A matter of life or death:
targeting MCL-1 in multiple myeloma

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MCL-1 als therapeutisch target in multipel myeloom

(met een samenvatting in het Nederlands)

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CHAPTER 1

General introduction

APOPTOSIS AND OTHER CELL DEATH MECHANISMS

Cell death plays a crucial role in multicellular organisms, from development into adulthood. The most prominent form of programmed cell death in animals is apoptosis, named after the Ancient Greek ἀπόπτωσις, which means "falling off", as leaves from a tree.[1] The resorption of a tadpole's tail upon metamorphosis into a frog occurs by apoptosis, as does the disappearance of webbing between fingers and toes of developing human embryos. Other examples of processes mediated by apoptosis are the elimination of self-reactive immune cells, the removal of neurons that do not form proper connections in the developing brain, and the shedding of old cells in the constantly renewing tissues of the skin and gut. In addition, apoptosis is important for prevention of disease, since infected cells and cells that have acquired DNA-damage are normally cleared by apoptosis.[2]

Apoptosis is characterized by cell shrinkage and condensation of chromatin, followed by plasma membrane blebbing, fragmentation of the nucleus, and formation of apoptotic bodies (Figure 1).[1] These apoptotic bodies, surrounded by an intact plasma membrane, contain cytoplasm, tightly packed organelles, and varying amounts of nuclear debris. Apoptotic bodies are phagocytosed by macrophages and digested in the phagolysosomes of these cells.[1,3] Throughout the process of apoptosis, no spillage of cellular content into the surrounding tissue takes place. Therefore, apoptosis generally does not provoke an inflammatory reaction.[4]

Apoptosis can be initiated by two pathways: the intrinsic and the extrinsic pathway (Figure 2). Both accumulate in activation of initiator caspases (caspases 8, 9, and 10), which activate effector caspases (caspases 3, 6, and 7).[5,6] Caspases are cysteine/aspartic proteases, meaning that they depend on a cysteine side chain to cleave proteins specifically after aspartic acid residues. Cleavage of cellular components by caspases results in the apoptotic phenotype.

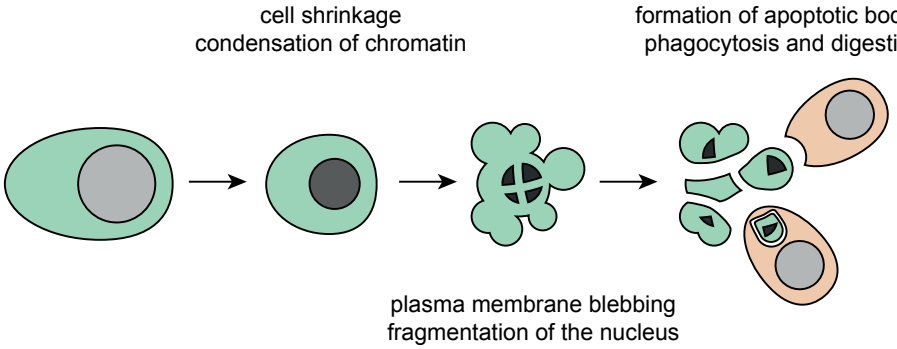

Figure 1. Schematic representation of the different morphologic steps of apoptosis.

Although apoptosis plays an essential role in development and tissue homeostasis, there are many more ways for a cell to die. Classically, besides apoptosis, two other major forms of cell death were recognized, based on morphology of the dying cell.[7] These were necrosis, which is characterized by cell swelling and bursting, resulting in release of cellular contents into the extracellular space which leads to an inflammatory response and tissue damage; and autophagy, where a cell digests part of its own content upon starvation, often resulting in cell survival rather than elimination. More recently, many other forms of cell death have been described, with varying degrees of cellular regulation and inflammatory effect. Examples are necroptosis, pyroptosis, ferroptosis, and lysosome-dependent cell death.[8]

Evasion of apoptosis is a hallmark of cancer

Cancer cells are characterized by alterations that allow for malignant growth. Besides sustained proliferative signaling, limitless replicative potential, evasion of the immune system, angiogenesis, and tissue invasion and metastasis, the ability to resist apoptosis is a hallmark of cancer.[9] Apoptosis is a protection mechanism against disruptions in homeostasis that are essential for carcinogenesis, and is therefore a major barrier for malignant transformation. Thus, malignant cells often have defective apoptosis-inducing mechanisms, or overexpression of downstream anti-apoptotic proteins acting as oncogenes. In addition, resistance to apoptosis hampers effectivity of anti-cancer treatments, such as chemotherapy and radiation, which normally kill cells via apoptosis. Investigating strategies to activate apoptosis despite resistance mechanisms, or even by taking advantage of these resistance mechanisms, is an important step towards better treatment of cancer.

THE EXTRINSIC APOPTOSIS PATHWAY

The extrinsic apoptosis pathway starts with engagement of a death receptor by its extracellular ligand (Figure 2). Therefore, this pathway is also named death receptor pathway. Death receptors are members of the TNF receptor superfamily that contain an intracellular death domain.[10] The best-described death receptors are TNF receptor 1 (TNFR1), Fas (CD95), and TNF-related apoptosis-inducing ligand receptors 1 and 2 (TRAIL-R1 and TRAIL-R2), whose ligands are TNF, Fas ligand (FasL), and TRAIL, respectively.[11,12] Upon binding of the death ligand, death domain-containing intracellular adaptor proteins, such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), are recruited.[10] Homotypic aggregation of receptor and adaptor death domains leads

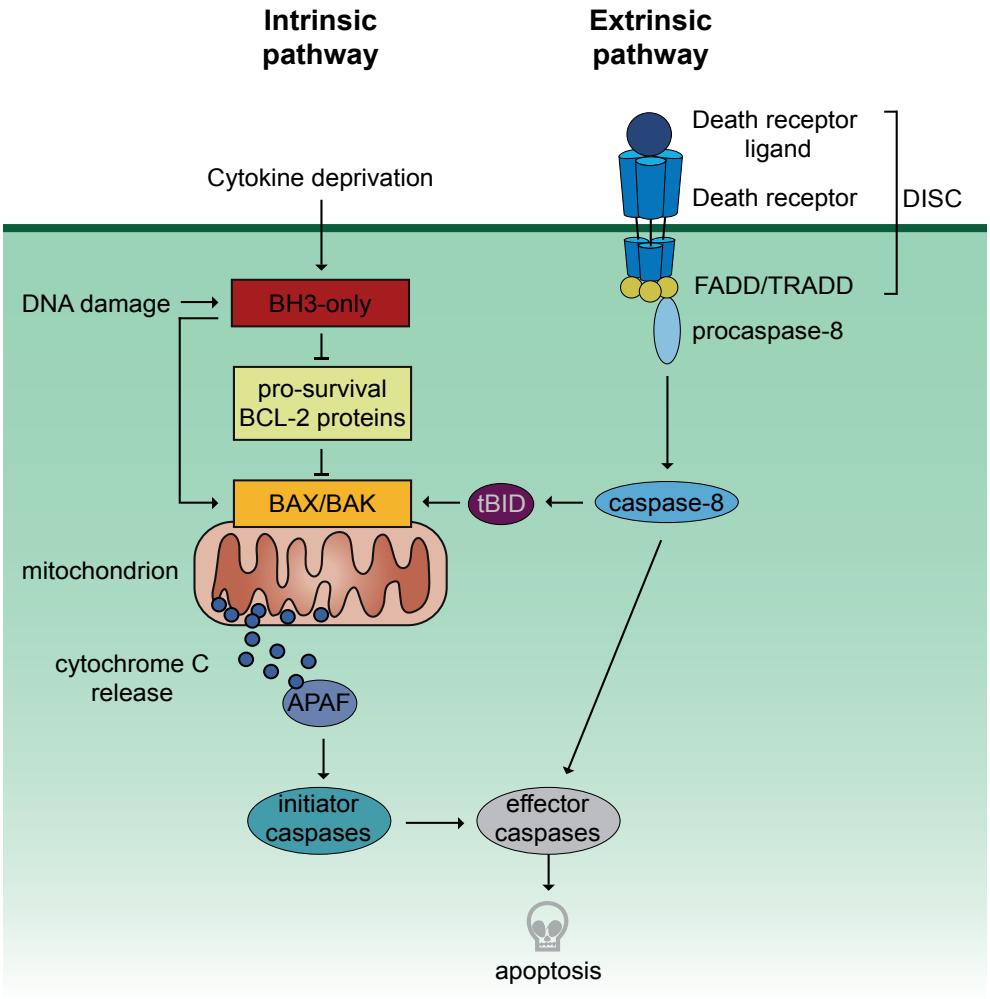

Figure 2. The intrinsic and extrinsic apoptosis pathways. The intrinsic apoptosis pathway is regulated by the BCL-2 protein family, which consists of pro-apoptotic and pro-survival proteins. Intracellular stress signals induce expression of pro-apoptotic BH3-only proteins, which sequester pro-survival BCL-2 family proteins and induce activation of BAX and BAK. BAX and BAK permeabilize the mitochondrial outer membrane, leading to cytochrome C release, APAF multimerization, and subsequent activation of initiator and effector caspases. The extrinsic apoptosis pathway is activated by binding of extracellular death receptor ligands to death receptors, after which intracellular domains support activation of caspase-8 and effector caspases. Cleavage of intracellular components by caspases leads to the apoptotic phenotype. Cleavage of BCL-2 protein BID into tBID by caspase-8 is a mechanism of crosstalk between the extrinsic and intrinsic apoptosis pathways. APAF, apoptotic protease activating factor; BH3, BCL-2 homology 3 domain; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; TRADD, tumor necrosis factor receptor type 1-associated death domain.

to formation of the death-inducing signaling complex (DISC), which then induces activation of pro-caspase 8 into caspase 8.[12,13] As an initiator caspase, caspase 8 can subsequently cleave downstream effector caspases and induce cell death.

Most death ligands are expressed on the membrane or produced in soluble form by immune cells. Examples of apoptosis induction by the extrinsic apoptosis pathway are activation-induced cell death of T cells and perforin-independent cell killing by cytotoxic T cells and NK cells.[12,14] Although death receptor stimulation as of now has not led to clinical approval, inhibition of the death ligand-receptor interaction using TNF blockers has proven to be very effective against inflammatory diseases.[14]

THE INTRINSIC APOPTOSIS PATHWAY

The intrinsic apoptosis pathway, or mitochondrial pathway, is mediated by the BCL-2 protein family. Its first member, BCL-2, was discovered in 1984 as a potential oncogene at the breakpoint of a recurrent chromosomal translocation in follicular lymphoma.[15] In the following decade, experiments revealed that BCL-2 was different from other oncogenes known at that time. Instead of promoting cell growth or proliferation, BCL-2 promoted cell survival by preventing apoptosis.[16] After identification of the closely related proteins MCL-1[17] and BCL-XL[18] in 1993, the BCL-2 family was established. In the same year, BAX was identified as the first BCL-2 family member that did not protect from apoptosis, but rather promoted it. Since then, a total of 17 human BCL-2 family proteins have been described, all containing one or more BCL-2 homology (BH) domain (Figure 3). The BCL-2 protein family can be divided into three groups: pro-survival proteins (BCL-2, BCL-XL, MCL-1, BCL-W, BCL-B, and BFL-1), pro-apoptotic effectors (BAX, BAK, and possibly BOK), and pro-apoptotic BH3-only proteins (BAD, BIK, BID, BMF, HRK, BIM, NOXA, and PUMA).

When BCL-2 was discovered in human cells, programmed cell death had already been well described in *Caenorhabditis elegans*, and it was shown that human BCL-2 was able to reduce cell death in these nematodes, suggesting that both organisms applied the same mechanism of programmed cell death, which thus has been conserved for more than 500 million years of evolution.[19] Yet, there are many differences between apoptosis in humans and in *C. elegans*. Within vertebrates, on the other hand, the process of cell death by intrinsic apoptosis is so conserved that orthologs of BCL-2 family members can be used interchangeably in many cases, allowing for rapid progress in the research field.[19]

Regulation of apoptosis by BCL-2 proteins

Activation of caspases through the intrinsic apoptosis pathway is dependent on mitochondrial outer membrane permeabilization (MOMP) by BAX and BAK (Figure 2). This results in leakage of cytochrome C, which activates caspase activator APAF1.[20] To explain the mechanism of BAX and BAK activation, three models of interactions between BCL-2 proteins have been described.[21] According to the direct activation model, some BH3-only proteins ('activators') directly bind and activate effectors BAX and BAK. Other BH3-only proteins ('sensitizers') are unable to do this, but in turn function by binding to pro-survival proteins, which then release bound activators. In the indirect activation model, BH3-only proteins solely function as neutralizers of pro-survival proteins, which then release BAX and BAK. As a result, BAX and BAK are activated spontaneously or by an unknown modification, which leads to apoptosis. These two models are not mutually exclusive, and currently a third and unified model is considered most likely. In this unified model, pro-survival proteins sequester both BH3-only proteins and BAX and BAK. When upregulated, BH3-only proteins act by neutralizing pro-survival proteins as well as by activating BAX and BAK, leading to MOMP and subsequent caspase activation.[21,22]

The common factor among all BCL-2 family proteins is the presence of BH domains (Figure 3). BH3-only proteins are named thus because they only contain a BH3-domain. Pro-survival BCL-2 proteins, as well as BAX and BAK, contain four BH domains and therefore have a similar overall structure.[21,23] Most BCL-2 proteins contain a transmembrane domain that allows them to localize to intracellular membranes. In some cases, BCL-2 proteins can receive protein modifications that lead to differences in activity, binding affinity, or degradation.[24] MCL-1 is exemplary in this regard, as it is the only BCL-2 family member whose N-terminal tail is rich in putative and experimentally confirmed modification sites, which are targets for phosphorylation, ubiquitination, and cleavage.[25]

Pro-apoptotic BH3-only proteins

BH3-only proteins are sensors of cellular stress. Under unstressed conditions, the expression level of many BH3-only proteins is low. Signals such as cytokine deprivation, DNA-damage, ER stress, hypoxia, and anoikis lead to transcriptional or post-translational upregulation of BH3-only proteins.[26] BH3-only proteins have distinct yet overlapping functions, and their expression depends on cell type and cytotoxic condition.[26] For example, BIM can be transcriptionally induced by FOXO3a upon cytokine deprivation, and phosphorylation of BIM can either increase or decrease its activity, depending on the site. BIM regulates apoptosis of

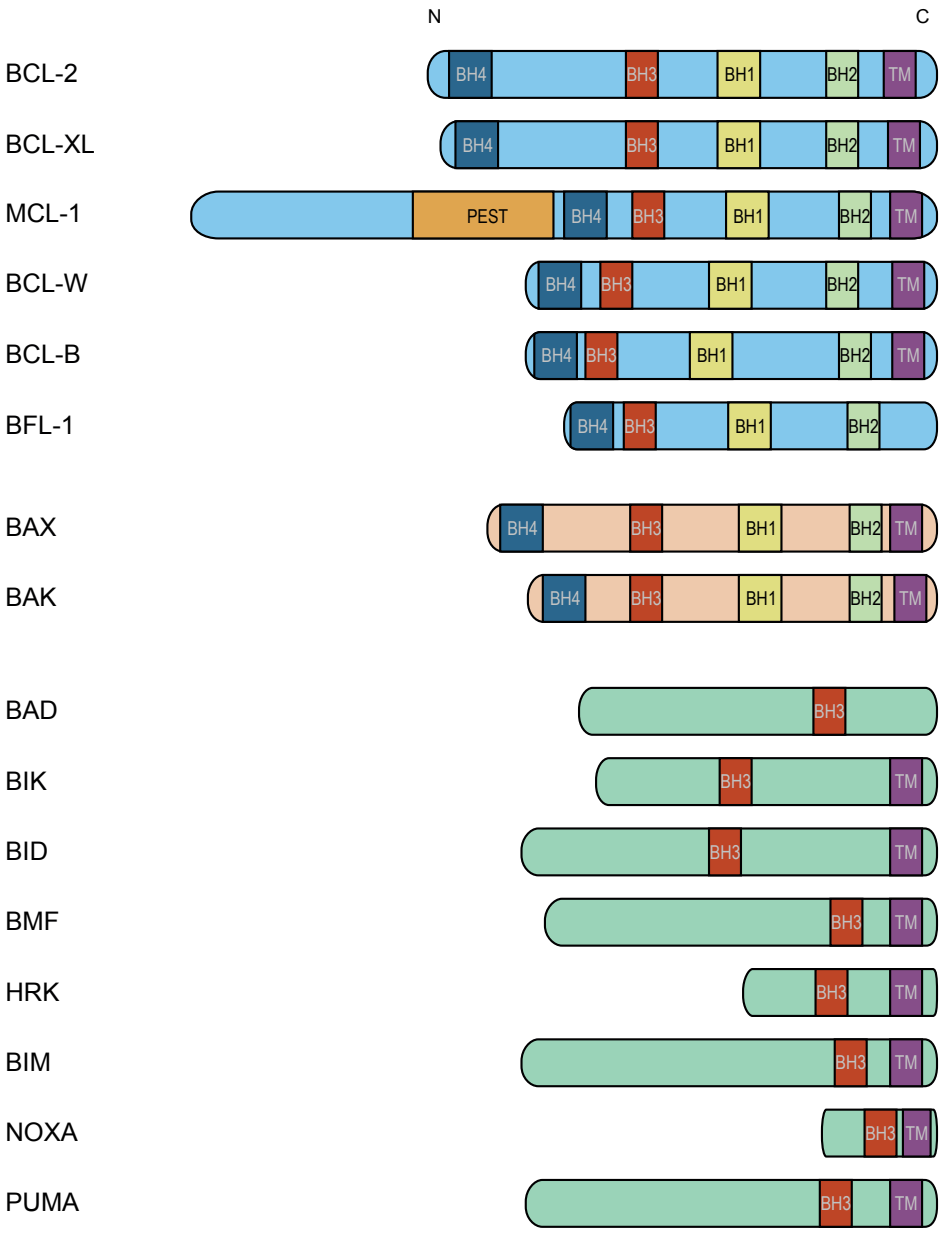

Figure 3. The BCL-2 protein family. Schematic representation of the pro-survival proteins (blue), pro-apoptotic effector proteins (orange), and pro-apoptotic BH3-only proteins (green) of the BCL-2 family. The presence and location of BH and transmembrane (TM) domains are indicated in the proteins. MCL-1 is a unique member of the BCL-2 protein family because it contains a high number of proline, glutamic acid, serine, and threonine (PEST) residues on its relatively long N-terminal tail. These residues are targets for post-translational modification.

autoreactive thymocytes and B cells, and its loss leads to decreased sensitivity to cytokine withdrawal, taxol, glucocorticoids, and DNA-damage-inducing agents.[27,28] In approximately 20% of mantle cell lymphoma cases, homozygous deletion of BIM is found,[29] and epigenetic silencing of BIM leads to its downregulation in Burkitt lymphoma.[30,31] NOXA and PUMA are direct transcriptional targets of DNA damage response protein p53 as well as transcription factor E2F1.[26] Over 50% of human tumors bear mutations in *P53*, resulting in impaired induction of NOXA and PUMA in response to DNA damage. BID is a unique BH3-only protein because it is ubiquitously expressed, but remains inactive until it is cleaved into tBID by proteases such as caspase-8 or granzyme B.[26,32] Since caspase-8 is an initiator caspase of the extrinsic apoptosis pathway, and granzyme B is a protease inserted into target cells by cytotoxic cells, tBID appears to function by amplifying non-intrinsic apoptosis signals through activation of intrinsic apoptosis.[32,33]

In contrast to other BCL-2 family proteins, BH3-only proteins only contain a single BH domain. This BH3 domain is an amphipathic α-helix of approximately 26 amino acids, which has the ability to bind in the hydrophobic surface groove of pro-survival BCL-2 family proteins.[26] As a result, pro-survival proteins are neutralized and can no longer bind to BAX and BAK. Some BH3-only proteins (BIM, PUMA, tBID) bind to all pro-survival BCL-2 proteins, whereas others have more selective binding specificity (Figure 4).[21,26,34] For example, BAD only binds with high specificity to BCL-2, BCL-XL and BCL-W, while NOXA selectively binds to MCL-1 and BFL-1.[34] In addition to binding pro-survival BCL-2 proteins, some BH3-only proteins, such as BIM, tBID, and PUMA, have also been reported to bind and activate pro-apoptotic effector proteins BAX and BAK.[35]

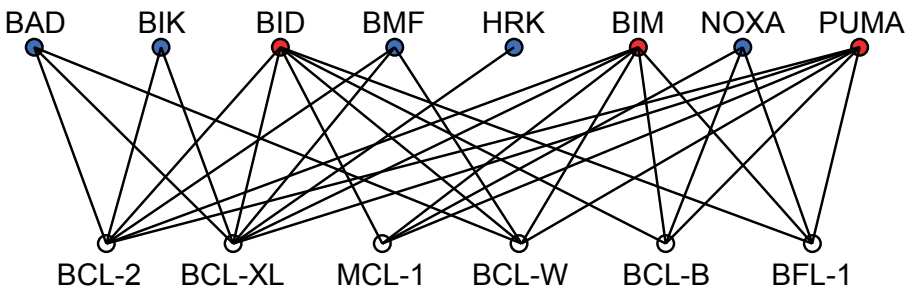

Figure 4. Binding selectivity of BH3-only proteins towards pro-survival BCL-2 proteins. Lines indicate binding selectivity. Some BH3-only proteins (red circles) bind to all pro-survival proteins, whereas others (blue circles) have more selective binding specificity.

Pro-apoptotic effector proteins

Induction of MOMP is generally considered the essential gateway to mitochondrial apoptosis.[36] The main players in this process, BAX and BAK, each contain four BH domains and are therefore structurally similar to pro-survival BCL-2 proteins. In contrast to the intrinsically unstructured BH3-only proteins, the structure of BCL-2 proteins with four BH domains is globular, consisting of a hydrophobic central core surrounded by amphipathic α-helices.[21] The folding of these helices generates a hydrophobic surface cleft that forms the binding site for BH3-domains of other BCL-2 proteins.[23] In healthy cells, BAX mainly resides in the cytosol and BAK at the mitochondrial outer membrane, although both cycle between these locations.[37–40] Upon apoptotic activation, they oligomerize in the mitochondrial outer membrane and induce MOMP. Until recently, the molecular mechanism of activation and oligomerization of BAX and BAK remained an unresolved question in the apoptosis research field. Structural studies have now revealed how BAX undergoes conformational changes to insert itself into the mitochondrial outer membrane and oligomerize into pores.[41–44]

A third BAX- and BAK-homologous BCL-2 protein, BOK, remains poorly understood.[45] BOK has been proposed to function in ER-stress-induced cell death, functions independent of other BCL-2 proteins, but nonetheless has pore-forming activity that can trigger MOMP in the absence of BAX and BAK.[46,47] Studies with Bax loss-of-function mutant mice revealed an increase in neurons and lymphocytes, male infertility, and behavioral deficits.[50] Loss of Bak or Bok, on the other hand, did not lead to apparent developmental abnormalities. Of *Bax*$^{-/-}$*Bak*$^{-/-}$ double knockout mice, >90% died perinatally, and the surviving mice had severe developmental phenotypes associated with defective apoptosis.[50] *Bok*$^{-/-}$*Bax*$^{-/-}$ and *Bok*$^{-/-}$*Bak*$^{-/-}$ mice did not have an exacerbated phenotype compared to *Bax*$^{-/-}$ and *Bak*$^{-/-}$ single knockouts, except for increased oocyte numbers in *Bok*$^{-/-}$*Bax*$^{-/-}$ mice.[51] *Bax*$^{-/-}$*Bak*$^{-/-}$*Bok*$^{-/-}$ triple knockout mice had a mildly more pronounced phenotype in lymphocytes compared to *Bax*$^{-/-}$*Bak*$^{-/-}$ double knockout mice.[52] Taken together, these studies suggest that BAX and BAK have highly redundant functions and play an essential role in development of many tissues. The role of BOK seems to be more limited, but perhaps confined to specific tissues or to processes in the adult organism.

Pro-survival BCL-2 proteins

Counterbalancing pro-apoptotic signals by pro-survival BCL-2 family proteins is very important for maintaining homeostasis. This is illustrated by constitutive and conditional knockout studies in mice, which showed varying functional specificity

of the different pro-survival depending on cell type. For example, *Bcl2*$^{-/-}$ mice suffer from fatal polycystic kidney disease, depletion of mature lymphoid cells due to apoptosis in the thymus and spleen, and defects in melanin-production in the hair follicles.[53] *Bclxl* (*Bcl2l1*)$^{-/-}$ mice die at around 13 days of gestation due to extensive neuronal degeneration and apoptosis of immature hematopoietic cells.[54] In mice with loss of only one allele, apoptosis of germ cells during development leads to reduced fertility in males.[55] In addition, BCL-XL was shown to be essential for survival of platelets[56] and differentiated neurons,[57] and for mammary involution.[58] Deletion of Mcl-1 leads to peri-implantation lethality.[59] Conditional knockout studies revealed that MCL-1 is essential for survival of a wide variety of cell types, including B cells,[60,61] plasma cells,[62] hematopoietic stem cells,[63] cardiomyocytes,[64] and neural precursor cells.[65] Bcl-w deficiency in mice leads to germ cell loss and testicular degeneration, probably resulting from Sertoli cell depletion.[66] The mouse ortholog of BFL-1 is A1, which has four isoforms and is only expressed in the hematopoietic compartment. In humans, expression is more widespread, though predominantly hematopoietic as well.[67] Complete loss of all A1 isoforms only causes minor defects in mice, with small decreases in γδT cells, memory CD4+ T cells, and conventional dendritic cells, suggesting functional redundancy with other pro-survival BCL-2 proteins.[68] The sixth pro-survival BCL-2 protein, BCL-B, is in amino acid sequence homology closest to the murine Boo/Diva, but the differences in function, expression, and protein interaction partners suggest that BCL-B and Boo/Diva are no genuine orthologs.[69,70] As a result, no murine studies are available to investigate the role of BCL-B.

Given the fact that many adult cell types depend on pro-survival BCL-2 proteins for survival, overexpression of pro-survival BCL-2 proteins can contribute to malignant transformation of cells that would otherwise be removed by apoptosis. In follicular lymphoma, overexpression of BCL-2 as a consequence of the t(14;18) hallmark chromosomal translocation occurs in 85-90% of cases.[15,71] In addition, high levels of BCL-2 are detected in chronic lymphocytic leukemia (CLL), diffuse large B cell lymphoma (DLBCL), mantle cell lymphoma, and solid tumors including breast and lung cancer.[72–76] Other pro-survival BCL-2 proteins have been strongly associated with cancer as well. High expression of BCL-XL and BCL-W has been reported in subsets of Burkitt lymphoma, follicular lymphoma, DLBCL, and mantle cell lymphoma.[77] MCL-1 overexpression is reported in multiple myeloma (MM),[78] acute myeloid leukemia,[79] hepatocellular carcinoma,[80] non-small cell lung cancer,[81] and breast cancer.[82] Notably, in MM, non-small cell lung cancer, and breast cancer, high MCL-1 expression has also been associated with poor disease prognosis.[78,81,82]

SCOPE OF THIS THESIS

The research described in this thesis focuses on pro-survival BCL-2 family protein MCL-1 as therapeutic target in MM, a plasma cell (PC) malignancy. Resistance to apoptosis enables malignant cells to survive in the presence of otherwise lethal chromosomal, cellular and metabolic alterations. As a consequence, from a therapeutic point of view, dependence on pro-survival proteins is a potentially targetable weakness of these cancer cells.

Chapter 2 serves as an introduction into the regulation and targetability of MCL-1, BCL-2, and BCL-XL in healthy PC differentiation and in the malignancies that can arise during this process, thereby focusing on multiple myeloma. In this review, current preclinical and clinical research into small-molecule inhibitors targeting BCL-2 family members (BH3-mimetics) is summarized.

In **Chapter 3**, the relative roles of MCL-1, BCL-2, and BCL-XL during the different stages of healthy B cell development and PC differentiation are studied, by combining inducible knockout mouse models and BH3-mimetics.

In MM, resistance to apoptosis is often mediated by overexpression of MCL-1, and to a lesser extent BCL-2 or BCL-XL. Due to heterogeneity between patients and redundancy of these proteins, it is difficult to predict which patients will benefit from treatment with a certain BH3-mimetic. Since MCL-1 is located on chromosome locus 1q21, a region that is amplified in ~30% of newly diagnosed and ~70% of relapsed MM, we hypothesize that MM with 1q21 amplification has increased dependence on MCL-1. In **Chapter 4**, this hypothesis is addressed using 31 human MM cell lines and 47 bone marrow aspirates from newly diagnosed MM patients.

In addition to PC, many other tissues have been reported to rely on MCL-1 for survival. Therefore, clinical targeting of MCL-1 using specific MCL-1 inhibitors may lead to side-effects. One way to circumvent these possible side-effects is by targeting MCL-1 indirectly, thereby exploiting cancer- or tissue-specific characteristics of MCL-1 regulation in MM. Proteasome inhibitors such as bortezomib, which is often used in first-line treatment of MM, are frequently considered to act as indirect MCL-1 inhibitors, due to their upregulation of MCL-1 antagonist NOXA. **Chapter 5** addresses the importance of NOXA expression in bortezomib-induced cell killing using NOXA knockout MM cell lines. Besides by interplay with pro-survival and pro-apoptotic BCL-2 family proteins, MCL-1 function is also regulated by post-translational modifications. In **Chapter 6**, molecular regulation of MCL-1 protein stability is studied, with the aim to identify phosphatases that upregulate MCL-1 protein levels in MM.

Inhibition of MCL-1 is a therapeutic strategy for cancers that depend on MCL-1 for survival, but this strategy may also be used for cells that have become resistant

to other killing pathways. For example, overexpression of protease inhibitor SerpinB9 confers resistance to granzyme B-mediated killing by T and NK cells. **Chapter 7** describes the widespread expression of SerpinB9 across cancers, and strategies to target the intrinsic and extrinsic apoptosis pathways in order to overturn SerpinB9-mediated resistance to T cell immunotherapy.

In **Chapter 8**, the results and insights obtained during these research projects are discussed and future perspectives are outlined.

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CHAPTER 2

Role and regulation of pro-survival BCL-2 proteins in multiple myeloma

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ABSTRACT

Apoptosis plays a key role in protection against genomic instability and maintaining tissue homeostasis, and also shapes humoral immune responses. During generation of an antibody response, multiple rounds of B cell expansion and selection take place in germinal centers (GC) before high antigen affinity memory B cells and long-lived plasma cells (PC) are produced. These processes are tightly regulated by the intrinsic apoptosis pathway, and malignant transformation throughout and following the GC reaction is often characterized by apoptosis resistance. Expression of pro-survival BCL-2 family protein MCL-1 is essential for survival of malignant PC in multiple myeloma (MM). In addition, BCL-2 and BCL-XL contribute to apoptosis resistance. MCL-1, BCL-2, and BCL-XL expression is induced and maintained by signals from the bone marrow microenvironment, but overexpression can also result from genetic lesions. Since MM PC depend on these proteins for survival, inhibiting pro-survival BCL-2 proteins using novel and highly specific BH3-mimetic inhibitors is a promising strategy for treatment. This review addresses the role and regulation of pro-survival BCL-2 family proteins during healthy PC differentiation and in MM, as well as their potential as therapeutic targets.

INTRODUCTION

Multiple myeloma (MM) is a malignancy of clonal long-lived plasma cells (PC) residing in the bone marrow (BM).[1] The malignancy arises as a result of genetic changes that occur during differentiation of B cells into PC.[2,3] MM is characterized by resistance against the intrinsic apoptosis pathway, which is regulated by proteins of the BCL-2 family.[4]

The BCL-2 protein family consists of pro-survival BCL-2-like proteins (BCL-2, BCL-B, BCL-W, BCL-XL, BFL-1/A1, and MCL-1), pro-apoptotic BH3-only proteins (initiators), and pro-apoptotic effectors BAX, BAK,[5] and possibly BOK.[6–8] Cytotoxic stimuli such as DNA damage, chemotherapeutic agents, or cytokine deprivation promote upregulation of BH3-only proteins, which inhibit pro-survival BCL-2 family member.[5] In addition, post-translational modification of BH3-only proteins can affect their stability, activity, and subcellular localization.[9] BH3-only proteins vary in their affinities for different pro-survival proteins. For instance, BAD only binds with high affinity to BCL-2, BCL-XL, and BCL-W, while NOXA selectively inhibits MCL-1 and BFL-1/A1. BIM, PUMA, and BID have high affinity for all pro-survival proteins.[10,11] If all available pro-survival proteins are sequestered by BH3-only proteins, BAX and BAK can disrupt the mitochondrial outer membrane, leading to cytochrome C release, caspase activation and execution of apoptosis.[12] In addition, some BH3-only proteins, including BIM, PUMA and BID, can directly bind to BAX or BAK and induce conformational changes that contribute to BAX/BAK activation.[13–15] Regulation of apoptosis is essential for generation and selection of high-affinity PC, and malignant transformation of cells in this process often coincides with defects in apoptosis.

HEALTHY PC DIFFERENTIATION

Long-lived PC originate from germinal centers (GC), which are dynamic structures that develop in secondary lymphoid organs upon antigen stimulation and helper T cell activation. Clonal expansion, somatic hypermutation, class switch recombination, and affinity-based selection of B cells take place in GCs, resulting in the production of high-affinity antibodies.[16] GCs contain a dark zone (DZ), consisting of dividing B cells, and a light zone (LZ), in which B cells are selected based on antigen affinity through B cell receptor (BCR) signaling and CD40-CD40L interactions.[17–19] B cells with low antigen affinity undergo apoptosis, and B cells with high antigen affinity either return to the DZ for another round of mutation and expansion, or differentiate and move out of the GC as memory B cells or PC. Somatic hypermutation and class switch recombination take place during proliferation in the DZ and are mediated by activation-induced cytidine

deaminase (AID).[20] Most GC-derived PC are recruited into the BM, where stromal cells provide signals for long-term survival.[21,22]

The BCL-2 family in PC differentiation

Apoptosis regulation plays a central role in the cycle of expansion, selection, and differentiation that eventually produces mature PC. Expression of BCL-2 family proteins during PC differentiation and after malignant transformation of (post-) GC B cells is highly variable and shown in Figure 1. MCL-1 is essential for GC formation and maintenance, memory B cell development,[23] and survival of existing PC.[24] In fact, B cells are dependent on MCL-1 throughout development.[25] BCL-2, which is important for naïve and memory B cells, is downregulated in the GC.[26,27] In contrast, MCL-1, BCL-XL, and BFL-1 are upregulated. BH3-only proteins BIM and BIK are also upregulated in the GC, but this upregulation was shown to be countered by MCL-1 and BCL-XL, respectively.[27] Apoptosis of low-affinity B cells in the GC is dependent on the interplay between pro-survival and pro-apoptotic BCL-2 proteins. In mice, overexpression of Bcl-2 was shown to disrupt GC selection of memory B cells, but not of high-affinity plasmablasts.[28] Knockout of Bim[29] or Noxa[30] resulted in increased amounts of low-affinity B cells, suggesting that these BH3-only proteins play a critical role in elimination of low-affinity B cell and PC clones. Puma was shown to be essential for regulation of memory formation in mice, since its loss resulted in accumulation of memory B cells.[31] Fully differentiated GC-derived PC are characterized by high expression of transcriptional regulator BLIMP-1, which promotes MCL-1 expression and represses BIM.[32]

MALIGNANT TRANSFORMATION OF GC B CELLS

In the GC, somatic hypermutation and class switch recombination are mediated by AID, which functions by deaminating cytidine residues to uracil.[20] AID is targeted to the variable immunoglobulin (Ig) regions, as well as the Ig switch regions. As a result of AID activity, the mutation rate in the variable Ig regions is estimated to increase to between 10^{-2} and 10^{-3} mutations per bp.[33] In addition to its function in the Ig gene, AID can also be erroneously targeted to other genomic loci, introducing mutations and Ig translocations that can contribute to malignant transformation.[34,35] Many different malignancies, some of which dependent on BCL-2 family proteins for survival, arise from (post-) GC B cells (Figure 1). These include B cell chronic lymphocytic leukemia (CLL),[36] follicular lymphoma,[37] diffuse-large B cell lymphoma (DLBCL),[38] Waldenström macroglobulinemia (WM),[39] and multiple myeloma (MM).[40]

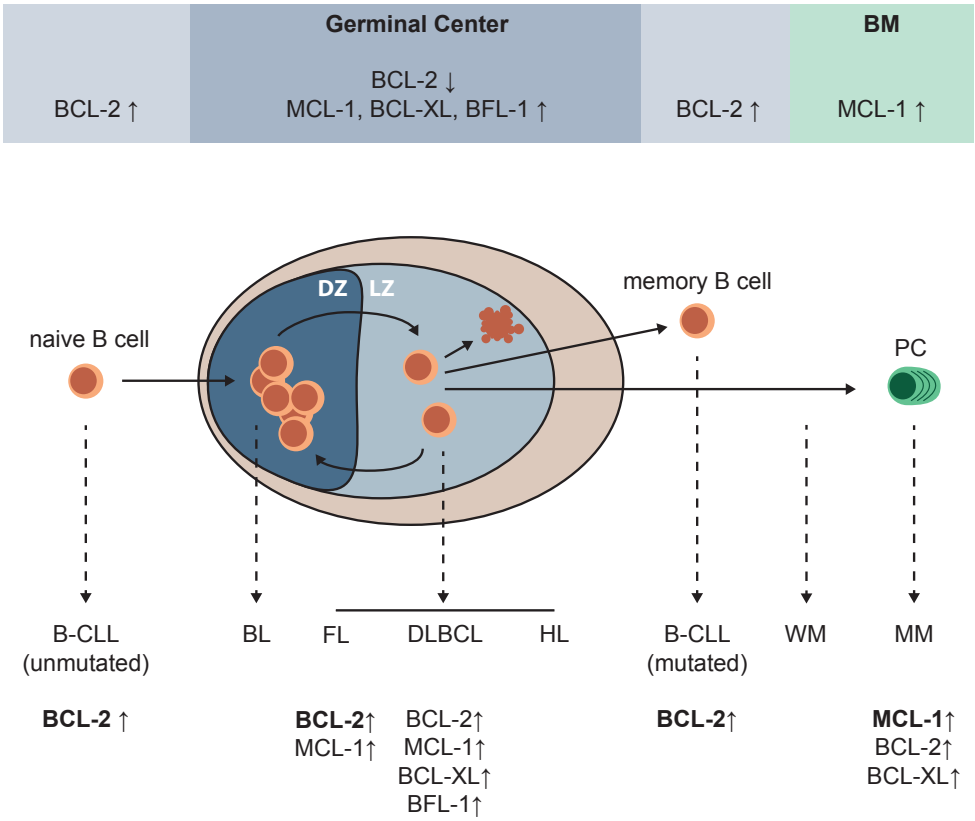

Figure 1. Expression of pro-survival BCL-2 family proteins during PC differentiation and after malignant transformation of (post-) GC B cells. Upon encounter of a naïve B cell with its cognate antigen, and in the presence of adequate T cell help, a germinal center (GC) is formed where the B cell undergoes multiple cycles of expansion and hypermutation in the dark zone (DZ), and affinity-based selection in the light zone (LZ). Low-affinity B cells undergo apoptosis, while high-affinity B cells can undergo further selection, or exit the GC as a memory B cell or plasma cell (PC). In the GC, BCL-2 expression is strongly repressed and expression of MCL-1, BCL-XL, and BFL-1 is increased. MCL-1, but not BCL-XL, was shown to be essential for survival of GC B cells. Naïve and memory B cells have high expression of BCL-2 and are sensitive to its inhibition, and PC depend on MCL-1 expression for survival. Erroneous targeting of activation-induced cytidine deaminase (AID) during somatic hypermutation and class switch recombination can lead to mutations that promote malignant transformation, resulting in a variety of GC-derived malignancies (dashed lines). Multiple GC-derived malignancies, such as follicular lymphoma (FL), diffuse-large B cell lymphoma (DLBCL), some B cell chronic lymphocytic leukemias (B-CLL), and multiple myeloma (MM) depend on overexpression of BCL-2 family proteins for survival. Abbreviations: BL, Burkitt lymphoma; BM, bone marrow; CLL, chronic lymphocytic leukemia; DLBCL, diffuse-large cell B cell lymphoma; DZ, dark zone; FL, follicular lymphoma; HL, Hodgkin lymphoma; LZ, light zone; MCL, mantle cell lymphoma; MM, multiple myeloma; PC, plasma cell; WM, Waldenström macroglobulinemia.

Pro-survival BCL-2 proteins in GC B cell malignancies

Pro-survival BCL-2 proteins contribute to apoptosis resistance of malignant B cells, and their overexpression can be regulated in different ways. In 60-65% of CLL cases, the BCR is hypermutated, indicating that the malignancy originates from post-GC B cells. Conversely, in the remaining 35-40% of cases, the BCR lacks signs of hypermutation and the disease presumably originates from B cells that have differentiated independently of the GC.[36] In both types, apoptosis resistance is mediated by overexpression of BCL-2.[41] This overexpression is due to *BCL2* gene hypomethylation and genetic loss of microRNA loci that normally inhibit BCL-2 expression.[42,43] Inhibition of BCL-2 using specific BH3-mimetic inhibitor venetoclax efficiently induces apoptosis in CLL cells in circulation, and is also promising for other BCL-2 dependent malignancies such as follicular lymphoma and a subset of DLBCL.[44–46]

Follicular lymphoma originates from GC B cells and is characterized by the hallmark chromosomal translocation t(14;18), which is present in 85% of patients and results in overexpression of BCL-2 due to juxtaposition of the Ig heavy chain (*IGH*) and *BCL2* loci.[37] In addition, MCL-1 is highly expressed in some follicular lymphomas, and its expression correlates with disease grade.[47]

DLBCL has distinct subtypes, including germinal center B cell-like (GCB-) DLBCL, which is derived from normal GC B cells; and activated B cell-like (ABC-) DLBCL, originating from B cells that have completed the GC reaction.[48] T(14;18) is present in 45% of GCB-DLBCL, but does not occur in ABC-DLBCL.[49] Still, *BCL2* expression is high in many cases of ABC-DLBCL, as a result of gain or amplification of the 18q chromosome arm on which *BCL2* is located.[50] MCL-1 expression is also frequently high in ABC-DLBCL and sometimes in GCB-DLBCL, possibly as a result of chromosomal amplification or transcriptional regulation.[51] In addition, ABC-DLBCL is characterized by constitutively high NF-κB activity. Among the targets of NF-κB are BCL-XL, BFL-1/A1, and possibly BCL-2, whose high expression as a result of NF-κB signaling may contribute to apoptosis resistance in ABC-DLBCL.[52–54]

MM and WM are malignancies that contain a clonal PC population residing in the bone marrow. Both are preceded by monoclonal gammopathy of undetermined significance (MGUS), which is characterized by presence of less than 10% clonal PC in the BM, presence of monoclonal Ig in the blood, and lack of clinical symptoms.[55,56] WM originates from post-GC B cells that have undergone somatic hypermutation but did not undergo class switching, whereas MM originates from post-GC B cells after class switching.[39] As a result, the serum Ig in WM is of the IgM type, and IgH translocations do not occur.[57] The cellular phenotype is mixed, ranging from B cells to PC.[58] Possibly, malignancy is acquired during the B cell or

plasmablast stage, with some malignant cells continuously differentiating into PC. MM, on the other hand, consists of fully differentiated PC and is characterized by frequent IgH translocations and genomic instability.[59] MM cells most frequently produce IgG or IgA, although IgM or IgD have been observed in rare cases.[60] In WM, pro-apoptotic and pro-survival BCL-2 family proteins are expressed at low levels similar to non-malignant B cells and PC. It is therefore expected that WM will only be sensitive to BH3-mimetic drugs if these are combined with other treatments that increase pro-apoptotic protein levels and mitochondrial priming.[61] In contrast, MM cells are highly dependent on BCL-2 family proteins for survival, with MCL-1 as the essential player.[62,63]

THE BCL-2 FAMILY IN MULTIPLE MYELOMA

MCL-1 protein expression is increased in newly diagnosed MM compared to healthy PC, and protein levels are even higher at relapse.[64] In addition, overexpression of MCL-1 is associated with shorter patient survival.[64] Using RNA interference lethality screening in cell lines, MCL-1 was also identified as one of the most important and selective survival genes for MM.[65] In subsets of MM cell lines and patient samples, BCL-2 and BCL-XL expression is also high,[66] suggesting that these three proteins may act redundantly in preventing apoptosis. Since expression of both pro-survival and pro-apoptotic BCL-2 family members is heterogeneous, and the interplay between them is complex and dynamic, dependence on MCL-1, BCL-2, and BCL-XL is likely to differ between patients.[66–68] Signals and cellular processes that may lead to overexpression of MCL-1, BCL-2, and BCL-XL in MM are indicated in Figure 2.

Survival signals from the BM microenvironment

MM cells reside in the BM, where they interact with extracellular matrix proteins and cells from the BM microenvironment, which include stromal cells, osteoblasts, osteoclasts, endothelial cells, fibroblasts, adipocytes and cells of hematopoietic origin.[40] MM cells promote neighboring cells to produce IL-6,[69] which induces JAK/STAT3 signaling in MM, leading to transcription of MCL-1 and BCL-XL.[70–73] MCL-1 expression in MM can also be IL-6-independent,[74] or occur via other signals from the BM microenvironment.[75] For instance, signaling through BAFF (B cell activating factor) and APRIL (a proliferation-inducing ligand), whose levels are increased in MM patients compared to healthy controls, induces expression of both MCL-1 and BCL-2 and promotes PC survival.[24,76] Other survival signals from the bone marrow environment include interferon α (IFN-α), which induces MCL-1

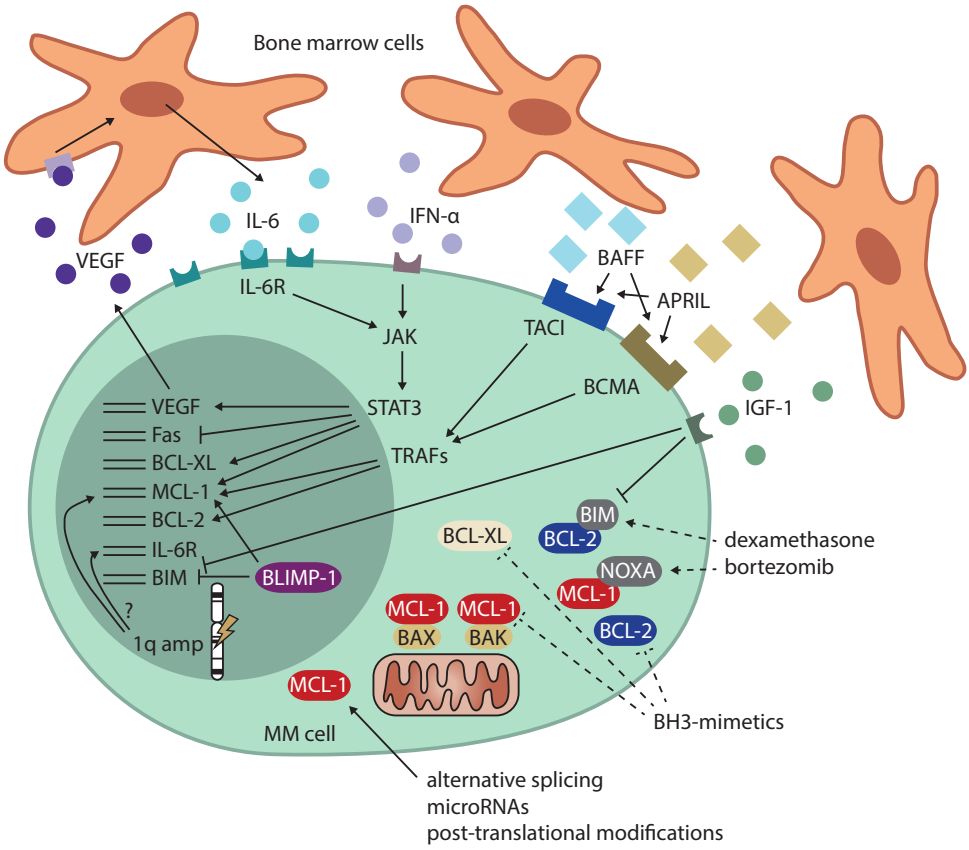

Figure 2. Signals and cellular processes that mediate apoptosis resistance in MM. MM cells receive signals from the bone marrow microenvironment that stimulate their survival. These signals include IL-6 and IFN-α, leading to JAK/STAT signaling and expression of MCL-1, BCL-XL, and VEGF. VEGF, in turn, promotes IL-6 production by neighboring cells. Other signals from the bone marrow microenvironment include BAFF and APRIL, which signal via TRAFs and induce expression of MCL-1 and BCL-2. IGF-1 signaling downregulates BIM, transcriptionally as well as post-translationally. MM cells also have high expression of the PC transcriptional regulator BLIMP-1, which promotes MCL-1 and represses BIM expression. Amplification of the 1q chromosome arm often occurs in MM. The genes for both MCL-1 and the IL-6 receptor (IL-6R) are present on this locus, possibly leading to overexpression in 1q-positive MM. In addition to transcriptional regulation, MCL-1 is heavily regulated post-transcriptionally, which may contribute to the high MCL-1 protein levels found in MM. Dashed lines represent methods for interference in apoptosis resistance by MM drugs dexamethasone and bortezomib, and by BH3-mimetics. Abbreviations: APRIL, a proliferation-inducing ligand; BAFF, B cell activating factor; BCMA, B cell maturation antigen; BH-3, BCL-2 homolog 3; BLIMP-1, B lymphocyte-induced maturation protein 1; IFN-α, interferon alpha; IGF-1, insulin-like growth factor 1; IL-6, interleukin 6; IL-6R, interleukin 6 receptor; JAK, janus kinase; STAT3, signal transducer and activator of transcription 3; TACI, transmembrane activator and calcium-modulating ligand interactor; TRAF, TNF receptor-associated factor; VEGF, vascular endothelial growth factor

in a STAT3-dependent manner,[77] and insulin-like growth factor 1 (IGF-1), which downregulates expression of BIM.[78]

Genetic lesions

MM is characterized by recurrent chromosomal aberrations, some of which may be linked to apoptosis pathways. Translocations or chromosomal amplifications and gains involving 18q are rare in MM,[79] suggesting that *BCL2* overexpression is not a key event in malignant transformation. No other genetic lesions in MM have directly been correlated to overexpression of a BCL-2 family member. Nevertheless, gain or amplification of 1q21, the chromosome region containing the *MCL1* gene, occurs in approximately 40% of MM cases and correlates with poor disease prognosis.[80] Notably, *IL6R*, the gene encoding the IL-6 receptor, is also located on 1q21, as are several other candidate drivers of high-risk disease.[81]

T(4;14), which is present in 10-15% of MM patients,[80] may lead to disruption and subsequent overexpression of fibroblast growth receptor 3 (*FGFR3)*, which is considered an oncogene.[79] In a murine IL-6-dependent hybridoma cell line, FGFR3 was shown to signal through STAT3 and substitute IL-6 signaling, leading to increased BCL-XL expression and decreased apoptosis.[82] Correspondingly, specific tyrosine kinase inhibitors with known anti-FGFR3 activity induced apoptosis in t(4;14)-positive cell lines.[83]

MCL-1 stabilization

Unlike for BCL-2 and BCL-XL,[66] transcriptional activity of *MCL1* does not directly correlate to protein levels. MCL-1 is unique within the BCL-2 family because it has a large N-terminal domain that allows for post-translational modification.[84,85] Proteasomal degradation of MCL-1 occurs upon phosphorylation and subsequent poly-ubiquitination of this N-terminal region. Kinases associated with phosphorylation of MCL-1 include JNK, GSK-3, and ERK-1.[86] Ubiquitin ligases Mule, $SCF^{\beta\text{-}TrCP}$, SCF^{Fbw7}, and APC/C^{Cdc20} were shown to target MCL-1 for proteasomal degradation after recognizing specific phosphorylated residues.[87] This process can be reversed by deubiquitinases, such as USP9X.[88] The contribution of these kinases and ubiquitin modifiers to MCL-1 regulation in MM is currently unknown. If the key players in MCL-1 regulation can be identified for MM, these MCL-1-modifying proteins may be interesting targets for therapeutic intervention.

OVERCOMING APOPTOSIS RESISTANCE: BCL-2 PROTEINS AS THERAPEUTIC TARGETS IN MM

As apoptosis resistance in B cell malignancies often results from overexpression of pro-survival BCL-2 family proteins, inhibiting these proteins is a promising strategy for development of targeted therapeutics. Several BCL-2 family inhibitors, also named BH3-mimetics because of their structural and functional resemblance to the BH3 domain of BH3-only proteins, are currently in clinical development. BCL-2 inhibitor venetoclax is the first BH3-mimetic approved by the Food and Drug Administration. It was approved in 2016 for treatment of CLL with a 17p deletion.[46] Additionally, venetoclax was tested in phase I clinical trials with relapsed and refractory MM patients, where monotherapy was particularly effective when the t(11;14) mutation was present.[89] T(11;14) is associated with an increased *BCL2*/*MCL1* mRNA ratio, but the mechanism behind this is unknown.[90] When MM patients were treated with venetoclax in combination with conventional MM drugs bortezomib (a proteasome inhibitor) and dexamethasone, it was well tolerated and the response rate was highest in patients with high *BCL2* expression.[91] Experiments in cell lines even indicate more-than-additive effects when venetoclax is combined with proteasome inhibitor carfilzomib or dexamethasone, due to upregulation of NOXA and BIM, respectively.[92] If conventional treatment increases availability of BH3-only proteins and their distribution towards pro-survival target proteins, this may increase sensitivity to BH3-mimetic drugs.

While the results of MM treatment with venetoclax underline the potential of using BH3-mimetics in MM, they also suggest that venetoclax may only be effective in a subset of patients, namely those who have relatively high BCL-2 and relatively low MCL-1. Based on *in vitro* and xenograft experiments, MCL-1 is often shown to be essential for MM survival and its generally high expression may confer resistance to venetoclax.[66,93,94] Therefore, MCL-1 itself is a very promising therapeutic target in MM, and multiple MCL-1 inhibitors are currently under development.[95] MCL-1 inhibitor S63845 efficiently kills MM and other MCL-1-dependent cancer cell lines.[96] Its derivate S64315/MIK665 is currently being tested in phase I clinical trials by Servier for acute myeloid leukemia and myelodysplastic syndrome (NCT02979366), and by Novartis for MM and DLBCL (NCT02992483). In addition, clinical testing in MM patients has started with MCL-1 inhibitors developed by Amgen, named AMG 176 and AMG 397 (NCT02675452 and NCT03465540, respectively),[97] and by AstraZeneca, named AZD5991 (NCT03218683).[98]

Simultaneous targeting of multiple BCL-2 family proteins may be a solution to resistance in case of redundancy between MCL-1, BCL-2, and BCL-XL in MM. Before the development of venetoclax, BH3-mimetics with broader protein specificity have been studied, such as navitoclax.[99] Navitoclax (ABT-

263) mimics the selectivity of BAD, thereby inhibiting only BCL-2, BCL-XL, and BCL-W. When tested in CLL patients, results were promising, but dose-limiting thrombocytopenia was observed as a result of BCL-XL inhibition.[100–102] This led to the development of BCL-2-selective BH3-mimetic venetoclax.[44] Other putative BCL-2 family inhibitors with broad target specificity, such as obatoclax (GX15-070), were shown to function partly or completely in a BAX/BAK-independent manner, and are therefore no longer considered BH3-mimetics.[103] The results with navitoclax indicate that potential side-effects of BCL-2 family inhibitors may be dose-limiting, and that combined inhibition of BCL-2 family members may only be possible if the concentration of each specific inhibitor remains below the threshold of toxicity.

MCL-1 is not only essential for B cells and PC, it is also essential in other cell types, including hematopoietic stem cells,[104] cardiomyocytes,[105] and neural precursor cells.[106] In contrast to healthy cells, increased expression of pro-apoptotic molecules ('priming') renders malignant cells more susceptible to apoptosis upon inactivation of pro-survival proteins.[107] Since MCL-1 is the most dominant pro-survival protein in MM, its inhibition leads to release of a large proportion of pro-apoptotic proteins present in MM cells, thereby promoting apoptosis induction. In mice, MCL-1 inhibitor S63845 was tolerated well at concentrations that killed cancer cells,[96] even when murine MCL-1 was replaced by its human ortholog, thereby increasing inhibitor sensitivity of all cells.[108] This may yield a therapeutic window for targeting MCL-1, especially if MCL-1 inhibitors are combined with existing treatments that increase pro-apoptotic protein expression.

CONCLUSION

High expression of pro-survival BCL-2 family proteins contributes to outgrowth and drug resistance of malignant B cell clones. While beneficial for cell survival, addiction to high levels of specific pro-survival BCL-2 proteins also makes cells vulnerable to BCL-2 family inhibition using BH3-mimetic drugs. MM is characterized by high expression of MCL-1, and overexpression of BCL-2 and BCL-XL is observed in subsets of patients. Constitutive overexpression of these pro-survival proteins in MM results from a range of microenvironmental signals and different genetic lesions. This complex regulation of MCL-1, BCL-2, and BCL-XL offers multiple direct and indirect targets for therapeutic intervention. Recent development of BH3-mimetic drugs, that specifically target MCL-1, BCL-2, or BCL-XL, may contribute to overcoming apoptosis resistance and improving treatment for MM.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

AS and VP wrote the manuscript and designed the figures. Both authors read and approved the final manuscript.

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ABBREVIATIONS

ABC-DLBCL, activated B cell-like diffuse-large B cell lymphoma; AID, activation-induced cytidine deaminase; APRIL, a proliferation-inducing ligand; BAFF, B cell activating factor; BCL-2, B cell lymphoma 2; BCR, B cell receptor; BH3, BCL-2 homology 3; BM, bone marrow; CLL, (B cell) chronic lymphocytic leukemia; DLBCL, diffuse-large B cell lymphoma; DZ, dark zone; FGFR3, fibroblast growth factor receptor 3; GC, germinal center; GCB-DLBCL, germinal center B cell-like diffuse-large B cell lymphoma; IFN-α, interferon α; Ig, immunoglobulin; IGF-1, insulin-like growth factor 1; IgH, immunoglobulin heavy chain; IL-6, interleukin 6; LZ, light zone; MCL-1, myeloid cell leukemia 1; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PC, plasma cell; WM, Waldenström macroglobulinemia.

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CHAPTER 3

MCL-1 is required throughout B cell development and its loss sensitizes specific B cell subsets to inhibition of BCL-2 or BCL-XL

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ABSTRACT

Pro-survival BCL-2 family members protect cells from programmed cell death that can be induced by multiple internal or external cues. Within the hematopoietic lineages, the BCL-2 family members BCL-2, BCL-XL, and MCL-1 are known to support cell survival but the individual and overlapping roles of these pro-survival BCL-2 proteins for the persistence of individual leukocyte subsets *in vivo* has not yet been determined. By combining inducible knockout mouse models with the BH3-mimetic compound ABT-737, which inhibits BCL-2, BCL-XL, and BCL-W, we found that dependency on MCL-1, BCL-XL, or BCL-2 expression changes during B cell development. We show that BCL-XL expression promotes survival of immature B cells, expression of BCL-2 is important for survival of mature B cells and long-lived plasma cells (PC), and expression of MCL-1 is important for survival throughout B cell development. These data were confirmed with novel highly specific BH3-mimetic compounds that target either BCL-2, BCL-XL, or MCL-1. In addition, we observed that combined inhibition of these pro-survival proteins acts in concert to delete specific B cell subsets. Reduced expression of MCL-1 further sensitized immature as well as transitional B cells and splenic PC to loss of BCL-XL expression. More markedly, loss of MCL-1 greatly sensitizes PC populations to BCL-2 inhibition using ABT-737, even though the total wild-type PC pool in the spleen is not significantly affected by this drug and the bone marrow (BM) PC population only slightly. Combined loss or inhibition of MCL-1 and BCL-2 reduced the numbers of established PC >100-fold within days. Our data suggest that combination treatment targeting these pro-survival proteins could be advantageous for treatment of antibody-mediated autoimmune diseases and B cell malignancies.

INTRODUCTION

Studies in cell lines and primary cells have revealed that when overexpressed, all pro-survival BCL-2 family members, BCL-2, BCL-XL, BCL-W, A1, and MCL-1, are capable of inhibiting the mitochondrial apoptotic pathway.[1] Transgenic expression (using the immunoglobulin heavy chain gene enhancer, *Eμ*, or the *Vav* gene promoter, *VavP*) of BCL-2, BCL-XL, or MCL-1 results in increased abundance of immature, transitional and mature B cell subsets,[2,3,4] although the marginal zone B cells were not affected by excess BCL-2 or BCL-XL.[5] A detailed analysis of the individual and overlapping contributions of pro-survival BCL-2 family members to normal lymphocyte persistence *in vivo*, however, has been hampered by their necessity during embryonic development. Complete loss of MCL-1 in mice results in embryonic lethality prior to day 3.5,[6] whereas BCL-XL-deficient mice die around embryonic day 14.[7] BCL-2-deficient mice, although born at the expected frequency, succumb to polycystic kidney disease, which commences during embryogenesis.[8,9] The relevance of BCL-W expression to lymphocyte differentiation appears limited, as lymphoid development in BCL-W-deficient mice is normal.[10] This is probably due to the low expression of BCL-W in normal as well as malignant lymphoid cells.[11] The generation of A1-deficient mice has proven difficult owing to quadruplication at the gene locus in mice, but transgenic RNA interference-mediated knockdown of A1 in mice revealed a role for A1 in the maintenance of mature follicular B cells.[12] The recent use of the BH3-mimetic compounds (ABT-263/navitoclax: inhibits BCL-2, BCL-XL, and BCL-W and ABT-199/venetoclax: inhibits BCL-2) to treat B cell malignancies, such as chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma,[13,14] and programs to develop MCL-1-specific BH3-mimetics, suggest that the detailed analysis of the role of individual BCL-2 family proteins in the maintenance of healthy B cells should be useful in aligning the use of such drugs with particular disorders and in predicting damage to normal B cell subsets imposed by such therapies.

We have previously shown that, with the appropriate genetic configuration, the *Mcl1* gene can be efficiently deleted in the B cell lineage *in vivo*.[15,16] In the current study we used this strategy to delete the *Mcl1* and/or *Bclx* genes in an inducible fashion, with and without concomitant treatment with the BH3-mimetic compound ABT-737, to assess the individual and overlapping roles of these pro-survival BCL-2 family members in the maintenance of different B cell subsets. From these experiments, we have learnt that BCL-XL is of only limited importance in B cell development, that MCL-1 is required at multiple stages and that BCL-2, the main target of ABT-737 in mice *in vivo*,[17,18] promotes survival of some terminally differentiated Blimp-1^{hi} PC, in addition to promoting survival of mature follicular and recirculating B cells. Moreover, we found that deletion of

one allele of *Mcl1* sensitizes transitional and mature B cells to loss of BCL-XL. Furthermore, deletion of both alleles of *Mcl1* greatly sensitized established plasma cells (PC) to treatment with ABT-737, even though the total PC pool in wild-type mice was not significantly affected by exposure to this drug.[19]

RESULTS

The importance of MCL-1 for B-lymphocyte development in mice has been demonstrated by others using CD19-Cre-mediated deletion of $Mcl1^{fl}$ alleles. These studies revealed a significant reduction in all B cell subsets from the early pro-B cell stage onwards,[20] this being the stage of Cre-recombinase expression and thus deletion of $Mcl1^{fl}$. These experiments, however, could not reveal a role for MCL-1 beyond the pro-B cell stage as differentiation was halted at this point. To determine if there is differentiation stage-specific sensitivity to *Mcl1* deletion in the B cell lineage specifically, we used a mouse model in which $Mcl1^{fl}$ could be efficiently deleted in established B cell populations of adult mice by delivery of tamoxifen to activate a conditional Cre^{ERT2} recombinase.[16] Deletion of both $Mcl1^{fl}$ alleles in this way rapidly reduced the absolute number of cells in transitional and mature B cell subsets in the spleen and bone marrow (BM) (Figures 1A–D). The impact of $Mcl1^{fl}$ deletion in PC in this setting has been previously reported[16] and the fold difference in PC number after tamoxifen-mediated $Mcl1^{fl}$ deletion is included (Figure 1D).

To determine the contribution of BCL-XL to the sustained survival of B cell subsets, we created BM chimeric mice in which $Bclx^{fl}$ alleles could be inducibly deleted in the B cell lineage.[16] Loss of BCL-XL significantly reduced only the BM immature B cell population (Figures 2A-B). Because of the relatively modest impact of $Bclx^{fl}$ deletion, we next examined the consequences of combined inducible deletion of both alleles of $Bclx^{fl}$ plus one allele of $Mcl1^{fl}$. In addition to a reduction in the numbers of immature B cells caused by loss of BCL-XL expression, additional deletion of one $Mcl1^{fl}$ allele also significantly reduced the numbers of transitional B cells in the BM as well as T1, follicular B and PC in the spleen (Figures 2C-D). The fold differences shown are calculated comparing $Mcl1^{fl/+}Bclx^{fl/fl}ER^{T2}Cre$ with $Mcl1^{fl/+}Bclx^{+/+}ER^{T2}Cre$, thus revealing the impact of *Bclx* deletion in a setting in which MCL-1 expression is reduced with 50% (Figure 2D).

As BCL-XL is mainly important for cell survival during early B cell development, *Bclx* gene expression was measured and related to gene expression of *Mcl1*, *Bcl2* and *A1*. This analysis revealed that, contrary to expression of *Mcl1* and *Bcl2*, *Bclx* transcription peaks early during B cell development and is highest in immature B cells (Figure 3). Expression of both *Mcl1* and *Bcl2* peaks in terminally differentiated PC, whereas expression of *A1* is relatively low in immature

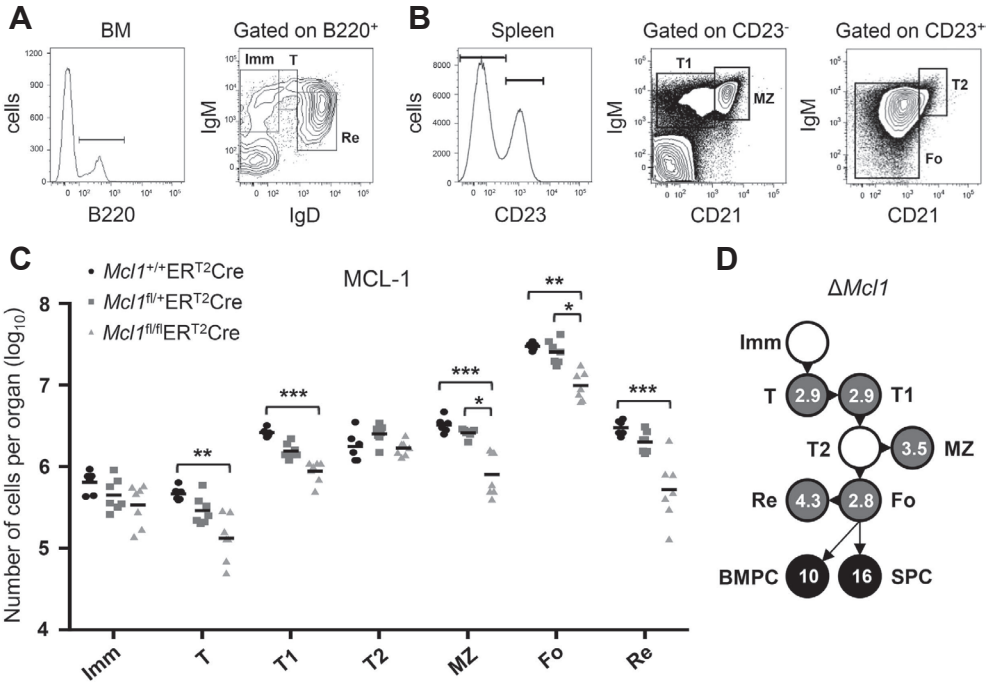


Figure 1. B-lymphoid cells are reliant on MCL-1 expression throughout development. (A and B) Flow cytometric analysis of cells from the BM and spleen of a control (*Mcl1*$^{+/+}$ER^{T2}Cre) mixed-BM chimeric mouse. (A) Subset representation in the BM. Abbreviations adjacent to outlined areas indicate the corresponding B cell population with immature (Imm; B220^{+}IgM^{+}IgD^{-}), transitional (T; B220^{+}IgM^{hi}IgD^{int}) or recirculating mature (Re; B220^{+}IgM^{int}IgD^{hi}) B cells in BM. (B) Subset representation in the spleen. Abbreviations adjacent to outlined areas indicate the corresponding B cell population with transitional type 1 (T1; CD23^{-}IgM^{+}CD21^{lo}), transitional type 2 (T2; CD23^{+}IgM^{hi}CD21^{hi}), marginal zone (MZ; CD23^{-}IgM^{hi}CD21^{hi}) or mature follicular (Fo; CD23^{+}IgM^{+}CD21^{+}) B cells. (C) Absolute numbers of B cell populations from the BM and spleen of mixed-BM chimeras after induced deletion of one (*fl*/+) or two (*fl*/*fl*) alleles of *Mcl1*. *In vivo* Cre-mediated gene deletion was induced by two tamoxifen treatments on consecutive days by oral gavage, with mice sacrificed 48 h after the first dose. Cell numbers were calculated based on total cell numbers per organ and gates of dot plots from flow cytometric analysis as shown in (A) or (B). Efficiency of *Mcl1* gene deletion was shown previously.[15] Data represent two independent experiments and six or seven animals per group. (D) Fold reduction of B cell numbers throughout development comparing control *Mcl1*$^{+/+}$ER^{T2}Cre to *Mcl1*$^{fl/fl}$ER^{T2}Cre mixed-BM chimeras, based on numbers shown in (C) and on previously published data.[16] *P≤0.05, **P≤0.01, ***P≤0.001 (statistical significance was calculated between the groups using a Kruskal–Wallis test followed by a *post hoc* Dunn's multiple comparisons test).

B cells and PC but high in T1 and T2 transitional cells and mature marginal zone and follicular B cells (Figure 3).

ABT-737 potently binds to BCL-2, BCL-XL, and BCL-W in Biacore assays, but has a clear preference for blocking BCL-2 in living cells in culture and within the whole mouse.[17,18] Thus, its effects on cell survival *in vivo* can mainly be attributed

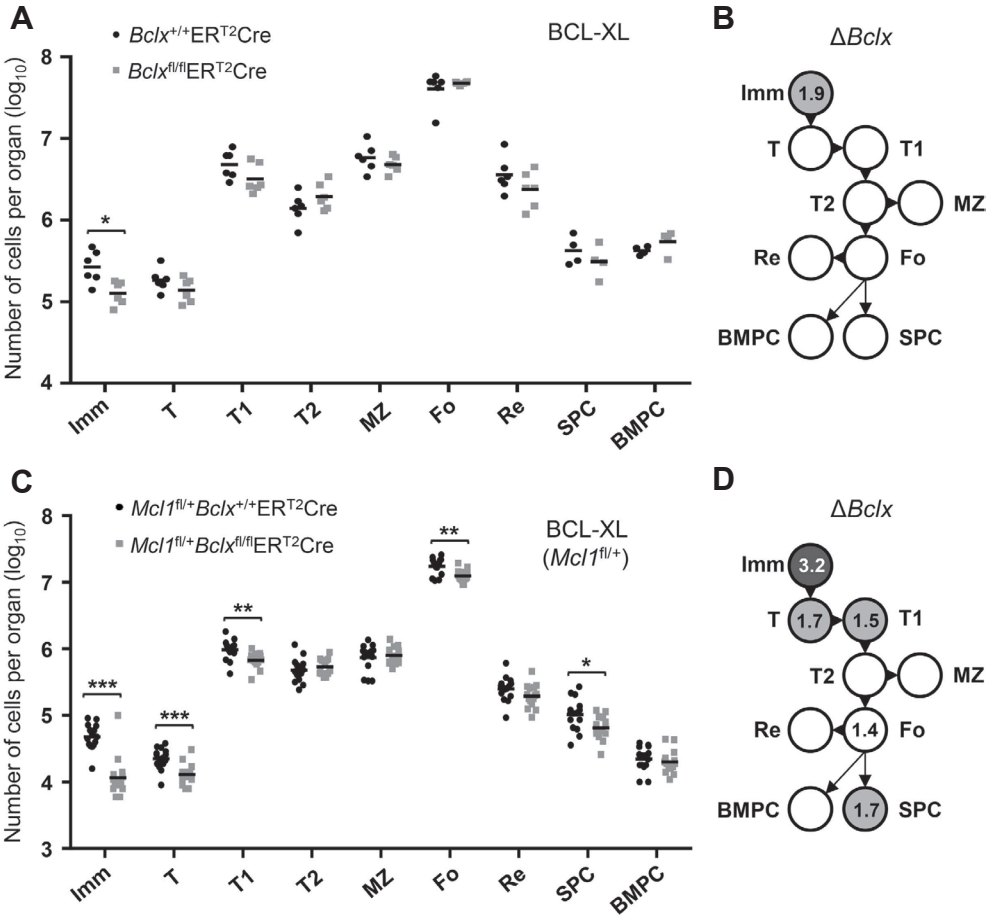


Figure 2. Reduced expression of MCL-1 sensitizes B cells to deletion of *Bclx*. (A) Absolute numbers of cells from the spleen and BM of mixed-BM chimeras after induced deletion of both (*fl*/*fl*) alleles of *Bclx* calculated as in Figure 1. *In vivo* Cre-mediated gene deletion was induced by two tamoxifen treatments on consecutive days by oral gavage, with mice killed 4 days after the first dose. This longer interval allowed for the loss of BCL-XL protein, which has a longer half-life (~24 h) than MCL-1 (<1 h).[15] Efficiency of *Bclx* gene deletion was shown previously.[16] Data represent two independent experiments and six animals per group. (B) Fold reduction of B cell numbers throughout development comparing control *Bclx*$^{+/+}$ER^{T2}Cre and *Bclx*$^{fl/fl}$ER^{T2}Cre mixed-BM chimaeras, based on numbers shown in (A). (C) Absolute numbers of cells after induced deletion of one (*fl*/+) allele of *Mcl1* and both (*fl*/*fl*) alleles of *Bclx*. Mice were killed 4 days after the first tamoxifen treatment. Data represent three independent experiments and 10–15 animals per group. (D) Fold reduction of B cell numbers comparing *Mcl1*$^{fl/+}$*Bclx*$^{+/+}$ER^{T2}Cre and *Mcl1*$^{fl/+}$*Bclx*$^{fl/fl}$ER^{T2}Cre mixed-BM chimeras, based on numbers shown in (C). *P≤0.05, **P≤0.01, ***P≤0.001 (statistical significance was calculated between the groups using a Mann–Whitney test).

to inhibition of BCL-2. ABT-737 was chosen for reasons of cost and availability, and when administered by intraperitoneal injection, becomes available to all relevant organs.[19] ABT-737 has a very similar binding specificity and affinity as the BH3-mimetic ABT-263/navitoclax that has improved bioavailability.[21] Treatment of mice with ABT-737 promotes apoptosis of mature follicular B cells in the spleen and recirculating B cells in the BM.[19,22] It also affects the survival of newly generated PC *en route* to the BM, but does not markedly affect preexisting PCs.[19] We examined the impact of ABT-737 on PC in more detail and found that the total PC population in the BM, but not the spleen, was mildly, but significantly, reduced upon treatment (Figures 4A-B). In addition, we found that mature follicular and

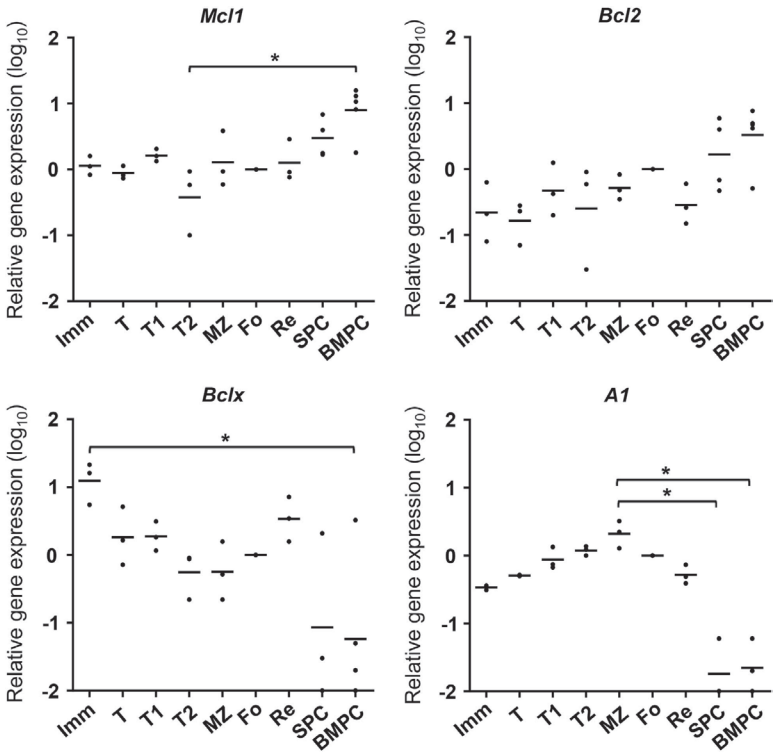


Figure 3. Gene expression of *Mcl1*, *Bcl2*, *Bclx* (*Bcl2l1*), and *A1* (*Bcl2a1*) throughout B cell development. Quantitative PCR analysis of mRNA encoding pro-survival members of the BCL-2 family in different B cell populations. FACS-sorting was performed as shown in Figures 1A and B. Data are normalized to expression of the housekeeping gene *Hprt* and presented relative to expression in Fo B cells, set as 0 (log_{10} of 1). Numbers in the graph indicate relative expression and represent three to five independent experiments, each performed in duplicate. *P≤0.05 (statistical significance was calculated between the groups using a Kruskal–Wallis test followed by a *post hoc* Dunn's multiple comparisons test).

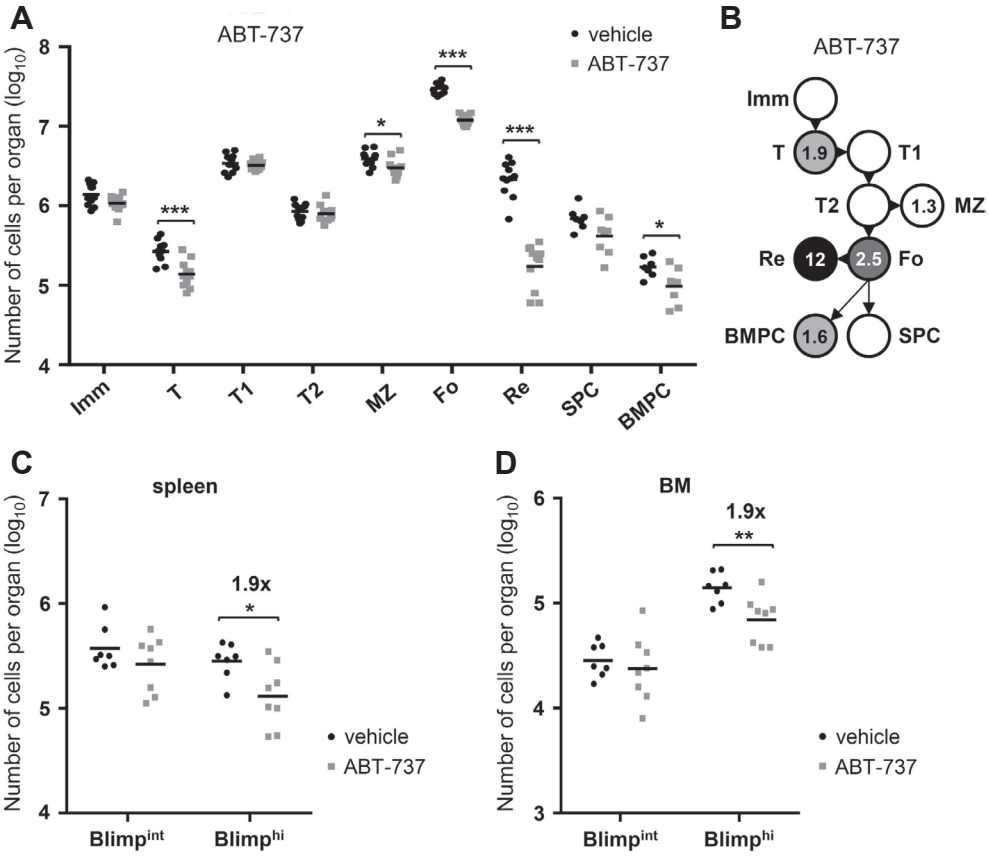
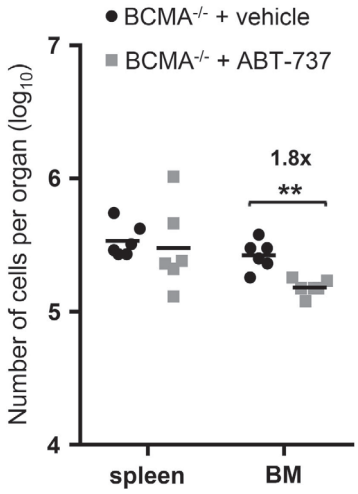


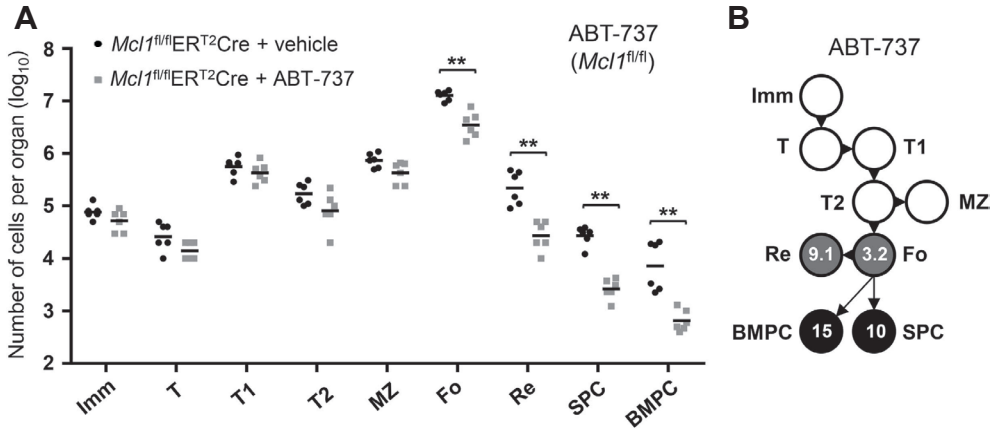
Figure 4. Treatment with BH3-mimetic ABT-737 reduces Blimp-1^{hi} plasma cells. (A) Absolute numbers of cells after treatment of wild-type mice with 75 mg/kg ABT-737 for 5 days, calculated as in Figure 1. Data represent three experiments and 7–11 animals per group. (B) Fold reduction of B cell numbers comparing wild-type+vehicle and wild-type+ABT-737, based on numbers shown in (A). (C–D) Absolute numbers of Blimp-1^{int} and Blimp-1^{hi} PC after treatment of Blimp-1-GFP reporter mice (*Prdm1*^{GFP})[21] with ABT-737 as in (A). Shown are PC in spleen (C) and BM (D). Data in (C) and (D) represent two experiments and 7–8 animals per group. *P≤0.05, **P≤0.01, ***P≤0.001 (statistical significance was calculated between the groups using a Mann–Whitney test).

recirculating B cells were strongly reduced after treatment, as expected from previous studies. We also observed a significant reduction in the numbers of marginal zone B cells and transitional B cells in the BM (Figures 4A-B). Next, we compared short-lived (Blimp-1^{int}) with long-lived (Blimp-1^{hi}) PC for sensitivity to ABT-737 by treating Blimp-1-GFP reporter mice (in which GFP is expressed from the gene encoding Blimp-1 (*Prdm1*^{GFP}))[23] with this drug. Interestingly, we observed that in both the spleen and BM, only the Blimp-1^{hi} PC were affected by ABT-737 treatment (Figures 4C-D). As, in contrast to the spleen, the majority of PC in the



◄Figure 5. Plasma cells that persist in the absence of TNF receptor BCMA remain sensitive to ABT-737. Absolute numbers of PC ($CD138^{+}B220^{lo}$) after ABT-737 treatment of lethally irradiated mice reconstituted with *Bcma*$^{-/-}$ BM, performed as in Figure 4. Data represent one experiment and six animals per group. **P≤0.01 (statistical significance was calculated between the groups using a Mann–Whitney test).

▼Figure 6. Reduced expression of MCL-1 sensitizes plasma cells to BCL-2 inhibition with ABT-737. (A) Absolute numbers of cells after induced deletion of both (*fl*/*fl*) alleles of *Mcl1* and treatment with ABT-737. Mice were killed 2 days after the first treatment with tamoxifen (treated for 2 consecutive days) and 5 days after treatment with 75 mg/kg ABT-737 (treated for 5 consecutive days). Data represent two experiments and six animals per group. (B) Fold reduction of B cell numbers comparing *Mcl1*$^{fl/fl}$ER^{T2}Cre+vehicle and *Mcl1*$^{fl/fl}$ER^{T2}Cre+ABT-737, based on numbers shown in (A). **P≤0.01 (statistical significance was calculated between the groups using a Mann–Whitney test).



BM are long-lived Blimp-1^{hi} PC,[23] these observations are in line with the results on the total PC population in Figure 4A.

We have shown previously that MCL-1 expression is essential for PC survival and that signaling through the TNF receptor (TNFR) family member BCMA promotes *Mcl1* transcription in BM PC.[16] In fact, we have shown that *Mcl1* gene expression was 3.4-fold lower in *Bcma*$^{-/-}$ BM PC as compared with wild-type BM PC.[16] Mice deficient for BCMA in hematopoietic cells were treated with ABT-737 as before and the impact on PC maintenance was measured. Although PC numbers

in the BM were reduced in *Bcma*$^{-/-}$ mice,[16,24] those PC that survive in the absence of BCMA-signaling remained as sensitive to treatment with ABT-737 as do those BM PC that survive in its presence (Figure 5). These results suggest the existence of non-overlapping roles between MCL-1, albeit reduced in BCMA-deficient BM PC, and BCL-2, the main target of ABT-737 in mice.[17,18] Therefore, we further tested the combinatorial dependence of MCL-1 and BCL-2 expression on PC survival. The combined impact of ABT-737 treatment with deletion of both *Mcl1*^{fl} alleles was examined. Treatment with ABT-737 in the absence of MCL-1 revealed a similar impact on follicular and recirculating B cells as compared with ABT-737 treatment in wild-type mice (Figures 6A-B). However, ABT-737 treatment in the absence of MCL-1 greatly augmented the reduction in PC populations in both the spleen and BM owing to MCL-1 (Figures 6A-B).

To complement our *in vivo* studies, we examined dependence on BCL-2, BCL-XL or MCL-1 expression using novel and highly specific BH3-mimetics with murine splenocytes and BM cells in culture. Identical to *Bclx* gene deletion experiments *in vivo* (Figures 2A-B), treatment with the BCL-XL-specific inhibitor, A-1155463,[25] only reduced the viability of immature B cells (Figure 7A). Sensitivity to the BCL-2-specific inhibitor ABT-199/venetoclax[13] was comparable to our observations with *in vivo* experiments using ABT-737 (Figures 4A-B), promoting apoptosis of mature marginal zone, follicular and recirculating B cells. In addition, we observed impaired survival of transitional type 2 cells from the spleen (Figure 7B). The latter was not observed in our *in vivo* experiments with ABT-737, but was previously described in an identical experimental set-up using ABT-199.[26] As expected from our experiments in Figures 4C and D, ABT-199 promoted apoptosis of fully differentiated PC, whereas early plasmablasts (PB, comparable to Blimp-intermediate PC) were unaffected by treatment with this drug (Figure 7B). Next we tested the role of MCL-1 in survival of B cell subsets using the MCL-1 inhibitor A-1210477. Although A-1210477 is highly specific for MCL-1, it lacks potency to reveal subtle differences in MCL-1 dependence.[27] In line with our gene deletion experiments presented in Figure 1 and previous findings,[16] we observed that the transitional as well as mature B cell- and PC-subsets, but not immature B cells, were sensitive to treatment with this drug (Figure 7C). In contrast to our *in vivo* experiments, we found that transitional type 2 cells in the spleen were sensitive, but transitional B cells from the BM were insensitive to A-1210477. The differential dependence on BCL-2 or MCL-1 expression in transitional B cells from the BM or transitional type 2 B cells from the spleen between our *in vitro* and *in vivo* models may result from the potency of the BH3-mimetics or the role of the protective microenvironment *in vivo*.

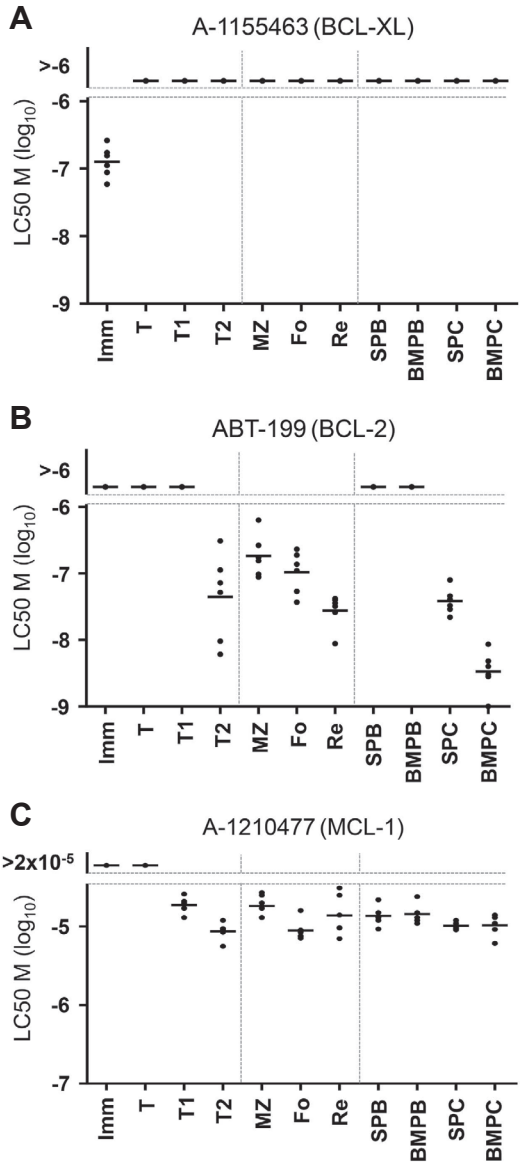


Figure 7. Sensitivity of B cells to highly specific BH3-mimetics that target BCL-XL, BCL-2, or MCL-1. Response of diverse freshly isolated mouse B cell subsets to (A) the BCL-XL-selective BH3-mimetic A-1155463, (B) the BCL-2-selective BH3-mimetic ABT-199, or (C) the MCL-1-selective BH3-mimetic A-1210477. Data represent cells from the spleen or bone marrow of six individual mice.

DISCUSSION

Selective targeting of pro-survival BCL-2 family members can provide an attractive strategy for treating B cell malignancies, either as single treatment or in combination with additional treatment modalities. This is exemplified by the recent discovery and use of the BCL-2-specific inhibitor ABT-199 (venetoclax).[13] Venetoclax has proven successful in multiple clinical trials and is a promising new treatment for CLL, certain non-Hodgkin lymphomas and other malignancies.[14,28] Its predecessor, navitoclax/ABT-263 (the orally bioavailable form of ABT-737), targets not only BCL-2, but also BCL-XL and BCL-W. As expression of BCL-XL is required for maintenance of platelets, navitoclax evoked severe thrombocytopenia in clinical trials as an unwanted side-effect.[28] The main target of ABT-737 in mice, however, was shown to be BCL-2 and not BCL-XL or BCL-W.[17,18] In addition to BCL-2 inhibition, novel drugs have been described that more specifically inhibit only BCL-XL (WEHI-539 and A-1155463)[25,29] or MCL-1 (A-1210477).[27] As various tumor types depend on MCL-1 or BCL-XL expression,[14] it is only a matter of time before specific inhibitors targeting these molecules will also find their way into the clinic. However, the importance of MCL-

1, BCL-2, and BCL-XL expression for the survival of healthy cell subsets *in vivo* is still incompletely understood. Thus, treatment with these specific inhibitors used alone or in combination may elicit unwanted side-effects, as seen with the impact of BCL-XL inhibition on platelets. A thorough understanding of the effects of BH3-mimetic drugs on normal cells should aid the development of a tailored treatment design that minimizes side-effects. Conversely, knowing which cell subsets are sensitive to the different inhibitors may provide clues on how to treat their malignant counterparts.

To assess the individual and overlapping roles of MCL-1, BCL-2, and BCL-XL in the maintenance of different B cell subsets, we examined mice where the *Mcl1* and/or *Bclx* genes were inducibly deleted in the B cell lineage. This was performed in combination with ABT-737 treatment. Our findings demonstrate a reliance on MCL-1 expression throughout the B cell lineage and indicate that even short-term loss of MCL-1 expression – for <2 days – will deplete B cell subsets at multiple stages of development (Figures 1 and 7C). In contrast, loss of BCL-XL expression, even up to 4 days, only affects the maintenance of immature B cells. The specific dependence of this B cell subset on expression of BCL-XL was confirmed using the BCL-XL-specific inhibitor, A-1155463 (Figure 7A). Hemizygous deletion of *Mcl1* did, however, sensitize transitional B cells and splenic PC to loss of BCL-XL (Figure 2). Previous reports have shown that treatment of mice with ABT-737 results in a decrease of follicular and recirculating mature B cells.[19,22] It inhibited the induction of newly generated PC, but left preexisting PC intact.[19] We show here that also long-lived terminally differentiated (Blimp-1^{hi}) PC, but not short-lived Blimp-1^{int} PC, are reduced after treatment with ABT-737 (Figure 4). In the above-mentioned experiments ABT-737 was chosen to study the role of BCL-2, its main *in vivo* target, for reasons of cost and availability. We did, however, also use the BCL-2-specific BH3-mimetic ABT-199 on isolated BM and splenic B cells in culture. These experiments confirmed to a large extend our *in vivo* findings when using ABT-737, with mature marginal zone, follicular, and recirculating B cells sensitive to BCL-2 inhibition (Figure 7B). We also confirmed our *in vivo* findings, proving that long-lived (B220^{low} or Blimp-1^{hi}) PC depend on BCL-2 expression, whereas recently generated, or short-lived (B220^{hi} or Blimp-1^{int}) PC do not (Figure 7B). The notion that mainly Blimp-1^{hi} PC are sensitive to this treatment correlates with previous findings showing that *Bcl2* expression is higher in these cells compared to Blimp-1^{int} PC.[16] As PC seem to be affected both by deletion of *Mcl1* and treatment with ABT-737, the combined impact on cell survival was examined. BCMA, a TNFR family member expressed specifically on PC, promotes survival of BM PC via transcriptional induction of *Mcl1* and, as a consequence, the number of BM PC in *Bcma*$^{-/-}$ mice is significantly reduced.[16,24] ABT-737 treatment of mice

reconstituted with *Bcma*$^{-/-}$ BM showed a similar fold reduction in the numbers of BM PC as seen in wild-type mice treated with ABT-737 (Figures 4 and 5). This means that reduced MCL-1 expression, caused by the absence of BCMA-signaling, and inhibition of BCL-2 with ABT-737 have additive effects on the reduction of BM PC. The combined effects of BCL-2 inhibition and reduced MCL-1 expression were further examined by treating mice with ABT-737 together with genetically deleting one or both alleles of *Mcl1*. These experiments revealed that B cell populations in mice with hemizygous deletion of *Mcl1* are equally sensitive to treatment with ABT-737 compared with wild-type mice (data not shown). However, PC in mice with homozygous deletion of *Mcl1* are strikingly sensitive to ABT-737 (Figure 6). These synergistic effects between inhibited BCL-2 and loss of MCL-1 expression seem to be specific for PC and open up a potential therapeutic strategy for deletion of auto-reactive or malignant PC in patients. This finding does, however, argue that

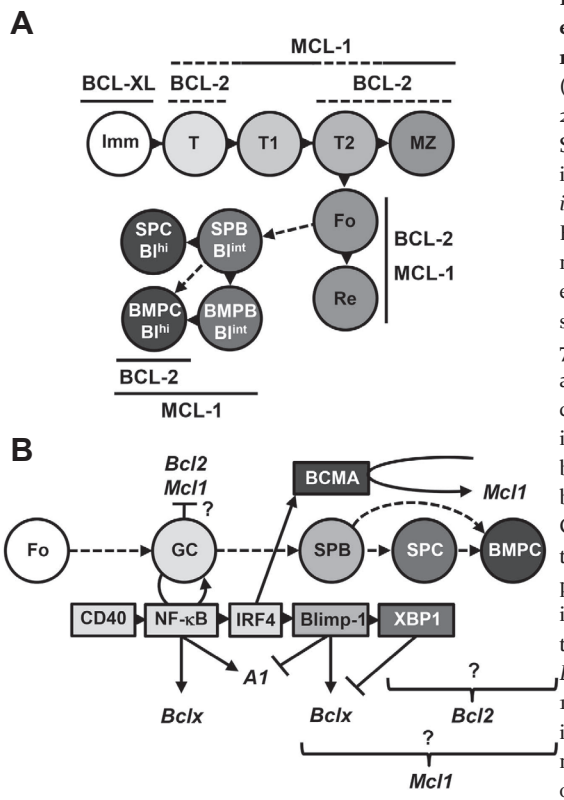


Figure 8. Differential dependence on - and expression of - pro-survival BCL-2 family members throughout B cell development. (A) Dependence on expression of MCL-1, BCL-2, and BCL-XL throughout B cell development. Solid lines indicate direct reliance on indicated BCL-2 family protein based on both *in vivo* data and *ex vivo* experiments using BH3-mimetics, whereas dotted lines indicate reliance on either *in vivo* data or *ex vivo* experiments with BH3-mimetics. Data are a summary of Figures 1C, 2A, 4A, 4C-D, 6A, and 7. (B) Regulation of *Mcl1*, *Bcl2*, *Bclx* (*Bcl2l1*), and *A1* (*Bcl2a1*) gene expression in germinal center (GC) B cells and PC. BCL-6-mediated inhibition of PC master regulator Blimp-1 can be abrogated by activation of IRF4.[33] This can be achieved by NF-κB activation following CD40 ligation.[34] Activated NF-κB can promote transcription of pro-survival BCL-2 family protein A1 (BFL-1),[35] which is subsequently inhibited by Blimp-1 when cells differentiate to PC.[16] Blimp-1 can promote transcription of *Bclx*, which is subsequently repressed by XBP-1.[30,31] Expression of *Mcl1* is transcriptionally induced by stimulation of BCMA in the BM microenvironment.[16] However, the mechanism of increased Mcl1 transcription in splenic PC remains unclear. Repression of Bcl2 has been observed in the early GC but is subsequently re-expressed in mature PC,[16] although the upstream mediators are currently unknown.

the reduction in MCL-1 expression needs to be substantial (more than 3.4-fold based on experiments with *Bcma*$^{-/-}$ PC) to observe synergism with ABT-737 (BCL-2 inhibition). As the number of PC is already strongly decreased in the absence of MCL-1,[16] the additional impact of ABT-737 results in a >100-fold decrease in the number of PC in both the spleen and BM (Figure 6A).

Collectively, these studies reveal the differential requirement and expression of specific anti-apoptotic BCL-2 family proteins during different stages of B cell development (Figure 8A). It also shows that reduced expression of MCL-1 sensitizes multiple B cell subsets to inhibition of BCL-XL or BCL-2. Recent publications shed light on the mechanism of the transient BCL-XL expression in newly generated PC, that has been described previously.[16] Expression of Blimp-1 represses A1, but has also been shown to bind to the *BCLX* gene promoter and promote its transcription.[30,31] Activation of XBP-1, downstream of Blimp-1, subsequently represses *BCLX* (Figure 8B).[31] The transient expression of BCL-XL is thought to safeguard PC *en route* to protective BM niches,[15,20] and we show here that BCL-XL does contribute significantly to the survival of splenic PC when MCL-1 expression is limited. The mechanism(s) that promote(s) *BCL2* transcription in Blimp-1^{hi} PC[16] is currently unresolved. Our results suggest that combining inhibition of BCL-2 with drugs that reduce the expression or activity of MCL-1 may prove valuable for targeting auto-reactive or malignant PC in autoimmune disease or multiple myeloma, respectively.

MATERIALS AND METHODS

Mice

C57BL/6 mice, *Prdm1*$^{GFP/+}$ mice,[23] *Mcl1*^{flox} mice,[16] *Bclx*^{flox} mice,[16] Rosa26-Cre^{ERT2} (hereafter, called Cre^{ERT2}) mice[16] (Taconic Artemis, Hudson, NY, USA), and μMT-deficient mice[32] were bred and maintained at the animal facilities of the Walter and Eliza Hall Institute (WEHI). All gene-targeted mice were maintained on a C57BL/6 background. *Bcma*$^{-/-}$ mice[16] were on a C57BL/6-and-C3H/He mixed genetic background. All animal procedures were approved by the WEHI Animal Ethics Committee. BM-reconstituted mice were generated as described.[16] In brief, lethally irradiated C57BL/6 (Ly5.1^{+}) mice were reconstituted with 80% B cell-deficient (μMT) BM plus 20% of either (Ly5.2^{+}) Cre^{ERT2}, *Mcl1*$^{fl/+}$Cre^{ERT2}, *Mcl1*$^{fl/fl}$Cre^{ERT2}, *Bclx*$^{fl/fl}$Cre^{ERT2}, or *Mcl1*$^{fl/+}$*Bclx*$^{fl/fl}$Cre^{ERT2} BM. Activation of the Cre^{ERT2} conditional recombinase to achieve deletion of *lox*P-flanked *Mcl1* or *Bclx* alleles was performed by oral gavage of tamoxifen on 2 successive days, as described.[16] Alternatively, mice deficient for BCMA were

generated by reconstitution of lethally irradiated C57BL/6 (Ly5.1$^+$) mice with 100% *Bcma*$^{-/-}$ BM (Ly5.2$^+$). ABT-737 (Abbott Laboratories, Abbott Park, IL, USA) or vehicle control were prepared and administered at 75 mg/kg body weight for 5 consecutive days as described.[19]

Flow cytometry

Single-cell suspensions were stained with the following fluorochrome-conjugated monoclonal antibodies: anti-B220 (RA3-6B2), anti-IgM (331.12), anti-IgD (11.26C), anti-CD23 (B3B4), anti-CD21 (7E9), anti-FcγR (2.4G2), anti-human CD4 (OKT4) all produced in-house, anti-CD138 (281.2; BD Biosciences, San Jose, CA, USA), and anti-CD45.2 (104; BD Biosciences). Stained cells were analyzed on a FACSCanto II cytometer (BD Biosciences). B cell populations were sorted from spleen and BM using a MoFlo cytometer (DAKO Cytomation Ltd, Ely, UK) to a purity of 98%.

Quantitative PCR

RNA isolation, quantitative real time PCR, and primer sequences are as described.[15,16]

BH3-mimetics and apoptosis assays

Single-cell suspensions from the spleen or BM were cultured with A-1210477 (catalog number CT-A121, ChemieTek, Indianapolis, IN, USA) at a concentration range of 0, 1, 5, or 20 μM; A-1155463 (catalog number CT-A115, ChemieTek) at 0, 10, 100, or 100 nM; or ABT-199 (catalog number A0776, LKT Laboratories, St Paul, MN, USA) at 0, 10, 100, or 1000 nM for 20 h at 37°C. Cell viability after treatment was assessed by flow cytometry using the TO-PRO-3 dye (catalog number T3605, ThermoFisher, Waltham, MA, USA) after gating on specific B cell subsets using the gating strategy as shown in Figures 1A and B or as previously published for PC.[16] Specific apoptosis was calculated by measuring the altered percentage of TOPRO3$^-$ (live) cells within indicated B cell populations, compared with untreated cells. LC50 values were subsequently calculated using Excel and Graphpad Prism software, and specific apoptosis values after incubation with a concentration range of the inhibitors.

Statistical analysis

Statistical significance was determined using a Mann–Whitney test when comparing two groups of mice (Figures 2A, 2C, 4A, 4C-D, 5, and 6A), or a Kruskal–

Wallis test followed by a *post hoc* Dunn's multiple comparisons test in case multiple groups of mice or cell subsets were compared (Figures 1C and 3).

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CHAPTER 4

Multiple myeloma with 1q21 amplification is highly sensitive to MCL-1 targeting

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ABSTRACT

Pro-survival BCL-2 family proteins are potent inhibitors of apoptosis and often overexpressed in lymphoid malignancies. In multiple myeloma (MM), MCL-1 expression contributes to survival of malignant plasma cells, and overexpression correlates with poor prognosis. In this study, we investigated whether sensitivity to the novel MCL-1 inhibitor S63845 could be predicted using cytogenetics, focusing on amplification of 1q21, the chromosomal region that contains the MCL1 locus. In addition, we studied the relation of MCL-1 inhibitor sensitivity with other diagnostic characteristics and BCL-2 family protein expression. In 31 human myeloma cell lines and in bone marrow aspirates from 47 newly diagnosed MM patients, we measured the effect of S63845 alone, or combined with BCL-2 inhibitor ABT-199 (venetoclax), and BCL-XL inhibitor A-1155463 or A-1331852 on cell viability. We demonstrated for the first time that MM cells from patients with 1q21 amplification are significantly more sensitive to inhibition of MCL-1. We suggest that this increased sensitivity results from high relative MCL1 expression resulting from amplification of 1q21. Additionally, and partially independent from 1q21 status, high serum β2 microglobulin level and presence of renal insufficiency correlated with increased sensitivity to MCL-1 inhibitor treatment. Combining S63845 with other BH3-mimetics synergistically enhanced apoptosis compared with single inhibitors, and sensitivity to inhibitor combinations was found in a large proportion of MM insensitive to MCL-1 inhibition alone. Collectively, our data indicate that amplification of 1q21 identifies an MM subset highly sensitive to MCL-1 inhibitor treatment and can be used as a predictive marker to guide selection of therapy.

INTRODUCTION

Despite recent advances in treatment, multiple myeloma (MM) is considered incurable, with most patients relapsing and eventually becoming refractory to therapy.[1] Treatment regimens generally consist of triple-drug combinations including a proteasome inhibitor, dexamethasone, and an immunomodulatory drug or chemotherapeutic agent with or without autologous stem cell transplantation.[2,3] At relapse, patients receive next-generation proteasome inhibitors and immunomodulatory drugs, and recently, anti-CD38 monoclonal antibody daratumumab was approved for use in relapsed and/or refractory MM.[4] Several novel therapies for MM with different mechanisms of action are currently being studied, including BCL-2 homology domain 3 (BH3) mimetics.[1,5]

BH3-mimetics overcome apoptosis resistance by binding and inhibiting select pro-survival BCL-2 family proteins.[6,7] BCL-2 family proteins are key mediators of the intrinsic apoptosis pathway. Whether a cell undergoes apoptosis is determined by the availability of both pro-survival (e.g. BCL-2, MCL-1, BCL-XL) and pro-apoptotic proteins (e.g. BAX, BAK, BIM, PUMA, BID, NOXA). In MM, overexpression of MCL-1 leads to apoptosis resistance and is associated with shorter patient survival.[8] In addition to MCL-1, overexpression of BCL-2 and/or BCL-XL has also been observed in MM, suggesting that these 3 pro-survival proteins are promising targets for therapy.[9,10] ABT-199 (venetoclax) is the first BCL-2–specific BH3 mimetic approved by the US Food and Drug Administration for use in chronic lymphocytic leukemia patients with a 17p chromosomal deletion.[11] MM patients with an (11;14) translocation [t(11;14)] have a relatively high *BCL2* gene expression level compared with gene expression of *BCL2L1* (BCL-XL) or *MCL1* and respond to ABT-199 as single treatment.[12,13] Consistent with these findings, MM cells with high gene expression of *MCL1* or *BCL2L1* are less sensitive to venetoclax.[9] Instead, MCL-1–specific BH3-mimetics may be effective, and multiple MCL-1–specific BH3-mimetics, such as S63845,[14] have been developed and are currently being tested in phase 1 clinical trials for MM.[15]

The heterogeneity of MCL-1, BCL-2, and BCL-XL expression in MM patients suggests that BH3-mimetics targeting each of these proteins may be more effective in specific patient groups. Expression of single pro-survival proteins may not directly correlate with inhibitor sensitivity, which rather seems to be a consequence of the relative expression and distribution of multiple pro- and anti-apoptotic BCL-2 family members.[9,16,17] Finding tumor characteristics that predict inhibitor responses is therefore key for finding optimal therapy combinations. In addition to age, fitness, and tumor stage, several genetic aberrations are strongly associated with treatment response and patient survival.[18] Of these genetic lesions,

1q21 amplification, 17p13 deletion, t(4;14), t(14;16), and t(14;20) are associated with poor prognosis.[18,19]

Because *MCL1* is one of the genes located on 1q21, we hypothesized that amplification of 1q21 would lead to increased MCL-1 expression, possibly conferring increased sensitivity to MCL-1 targeting. In 47 primary MM bone marrow (BM) samples and 31 human MM cell lines (HMCLs), we determined dependence on MCL-1, BCL-2, and BCL-XL by treatment with specific BH3-mimetics and investigated whether this correlated with tumor cytogenetics, disease stage, or protein expression. In addition, combinations of BH3-mimetics were used to determine whether they acted in synergy. We found that plasma cells (PCs) from MM patients with 1q21 amplification are markedly more sensitive to MCL-1 inhibition, and the subgroup with both 1q21 amplification and increased serum levels of β2 microglobulin (β2m) has highest sensitivity. Therefore, 1q21 amplification is a possible new patient-specific marker for the selection of targeted therapy in MM.

MATERIALS AND METHODS

Cell culture and chemicals

HMCLs were cultured in RPMI 1640 GlutaMAX HEPES culture medium (Life Technologies) or Iscove modified Dulbecco medium (Life Technologies) for LME-1, supplemented with 10% fetal bovine serum (FBS; Biowest) and 100 μg/mL of penicillin-streptomycin (Gibco/Life Technologies). For NCI-H929, RPMI 1640 medium was supplemented with 20% FBS, 1 mM of sodium pyruvate (Thermo Fisher), and 50 μM of β-mercaptoethanol (Life Technologies). MS-5 cells were cultured in MEM α (Life Technologies) with 10% FBS, 2 mM of L-glutamine (Life Technologies), and 100 μg/mL of penicillin-streptomycin. Human mesenchymal stromal cells (MSCs) were isolated from (allogeneic) MM BM by adherence to tissue culture plastic and cultured in MEM α supplemented with 10% FBS, 0.2 of mM L-ascorbic acid 2-phosphate (Cayman Chemical), and 100 μg/mL of penicillin-streptomycin.

Patient samples

Primary human MM samples of newly diagnosed patients were obtained from the Parelsnoer Institute biobank. All samples were obtained after written informed consent, and protocols were approved by the local ethics committee of the University Medical Center Utrecht and contributing partners of the Parelsnoer

Institute. After thawing, primary MM samples were plated on monolayers of near-confluent MS-5 feeder cells and recovered for 24 hours. In experiments comparing culture systems, MM cells were plated without supporting cells, with MS-5 cells, with MSCs in 2-dimensional (2D) culture, or with MSCs in 3D culture in 0.5% PuraMatrix hydrogel (Corning) (3D-MSCs), as described elsewhere.[20] In flow cytometric experiments, MM cells were distinguished by positive surface staining with CD38-PerCP.CY5.5 (BioLegend). Clinical data and cytogenetic information of MM patients were collected by the University Medical Center Utrecht and Leiden University Medical Center. Clinical data included age, sex, International Staging System (ISS) stage, PC percentage, β2m level, and occurrence of bone lesions, anemia, and renal insufficiency. Cytogenetic information included hyperdiploidy, 13q deletion, 17p deletion, 1q21 gain, t(4;14), t(11;14), t(14;16), and t(14;20).

Apoptosis assays

To determine 50% inhibitory concentrations (IC_{50}s), cells were treated with serially diluted individual or combined BH3-mimetics S63845 (MCL-1i; Servier), ABT-199 (BCL-2i; LKT Laboratories), and A-1331852 (BCL-XLi; gift of G. Lessene, Walter and Eliza Hall Institute of Medical Research; Figure 1A-B; Supplementary Figure 1). Cell viability 6 hours after treatment was determined using CellTiter-Glo assay (Promega, category #G9241) and normalized dimethyl sulfoxide controls. Specific apoptosis and IC_{50}s, as shown in Figures 1C, 2-4, and 6-7 and Supplementary Figures 2, 4-5, and 7-9, were determined after 24 hours of treatment with S63845, ABT-199, and A-1155463 (BCL-XLi; MedChemExpress) by staining with 15 nM of DiOC6 (Thermo Scientific) and 20 nM of TO-PRO-3 (Thermo Scientific). Specific apoptosis was calculated using flow cytometric analysis (FACSCanto II; BD Biosciences) by measuring the altered percentage of $DiOC6^+/TO\text{-}PRO\text{-}3^-$ (live) cells compared with untreated cells and was defined as follows: ([% cell death in treated cells – % cell death in control]/% viable cells control) × 100. Synergy was assessed by treating cells with a dilution series of 2 drugs, single and in combinations of concentrations. Inhibitor concentrations that induce 50% specific apoptosis were used to make isobolograms. CIs were calculated using the Chou-Talalay method.[21]

Intracellular staining

For intracellular BCL-2 family protein staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) and stained with mouse anti–BCL-2 phycoerythrin (PE; BD Biosciences), rabbit anti–BCL-XL (Cell Signaling), isotype controls mouse IgG1K PE (Sony Biotech) and rabbit IgG (Santa Cruz), and goat anti-

rabbit IgG1 fluorescein isothiocyanate (Santa Cruz). Flow cytometry (FACSCanto II; BD Biosciences) was performed and staining intensity was analyzed using FACSDiva and FlowJo software.

siRNA knockdown, coimmunoprecipitation, and immunoblotting

siRNA-mediated knockdown of BCL-2 and BCL-XL was performed using ON-TARGETplus human nontargeting, BCL2, and BCL2L1 SMARTpools (Horizon) and Neon Transfection System (Thermo Fisher) with 2 × 30 ms pulses of 1050 (UM9) or 1150 V (MM1.S). Lysis buffer containing 1% NP-40 was used to obtain lysates for coimmunoprecipitation and western blotting. Coimmunoprecipitation was performed using Dynabeads Protein G kit (Invitrogen), with rabbit IgG (R&D), rabbit anti–MCL-1 (Abcam), mouse anti–BCL-2 (Cell Signaling), and rabbit anti–BCL-XL (Cell Signaling) antibodies. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to low-fluorescence polyvinylidene difluoride membranes (Bio-Rad), blocked in PBS containing 2% nonfat dry milk, and stained using the aforementioned antibodies, as well as mouse anti–α-tubulin, rabbit anti-BIM (Cell Signaling), goat anti-mouse 680RD, and goat anti-rabbit 800CW (LI-COR Biosciences). Infrared imaging was used for detection (Odyssey Sa; LI-COR Biosciences). Analysis and quantification were performed using LI-COR Image Studio and ImageJ 1.47V software.

Immunohistochemistry

Paraffin-embedded 4-µm tissue sections of BM biopsies from MM patients (n = 11) were stained with anti-CD138 (Dako) and anti–MCL-1 (Dako) antibodies and detected using a Ventana Benchmark ULTRA and BrightVision poly-HRP anti-mouse/rabbit IgG (VWR), respectively. Visualization was performed with diaminobenzidine as chromogen. Results were evaluated by 2 experienced observers, who were blinded to the clinical characteristics and experimental results. Sections were evaluated for CD138 expression, after which the MCL-1 staining intensity on a sequential section was scored (weak staining, 1; moderate, 2; strong, 3).

Copy-number variation and gene expression data analysis

Using published data sets, *MCL1* gene expression was assessed in different MM disease stages and in patients with or without 1q amplification. The following data sets from the National Center for Biotechnology Information Gene Expression Omnibus were used: GSE2658[22] and GSE6401.[23] Data sets were analyzed using the R2 Genomics Analysis and Visualization Platform.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0.1. Data are represented as mean or mean ± standard error of the mean, unless otherwise stated. For experiments with cell lines, data points are the mean of ≥3 independent experiments or representative of at least 2 experiments. Groups were compared with Student *t* test (2 groups), Kruskall-Wallis test (multiple groups), mixed-effects or 2-way analysis of variance (>2 groups at multiple concentrations), or χ^2 test (frequencies in categories).

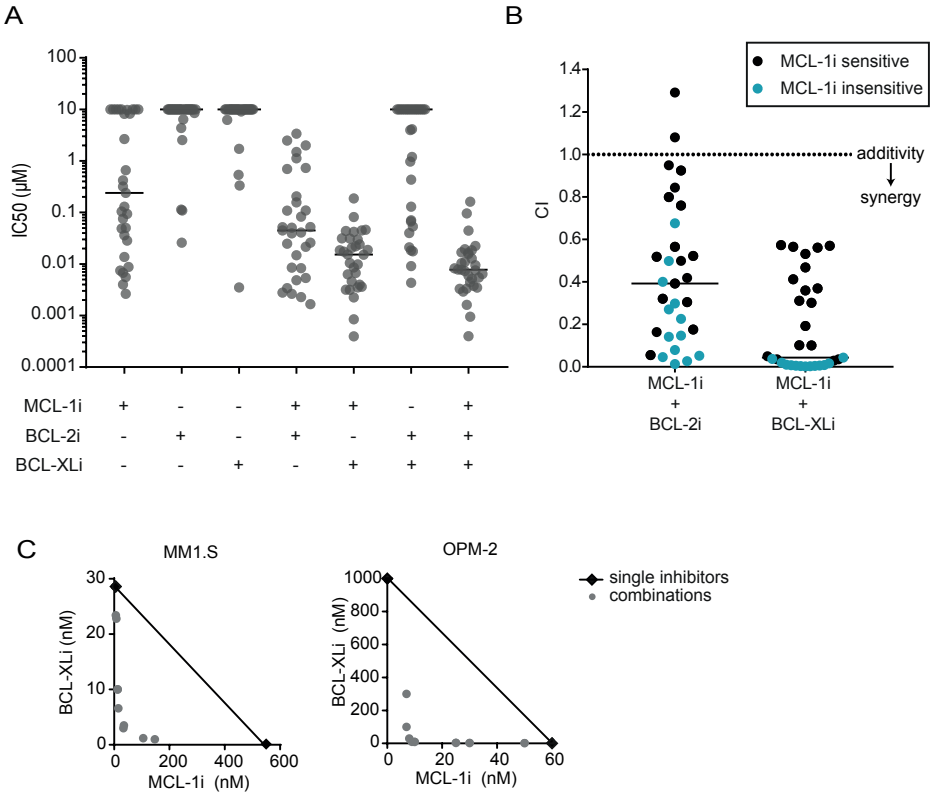


Figure 1. MCL-1i has single-drug activity and synergizes with BCL-2i and BCL-XLi in HMCLs. (A) IC50 (μM) of BH3-mimetics in HMCLs, single and combined (ratios of combined BH3-mimetics are 1:1 or 1:1:1) after treatment for 6 hours and determined by Celltiter Glo assay. Black lines indicate median values. (B) For all 31 HMCLs, combination indices (CIs) were calculated for the indicated inhibitor combinations. CI <1 indicates synergy (dotted line). Cell lines insensitive to MCL-1i (i.e. IC50 > 1 μM) are shown in blue. Solid lines indicate median values. (C) Synergy between MCL-1i and BCL-XLi was studied by combining concentration series of the 2 inhibitors in a matrix after treatment for 24 hours using DiOC6/TO-PRO-3 flow cytometric staining as readout. The isobolograms show concentrations at which the combined inhibitors induced 50% specific apoptosis (IC50; grey points). Black diamonds indicate the IC50s of the single drugs. Black lines illustrate where CI = 1. For MM1.S, CI < 1, indicating synergy of MCL-1i and BCL-XLi. In OPM-2, the IC50 of BCL-XLi was >1000, but it was set to 1000 to be able to calculate CI values. The resulting CI (<1) therefore underestimates the synergistic effect in OPM-2.

RESULTS

MCL-1i has single-drug activity and synergizes with BCL-2i and BCL-XLi in HMCLs

A panel of 31 HMCLs was used to investigate sensitivity to specific MCL-1, BCL-2, and BCL-XL BH3-mimetics. The cell lines were treated with BH3 mimetic S63845 (MCL-1i),[14] venetoclax (BCL-2i),[24] and A-1331852 (BCL-XLi),[25] and IC_{50} values were calculated for single inhibitors and inhibitor combinations (Figure 1A; Supplementary Figure 1). Most HMCLs were relatively insensitive to BCL-2i or BCL-XLi (median IC_{50}, ≥10 μM for both). In contrast, MCL-1i resulted in a wide range of IC_{50} values within the cell line panel (median IC_{50}, 239 nM), suggesting that a selection of cell lines is solely dependent on MCL-1 expression for survival. When BCL-2i or BCL-XLi was added to MCL-1i treatment, median IC_{50} values decreased compared with single-inhibitor treatment (median IC_{50}, 45 nM for MCL-1i + BCL-2i and 15 nM for MCL-1i + BCL-XLi). This effect was strongest for combined inhibition of MCL-1 and BCL-XL, to which all cell lines were sensitive. Calculation of CIs showed that combining MCL-1i with BCL-2i or BCL-XLi results in apoptosis in a synergistic manner, even in cell lines that are insensitive to MCL-1i single treatment (Figure 1B). To further investigate synergy of the MCL-1i and BCL-XLi combination, isobolograms were made for 2 HMCLs (Figure 1C). The observed CIs of <0.2 indicate strong synergy for the MCL-1i and BCL-XLi combination, in an MCL-1i–sensitive cell line (OPM-2) as well as in an MCL-1i–insensitive cell line (MM1.S).

On the basis of these observations, we investigated whether BCL-2 or BCL-XL protein expression levels correlated with sensitivity to MCL-1i (Figure 2A). MCL-1i IC_{50} correlated with expression of both BCL-2 and BCL-XL (R^2 = 0.46 and 0.66; P = .04 and .008, respectively), suggesting that high expression of these proteins may render MM cells more resistant to MCL-1 inhibition. A correlation between MCL-1 inhibitor sensitivity and BCL-2 protein expression was also recently observed by others using the MCL-1–specific BH3 mimetic AZD5991.[26] MCL-1i IC_{50} did not correlate with MCL-1 protein expression (Supplementary Figure 2). In this HMCL panel, BCL-XL expression seemed to be the strongest predictor for MCL-1i sensitivity, which may explain the high killing potential of combined MCL-1 and BCL-XL inhibitors, shown in Figure 1A-B. On the basis of these findings, we tested the relation between BCL-2 or BCL-XL expression and MCL-1i sensitivity. We selected an MCL-1i–sensitive (L363) and 2 MCL-1i–insensitive HMCLs (UM9 and MM1.S) expressing high BCL-2 or BCL-XL, respectively, and tested the binding pattern of pro-apoptotic BIM. This revealed that, among these 3 pro-survival BCL-

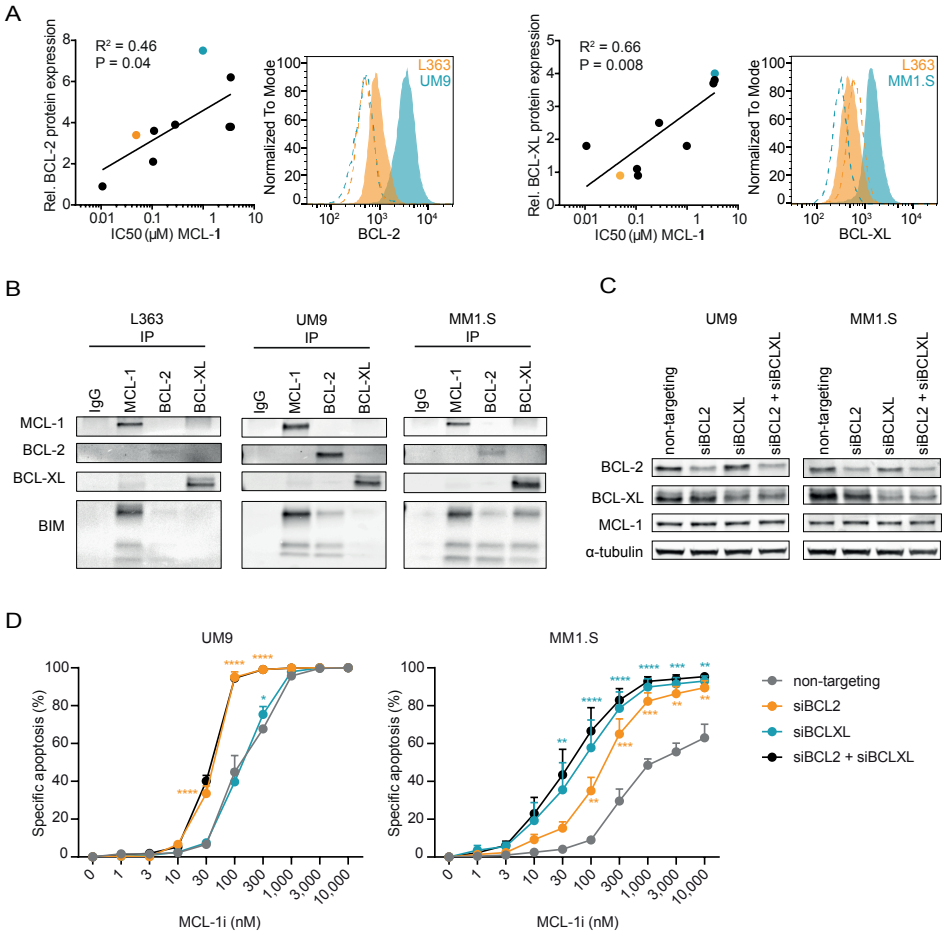


Figure 2. High BCL-2 or BCL-XL expression confers resistance against MCL-1i in HMCLs. (A) The correlation between BCL-2 or BCL-XL expression (fold over isotype control) as determined by flow cytometry and IC50s of MCL-1i in 9 HMCLs (RPMI-8226, NCI-H929, L363, LME-1, MM1.S, OPM-2, U266, UM1, and UM9). Representative histograms from BCL-2 or BCL-XL (filled) and isotype (dotted lines) staining of high- (blue) and low-expressing (orange) cell lines are shown, with the cell lines marked in the same colors in the adjacent correlation plot. (B) Coimmunoprecipitation of MCL-1, BCL-2, and BCL-XL and staining for BIM in L363 (MCL-1i sensitive), UM9 (MCL-1i insensitive, high BCL-2), and MM1.S (MCL-1i insensitive, high BCL-XL). (C) Protein expression 48 hours after small interfering RNA (siRNA)–mediated knockdown of BCL-2, BCL-XL, or both. (D) Specific apoptosis after 24 hours of treatment of cells from panel C with MCL-1i. Statistical significance is shown for siBCL-2 and siBCL-XL compared with the nontargeting control. *P < .05, **P < .01, ***P < .001, ****P < .0001. IgG, immunoglobulin G.

2 family proteins, BIM was exclusively bound to MCL-1 in L363 cells, suggesting that MCL-1 inhibition releases the total BIM pool. In contrast, BIM was bound to both MCL-1 and BCL-2 in UM9 cells and to MCL-1, BCL-XL, and BCL-2 in MM1.S cells, suggesting that MCL-1 inhibition in these cells only partially releases cellular BIM (Figure 2B; Supplementary Figure 3). In line with these results, knocking

Table 1. Clinical characteristics and frequency of cytogenetic and diagnostic determinants of MM patients (N = 47)

Characteristic	n (%) or n/N (%)
Age at diagnosis, y	
Median	63
Range	40-87
Sex	
Male	33 (70)
Female	14 (30
PCs, %	
Median	45
Range	4-100
Immunoglobulin type	
IgG	24 (51)
Non-IgG	23 (49)
Light chain type	
κ	26 (55)
λ	21 (45)
Cytogenetics	
Standard risk	32/47 (68)
High risk [1q amp, 17p del, t(4;14), or t(14;16)]	15/47 (32)
Hyperdiploidy	20/40 (50)
13q del	16/40 (40)
1q amp	12/44 (27)
t(11;14)	7/38 (18)
t(4;14)	3/41 (7)
t(14;16)	2/42 (5)
17p del	0/26 (0)
ISS stage at diagnosis	
1	16 (34)
2	14 (30)
3	13 (28)
ND	4 (9)
Serum ß2m ≥ 5.5 mg/L	16/39 (41)
Serum albumin < 3.5 g/L	32/47 (68)
Bone lesions	34/47 (72)
Anemia	15/47 (32)
Renal insufficiency (serum creatinine levels > 173 mmol/L)	9/47 (19)

ND, no data

down BCL-2 and/or BCL-XL expression in UM9 and MM-1S resensitized these HMCLs to single MCL-1i treatment (Figure 2C-D). Overall, our data show that sensitivity to MCL-1i depends to a large extent on expression of BCL-2 and BCL-XL, where high expression of BCL-2 and/or BCL-XL results in reduced sensitivity to MCL-1i, and low expression results in high sensitivity to MCL-1i, as shown previously,[9,11] and that inhibition of MCL-1, especially in combination with BCL-XL inhibition, efficiently induces apoptosis in HMCLs.

Ex vivo cultured MM PCs vary in sensitivity to BH3-mimetics

Our experiments with HMCLs show that MCL-1 inhibitor S63845 is indeed a promising drug for promoting apoptosis in MM, either alone or in combination with BCL-2 or BCL-XL inhibition. Next, we examined the effect of BH3-mimetics in primary MM cells from 47 newly diagnosed patients and tested whether we could predict sensitivity to MCL-1i in these samples. We received corresponding patient characteristics and cytogenetics (summarized in Table 1).

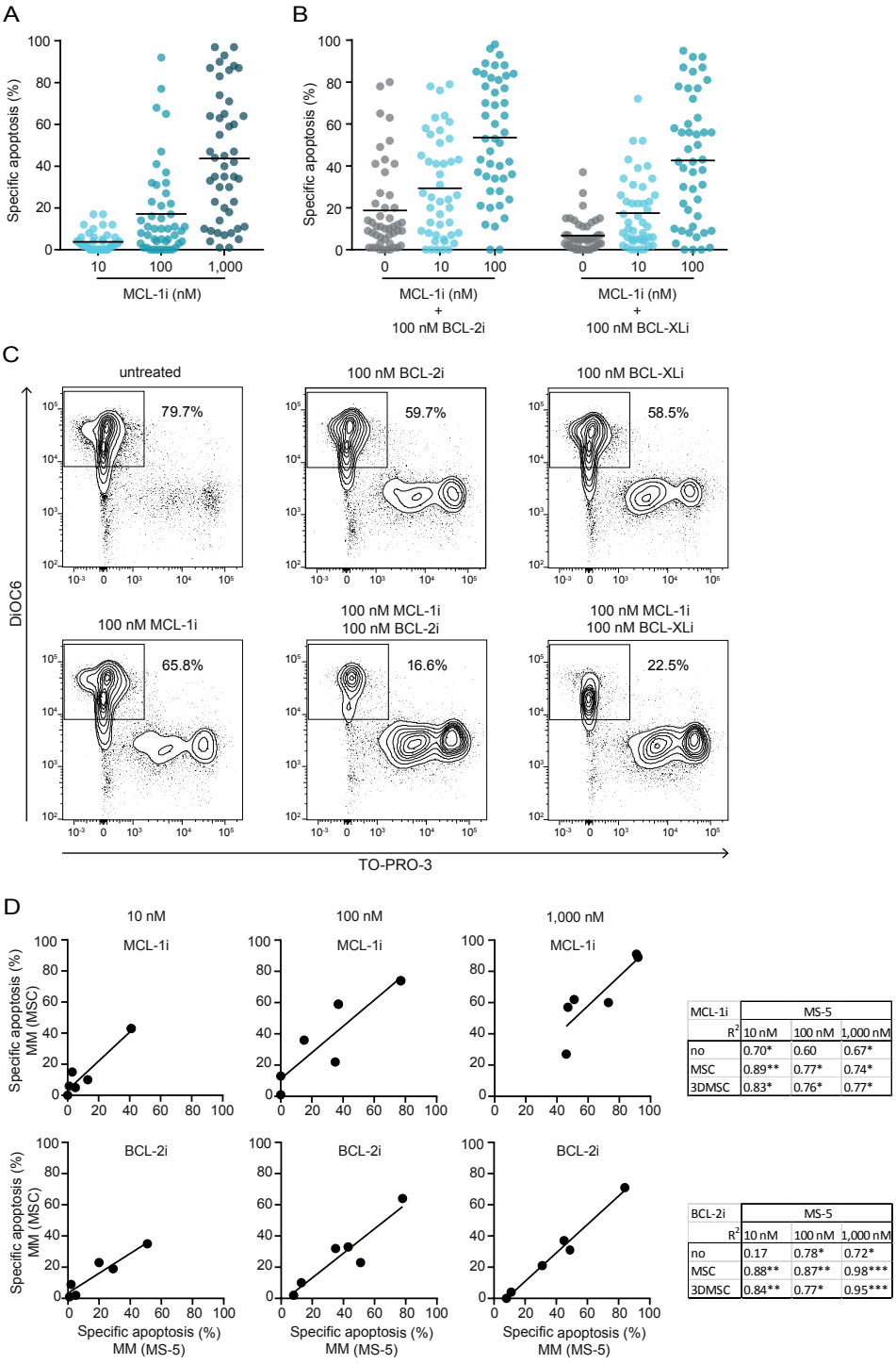
Part of each MM sample was treated with single or combined BH3-mimetics, and the remaining cells were used for measurement of intracellular BCL-2 and BCL-XL protein expression. Using this method, we confirmed previously reported increased sensitivity to venetoclax of MM PCs with t(11;14) or high BCL-2 protein expression (Supplementary Figure 4).[12,27] Notably, when MCL-1i was added to BCL-2i, no increased sensitivity was seen for t(11;14) MM compared with non-t(11;14), whereas MM with high expression of BCL-2 was significantly more sensitive to this inhibitor combination compared with MM with low BCL-2.

After treatment of the whole cohort with BH3-mimetics, we observed that MCL-1i sensitivity varied greatly between patients, with average specific apoptosis of 44% (range, 1%-97%) after treatment with 1000 nM of MCL-1i (Figure 3A; Supplementary Figure 5). As observed with HMCLs, addition of BH3-mimetics against BCL-2 or BCL-XL further increased sensitivity to MCL-1i (Figure 3B-C).

In this experimental setup, the MM cells were supported by MS-5 feeder cells, which support MM viability in short-term cultures. Additionally, we validated the results obtained from MS-5 coculture in a set of other culture systems. To this end, 6 MM samples were cultured with MS-5 cells, MSCs, or 3D-MSCs[20] or without feeder cells. Baseline viability of samples decreased most strongly after 48 hours without feeder cells, and viability was maintained to the highest extent in coculture with MS-5 cells (Supplementary Figure 6). A strong correlation in MCL-1i and BCL-2i sensitivity was observed between the MS-5 culture system and all other culture systems (Figure 3D), indicating that the relative sensitivity to BCL-2 family inhibitors in MM samples can be determined regardless of the culture system.

1q21 amplification correlates with increased MCL-1i sensitivity

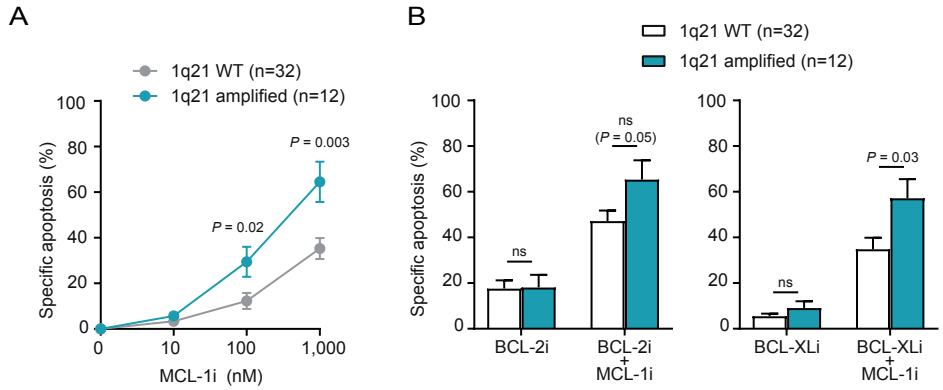
To address the hypothesis that 1q21 amplification leads to high MCL-1 expression and therefore confers cellular dependence on MCL-1, we compared MCL-1i sensitivity of patient samples with and without 1q21 amplification. We demonstrated that MCL-1i sensitivity was significantly increased (2.4-fold and 1.8-fold at 100 and 1000 nM, respectively) in 1q21-amplified PCs (Figure 4A). In addition, although



MCL-1i	MS-5		
R^2	10 nM	100 nM	1,000 nM
no	0.70*	0.60	0.67*
MSC	0.89**	0.77*	0.74*
3DMSC	0.83*	0.76*	0.77*

BCL-2i	MS-5		
R^2	10 nM	100 nM	1,000 nM
no	0.17	0.78*	0.72*
MSC	0.88**	0.87**	0.98***
3DMSC	0.84**	0.77*	0.95***

◄Figure 3. MM cell sensitivity to MCL-1i treatment is highly variable, and combination of MCL-1i with other BH3-mimetics synergistically induces cell death. (A) Specific apoptosis induced in MM PCs (n = 47), distinguished by their CD38+ phenotype, after 24 hours of treatment with the indicated concentrations of MCL-1i S63845. (B) Specific apoptosis induced in MM PCs (n = 47) after combination of MCL-1i treatment (at indicated concentrations) with 100 nM of BCL-2i or BCL-XLi. Black lines indicate average values. (C) Representative example of flow cytometric analysis of apoptosis induction in CD38+ (MM) cells after 24 hours of treatment with the indicated BH3-mimetics. Outlined areas indicate the populations of viable (DiOC6+/TO-PRO-3–) cells. (D) Linear regression analysis between specific apoptosis of MM samples (n = 6) cultured on MS-5 cells and MSCs, after treatment with indicated concentrations of MCL-1i (upper) or BCL-2i (lower). Tables show all coefficients of determination (R^2 values) for MS-5 and other culture systems, after treatment with MCL-1i or BCL-2i. *P < .05, **P < .01, ***P < .001.



▲Figure 4. 1q21 amplification correlates with increased MCL-1i sensitivity. (A) Differences in specific apoptosis of patients with wild-type (WT) 1q21 (n = 32) or amplification of 1q21 (n = 12), after treatment with MCL-1i for 24 hours. (B) Differences in specific apoptosis of patients with WT and amplified 1q21 after treatment with BCL-2i (left) or BCL-XLi (right) and their combinations with MCL-1i (all inhibitors, 100 nM). ns, nonsignificant.

sensitivity to BCL-2i or BCL-XLi single treatment was not affected by 1q21 status, we observed a strong trend toward increased sensitivity to MCL-1i + BCL-2i treatment of 1q21-amplified MM, as well as significantly increased apoptosis after MCL-1i + BCL-XLi treatment (Figure 4B). Despite the limited sample size, increased MCL-1i sensitivity of 1q-amplified MM was validated in culture with MSCs, and a similar trend was observed for other culture systems (Supplementary Figure 7). In contrast to the results in HMCL, BCL-2 and BCL-XL expression in primary MM cells did not correlate with MCL-1i sensitivity (Supplementary Figure 8).

1q21-amplified MM is characterized by high *MCL1* expression

Because MM with 1q21 amplification had increased sensitivity to MCL-1i, we investigated whether this subgroup was characterized by high *MCL1* expression. To test whether 1q21-amplified MM correlated with high MCL-1 protein expression,

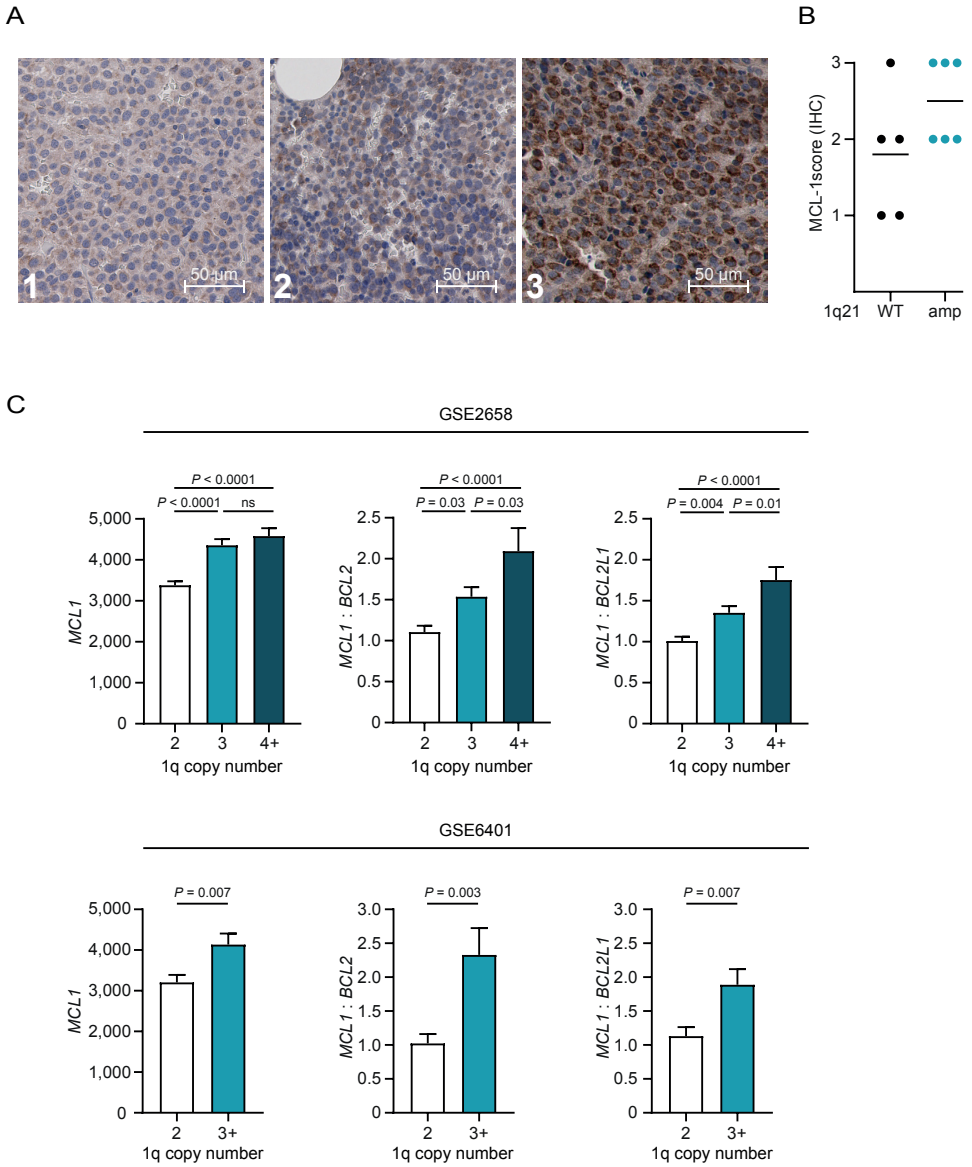


Figure 5. 1q21-amplified MM is characterized by high MCL1 expression. (A) Representative images of low (left; 1), moderate (center; 2), and high (right; 3) MCL-1 staining of MM BM biopsies by immunohistochemistry (IHC) (original magnification ×400). (B) MCL-1 staining intensity in 1q21 WT (n = 5) and amplified (n = 6) patient samples, as determined by IHC. (C) *MCL1* gene expression and *MCL1*/*BCL2* and *MCL1*/*BCL2L1* (BCL-XL) ratios in patients without and with 1q amplification (GSE2658: 2 copies, n = 131; 3 copies, n = 69; ≥4 copies, n = 43; and GSE6401: 2 copies, n = 37; ≥3 copies, n = 40). Ratios were calculated based on normalized gene expression values. ns, nonsignificant.

we performed immunohistochemical staining for MCL-1 on 11 BM biopsies, of which 6 had 1q21 amplification. We observed strong differences in MCL-1 staining intensity in MM cells between samples (Figure 5A), with generally higher MCL-1 staining in the 1q21-amplified samples (Figure 5B). Interestingly, the only 1q21$^-$ sample that had high (level 3) MCL-1 staining was also very sensitive to MCL-1i treatment (MMP1; Supplementary Figure 5). High MCL-1 protein expression and MCL-1i sensitivity in this sample may result from posttranscriptional and/or posttranslational regulation of MCL-1. By analyzing published messenger RNA expression data, we found significantly increased *MCL1* gene expression in patients with 1q amplification in 2 independent data sets (Figure 5C). Additionally, *MCL1*/*BCL2* and *MCL1*/*BCL2L1* ratios were increased in the groups with 1q amplification (Figure 5C).

Presence of 1q21 in combination with other poor-prognosis diagnostic markers identifies patient subsets of MM most sensitive to MCL-1i

In addition to patients with 1q21 amplification, we also found enhanced MCL-1i sensitivity in patients with increased (≥5.5 mg/L) serum β2m level (1.6-fold at 1000 nM; P = .03; Figure 6A). Elevated serum β2m levels are associated with poor prognosis of MM,[28,29] and the ISS for MM assigns stage 3 to patients with serum β2m levels >5.5 mg/L.[30] We analyzed the relative abundance of 1q21 amplification in relation to serum β2m levels and found enrichment of 1q21-amplified patients in the high β2m group (45% vs 19%; Figure 6B). Next, combining β2m status and 1q21 amplification enabled identification of a patient subset with the highest sensitivity to MCL-1i, namely patients who had both 1q21 amplification and high β2m levels (Figure 6C). This patient subset has 1.9-fold increased specific apoptosis after treatment with 1000 nM of MCL-1i compared with the average of the whole cohort (84% specific apoptosis, n = 5 vs 44% in the whole cohort, N = 47). Moreover, specific apoptosis in this patient subset was increased 2.7-fold compared with the patient group that had no 1q21 amplification and normal β2m levels (84% vs 31%). Within 1q21-amplified MM, patients with normal β2m levels were less sensitive to MCL-1i compared with the group with increased β2m, suggesting that enrichment of 1q21 amplification does not fully explain increased MCL-1i sensitivity of MM cells from patients with increased β2m.

Increased β2m serum levels can be the result of impaired renal filtration, which is often seen in MM. In our cohort, presence of renal insufficiency or ISS stage was indeed also linked to increased sensitivity to MCL-1i (Supplementary Figure 9A-B). Presence of bone lesions, anemia, or high PC percentage did not correlate with MCL-1i sensitivity (Supplementary Figure 9C).

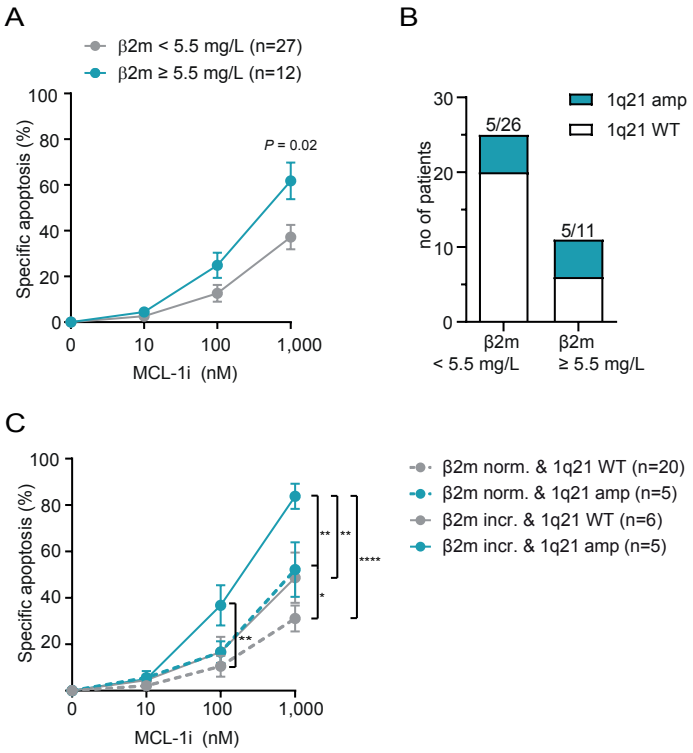


Figure 6. Presence of 1q21 in combination with other poor-prognosis diagnostic markers identifies patient subsets of MM most sensitive to MCL-1i. (A) Specific apoptosis of patients with normal (<5.5 mg/L; n = 23) or increased (≥5.5 mg/L; n = 15) serum β2m levels, after treatment with MCL-1i for 24 hours. (B) Fraction of patients with 1q21 amplification in the groups shown in panel A. (C) As in panel A, but with stratification for 1q21 amplification status. Blue lines indicate patient groups with 1q21 amplification, and grey lines show groups with WT 1q21. Dotted lines indicate normal levels of β2m, and filled lines indicate increased β2m. Group sizes are shown in the figure legend. *P < .05, **P < .01, ****P < .0001.

Subset of patients without 1q21 amplification shows sensitivity toward MCL-1 inhibition

To further evaluate MCL-1i sensitivity of 1q21-amplified MM, patients were divided into equally sized subgroups of high or low sensitivity to MCL-1i (Figure 7A-B). Subsequently, the abundance of these groups within 1q21 WT and 1q21-amplified MM was determined. Within 1q21-amplified MM, 75% had high sensitivity to MCL-1i, whereas this was only 19% within 1q21 WT MM (Figure 7A-B). Even though this analysis underlines the sensitivity of 1q21 amplification as a marker for susceptibility to MCL-1i, it also shows that a subset of 1q21 WT MM may benefit from MCL-1i treatment.

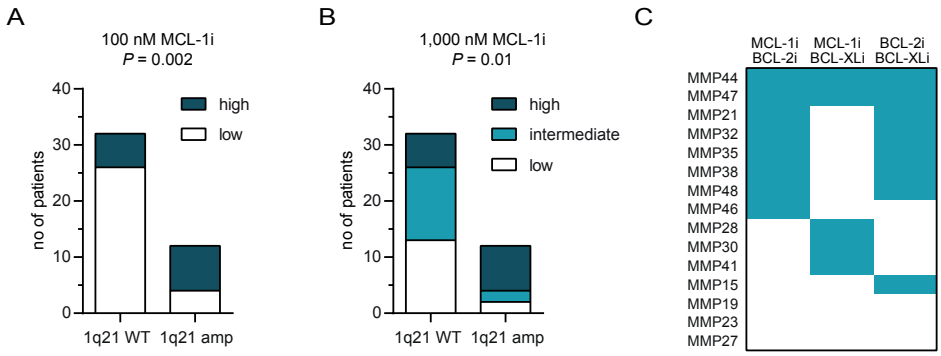


Figure 7. A subset of patients without 1q21 amplification shows sensitivity to MCL-1 inhibition. (A) Fraction of patients within 1q21 WT and amplified groups with high (≥20%; n = 14) or low (<20%; n = 30) sensitivity to MCL-1i at 100 nM. Significance by χ^2 test is shown in the graph. (B) As in panel A, but at 1000 nM of MCL-1, where high (≥60%; n = 14), intermediate (23%-60%; n = 15), and low (<23%; n = 15) sensitivity groups could be identified. Cutoff values were based on terciles of specific apoptosis after 24 hours of treatment with MCL-1i. (C) Inhibitor combination sensitivity of patients with low sensitivity to single MCL-1i. Blue boxes indicate at least intermediate sensitivity, after 24 hours of treatment with indicated inhibitor combinations (all at 100 nM). Two patients in this analysis had amplification of 1q21, namely MMP27 and MMP44.

Next, we examined whether MCL-1i–insensitive patients can be resensitized by cotreatment with BCL-2i or BCL-XLi (Figure 7C). In this group, 73% showed sensitivity to a combination of MCL-1i with BCL-2i or BCL-XLi, suggesting that the synergistic effect between S63845 and other BH3-mimetics found in HMCL is replicated in primary samples insensitive to MCL-1i alone.

DISCUSSION

MCL-1, BCL-2, and BCL-XL seem promising therapeutic targets in MM, but dependence on these 3 pro-survival proteins varies between patients. In fact, using BH3 profiling or a BH3 mimetic toolkit, others have shown that dependence on these proteins is highly variable.[31,32] Our study aimed to identify groups of patients sensitive to MCL-1–specific BH3-mimetics. To this end, we analyzed sensitivity of HMCLs and primary MM samples to MCL-1i alone or in combination with BCL-2i and BCL-XLi and searched for patient characteristics that correlated with inhibitor sensitivity.

As more MM treatment options become available, it is increasingly important to be able to predict which patient will benefit from which therapy. It has previously been shown that venetoclax as monotherapy is effective in MM patients with t(11;14), who were reported to have relatively high *BCL2* gene expression compared

with *BCLXL* and *MCL1*.[12] Additionally, venetoclax, in combination with bortezomib and dexamethasone, was shown to be particularly effective in MM patients with high *BCL2* gene expression.[27] We were able to reproduce these findings in our experimental setup, supporting the validity of our approach. Our data indicate that at time of diagnosis, MM cells from more than half of all patients are sensitive to MCL-1 inhibition, underlining the potential of MCL-1–specific BH3-mimetics as first-line therapy. Importantly, we showed that MM with 1q21 amplification was the patient subgroup most likely to benefit from MCL-1i treatment. Reported incidence of 1q amplification in large cohorts of newly diagnosed MM ranges between 34.5% and 43%.[19,33] At relapse, 1q amplification occurs in 72% of MM patients.[32] In our cohort of newly diagnosed MM, 1q amplification was present in 28.5% of patients, and most of them (75%) showed high sensitivity to MCL-1i. Combined, this shows that amplification of 1q21 identifies a large patient subset that may be treated more effectively with MCL-1i. Notably, a reasonable proportion (19%) of MM without 1q21 amplification is highly sensitive to MCL-1i as well, suggesting that effectiveness of MCL-1i treatment may not be limited to 1q21-amplified MM. Besides by increased transcription, MM cells can also overexpress MCL-1 through posttranscriptional regulation. Because MCL-1 can be subject to complex modification at the protein level as well,[34] studying MCL-1 protein expression and stability may provide mechanisms that explain MCL-1i sensitivity in 1q21 WT MM.

To support primary MM cells *ex vivo*, we used MS-5 feeder cells, because they promote MM viability in short-term cultures without producing interleukin-6. Other published culture systems for MM cells include MSCs in 2D or 3D culture,[20] the latter of which was set up to mimic the structure and cellular support of the BM microenvironment. Our MM material consisted of frozen biobanked samples, which were shown to maintain highest cell viability when cultured on MS-5 cells. We observed strong correlations between results obtained with the murine BM-derived stromal cell line MS-5 and with primary MSCs derived from BM of MM patients, in either a 2D or more protective 3D setting, suggesting that MCL-1i–sensitive and –insensitive MMs can be identified regardless of the culture system. Moreover, our findings using these different culture methods are in line with a recent publication where increased MCL-1i sensitivity was observed in a small cohort of directly isolated MM patient samples containing 1q amplification compared with samples without this chromosomal gain.[35] Although we used different culture methods, a common pro-survival factor that may skew sensitivity toward MCL-1i in certain MM samples cannot be ruled out. Therefore, it remains important to validate the increased MCL-1i sensitivity of 1q-amplified MM using a large cohort of freshly isolated MM BM samples.

Although 1q21 amplification is considered a poor prognosis marker in MM, the responsible genes located on 1q21 that underlie this poor prognosis remain debatable. Besides *MCL1*, other potentially responsible genes in this region include the cell-cycle progression genes *CKS1B*[20] and *PMSD4*,[21] the latter of which has been associated with bortezomib resistance. Here, we report increased *MCL1* expression and MCL-1i sensitivity in MM with 1q21 amplification. In contrast to BCL-2 and BCL-XL, MCL-1 protein expression could not be quantitatively measured by flow cytometry. Therefore, it was not possible to correlate MCL-1 protein levels with 1q21 amplification status in the complete patient cohort. Alternatively, we were able to measure MCL-1 protein expression using immunohistochemistry for a limited group of MM patient samples that were also used throughout the study and for which material was available. These data indeed suggest a positive correlation between 1q21 amplification status and MCL-1 protein expression. In HMCLs, there was no correlation between MCL-1i sensitivity and MCL-1 protein expression as determined by western blotting, but we did observe inverse correlations between BCL-2 and BCL-XL expression and MCL-1i sensitivity. It is not surprising that expression of single pro-survival proteins does not always directly correlate with inhibitor sensitivity. Inhibitor sensitivity rather seems to be a consequence of the relative expression and distribution of multiple pro- and anti-apoptotic BCL-2 family members, as we also observed by examining interaction patterns of BIM in MCL-1i–sensitive and –insensitive HMCLs.[9,16,17]

Recently, sensitivity to MCL-1i was shown to be increased in MM cells from relapsed compared with newly diagnosed patients.[32] This may be explained by increased incidence of 1q21 amplification at relapse.[33] Investigating the effect of current treatment regimens as well as BH3 mimetic treatment on MCL-1i sensitivity during the whole course of disease will be very important for optimal implementation of BH3 mimetic treatment in clinical practice.

When investigating the enrichment of 1q21-amplified MM within the patient subset with increased β2m, we found that both these parameters independently correlated with MCL-1i sensitivity. Patients who had both increased β2m and 1q21 amplification were identified as the patient subset most sensitive to MCL-1i. Both increased β2m levels and 1q21 amplification correlate with poor prognosis of MM, suggesting that MCL-1i treatment can be particularly effective in poor prognosis patients.[18,19,28,29] Finding new and effective treatment options for poor prognosis patients is essential for extending overall patient survival.

Previous research in mice showed that *MCL1* knockout leads to peri-implantation embryonic lethality.[36] Conditional gene knockout of *MCL1* has lethal effects in multiple cell types, including hematopoietic stem cells,[37] cardiomyocytes,[38] neural precursor cells,[39] B cells,[40,41] and healthy PCs.[42] However, when S63845 was tested

in mice, the drug was well tolerated at doses that were lethal for MM, lymphoma, and acute myeloid leukemia cells,[14] suggesting that the effects of pharmacological inhibition of MCL-1 are not as detrimental as the effects of *MCL1* gene knockout. This illustrates that there may be a therapeutic window for direct MCL-1 inhibition in MM and other high MCL-1–expressing tumors. Phase 1 clinical trials with MCL-1i, which are currently under way, will clarify whether these preclinical results can be recapitulated in humans and to what extent adverse effects limit the dosage of MCL-1i.

In HMCLs as well as primary MM samples, we found that inhibitor combinations induced apoptosis in a more-than-additive fashion when compared with the expected effect from single drugs. This suggests that combinations of BH3-mimetics may be very powerful in treatment of MM. Adverse effects of individual BH3-mimetics could be reduced by combining 2 inhibitors at much lower concentrations. Clinical targeting of BCL-XL may be difficult because of the dependence of thrombocytes on BCL-XL, which was dose-limiting in trials with BCL-2 and BCL-XL inhibitor navitoclax.[43-45] Combinations of MCL-1i and BCL-2i were well tolerated in mice and showed synergistic antitumor activity in MM, mantle cell lymphoma, and acute myeloid leukemia.[35,46-49] Nevertheless, when MM patients were treated with venetoclax, various cytopenias were observed, including thrombocytopenia and neutropenia.[12]

Because *MCL1* is located on 1q21, a region often amplified in MM, we hypothesized that presence of this amplification would result in enhanced MCL-1 expression and MCL-1i sensitivity. This hypothesis was tested and confirmed in this study using 47 BM aspirates from newly diagnosed MMs. Our results identify the patient subpopulation most sensitive to MCL-1 inhibition, which may guide further testing of MCL-1 inhibitors in future clinical trials.

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AUTHORSHIP

Contribution: V.P., M.C.M., and A.S. designed the research; A.S., L.M.M., J.G., and M.C. did experiments and contributed to interpretation and discussion; V.P., A.S., L.M.M., J.G., M.C., P.A.v.d.B., P.S., D.C.S.H., and M.C.M. contributed to the design of experiments, interpretation of results, and drafting of the manuscript; V.P., A.S., L.M.M., M.C., and J.G. analyzed data and prepared figures; and A.S. and V.P. wrote the manuscript.

Conflict-of-interest disclosure: V.P. received research funding from Philips and royalty payments related to venetoclax. M.C.M. received research funding from Celgene and honoraria from Celgene, Takeda, BMS, and Servier. P.S. received research funding from Amgen, Celgene, Janssen, Takeda, and SkylineDx and honoraria from Amgen, Celgene, Janssen, Takeda, and Karyopharm. J.G. and D.C.S.H. are employees of the Walter and Eliza Hall Institute, which receives milestones and royalty payments related to venetoclax. The remaining authors declare no competing financial interests.

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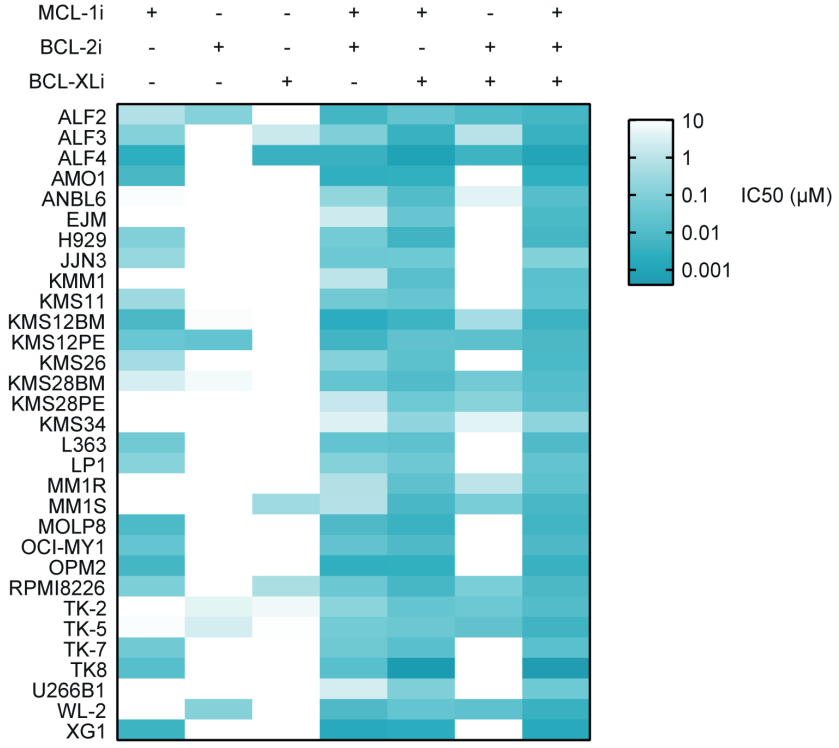
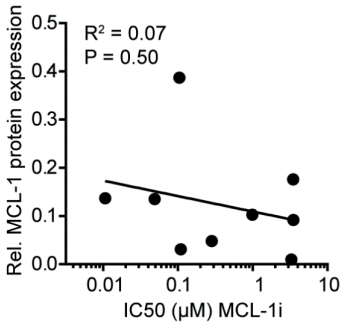
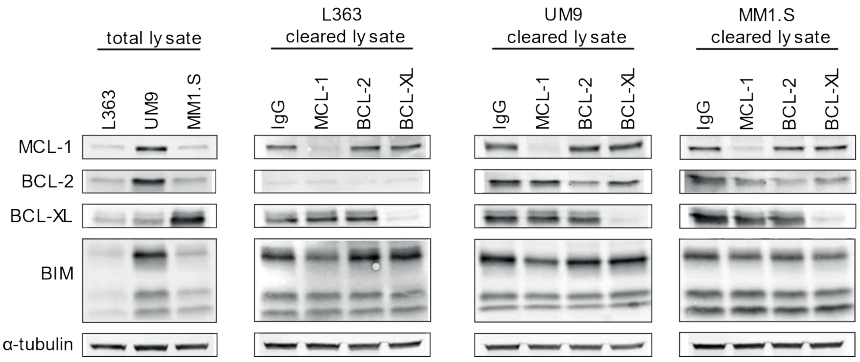
SUPPLEMENTARY FIGURES



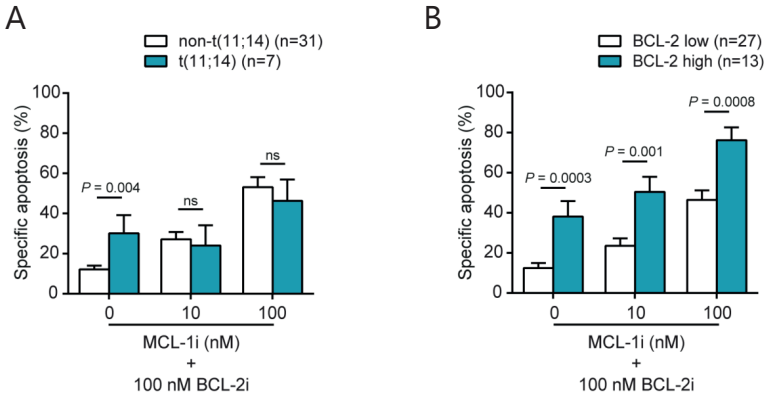
▲Supplementary Figure 1. Heatmap showing IC50s of single and combined BH3-mimetics in 31 HMCL. IC50s, defined as the drug concentrations that induce 50% killing, were determined after 6h treatment with individual and combined BH3-mimetics, by using a CellTiter-Glo assay. For double and triple combinations, BH3-mimetics were used at concentration ratios of 1:1 or 1:1:1.



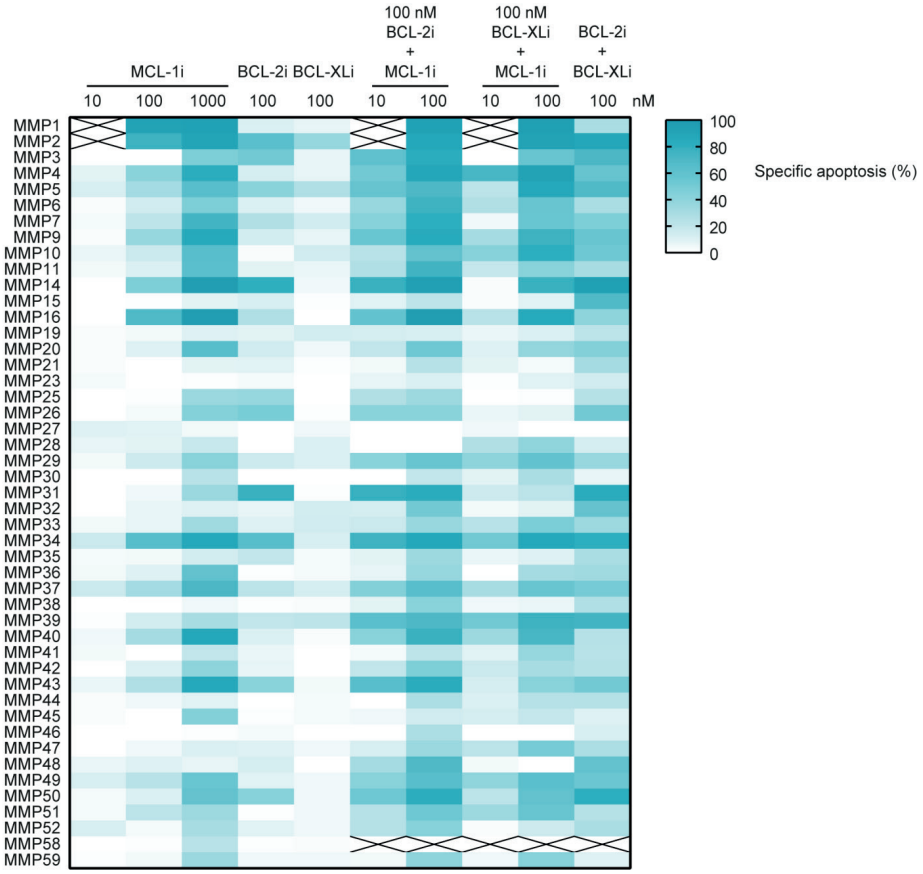
◄Supplementary Figure 2. MCL-1 expression does not correlate to MCL-1i sensitivity in HMCL. Protein expression of MCL-1 (fold over loading control) was determined for each cell line by immunoblotting, and compared to MCL-1i IC50s. No correlation was found.



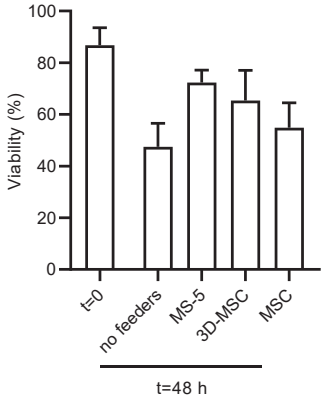
Supplementary Figure 3. Total and cleared lysates of MCL-1, BCL-2, and BCL-XL co-immunoprecipitation. From the same experiment as shown in Figure 2B, expression of MCL-1, BCL-2, BCL-XL, BIM, and α-tubulin in total lysate (input) and cleared lysate (flow-through after co-immunoprecipitation) of L363, UM9, and MM1.S.



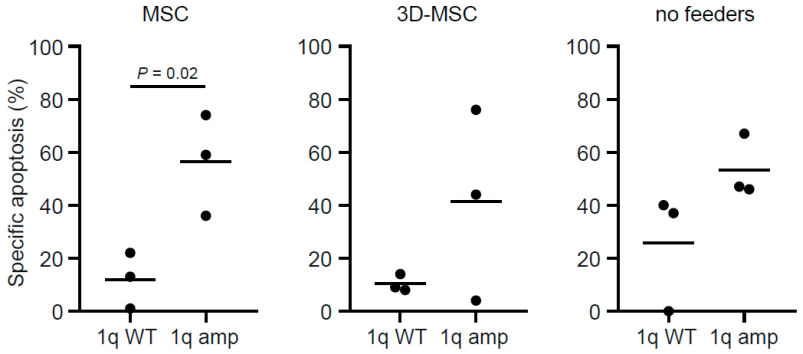
Supplementary Figure 4. T(11;14) and high BCL-2 expression correlate with increased venetoclax sensitivity. Differences in specific apoptosis between cells from non-t(11;14) and t(11;14) patients after treatment with BCL-2i alone or combined with MCL-1i (B) Differences in specific apoptosis between cells from patients with low (below average) and high (above average) BCL-2 protein expression after treatment with BCL-2i alone or combined with MCL-1i.



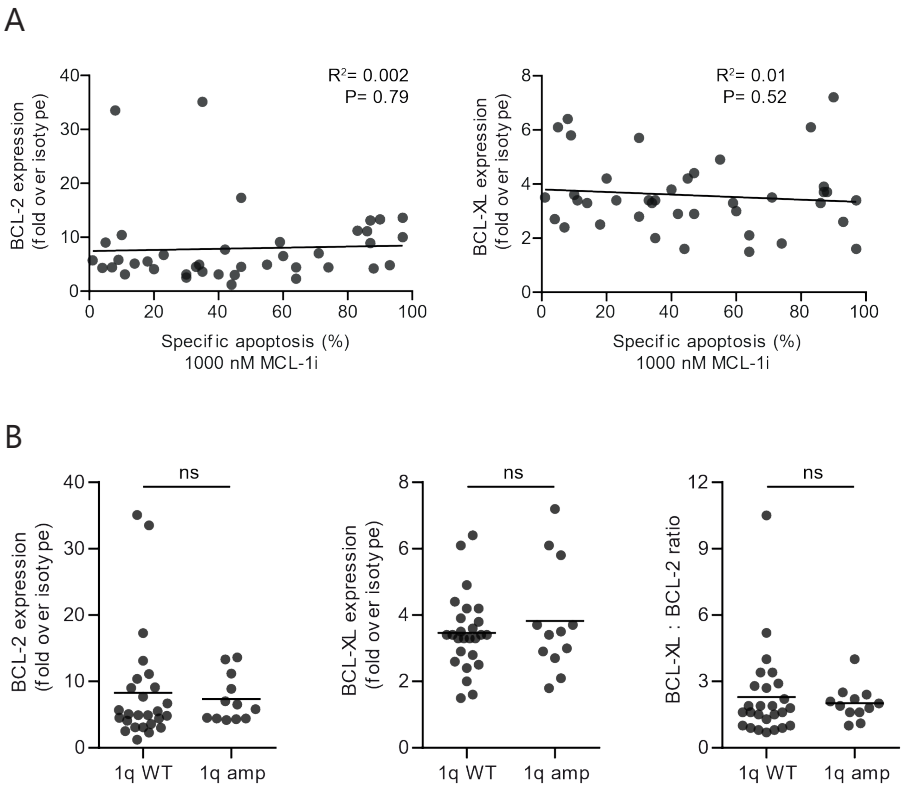
▲Supplementary Figure 5. Heatmap of primary MM sensitivity to single and combined BH3-mimetics. Specific apoptosis induced by 24h treatment with BH3-mimetics at indicated concentrations is shown for all MM patient samples included in the study. Crosses indicate missing values.



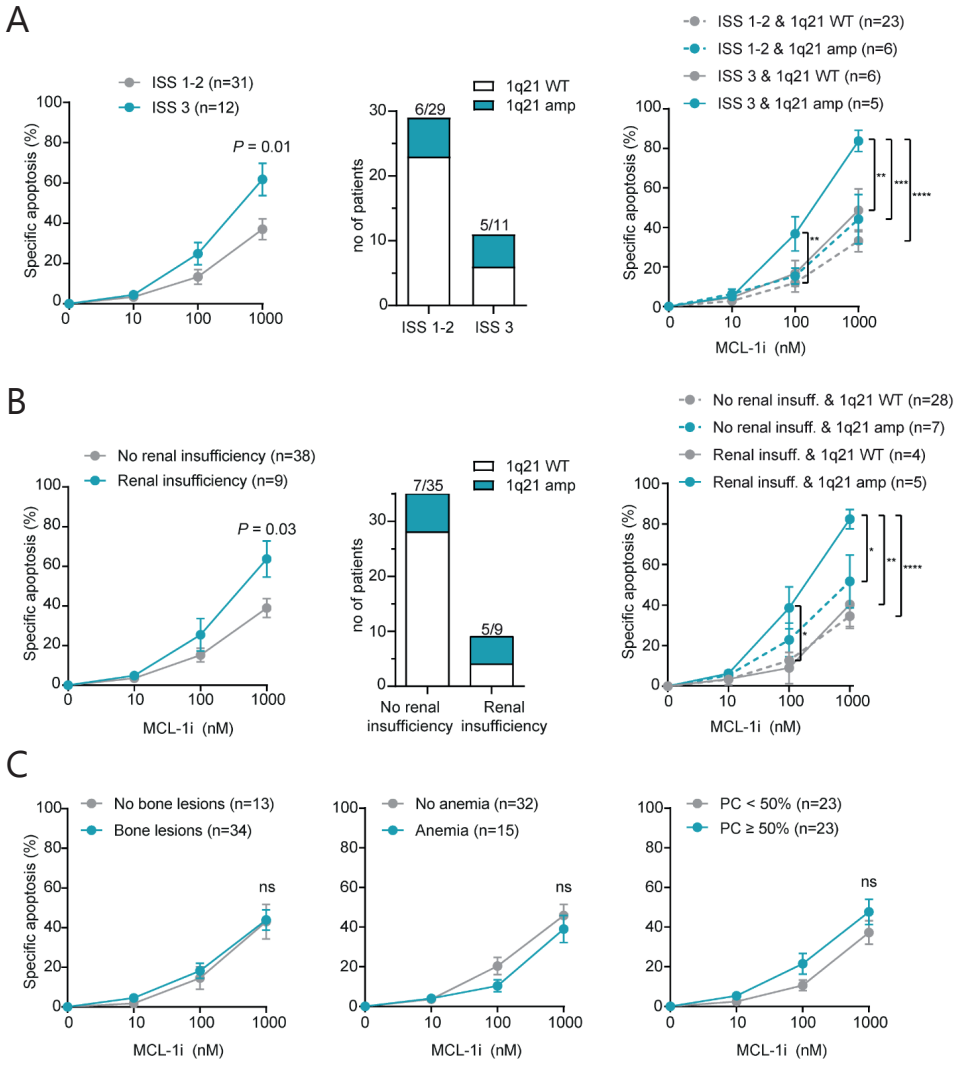
◄Supplementary Figure 6. Viability of MM cells after culture for 48 h in different culture systems. Viability of untreated pMM samples (n=6) was determined after thawing (0 h) and after 48 hours without feeder cells or in culture with MS-5 cells, MM-derived MSC, or MM-derived MSC in 3D hydrogel (3D-MSC).



Supplementary Figure 7. Increased MCL-1i sensitivity of 1q amplified MM was confirmed in MSC culture systems. Specific apoptosis of 1q WT (n=3) and 1q amplified (n=3) MM samples in culture with MSC, 3D-MSC, or without feeders, after treatment with 100 nM MCL-1i.



Supplementary Figure 8. BCL-2 and BCL-XL expression in pMM cells do not correlate with MCL-1i sensitivity or 1q status. (A) Correlation between BCL-2 or BCL-XL expression and MCL-1i sensitivity in pMM. (B) BCL-2 and BCL-XL expression, and BCL-2 : BCL-XL ratio in 1q WT and 1q amplified MM groups.



Supplementary Figure 9. ISS stage and renal function of MM patients correlate with MCL-1i sensitivity. (A) Specific apoptosis of ISS 1-2 and ISS 3 patients after treatment with MCL-1i for 24 hours (left). Fraction of patients with 1q21 amplification in the ISS 1-2 and ISS 3 groups (middle). Stratification of ISS groups for 1q21 amplification status (right). Blue lines indicate patient groups with 1q21 amplification, and grey lines show groups with WT 1q21. Dotted lines indicate normal levels of β2m or albumin, and filled lines indicate increased β2m (left graph) or decreased albumin (right graph). Group sizes are shown above the graphs. Differences between groups were statistically assessed for each concentration using mixed-effects analysis, and statistically significant results are indicated with stars: * P ≤ .05; ** P ≤ .01; *** P ≤ .001; **** P ≤ .0001. (B) As in (A), but for patients with renal insufficiency, which was defined by presence of serum creatinine levels >173 mmol/L. (C) Specific apoptosis of the indicated patient groups after treatment with MCL-1i for 24 hours.

CHAPTER 5

Bortezomib-induced apoptosis in multiple myeloma does not require NOXA

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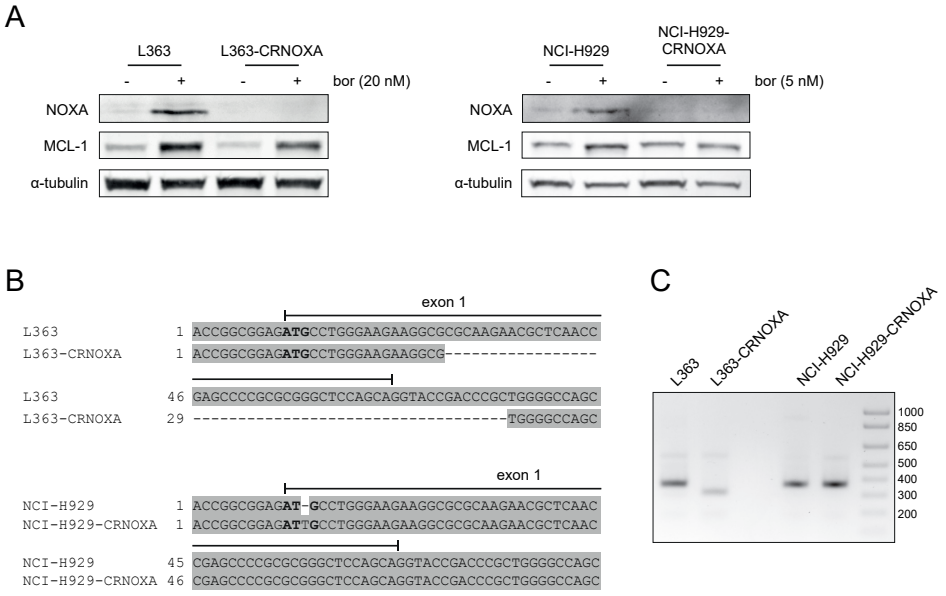
ABSTRACT

Proteasome inhibitors (PI) are essential for the treatment of multiple myeloma (MM), an incurable plasma cell malignancy. Although proteasomal dysfunction induced by proteasome inhibitors can have diverse roles on cellular functioning, a main role has been ascribed to upregulation of the pro-apoptotic BCL-2 family protein NOXA (alternatively named phorbol-12-myristate-13-acetate-induced protein 1 / PMAIP1). We demonstrate in contrast to previous publications that NOXA is not required for apoptosis induced by proteasomal inhibition in MM.

The ubiquitin-proteasome pathway is essential for maintaining cellular homeostasis. Many proteins that regulate processes such as cell-cycle progression, apoptosis, and response to cellular stress, are poly-ubiquitinated and subsequently degraded by the proteasome.[1] MM is characterized by clonal expansion of malignant plasma cells that produce a large amount of immunoglobulins. To prevent protein overload and ER stress resulting from the accumulation of misfolded proteins, MM cells highly depend on proteasomal function, which can be blocked with PI treatment.[2] Besides ER stress, PI induce p53, cell cycle arrest, and oxidative stress.[3] Moreover, PI inhibit the NFkB pathway, which is thought to be constitutively active in MM.[3] All these effects of PI ultimately result in apoptosis in MM cells. PI are generally included in first-line treatment of MM, in combination with immunomodulatory drugs and dexamethasone.[4] So far, three PI have been FDA-approved: bortezomib (2005), carfilzomib (2012), and ixazomib (2015), and several others are still under development.[3]

Upon treatment of MM cells with PI, protein levels of NOXA increase, and cleavage of pro-survival BCL-2 family protein MCL-1 takes place.[5] MCL-1, that binds to and is neutralized by NOXA, is essential for MM cell survival.[6,7] In multiple studies, knockdown of NOXA indeed reduced efficacy of bortezomib.[5,8,9] Based on these data, PI such as bortezomib are often considered to be indirect MCL-1 inhibitors through upregulation of NOXA, although direct evidence was lacking. In this study, NOXA-deficient MM cell lines are used to revisit the role of NOXA in apoptosis induced by PI.

We used a lentiviral-based CRISPR strategy to generate NOXA knockout MM cell lines. The resulting clonal cell lines, L363-CRNOXA and NCI-H929-CRNOXA, lack NOXA expression, as shown by bortezomib treatment for 24 hours (Figure 1A). Sequencing of *PMAIP1*, the gene encoding NOXA, in wild type (WT) and knockout (KO) cell lines revealed a 52-nt deletion in L363-CRNOXA, spanning a large fraction of the first exon of the protein (Figure 1B). In NCI-H929-CRNOXA, a single nucleotide insertion in the start codon of the gene resulted in disruption of the gene (Figure 1B). Gel electrophoresis of exon 1 (and flanking regions) indeed indicates reduction in PCR product size for L363-CRNOXA, whereas the NCI-H929-CRNOXA PCR product has a similar size to that in WT cells (Figure 1C). In WT L363 and NCI-H929, treatment with bortezomib results in 12.4-fold and 5.7-fold increase of NOXA, respectively (Figure 1A). This suggests that PI-induced NOXA-upregulation occurs at least in part independent of p53 signaling, since L363 is p53-mutant and NCI-H929 has functional p53. This is in concordance with other studies, which showed that PI-mediated NOXA upregulation can take place in the absence of functional p53.[8,10] In addition to the p53 pathway, NOXA induction was previously shown to result from a variety of cellular stimuli,

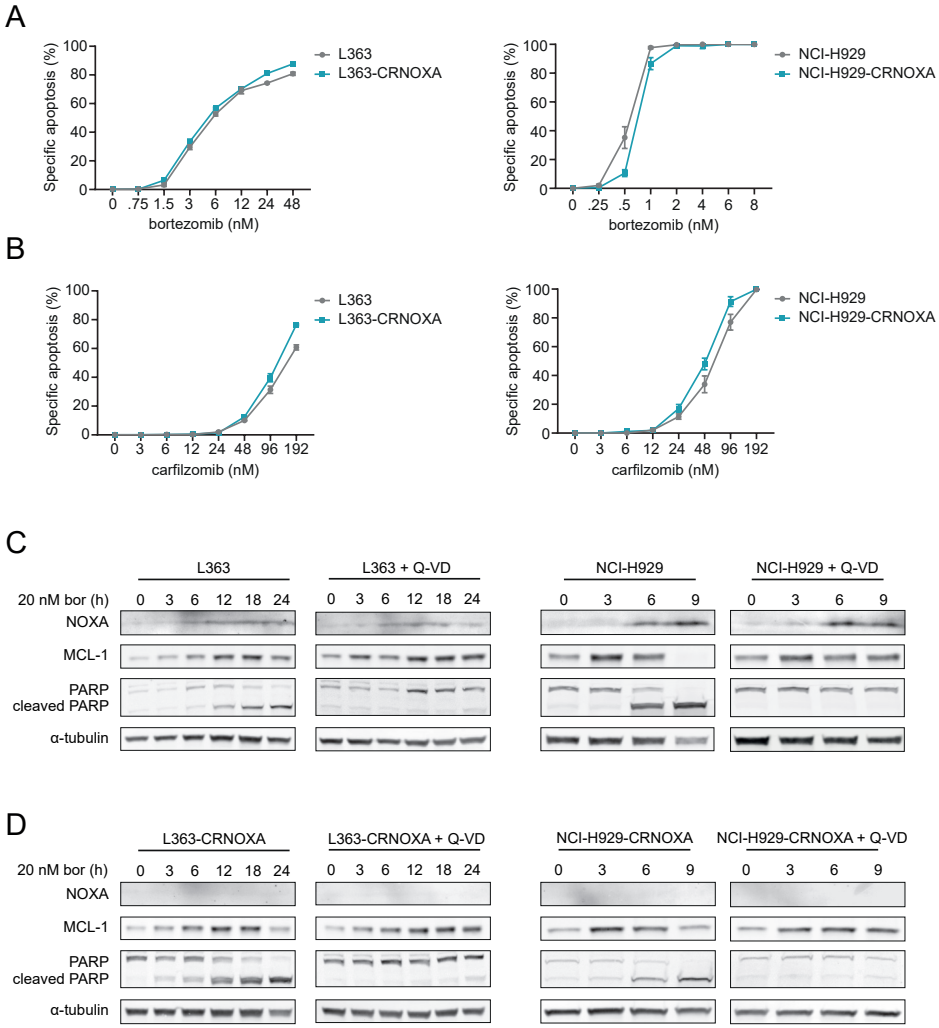


including hypoxia (through HIF1a), p73, and c-Myc signaling.[10,11] Proteasome inhibition induces many of these stimuli, thus PI-induced NOXA upregulation may occur through multiple parallel mechanisms.

If PI induce apoptosis through upregulation of NOXA, the NOXA knockout cell lines are expected to have reduced sensitivity to bortezomib and carfilzomib treatment. Surprisingly, treatment with either PI resulted in apoptosis regardless of NOXA expression (Figure 2A-B). These results indicate that PI do not function in a NOXA-dependent manner.

In order to further investigate the effect of NOXA deficiency on apoptosis induction, the WT and KO MM cell lines were treated with bortezomib for different time periods up to 24 (L363) or 9 (NCI-H929) hours. Pan-caspase inhibitor Q-VD-OPh was used to distinguish caspase-dependent and –independent effects of bortezomib. In both WT cell lines, bortezomib led to an accumulation of NOXA protein over time, regardless of caspase activity (Figure 2C). MCL-1, on the other hand, only stably increased when caspase activity was prevented. In the absence of Q-VD-OPh, MCL-1 cleavage took place simultaneous to cleavage of apoptosis marker PARP. These results indicate that cleavage of MCL-1 occurs after activation of caspases and therefore is a consequence, rather than the cause, of apoptosis induction in MM cells. Indeed, MCL-1 is a known caspase substrate, and its cleavage during apoptosis may generate a product that inhibits the function of full-length MCL-1.[12]

◄Figure 1. Characterization of NOXA-knockout MM cell lines. (A) NOXA and MCL-1 expression in WT and NOXA-KO MM cell lines after 24 hours of treatment with 20 nM (L363) or 5 nM (NCI-H929) bortezomib, in the presence of 10 μM Q-VD-OPh. Data are representative of 3 independent experiments. (B) Genomic sequence of exon 1 of *PMAIP1*, the gene encoding NOXA, in WT and NOXA-KO MM cell lines. The span of exon 1 is marked in the sequence, and the start codon is shown in bold text. (C) Gel electrophoresis of *PMAIP1* exon 1 and flanking regions. Expected product size in the WT sequence is 363 nucleotides.



▲Figure 2. NOXA is not required for bortezomib-induced apoptosis. (A-B) Specific apoptosis of WT and KO cell lines after treatment with a concentration series of proteasome inhibitors bortezomib (A) and carfilzomib (B). Graphs show technical triplicates (±S.D.) representative of four independent experiments. (C-D) Immunoblot analysis of WT and KO cell lines after treatment with 20 nM bortezomib for different time periods up to 24 hours (L363) or 9 hours (NCI-H929). Where indicated, bortezomib treatment took place in the presence of 10 μM caspase inhibitor Q-VD-OPh. Images are representative of at least 2 independent experiments.

In the NOXA KO cell lines, as expected, no NOXA expression was observed upon bortezomib treatment (Figure 2D). MCL-1 upregulation and caspase-dependent cleavage took place in NOXA-WT or –deficient MM cells in a similar manner. Therefore, we conclude that NOXA is not involved in the degradation of MCL-1 that occurs after bortezomib treatment. Notably, knockout of NOXA does not prevent PARP cleavage in absence of Q-VD-OPh (Figure 2C). Combined, these data show that proteasome inhibitor-induced apoptosis in MM cell lines does not depend on NOXA.

To our knowledge, this is the first time PI function was studied in NOXA knockout MM cells. Yet, other p53-dependent and –independent cell death stimuli were previously studied in *Noxa*$^{-/-}$ mice, which were characterized by normal development and physiology.[13] In pre-B cells and mature B cells from these mice, Noxa loss did not protect against cell death induced by γ irradiation and etoposide (p53-dependent), nor by cytokine withdrawal, glucocorticoid dexamethasone, ionomycin, and phorbol 12-myristate 13-acetate (PMA) (p53-independent).[13] Redundant function of other pro-apoptotic BCL-2 family proteins, such as PUMA or BIM, may explain why NOXA is not essential for cell death induced by PI or other cell death stimuli. Possibly, in previous studies using RNA interference to reduce NOXA expression, no redundant mechanisms had been established in the cell due to the transient nature of these experiments. Stable knockout of NOXA in this study, on the other hand, shows that NOXA is not required for execution of PI-induced cell death.

Over the past 15 years, the introduction of proteasome inhibitors has revolutionized the therapeutic landscape for MM. In addition, trials combining bortezomib with novel agents such as anti-CD38 antibody daratumumab and histone deacetylase inhibitors have shown improved results.[3] In preclinical studies, simultaneous inhibition of MCL-1 and BCL-2 was shown to synergistically induce cell death in MM.[14] Since bortezomib is often considered an indirect MCL-1 inhibitor, this led to the investigation of the combination of bortezomib and BCL-2 inhibitor venetoclax. In a phase 3 clinical trial, the MM subgroup with a t(11;14) chromosomal translocation was recently reported to have consistent clinical benefit from the combination of bortezomib, dexamethasone, and venetoclax, compared to bortezomib and dexamethasone alone.[15] Although, we demonstrate that apoptosis induction by bortezomib does not require NOXA, and that bortezomib-induced degradation of MCL-1 is also independent of NOXA, the observed increase in NOXA protein by bortezomib treatment may indirectly promote the apoptotic effect of venetoclax. Conversely, knockdown of MCL-1 was previously shown to increase bortezomib-induced apoptosis in MM.[5] Since MM cells are highly dependent on MCL-1 for survival, modulation of MCL-1 is likely to

affect sensitivity to other apoptosis-inducing agents such as bortezomib. Hence, an interplay between bortezomib and BCL-2 family proteins may still be in place.

Based on our results we conclude that NOXA induction is not required for PI-mediated apoptosis in MM. In order to optimize rational drug combinations in the expanding landscape of available MM treatments, it is essential to expose the exact mechanism of apoptosis induced by bortezomib and other PI. Ultimately, this may lead to identification of novel synergistic drug combinations and improve treatment of MM.

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SUPPLEMENTARY METHODS

Cell culture and chemicals

Human multiple myeloma cell lines NCI-H929 and L363 were purchased from DSMZ. NCI-H929 was cultured in RPMI 1640 GlutaMAX HEPES culture medium (Life Technologies) supplemented with 20% fetal bovine serum (FBS) (Sigma-Aldrich), 1 mM sodium pyruvate (ThermoFisher), 50 μM β-mercaptoethanol (Life Technologies), and 100 μg/ml penicillin-streptomycin (Gibco/Life Technologies). L363 was cultured in RPMI 1640 GlutaMAX HEPES culture medium supplemented with 10% FBS and 100 μg/ml penicillin-streptomycin. P53-status of these MM cell lines is as follows: WT p53 in NCI-H929 and a missense mutation leading to a disrupted splicing site in L363.[1,2] Hek293T cells were cultured in Dolbecco's Modified Eagle Medium with 10% FBS and 100 μg/ml penicillin-streptomycin. In order to assess the effect of proteasome inhibitors, cells were treated with bortezomib (Selleckchem) or carfilzomib (MedChem Express), serially diluted or at a single dose of indicated concentration. Where indicated, caspase activity was blocked using 10 μM Q-VD-OPh hydrate (Sigma-Aldrich).

Generation of CRISPR knockout cell lines

In order to generate NOXA knockout cell lines, anti-NOXA gRNAs (PMAIP1_1: 5'-GCGGCACCGGCGGAGATGCC-3' and PMAIP1_2: 5'-ACGCTCAACCGAGCCCCGCG-3') were designed using the "Optimized CRISPR Design tool" from the Zhang lab (crispr.mit.edu), and subsequently cloned in a lentiviral vector that was described previously.[3] Hek293T cells were transfected with the lentiviral vector, BaEVTR baboon envelope vector,[4] and psPAX2 packaging vector using Mirus LT1 (Mirus Bio) or FugeneHD (Promega) transfection reagent. Lentivirus was harvested 72 hours after transfection. NCI-H929 and L363 cell lines were transduced with lentivirus by spinoculation at 1,000 g for 90 minutes at 33 °C, in the presence of 4 μg/ml polybrene (Sigma). After 72 hours, successfully transduced cells were selected using 2 μg/ml puromycin (Sigma). Single cell clones were expanded and screened for absence of NOXA protein after bortezomib treatment. The resulting knockout cell lines (NCI-H929-CRNOXA from gRNA PMAIP1_1 and L363-CRNOXA from gRNA PMAIP1_2) were validated by sequencing of the *PMAIP1* (NOXA) gene. Briefly, genomic DNA was isolated (QIAamp DNA mini kit, Qiagen), followed by PCR amplification and sequencing of *PMAIP1* exon 1. Primers 5'-AGTTGGAGGCTGAGGTTCC-3' (forward) and 5'-CATCCCAATCGCAAATCCGG-3' (reverse) were used for PCR and sequencing.

Immunoblotting

For immunoblotting analysis, cells were lysed in lysis buffer containing 1% NP-40 and 1X protease/phosphatase inhibitor cocktail (Cell Signaling). Proteins were separated using SDS-PAGE, transferred to low florescence PVDF membranes (Bio-rad), and blocked in PBS containing 2% non-fat dry milk. The following antibodies were used for staining: mouse anti-NOXA (Novus Biologicals), rabbit anti-MCL-1 (Abcam), mouse anti-α-tubulin, rabbit anti-PARP (Cell Signaling), goat anti-mouse-680RD, and goat anti-rabbit-800CW (LI-COR Biosciences). Infrared imaging was used for detection (Odyssey Sa, LI-COR Biosciences). Analysis and quantification were performed using LI-COR Image Studio and ImageJ 1.47V software.

Flow cytometry

To quantify apoptosis induction, cells were stained with 20 nM DiOC6 (Thermo Scientific) and 20 nM TO-PRO-3 (Thermo Scientific), and staining intensity was measured using flow cytometric analysis (FACSCanto II, BD Biosciences). Specific apoptosis was determined by measuring the altered percentage of $DiOC6^{+}$/$TO\text{-}PRO\text{-}3^{-}$ (live) cells compared with untreated cells, using the formula (% cell death in control – % cell death in treated cells) / (% viable cells in control) × 100. Data analysis was performed using FlowJo software.

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CHAPTER 6

MCL-1 is stabilized by PP2A in multiple myeloma

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Manuscript submitted

ABSTRACT

Multiple myeloma (MM) cells depend on expression of BCL-2 family proteins, in particular MCL-1, for survival. The cellular regulation of MCL-1 is complex and cell type-dependent. Unraveling the exact mechanism by which MCL-1 is overexpressed in MM may provide with new strategies to inhibit MCL-1 in malignant cells, preferably limiting side-effects on healthy cells. In this study, we show that MCL-1 protein is stabilized in a subset of MM and diffuse large B cell lymphoma (DLBCL) cell lines and MM patient samples. Hence, high MCL-1 protein levels in MM and DLBCL result not only from a high transcription rate, but rather seem to be a product of both transcription and protein stability. We took an unbiased approach to identify the phosphatase responsible for MCL-1 stabilization in MM, and identified PP2A as the MCL-1 stabilizing phosphatase in a phosphatase siRNA screen. Using PP2A inhibitor okadaic acid, we validated that PP2A dephosphorylates MCL-1 at Ser159 and/or Thr163, and thereby stabilizes MCL-1 in MM with long MCL-1 half-life, but not in DLBCL cell lines. Combined kinase and phosphatase inhibition experiments suggest that MCL-1 half-life in MM is regulated by the counteracting functions of JNK and PP2A. These findings increase the understanding of the mechanisms by which MCL-1 is post-translationally regulated, which may provide novel strategies to inhibit MCL-1 in MM cells.

INTRODUCTION

Pro-survival B cell lymphoma 2 (BCL-2) family proteins are potent inhibitors of programmed cell death and are often overexpressed in malignant cells, enabling these cells to survive in the presence of DNA damage, irregular growth signaling, and chemotherapeutic agents.[1] MCL-1 is overexpressed in many germinal center-derived malignancies, including multiple myeloma (MM), diffuse large B cell lymphoma (DLBCL), and follicular lymphoma.[2] MCL-1 expression is essential for survival of MM,[3–5] and its overexpression in MM is associated with relapse and shorter survival,[6] making MCL-1 an attractive therapeutic target. Multiple MCL-1-specific BH3-mimetics have shown effectivity in preclinical models and are now being evaluated in clinical trials for MM and other hematologic malignancies.[2,7–9] Thus, targeting MCL-1 may be beneficial for the treatment of B cell malignancies such as MM. However, in addition to its importance for malignant cells, MCL-1 expression is important for the survival of many healthy cells and tissues, such as plasma cells,[10] B cells,[11,12] hematopoietic stem cells,[13] cardiomyocytes,[14] and neural precursor cells.[15] In order to prevent unwanted side effects of MCL-1 inhibition on healthy tissues, it is important to unravel the exact mechanism of MCL-1 upregulation in MM, because this may provide new strategies to inhibit MCL-1 in malignant cells.

MCL-1 distinguishes itself from other BCL-2 family members by its size and structure. Although the C-terminal part of MCL-1, which contains a BH3 domain that is required for its anti-apoptotic function, closely resembles other pro-survival BCL-2 family members, MCL-1 is considerably larger and has an N-terminus that is subject to heavy post-translational modification. Presence of PEST regions (enriched in proline (P), glutamic acid (E), serine (S), and threonine (T) residues), as well as presence of four arginine pairs, allow rapid turnover of MCL-1,[16,17] resulting in a half-life of 30-40 minutes in many cell types.[18,19] Proteasomal degradation of MCL-1 occurs upon phosphorylation[20] and subsequent poly-ubiquitination[21] of its PEST regions.

In its PEST regions, MCL-1 contains at least 13 putative phosphorylation sites, some of which have been experimentally studied and verified.[20] Kinases GSK-3β, ERK-1, and JNK have been shown to phosphorylate MCL-1 at Thr163, and subsequently Ser155 and Ser159, resulting in MCL-1 destabilization.[20,22–25] Multiple ubiquitin ligases have been associated with MCL-1 destabilization, including Mule, $SCF^{\beta\text{-}TrCP}$, SCF^{Fbw7}, APC/C^{Cdc20}, and Trim17. [21,25–29] Deubiquitinases USP9X,[30] Ku70,[31] and USP13[32] were shown to counteract this process and thereby stabilize MCL-1. Modification of MCL-1 by kinases and ubiquitin modifiers is highly dependent on cellular context, and the exact role and mechanism of MCL-1 stabilization in MM is unknown.

In healthy germinal center (GC) B cells, Tyrosine and Serine/Threonine phosphatase activity was shown to be increased compared to non-GC and naïve B cells.[33] It was also observed that stability of MCL-1 is increased in GC B cells, resulting in increased protein levels, and that this increased MCL-1 stability could be reversed by treatment with okadaic acid, an inhibitor targeting the PP2A phosphatase complex.[34] We hypothesize that MCL-1 overexpression in post-GC B cell malignancies is the result of increased dephosphorylation of MCL-1, which leads to stabilization of the protein. Identifying the specific phosphatase responsible for MCL-1 dephosphorylation would increase insight into MCL-1 protein regulation and may result in identification of new treatment targets for B cell malignancies.

In this study, we show that MCL-1 is stabilized in a subset of DLBCL and MM cell lines and patient cells. By using a phosphatase siRNA screen, we identify PP2A as MCL-1 stabilizing phosphatase in MM, but not in DLBCL. Moreover, we show that in MM, PP2A activity reverses phosphorylation of MCL-1 by JNK.

MATERIALS AND METHODS

Cell culture

Cell lines L363, OPM-2, MM1.S, RPMI-8226, and SU-DHL-2 were cultured in RPMI 1640 GlutaMAX HEPES culture medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Biowest) and 100 µg/ml penicillin-streptomycin (Gibco/Life Technologies). UM9 was cultured with 20% FBS, and NCI-H929 with 20% FBS, 1 mM sodium pyruvate (Thermo Fisher), and 50 µM β-mercaptoethanol (Life Technologies). OCI-Ly1, OCI-Ly3, OCI-Ly7, and OCI-Ly10 were cultured in Iscove's Modified Dulbecco's Medium (Life Technologies) supplemented with 20% FBS and 100 µg/ml penicillin-streptomycin.

Chemicals

In order to assess the effect of PP1 and PP2A phosphatases, cells were treated for 2 hours (unless otherwise stated) with 1 uM tautomycetin (2305, Tocris) or 100 nM okadaic acid (OA) (ALX-350-003, Enzo Life Sciences), respectively. When phosphorylation of MCL-1 was studied, all samples were treated with 20 nM bortezomib (S1013, Selleckchem) and 10 uM Q-VD-OPh hydrate (SML0063, Sigma-Aldrich). Protein half-life determination was performed using 25 ug/ml cycloheximide (HY-12320, MedChemExpress) for 0, 1, 2, and 4 hours or for 0.5, 1, and 2 hours. In case of CHX experiments with OA, cells were pretreated for 2 hours

with OA, and OA remained present during CHX treatment. In order to determine MCL-1 half-life in primary MM samples, CD138+ cells were purified from MM bone marrow aspirates by magnetic activated cell sorting using CD138 microbeads (Miltenyi Biotec), followed by CHX treatment as described above. CDK7 inhibitor THZ1 (MedChemExpress) was used at 200 nM for 8 hours in order to inhibit *MCL1* transcription. 10 μM of GSK-3β inhibitor CHIR99021 (Tocris Bioscience), 250 nM of MEK/ERK-1 inhibitor trametinib/GSK1120212 (Selleckchem), and 20 μM of JNK inhibitor SP600125 (Merck) were used to assess the role of kinases in MCL-1 degradation.

Immunoblotting

For western blot analysis, cells were lysed in buffer containing 1% NP-40 and proteins were separated using SDS-PAGE (Mini-PROTEAN® TGX™ Precast Gels, Bio-rad), transferred to low fluorescence PVDF membranes (Bio-rad), blocked in PBS containing 2% non-fat dry milk, and stained using the following antibodies: mouse anti-α-tubulin, rabbit anti-phospho-MCL-1 (Ser159/Thr163), rabbit anti-PP2A A subunit (PPP2R1A/B, Cell Signaling), mouse anti-PP2A C subunit, alpha isoform (PPP2CA, Merck), rabbit anti-MCL-1 (Abcam), mouse anti-MCL-1 (Santa Cruz), goat anti-mouse-680RD, and goat anti-rabbit-800CW (LI-COR Biosciences). For phospho-MCL-1 immunoblotting, all PBS in washing and staining buffers was replaced with TBS. Infrared imaging was used for detection (Odyssey Sa, LI-COR Biosciences). Analysis and quantification were performed using LI-COR Image Studio and ImageJ 1.47V software.

qPCR

Total RNA for quantitative real-time PCR was extracted using RNA-Bee (Tel-Test, Inc.) according to the manufacturer's protocol. cDNA was produced using the RevertAid H minus first strand cDNA synthesis kit (K1632, Thermo Fisher Scientific), and qPCR was performed with SYBR Green PCR master mix (4309155, Life Technologies) using the ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). The following primers were used: MCL1 forward 5'-TCGTAAGGACAAAACGGGAC-3', MCL1 reverse 5'-CATTCCTGATGCCACCTTCT-3', HPRT forward 5'-CCTGGCGTCGTGATTAGTGA-3', HPRT reverse 5'-CGAGCAAGACGTTCAGTCCT-3', UBC forward 5'-ATTTGGGTCGCGGTTCTTG-3', UBC reverse 5'-TGCCTTGACATTCTCGATGGT-3', YWHAZ forward 5'-ACTTTTGGTACATTGTGGCTTCAA-3', and YWHAZ reverse 5'-CCGCCAGGACAAACCAGTAT-3'. Results were normalized to expression of HPRT, UBC, and YWHAZ using the ΔΔCt method.[35]

Phosphatase siRNA screen

A library of siRNAs against 188 human phosphatases (Dharmacon ON-TARGET*plus*® SMARTpool® siRNA Library - Human Phosphatase) was transfected into MM1.S cells using Lipofectamine RNAiMAX and 50 nM siRNA. 66 hours after transfection, lysates were made for detection of MCL-1 protein levels by immunoblotting.

Statistical analysis

MCL-1 half-life was determined using a one-phase exponential decay non-linear regression analysis model (GraphPad Prism version 8.0.1). Statistical analysis was performed using GraphPad Prism version 8.0.1.

RESULTS

MCL-1 is stabilized in post-GC B cell malignancies

MCL-1 protein half-life is reported to be 30-40 minutes in many cell types and tissues.[18,19] By treating cell lines with CHX for different time periods, we determined half-life of MCL-1 in a panel of MM and DLBCL cell lines (Figure 1A-C). In some cell lines, such as MM cell lines L363, NCI-H929, OPM-2, and DLBCL cell lines OCI-Ly7, OCI-Ly1, and SU-DHL-2, MCL-1 half-life is relatively short and in line with published half-lives. In contrast, MM cell lines MM1.S, RPMI-8226, and UM9, as well as DLBCL cell lines OCI-Ly10 and OCI-Ly3 are characterized by more stable MCL-1, with a half-life significantly longer than 40 minutes. In order to determine whether stabilization of MCL-1 is also observed in primary MM cells, half-life of MCL-1 was determined in CD138-purified cells from MM bone marrow aspirates (Figure 1D). In 5 tested patient samples, MCL-1 half-life ranged from 32 to 177 minutes.

MCL-1 protein level reflects transcriptional activity as well as protein half-life

In order to investigate whether increased MCL-1 stability is mirrored by an increased amount of protein, we determined MCL-1 protein expression across the MM and DLBCL cell line panel (Figure 2A). In the DLBCL cell lines, the amount of MCL-1 protein seems to correlate well with protein stability (Figure 2B). This was not the case in the MM cell lines, where high MCL-1 protein levels were observed in NCI-H929, while this cell line has a normal MCL-1 half-life. If high MCL-1 protein levels in NCI-H929 are not caused by protein stabilization,

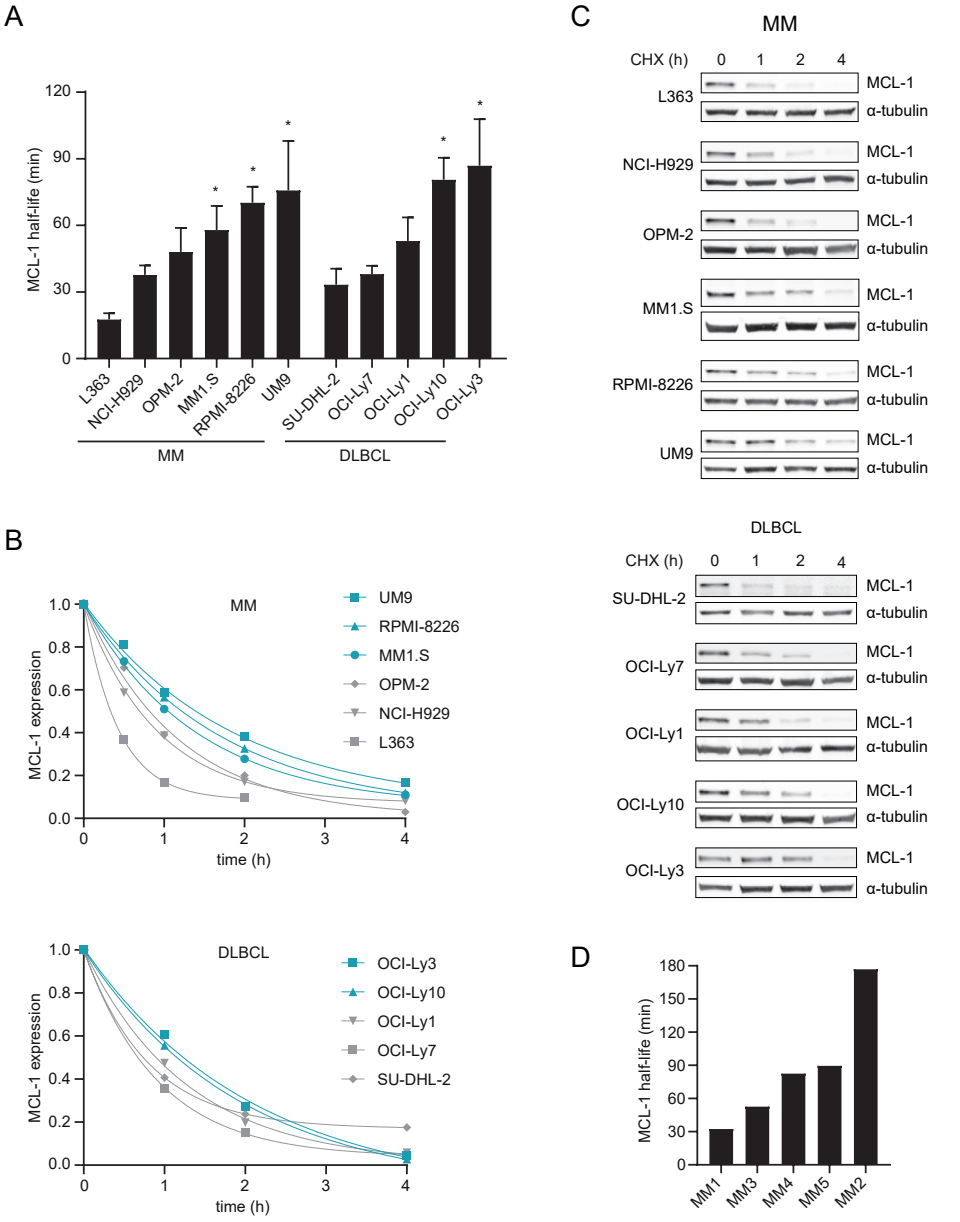
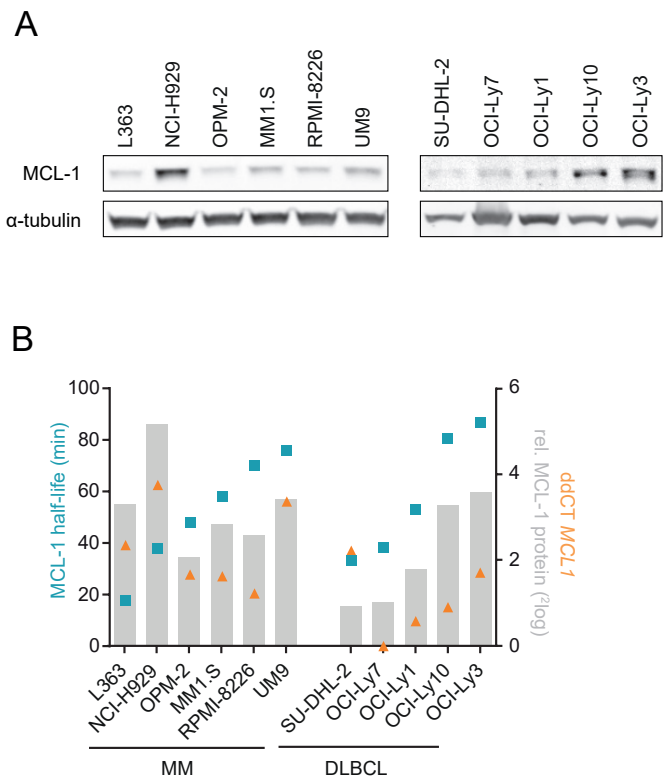


Figure 1. MCL-1 is stabilized in post-GC B cell malignancies. (A-C) MCL-1 protein half-life as determined by treatment of MM and DLBCL cell lines with cycloheximide (CHX). Presented data are averages of 3-6 independent replicates per cell line. (A) Summarized data (mean + S.E.M.) of MCL-1 half-life in 6 MM and 5 DLBCL cell lines. * indicates half-life significantly longer than 40 minutes, based on the 95% confidence interval. (B) Non-linear regression analysis (one-phase decay) of MCL-1 degradation over time after CHX treatment. (C) Representative immunoblot images showing MCL-1 levels after CHX treatment for the indicated time periods. (D) MCL-1 half-life in primary (CD138-purified) cells from 5 MM bone marrow aspirates, analyzed by non-linear regression analysis.



◀Figure 2. MCL-1 protein level reflects transcriptional activity as well as protein half-life (A) MCL-1 protein expression in the MM and DLBCL cell line panel. (B) Relative MCL-1 protein expression (over α-tubulin) in the MM and DLBCL cell line panel as determined by immunoblotting (grey bars, right y-axis), compared to MCL-1 protein half-life (blue squares, left y-axis) and *MCL1* gene expression as determined by RT-PCR (orange triangles, right y-axis). MCL-1 gene and protein expression values are averages of two experiments. MCL-1 half-lives are averages of 3-6 independent replicates per cell line.

they are likely to result from high *MCL1* transcription. To address this, the rate of *MCL1* transcription was determined by RT-PCR analysis for each cell line, and both the rate of transcription and protein half-life of MCL-1 were compared to the total MCL1 protein level (Figure 2B). This analysis reveals that high MCL-1 protein expression in MM cells is the product of transcription level combined with protein half-life. In DLBCL, MCL-1 protein level correlates with half-life. Due to the limited number of purified MM cells from some patients, MCL-1 protein and transcription analysis in primary MM cells is incomplete. However, the available data suggests that also in primary cells, high MCL-1 protein level can result from either high transcription or long protein half-life (Supplementary Figure 1).

Phosphatase siRNA screen identifies PP2A as MCL-1-stabilizing phosphatase complex

Phosphatases stabilizing MCL-1 may be interesting therapeutic targets because their inhibition promotes proteasomal degradation of MCL-1. Since the function of reported MCL-1-modifying proteins is highly cell type-specific, and no specific

phosphatases have yet been linked to MCL-1 stabilization in MM, an unbiased approach was taken to identify phosphatases that dephosphorylate MCL-1 in MM. To this end, an siRNA screen targeting 188 phosphatases and phosphatase subunits was performed in MM cell line MM1.S (Figure 3A). The screen was performed in three independent experiments, using altered MCL-1 protein expression as readout.

The phosphatase screen contained siRNAs against 188 phosphatases, 117 (62%) of which were protein phosphatases (Supplementary Figure 2A). The top 10 hits identified in the screen (Figure 3A) contained 7 protein phosphatases, of which 5 had Ser/Thr and 2 had dual phosphatase specificity (Supplementary Figure 2B). MCL-1 contains 13 reported putative phosphorylation sites in the PEST regions of the protein (Supplementary Figure 2C).[20,36] Since 12 of these sites are Ser or Thr

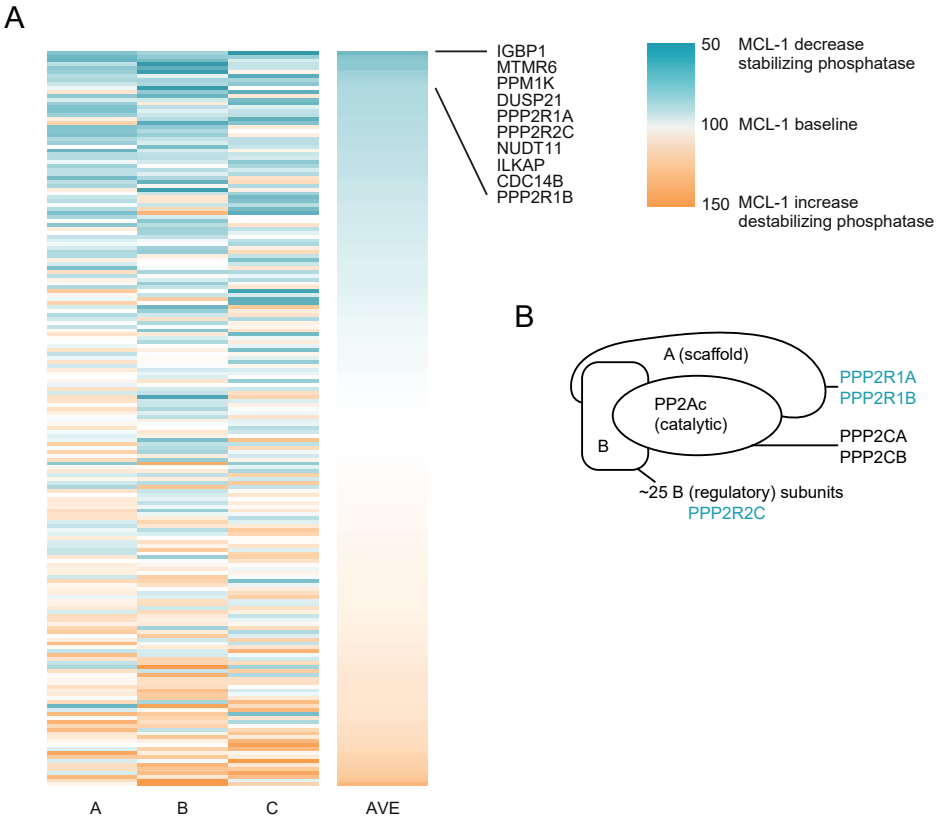


Figure 3. Phosphatase siRNA screen identifies PP2A as MCL-1 stabilizing phosphatase (A) Heatmap indicates changes in MCL-1 level after phosphatase knockdown (t=66h). The phosphatase siRNA screen was performed in 3 independent replicates (A-C), after which the 188 included siRNA pools were ranked based on average MCL-1 expression (AVE). The top 10 hits are named in the figure. (B) Schematic presentation of the 3 subunits of PP2A, with subunits that were identified in the phosphatase screen shown in blue.

residues, the phosphatase that dephosphorylates MCL-1 is most likely to be of Ser/Thr or dual specificity. Among the top 10 phosphatases whose knockdown diminished MCL-1 protein levels were three subunits of phosphatase complex PP2A (PPP2R1A, PPP2R2C, and PPP2R1B), and a PP2A-interacting protein (IGBP1[37]). Therefore, PP2A was identified as potential MCL-1-stabilizing phosphatase. PP2A is a Ser/Thr phosphatase complex consisting of three subunits: a catalytic,

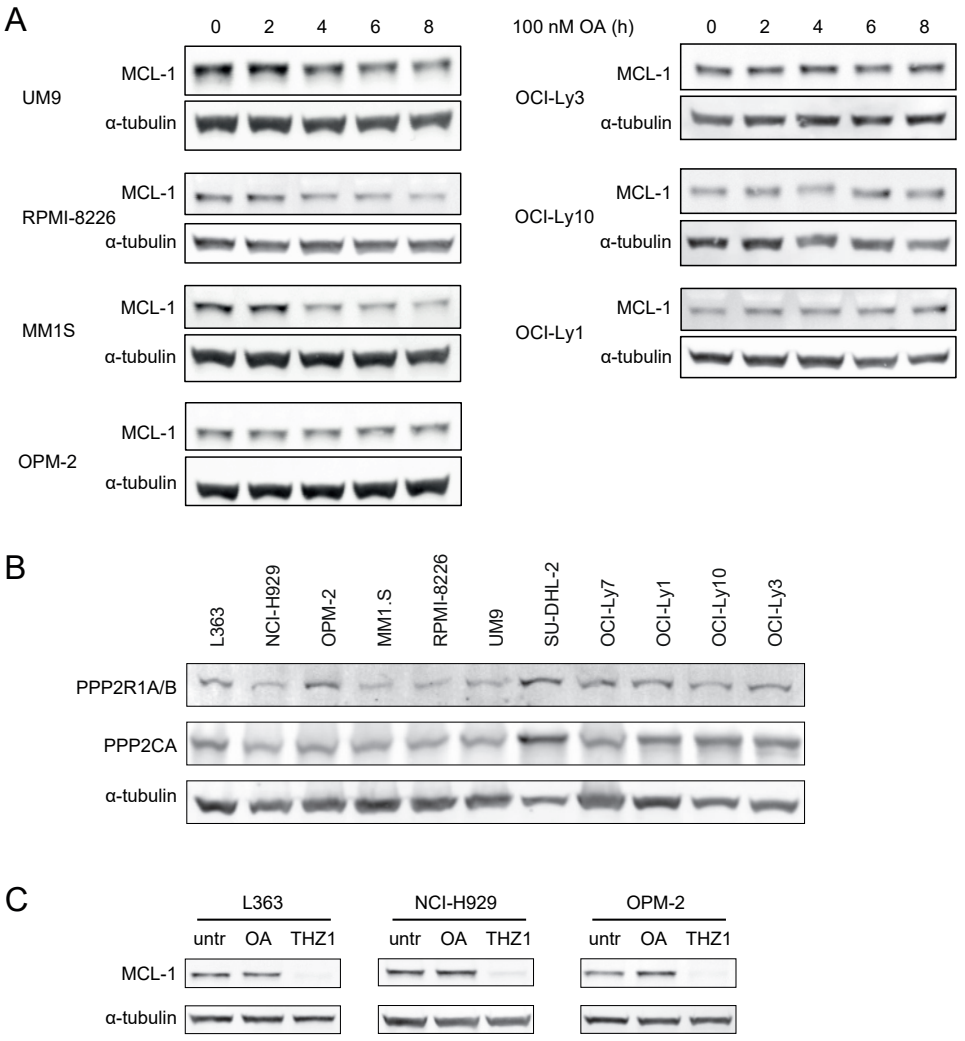


Figure 4. PP2A prevents MCL-1 degradation in MM with long MCL-1 half-life, but not in DLBCL (A) MCL-1 protein levels after treatment with 100 nM PP2A inhibitor okadaic acid (OA) for the indicated time periods in MM cell lines (left) and DLBCL cell lines (right), in the presence of 10 μM pan-caspase inhibitor Q-VD-OPh hydrate. (B) Expression of the PP2A catalytic subunit PPP2CA and scaffold subunits PPP2R1A/B in the MM and DLBCL cell line panel. (C) MCL-1 protein levels after 8h treatment with 100 nM of OA or 200 nM of CDK7 inhibitor THZ1 in MM cell lines with short MCL-1 half-life.

scaffold, and a regulatory subunit (Figure 3B). Substrate specificity and affinity of PP2A is determined by the specific regulatory subunit present in the complex.[38] In the siRNA screen, both scaffold subunits PPP2R1A and PPP2R1B were identified, as well as one regulatory subunit (PPP2R2C).

PP2A prevents MCL-1 degradation in MM with long MCL-1 half-life, but not in DLBCL

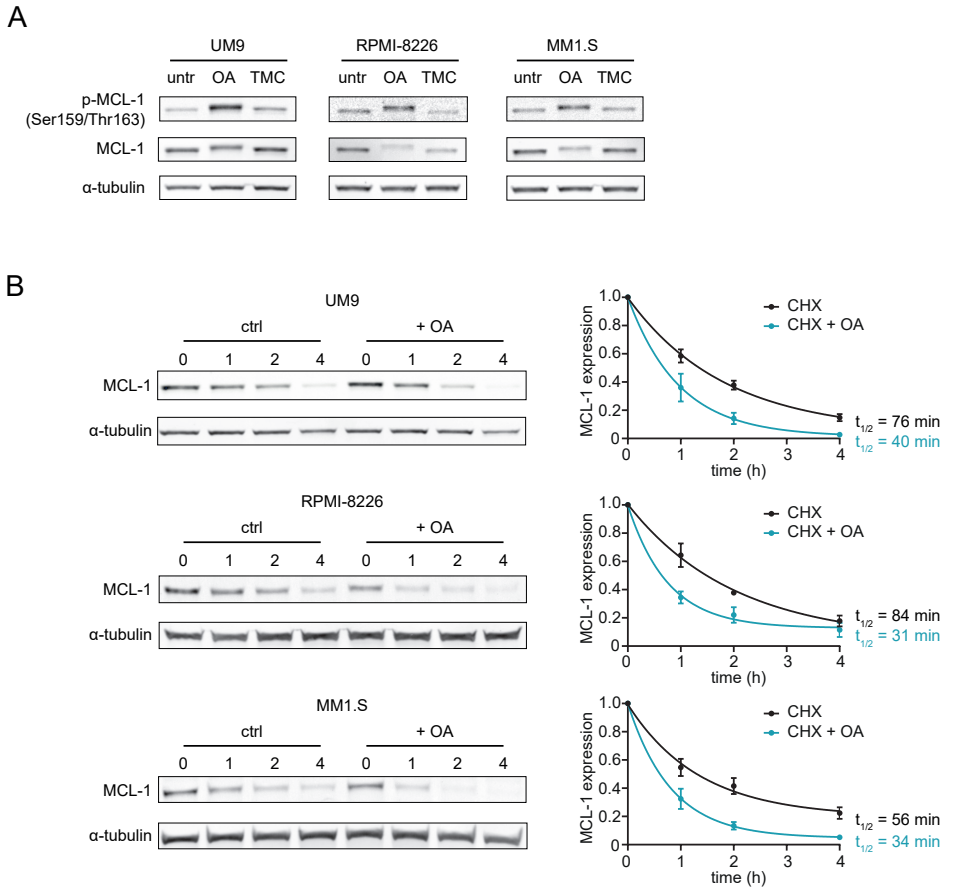
To explore PP2A as potential MCL-1-stabilizing phosphatase, this phosphatase complex was further studied using okadaic acid (OA), a small molecule PP2A inhibitor. Treatment of MM and DLBCL cell lines with OA resulted in decreased MCL-1 levels in MM cell lines where MCL-1 is stabilized, but not in cell lines where MCL-1 has a short half-life (Figure 4A). Moreover, the reduction in MCL-1 was observed in none of the tested DLBCL cell lines, regardless of MCL-1 half-life, suggesting that MCL-1 stabilization in DLBCL may result from the action of other phosphatases or another mechanism altogether.

Immunoblot analysis revealed that the PP2A catalytic (C) subunit PPP2CA, and scaffold (A) subunits PPP2R1A and PPP2R1B are present in all tested MM and DLBCL cell lines (Figure 4B). Due to the absence of MCL-1 degradation upon OA treatment in DLBCL, the effect of OA on MCL-1 was further studied only in MM.

RT-PCR analysis of OA-treated MM cell lines indicates that OA does not decrease transcriptional levels of *MCL1* (Supplementary Figure 3), suggesting that the reduction of MCL-1 in Figure 4A results from protein-level rather than transcriptional regulation. In MM cell lines with short MCL-1 half-life, which are expected to rely more on *MCL1* transcription than on protein stabilization, no reduction in MCL-1 was seen following OA treatment (Figures 4A and 4C). To further show that MCL-1 protein expression in these cell lines is determined by gene transcription we tested the impact of an inhibitor of *MCL-1* transcription. Cyclin dependent kinase (CDK) 7 inhibition by THZ1 was previously shown to inhibit transcription of *MCL1*, among other genes.[39] As expected, treatment with THZ1 strongly reduced MCL-1 protein levels in L363, NCI-H929, and OPM-2 cells (Figure 4C).

PP2A inhibition leads to phosphorylation and destabilization of MCL-1

MCL-1 degradation is thought to be preceded by phosphorylation, which may be reversed by PP2A. In concordance with this hypothesis, OA treatment increased MCL-1 phosphorylation at Ser159/Thr163 in MM cell lines with stabilized MCL-1, when the final step of MCL-1 degradation was prevented using a proteasome



inhibitor (Figure 5A). No enhanced MCL-1 phosphorylation was observed after treatment with tautomycetin (TMC), a PP1 inhibitor.

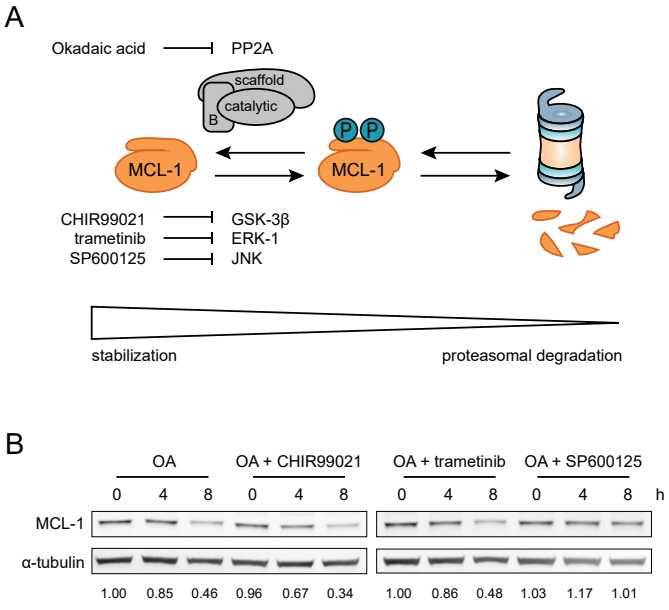
Since these results indicate that PP2A dephosphorylates MCL-1, leading to increased MCL-1 protein levels, it is expected that OA treatment shortens MCL-1 half-life. Indeed, CHX experiments reveal that OA reduces MCL-1 half-life, confirming that PP2A activity leads to increased MCL-1 stability in a subset of MM (Figure 5B).

JNK inhibition rescues MCL-1 destabilization after OA treatment

Phosphorylation of MCL-1 on Ser155, Ser159, and Thr163 was previously reported to target MCL-1 for degradation. Our results indicating that PP2A inhibition leads to increased phosphorylation at Ser159/Thr163 (Figure 5A) suggest that PP2A can

◄Figure 5. PP2A inhibition leads to phosphorylation and destabilization of MCL-1. (A) Phosphorylation of MCL-1 (residues Ser159/Thr163) after treatment of MM cell lines with long MCL-1 half-life for 2 hours with either 100 nM OA or 1 μM PP1 inhibitor tautomycetin (TMC). All samples were treated in the presence of 20 nM proteasome inhibitor bortezomib and 10 uM Q-VD-OPh hydrate in order to prevent proteasomal degradation of phosphorylated MCL-1, while maintaining cell viability. (B) MCL-1 half-life after treatment with OA. Experiments and analyses were performed as in Figure 1A-C. OA-treated samples received 2 hours pretreatment with OA, and OA remained present during CHX treatment. Graphs show averages of 3 independent replicates ±S.E.M.

►Figure 6. PP2A reverses MCL-1 phosphorylation by JNK. (A) Model of MCL-1 phosphorylation leading to proteasomal degradation, which is counteracted in a subset of MM by PP2A phosphatase activity. Potential kinases phosphorylating and destabilizing MCL-1 are GSK-3β, ERK-1, and JNK. The PP2A and kinase inhibitors used in this study are indicated. (B) MCL-1 expression in MM1.S after treatment for 0, 4, and 8 hours with 100 nM OA, either alone or combined with 10 μM CHIR99021, 250 nM trametinib, or 20 μM SP600125. Data represent one experiment. Quantified MCL-1 levels (normalized to α-tubulin level) are shown below the bands.



counteract kinase activity at these particular phosphorylation sites. Kinases that have been shown to phosphorylate MCL-1 at Ser155, Ser159, and Thr163 are GSK-3β, ERK-1, and JNK. In order to investigate whether one or more of these kinases is responsible for MCL-1 phosphorylation that is subsequently counteracted by PP2A, we combined OA treatment of MM1.S cells with specific kinase inhibition (Figure 6A). GSK-3β, ERK-1, and JNK were inhibited using CHIR99021, trametinib, or SP600125, respectively. Of these inhibitors, only JNK inhibitor SP600125 was able to rescue MCL-1 stability during OA treatment (Figure 6B). Hence, PP2A stabilizes MCL-1 by counteracting phosphorylation by JNK, but not by GSK-3β or ERK-1.

DISCUSSION

Overexpression of pro-survival BCL-2 proteins contributes to malignant transformation and cancer persistence, but it also renders cancer cells sensitive to therapeutic targeting of BCL-2 proteins using BH3-mimetics.[40] In MM, which predominantly depends on MCL-1 for survival, inhibition of MCL-1 potently induces apoptosis in preclinical models, and multiple MCL-1-specific BH3-mimetics are currently being tested in clinical trials.[3,6–9,41] The cellular regulation of MCL-1 is complex and cell type-dependent. In this study, we aimed to unravel the exact mechanism that results in MCL-1 overexpression in MM. Here, we show that MCL-1 is stabilized in a subset of MM and DLBCL cell lines, as well as in some primary MM samples (Figure 1). High MCL-1 protein levels in MM result not only from high transcription, but rather seem to be a product of both transcription and protein stability (Figure 2). Since phosphatase activity was previously shown to be increased in the GC,[33] we hypothesized that MCL-1 stabilization in post-GC B cell malignancies may also result from increased phosphatase activity. By using a phosphatase siRNA screen, we took an unbiased approach to identify the phosphatase responsible for MCL-1 stabilization in MM, and found three PP2A subunits among the top 10 hits (Figure 3). Using PP2A inhibitor OA, we further validated that PP2A dephosphorylates and destabilizes MCL-1 in MM with long MCL-1 half-life, but not in DLBCL (Figures 4-5). Combined kinase and phosphatase inhibition experiments suggest that MCL-1 half-life in MM is regulated by the counteracting functions of JNK and PP2A (Figure 6).

Protein expression of MCL-1 in MM is regulated at multiple levels. Firstly, overexpression can occur at the transcriptional level, resulting from signals from the bone marrow microenvironment or from amplification of 1q21, the locus that contains the *MCL1* gene.[2,42] 1q21 amplification occurs in ~30% of newly diagnosed and ~70% of relapsed MM patients, and is associated with increased *MCL1* expression as well as enhanced MCL-1 inhibitor sensitivity.[41,43] Secondly, alternative splicing and mRNA-regulation can influence the amount of MCL-1 protein translation.[44,45] Thirdly, MCL-1 is subject to heavy post-translational regulation, which determines its stability and function.[20]

Some phosphorylation sites of MCL-1 have been experimentally studied in cell types other than MM. In general, phosphorylation of MCL-1 at Thr163 is considered instrumental for regulation of protein stability and function, since this modification appears to be necessary for phosphorylation of other residues.[23] In conjunction with phosphorylation at Thr163, residues Thr92, Ser121, Ser155, and Ser159 can be phosphorylated. Phosphorylation of these sites has been associated with stabilization or destabilization of MCL-1.[19,22–25,46] Based on our observation that PP2A dephosphorylates MCL-1 at Ser159/Thr163 (Figure 5A), which was

also shown by others in lymphoma cells,[47] we studied the kinases that can phosphorylate MCL-1 at these residues: GSK-3, ERK-1, and JNK. Of these kinases, only JNK inhibition was able to rescue MCL-1 stability upon OA treatment in MM (Figure 6). Therefore, we conclude that JNK and PP2A together regulate MCL-1 protein stability in MM.

In contrast to the extensively studied MCL-1-modifying kinases, not much is known about the mechanisms of MCL-1 dephosphorylation. Classically, phosphatases are considered promiscuous and therefore less relevant to study in the context of specific cellular pathways than kinases. The genome encodes approximately 3-fold less phosphatases than kinases,[48,49] and phosphatases were thought to have a non-specific function in phospho-protein homeostasis.[50] More recent findings, however, have shown that phosphatases play an active role in regulating cellular levels of tyrosine, threonine, and serine phosphorylation. Serine/threonine phosphatases such as PP2A act as holoenzymes containing a catalytic subunit, sometimes a scaffold subunit, and a regulatory subunit that recruits substrates to the complex.[38,51] As a result, PP2A can have many specific targets, depending on the regulation and availability of its regulatory subunits.[51]

We identified PP2A as the phosphatase that stabilizes MCL-1 in a subset of MM. In DLBCL, MCL-1 stabilization was observed in a subset of cell lines, but this did not result from PP2A activity. Immunoblotting indicated that the catalytic and scaffold subunits of PP2A are expressed in all tested MM and DLBCL cell lines (Figure 4B). It is possible that the regulatory PP2A subunit responsible for MCL-1 targeting is lacking in DLBCL cells. The phosphatase siRNA screen identified one regulatory PP2A subunit, PPP2R2C. Unfortunately, we were unable to stain this subunit in immunoblot experiments, and we can therefore not validate whether PPP2R2C was a true hit from the screen responsible for MCL-1-specificity of PP2A. Regardless, MCL-1 stabilization in DLBCL likely results from a PP2A-independent mechanism, possibly by other phosphatases, by kinases, or at the ubiquitin level.

We observed increased MCL-1 stability in 3 out of 6 MM cell lines and at least 3 out of 5 tested primary MM samples. In two cell lines with shortest MCL-1 half-life, L363 and NCI-H929, the MCL-1 protein levels likely result from high transcription of *MCL1*. It is conceivable that different processes contribute to high MCL-1 protein levels in MM patients as well. Thus, besides 1q21-amplified MM, where *MCL1* transcription is increased, there may also be MM patients with increased MCL-1 stability, as can be expected based on our findings with primary MM cells *ex vivo*.

In addition to PP2A subunits, IGBP1 (Immunoglobulin-Binding Protein 1, alternatively named Alpha 4), was identified in the screen. IGBP1 does not have intrinsic phosphatase activity but is a regulator of PP2A activity. IGBP1 binds

catalytic PP2A subunits and protects them from proteasomal degradation, and removal of IGBP1 was shown to result in loss of PP2A expression, leading to apoptosis.[37,52,53] The observation that IGBP1 knockdown in our screen consistently led to reduced MCL-1 levels therefore supports the hypothesis that PP2A stabilizes MCL-1.

Despite PP2A often being considered a tumor suppressor that is downregulated or mutated in cancer,[54–57] PP2A activity is essential for cell survival and its inhibition is thus a promising anti-cancer strategy.[58] This paradoxical role of PP2A in cancer likely results from the plethora of different cellular functions in which PP2A is involved, depending on the exact composition of catalytic, scaffold, and regulatory domains. Although the development of specific phosphatase inhibitors lags behind kinase inhibitor development, increasing knowledge about phosphatase biology shows that it is possible to generate small-molecule phosphatase inhibitors.[59] In a phase 1 clinical trial, PP2A inhibitor LB100 was deemed safe to continue further development.[60] LB100 has strong chemo- and radiosensitizing potential in cancer,[58,61] indicating that PP2A inhibition is a realistic therapeutic strategy. LB100 was shown to overcome cellular senescence and promote mitotic catastrophe and apoptosis.[58] Because of the chemosensitizing potential of LB100, it would be highly relevant to address the interplay between PP2A inhibitors and established MM drugs. As alternative to systemic inhibition of all PP2A, generation of a PP2A inhibitor that only inhibits the specific MCL-1-targeting regulatory subunit would be a promising future option.

In conclusion, we show that MCL-1 is stabilized in a subset of MM and DLBCL, and that PP2A is the phosphatase responsible for MCL-1 dephosphorylation and stabilization in MM. This finding increases the understanding of post-translational regulation of MCL-1, which may provide novel strategies to inhibit MCL-1 in MM cells.

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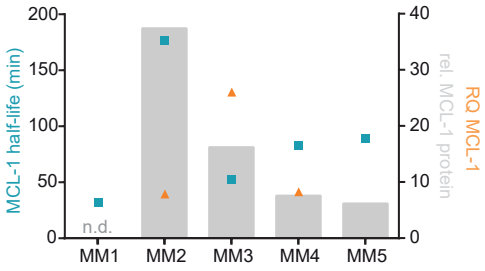
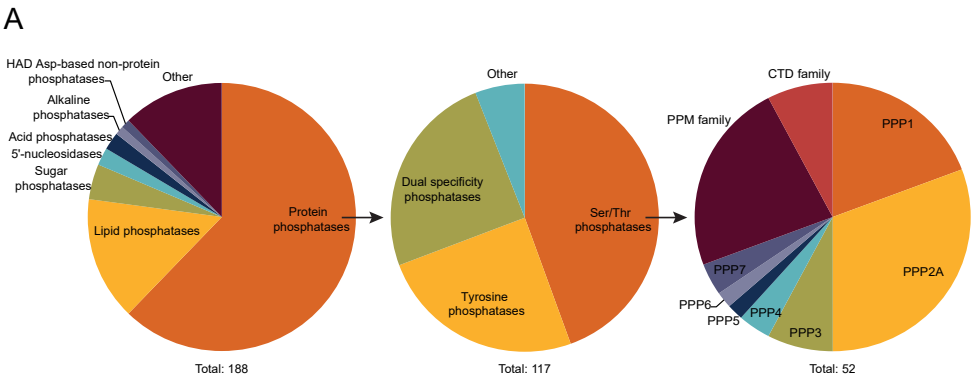
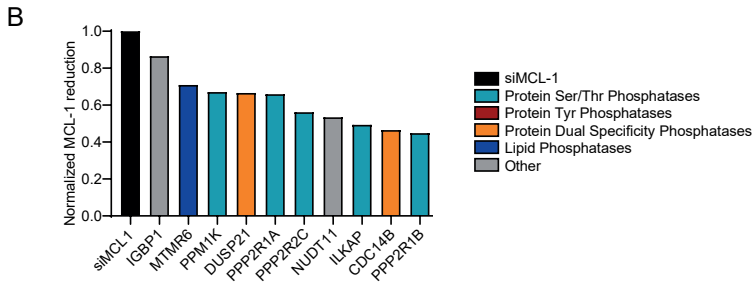
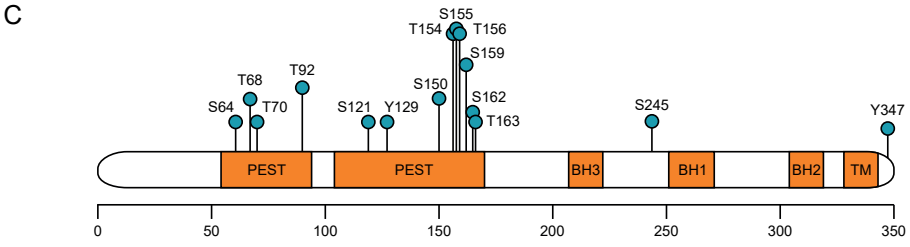
SUPPLEMENTARY FIGURES



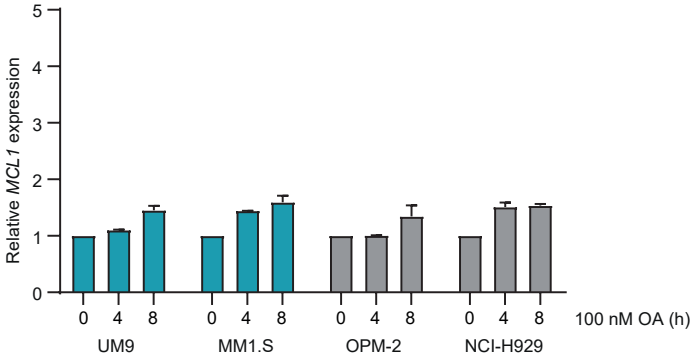
◄Supplementary Figure 1. Transcription and protein stability contribute to high MCL-1 protein level in MM patient cells. Relative MCL-1 protein expression (over β-actin) in CD138-purified cells from MM patients as determined by immunoblotting (grey bars, right y-axis), compared to MCL-1 protein half-life (blue squares, left y-axis) and *MCL1* gene expression as determined by RT-PCR (orange triangles, right y-axis). "n.d." or absence of an orange triangle indicates that MCL-1 protein or transcription levels could not be determined.







◄Supplementary Figure 2. A phosphatase siRNA screen was used to determine which phosphatases stabilize MCL-1. (A) Pie-charts indicating the composition of the phosphatase siRNA screen that was used to identify MCL-1-stabilizing phosphatases. The majority (117/188) of siRNA pools included in the screen targeted protein phosphatases (left), of which Ser/Thr, Tyr, and dual specificity phosphatases were all represented (middle). Among the 52 targeted Ser/Thr phosphatases and phosphatase subunits, half were PP1 and PP2A subunits (right), indicated as "PPP1" and "PPP2A" in the graph. (B) Types of phosphatases identified as top 10 MCL-1-stabilizing phosphatases from the phosphatase siRNA screen, and the reduction in MCL-1 level that results from knockdown of these phosphatases, relative to an siRNA pool that targets MCL-1 (siMCL1). (C) Schematic representation of MCL-1, its domains, and its predicted phosphorylation sites, some of which have been experimentally confirmed. The scale below indicates the size of MCL-1 in amino acids.



▲Supplementary Figure 3. Okadaic acid treatment does not reduce *MCL1* transcription in MM cell lines with short or long MCL-1 half-life. Relative *MCL1* gene expression levels as determined by RT-PCR, after treatment for 4 or 8 hours with 100 nM OA, in MM cell lines with long (blue bars) or short (grey bars) MCL-1 protein half-life. Bars represent the average of 3 independent experiments (+ S.E.M.).

CHAPTER 7

SerpinB9 overexpression in malignant cells confers resistance to granzyme B-mediated killing by gene engineered T cells

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Manuscript in preparation

ABSTRACT

Adoptive cellular therapies, such as chimeric antigen receptor (CAR) T cell therapies, are an effective form of immune therapy. These gene engineered T cells use granzyme B (GrB) to kill tumor cells. Although this approach is effective in multiple types of cancer and tumor cell killing is observed, relapse after initial response remains a problem. It was recently reported that expression of GrB-inhibitor SerpinB9 in cancer cells promotes T cell dysfunction. The goal of our study is to investigate the role of SerpinB9 expression on the killing capacity of gene engineered T cells. To that end, we analyzed expression of SerpinB9 in a panel of human cancer cell lines by western blot and reveal expression in a range of cancer types, including B cell lymphoma and melanoma. Next, cell lines were generated where SerpinB9 was either inhibited or overexpressed. Using these modified cell lines, we show that SerpinB9-overexpressing melanoma cells are less sensitive to exogenously delivered GrB or to GrB-mediated killing by NK cells, as compared to melanoma cells lacking SerpinB9. Conversely, knockdown of SerpinB9 in diffuse large B cell lymphoma (DLBCL) renders these cells more sensitive towards killing by anti-CD20 CAR T cells, as compared to DLBCL with high expression of SerpinB9. Combined, these data expose SerpinB9 as a potential mediator of resistance towards gene engineered T cells and provide a rationale to improve the killing capacity of T cells by circumventing SerpinB9-mediated inhibition of GrB.

INTRODUCTION

Adoptive T cell transfer is a form of personalized cancer therapy where a patient receives autologous lymphocytes that have antitumor activity.[1] After isolating immune cells from a patient, *ex vivo* activation, selection, and expansion techniques allow for production of large numbers of tumor-reactive T cells. In addition, gene engineering strategies can be used to redirect T cells towards a specific antigen. Examples of gene engineered T cells that are currently being developed for clinical use as anticancer therapy are T cell receptor (TCR)-modified T cells and chimeric antigen receptor (CAR) T cells.[1,2]

TCR engineering strategies allow for gene transfer of known tumor-specific antigen-reactive TCRs into peripheral blood T cells of patients. The advantage of such TCR T cells is that the same receptor sequence can be used for many patients, and there is no need for isolation of T cells from tumor tissue.[3] Clinical trials using TCR T cell therapy directed against the NY-ESO-1 cancer/testis antigen reported objective responses in patients with synovial cell carcinoma, melanoma, and multiple myeloma.[4–6] TCR T cells are generally HLA-restricted, because they recognize intracellular peptides presented in MHC molecules. CAR T cells, on the other hand, recognize extracellular surface targets in an MHC-independent manner. A CAR consists of an antigen-binding single-chain variable fragment (scFv) derived from the variable domains of an antibody, combined with signaling domains of the TCRζ chain and costimulatory domains from receptors such as CD28 or CD137.[2] CAR T cells directed against CD19 have shown remarkable effectivity in lymphomas and leukemias, where response rates of 60-100% have been reported in multiple clinical trials.[7–12] As a result, two forms of CD19 CAR T cell therapy have been approved by the U.S. Food and Drug Administration (FDA) for treatment of refractory B cell precursor acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma (DLBCL).[13] Additionally, promising response rates have been reported for B Cell Maturation Antigen (BCMA)-specific CAR T cells in patients with heavily pretreated multiple myeloma.[14–16]

Despite the success of CAR T cell treatment in tumors of the B cell lineage, major challenges have been encountered that limit the effectivity of TCR engineered T cells, in particular in solid cancers. The lack of highly tumor-specific markers, as well as limited recruitment and persistence at the tumor site, limit the effectivity of TCR engineered T cells in solid cancers. Other immune evasion strategies, such as epitope loss or defects in presentation of antigenic epitopes, have led to relapse after T cell therapy and resistance towards gene engineered T cells.[2,17]

Resistance of tumor cells to undergo apoptosis is another immune escape mechanism, and can be mediated by downregulation of death receptors such as Fas (CD95).[18] The main killing mechanism of T cells and NK cells, however,

is by releasing cytotoxic granules containing perforin and granzymes into the target cell.[19] There, granzymes cleave intracellular targets including Bid, leading to intrinsic apoptosis pathway activation and caspase-mediated apoptosis.[20] Cytotoxic cells are protected from endogenously produced granzyme-mediated cell death by expression of Serine protease inhibitors (Serpins), such as SerpinB9 (also named Serpin PI9), which binds and neutralizes granzyme B (GrB).[21–23] Interestingly, SerpinB9 expression has also been reported in cancer cells, suggesting a mechanism of immune evasion. For example, in metastatic melanoma patients, clinical outcome was associated with elevated SerpinB9 expression in tumor cells.[24] Recently, Jiang et al. developed a computational model to predict factors that are responsible for immune evasion of tumors, and found SerpinB9 expression to be a critical factor for immunotherapy resistance.[25]

Since therapeutic strategies using gene engineered T cells are widely emerging for hematological as well as solid tumors, it is important to assess the role of SerpinB9 in resistance against these novel therapies. In this study, we investigate whether expression of SerpinB9 by cancer cells leads to resistance towards CAR T cells. We report that SerpinB9 expression is present in most lymphoma cell lines, and in a selection of solid cancer cell lines. Knockdown of SerpinB9 in lymphoma results in increased sensitivity to anti-CD20 CAR T cell-mediated killing, whereas overexpression of SerpinB9 in melanoma results in resistance to GrB-mediated killing by NK cells. We conclude that SerpinB9 expression by cancer cells is a resistance mechanism against novel immunotherapeutic strategies, including CAR T cells.

METHODS

Cell culture and chemicals

Cell lines were cultured in Dulbecco´s Modified Eagle Medium (DMEM, Life Technologies), Iscove´s Modified Dulbecco´s Medium (IMDM, life Technologies), or RPMI 1640 GlutaMAX HEPES culture medium (Life Technologies), supplemented with 10-20% fetal bovine serum (FBS, Sigma) and 100 µg/ml penicillin-streptomycin (p/s, Gibco/Life Technologies). For a complete list of the cell lines used in this study, see Supplementary Table 1. Human healthy donor peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin, Amsterdam, the Netherlands) using Ficoll-Paque according to the manufacturer´s protocol. PBMCs were cultured in RPMI with 2.5% pooled AB+ human serum (IPLA-CSER, Innovative Research), 50µM β-mercaptoethanol (Life Technologies) and 100 µg/ml p/s.

Immunoblotting

For western blot analysis, cells were lysed in buffer containing 1% Nonidet P-40 and proteins were separated using SDS-PAGE (Mini-PROTEAN® TGX™ Precast Gels, Bio-Rad), transferred to low florescence PVDF membranes (Bio-Rad), blocked in PBS containing 2% non-fat dry milk, and stained using the following antibodies: mouse anti-SerpinB9 (Invitrogen), mouse anti-α-tubulin (Cell Signaling), and goat anti-mouse-680RD (LI-COR Biosciences). Infrared imaging was used for detection (Odyssey Sa, LI-COR Biosciences). Analysis and quantification were performed using LI-COR Image Studio software.

Generation of anti-CD20 CAR T cells

The anti-CD20 CAR construct (pBu-CD20-CAR) was generated by cloning single chain variable fragments from anti-CD20 antibody Rituximab into a pBullet vector containing a D8α-41BB-CD3-ζ signaling cassette. Phoenix-Ampho packaging cells were transfected with gag-pol (pHit60), env (P-COLT-GALV) and pBu-CD20-CAR, using FugeneHD transfection reagent (Promega). Human PBMCs were pre-activated with 30 ng/ml anti-CD3 (OKT3, Miltenyi) and 50 IU/ml IL-2 (Sigma) and subsequently transduced two times with viral supernatant in the presence of 6 ug/ml polybrene (Sigma) and 50 U/ml IL-2. Transduced T cells were expanded using 50 U/ml IL-2 and anti CD3/CD28 dynabeads (Thermo Fisher), and anti-CD20 CAR-expressing cells were selected by treatment with 80 µg/ml neomycin. T cells were further expanded using a rapid expansion protocol as described elsewhere.[26]

Overexpression and knockdown of SerpinB9

Generation of the retroviral vector pMSCV-SerpinB9 is described elsewhere.[27] Virus production was performed as described for the anti-CD20 CAR construct. Subsequently, Mewo cells were transduced two times with viral supernatant in the presence of 6 ug/ml polybrene, and stably overexpressing cells were selected using 1 µg/ml puromycin (Sigma). In order to knock down SerpinB9 expression, the ON-TARGETplus Human SERPINB9 siRNA SMARTpool (L-015400-00-0005, Dharmacon) was electroporated into OCI-Ly7 cells using a Neon transfection system 10 µl kit (Thermo Fischer Scientific), at 1150 V, with 2 x 30 ms pulses.

Co-culture experiments

The effect of SerpinB9 overexpression in Mewo cells on killing by cytotoxic cells was determined by co-culturing WT and SerpinB9-overexpressing Mewo with the YT-Indy NK cell line. Similarly, the effect of SerpinB9 knockdown in OCI-Ly7 cells was investigated by co-culture with YT-Indy cells or anti-CD20 CAR T cells. Effector and target cells were combined in ratios 1:1, 3:1, and 6:1, and co-culture took place for 4 hours (lymphoma cell lines) or 24 hours (Mewo).

Apoptosis staining and flow cytometry

Assessment of cell viability took place by staining with 15 nM DiOC6 (Thermo Scientific) and 20 nM TO-PRO-3 (Thermo Scientific), followed by flow cytometric analysis (BD FACSCanto II or BD LSRFortessa, BD Biosciences). Specific apoptosis was calculated by determining the altered percentage of $DiOC6^{+}/TO\text{-}PRO\text{-}3^{-}$ (live) cells compared to untreated cells, using the formula (% cell death in treated cells - % cell death in control) / % viable cells control * 100. In co-culture experiments, target cells were identified by flow cytometric surface staining with anti-CD19-BV421 (Sony Biotechnology) (lymphoma cell lines), or by staining with Cell Trace Violet (Invitrogen) (Mewo) prior to adding effector cells. Anti-CD20 CAR T cells were characterized by staining with anti-CD3-BV510, anti-CD4-Pacific Blue, anti-CD8-PerCP-Cy5.5 (Biolegend), and biotinylated protein L (Genscript) with streptavidin-PE (Thermo Fisher). Expression of Fas and Fas ligand was assessed by surface staining with anti-CD95-PE (BD Biosciences) and anti-CD178-APC (Miltenyi), respectively. For intracellular staining of SerpinB9, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences), and stained with mouse anti-serpinB9 (Invitrogen), mouse anti-IgG Isotype (Southern Biotech), and goat anti-mouse IgG-FITC (Santa Cruz). Flow cytometry data analysis took place using FlowJo software.

Introduction of exogenous GrB into target cells

In order to determine the effect of SerpinB9 overexpression or knockdown on GrB-mediated killing, the pore forming Streptolysin O (Sigma) was used to facilitate entry of exogenous GrB (Enzo) into target cells. SLO was activated with 10 mM DTT for 20 minutes at RT, and subsequently diluted in serum-free DMEM to a final concentration of 4 U/ml. Target cells were incubated with SLO and 200 nM GrB for 30 minutes at 37 °C, after which FBS-containing medium was added to inactivate SLO. After 24 hours, apoptosis staining was performed to measure target cell viability using flow cytometry.

Datasets and statistical analysis

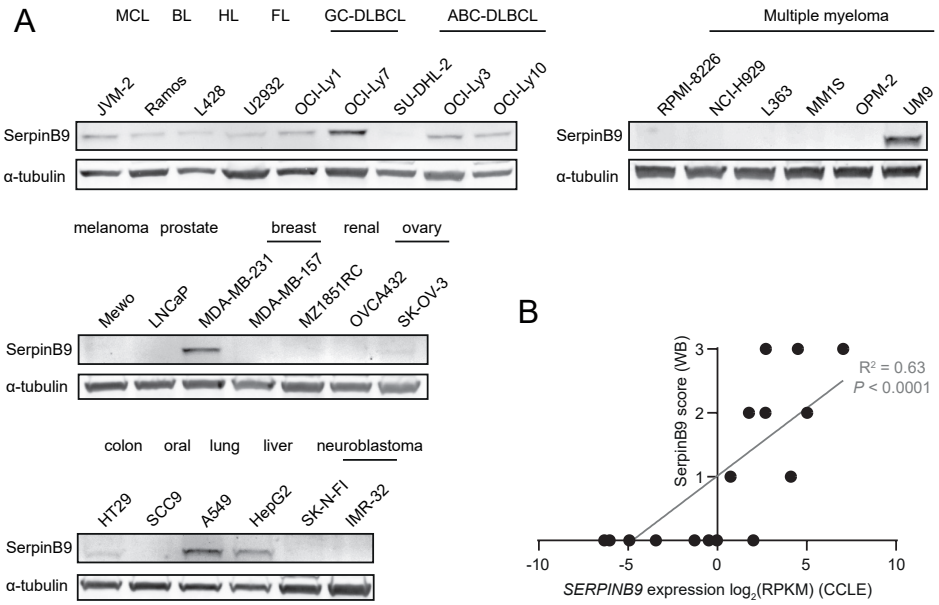
SERPINB9 gene expression levels of different cancer types were obtained from the Cancer Cell Line Encyclopedia (CCLE)[28] and The Cancer Genome Atlas (TGCA: https://www.cancer.gov/tcga) for cell lines and primary cancer, respectively.

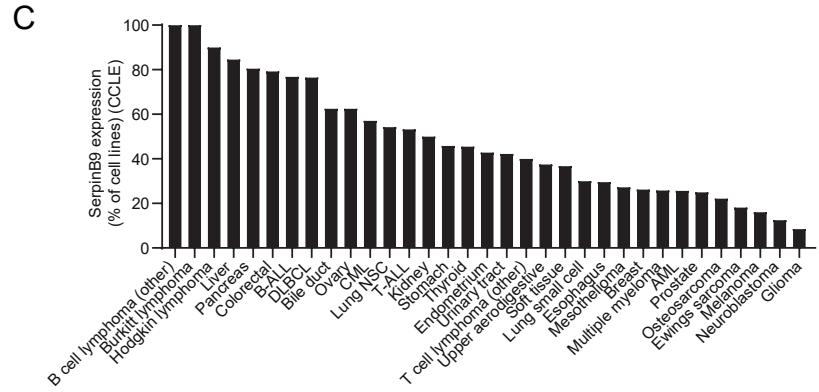
Statistical analysis was performed using GraphPad Prism version 8.3. Unpaired groups were compared with a Student's *t*-test. For comparison of more than two groups, a two-way ANOVA was used.

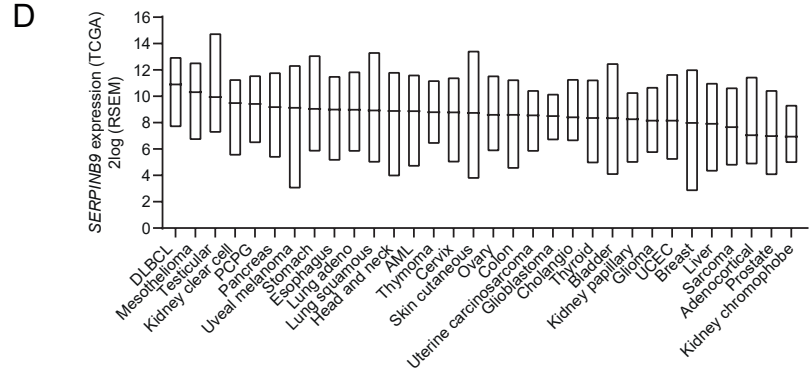
RESULTS

SerpinB9 is expressed across lymphoma and in some multiple myeloma and solid cancer cell lines

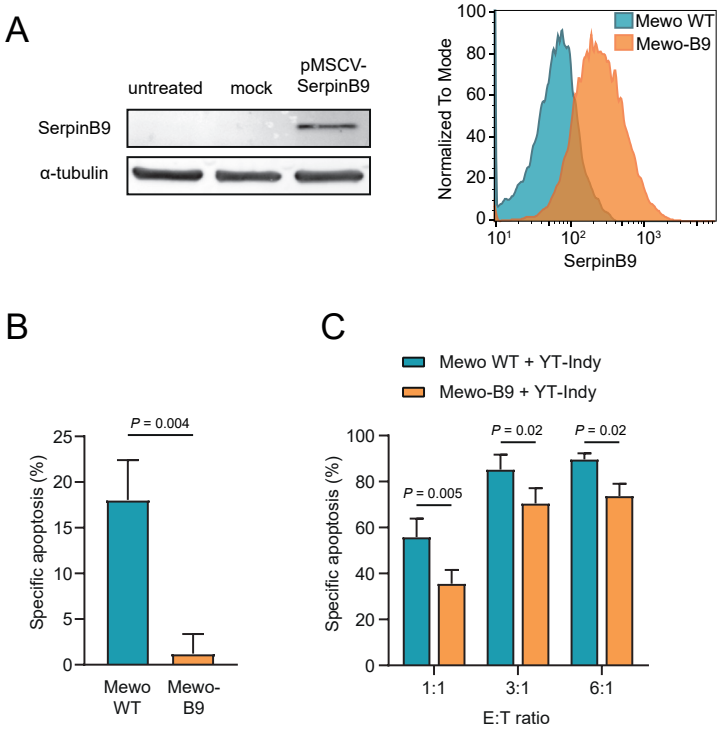
SerpinB9 is normally expressed in CTL, NK cells, antigen-presenting cells, endo- and mesothelial tissues, and immune privileged tissues.[23,29] In order to determine the frequency of SerpinB9 expression in malignant cells of different tumor types, we measured SerpinB9 expression in panels of lymphoma, multiple myeloma and solid cancer cell lines (Figure 1A). In lymphoma, SerpinB9 was ubiquitously expressed, with SerpinB9 levels varying from almost undetectable (SU-DHL-2) to very high (OCI-Ly7). In contrast, in multiple myeloma, only one (UM9) out of six tested cell lines expressed SerpinB9. In a panel of solid cancer cell lines, clear SerpinB9 expression was observed in breast cancer cell line MDA-MB-231, lung cancer cell line A549, and liver carcinoma cell line HepG2, and lower levels were found in SK-OV-3 (ovary carcinoma) and HT29 (colon carcinoma). Together, these data indicate that SerpinB9 is expressed across lymphomas, and in some multiple myeloma and solid cancers.


A
MCL
BL
HL
FL
GC-DLBCL
ABC-DLBCL
JVM-2
Ramos
L428
U2932
OCI-Ly1
OCI-Ly7
SU-DHL-2
OCI-Ly3
OCI-Ly10
SerpinB9
α-tubulin
Multiple myeloma
RPMI-8226
NCI-H929
L363
MM1S
OPM-2
UM9
melanoma
prostate
breast
renal
ovary
Mewo
LNCaP
MDA-MB-231
MDA-MB-157
MZ1851RC
OVCA432
SK-OV-3
colon
oral
lung
liver
neuroblastoma
HT29
SCC9
A549
HepG2
SK-N-FI
IMR-32
B
SerpinB9 score (WB)
R² = 0.63
P < 0.0001
SERPINB9 expression log2(RPKM) (CCLE)


C
SerpinB9 expression (% of cell lines) (CCLE)
B cell lymphoma (other)
Burkitt lymphoma
Hodgkin lymphoma
Liver
Pancreas
Colorectal
B-ALL
DLBCL
Bile duct
Ovary
CML
Lung NSC
T-ALL
Kidney
Stomach
Thyroid
Endometrium
Urinary tract
T cell lymphoma (other)
Upper aerodigestive
Soft tissue
Lung small cell
Esophagus
Mesothelioma
Breast
Multiple myeloma
AML
Prostate
Osteosarcoma
Ewings sarcoma
Melanoma
Neuroblastoma
Glioma


D
SERPINB9 expression (TCGA) 2log (RSEM)
DLBCL
Mesothelioma
Testicular
Kidney clear cell
PCPG
Pancreas
Uveal melanoma
Stomach
Esophagus
Lung adeno
Lung squamous
Head and neck
AML
Thymoma
Cervix
Skin cutaneous
Ovary
Colon
Uterine carcinosarcoma
Glioblastoma
Cholangio
Thyroid
Bladder
Kidney papillary
Glioma
UCEC
Breast
Liver
Sarcoma
Adenocortical
Prostate
Kidney chromophobe

◄Figure 1. SerpinB9 is expressed across lymphoma and in some multiple myeloma and solid cancer cell lines. (A) SerpinB9 protein expression in panels of lymphoma, multiple myeloma, and solid cancer cell lines. (B) SerpinB9 expression from (A), normalized to α-tubulin level, was quantified per cell line on a scale of 0 (no expression) to 3 (high expression), and compared to *SERPINB9* gene expression reported in the Cancer Cell Line Encyclopedia database. The coefficient of determination (R^2) and *P*-value of the linear regression analysis of these two variables are shown in the top right. (C) Fraction of cell lines in the CCLE database with SerpinB9 expression, per cancer type. Based on the analysis in (B), the $\log_2$ RPKM value of 0 was chosen as cut-off value indicating whether a cell line expresses SerpinB9. (D) Levels of *SERPINB9* expression in human cancers of The Cancer Genome Atlas, per cancer type. ABC-DLBCL, diffuse large B cell lymphoma, activated B cell subtype; AML, acute myeloid leukemia; B-ALL, B cell acute lymphocytic leukemia; BL, Burkitt lymphoma; CCLE, Cancer Cell Line Encyclopedia; CML, chronic myeloid leukemia; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; GC-DLBCL, diffuse large B cell lymphoma, germinal center subtype; HL, Hodgkin lymphoma; MCL, mantle cell lymphoma; NSC, non-small cell (lung cancer); PCPG, pheochromocytoma and paraganglioma; T-ALL, T cell acute lymphocytic leukemia; TCGA, The Cancer Genome Atlas; UCEC, uterine corpus endometrial carcinoma; WB, western blot



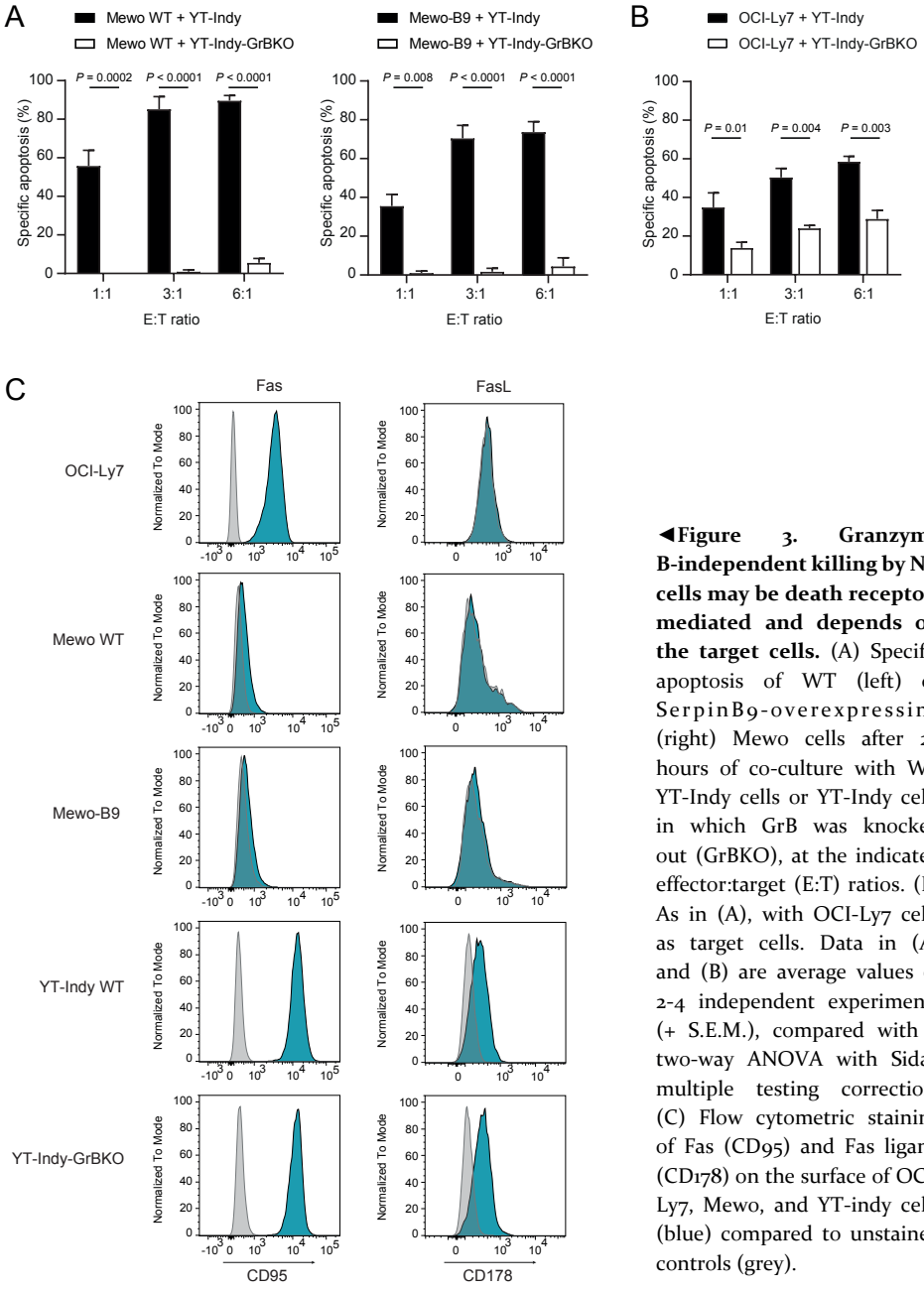
▲Figure 2. Overexpression of SerpinB9 impairs sensitivity to granzyme B-induced cell death. (A) SerpinB9 expression in untreated (WT), mock-transduced, and SerpinB9 overexpressing (pMSCV-SerpinB9) Mewo cells, as determined by immunoblotting and flow cytometry. (B) Specific apoptosis induced by streptolysin O (SLO)-mediated introduction of 200 nM recombinant GrB into WT and SerpinB9-overexpressing Mewo cells, normalized to the conditions with SLO only. (C) Specific apoptosis in WT and SeprinB9-overexpressing Mewo cells after 24 hours of co-culture with NK cell line YT-Indy at the indicated effector:target (E:T) ratios. Data in (B) and (C) are average values of 3-4 independent experiments (+ S.E.M.), compared with an unpaired student's t-test (B) or two-way ANOVA with Sidak multiple testing correction (C).

Next, SerpinB9 protein expression from Figure 1A was quantified and correlated to *SERPINB9* gene expression in the same cell lines, as reported in the Cancer Cell Line Encyclopedia (CCLE). As a result, a strong correlation was observed between SerpinB9 mRNA and protein levels (Figure 1B). In addition, by combining immunoblot and gene expression data, a cutoff (log_2 RPKM 0) could be set to distinguish cell lines that express *SERPINB9* from cell lines lacking gene and protein expression. Based on this cutoff, the fraction of SerpinB9-expressing cell lines within each cancer type included in the CCLE dataset was determined (Figure 1C). This analysis predicts expression of SerpinB9 in 75-100% of B cell lymphoma and leukemia (Burkitt lymphoma, Hodgkin lymphoma, B-ALL, DLBCL, "B cell lymphoma other") cell lines. Additionally, >75% SerpinB9 expression was observed in liver, pancreas, and colorectal cancer. In other cancer types, the fraction of SerpinB9 expression was lower, with large variation of expression within cancer types (Figure 1C and Supplementary Figure 1). In primary human cancers (TCGA), *SERPINB9* expression was highly variable as well, with highest expression in DLBCL (Figure 1D). Together, these data show high expression of SerpinB9 across lymphomas, and variable expression in other cancer types.

Overexpression of SerpinB9 impairs sensitivity to granzyme B-induced cell death

In order to examine the role of SerpinB9 in GrB-mediated killing, SerpinB9 was stably overexpressed in Mewo, a melanoma cell line lacking endogenous SerpinB9 expression (Figure 2A). Sensitivity of the WT and SerpinB9-overexpressing Mewo cell lines to GrB was first assessed by introducing recombinant GrB using streptolysin O (SLO), a bacterial pore-forming exotoxin. In this experiment, SerpinB9 overexpression rendered the cell line completely resistant to GrB-mediated killing (Figure 2B). Next, the cell lines were co-cultured with NK cell line YT-Indy, and significantly reduced killing was observed in SerpinB9-overexpressing cells (Figure 2C).

Co-culture of Mewo with a GrB-knockout YT-Indy cell line (YT-Indy-GrBKO) completely abrogated cell death (Figure 3A), indicating that the observed target cell killing was fully dependent on GrB. In contrast to Mewo, DLBCL cell line OCI-Ly7 has high endogenous expression of SerpinB9 (Figure 1A). Specific apoptosis observed upon co-culture of OCI-Ly7 was not fully abrogated when using YT-Indy-GrBKO (Figure 3B), suggesting that other cell death pathways are still effectively engaged in OCI-Ly7 in the absence of GrB. Besides GrB, NK cells kill by expressing death receptor ligands, such as Fas ligand, which may induce extrinsic apoptosis in target cells. Indeed, surface staining of Mewo and OCI-Ly7 cells for expression of death receptor Fas (CD95) shows abundant Fas expression on OCI-Ly7 and lack



◀Figure 3. Granzyme B-independent killing by NK cells may be death receptor-mediated and depends on the target cells. (A) Specific apoptosis of WT (left) or SerpinB9-overexpressing (right) Mewo cells after 24 hours of co-culture with WT YT-Indy cells or YT-Indy cells in which GrB was knocked out (GrBKO), at the indicated effector:target (E:T) ratios. (B) As in (A), with OCI-Ly7 cells as target cells. Data in (A) and (B) are average values of 2-4 independent experiments (+ S.E.M.), compared with a two-way ANOVA with Sidak multiple testing correction. (C) Flow cytometric staining of Fas (CD95) and Fas ligand (CD178) on the surface of OCI-Ly7, Mewo, and YT-indy cells (blue) compared to unstained controls (grey).

of Fas expression on Mewo cells (Figure 3C). Additionally, expression of Fas ligand (CD178) on the surface of the YT-Indy cell lines is confirmed. This may explain why YT-Indy-GrBKO does not kill Mewo, but is still capable of inducing cell death in OCI-Ly7.

Knockdown of SerpinB9 expression in lymphoma cells increases killing by CAR T cells

To study the role of SerpinB9 in CAR T cell therapy resistance, we performed siRNA-mediated knockdown of SerpinB9 in OCI-Ly7 (Figure 4A). In addition, we stably expressed an anti-CD20 CAR in T cells from healthy donors. The used CAR contains the scFv sequence from anti-CD20 antibody rituximab, as well as a 4-1BB co-stimulatory domain. Characterization of the transduced and expanded T cells revealed that most T cells (70-90%) were CD8-positive, and that all T cells expressed the CAR, as shown by positive protein L staining (Figure 4B). When OCI-Ly7 cells expressing normal or reduced levels of SerpinB9 were cultured in the presence of anti-CD20 CAR T cells from three healthy donors, the OCI-Ly7 cells underwent apoptosis in a concentration-dependent manner (Figure 4C). Notably, knockdown of SerpinB9 significantly increased anti-CD20 CAR T cell-mediated killing of OCI-Ly7 cells (Figure 4C). These results indicate that SerpinB9 expression, which is abundant in lymphomas, impairs killing by CAR T cells.

SerpinB9 expression does not impair the intrinsic apoptosis pathway

We have shown that SerpinB9 expression, which is widespread across cancers, confers resistance of cancer cells against GrB-mediated killing by gene-engineered T cells as well as NK cells. Besides by GrB, apoptosis can be induced in tumor cells through activation of the extrinsic and intrinsic apoptosis pathways. Although Bid cleavage by GrB can indirectly lead to intrinsic apoptosis, activation of both extrinsic and intrinsic apoptosis pathways does not depend on GrB functionality (Supplementary Figure 2). Indeed, when OCI-Ly7 cells were treated with MCL-1 inhibitor S63845, apoptosis was induced regardless of SerpinB9 expression (Figure 5A). Treatment of Mewo cells with MCL-1, BCL-2 (ABT-199), and BCL-XL (A-1155463) inhibitors reveals high sensitivity to combined MCL-1 and BCL-2 or MCL-1 and BCL-XL inhibitor treatment (Figure 5B-C). No difference between WT and SerpinB9-overexpressing Mewo with regard to BCL-2 family inhibitor sensitivity was observed. These results suggest that GrB-resistance resulting from SerpinB9 expression may be circumvented by activating the intrinsic or extrinsic apoptosis pathways directly.

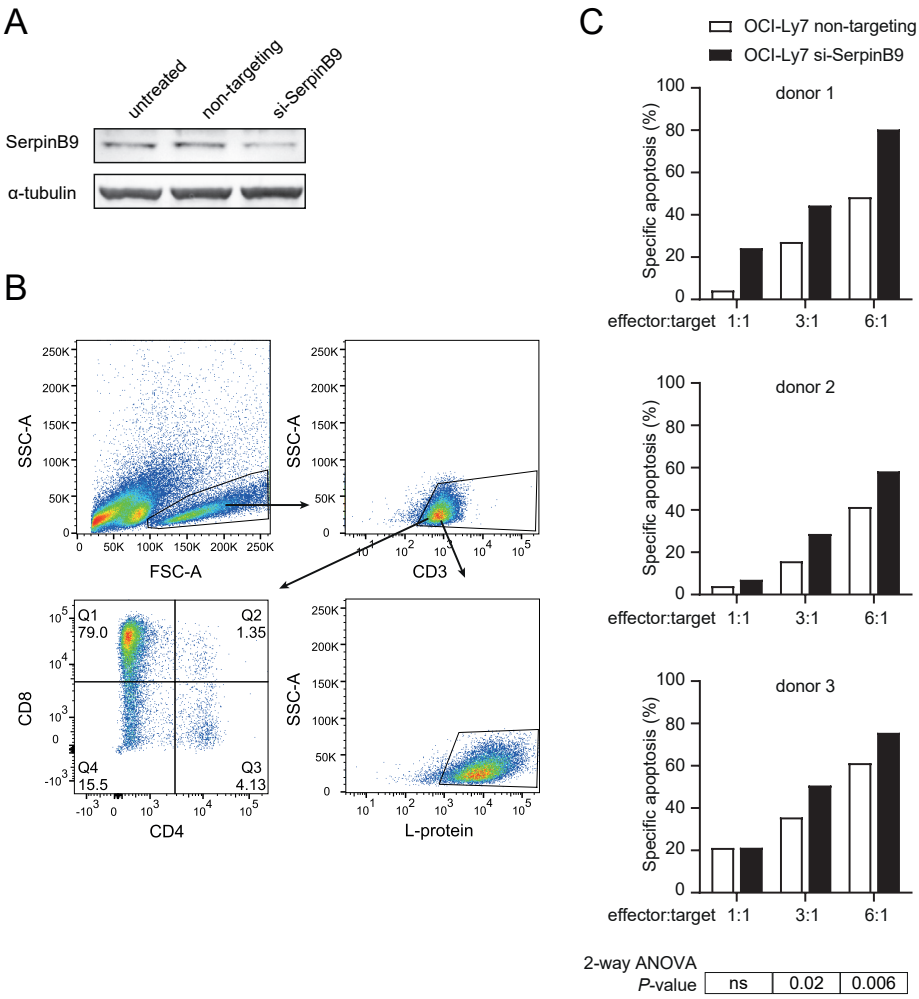


Figure 4. Knockdown of SerpinB9 expression in lymphoma increases killing by CAR T cells. (A) Representative staining of SerpinB9 expression in OCI-Ly7 cells after siRNA-mediated knockdown of SerpinB9, compared to untreated cells and cells electroporated with non-targeting siRNA. (B) Representative flow cytometric analysis of CD3, CD4, and CD8 expression on the surface of healthy donor T cells after transduction with an anti-CD20 CAR. Protein L staining indicates the fraction of lymphocytes expressing the CAR. (C) Specific apoptosis of OCI-Ly7 cells electroporated with non-targeting or SerpinB9 siRNA, after 4 hours co-culture with anti-CD20 CAR expressing T cells from three healthy donors at the indicated effector:target (E:T) ratios. A 2-way ANOVA with Sidak multiple testing correction was performed after combination of the three independent experiments, and the resulting *P*-values are shown below the graphs.

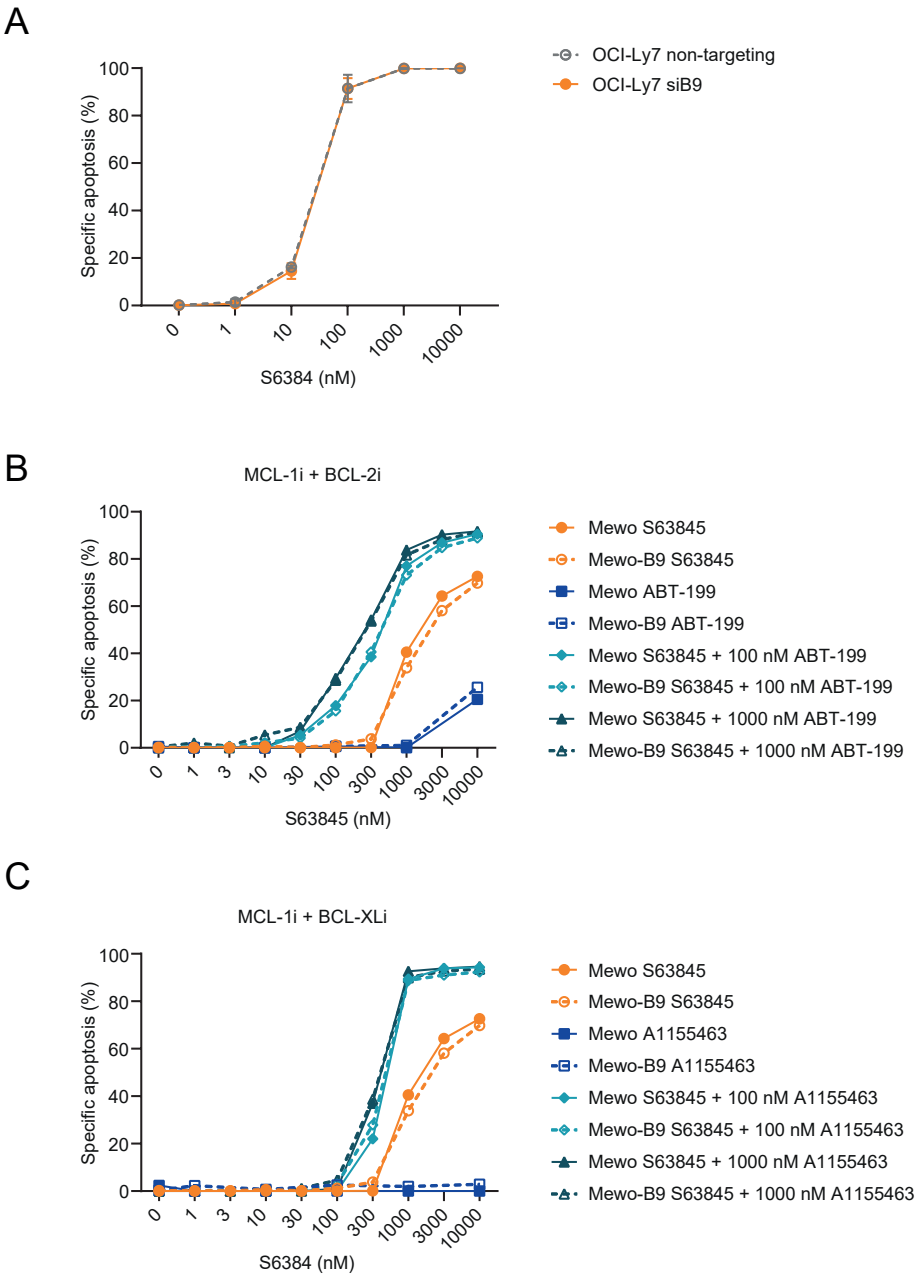


Figure 5. SerpinB9 expression does not affect the intrinsic apoptosis machinery. (A) Specific apoptosis of OCI-Ly7 cells electroporated with non-targeting or SerpinB9 siRNA, after 24 hours treatment with the indicated concentrations of MCL-1 inhibitor S63845. Data are averages of 3 independent experiments. (B-C) Specific apoptosis of WT Mewo (solid lines) and Mewo overexpressing SerpinB9 (Mewo-B9, dotted lines) after 24 hours treatment with the indicated concentrations of MCL-1 inhibitor S63845, BCL-2 inhibitor venetoclax (ABT-199) (B), or BCL-XL inhibitor A-1155463 (C), alone or combined.

DISCUSSION

Gene engineered T cell therapies are widely emerging and show promising clinical responses, particularly in hematological cancers. All gene engineered T cells make use of GrB to kill cancer cells. In this study, we show that expression of SerpinB9 is a resistance mechanism of cancer cells towards GrB-mediated killing by gene engineered T cells.

We show that expression of SerpinB9 expression can be found across hematological and solid cancers, with particular high and ubiquitous expression in lymphomas (Figure 1). By siRNA-mediated knockdown of SerpinB9 in high-expressing DLBCL cell line OCI-Ly7, and subsequent co-culture with anti-CD20 CAR T cells, we show that SerpinB9 expression promotes resistance to killing by CAR T cells (Figure 4). In addition, overexpression of SerpinB9 in the melanoma cell line Mewo rendered these cells significantly more resistant to GrB-mediated killing by NK cells (Figure 2). Additional experiments will be performed to investigate whether overexpression of SerpinB9 in melanoma cells also protects these cells from killing by gene engineered T cells, such as NY-ESO-1 TCR expressing T cells.

In immune cells, SerpinB9 acts in a cytoprotective manner by preventing proteolysis of cytotoxic cells by their endogenously produced GrB.[30] Besides, SerpinB9 expression has been reported in a wide range of other healthy cell types, including many endothelial and mesothelial cells as well as cells in immune-privileged sites such as the testis.[23,29] In cases where SerpinB9 and GrB expression do not overlap, the likely function of SerpinB9 expression is to protect bystander cells from damage caused by cytotoxic cells.[23,31] Thus, SerpinB9 expression can be induced in many different cell types, and may therefore be a widespread mechanism of immune evasion.

SerpinB9 expression has previously been reported to protect cancer cells from T or NK cell killing[32–34] and to predict clinical outcome.[24,35,36] In clinical cohorts of immune checkpoint blockade, SerpinB9 gene expression was found to be higher in non-responders than in responders, and high SerpinB9 expression correlated with worse overall survival.[25] These results indicate that SerpinB9 may be a powerful predictor of effectiveness of gene engineered T cell therapies as well as other immune therapies relying on cytotoxic cells. Therefore, measuring SerpinB9 gene or protein expression in patients may guide the selection of treatment.

Expression of SerpinB9 by tumor cells is not the only factor limiting effectivity of gene engineered T cell therapy. Modulation of the immune microenvironment, promoting recruitment and survival of gene engineered T cells at the tumor site, as well as the identification of new tumor antigens and antigen combinations, are

examples of strategies that are likely to improve effectiveness of gene engineered T cells.[1,2,17]

Additionally, gene engineered T cells may be modified to contain an optimized killing machinery that circumvents GrB blockade by SerpinB9.[38] Since SerpinB9 expression does not impair the downstream apoptotic machinery of cancer cells (Figure 5), SerpinB9-expressing cancer cells can still be sensitive to direct engagement of the extrinsic and intrinsic apoptosis pathways.

In conclusion, SerpinB9 expression is found across cancer types and is a resistance mechanism against novel immunotherapeutic strategies, including CAR T cells. These data provide a rationale to improve the killing capacity of T cells by circumventing SerpinB9-mediated inhibition of GrB.

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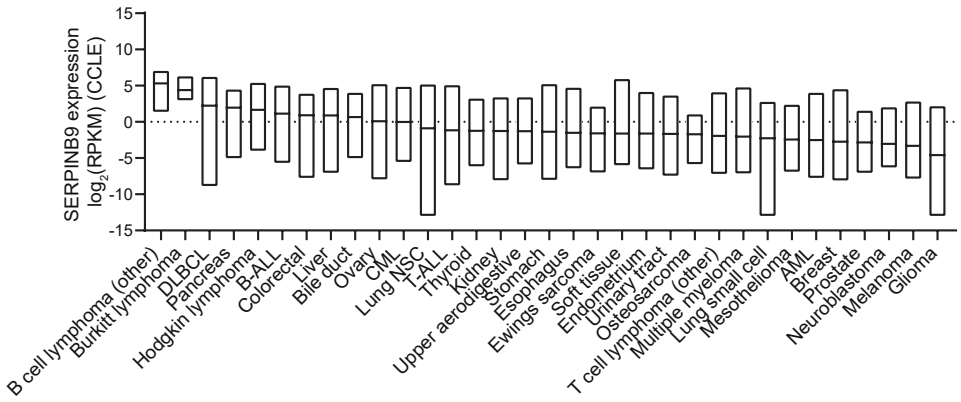
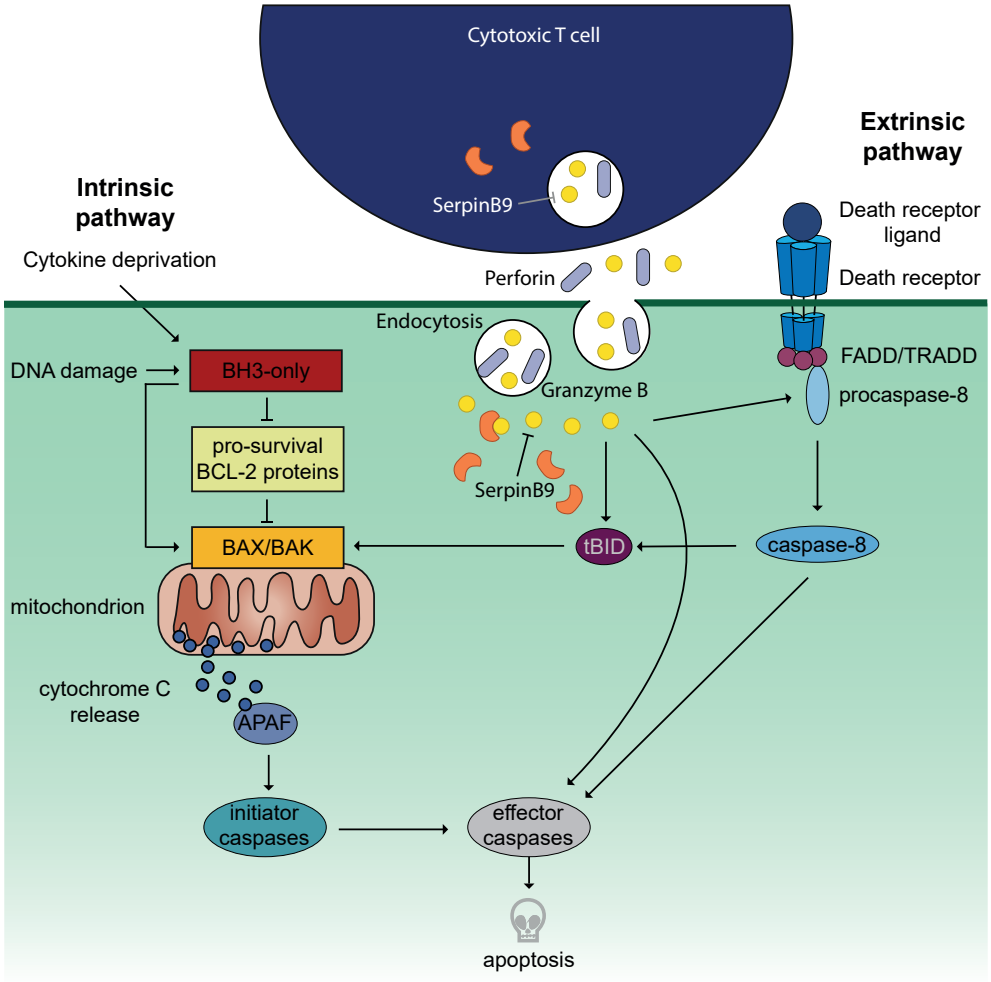
SUPPLEMENTARY FIGURES AND TABLE



Supplementary Figure 1. *SERPINB9* expression in cell lines of different tumor types. *SERPINB9* expression (log_2 RPKM) in cell lines in the Cancer Cell Line Encyclopedia (CCLE), per tumor type. Bars indicate range (min to max), with a line at the mean value of each group. AML, acute myeloid leukemia; B-ALL, B cell acute lymphocytic leukemia; CML, chronic myeloid leukemia; DLBCL, diffuse large B cell lymphoma; T-ALL, T cell acute lymphocytic leukemia.



Supplementary Figure 2. Schematic representation of granzyme B-mediated killing in the context of other cell death pathways. Cytotoxic T cells are protected from killing by their endogenous granzyme B by expression of SerpinB9. In tumor cells, expression of SerpinB9 protects against killing by perforin-mediated introduction of granzyme B, by preventing cleavage of BID and activation of caspases by granzyme B. Independent of granzyme B-mediated cell death, the intrinsic (left) and extrinsic (right) apoptosis pathways can induce cell death by activation of caspases. These pathways remain intact when SerpinB9 is overexpressed in the tumor cell. APAF, apoptotic protease activating factor; FADD, Fas-associated death domain; TRADD, tumor necrosis factor receptor type 1-associated death domain.

Supplementary Table 1. Overview of cell lines, including tumor type and culture medium.

Cell line	Cancer type	Category	Culture medium
A549	Lung carcinoma	Solid cancers	DMEM + 10% FBS + p/s
HepG2	Liver carcinoma	Solid cancers	DMEM + 10% FBS + p/s
HT29	Colon carcinoma	Solid cancers	DMEM + 10% FBS + p/s
IMR-32	Neuroblastoma	Solid cancers	DMEM + 10% FBS + p/s
JVM-2	Mantle cell lymphoma	Lymphomas	RPMI + 10% FBS + p/s
L363	Multiple myeloma	Multiple myeloma	RPMI + 10% FBS + p/s
L428	Hodgkin lymphoma	Lymphomas	RPMI + 10% FBS + p/s
LNCaP	Prostate carcinoma	Solid cancers	DMEM + 10% FBS + p/s
MDA-MB-157	Breast medullary carcinoma	Solid cancers	DMEM + 10% FBS + p/s
MDA-MB-231	Breast adenocarcinoma	Solid cancers	DMEM + 10% FBS + p/s
Mewo	Melanoma	Solid cancers	DMEM + 10% FBS + p/s
MM1.S	Multiple myeloma	Multiple myeloma	RPMI + 10% FBS + p/s
MZ1851RC	Renal carcinoma	Solid cancers	DMEM + 10% FBS + p/s
NCI-H929	Multiple myeloma	Multiple myeloma	RPMI + 20% FBS + p/s + 1 mM sodium pyruvate + 50 uM β-mercaptoethanol
OCI-Ly1	GC-DLBCL	Lymphomas	IMDM + 20% FBS + p/s
OCI-Ly3	ABC-DLBCL	Lymphomas	IMDM + 20% FBS + p/s
OCI-Ly7	GC-DLBCL	Lymphomas	IMDM + 20% FBS + p/s
OCI-Ly10	ABC-DLBCL	Lymphomas	IMDM + 20% FBS + p/s
OPM-2	Multiple myeloma	Multiple myeloma	RPMI + 10% FBS + p/s
OVCA432	Ovarian adenocarcinoma	Solid cancers	DMEM + 10% FBS + p/s
Phoenix-AMPHO	Kidney	Virus production	DMEM + 10% FBS + p/s
Ramos	Burkitt lymphoma	Lymphomas	RPMI + 10% FBS + p/s
RPMI-8226	Multiple myeloma	Multiple myeloma	RPMI + 10% FBS + p/s
SCC9	Oral squamous cell carcinoma	Solid cancers	DMEM + 10% FBS + p/s
SK-N-FI	Neuroblastoma	Solid cancers	DMEM + 10% FBS + p/s
SK-OV-3	Ovarian adenocarcinoma	Solid cancers	DMEM + 10% FBS + p/s
SU-DHL-2	ABC-DLBCL	Lymphomas	RPMI + 10% FBS + p/s
U2932	Follicular lymphoma	Lymphomas	RPMI + 10% FBS + p/s
UM9	Multiple myeloma	Multiple myeloma	RPMI + 20% FBS + p/s
YT-Indy	NK-ALL	NK cell line	RPMI + 10% FBS + p/s

ABC-DLBCL, diffuse large B cell lymphoma, activated B cell subtype; DMEM, Dulbecco′s Modified Eagle Medium; FBS, fetal bovine serum; GC-DLBCL, diffuse large B cell lymphoma, germinal center B cell subtype; IMDM, Iscove′s Modified Dulbecco′s Medium; NK-ALL, natural killer cell acute lymphocytic leukemia; p/s, 100 µg/ml penicillin-streptomycin; RPMI, Roswell Park Memorial Institute 1640 GlutaMAX HEPES medium.

CHAPTER 8

General discussion

Despite the approval of many new therapies for multiple myeloma (MM) in recent decades, the disease is still incurable. This thesis focuses on the anti-apoptotic protein MCL-1 as novel therapeutic target in MM. To this end, the role and regulation of MCL-1 have been studied, such as dependence of normal and malignant B cells and PC on MCL-1; sensitivity of MM cells from patients to MCL-1 inhibition; molecular regulation of MCL-1 expression in MM; and the potential role of MCL-1 in established and future cancer treatments. In this chapter, I will summarize the key findings of thesis, and discuss the results in relation to current literature.

BCL-2 PROTEIN EXPRESSION IN HEALTHY AND MALIGNANT B CELLS

The origin of MCL-1 overexpression in MM partly lies in the processes that regulate healthy B cell development and plasma cell (PC) differentiation. In order to differentiate into long-lived PC, B cells undergo multiple rounds of expansion and selection in germinal centers.[1] These processes are tightly regulated by the intrinsic apoptosis pathway, and malignant transformation of cells during and following the germinal center reaction is often characterized by apoptosis resistance.

In many post-germinal center B cell malignancies, apoptosis resistance occurs by continued overexpression of specific BCL-2 proteins that were already essential during the stage of B cell differentiation from which the malignancy arises. Therefore, we investigated the individual and overlapping roles of MCL-1, BCL-2, and BCL-XL in the maintenance of healthy B cell subsets in **Chapter 3**. Different B cell populations were isolated from the spleen and bone marrow of mice with inducible deletion of the *Mcl1* and/or *Bclx* genes. To study the role of BCL-2, mice were treated with ABT-737, a BH3-mimetic that inhibits BCL-2, BCL-XL, and BCL-W, but has preference for blocking BCL-2 in mice.[2,3] Using this approach, we discovered that BCL-XL is only important for survival of immature B cells, while BCL-2 is important for survival of mature B cells and terminally differentiated BLIMP-1^{hi} (B-Lymphocyte-Induced Maturation Protein 1) PC. Notably, MCL-1 is important for survival throughout B cell development, and reduced expression of MCL-1 sensitizes PC to BCL-2 and BCL-XL inhibition. These results suggest that combined inhibition of MCL-1 and BCL-2 may prove valuable for targeting malignant PC in multiple myeloma or auto-reactive PC in autoimmunity.

In **Chapter 2** I reviewed the role and regulation of pro-survival BCL-2 proteins in malignant B cells, thereby focusing on multiple myeloma. High expression of pro-survival BCL-2 family proteins contributes to outgrowth and drug resistance of malignant clones, but it also makes malignant cells vulnerable to BCL-2 family

inhibition using BH3-mimetic drugs. MM is characterized by high expression of MCL-1, while additional overexpression of BCL-2 and BCL-XL is observed in subsets of patients.[4,5] This was experimentally confirmed in **Chapter 4**, where a large panel of human MM cell lines (n=31) and patient samples (n=47) was treated with specific MCL-1, BCL-2, and BCL-XL inhibitors. These experiments showed that sensitivity to single BCL-2 and BCL-XL inhibitors is limited in cell lines as well as primary MM cells. MCL-1 inhibitor sensitivity, on the other hand, was observed in a large fraction of cell lines and primary MM cells. Importantly, strong synergistic cell death occurred when MM cells were treated with combined MCL-1 and BCL-XL or MCL-1 and BCL-2 inhibitors. When comparing BCL-2 protein dependency of healthy and MM PC (**Chapters 2** and **4**), I conclude that MCL-1 expression is essential for survival of both healthy and malignant PC. BCL-2 plays a role – though inferior to MCL-1 – in healthy as well as malignant PC, whereas BCL-XL co-dependency arises in MM but is not present in healthy PC.

THE INTERPLAY BETWEEN BCL-2 PROTEINS IN MM

Due to the complex interplay between BCL-2 proteins, expression of a certain pro-survival BCL-2 protein does not automatically translate to high sensitivity to its specific inhibitor. Rather, inhibitor sensitivity seems to be a consequence of the relative expression and distribution of multiple pro-apoptotic and pro-survival BCL-2 proteins. In MM, interactions of pro-survival BCL-2 proteins with BIM were previously found to determine MCL-1 dependence or co-dependence with BCL-2 or BCL-XL.[6] Indeed, I showed in **Chapter 4** that in an MCL-1-dependent MM cell line, BIM is bound to MCL-1 but not to BCL-2 or BCL-XL. Contrarily, in BCL-2 and BCL-XL co-dependent cell lines, BIM is bound to MCL-1 as well as BCL-2 or BCL-XL. siRNA-mediated knockdown of BCL-2 or BCL-XL in these co-dependent cell lines increases sensitivity to MCL-1 inhibition, probably due to redistribution of BIM towards MCL-1. In MM cell lines, MCL-1 expression did not correlate with MCL-1 inhibitor sensitivity, but BCL-2 and BCL-XL expression did, in an inverse way. This is in line with observations from others, who showed that BCL-XL can act as a sink to capture pro-apoptotic BCL-2 proteins released by MCL-1 upon MCL-1 inhibitor treatment.[7]

In contrast to these results from MM cell lines, I did not observe an inverse correlation between BCL-2 or BCL-XL expression and MCL-1 inhibitor sensitivity in PC from MM patients. In our cohort of newly diagnosed MM patients, high BCL-2 expression correlated with venetoclax sensitivity *ex vivo*. Additionally, 1q21 amplification, which is associated with high *MCL1* gene expression, correlated with increased MCL-1 inhibitor sensitivity. This discordance between cell lines and primary cells may result from the highly malignant and advanced-stage

nature of MM cell lines, most of which are produced from extramedullary disease. Unlike MM cells in most newly-diagnosed patients, cell lines are not dependent on the BM microenvironment. In addition, most MM cell lines have amplification of chromosome 1q.[8] MM cell lines thus do not represent the population of newly-diagnosed MM patients, and the role of 1q21 amplification could not be studied in MM cell lines. Still, many observations from MM cell lines, such as BIM binding[6] and single and combined BCL-2 family inhibitor sensitivity, are very similar in cell lines and primary cells *ex vivo*.

Besides main players MCL-1, BCL-2, BCL-XL, and BIM, other BCL-2 family proteins have been studied in the context of MM. Pro-survival protein BFL-1, despite its important role in mature B cells, is not expressed in healthy PC or MM, but retroviral overexpression of BFL-1 does promote survival of MM cells.[9] BCL-W has scarcely been studied in MM, and in preclinical and clinical studies with combined BCL-2/BCL-XL/BCL-W inhibitors ABT-737 and ABT-263 (navitoclax), neither we nor others have identified BCL-W to be relevant in MM (data not shown). BCL-B, in contrast, is expressed in MM.[10,11] Due to the lack of a murine ortholog or specific BH3-mimetic drug, the role of BCL-B in MM is poorly understood. Yet, Eμ-directed overexpression of BCL-B in mice drives a phenotype that accurately mimics human MM.[11] BCL-B therefore could play a role in MM development, but further studies are needed to confirm whether this is indeed the case.

Next to BIM, the BH3-only protein that is considered most interesting in MM is NOXA, due to its selective preference for MCL-1 binding (and BCL-B and BFL-1). Expression of NOXA in unstimulated cells is very heterogeneous among MM cell lines as well as primary MM cells.[12,13] The CCND1 subtype of MM patients is characterized by high NOXA expression, concomitant with high BCL-2 and low MCL-1 expression. This subgroup is characterized by a high fraction of venetoclax-sensitive patients, possibly because the limited amount of MCL-1 is completely neutralized by NOXA.[14] NOXA, as well as PUMA, are highly upregulated following cellular stress signals, including those produced by drug treatment.[15] Under these conditions, NOXA and PUMA may act together with BIM in neutralizing MCL-1. For instance, NOXA was often considered to play a key role in proteasome-induced apoptosis in MM, because its upregulation upon bortezomib treatment coincides with MCL-1 degradation and apoptosis induction.[16–18] Yet, we discovered in **Chapter 5**, by generating NOXA knockout cell lines, that NOXA is in fact not required for bortezomib-induced apoptosis in MM. In addition, NOXA is not essential for bortezomib-induced MCL-1 degradation, which I showed to be a consequence of caspase activation rather than the cause of apoptosis. The role of NOXA in MM thus remains controversial. The observation that proteasome

inhibitors induce MCL-1 accumulation but nevertheless induce apoptosis, is another aspect that remains a paradox.

Other BH3-only proteins, such as BIK and BAD, are expressed in MM, but these do not bind to MCL-1.[12] The role of these proteins will therefore be limited to neutralization of BCL-2 or BCL-XL, possibly followed by release of BIM. The two pro-apoptotic effector BCL-2 proteins BAX and BAK, although having highly redundant functions, have different interaction preference towards pro-survival proteins: BAK is sequestered by MCL-1 and BCL-XL, but not BCL-2, whereas BAX can be neutralized by all pro-survival proteins.[19] BAK is therefore potentially more important than BAX for apoptosis induced by MCL-1 inhibitor treatment.[7]

CLINICAL DEVELOPMENT OF MCL-1 INHIBITORS: CURRENT STATUS

Currently, MCL-1 inhibitors from multiple pharmaceutical companies are being evaluated in no less than 8 phase 1 clinical trials (Table 1). These trials include patients with hematological malignancies, predominantly MM and acute myeloid leukemia (AML), the latter of which also strongly depends on MCL-1 for survival.[20] None of these trials has been completed yet, but preliminary results suggest that AMG 176 may have acceptable tolerability in relapsed and refractory MM patients.[21] The biggest concern regarding MCL-1 inhibitors in clinical studies is the possibility of side-effects caused by MCL-1 inhibition in healthy tissues. Full

Table 1. MCL-1 inhibitors in phase 1 clinical trials

Compound	Sponsor	Indication(s)	ClinicalTrials.gov Identifier
AMG 176	Amgen	MM, AML	NCT02675452
AMG 176 (+ven)	Amgen	AML, NHL, DLBCL	NCT03797261
S64315*	Servier	AML, MDS	NCT02979366
S64315* (+ven)	Servier	AML	NCT03672695
MIK665*	Novartis	MM, DLBCL	NCT02992483
AZD5991 (single and +ven)	AstraZeneca	MM, CLL, AML, MDS, other hematological malignancies	NCT03218683
AMG 397	Amgen	MM, NHL, AML, DLBCL	NCT03465540
ABBV-467	AbbVie	MM	NCT04178902

* S64315 and MIK665 are different names for the same drug. This drug is a close derivate of S63845, the MCL-1 inhibitor that is used throughout this thesis.

+ven indicates a combination treatment with venetoclax (ABT-199).

AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin lymphoma.

knockout of *Mcl-1* in mice leads to peri-implantation embryonic lethality.[22] Using inducible knockout experiments in mice, MCL-1 was shown to be essential for survival of multiple cell types, including B cells,[23] healthy PC,[24] hematopoietic stem cells,[25] neural precursor cells,[26] and cardiomyocytes.[27] In fact, I showed in **Chapter 3** that MCL-1 is required throughout B cell development. However, in mouse experiments, MCL-1 inhibitors S63845, AMG 176, and AZD5991 were well tolerated at concentrations that effectively killed MM and AML cells.[28–30] These results suggest that irreversible loss of MCL-1 by gene knock-out is more detrimental to healthy cells than intermittent periods of pharmacological MCL-1 inhibition. Moreover, depletion of the B cell compartment is known to be tolerable, as shown by therapeutic antibodies and chimeric antigen receptor T cells targeting B cell antigens. B cell aplasia and resulting hypogammaglobulinemia can be treated with intravenous immunoglobulin replacement therapy.[31] The ongoing clinical trials will reveal whether any dose-limiting toxicities occur upon treatment, and whether there is a therapeutic window for MCL-1 inhibition, either as single treatment or in combination with other drugs.

Before development of MCL-1-specific BH3-mimetics, multiple BCL-2 and/or BCL-XL-specific BH3-mimetics have been produced. Combined BCL-2 and BCL-XL inhibitor navitoclax (ABT-263) initially showed promising effects when tested in CLL patients, but dose-limiting thrombocytopenia occurred due to BCL-XL inhibition.[32–34] Subsequently, specific BCL-2 inhibitor venetoclax was developed, and has since been approved by the Food and Drug Administration (FDA) for use in CLL and AML patients.[35–38] In MM, which generally depends more on MCL-1 than on BCL-2, venetoclax has limited effectivity. Only in the MM population with t(11;14), representing approximately 20% of newly-diagnosed MM,[39] venetoclax has a favorable benefit-risk profile.[40–42] In **Chapter 4**, I confirmed that indeed venetoclax has highest cytotoxicity in MM with t(11;14) or high expression of BCL-2 protein. More importantly, I showed that MCL-1 inhibition is highly effective in MM, in particular in the patient subset with 1q21 amplification. 1q21 amplification, which is present in approximately 40% of newly diagnosed and 70% of relapsed MM patients, is correlated with poor prognosis.[7,43] In addition, and partly independent of 1q21, serum β2m levels correlate with MCL-1 inhibitor sensitivity. Together, 1q21 amplification and high serum β2m level – both poor prognosis markers – identify a patient subset that has highest sensitivity to MCL-1 inhibition. Besides t(11;14) for venetoclax, it is conceivable that 1q21 amplification and serum β2m level can be used as biomarkers to predict MCL-1 inhibitor sensitivity. This finding may guide further clinical testing of MCL-1 inhibitors in MM.

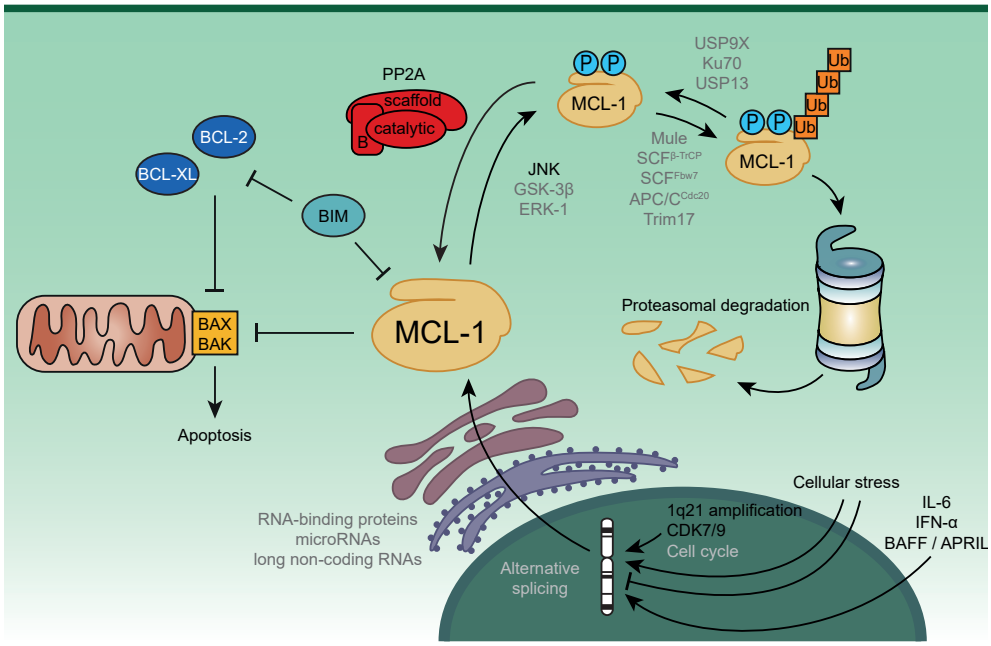


Figure 1. Multilateral MCL-1 regulation in MM. MCL-1 is continuously being produced and proteasomally degraded in MM. MCL-1-regulating signals and processes that are confirmed in MM are shown in black text, while signals reported in other cell types are written in grey. Transcriptional upregulation of *MCL1* can occur through signals from extracellular molecules such as IL-6, IFN-α, BAFF, and APRIL. Intracellular stress signals can either positively or negatively regulate *MCL1* transcription, depending on the signal and the cellular state. In addition, 1q21 amplification, CDKs and cell cycle phase can upregulate *MCL1* transcription. Following transcription, alternative splicing and regulation of mRNA half-life by RNA-binding proteins, microRNAs, and long non-coding RNAs influence the amount of MCL-1 protein that is produced. Dependence of an MM cell on MCL-1 depends on the presence of pro-survival proteins BCL-2 and BCL-XL, as well as the distribution of pro-apoptotic BIM towards these pro-survival proteins. Besides by high transcription, high cellular levels of MCL-1 can be reached by stabilization of MCL-1 protein, which otherwise has a very short half-life. Kinases JNK, GSK-3b, and ERK-1, and ubiquitin ligases Mule, $SCF^{\beta\text{-}TrCP}$, SCF^{Fbw7}, APC/C^{Cdc20}, and Trim17 were previously reported to promote proteasomal degradation of MCL-1. Deubiqutinases USP9X, Ku70, and/or USP13 were shown to counteract MCL-1 breakdown. The phosphatase complex PP2A stabilizes MCL-1 in a subset of MM. Due to this multilateral regulation of MCL-1 expression, the complex interplay of MCL-1 with other BCL-2 proteins, and the prominent role of MCL-1 in apoptosis resistance, MCL-1 is an interesting target for therapy of MM.

REGULATION OF MCL-1 EXPRESSION

Expression of MCL-1 is regulated by numerous mechanisms at transcriptional, post-transcriptional, and post-translational levels (Figure 1).[44] Understanding the molecular processes that underlie MCL-1 dysregulation in cancer can be of great therapeutic significance.

Transcriptional regulation of MCL-1

As reviewed in **Chapter 2**, constitutively high transcription of MCL-1 in MM results from extracellular and intracellular signals. IL-6 and IFN-α, produced by cells in the bone marrow microenvironment, induce JAK/STAT3 signaling in MM cells, which results in transcription of *MCL1*, *BCLX* and *VEGF* (Vascular Endothelial Growth Factor), the latter of which produces a positive feedback loop by stimulating IL-6 production in neighboring cells. Additionally, BAFF (B cell Activating Factor) and APRIL (A Proliferation-Inducing Ligand) induce *MCL1* transcription by signaling via TRAFs (TNF Receptor-Associated Factors). Translocations and point mutations of *MCL1* are rarely found,[44] but amplification of 1q21, the chromosome locus that contains *MCL1*, frequently occurs in MM. *MCL1* was previously identified as the most likely amplification target gene of 1q21 in a somatic copy number alteration analysis across human cancers,[45] and we proved for the first time that 1q21-amplified MM is associated with increased *MCL1* transcription and MCL-1 inhibitor sensitivity (**Chapter 4**). In addition to the stimuli described above, putative transcription factor binding elements in the *MCL1* locus indicate that *MCL1* may be transcriptionally regulated by cellular stresses such as hypoxia, ER stress, and microtubule disruption, but the exact role of these transcription factors in MM is unclear.[44] It is known, however, that cyclin-dependent kinases (CDKs), in particular CDK7 and CDK9, influence the function of RNA polymerase II and that inhibition of CDK7 and CDK9 leads to reduced transcription of *MCL1* mRNA.[46–48]

Post-transcriptional regulation of MCL-1

Although not experimentally addressed in this thesis, alternative splicing, mRNA regulation, and translational control have been reported to influence MCL-1 expression. The *MCL1* gene consists of three exons, which together form the 350 amino-acid full-length protein. Alternatively, a splicing variant lacking the second exon can be produced, resulting in a 272 amino-acid protein that structurally only contains the BH3 domain.[49] This short variant of MCL-1, also named MCL-1S, has been reported to have a pro-apoptotic function by neutralizing full-length MCL-1.[50] The relevance of MCL-1S in MM pathogenesis is unknown, and although the antibody used in our studies is expected to stain MCL-1S as well, I did not observe any potentially relevant changes in a band of the appropriate size for MCL-1S on immunoblot.

Not only does the MCL-1 protein have a short half-life, its encoding mRNA has a very short half-life as well (~2 hours).[51,52] Multiple RNA-binding proteins, microRNAs, and long non-coding RNAs have been identified to modulate *MCL1*

mRNA stability in a variety of cancer types.[44,52] These processes are highly cell type-specific, so not all reported mechanisms of *MCL1* mRNA regulation are expected to be relevant for MM.

Post-translational regulation of MCL-1

The N-terminus of MCL-1 is unique among BCL-2 family proteins because it contains a large number of proline, glutamic acid, serine, and threonine (PEST) residues, which are targets for post-translational modification and allow for rapid protein turnover.[53,54] Proteasomal degradation of MCL-1 occurs upon phosphorylation and subsequent poly-ubiquitination of its PEST regions.[44] Many kinases, ubiquitin ligases, and deubiquitinases have been shown to modify MCL-1 and thereby regulate its apoptotic function or stability (Figure 1).[44,55,56] Yet, activity and function of such modifying proteins is highly dependent on cellular context, and the exact processes determining turnover of MCL-1 in MM were unknown. It was previously reported that MCL-1 is stabilized in healthy germinal center B cells,[57] and that phosphatase activity is increased in germinal center B cells compared to other B cells.[58] We therefore set out to investigate whether MCL-1 is also stabilized in MM and diffuse large B cell lymphoma (DLBCL), which are both post-germinal center B cell malignancies.

In **Chapter 6**, I showed that MCL-1 is indeed stabilized in a subset of MM and DLBCL cell lines and MM patient samples. MCL-1 protein levels in MM and DLBCL seem to be a product of transcriptional activity and protein stability, with some cell lines relying more on the first process and other cell lines depending more on the latter. We took an unbiased approach to study the phosphatases that may be responsible for stabilization of MM, and identified multiple subunits of the Ser/Thr phosphatase complex PP2A among the hits from a phosphatase siRNA screen. By using PP2A inhibitor okadaic acid, PP2A was conformed to stabilize MCL-1 in MM, but not in DLBCL. Based on literature research, I identified GSK-3β, ERK-1, and JNK to be the most likely candidate kinases responsible for the MCL-1 phosphorylation that is reversed by PP2A. Preliminary results from our study indicate that JNK and PP2A have opposing functions in respectively destabilizing and stabilizing MCL-1 in MM.

PP2A plays an important role in mitotic progression and cellular responses to DNA damage. Due to its broad and complex function, PP2A is sometimes being reported as a tumor suppressor and sometimes as an oncogene, since its inhibition can have tumorigenic as well as tumoricidal effects.[59–61] In various preclinical studies using solid and hematological cancer models, PP2A inhibitor LB100 was shown to have strong chemo- and radiosensitizing potential.[60,62,63] In a phase I clinical trial, LB100 safety and efficacy were deemed favorable for further testing

alone and in combination with other therapies.[64] This shows that pharmacological inhibition of PP2A is feasible despite its important role in many cellular processes.

Indirect inhibition of MCL-1 by targeting its regulation

Our results provide a rationale for further investigating whether MM with stabilized MCL-1 can be effectively targeted with PP2A inhibitors. If direct inhibition of MCL-1 turns out to cause dose-limiting toxicities, PP2A inhibition may be a means to indirectly inhibit MCL-1 in MM cells only. Generating an inhibitor selective for the MCL-1-specific regulatory PP2A subunit would be a desirable approach to limit unwanted inhibition of other functions of PP2A. Alternatively, in MM that depends on high *MCL1* transcription rather than protein stability, transcription-inhibiting drugs may be an option. For example, CDK7 and CDK9 inhibitors have been shown to reduce *MCL1* transcription.[48,65]

Besides its evident importance in apoptosis, MCL-1 has also been ascribed a role in cell cycle progression. The tight cell cycle-dependent regulation of many MCL-1-modifying proteins, as well as reports that expression of MCL-1 itself is cell cycle-dependent, suggest that indirect MCL-1 targeting might be possible by interfering with cell cycle mechanisms.[44,56,66,67] Further studying the mechanisms by which MCL-1 is embedded in cellular processes besides apoptosis may provide new hypotheses for synergistic drug combinations in MCL-1-dependent MM.

COMBINING MCL-1 INHIBITORS WITH OTHER DRUGS

Due to the multilateral regulation of MCL-1 expression (Figure 1), the complex interplay of MCL-1 with other BCL-2 proteins, and its prominent role in apoptosis resistance, MCL-1 is an interesting candidate target for combined treatments. In **Chapter 4**, I showed the profound synergistic cell death that takes place when MCL-1 inhibitors are combined with BCL-2 or BCL-XL inhibitors in MM. This was also recently shown by others in preclinical models of MM, AML, mantle cell lymphoma, and melanoma.[29,68–72] This strong synergy likely allows for reduced drug dosing in patients when different BH3-mimetics are combined, possibly leading to a decrease in side-effects. The combination of MCL-1 inhibitors with venetoclax is now being evaluated in three clinical trials (Table 1).

After dose-escalation and safety assessment of single MCL-1 inhibitors, they will likely be combined with other established therapies. If conventional treatment increases availability of BH3-only proteins and their distribution towards pro-survival target proteins, this may increase sensitivity to BH3-mimetic drugs. In MM, proteasome inhibitors and dexamethasone are considered key agents to combine with MCL-1 – and other BH3-mimetic – treatment. I showed in **Chapter**

5 that bortezomib is not an indirect MCL-1 inhibitor through upregulation of NOXA, and I did not observe synergy between bortezomib and MCL-1, BCL-2, or BCL-XL inhibitors in MM cell lines (data not shown). Despite this, combinations of proteasome inhibitors with MCL-1 inhibitors or venetoclax have strong antitumor effects in mouse models.[5,29,30] Dexamethasone synergizes with MCL-1 inhibitors and venetoclax *in vitro*, but the exact mechanism of this synergy is unknown.[29,73] Other novel MM treatments, such as anti-CD38 monoclonal daratumumab or chimeric antigen (CAR) T cell therapies would also be interesting to test in combination with MCL-1 inhibitors, because the cell death mechanisms activated by those treatments may be provoked even stronger in presence of another apoptosis-inducing agent.

Exemplary of the potential role of BH3-mimetics in immunotherapy is the study shown in **Chapter 7**, where I showed that expression of granzyme B inhibitor SerpinB9 is widespread across hematological and solid cancers. SerpinB9 overexpression renders tumor cells resistant to granzyme B-mediated killing by NK cells and gene engineered T cells such as CAR T cells, and expression of SerpinB9 may thus predict effectivity of immunotherapy. Importantly, I showed that SerpinB9 overexpression does not alter sensitivity to intrinsic apoptosis induction by BH3-mimetics. Therefore, BH3-mimetics can potentially be used in combination with immunotherapy, to prevent or overcome SerpinB9-mediated granzyme B-resistance.

CONCLUSION

Taken together, the research conducted in this thesis indicates that MCL-1 inhibition is a promising therapeutic strategy in MM, and that amplification of 1q21 may be a diagnostic marker to predict MCL-1 inhibitor sensitivity in MM patients. Additionally, high MCL-1 expression can result from protein stabilization at the post-translational level. Further studying the complex regulation of MCL-1 will aid in finding new rational drug combinations that can exploit MCL-1 dependence and induce apoptosis in MM, and potentially in other cancer types.

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Appendices

SUMMARY

Resistance to apoptosis enables malignant cells to survive in the presence of otherwise lethal chromosomal, cellular, and metabolic alterations. As a consequence, dependence on pro-survival proteins is a potentially targetable weakness of these cancer cells. The research described in this thesis focuses on pro-survival BCL-2 family protein MCL-1 as therapeutic target in multiple myeloma (MM). Here, the key findings are summarized.

In **Chapter 2**, I reviewed current literature concerning the role and regulation of pro-survival BCL-2 proteins in MM. MM is characterized by high expression of MCL-1, while additional overexpression of BCL-2 and BCL-XL is observed in subsets of patients. Constitutive overexpression of pro-survival BCL-2 family proteins in MM results from a range of microenvironmental signals and genetic lesions. The complex regulation of MCL-1, BCL-2, and BCL-XL offers multiple direct and indirect targets for therapeutic intervention, for example using BH3-mimetic drugs.

Chapter 3 addressed the relative roles of MCL-1, BCL-2, and BCL-XL during the different stages of healthy B cell development and plasma cell (PC) differentiation. BCL-XL was found to be important for survival of immature B cells, while BCL-2 is important for survival of mature B cells and long-lived PC. MCL-1, in contrast, is important for survival throughout B cell development, and reduced expression of MCL-1 sensitizes PC to BCL-2 and BCL-XL inhibition.

In **Chapter 4**, I showed that MM cells from patients with amplification of 1q21, the locus that contains the MCL1 gene, are significantly more sensitive to treatment with MCL-1 inhibitor S63845 than MM cells without this chromosomal aberration. This finding indicates that 1q21 amplification can be used as a predictive marker to guide selection of therapy. Besides 1q21 amplification, serum β2m levels correlate with S63845 sensitivity. Together, 1q21 amplification and high serum β2m level – both poor prognosis markers – identify a patient subset that has highest sensitivity to MCL-1 inhibition. Combining S63845 with BCL-2 inhibitor venetoclax or BCL-XL inhibitors synergistically enhances apoptosis compared to the single inhibitors. In some MM cell lines, high expression of BCL-2 or BCL-XL confers resistance to MCL-1 inhibitor treatment, but these cell lines are highly sensitive to inhibitor combinations.

Because MCL-1 is important for survival of many cell types besides MM cells, clinical targeting of MCL-1 using specific MCL-1 inhibitors may lead to undesired side-effects. One way to circumvent these possible side-effects is by targeting MCL-1 indirectly, thereby exploiting cancer- or tissue-specific characteristics of MCL-1 regulation in MM. Proteasome inhibitors, such as bortezomib, are often considered indirect MCL-1 inhibitors because they induce upregulation of NOXA,

a pro-apoptotic BCL-2 protein selective for MCL-1. In contrast, I showed that bortezomib-induced apoptosis in MM does in fact not require NOXA (**Chapter 5**). In addition, degradation of MCL-1 upon bortezomib treatment was found to be independent from NOXA expression and a consequence, rather than the cause, of caspase activation.

In **Chapter 6**, molecular regulation of MCL-1 was studied, with the aim to identify phosphatases that stabilize MCL-1 in MM. MCL-1 half-life was shown to be increased in a subset of MM and diffuse large B cell lymphoma (DLBCL) cell lines, and in MM patient samples. MCL-1 protein levels in MM and DLBCL reflect transcriptional activity as well as protein half-life, and the relative contribution of these processes differs per cell line. In MM, the Serine/Threonine phosphatase complex PP2A dephosphorylates and stabilizes MCL-1, as shown by okadaic acid (a PP2A inhibitor) treatment. This dephosphorylation at Ser159 and/or Thr163 counteracts phosphorylation by c-Jun N-terminal kinase (JNK). These findings increase our understanding of post-translational regulation of MCL-1 in MM, which may provide novel strategies to indirectly inhibit MCL-1.

Inhibition of MCL-1 is a strategy for treatment of cancers that depend on MCL-1 for survival, but this strategy may also be used for cells that have become resistant to other killing pathways. **Chapter 7** describes the widespread expression of SerpinB9 across lymphomas and other cancers. SerpinB9 expression renders tumor cells resistant to granzyme B-mediated killing by NK cells and gene engineered T cells, such as chimeric antigen receptor T cells, and expression of SerpinB9 may thus predict effectivity of immunotherapy. SerpinB9 expression does not alter sensitivity to intrinsic apoptosis induction by BH3-mimetics. Therefore, BH3-mimetics can potentially be used to overcome SerpinB9-mediated granzyme B-resistance.

In **Chapter 8**, the final chapter of this thesis, the results and insights obtained during these research projects were discussed and future perspectives were outlined. In conclusion, MCL-1 inhibition is a promising therapeutic strategy in MM, and amplification of 1q21 may be a diagnostic marker to predict MCL-1 inhibitor sensitivity in MM patients. Additionally, high MCL-1 expression can result from protein stabilization at the post-translational level. Further studying the complex regulation of MCL-1 will aid in finding new rational drug combinations that can exploit MCL-1 dependence and induce apoptosis in MM, and potentially in other cancer types.

NEDERLANDSE SAMENVATTING

Apoptose is een biologisch proces waarbij oude, defecte of overbodige cellen gereguleerd doodgaan. Het is daarmee een zelfvernietigingsmechanisme van de cel. Voorbeelden van apoptose zijn het verdwijnen van de staart van een kikkervisje en de vliezen tussen vingers en tenen van ontwikkelende embryo's, maar ook in volwassen mensen en dieren speelt apoptose een belangrijke rol. Zo worden bepaalde weefsels, zoals de huid en de binnenkant van de darmen, constant vernieuwd en worden oude cellen afgebroken door middel van apoptose. Ook is apoptose belangrijk voor de preventie van ziekte, omdat geïnfecteerde cellen en cellen die mutaties hebben opgelopen normaal gesproken apoptose ondergaan en vervolgens opgeruimd kunnen worden door cellen van het immuunsysteem.

Apoptose in kanker

Kanker kan ontstaan wanneer een cel mutaties oploopt die ervoor zorgen dat de cel ongeremd gaat delen en zo het omliggende weefsel overgroeit. Apoptose is een belangrijke barrière tegen het ontstaan van kanker, omdat gemuteerde cellen normaal gesproken in apoptose gaan en daarmee niet kunnen uitgroeien tot kanker. In sommige gevallen, echter, treffen de mutaties in een cel ook het zelfvernietigingsmechanisme en wordt de cel resistent tegen apoptose. Hiermee vergroot de cel zijn kansen om uit te groeien tot een kwaadaardige tumor aanzienlijk. Kankercellen hebben vaak defecten die ervoor zorgen dat apoptose niet meer geactiveerd kan worden, of dat apoptose na activatie niet meer uitgevoerd wordt. Dit vergroot niet alleen de overlevingskansen van de kanker, maar het zorgt er ook voor dat bepaalde therapieën minder goed werken. Chemotherapie en bestraling activeren apoptose in kankercellen, dus apoptose-resistente kankercellen gaan minder goed dood door deze therapieën. Het is dus belangrijk om te onderzoeken hoe apoptose-resistentie precies werkt en hoe apoptose in kankercellen weer aangezet kan worden.

De BCL-2 eiwitten

Er zijn meerdere mechanismen die apoptose kunnen activeren. Het mechanisme dat wordt geactiveerd door mutaties en veranderingen in de cel draait om BCL-2 eiwitten. Van deze eiwitten bestaan twee typen: pro-apoptotische en anti-apoptotische. De pro-apoptotische BCL-2 eiwitten worden geproduceerd als de cel een stress- of schadesignaal oppikt. De anti-apoptotische BCL-2 eiwitten worden geproduceerd als de cel signalen heeft opgevangen die aangeven dat de cel moet blijven leven. Deze eiwitten zijn een soort rem op het zelfvernietigingsmechanisme van de cel, zodat apoptose alleen geactiveerd wordt als de cel écht moet doodgaan.

Wanneer de hoeveelheid pro-apoptotische eiwitten de overhand krijgt in de cel, vindt activatie van apoptose plaats en begint de cel zichzelf van binnenuit af te breken in kleine stukken die ten slotte worden opgeruimd door immuuncellen. In sommige kankers is het stresssignaal onwerkzaam gemaakt; in dat geval kunnen er geen pro-apoptotische BCL-2 eiwitten meer worden gemaakt. In andere gevallen produceren kankercellen extreem veel anti-apoptotische BCL-2 eiwitten; dan is de rem constant zo hard ingedrukt dat apoptose niet meer geactiveerd kan worden. Dit laatste is vaak het geval in de beenmergkanker multipel myeloom.

Multipel myeloom

Multipel myeloom, ook bekend als “de ziekte van Kahler” of kortweg “myeloom”, is een vorm van kanker die ontstaat uit plasmacellen, de immuuncellen die antistoffen produceren. Myeloom bevindt zich in het beenmerg, het sponzige weefsel in de kern van botten. In het beenmerg vindt normaal gesproken de aanmaak van nieuwe immuuncellen en andere bloedcellen plaats. De groei van myeloomcellen in het beenmerg verstoort deze aanmaak van bloedcellen en ontregelt de normale samenstelling en stevigheid van het bot. Daardoor hebben myeloompatiënten vaak last van bloedarmoede, botbreuken, pijn aan de botten, en nierschade door de grote hoeveelheid calcium die vrijkomt uit de beschadigde botten. Myeloom komt voornamelijk voor bij ouderen, de gemiddelde leeftijd bij diagnose is 70 jaar. In Nederland wordt jaarlijks bij ongeveer 1200 personen, waarvan iets meer dan de helft mannen, de diagnose myeloom gesteld. Ondanks sterke recente verbeteringen in behandeling van myeloom is de ziekte nog niet te genezen. Dit wil zeggen dat myeloom altijd terugkeert en uiteindelijk niet meer reageert op behandelingen.

Een kenmerk van myeloom is de resistentie tegen apoptose door hoge expressie van bepaalde anti-apoptotische BCL-2 eiwitten. Vooral het eiwit MCL-1 is vaak zo hoog aanwezig dat myeloomcellen geen apoptose meer kunnen ondergaan, ongeacht de stresssignalen die aanwezig zijn in deze cellen. Dit maakt MCL-1 een interessant potentieel doelwit voor nieuwe medicijnen tegen myeloom. Het weghalen van MCL-1 zorgt er namelijk voor dat de rem van het apoptosemechanisme verdwijnt, waardoor pro-apoptotische BCL-2 eiwitten ineens de overhand krijgen. Hierdoor kan apoptose weer geactiveerd worden, met uitschakeling van de myeloomcellen als gevolg. Dit proefschrift richt zich op MCL-1 als nieuw therapeutisch doelwit in multipel myeloom.

Samenvatting van de belangrijkste resultaten

Hoofdstuk 2 bestaat uit een review artikel over de rol en regulatie van anti-apoptotische BCL-2 eiwitten in de ontwikkeling van gezonde plasmacellen en in multipel myeloom. De ontwikkeling van plasmacellen vindt plaats in de lymfeklieren. Tijdens de ontwikkeling van plasmacellen vinden meerdere rondes van selectie plaats waarin cellen die slecht werkende antilichamen produceren, doodgaan door apoptose. Multipel myeloom komt voort uit plasmacellen die helemaal tot het einde van de ontwikkeling zijn gekomen en dus functionele antilichamen produceren. Net zoals myeloomcellen zijn plasmacellen afhankelijk van MCL-1 voor overleving, en ze bevinden zich in het beenmerg. Het beenmerg geeft verschillende signalen af waardoor myeloomcellen veel MCL-1 produceren. Behalve MCL-1 kunnen ook BCL-2 en BCL-XL (twee andere anti-apoptotische BCL-2 eiwitten) belangrijk zijn voor apoptoseresistentie in multipel myeloom. Zeer recentelijk zijn nieuwe medicijnen ontwikkeld die MCL-1, BCL-2, of BCL-XL kunnen remmen en daarmee apoptose kunnen activeren. Deze medicijnen hebben grote potentie in multipel myeloom en andere kankers die afhankelijk zijn van deze BCL-2 eiwitten.

In **Hoofdstuk 3** onderzoek ik de rol van MCL-1, BCL-2 en BCL-XL tijdens de ontwikkeling van gezonde B-cellen, de voorlopers van plasmacellen. Vanwege de grote overeenkomsten tussen het apoptosemechanisme van mensen en muizen kan dit erg goed onderzocht worden in muizen. Voor dit onderzoek maakten we gebruik van muizen die MCL-1, BCL-2 of BCL-XL niet kunnen produceren in hun B-cellen, waarna we bestudeerden tijdens welke stadia in de ontwikkeling van B-cellen tot plasmacellen defecten optraden. Uit dit onderzoek bleek dat BCL-XL belangrijk is voor jonge B-cellen, en BCL-2 juist voor terminaal ontwikkelde B-cellen en plasmacellen. MCL-1 speelt een grote rol tijdens alle stadia van de ontwikkeling van B-cellen, inclusief plasmacellen. Bovendien zijn plasmacellen zonder MCL-1 extra gevoelig voor remming van BCL-2 en BCL-XL, een observatie die therapeutisch relevant kan zijn indien deze wordt bevestigd in multipel myeloom, de kwaadaardige tegenhanger van deze cellen.

Hoofdstuk 4 gaat over het induceren van apoptose in multipel myeloom door remming van MCL-1. In deze studie zijn myeloomcellen van 47 patiënten in het laboratorium behandeld met een nieuw ontwikkelde MCL-1-remmer, die we hebben verkregen door samenwerking met het bedrijf dat deze remmer produceert. We richtten ons hierbij onder meer op de groep patiënten waarvan de myeloomcellen een bepaalde mutatie hebben, namelijk een vermeerdering van chromosoomdeel 1q21. Deze mutatie is in verband gebracht met een slechte prognose van de ziekte, maar het mechanisme hierachter is onbekend. Aangezien het gen voor MCL-1 zich op dit specifieke chromosoomdeel bevindt dat vermeerderd is in deze patiënten,

vermoedden wij dat als gevolg van deze mutatie meer MCL-1 wordt geproduceerd en de cellen gevoeliger zijn voor MCL-1-remming. Deze hypothese hebben we kunnen bevestigen. Dit resultaat is belangrijk voor het klinisch onderzoek naar MCL-1-remmers, dat zich nog in een vroeg stadium bevindt. Het is belangrijk om vooraf te weten welke groep patiënten baat zal hebben bij behandeling met een bepaald medicijn. Op basis van onze resultaten is de MCL-1-remmer waarschijnlijk erg effectief in de groep patiënten met 1q21 vermeerdering, ongeveer 30% van de nieuw gediagnosticeerde patiënten. Klinisch onderzoek zal in de toekomst moeten bevestigen of dit inderdaad zo is, wat een grote verbetering zou kunnen zijn van de perspectieven voor deze tot dusver moeilijk te behandelen patiëntgroep.

Daarnaast laat ik in dit hoofdstuk zien dat het combineren van MCL-1- met BCL-2- of BCL-XL-remmers in de hele patiëntpopulatie zorgt voor grote toename van celdood in myeloomcellen. Waarschijnlijk versterken deze remmers elkaar, een proces dat in de farmacologie "synergie" wordt genoemd. Voor patiënten die niet reageren op de enkele remmers kan behandeling met gecombineerde remmers daarom een optie zijn.

Hoofdstuk 5 bestaat uit een kort artikel waarin ik de functie bestudeer van bortezomib, een veelgebruikt medicijn tegen multipel myeloom. Eerder onderzoek suggereert dat bortezomib apoptose activeert doordat het zorgt voor grote productie en ophoping van NOXA, een pro-apoptotisch BCL-2 eiwit dat specifiek MCL-1 blokkeert. Wij toonden echter aan, door NOXA compleet te verwijderen uit myeloomcellen, dat myeloomcellen zonder NOXA net zo snel doodgaan na behandeling met bortezomib. Deze resultaten bewijzen dat NOXA niet nodig is voor het functioneren van bortezomib en dat bortezomib dus een ander werkingsmechanisme moet hebben.

In **Hoofdstuk 6** richt ik me op de moleculaire regulatie van MCL-1. Behalve door hoge productie kan de grote hoeveelheid MCL-1 in myeloomcellen ook het gevolg zijn van langzame afbraak. We ontdekten in dit onderzoek dat in multipel myeloom, maar ook in een verwant type lymfoom, stabilisatie van MCL-1 plaatsvindt. Normaal wordt MCL-1 continu geproduceerd en weer afgebroken. Deze afbraak vindt plaats doordat bepaalde eiwitten fosfaatgroepen aan MCL-1 binden waarna MCL-1 in stukjes wordt geknipt. Ik beschrijf in dit hoofdstuk onze bevinding dat in myeloom een tegenwerkend eiwit, PP2A, de fosfaatgroepen weer van MCL-1 afhaalt waardoor MCL-1 stabiel blijft. Het ontrafelen van dit mechanisme vergroot de kennis over de totstandkoming van de hoge MCL-1 niveaus in multipel myeloom.

In **Hoofdstuk 7** onderzoek ik een ander mechanisme van celdood, namelijk het doodmaken van kankercellen door immuuntherapie. Deze immuuntherapie, CAR T-cellen, bestaat uit immuuncellen van patiënten die in het laboratorium dusdanig

worden bewerkt dat ze een specifiek kenmerk van kankercellen beter herkennen. Na toediening binden de CAR T-cellen aan tumorcellen, die ze doodmaken door granzym B in de tumorcel te injecteren. De resultaten in dit hoofdstuk laten zien dat veel tumoren een slim afweermechanisme tegen granzym B hebben: ze produceren het eiwit SerpinB9 dat granzym B onschadelijk maakt. Ik concludeer dat het belangrijk is voor onderzoek naar en uitvoering van immuuntherapie om te kijken naar SerpinB9 expressie in tumorcellen, omdat dit een mogelijk resistentiemechanisme is tegen immuuntherapie. Het werkingsmechanisme van de BCL-2 eiwitten wordt echter niet aangetast door SerpinB9, dus misschien kan in deze tumorcellen alsnog apoptose worden geactiveerd door MCL-1 te remmen.

Tenslotte bediscussieer ik in **Hoofdstuk 8** de hierboven beschreven resultaten in relatie tot de bestaande literatuur. Concluderend, MCL-1 remming is een veelbelovende om apoptose te induceren in multipel myeloomcellen. Vermeerdering van chromosoomdeel 1q21 is een mogelijke diagnostische marker om gevoeligheid van myeloompatiënten voor MCL-1-remming te voorspellen. Daarnaast kan sterke expressie van MCL-1 in myeloom het gevolg zijn van eiwitstabilisatie. De regulatie van MCL-1 is dus erg complex. Verder onderzoek hiernaar kan leiden tot nieuwe behandelmogelijkheden voor multipel myeloom en mogelijk ook voor andere soorten kanker die afhankelijk zijn van MCL-1.

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CURRICULUM VITAE

Anne Slomp was born on 20 July 1993 in Eindhoven, the Netherlands. In 2010, she finished her secondary education at the Van Maerlantlyceum in Eindhoven (VWO-Gymnasium). She then moved to Utrecht to study Biomedical Sciences at Utrecht University. In 2013, she completed her BSc in Biomedical Sciences *cum laude*, with addition of an extracurricular Honours Programme. Subsequently, she enrolled in the MSc program Infection & Immunity at Utrecht University, for which she did a 9-month internship on Influenza A Virus binding and entry characteristics at Utrecht University, under supervision of dr. Erik de Vries. After this, she did a 6-month internship with prof. dr. Glenn Dranoff at Dana-Farber Cancer Insititute in Boston, USA, focusing on GM-CSF signaling induced by tumor vaccines. Besides, she was enrolled in the Honours Programme of the Graduate School of Life Sciences. After obtaining her MSc degree *cum laude* in 2015, she started her PhD at the Center of Translational Immunology of the University Medical Center Utrecht, under supervision of dr. Victor Peperzak en prof. dr. Monique Minnema. There, she investigated apoptosis resistance in multiple myeloma, specifically focusing on regulation and targetability of anti-apoptotic protein MCL-1. The results of her research are described in this thesis, entitled "A matter of life or death: targeting MCL-1 in multiple myeloma".
Currently, Anne is working as an Associate Medical Publications Manager at Excerpta Medica in Amsterdam.