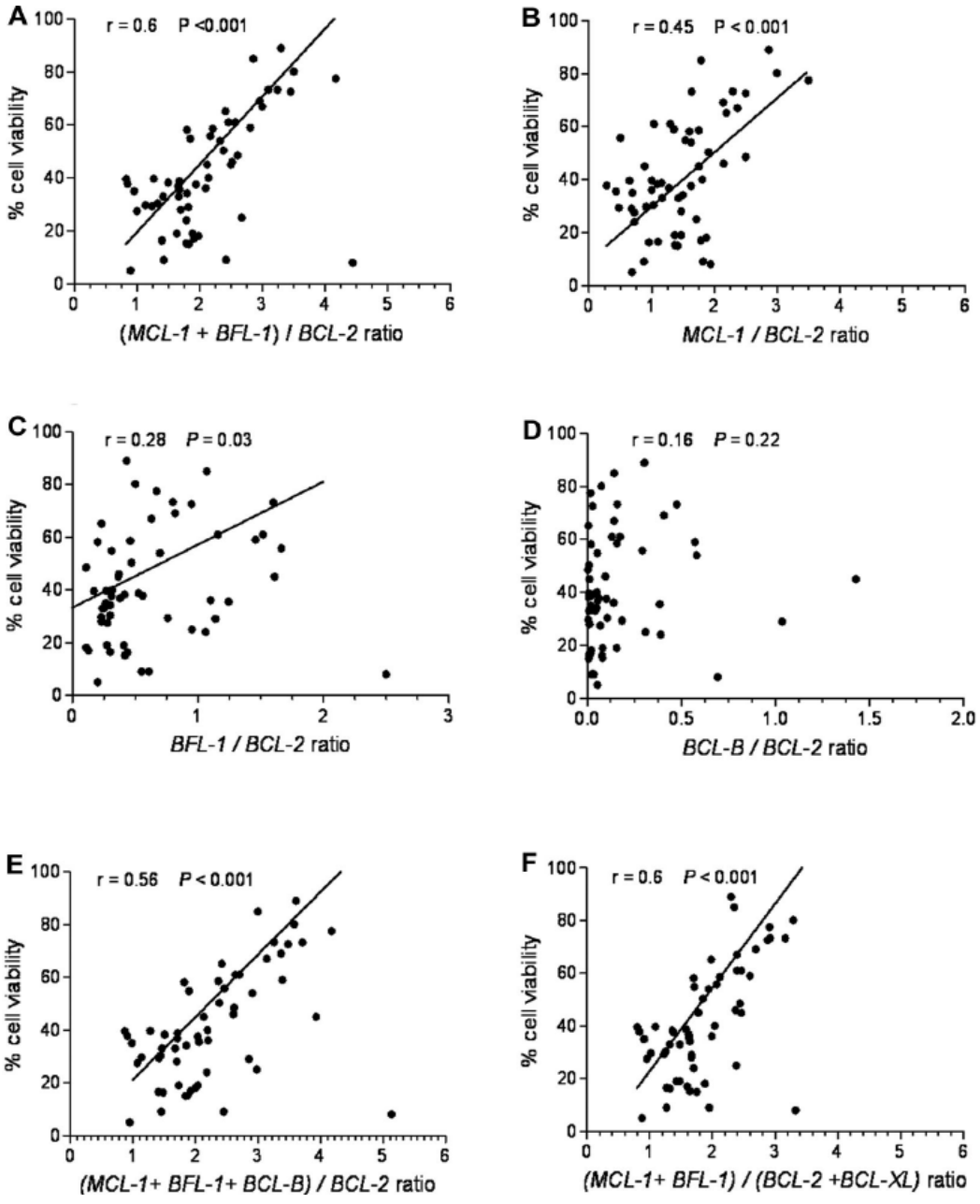


Figure 2.6. (Mcl-1 + Bfl-1)/Bcl-2 provides the most significant linear correlation for sensitivity to ABT-737. Cell viability after ABT-737 treatment was plotted against: (A) (Mcl-1+Bfl-1)/Bcl-2, (B) Mcl-1/Bcl-2, (C) Bfl-1/Bcl-2, (D) Bcl-b/Bcl-2, (E) (Mcl-1 + Bfl-1 + Bcl-b)/Bcl-2, and (F) (Mcl-1+Bfl-1)/(Bcl2+Bcl-xl) ratios. Spearman correlation (*r*) and *P*-values are shown.

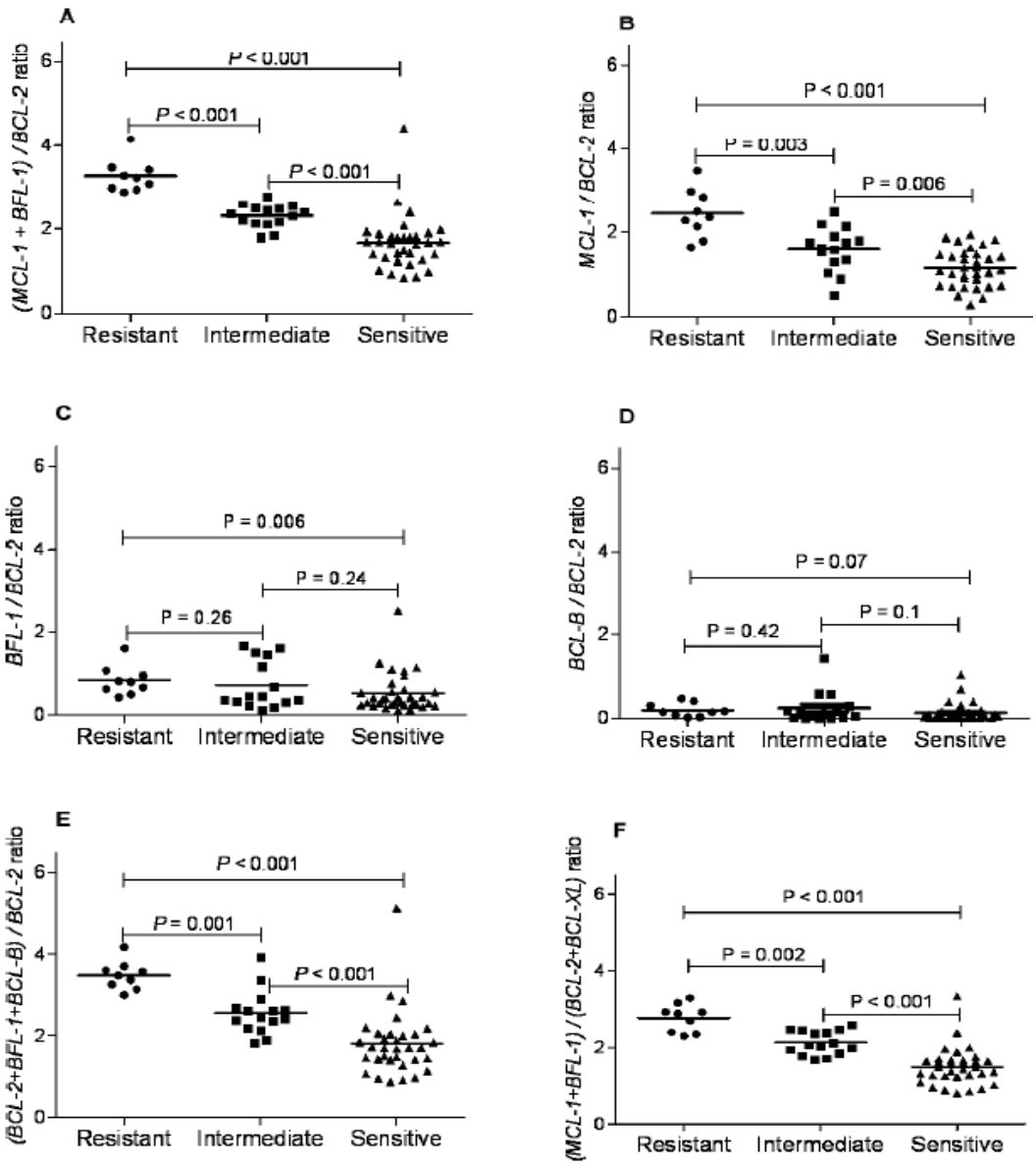


Figure 2.7. The relative ratio of (Mcl-1 + Bfl-1)/Bcl-2 represents the most informative predictive marker for the ABT-737 sensitivity. Relative expression values for: (A) (Mcl-1 + Bfl-1) / Bcl-2, (B) Mcl-1/Bc-l2, (C) Bfl-1/Bcl-2, (D) Bcl-b / Bcl-2, (E) (Mcl-1 + Bfl-1 + Bcl-b)/Bcl-2, and (F) (Mcl-1 + Bfl-1)/(Bcl-2 + Bcl-xl), (Mcl-1 + Bfl-1)/Bcl-2 are the most significant among the sensitive, intermediate, and resistant groups. *P*-values are indicated for comparison between groups.

2.4.5 The (Mcl-1 + Bfl-1) / Bcl-2 ratio is predictive of ABT-737 response in leukemic and lung carcinoma cells

To further address the generality of our predictive model in leukemic cells, the (Mcl-1 + Bfl-1) / Bcl-2 ratio was determined in a panel of leukemic tumor cell lines (Table 2.3), (Figure 2.8). Of the nine cell lines tested, five (NCI-H929, Nalm-6, Mec-1, Mec-2, and PTA-3920) were most resistant to ABT-737. They all had a very high (Mcl-1 + Bfl-1) / Bcl-2 ratio, ranging from 6.4 to 17.5, with EC_{50} > 1000 nM. Two other cell lines, Reh and Molt-4 displayed an intermediate sensitivity to ABT-737 (EC_{50}= 300 and 400) having a ratio of 2.5 and 2.3, respectively. IM-9 and Jurkat were the most sensitive (EC_{50} ≤100 nM) and had ratios of 1.2 and 1.8, respectively (Table 2.3). These data indicate that the (Mcl-1 + Bfl-1) / Bcl-2 ratio is predictive for the ABT-737 response in a broad range of leukemic tumor cells, both primary and established**.**

Table 2.3: The (Mcl-1 + Bfl-1) / Bcl-2 ratio in leukemic cell lines

Cell line	Response to ABT-737	(Mcl-1 + Bfl-1)/Bcl-2 ratio
NCI-H929	Resistant	17.5
Nalm-6	Resistant	12.5
MEC-1	Resistant	9.6
MEC-2	Resistant	9.4
PTA-3920	Resistant	6.4
Reh	Intermediate	2.5
MOLT-4	Intermediate	2.3
JURKAT	Sensitive	1.8
IM-9	Sensitive	1.2

The resistant group had an $EC_{50} > 1000nM$ for ABT-737, the intermediate group had an $EC_{50} = 300$ and 400nM, and the sensitive group had an $EC_{50} \leq 100nM$.

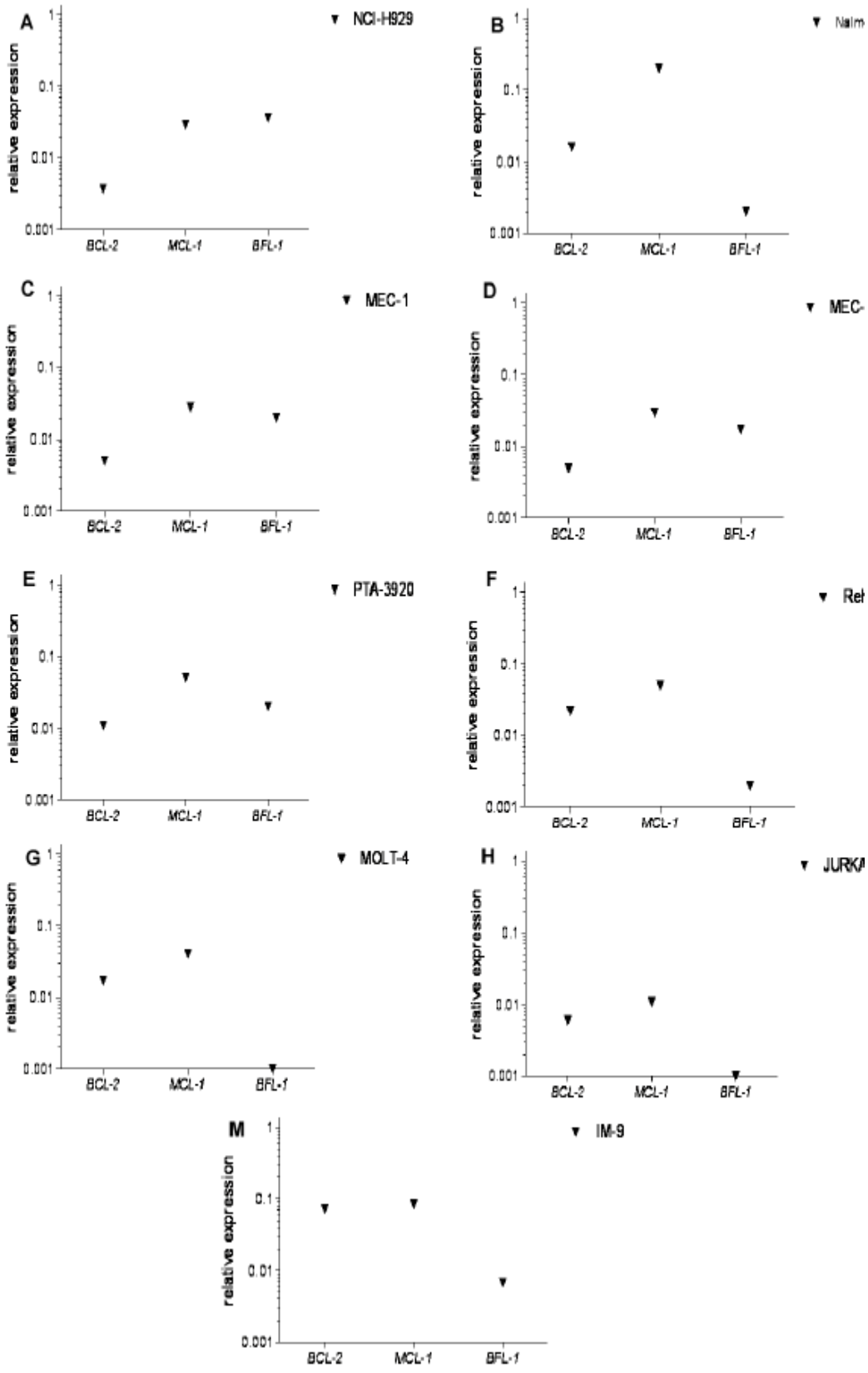

A
NCI-H929
relative expression
1
0.1
0.01
0.001
BCL-2
MCL-1
BFL-1
B
Naim
C
MEC-1
D
MEC-
E
PTA-3920
F
Rel
G
MOLT-4
H
JURK
M
IM-9

Figure 2.8. Relative expression of Bcl-2, Mcl-1, and Bfl-1 in a panel of leukemic cell lines. Bcl-2, Mcl-1, and Bfl-1 mRNA expression relative to β-actin in (A) NCI-H929, (B) Nalm-6, (C) Mec-1, (D) Mec-2, (E) PTA-3920, (F) Reh, (G) Molt-4, (H) Jurkat, and (I) IM-9 cells.

Next, we tested if our model can also be applied to cancer types other than of hematological origin. Small cell lung carcinoma (SCLC) is a solid tumor that has been reported to have a high expression level of anti-apoptotic proteins, such as Bcl-2. As navitoclax (ABT-263) is evaluated in clinical trials in SCLC patients, we reasoned that it would be informative to test whether our model can predict ABT-737 response in SCLC cell lines. By using ONCOMINE (Rhodes, Yu et al. 2004) as a cancer microarray database aimed to facilitate discovery from genome-wide expression analyses, we applied our model in SCLC cell lines previously categorized as ABT-737 resistant, intermediate, and sensitive groups (Olejniczak, Van Sant et al. 2007). We found that in the sensitive group the average ratio of Mcl-1 and Bfl-1 to Bcl-2 was 1.7, with the intermediate and resistant groups having a ratio of 2.1 and 2.8, respectively (Table 2.2). Interestingly, these ratios were very close and consistent with our findings in CLL patients. Of the different anti-apoptotic Bcl-2 family combinations evaluated, the (Mcl-1 + Bfl-1) /Bcl-2 ratio provided the highest predictive value for ABT-737 response in SCLC ($r=0.73$, $P < 0.001$) (Figure 2.9).

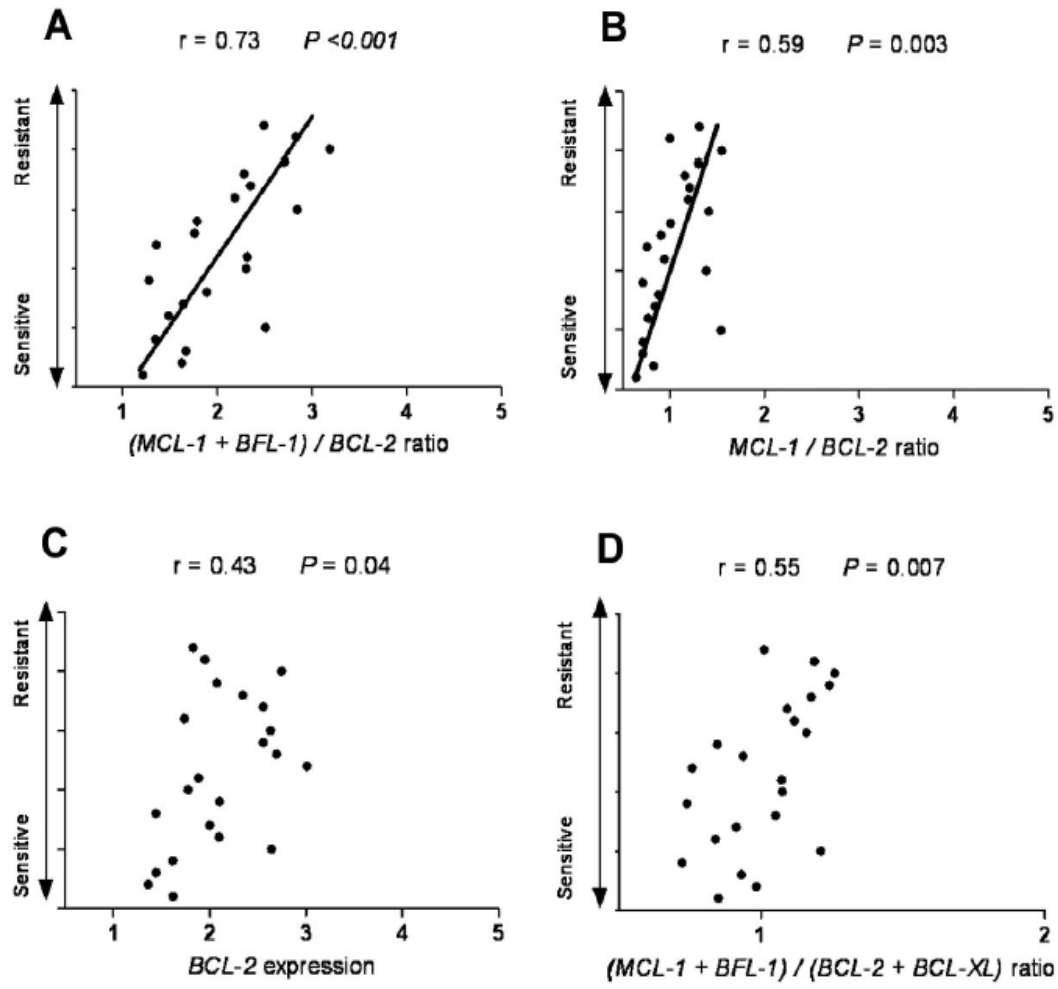


Figure 2.9. The (Mcl-1 + Bfl-1)/Bcl-2 represents the most significant linear correlation for sensitivity to ABT-737 in small cells lung carcinoma (SCLC). SCLC cell lines with a wide range of response (resistant, intermediate, and sensitive) to ABT-737 were plotted against: (A) (Mcl-1+Bfl-1)/Bcl-2, (B) Mcl-1/Bcl-2, (C) Bcl-2, (D) (Mcl-1+Bfl-1)/(Bcl-2+Bcl-xl) ratios. Spearman correlation (r) and *P*-values are shown. The Olejniczak dataset was used as extracted from the ONCOMINE database (Rhodes, Yu et al. 2004).

2.4.6 Decreased Mcl-1 and/or Bfl-1 levels enhance ABT-737 response in resistant CLL cell lines

The five cell lines that we found to be highly resistant to ABT-737 had also a high ratio of Mcl-1 and Bfl-1 to Bcl-2. We examined whether a decrease in Mcl-1 and/or Bfl-1 levels by a clinically relevant approach may improve ABT-737 response in resistant cell lines. Flavopiridol, which as a CDK9 inhibitor was shown to decrease Mcl-1 at transcriptional level, also demonstrated a high response rate in a phase II clinical trials in relapsed CLL patients (Lin, Ruppert et al. 2009). All ABT-737 resistant cell lines were treated with flavopiridol and then the Bcl-2, Mcl-1, and Bfl-1 levels were determined by quantitative RT-PCR (Figure 2.10A; only data for CLL cell lines are shown). A four-hour flavopiridol treatment decreased Mcl-1 and Bfl-1 levels, with little effect on Bcl-2 levels. Following pre-treatment with flavopiridol, the (Mcl-1 + Bfl-1)/ Bcl-2 predictive index values changed in all five ABT-737 resistant cell lines, shifting from highly resistant to an index similar to what we have established for the sensitive CLL group (Table 2.4). To determined whether this shift of the (Mcl-1 + Bfl-1) / Bcl-2 ratio by flavopiridol can convert the resistant cell lines to the sensitive group. Following flavopiridol pre-treatment and removal, cells were treated with ABT-737 for 18 hours (Figure 2.10B). There was a significant increase in cell death when cells were pretreated with flavopiridol prior to ABT-737 treatment as compared to single treatments with ABT-737 or flavopiridol for 18 hours (data for NCI-H929 and Nalm-6 are not shown).

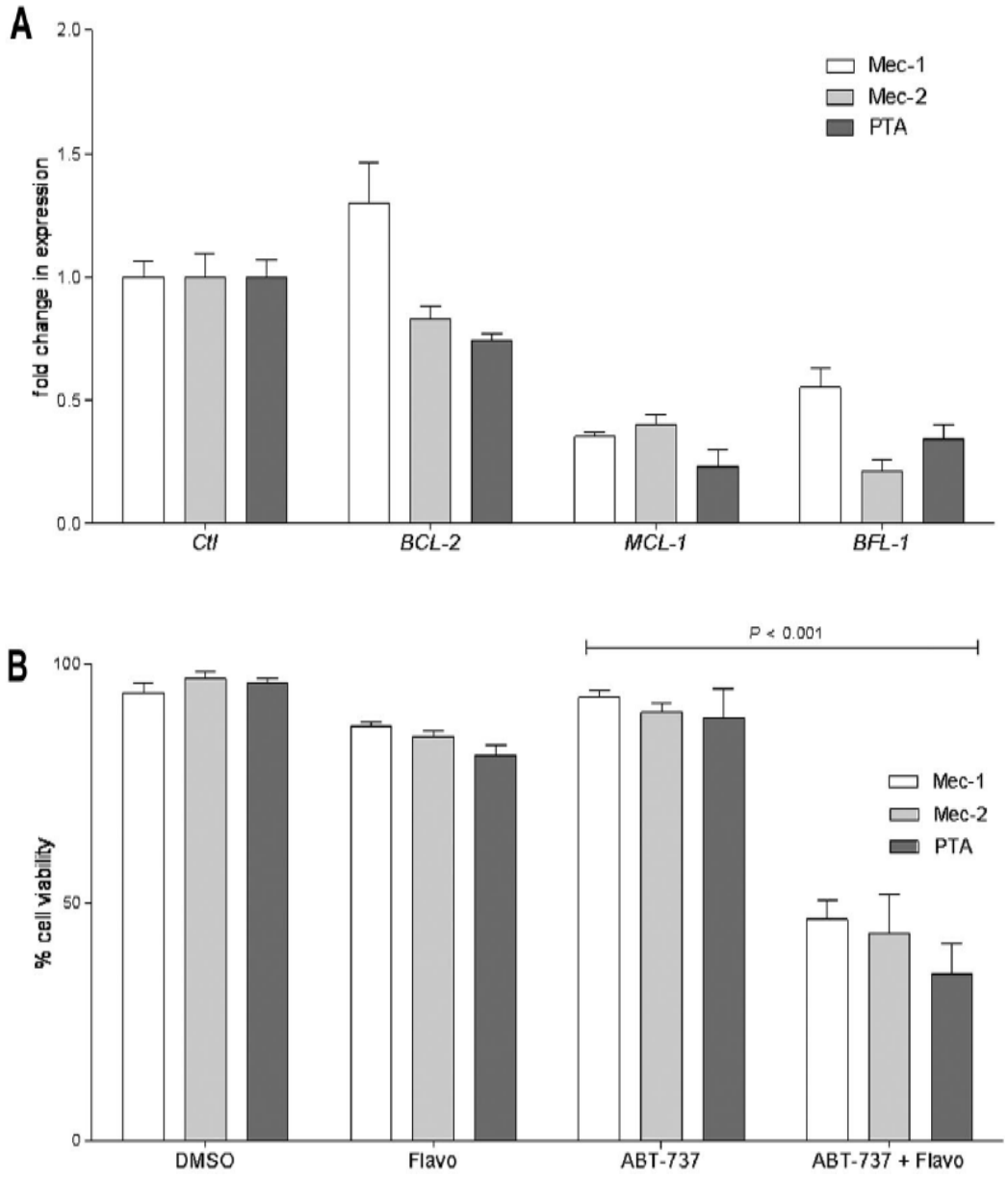


Figure 2.10. Flavopiridol decrease Mcl-1 and Bfl-1 levels and increase the response to ABT-737 in resistant CLL cell lines. (A) Mec-1, Mec-2, and PTA CLL cells were treated with flavopiridol for four hours and then Bcl-2, Mcl-1 and Bfl-1 levels were assessed by quantitative RT-PCR. (B) Mec-1, Mec-2 and PTA-3920 were treated with flavopiridol for four hours, then the drug was removed and cells were cultured without or with ABT-737(100 nM) for an additional 18 hours.

Cell viability was assayed by annexin-V–FITC and PI staining and analyzed by flow cytometry. Values are normalized to untreated cells and DMSO treatment served as a negative control. *P* values were determined by the 2-tailed *t*-test. The graphs presented are representative of two independent experiments with data shown as mean ± s.d.

Table 2.4. The (Mcl-1+Bfl-1)/Bcl-2 in ABT-737–resistant cell lines after treatment with flavopiridol for 4 hours.

Cell lines	(*MCL-1* + *BFL-1*)/*BCL-2*	(*MCL-1* + *BFL-1*)/*BCL-2* after flavopiridol treatment
Mec-1	9.5	1.3
Mec-2	9.4	1.7
PTA-3920	6.4	1.8
Nalm-6	12.5	2
NCI-H929	17.5	3.3

To further validate the pharmacologic inhibitor data, we applied a targeted genetic approach for reducing Mcl-1 or Bfl-1, using shRNAs transduced with lentivirus particles. Mec-1 was chosen since it is a highly ABT-737-resistant CLL cell line that has high Mcl-1 and Bfl-1 levels. Examining knockdown of Mcl-1 and Bfl-1 by quantitative RT-PCR indicated an ~ 50% and 45% reduction in Mcl-1 and Bfl-1 levels in shMcl-1 and shBfl-1-expressing Mec-1 cells, respectively (Figure 2.11A). Estimating the index in these two cell lines indicated that knockdown of Mcl-1 or Bfl-1 by shRNA reduced the index from 9.6 in parental cells to 3.4 in shMcl-1 and 3.8 in shBfl-1-expressing Mec-1 cells, respectively. Treating shMcl-1 and shBfl-1-expressing cells with ABT-737 increased cell death significantly as compared to parental Mec-1 cell or those expressing as a control scrambled shRNA (Figure 2.11B).

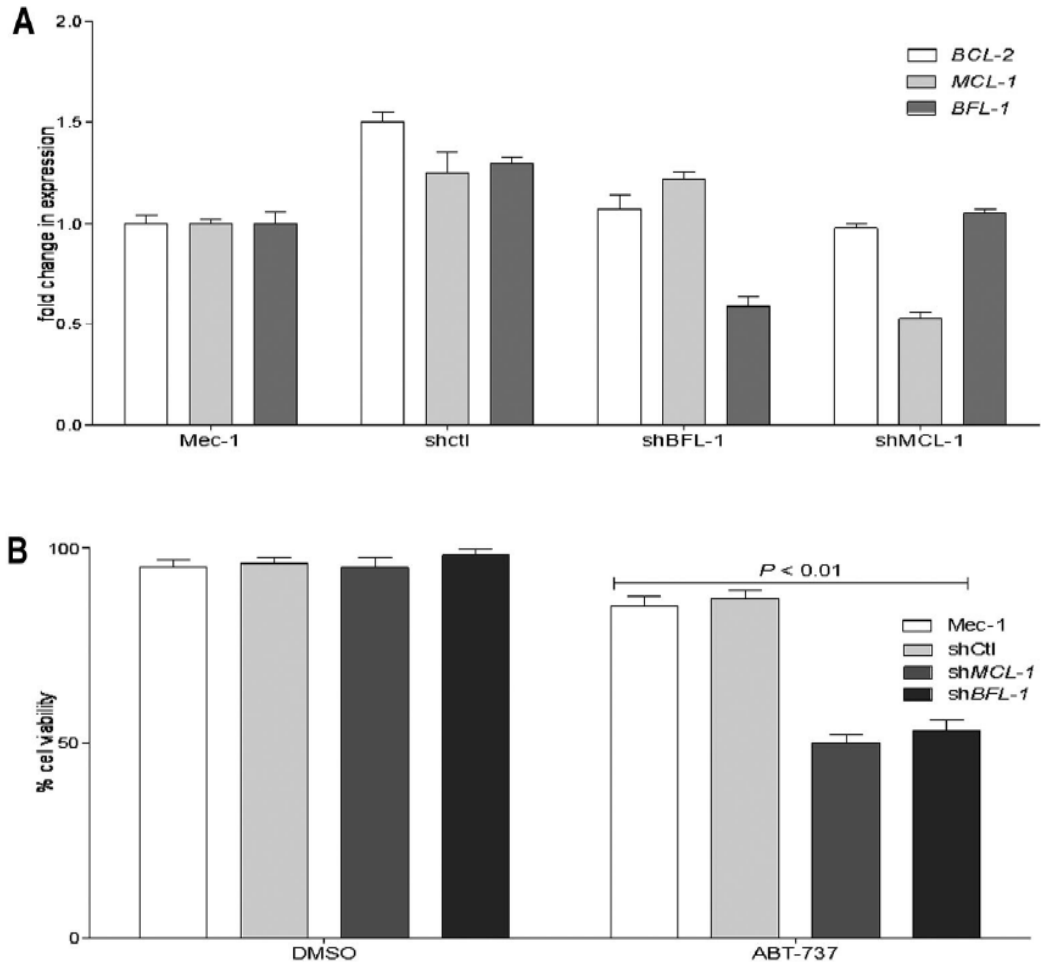


Figure 2.11. ShRNA decrease Mcl-1 and Bfl-1 levels and increase the response to ABT-737 in resistant CLL cell lines. (A) Quantitative RT-PCR analyses of Bcl-2, Mcl-1, and Bfl-1 levels in Mec-1 parental, shMcl-1, shBfl-1, and as a control, scrambled shRNA (shControl)-expressing cells. (B) Mec-1 parental, shControl, shMcl-1, and shBfl-1-expressing cells were treated with ABT-737 (1000 nM) for 18 hours. Cell viability was assayed by annexin-V–FITC and PI staining and analyzed by flow cytometry. Values are normalized to untreated cells and DMSO treatment served as a negative control. *P* values were determined by the 2-tailed *t*-test. The graphs presented are representative of two independent experiments with data shown as mean ± s.d.

2.4.7 Modulation of anti-apoptotic Bcl-2 family mRNA expression following ABT-737 treatment

As chemotherapeutics are known to modulate the expression of Bcl-2 family members in hematologic tumors, we next examined the changes in expression of anti-apoptotic Bcl-2 family mRNAs after ABT-737 treatment. In most primary CLL samples tested there was a significant decrease in Bcl-2 ($P < 0.001$) and Mcl-1 ($P = 0.003$) mRNA expression (Figure 2.12 A-B). In contrast, Bfl-1, Bcl-b, and Bcl-w expression levels were highly upregulated following ABT-737 treatment (Figure 2.12 C-E). Bfl-1 levels were upregulated 9-fold, ($P < 0.001$), with Bcl-w levels increased 6.5-fold ($P < 0.001$). Bcl-b levels were increased more than 10-fold following ABT-737 treatment ($P < 0.001$). There were no significant changes in Bcl-xl levels ($P = 0.26$) (Figure 2.12 F).

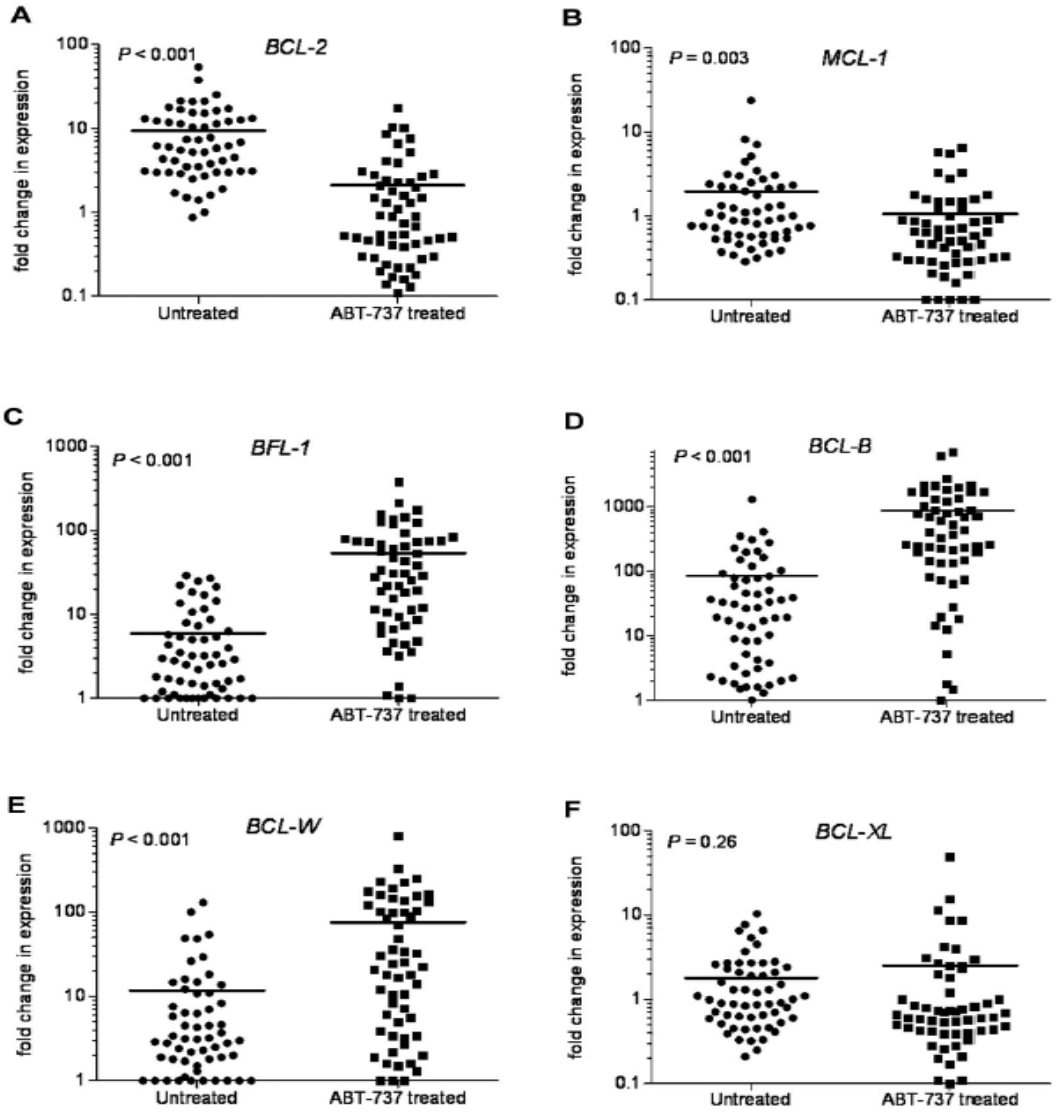


Figure 2.12. Changes in the levels of anti-apoptotic Bcl-2 family transcripts following ABT-737 treatment. Changes in mRNA expression levels of anti-apoptotic Bcl-2 family proteins after treatment with ABT-737 (50 nM) for 18 hours. Expression was determined by quantitative RT-PCR to compare fold difference in expression to levels prior to treatment that were normalized to those found in lymphocytes isolated from six healthy donors. Fold change for: (A) Bcl-2, (B) Mcl-1, (C) Bfl-1, (D) Bcl-b, (E) Bcl-w, and (F) Bcl-xl levels. *P*-values are shown.

2.5 Discussion

ABT-737 was rationally designed to bind selectively to the anti-apoptotic proteins Bcl-2, Bcl-xl, and Bcl-w that are known to be overexpressed in lymphoid malignancies (Kitada, Andersen et al. 1998; Pepper, Hoy et al. 1998). There is no available marker that directly predicts the sensitivity to ABT-737 based on anti-apoptotic protein expression levels. The inconsistency of reports on the correlation of the expression of anti-apoptotic proteins with the ABT-737 response might be due to the difficulty in detecting some of them by Western blotting in primary cells (Del Gaizo Moore, Brown et al. 2007; Mason, Khaw et al. 2009; Vogler, Butterworth et al. 2009). This may explain the emphasis that has been placed on individual anti-apoptotic proteins, such as Bcl-2 and Mcl-1, with no extensive studies available on the contribution of Bfl-1 and Bcl-b to the ABT-737 response in primary CLL. Moreover, the primary use of Western blotting in most of these studies further limited the development of quantitative approaches.

Here, we report that the anti-apoptotic Bcl-2 family mRNAs are expressed at different basal levels in lymphocytes from healthy donors. Therefore, comparing the basal expression levels of the anti-apoptotic transcripts in CLL to healthy donors could be misleading. In contrast, normalizing these mRNAs to β-actin provided an equal scale for comparison of their expression in untreated primary CLL and further establishes their contribution to the ABT-737 response. We found that Mcl-1, Bcl-2, Bfl-1, and Bcl-b transcripts were more abundant than

those of Bcl-xl and Bcl-w. However, Mcl-1, Bfl-1, and Bcl-2 appear to be the most critical anti-apoptotic factors that account for ABT-737 sensitivity. Applying RT-PCR as a highly sensitive and quantitative assay allowed us to establish a quantitative comparison between the expression levels of Bcl-2 family members. Bcl-2, Mcl-1, and Bfl-1 expression were higher in cells sensitive to ABT-737, with Bcl-2 expression being the highest. These findings may explain why the sensitivity to ABT-737 in primary CLL was correlated with high Mcl-1 levels in previous reports (Vogler, Butterworth et al. 2009).

In the moderately sensitive CLL group, Mcl-1 and Bfl-1 levels were higher than those of Bcl-2, whereas in the resistant group Mcl-1 and Bfl-1 expression were much higher compared to those of Bcl-2. Our findings reveal that individual or even combined levels of Mcl-1 and Bfl-1 could not predict ABT-737 sensitivity without accounting for their relative ratio to Bcl-2. This likely reflects the molecular abundance of Mcl-1, Bfl-1, and Mcl-1, as well as the cellular balance between low and high affinity ABT-737-binding proteins. Importantly, our model directly addresses the wide range of ABT-737 responses in primary CLL that were reported previously (Oltersdorf, Elmore et al. 2005; Mason, Khaw et al. 2009). The anti-apoptotic Bcl-2 family expression index in a panel of leukemic tumor cell lines was consistent with our CLL data. This model was also validated in SCLC as another tumor type. Recent data from a phase I study of navitoclax (ABT-263) has shown that a high Bcl-2 copy number in circulating tumor cells was associated with the best tumor response in SCLC patients.

Remarkably, our predictive model showed a higher correlation with ABT-737 response than expression of Bcl-2 alone in these SCLC cell lines based on data extracted from the ONCOMINE database (Rhodes, Yu et al. 2004). There are currently 17 clinical trials (http://clinicaltrials.gov/, 06-13-2011) with the Bcl-2-family inhibitor navitoclax, the oral derivative of ABT-737, for CLL, other hematologic malignancies, and solid tumors for which our predictive model could also become informative.

We also studied the expression of pro-apoptotic BH3-only Bcl-2 members Noxa, Bim, and Puma. These pro-apoptotic members are most likely to be sequestered by anti-apoptotic proteins as the primed for cell death model predicts (Deng, Carlson et al. 2007). This model posits that cells expressing significant amounts of BH3-only proteins, such as Bim, must sequester them with anti-apoptotic proteins to stay alive. The sensitive CLL group had increased levels of Noxa, Bim, and Puma. However, as some resistant and intermediate groups also had high Noxa, Bim, and Puma levels, we could not establish a predictive value for a BH3-only index and ABT-737 response in the CLL samples we have examined. Nevertheless, it is possible that including their expression in a Bcl-2 family index may help to predict clinical response to other therapeutics or in other tumor types.

We have applied both a clinically relevant pharmacologic (flavopiridol) as well as a genetic (shRNA) approach to reduce Mcl-1 and Bfl-1 levels to improve

the ABT-737 response in the resistant cell lines. shRNA-expressing cells, in which expression of either Mcl-1 or Bfl-1 was down regulated, were treated with a higher ABT-737 concentration as compared to flavopiridol in response to which both Mcl-1 and Bfl-1 levels were decreased. In addition, there was some compensation by an increase in the levels of alternative apoptosis-resistance determinants, Mcl-1 in shBfl-1 and Bfl-1 in shMcl-1-expressing cells. Nevertheless, these data indicate that clinical agents that reduce Mcl-1 and Bfl-1 levels can be used in combination with navitoclax to maximize their efficacy.

Following ABT-737 treatment, we observed induction of Bfl-1, Bcl-b, and Bcl-w expression and reduction of Bcl-2 and Mcl-1 levels in most CLL samples. Bfl-1 and Bcl-b are known to have very low binding affinity for ABT-737; therefore, their increased levels suggests that these proteins may account for resistance as a result of long-term ABT-737 exposure, as a published report indicates (Yecies, Carlson et al. 2010). Here we show that Bfl-1 levels can be upregulated following ABT-737 treatment in primary CLL not only, as previously reported, as a result of co-culture of primary CLL with fibroblasts that mimics the lymph node environment (Vogler, Butterworth et al. 2009). As this is the first report that reveals the elevated Bcl-b levels following ABT-737 treatment, this finding suggests a potential novel role for Bcl-b in developing ABT-737 resistance in CLL patients.

In summary, we have applied RT-PCR as a highly sensitive and quantitative assay to examine Bcl-2 family transcripts in primary CLL to develop a unique predictive marker for ABT-737 sensitivity. Our model provides highly predictive power for ABT-737 sensitivity in primary CLL. This approach requires a small number of primary cells and allows the study of comparative expression of all relevant genes. It can also identify whether CLL patients are dependent on Mcl-1/Bfl-1 or on Bcl-2 as Mcl-1-dependent leukemic cells have been reported to be more chemo-sensitive than those that are Bcl-2 dependent (Brunelle, Ryan et al. 2009). Establishing this dependency may guide appropriate ABT-737 administration for future development of novel combinations of ABT-737 with other targeted agents.

Acknowledgments

We thank Drs. Saul Rosenberg and Steven Elmore (Abbott Laboratories) for ABT-737. This work was supported by a research grant from National Institutes of Health (CA127264 to A. A) and Saudi Arabia Education Ministry Fellowship (to S.A). Elutriation was supported in part by NIH/NCRR, Cleveland CTSA UL1RR024989.

CHAPTER III

BCL-XL EXPRESSION IS A MARKER OF SHORT TIME TO TREATMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA

Sayer Al-harbi et al.

3.1 Abstract

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the western world. Whereas some patients may require immediate therapy and survive only months, others may live indefinitely untreated. The heterogeneous clinical course of CLL indicates the need for a prognostic marker that can be used to predict disease progression and allow better management of the disease. Common prognostic markers, such as p53, ZAP70, and CD38 are useful in CLL, but they fail to predict clinical outcome in a substantial number of CLL patients and limited usefulness in guiding therapeutic decisions. The anti-apoptotic Bcl-2 proteins are overexpressed in lymphoid malignancies, including chronic lymphocytic leukemia. While extensive studies have focused on the role of Bcl-2 or Mcl-1 in CLL, nothing is known about the role of other anti-apoptotic proteins and their contribution to clinical outcome in CLL patients. Therefore, we used RT-PCR as a highly quantitative and sensitive assay to assess expression of all

known anti-apoptotic Bcl-2 family genes to study their contribution to treatment free survival in CLL. Our finding indicate that only high Bcl-xl expression is strongly correlate (P=0.002) with short treatment-free survival. Strikingly, Bcl-xl was able to identify patients with high risk in the ZAP70 negative and CD38 negative groups as well as in patients with normal/unknown p53 showing a very powerful prognostic value in CLL.

3.2 Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world (Lee, Dixon et al. 1987). CLL patients have very wide overall survival; whereas some patients may survive for months, others may live for many years (Zwiebel and Cheson 1998; Chiorazzi, Rai et al. 2005). This heterogonous clinical course led to the search for a prognostic marker that can predict disease progression. The identification of such markers would enable individualized counseling and better management of the disease. Immunoglobulin heavy chain variable-region (VH) gene mutation status and zeta-associated protein 70 (ZAP70) and CD38 have been proposed to be prognostic markers in CLL (Damle, Wasil et al. 1999; Hamblin, Davis et al. 1999; Orchard, Ibbotson et al. 2004; Rassenti, Huynh et al. 2004). Despite their clinical value, there are some technical difficulties involved in these markers such as labor intensive, standardization and modification following chemotherapy (Shanafelt, Byrd et al. 2006; Van Bockstaele, Verhasselt et al. 2009). In addition, p53 deletion (del 17p)

is a well-established marker associated with short survival and chemoresistance. However, p53 deletion accounts only for a small percentage of CLL patients (Dohner, Stilgenbauer et al. 2000; Bottcher, Ritgen et al. 2012); One study found the prevalence of p53 mutation in a large CLL cohort to be less than 8% of the cases (Zenz, Krober et al. 2008; Gonzalez, Martinez et al. 2011). Overall, these established prognostic markers fail to predict clinical outcome in a considerable number of CLL patients (Bottcher, Ritgen et al. 2012). Moreover, it is difficult to combine the results of these variables markers to asses the risk in individual patients (Shanafelt, Byrd et al. 2006). Thus, additional prognostic markers for CLL patients are of interest.

Blocked apoptosis is a hallmark of treatment-resistant cancers and thus, may highlight the potential application of Bcl-2 family members as prognostic markers (Letai 2008; Hanahan and Weinberg 2011). In fact, several studies have linked Bcl-2 family expression and response to chemotherapy in different type of cancers. It has been recently reported that patients with cancers highly primed to cross the apoptotic threshold exhibited superior clinical responses to chemotherapy (Ni Chonghaile, Sarosiek et al. 2011). However, in CLL, high Bcl-2 and Mcl-1 levels have been reported to mediate resistance to chlorambucil, fludarabine, and rituximab (Thomas, Pepper et al. 2000; Hussain, Cheney et al. 2007; Pepper, Lin et al. 2008). Another report finds Mcl-1-dependent leukemias are more sensitive to a wide range of chemotherapeutic agents than Bcl-2 dependent (Brunelle, Ryan et al. 2009). In addition, we have recently shown that

relative expression of Mcl-1 and Bfl-1 to Bcl-2 predicts ABT-737 response in hematological cells, including CLL and solid malignancies (Al-Harbi, Hill et al. 2011). Nevertheless, while numerous studies have focused on the role of Bcl-2 or Mcl-1 in CLL, nothing is known about the role of other anti-apoptotic proteins and their contribution to clinical outcome in CLL patients. Here, we used RT-PCR as a highly quantitative and sensitive assay to assess expression of all anti-apoptotic genes, including Bcl-2, Mcl-1, Bcl-xl, Bcl-w, Bfl-1, Bcl-b and pro-apoptotic Bim, Puma and Noxa to study their contribution to treatment free survival in 76 CLL patients. Our data indicate that Bcl-xl was the only anti-apoptotic member to demonstrate a very strong correlation with treatment-free survival. Interestingly, Bcl-xl was able to identify patients with high risk in ZAP70 negative, CD38 negative groups as well as the p53 unmutated/unknown group, which indicates a superior and independent prognostic value.

3.3 Methods

3.3.1 Purification of primary CLL cells

Peripheral blood samples from 76 patients with CLL were obtained with the patients' informed consent according to protocols approved by the Cleveland Clinic Institutional Review Board according to the Declaration of Helsinki. All primary CLL cells were freshly processed without freezing. Lymphocytes were purified by Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation.

3.3.2 Flow cytometry

It has been previously described in 2.3.3 (Al-Harbi, Hill et al. 2011).

3.3.3 FISH

It has been previously described in 2.3.4

3.3.4 RNA isolation and quantitative RT-PCR

It has been previously described in 2.3.6 (Al-Harbi, Hill et al. 2011).

3.3.5 Statistical analysis

Cox-proportional hazard models were used to identify significant survival dependencies via slope coefficients differing significantly from zero. To graph these dependencies, Kaplan-Meier methods were applied with covariates dichotomized at their medians. P values shown in such plots were obtained using the log rank test. Two sample t-tests were used to compare Bcl-xl levels between low and high levels of ZAP70, CD38 and p53. All computations were performed in R.

3.4 Result and discussion

Baseline characteristics of the 76 CLL patients studied are summarized in (Table 3.1). We have used RT-PCR as a quantitative assay to assess expression all anti-apoptotic Bcl-2 family genes: Bcl-2, Mcl-1, Bcl-xl, Bcl-w, Bcl-b and Bfl-1 as well as pro-apoptotic members: Bim, Puma and Noxa. We tested the correlation of expression level of each member with treatment-free survival (Figure 3.1 and 3.2). To test the impact of each member on the treatment-free survival, the patients were categorized into two subsets, high (above) and low (below) the median expression value for each Bcl-2 family member. We also examined the combined expression of all anti-apoptotic members, all pro-apoptotic members, and the ratio of these, with treatment-free survival. From all these analyses only Bcl-xl expression emerged as significant and correlate with treatment-free survival (Figure 3.1A). Interestingly, Bcl-xl expression did not significantly correlate with Rai stage (Figure 3.3), CD38, ZAP70, and commonly detected chromosomal abnormalities (Table 3.1), indicating that Bcl-xl expression is independent of known CLL prognostic markers. We also assessed the correlation of common CLL prognostic markers: p53, CD38 and ZAP70 with treatment-free survival. Patients with p53 deletion also show very strong correlation with treatment-free survival, where ZAP70 and CD38 failed to show any correlation with treatment free-survival (Figure 3.4).

Interestingly, we have noticed that some patients in ZAP70 negative, CD38 negative, normal/unknown p53 categories also experience rapid progression and aggressive disease (Figure 3.4). This suggests that some patients in these categories are still at high risk, and the current prognostic markers may be limited in identifying those patients. Therefore, we wanted to test the ability of Bcl-xl to identify patients with high risk within ZAP70 negative, CD38 negative, and p53 normal/unknown categories. Surprisingly, Bcl-xl was able to identify patients with high risk in the ZAP70 negative and CD38 negative groups as well as patients with normal/unknown p53 showing a very strong prognostic value in CLL (Figure 3.5).

As Bcl-xl provides a comprehensive and powerful prognostic marker in CLL, it was not surprising that Bcl-2 and Mcl-1 failed to show significance in treatment-free survival since other studies report similar findings (Grever, Lucas et al. 2007; Pepper, Lin et al. 2008). There are conflicting reports on the significance of ZAP70 and CD38 in predicting the clinical outcome in CLL (Grever, Lucas et al. 2007). Lack of method standardization following chemotherapy have been associated with the use of these markers (Shanafelt, Byrd et al. 2006; Sheikholeslami, Jilani et al. 2006; Van Bockstaele, Verhasselt et al. 2009). Despite of these shortcomings, ZAP70, and CD38 are often performed as part of the diagnostic evaluation in CLL patients. An interesting finding of this study is that Bcl-xl was able to identify high-risk patients within ZAP70-negative and CD38-negative groups. More importantly, the prevalence of p53 deletion or mutation has been reported in only a small proportion of CLL patients.

Remarkably, Bcl-xl was able to mark patients with high risk in p53 undeleted/unknown group, which may indicate Bcl-xl as ideal prognostic marker in this group.

The role of bcl-xl and survival has not been investigated in CLL; however recent reports show that Bcl-xl is essential for lymphoma development (Kelly, Grabow et al. 2011). In support of our findings, the resistance to a group of compounds that repress Mcl-1 expression was correlated with high mRNA expression of Bcl-xl, indicating that patients-selection strategy for development of any Mcl-1 inhibitor in breast and non-small cells lung carcinoma (NSCLC) tumors should be based on patients with low Bcl-xl expression (Wei, Margolin et al. 2012). Resistance to 122 standard chemotherapy agents was also correlated with high Bcl-xl expression in the NCI 60 cell line panel (Amundson, Myers et al. 2000) which suggest its unique role in mediating general chemotherapy resistance.

In summary, in an extensive study that quantify all anti-apoptotic genes by using RT-PCR as a simple, highly sensitive, cost effective technique, we propose Bcl-xl as an independent prognostic marker that can predict high risk in diverse CLL patients.

Table 3.1: Baseline characteristic of CLL patients

	All Patients		Bcl XL < median (n=38)		Bcl XL > median (n = 38)		P
	Number	Value (range)	Number	Value (range)	No. (%)	Value (range)	
Age, median (range), years		64 (38 -90)		64.5 (38-90)		64 (48-87)	N.S.
Sex, male/female	52/24		22/16		30/8		0.08
Rai Stage	N = 72						
Early (Stage 0/I/II)	58		33 of 35		23 of 37		0.064
Advanced (Stage III/IV)	16		2 of 35		13 of 37		0.61
Zap70 positive	19 of 44		12 of 24		7 of 20		0.703
CD38 positive	22 of 54		9 of 26		13 of 26		0.324
FISH							
17p	7 of 35		1 of 19		6 of 16		0.235
11q	6 of 35		4 of 19		2 of 16		0.666
13q14	22 of 35		12 of 18		10 of 17		0.733
Trisomy 12	6 of 35		2 of 17		4 of 16		0.398

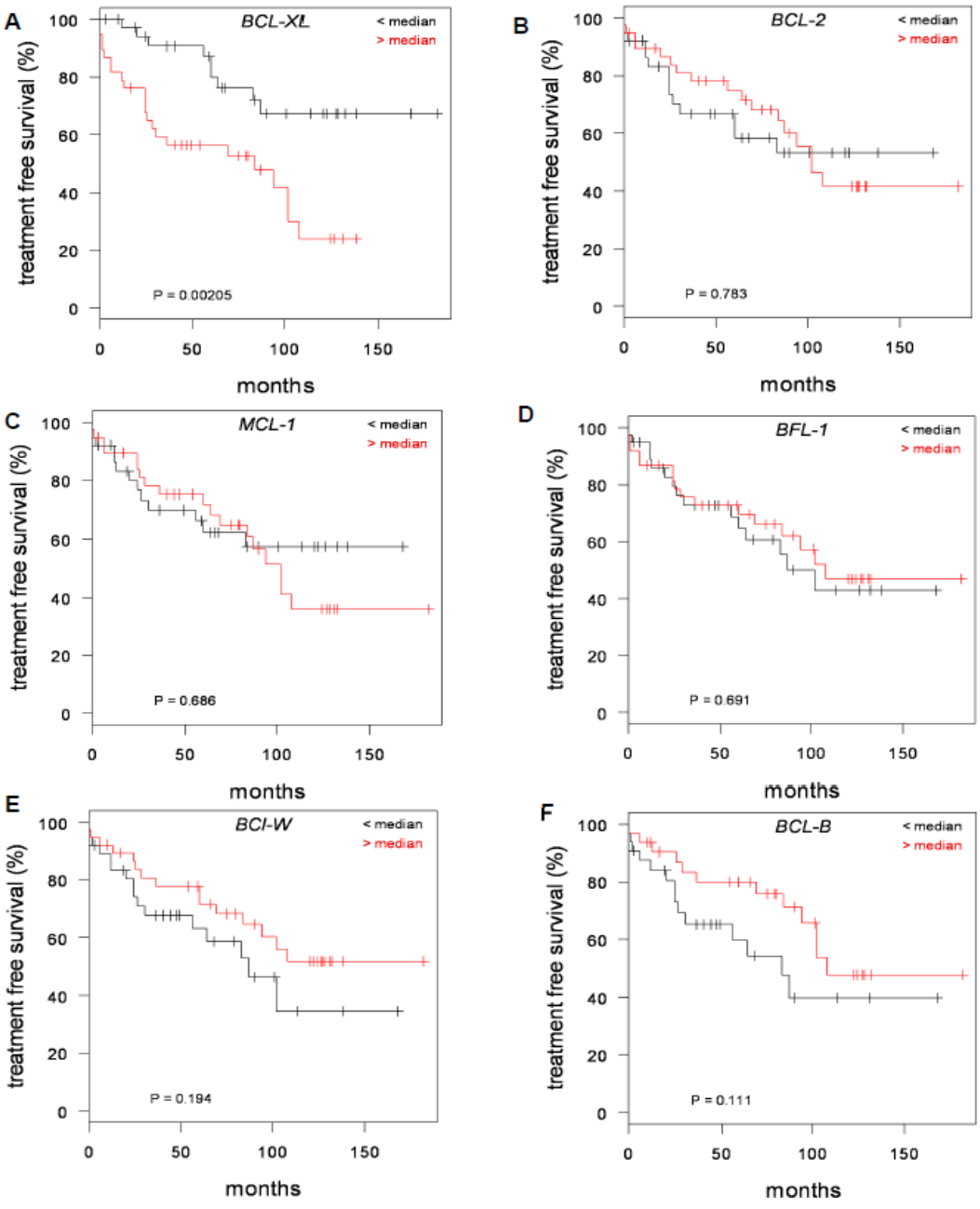


Figure 3.1: Kaplan-Meier curves for correlation of treatment-free survival with anti-apoptotic Bcl-2 expression levels. (A) BCL-XL, (B) BCL-2, (C) MCL-1, (D) BFL-1, (E) BCL-W, (F) BCL-B.

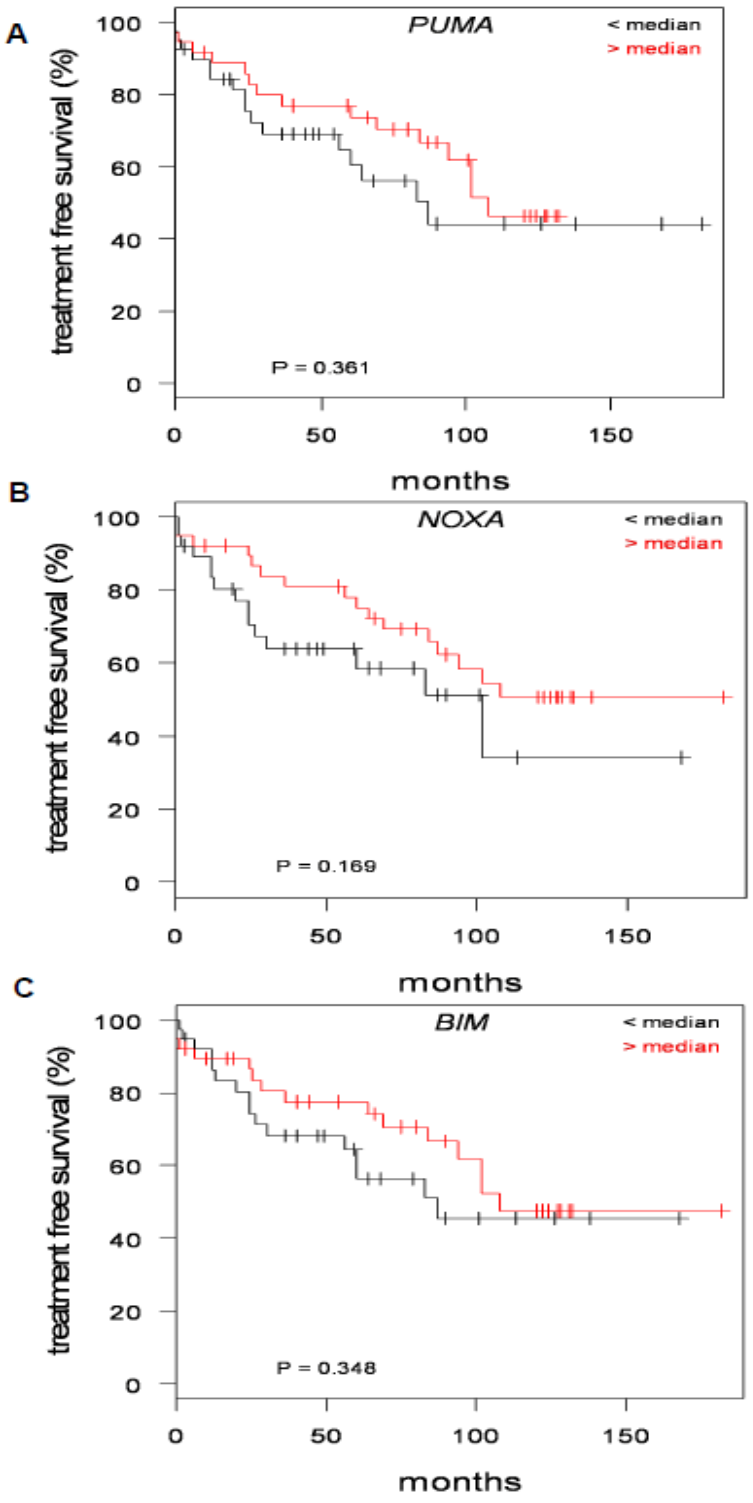


Figure 3.2: Kaplan-Meier curves for correlation of treatment-free survival with pro-apoptotic Bcl-2 expression levels. (A) PUMA, (B) NOXA, (C) BIM.

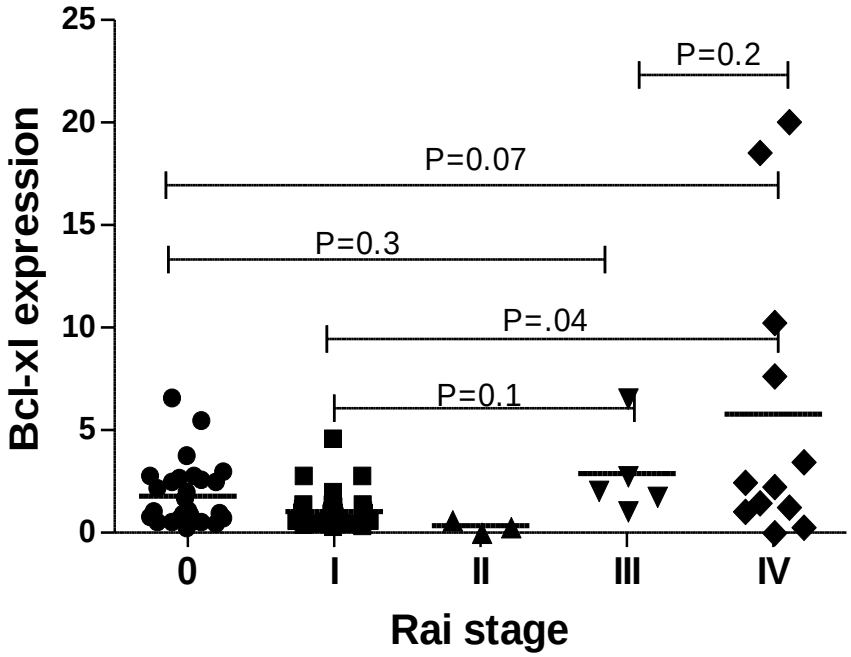


Figure 3.3: BCL-XL expression is independent of Rai stage. RNA expression of BCL-XL, as determined by quantitative RT-PCR in 0, I, II, III, and IV stages. P-values are indicated for comparison between groups.

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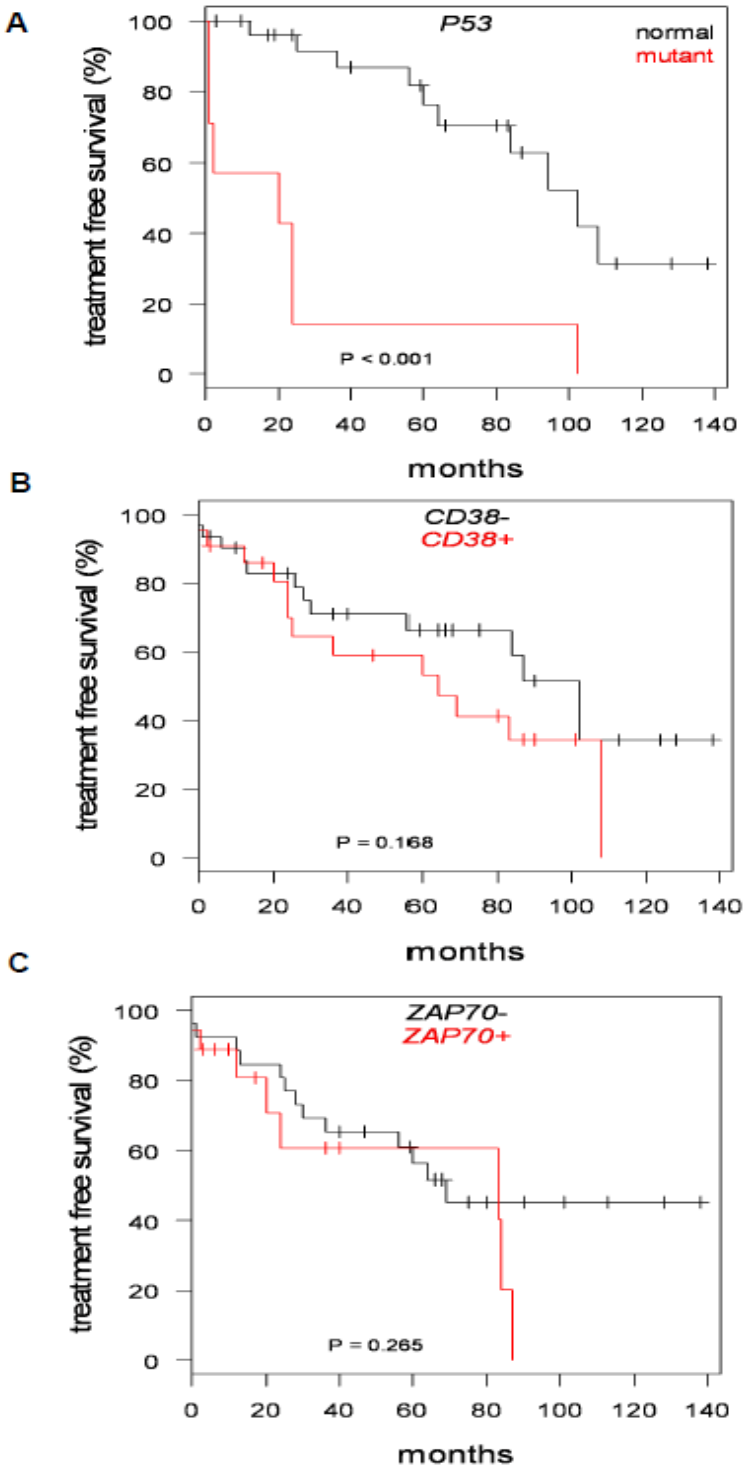

Figure 3.4: Kaplan-Meier curves for correlation of treatment-free survival with CLL prognostic markers. (A) 17p deletion, (B) CD38, (C) ZAP70.

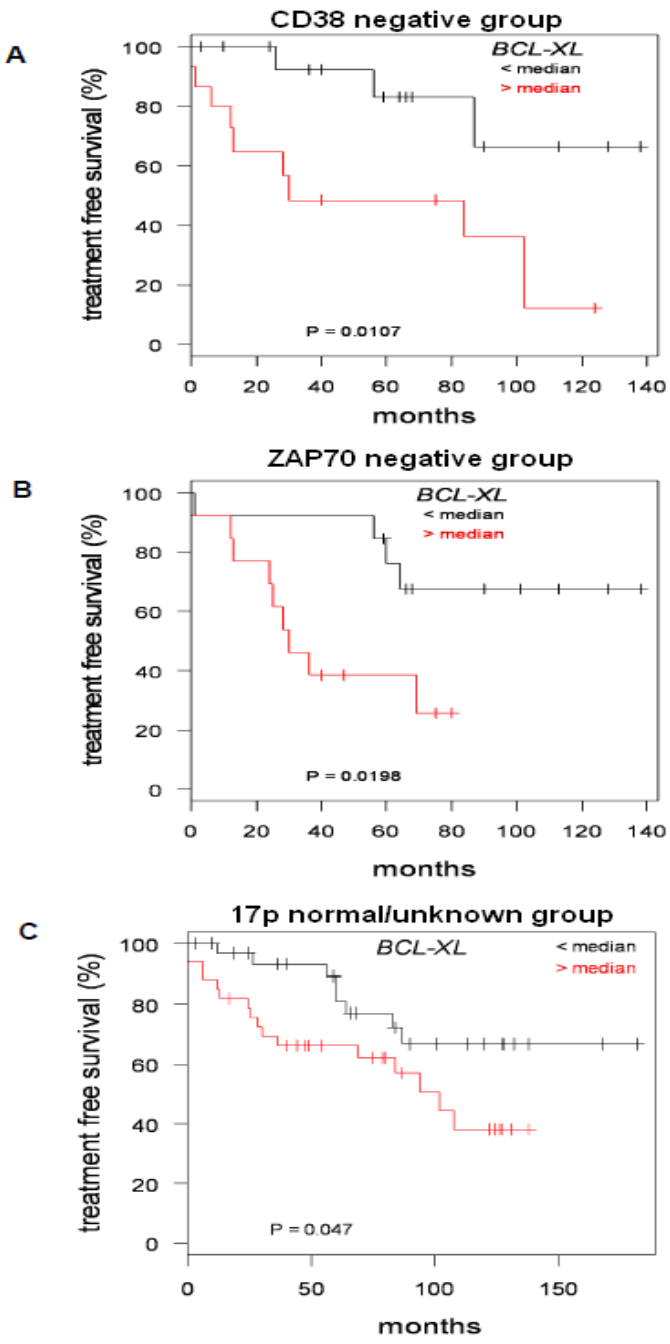

Figure 3.5: Kaplan-Meier curves for BCL-XL correlation with treatment-free survival in negative CLL prognostic marker groups. (A) CD38 negative group, (B) ZAP70 negative group, (C) 17p negative/unknown group.

CHAPTER IV

MCL-1 STABILITY DEFINES ABT-737 RESISTANCE IN LEUKEMIC B-CELLS

Mazumder S, Choudhary GS, Al-Harbi S, Almasan A. Cancer Res. 2012 Apr 23.

4.1 Abstract

ABT-737 is a small molecule Bcl-2 homology BH3 domain mimetic that binds to the Bcl-2 family proteins Bcl-2 and Bcl-xL. Recent clinical trials show that the drug is highly effective however, the most effective chemotherapeutics acquired resistance is a serious clinical problem. In order to investigate the potential mechanism responsible for ABT-737 resistance. We generated ABT-737 resistant (ABT-R) cell lines from initially sensitive Nalm-6 and Reh after chronic exposure to ABT-737. Compared with parental cells, cells that have developed acquired resistance to ABT-737 showed increased expression of Mcl-1. Mcl-1 was not transcriptionally upregulated as there was no difference in constitutive Mcl-1 mRNA levels in resistant and parental Nalm-6 and Reh cells. Our result indicates that increased Mcl-1 levels of ABT-R cells were due to its stabilization at the protein. We also show that increased Mcl-1 levels are associated with Bim in

ABT-R cells which mediate resistance to ABT-737. These data therefore reveal novel insights into mechanisms of acquired resistance to ABT-737.

4.2 Introduction

The interplay between Bcl-2 family members is essential for controlling the mitochondrial cell death pathway and thereby the survival of most cells, including those of hematopoietic origin. Based on their Bcl-2 homology (BH) domains, the Bcl-2 proteins have been grouped in three classes: anti-apoptotic, pro-apoptotic, and those with the BH3 domain-only (Chipuk, Moldoveanu et al. 2010; Elkholi, Floros et al. 2011). The members of this group regulate mitochondrial outer membrane permeabilization (MOMP), monitoring release of cytochrome c and activating downstream effector caspases (Chen, Gong et al. 2000). The imbalance in expression of these partners has been implicated in development of various tumor types and resistance to chemotherapeutic regimens (Chipuk, Moldoveanu et al. 2010). This often results from high level expression of anti-apoptotic members, such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1 that prevent cell death by sequestering BH3-only proteins, such as Bim, Puma, and Noxa, and regulate activation of the pro-apoptotic proteins Bax and Bak. In most of these cases, up-regulation and binding of significant amounts of anti-apoptotic proteins to activator proteins keeps these cells alive (Letai 2008; Al-Harbi, Hill et al. 2011).

ABT-737 is a small molecule inhibitor that is effective against certain Bcl-2 family members (Oltersdorf, Elmore et al. 2005). It has a strong affinity for Bcl-2,

Bcl-xL, and Bcl-w that are bound to Bim by releasing Bim from anti-apoptotic Bcl-2 partners, thereby initiating MOMP (Morales, Kurtoglu et al. 2011). The oral derivative of ABT-737, navitoclax (ABT-263) is currently under investigation in several clinical trials in lymphoid malignancies, such as chronic lymphocytic leukemia (CLL), and tumors, such as small cell lung cancer (Wilson, O'Connor et al. 2010; Gandhi, Camidge et al. 2011; Roberts, Seymour et al. 2012). Importantly, ABT-737 mediated cell death is Bax/Bak-dependent as Bax/Bak double knock-out mouse fibroblasts are resistant to this treatment (van Delft, Wei et al. 2006). However, it is expected that even for the most effective chemotherapeutics acquired resistance to be a serious clinical problem, hence investigate the potential mechanism responsible for acquired resistance is of special interest in cancer therapy (Vogler, Dinsdale et al. 2009; Yecies, Carlson et al. 2010; Shi, Zhou et al. 2011).

ABT-737 has very weak affinity for Mcl-1 (Lee, Czabotar et al. 2007). Various studies have shown that sensitivity to ABT-737 is decreased in cells expressing elevated levels of Mcl-1 (Konopleva, Contractor et al. 2006; Al-Harbi, Hill et al. 2011). Moreover, cells initially sensitive to ABT-737 become resistant by up-regulating Mcl-1 levels (Yecies, Carlson et al. 2010). To investigate the probable mechanisms of resistance to ABT-737, resistant cell lines were generated from pre-B tumor cells that developed increased levels of Mcl-1 protein. This study reveals novel insights into regulation and role of Mcl-1 in response to ABT-737 resistance.

4.3 Materials and Methods

4.3.1 Cell lines and reagents

Human B-cell acute lymphoblastic leukemia (ALL) cell lines Nalm-6 and Reh were obtained from ATCC (Manassas, VA). These pre-B cells express CD19 and CD127 surface markers with rearranged immunoglobulin heavy chains. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), L-glutamine, Antibiotic-antimycotic (Invitrogen). ABT-R cells were cultured in 5% FBS. Cell lines were routinely verified for growth rates, morphological characteristics, and response to stimuli using Annexin V/Propidium iodide staining. ABT-737 was provided by Abbott Laboratories (Abott Park, IL). Actinomycin D, and cycloheximide were from Sigma-Aldrich.

4.3.2 Generation of ABT-737-resistant cell lines

Nalm-6 and Reh cells were cultured in increasing concentrations of ABT-737 administered intermittently, with the drug being washed off to allow cells to recover. Gradually, the ABT-737 concentration was increased until cells remained viable when ABT-737 concentrations double to that of their IC_{50} value was administered continuously. Cells were treated with verapamil (Sigma-Aldrich) to exclude the possibility of acquiring resistance due to increase in expression of drug efflux pumps (Safa, Glover et al. 1987). The ABT-R cells were routinely monitored for resistance to ABT-737; they were cultured without drug for 72 hours before performing experiments.

4.3.3 Flow cytometry

Cell death was measured by phosphatidylserine externalization, by staining with fluorescein-conjugated Annexin V (BD Biosciences, San Jose, CA) and propidium iodide, and analyzed on a BD FACS Calibur flow cytometer. The raw data obtained was analyzed by CellQuest Version 5.2.1 software. The results were normalized to survival of control cells that have been treated with DMSO.

4.3.4 Immunoblotting and immunoprecipitation

Protein lysates were prepared with 1% NP-40 lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM Nacl; 1 mM EDTA; 1% NP-40) containing protease inhibitors (Roche) and phosphatase inhibitors cocktail 2 and 3 (Sigma). The cells were lysed for 30-45 min at 40C°. 50-60 μg of protein was resolved on 10%-12% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotting was performed with primary antibodies. For immunoprecipitation cells were lysed with CHAPS buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 2% CHAPS, Calbiochem) containing protease and phosphates inhibitors for 1 hour. Protein lysates were incubated with primary antibody overnight at 40 C° followed by 1 hour incubation of protein A agarose beads (eBioscience, Calbiochem) at 40C°. Immunoprecipitates were washed 3 times with CHAPS and eluted with loading buffer.

The co-immunoprecipitated proteins were resolved by SDS-PAGE. Primary antibodies used were for Mcl-1, Bim (BD-Biosciences), NOXA (Enzo Life

Sciences), Bcl-2, Bcl-xL (Santa Cruz Biotechnology), PUMA (ProSci Corporation), USP-9X (Abnova), PARP1 (Cell Signaling), and β-actin (Sigma).

4.3.5 RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from parental and ABT-R cells after ABT-737 treatment (500-1000 nM) for 4-24 hours using the Trizol method (Invitrogen); 2 μg of RNA was reverse transcribed using the TaqMan reverse transcription kit and amplified using the SYBR Green Master Mix (Applied Biosystems, N8080234 and 4309155) and examined on a 7500 Real-Time PCR system (Applied Biosystems). RNA isolated using primers for Mcl-1 and normalized for β-actin as described (Al-Harbi, Hill et al. 2011).

4.3.6 Confocal immunostaining

Cells were fixed with 2.0% paraformaldehyde/PBS for 15 min at room temperature, washed 3 times for 10 min each, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked in 10% fetal bovine serum in PBS for 1 hour. The coverslips were then immunostained using Mcl-1 antibody diluted in blocking buffer, followed by fluorescently conjugated secondary antibody. DAPI and Mito-tracker were added to stain Nucleus and Mitochondria respectfully. They were then mounted in Vectashield (Vector Laboratories, H-1000). Images were collected using an HCX Plan Apo 63X/1.4N.A. Oil immersion objective lens on a Leica TCS-SP2 confocal microscope (Leica Microsystems AG).

4.3.7 Statistical Analysis

Statistical comparisons between groups were conducted by 2-way ANOVA using Prism software. Standard deviation was calculated from experiments performed in triplicates and indicated as error bars. All experiments were repeated three times independently.

4.4 Results

4.4.1 Acquired resistance of B-cells to ABT-737 after prolonged exposure

ABT-737 is a BH3-mimetic that binds only to certain Bcl-2 family proteins, such as Bcl-2, Bcl-xL, and Bcl-w, but not Mcl-1. In our previous studies we have shown that patients with increased Mcl-1 levels do not respond to ABT-737 and thereby become resistant to this drug (Al-Harbi, Hill et al. 2011). Hence, in order to investigate the mechanism responsible for resistance, we generated ABT-737-resistant (ABT-R) cell lines from initially sensitive pre-B Nalm-6 and Reh cells. These cell lines were selected for their sensitivity to ABT-737, with IC_{50} values of ~1000 and ~500 nm, respectively. The Nalm-6 ABT-R and Reh ABT-R cells generated could tolerate continuous exposure to ABT-737 at a concentration double to that of their IC_{50} value (Fig. 4.1A). In addition, Parental cells were sensitive to ABT-737 and within 3 hours they were committed to cell death, as indicated by cleavage of poly-(ADP-ribose) polymerase 1(PARP1) (Fig. 4.1B). These results indicate that ABT-737 resistant cells are highly resistant to ABT-737.

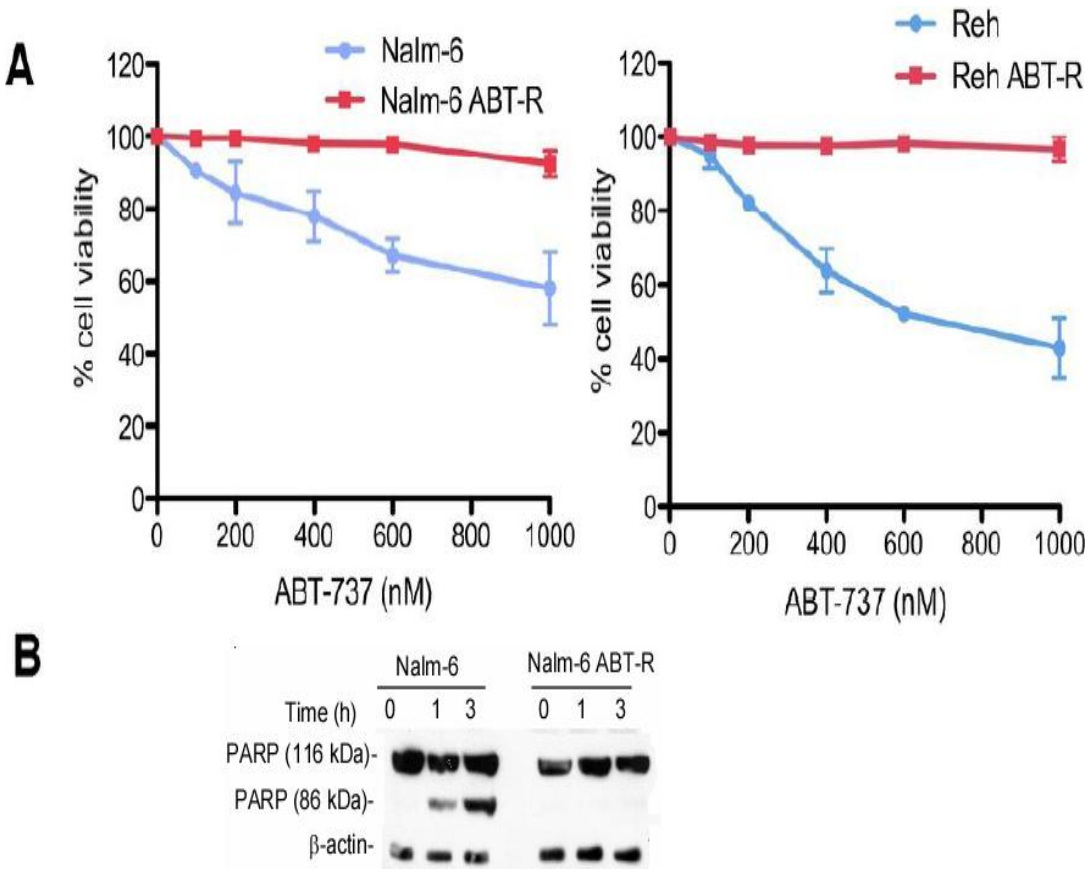

Figure 4.1. Acquired resistance development in leukemic cells. (A) Parental and ABT-737 resistant (ABT-R) Nalm-6 and Reh cells were treated with ABT-737 for 24 hours. Cell viability is shown as a percentage of Annexin V-FITC/PI-negative, relative to control cells treated with DMSO, as determined by flow cytometric analyses. (B) Parental and ABT-R Nalm-6 cells were treated with ABT-737 (1000 nM for 1-3 hours) and expression of PARP1, and β-actin was determined by immunobloting.

4.4.2 Mcl-1 levels are high in ABT-737 resistant cells

Further examination of Bcl-2 family proteins by immunoblotting in parental and ABT-R cells indicated increased Mcl-1 levels in ABT-R cells (Fig. 4.2A). Bfl-1 levels were below our detection limit. We could also observe decreased Bcl-2 and increased Bim levels in Reh ABT-R cells, which were comparable to those found in Nalm-6 ABT-R cells. Puma and NOXA expression was not markedly altered (Fig. 4.2A). Mcl-1 protein levels increased in parental but not in ABT-R cells following acute ABT-737 treatment (Fig. 4.2B).

Increased Mcl-1 levels were attributed to increased deubiquitination as a result of increased levels of the USP9X deubiquitinating enzyme (Schwickart, Huang et al. 2010), however, USP9X levels (Fig. 4.2B) and its association with Mcl-1 (data not shown) did not change in ABT-R cells. To validate our immunoblotting data, we performed immunostaning experiment to asses expression and localization of Mcl-1 in parental and ABT-737 resistant cells. Our data indicate that Mcl-1 expression is high in ABT-737 resistant cells and predominantly localized in the mitochondria (Figure 4.3). Similar results were found for Nalm6R (data not shown). Our results show that ABT-737-resistant cells could block apoptosis and develop resistance by elevating Mcl-1 levels.

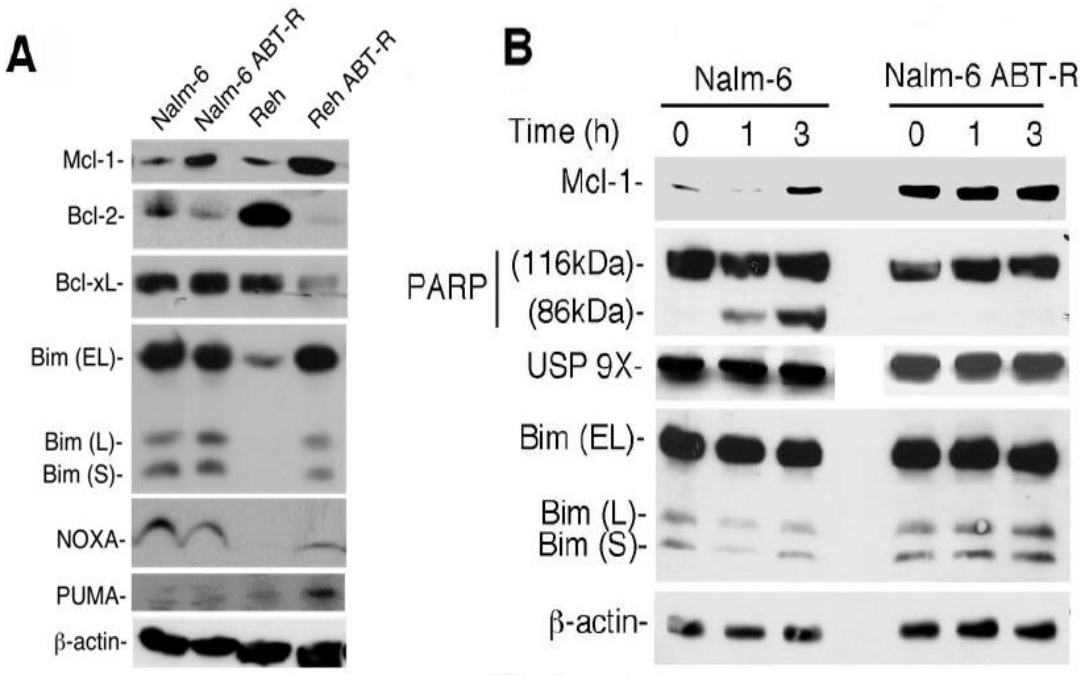

Figure 4.2. High Mcl-1 level in ABT-R cells. (A) Whole cell lysates were analyzed by immunoblotting for expression of Bcl-2 family proteins and β-actin by immunobloting with the indicated primary antibodies. (B) Parental and ABT-R Nalm-6 cells were treated with ABT-737 (1000 nM for 1-3 hours) and expressions of Bim, USP 9X, PARP, Mcl-1, and β-actin were determined by immunobloting. The results are representative of three independent experiments.

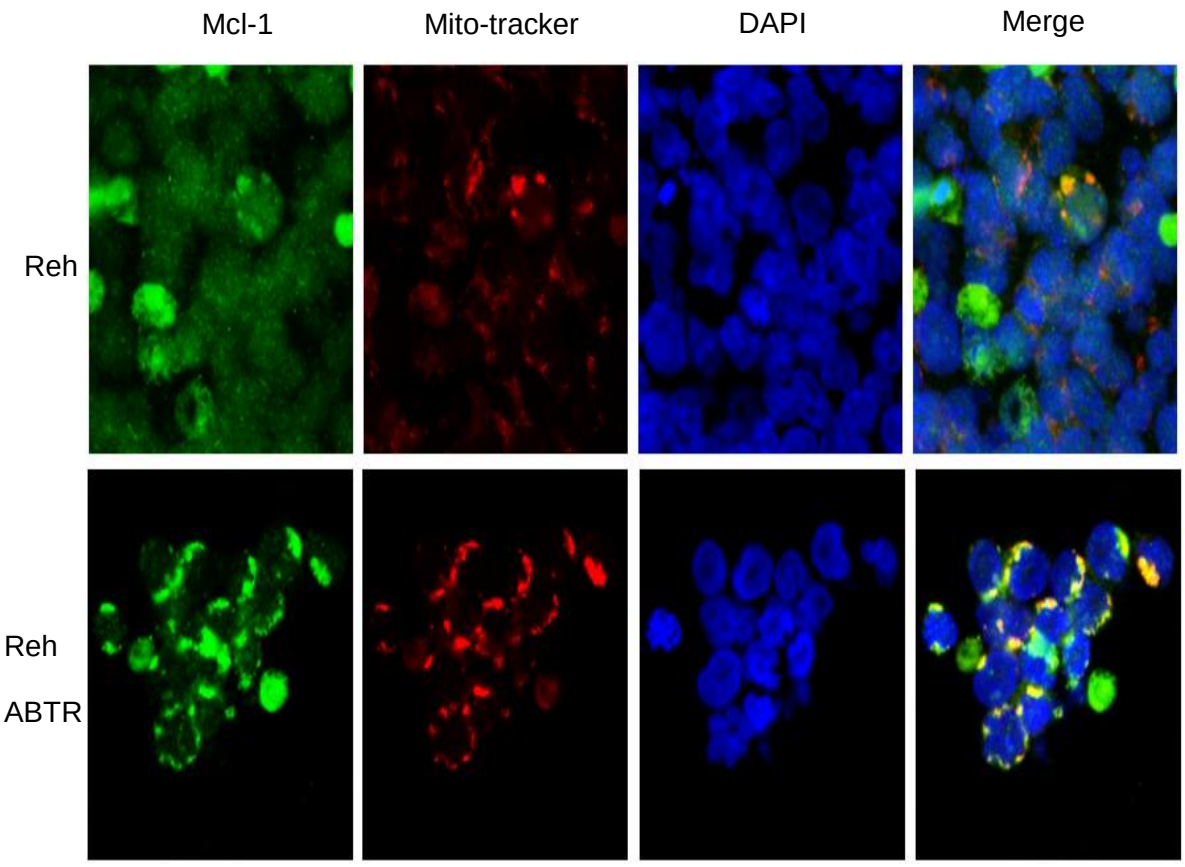

Figure 4.3 Expression and localization of Mcl-1 in parental and Reh ABT-737-resistant cells. Confocal co-immunostaining for Reh parental and Reh ABT-737-resistant cells stained with Mcl-1 (green), Mitochondria (red), and Nucleus (blue).

4.4.3 ABT-737 resistance is associated with increased Mcl-1 protein stability

Previous studies of acquired ABT-737-resistance reported that increased expression levels of Mcl-1 in ABT-R cells are a result of increased mRNA levels (Yecies, Carlson et al. 2010). However, there was no difference in constitutive Mcl-1 mRNA levels in resistant compared to parental Nalm-6 and Reh cells, as determined by qRT-PCR (Figure. 4.4 A-B). Similar results were found for Bfl-1 (data not shown). Most importantly, there was no change in Mcl-1 mRNA levels following chronic ABT-737 treatment in Nalm-6 (1000 nM) and Reh (500 nM) parental and ABT-R cells. These results indicate that Mcl-1 was not transcriptionally upregulated, however do not preclude the possibility of a reduced turnover of the Mcl-1 transcript in ABT-737-resistant cells. ABT-R Nalm-6 and Reh cells examined up to 2 hours following treatment with actinomycin D, a known transcriptional inhibitor, indicated that the half-life of Mcl-1 mRNA was not altered (data not shown). In addition, there was no further change of Mcl-1 proteins levels in ABT-R cells following 18 hours ABT-737 exposure (Figure 4.4C).

Since the Mcl-1 protein has a very short half-life, Mcl-1 protein stability was further evaluated. Immunoblotting following 30-90 min of cycloheximide treatment revealed that the half-life of Mcl-1 protein was increased considerably, to 60-90 min in ABT-R compared to 15-20 min in parental cells (Fig. 4.5A-C). These results indicate that increased Mcl-1 levels of ABT-R cells were due to its stabilization at the protein and not at mRNA level.

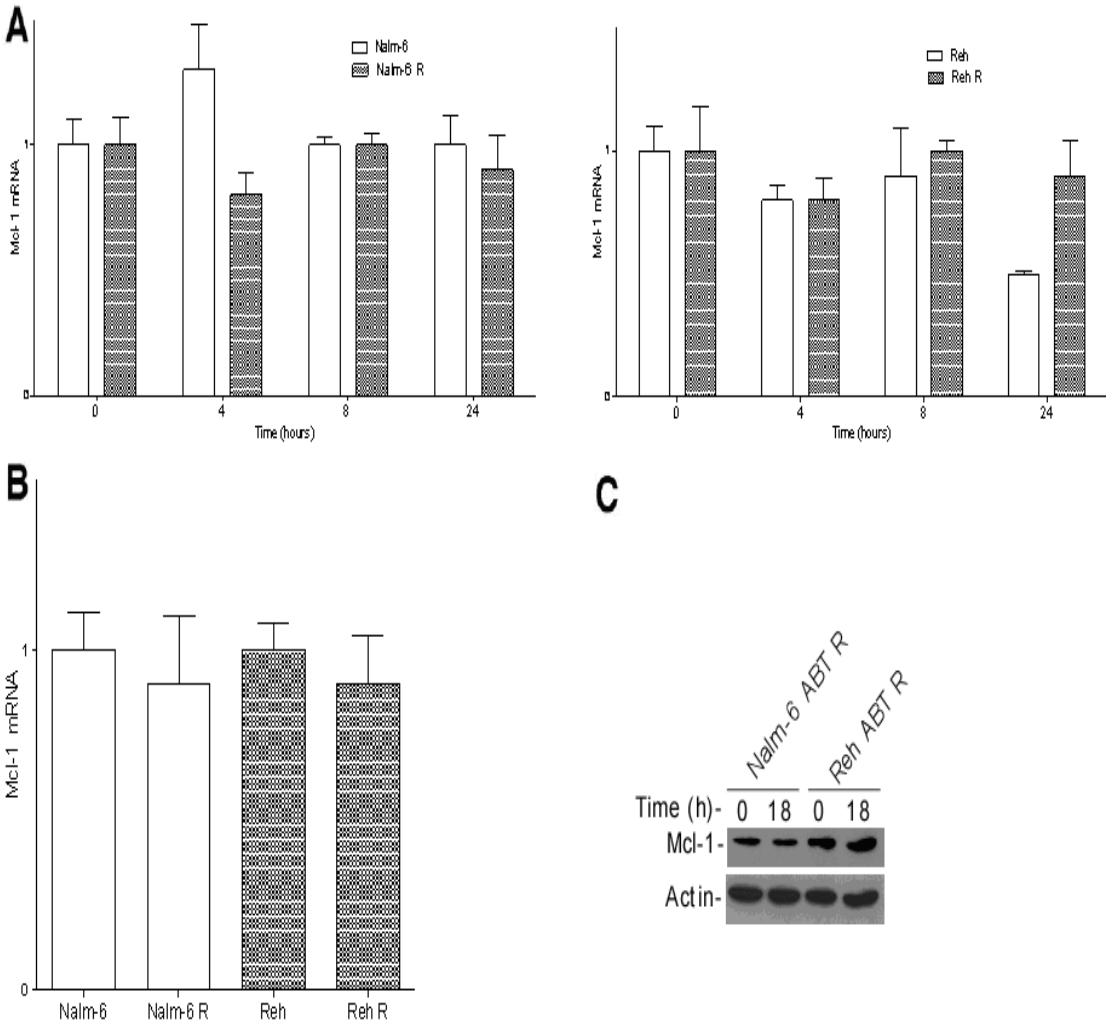

Figure 4.4. Increased Mcl-1 levels in ABT-737-resistant cells are not due to regulation of mRNA expression. (A) Mcl-1 mRNA levels were examined by quantitative real-time PCR in parental and ABT-R Nalm-6 and Reh cells following ABT-737 treatment (1000 nM for Nalm-6 ABT-R and 500 nM for Reh ABT-R) for 4, 8 and 24 hours. (B) Analyses of Mcl-1 mRNA basal levels were performed in parental and ABT-737-resistant Nalm-6 and Reh cells by quantitative real-time PCR. (C) ABT-R cells were treated with ABT-737 (1000 nM for Nalm-6 ABT-R and 500 nM for Reh ABT-R) for 18 hours and immunoblotted with Mcl-1 and β-actin primary antibodies.

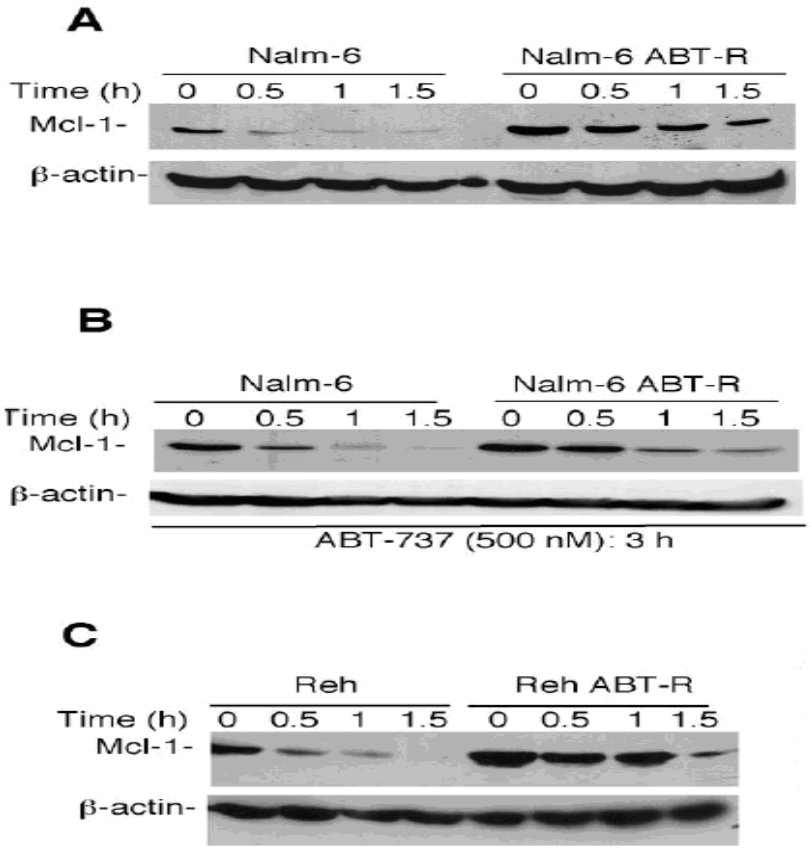

Figure 4.5. Mcl-1 levels are regulated by protein stabilization. (A) Mcl-1 protein stability was determined following 10 µg/ml cycloheximide (CHX) treatment in Nalm-6 and Nalm-6 ABT-R cells. (B) Nalm-6 and Nalm-6 ABT-R cells were pretreated with ABT-737 (500 nM) for 3 hours prior to exposure to CHX to determine Mcl-1 protein stability. (C) Mcl-1 protein half-life was determined in Reh and Reh ABT-R cells following CHX treatment.

4.4.4 Increased Mcl-1 levels sequester Bim following its displacement from Bcl-2 and Bcl-xl complexes in ABT-R cells

It has been reported that increased Mcl-1 levels in ABT-R cells associate with Bim after its competitive dissociation from Bcl-2/Bcl-xl complexes by ABT-737. Importantly, displacement of Bim from Bcl-2/Bcl-xl complexes by ABT-737 in sensitive cells was shown to be a critical event for committing cells to death (Yecies, Carlson et al. 2010). We investigated the interaction of Bim with Mcl-1, Bcl-2, and Bcl-xl in parental and ABT-R Nalm-6 cells. There was less association of Bim with Bcl-2/Bcl-xl in ABT-R compared to parental Nalm-6 cells, which was in contrast to binding of Bim to Mcl-1 (Figure 4.6A). Indeed, our co-immunoprecipitation analyses indicated that association of Mcl-1 with Bim in ABT-R cells was more pronounced compared to those in parental cells. These results were confirmed by reciprocal immunoprecipitation-immunoblot analyses with Mcl-1 and Bim, respectively (Figure 4.6B). On other hand, there was more Bim present that was not bound to Mcl-1 in parental cells, instead was found in the supernatant following immunodepletion of Mcl-1 (Figure 4.6B). Taken altogether, these data suggest that Bim was dissociated from Bcl-2/Bcl-xl complexes by competitive displacement by ABT-737. Moreover, this displaced Bim was bound to Mcl-1 in resistant but not in parental cells, even when ABT-737 was absent. Similar observations were made in Reh ABT-R cells (data not shown).

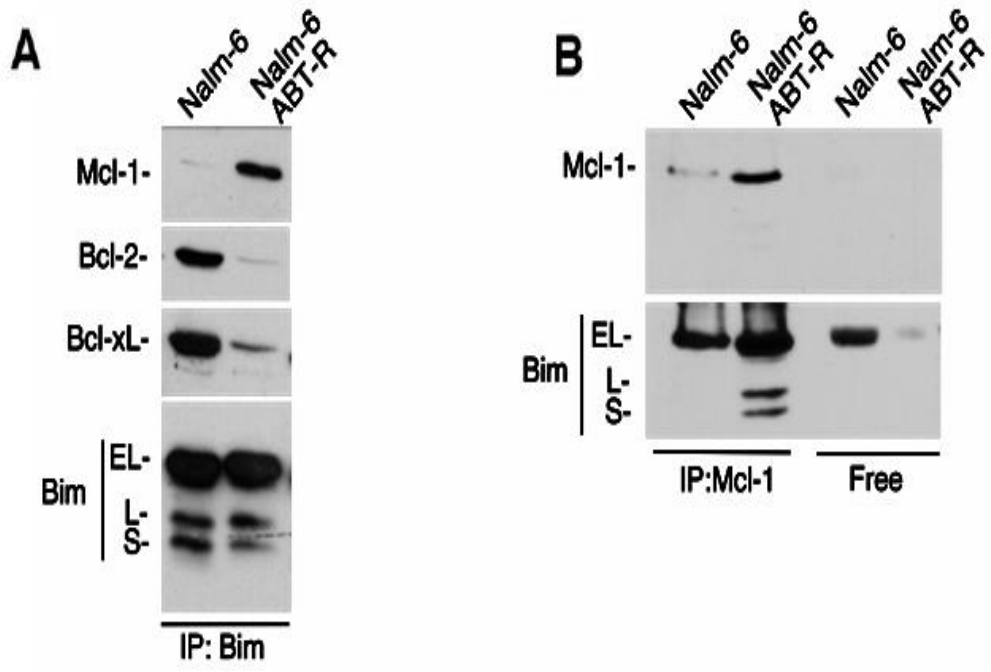

Figure 4.6. Increased Mcl-1 levels are associated with Bim in ABT-R cells. (A) Bim was immunoprecipitated and immunoblot analyses were performed for Mcl-1, Bcl-2, Bcl-xl, and Bim. (B) Reciprocal immunoprecipitation analysis was performed for Mcl-1 followed by immunoblotting for Bim to determine Mcl-1 bound and free Bim. Bim was immunoprecipitates from cells treated with ABT-737 (1000 nM) for 3 hours and immunoblotted for Mcl-1 and Bim to determine the proportion of Mcl-1-bound and Mcl-1-free Bim.

4.5 Discussion

Acquired resistance is a concern for chemotherapeutic treatments used for leukemia, and other malignancies. Although there have been basic advances in understanding the mechanisms for this resistance, few efforts have been made to study the contribution of post-translational regulation of proteins that leads to such resistance. The focus of our current studies was to understand the mechanism responsible for acquired ABT-737 resistance in leukemic cells. Nuclear magnetic resonance (NMR) studies have shown that ABT-737 binds to Bcl-2, Bcl-xl, and Bcl-w at sub-nM concentrations, but not to Mcl-1. Hence, Mcl-1 levels can determine ABT-737 sensitivity (Yecies, Carlson et al. 2010). Moreover, increased levels of these proteins can be a frequent cause of resistance. Various drugs, such as flavopiridol, R-roscovitine (Seliciclib), and PHA 767491 that downregulate Mcl-1 at mRNA level are being tested for sensitizing such tumor cells (Raje, Kumar et al. 2005; Yecies, Carlson et al. 2010).

Recently, sorafenib (BAY43-9006;Nexavar) was shown to induce apoptosis by downregulation of Mcl-1 at translational rather than post-translational level (Huber, Oelsner et al. 2011). This study has examined the molecular mechanism for acute versus chronic response to ABT-737 in leukemic cells. Recent studies and our previous report indicate that increased levels of Mcl-1 accumulate in ABT-R cells, with Mcl-1 being bound to Bim significantly more in resistant compared to parental cells (Yecies, Carlson et al. 2010; Al-Harbi, Hill et al. 2011). As in our leukemic model of ABT-737 resistance, Mcl-1 was not upregulated at transcriptional level, led us to explore its protein stability. Previous

studies have shown that post-translational modifications, such as phosphorylation of Mcl-1 at specific residues are important for it's binding to BH3-only proteins and its stability (Kobayashi, Lee et al. 2007; Liao, Zhao et al. 2011). Mcl-1 is a short-lived protein and it is a target of E3 ubiquitin ligases that mediate its proteasomal-mediated degradation. Mcl-1 is inaccessible to E3-ubiquitin ligases, FBW7 and β-Trcp when it is bound to BH3-only proteins, such as Bim, as more association of Bim with Mcl-1 prevents their access to Mcl-1. As there is competitive binding between these E3-ubiquitin ligases and BH3-only proteins because they share the same C-terminal-binding region of Mcl-1 (Hogarty 2010), most likely this could contribute towards Mcl-1 stability, as more Bim is associated with Mcl-1 in ABT-R cells. These findings provide insights into the molecular mechanism of ABT-737 resistance.

CHAPTER V

FUTURE DIRECTIONS

5.1 Introduction

Apoptosis is often blocked in cancer, which limits the response to conventional therapy. Resistance of tumor cells to conventional chemotherapy may be caused by a defect in the apoptosis machinery. It is clear that high levels of anti-apoptotic proteins have been reported in many human malignancies and generally correlate with aggressive tumors and chemotherapeutic resistance. However, it is not clear how to apply the expression of anti-apoptotic Bcl-2 genes in a rational way to predict the maximum response to current chemotherapy regimens in cancer patients.

We have developed a marker that can be used to predict responses in leukemia, including CLL as well as small cell lung carcinomas cells. We have also shown that combining ABT-737 with agents that target high Mcl-1 levels, such as flavopiridol, can improve the clinical response in highly ABT-737-resistant cells. However, data from the flavopiridol clinical trials indicate that the drug is not quite safe, as there is a substantial risk for tumor lysis syndrome (TLS) in the treated relapsed CLL patients (Blum, Ruppert et al. 2011). Therefore, we wanted

to test whether disrupting the proliferative signal that mediates the growth and survival of CLL cells, along with ABT-737, will lead to increased response rates in CLL patients.

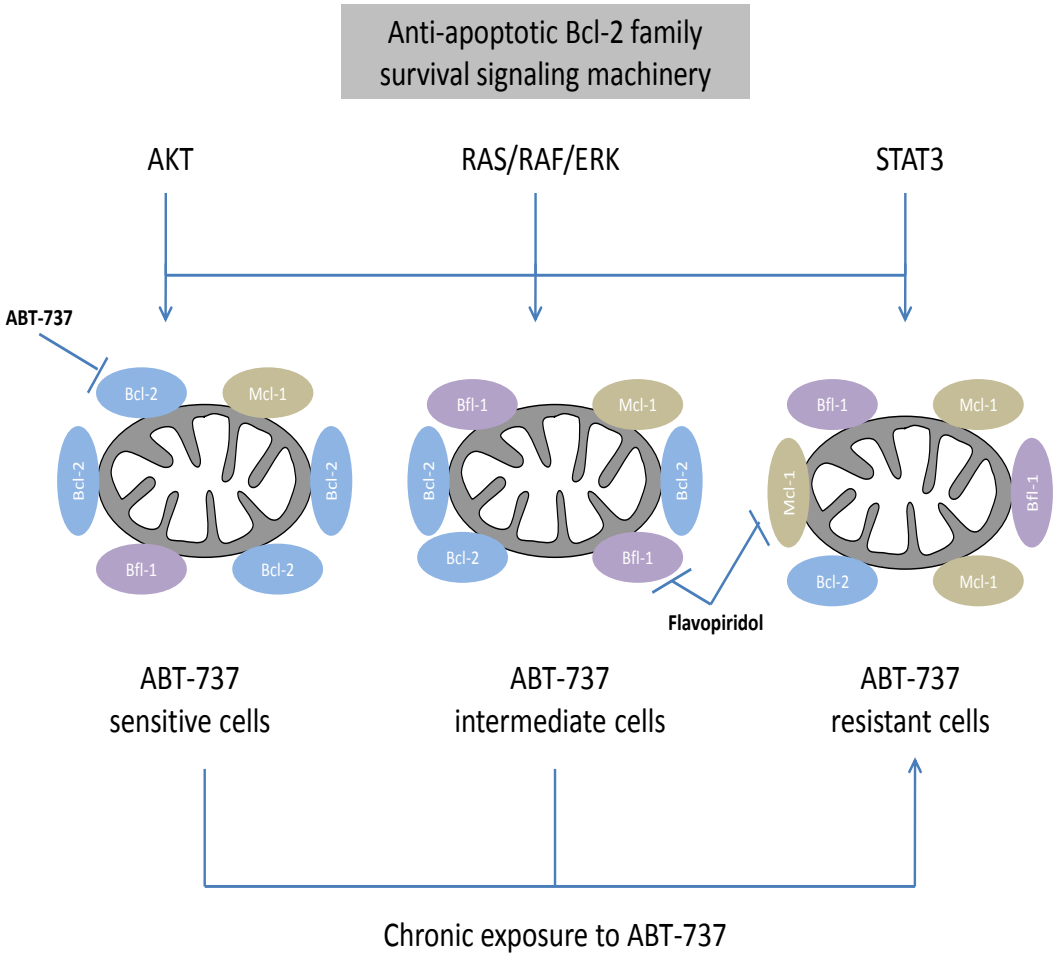

Figure 5.1 A model for the clinical response to ABT-737 in CLL cells

5.2 Effect of ABT-737 in combination with CAL-101 on ABT-737-resistant cells.

CAL-101 is a novel small molecule inhibitor of the delta isoform of phosphatidylinositol 3-kinase. CAL-101 targets the B-cell receptor that is reported to promote CLL cell growth and survival, which can block the interaction between the tumor microenvironment and CLL cells (Herman, Lapalombella et al. 2011; Hoellenriegel, Meadows et al. 2011). The drug is available for oral use, is well tolerated, and shows impressive clinical effects in phase I trials of refractory hematologic malignancies including CLL (Lin 2010).

To test whether combining ABT-737 and CAL-101 would improve the clinical response in CLL, we have treated several primary CLL cells with ABT-737 alone or in combination with CAL-101. Our data indicate that the combination treatment shows a synergistic effect more than a single treatment of ABT-737 or CAL-101. However, we need to collect more ABT-737-resistant cells to have an adequate number of CLL samples. We also want to investigate how cell death is mediated by the combination treatment and whether the response is Mcl-1 dependent.

5.3 Anti-apoptotic Bcl-2 profiling as a clinical tool in follow-up patients.

We have used RT-PCR as a highly quantitative assay to study the expression of the anti-apoptotic Bcl-2 family genes. Unlike other techniques that have been used in the clinic, our assay can quantify all known anti-apoptotic Bcl-2 or any

other gene transcripts. It is also highly sensitive, simple, cost-effective and most importantly, requires only small number of cells. As we have applied this assay to quantify the expression ratio of Mcl-1, Bfl-1, and Bcl-2 to predict response to ABT-737, we wanted to take this assay to the next level by assessing the expression level of anti-apoptotic genes in follow-up CLL patients. We have generated a large cDNA library of CLL patients, with complete Bcl-2 family expression profiling. The patients all come to the clinic for follow-up visit. Therefore, we wanted to compare the anti-apoptotic Bcl-2 profiling between the first and the second visit. We also wanted to test whether the difference can be correlated to any clinical outcome such as disease progression or clinical response. This assay can quantify the modulation in the anti-apoptotic Bcl-2 expression following chemotherapy treatment. Thus, it would allow us to identify whether CLL cells are Bcl-2 dependent or Mcl-1 dependent, which will provide a rational way to choose the ideal chemotherapy treatment in order to maximize the clinical response.

We have collected 17 follow-up cases. The period between the first and second sample collection ranges between four to 12 months. We compared the expression levels of Bcl-2, Mcl-1, and Bfl-1 in these samples. We did not detect any significant difference in the Mcl-1 expression levels between the first and the second samples. There was a significant increase in Bcl-2 and Bfl-1 expressions in 7 out of 17 samples (Figure 5.1), and 3 out 7 show some tumor progression such as lymph node enlargement. These preliminary data were not significant, as

more CLL samples as well as longer time to monitor are needed in order to have significant data on those patients. It is also important to expand the anti-apoptotic profiling to include other anti-apoptotic Bcl-2 family members, such as Bcl-xl, in the study.

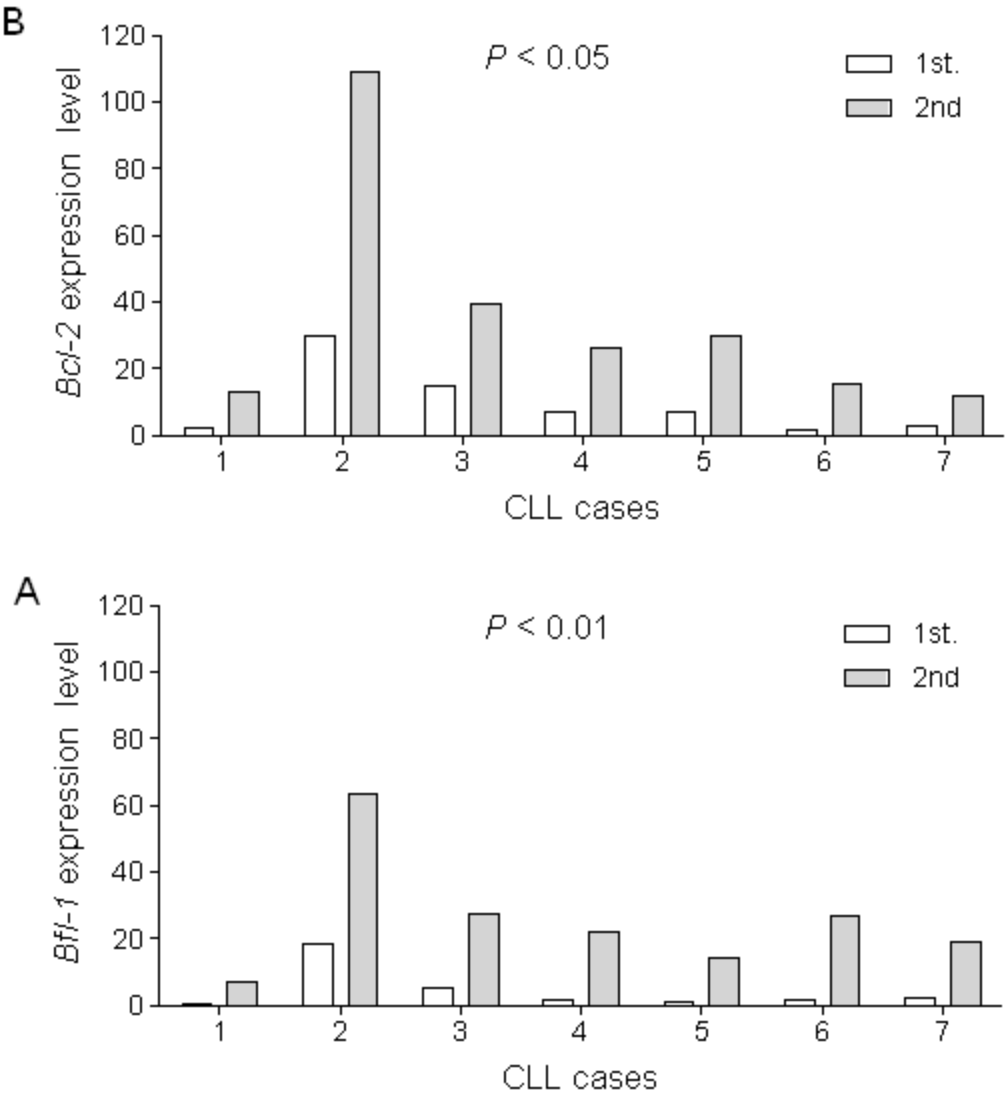

Figure 5.2 High expressions of Bcl-2 and Bfl-1 in follow-up CLL patients. Comparison in RNA expression of anti-apoptotic Bcl-2 family members (A) Bcl-2, and (B) Bfl-1 as determined by quantitative RT-PCR between 1st and 2nd CLL samples.

5.4 To investigate whether high Bcl-xl expression is mediated by somatic genetic alterations in CLL patients

We have shown that CLL patients with a high Bcl-xl level show significantly short treatment-free survival. It is well known that cancer progression is driven mainly through somatic genetic alterations including, gene amplification and translocations (Beroukhim, Mermel et al. 2010). It is highly valuable to identify any alteration within a key gene that has a critical role in oncogenesis, such as Bcl-xl. A recent report shows that Bcl-xl mediates general resistance to many chemotherapy regimens, however, no data to show whether Bcl-xl upregulation is due to genetic alterations in CLL patients. The ideal approach to address this issue is to sequence Bcl-xl gene. However, this would be costly and time consuming.

I hypothesize that high Bcl-xl expression is mediated by gene amplification or chromosomal translocation that can place the bcl-xl gene under the control of a highly expressed gene. To test my hypothesis, I will use newly custom-designed Bcl-xl probes to test whether high Bcl-xl is mediated by gene amplification or chromosomal translocation in a few CLL patients that are known to have high Bcl-xl using FISH technique.

The second hypothesize is that Bcl-xl could be upregulated due to deletion of any microRNA that negatively regulates the Bcl-xl transcript. To test this hypothesis, I will conduct miroRNA profiling in a small number of our cDNA samples to identify a miroRNA candidate that can be involved in Bcl-xl

regulation. These hypotheses do not exclude the possibility that high levels of Bcl-xl can be due to genetic alterations that constitutively activate its upstream signaling, such as NF-kb.

5.5 Conclusion

Apoptosis is often impaired in cancer, which can significantly limit the response to conventional therapy. Since many anti-cancer regimens kill tumor cells through the mitochondrial pathway, resistance of tumor cells to radiation or chemotherapy can be due to high levels of anti-apoptotic proteins. However, applying anti-apoptotic profiling as a diagnostic tool to predict clinical response is not yet feasible.

In an effort to accomplish this goal, we have shown that our anti-apoptotic index can identify which CLL patients are most likely to respond to ABT-737, which cannot be predicted using traditional prognostic markers. We have also shown that Bcl-xl can be used as a prognostic marker to identify CLL patients that can be at high risk. This study provides insight into the useful application of anti-apoptotic profiling as a clinical tool, and can be a step toward applying personalized treatment, which may maximize the clinical response in cancer patients.

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