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Brandon Chen April 9, 2018

Myeloma Patient-Derived MCL1 Point Mutations Can Influence MCL1-Inhibitor Function

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Multiple myeloma is a malignancy of long-lived plasma cells that is rarely curable. Despite recent drug development, additional approaches are required. We decided to investigate molecular vulnerabilities of the BCL2 family. Using the MMRF CoMMpass study, we determined nonsynonymous mutations frequency in the BCL2 family. Analysis from 982 patients revealed that mutations in the BCL2 family are relatively rare. Interestingly, MCL1 was mutated in 10 baseline samples (1.02%) and the frequency of the mutations in these samples was high (median 0.391, range 0.066-0.531). Therefore, we further investigated the functional consequences of the MCL1 mutation.

Of the 10 mutations detected, we focused on the 4 mutations that lie near the functional BH1 (V249L and L267V) and within the BH3 (N223S, and R214Q) domains. Wild-type (WT) MCL1 and the four mutant MCL1 constructs were introduced into murine B-ALL cell line that has endogenous murine MCL1 flanked with LoxP sites and confirmed expression by western blot analysis. Human MCL1 can replace murine MCL1 in this cell model, therefore we are determining if the myeloma-derived mutants of MCL1 can complement the loss of mouse Mcl1 and will report on these findings. We treated cells with MCL1 inhibitor S63845 and measured apoptosis (Annexin V/PI) at 24 hours. Cells with empty vector were highly resistant to death (less than 20% at 1000 nM) while cells expressing the human WT MCL1 were susceptible to the MCL1 inhibitor at 300 and 1000 nM. The V249L, N223S and R214Q mutations mimicked the sensitivity of the WT MCL1. However, L267V mutation resulted in a dose curve similar to the empty vector control suggesting this mutation was either resulted in the loss of function or impaired drug binding. Since drug binding stabilizes MCL1 by competing for E3 ligase, we determined the effect of S63845 on MCL1 protein levels. We found S63845 increased human MCL1 protein expression in all mutants, ruling out lack of drug binding. We next co-immunoprecipitated MCL1 and found that BIM release correlated with S63845 sensitivity. In addition to not releasing BIM, L267V did not effectively release NOXA and BAK after S63845 treatment. Taken together, the L267V mutation blocks the ability of the drug to displace pro-apoptotic proteins required to induce cell death.

We tested another MCL-1 inhibitor in clinical trials, AZD5991. Interestingly, all four mutations resulted in diminished killing activity when compared to cells expressing WT MCL1, suggesting that these mutations may also influence drug function. Together these data suggest that MCL1 mutations may not necessarily influence MCL1 function, yet they could alter responses to an emerging class of inhibitors where 3 drugs are currently in clinical trials.

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Acknowledgement

I will like to thank previous lab members Pamela Wong and Dr. Jason Conage-Pough for providing important data for the thesis, Dr. Joseph Opferman for kindly donating murine $Mcl1^{fl/fl}$ B-ALL cells, Dr. Michelle Lamb from AstraZeneca for generating crystal structures, and Dr. Stephan Fesik for his publication figure.

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Introduction

Multiple myeloma (MM) is a plasma cell malignancy and a genetically complex disease that has become increasingly more common in the aging population during the last 15 years[1,2]. It is also the second most common hematological malignancy and belongs to a group of related paraproteinaemias illustrated by abnormal clonal plasma cell infiltrations in the bone marrow[3]. A full-blown myeloma undergoes a multistep malignant transformation process. Characterization of the genetic architecture of multiple myeloma is an ongoing process. Various genomic events such as somatic mutations, epigenetic and chromosomal copy-number changes drive the progression from monoclonal gammopathy of unknown significance (MGUS) to symptomatic MM, and ultimately to aggressive extramedullary disease in some patients[4,5]. MM is classified with chromosomal rearrangements involving the immunoglobulin heavy (IGH) locus and hyperdiploidy, which harbors multiple trisomies on most odd number chromosomes[6]. A multitude of genes and pathways mediating oncogenic transformation of myelomas have been described, yet recent sequencing results elucidated that there is no single genetic change underlying the transformation that can be targeted therapeutically[7,8]. Corroborating this result, global sequencing initiatives in MM patients have been undertaken. Preliminary data suggested that MM shows a heterogeneous subclonal structure at diagnosis, and only few recurrent genetic mutations can attribute to the pathogenetic significance, including KRAS, NRAS, TP53, BRAF and FAM46C[4,9,10]. In conclusion, MM is not only a heterogenic malignancy with complex genetic architecture, but also a multiclonal disease that varies within a single patient.

Long-lived plasma cells can survive in the bone marrow microenvironment and produce antigen-specific antibodies for decades even in the absence of repeat antigen exposure[11]. Myeloma plasma cells, similar to normal plasma cells, reside in the bone marrow and heavily rely on similar survival cues from the microenvironment, such as Interleukin-6 (IL6)[12]. Bone marrow stromal cells (BMSC) sustain myeloma cell survival through secreting survival factors such as IL6, insulin-like growth factor (IGF1), and vascular endothelial growth factors (VEGF) to activate pro-survival signals[12]. In addition, surface receptor/ligand pair interaction such as CD86/CD28 can increase myeloma cell survival when stromal cell interactions such as dendritic cells are considered[13]. As plasma cells undergo malignant transformation into myeloma cells, they have to overcome various intrinsic/extrinsic stress to maintain their cancerous phenotype[13]. One hallmark of cancer is the evasion of apoptosis or programmed cell death[14], meaning myeloma cells have to counter pro-death signals in order to survive. Both long-lived plasma and myeloma cells require anti-apoptotic proteins MCL1 and/or BCL2/X_L to maintain viability and promote cell differentiation[15-17]. An amplification of MCL1 is also often seen in myeloma cells to drive drug resistance and survival. Therefore, inhibitors that target anti-apoptotic BCL2 proteins such as venetoclax (ABT-199), a BCL2 inhibitor, navitoclax, a BCL2/X_L inhibitor, and MCL1 inhibitors have been developed for potential multiple myeloma treatment[18].

The apoptosis pathway governed by the BCL2 family is also known as the ‘intrinsic’ or ‘mitochondrial’ pathway[19]. This family encompasses four subgroups that contain homologous protein domains: 1. anti-apoptotic BCL2-like protein (BCL2, BCLX_L , BCLW, MCL1, A1/BFL1), 2. pro-apoptotic effectors BAX/BAK, 3. pro-apoptotic BH3-only activator proteins (BIM, BID, BAD, BIK, BMF, HRK), and 4. sensitizer BH3 proteins (NOXA, PUMA)[20]. The pro-apoptotic

BH3-only activator proteins share the BH3 domain with other BCL2 family proteins. The BH3 domain of the BH3-only activators is important in interacting with anti-apoptotic Bcl-2 family proteins. The dynamic BCL2 protein interactions occur on the mitochondrial outermembrane (MOM). Upon cytotoxic stress such as DNA damage, pro-apoptotic effectors, activators, and sensitizers get induced and activated. Sensitizer proteins prime a cell to apoptosis by releasing activator proteins such as BIM from the anti-apoptotic proteins like MCL1[21]. After the pro-apoptotic activator proteins get released, they will activate pro-apoptotic effector proteins BAX and BAK. BAX/BAK oligomerize on the MOM and lead to mitochondria outer mitochondrial membrane permeabilization (MOMP)[22,23]. Subsequently, the release of cytochrome c from the mitochondria intermembrane space (IMS) to the cytosol recruits APAF1 and initiator pro-caspase 9 to form the apoptosome[24]. Activated caspase 9 cleaves other effector caspases such as caspase 3 and 7 to activate their protease activity and commit to irreversible apoptosis. The final step of apoptosis is accomplished by effector capases' activities to inflict intracellular damage by acting on their proteolytic enzymatic functions (Figure 1).

Among all anti-apoptotic BCL2 proteins, MCL1 is one of the most distinct members within this family. The *mcl1* locus is located on the chromosomal arm 1q21 and encodes a full-length protein with 350 amino acid. Close to the N-terminus is a proline (P), glutamate (E), serine (S), threonine(T)-rich PEST domain that regulates protein degradation[25]. The PEST domain subjects MCL1 to post-translational modification such as phosphorylation by various kinases, which signals for E3 ligases to engage in ubiquitin-mediated proteasome degradation[26,27,28]. This PEST region is absent in other BCL2 proteins, which make MCL1 the highest turnover protein in the whole family. Further down the PEST domain are the functional domains BH1, BH2, and BH3

domains. These three domains have anti-apoptotic capabilities[29] and form a binding pocket that interacts with other pro-apoptotic proteins[30]. The heterodimerization of anti and pro-apoptotic proteins sequesters pro-apoptotic activators from inducing BAX/BAK oligomerization[26]. Following the functional domain is a transmembrane domain that anchors MCL1 on the MOM.

In addition to its distinctive structures, MCL1 is indispensable in embryonic development and survival mechanisms in various cell lineage, including lymphocyte and hematopoietic stem cells[31,32]. It is also one of the most highly amplified genes in a variety of human cancers, and it promotes MM cells survival. In multiple myeloma, greater than 40% of the newly diagnosed patients have more than 2 copies of chromosomal arm 1q21, and the amplification of 1q21 is positively correlated with MCL1 mRNA expression level (Figure 2)[33]. Overall, MCL1 is an essential pro-survival protein in myeloma cell maintenance, development, and apoptosis regulation. Downregulation of MCL1 in MM cells sensitizes them to ABT-737, a BCL2, $BCLX_L$, and BCLW inhibitor, and thus inducing apoptosis[34].

A new class of drugs that target anti-apoptotic proteins are referred to as "BH3-mimetics", meaning the inhibitors are designed to have similar effects as the pro-apoptotic BH3-only sensitizer proteins[35]. These inhibitors target anti-apoptotic BCL2 proteins by displacing sequestered activators from anti-apoptotic proteins to force jumpstart the apoptotic programming. Currently, BCL2 inhibitor venetoclax (ABT-199) is FDA approved for treating several hematological malignancies[36], but we still lack federally approved inhibitors for MCL1 and other anti-apoptotic proteins. Since myeloma cells are highly dependent on MCL1 for survival, MCL1 specific inhibitors might be even more efficacious in their treatment. Currently, we have three

MCL1 inhibitors undergoing phase I clinical trials, and many studies on myeloma cell lines and patient samples indicated sensitivity towards these new MCL1 inhibitors[37].

Despite recent advances in the development of new therapies for multiple myeloma such as immunomodulatory drugs (iMiDs) and proteasome inhibitors, additional approaches are required[13]. The recent CoMMpass database is a longitudinal study conducted by Multiple Myeloma Research Foundation (MMRF) that monitors over 1000 multiple myeloma patients following diagnosis and records various genetic sequencing data (i.e. whole genome sequencing, long insert sequencing, bisulfite sequencing, etc). Using the MMRF CoMMpass study (Interin Analysis 13), we determined the frequency of nonsynonymous coding mutations in the BCL2 family. Analysis of baseline samples from 982 patients revealed that mutations in the BCL2 family are relatively rare events. No mutations were observed in the 3 pro-apoptotic effector genes BAX, BAK1 and BOK. Similarly, in the BH3-only genes, mutations were rare with no mutations in BCL2L11 (BIM), BAD, BID, HRK and BMF and only single mutations in BBC3 (PUMA) and PMAIP1 (NOXA). In the anti-apoptotic BCL2 genes, mutations were also rare with no mutations in BCL2, BCL2L1 (BCLX) and BCL2L10 (BCLB). A single sample had a mutation in BCL2A1 (A1) while 2 samples had mutations in BCL2L2 (BCLW). Interestingly, MCL1 was mutated in 10 baseline samples (1.02%) and the frequency of the mutations in these samples was high (median 0.391, range 0.066-0.531). Therefore, we further investigated the functional consequences of the 10 MCL1 mutant alleles.

Of the 10 mutations detected, one was in the N-terminal region (G32R) and 4 were in the PEST domain in the N-terminal half of MCL1 that is associated with regulating protein stability

(V140I, P142S, E149Q and E173K). An additional mutation was found in an uncharacterized region between the PEST and BH1 domains (L186F). We focused on the 4 mutations that lie within or near the functional BH1 (V249L and L267V) and within the BH3 (N223S, and R214Q) domains (Figure 2A). The SNVs observed in MCL1 occur in varying allelic frequency and most of the mutations are clonal population when copy number amplification is considered (Figure 2B). As for progression free survival and overall survival, there is not much difference between patients with wild type and mutant MCL1 (Figure 2C). In order to study these mutations, wild type MCL1 and the four mutant MCL1 constructs will be introduced into MCL1-dependent murine B cell acute lymphoblastic leukemia (B-ALL) cell line that has endogenous murine MCL1 flanked with LoxP. Human MCL1 can replace murine MCL1 in this cell model, therefore we can determine if the myeloma-derived mutants of MCL1 can complement loss of mouse MCL1 and will report on these findings (Figure 3)[38].

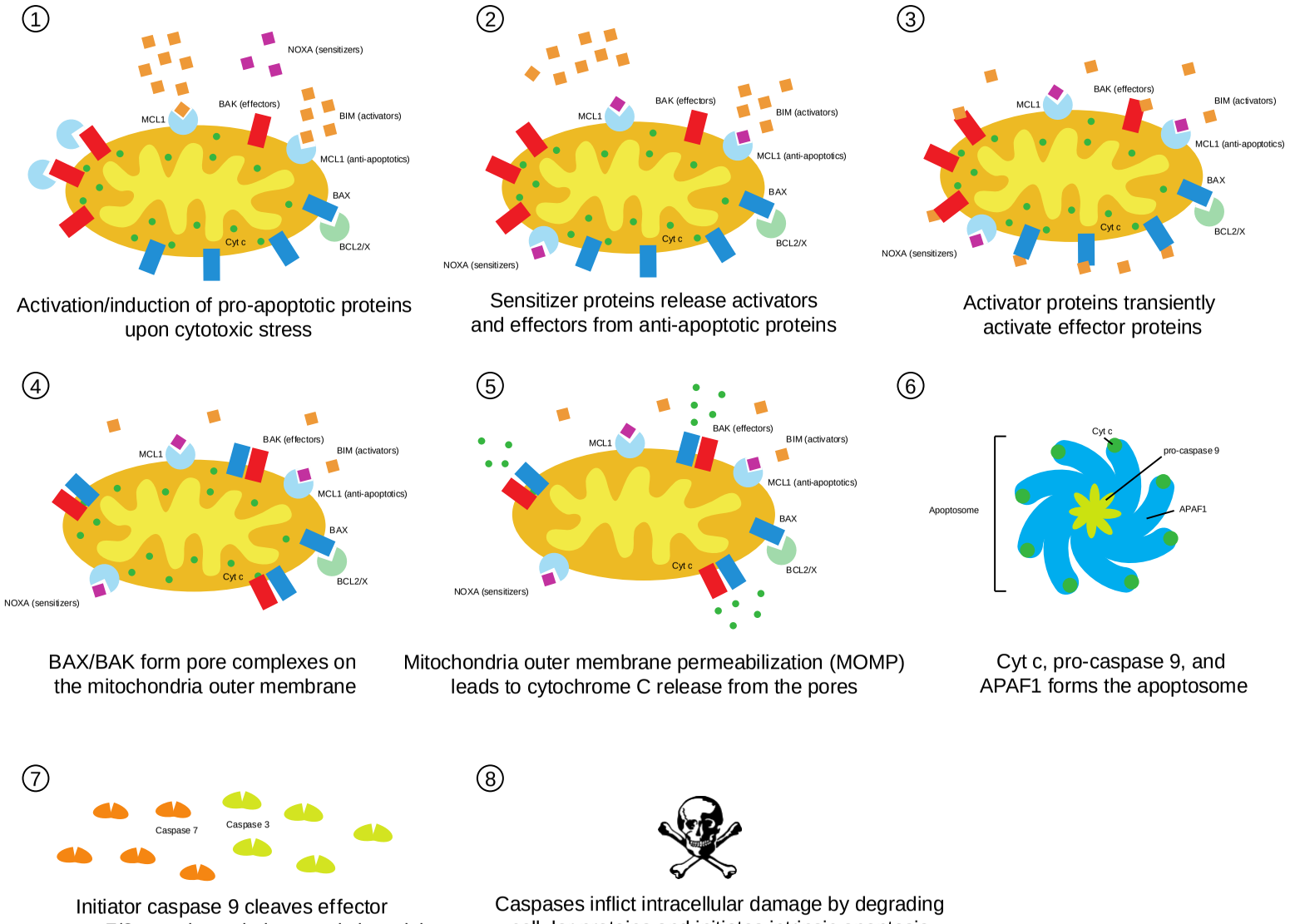
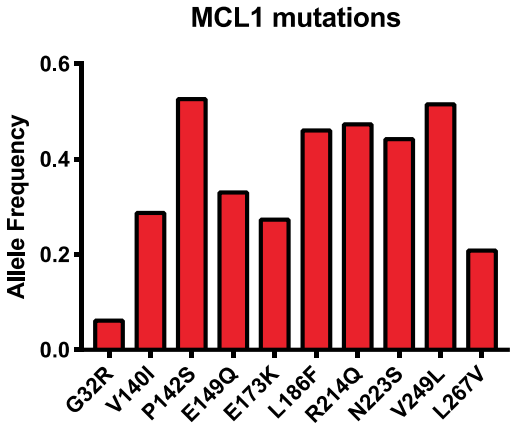
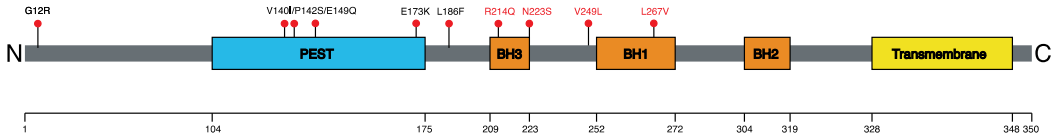


Figure 1. Schematic of the intrinsic apoptosis pathway.

A.


MCL1 mutations
Allele Frequency
0.6
0.4
0.2
0.0
G32R
V140I
P142S
E149Q
E173K
L186F
R214Q
N223S
V249L
L267V

B.


G12R
V140I/P142S/E149Q
E173K
L186F
R214Q
N223S
V249L
L267V
N
PEST
BH3
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Transmembrane
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223
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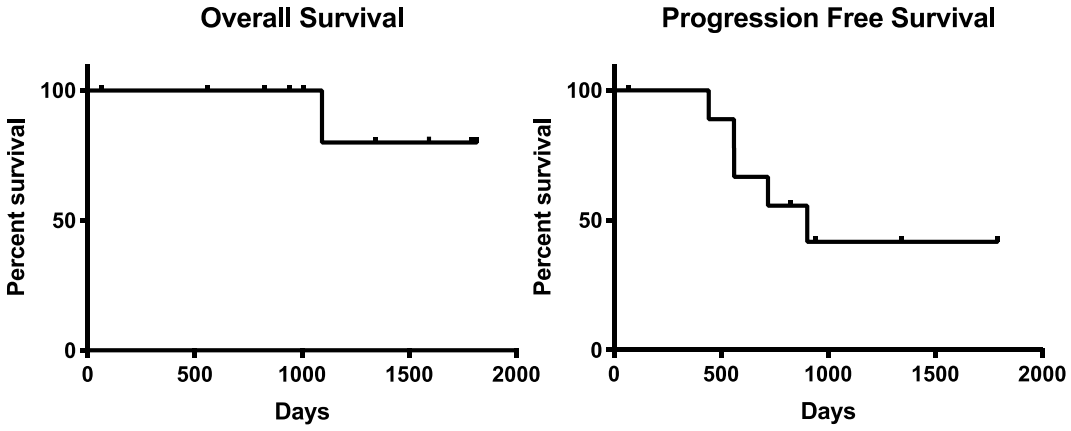
272
304
319
328
348
350

C.


Overall Survival
Percent survival
100
50
0
0
500
1000
1500
2000
Days
Progression Free Survival
Percent survival
100
50
0
0
500
1000
1500
2000
Days

D.

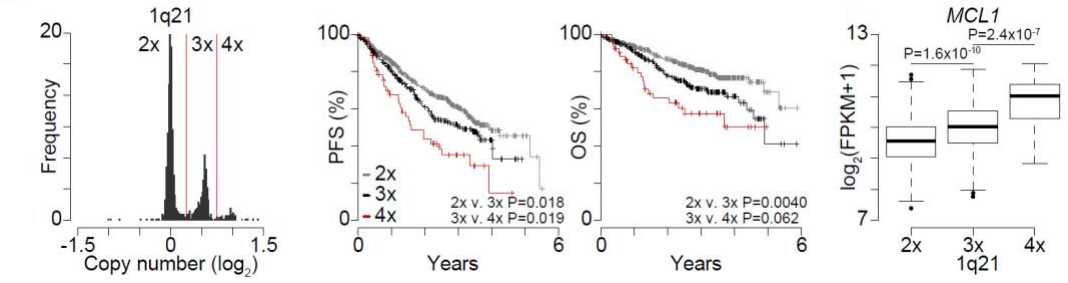


Figure 2. Single nucleotide variations (SNVs) in MCL1 from the CoMMpass database. (A) The 10 mutations and their respective allelic frequencies. (B) Schematic of MCL1 protein domains and relative locations of SNVs. (C) Overall survival (OS) and progression free survival (PFS) of patients with SNVs in MCL1. (D) 1q21 amplification is common in 40% of the patients, is associated with poor survival, and is correlated with MCL1 mRNA expression level.

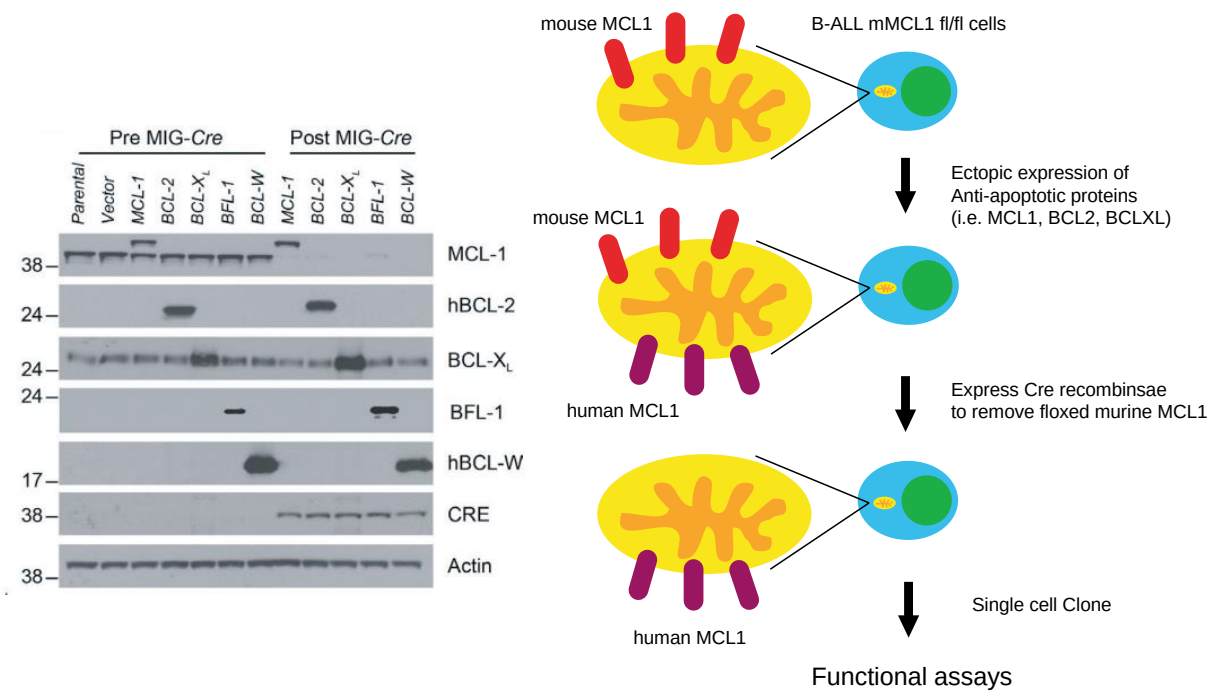


Figure 3. Schematic of the murine B-ALL MCL1$^{fl/fl}$ system. (Koss et al. *Oncotarget*, 2016)

Results

Cytotoxicity-inducing agents cannot differentiate between murine, wild type and mutant MCL1

Empty vector (EV), wild type (WT) and the four point-mutants (L267V, V249L, N223S, R214Q) MCL1 constructs were cloned into pBABE-puro retroviral vector then stably expressed in the murine $Mcl1^{fl/fl}$ B-ALL cells. Protein expression level was determined via western blot analyses, and all mutants had comparable level of MCL1 with the L267V mutant having slightly lower level (figure 4A). Next, the cell lines were subjected to increasing doses of cytotoxic drug treatments to induce apoptosis. We decided to use topoisomerase inhibitor etoposide, microtubule inhibitor vincristine and proteasome inhibitor bortezomib to induce cytotoxic stress. We treated EV, WT, and the four mutant MCL1 expressing cells with increasing concentrations of the three drugs and measured cell death (Annexin V/PI) at 24 hours. Nevertheless, we did not see any significant differences in cell viability across the control and experiment groups (Figure 4B/C/D). Since there was no difference even between EV and WT MCL1 expressing, we believed it was the presence of endogenous murine Mcl1 protecting the cell from apoptosis. Thus, murine Mcl1 confounded the effects of our ectopic mutant MCL1 expression. We will need to remove endogenous murine Mcl1 before fully understanding the effects of mutant MCL1 in protecting against cytotoxic stress.

Rodent MclL1 has decreased binding affinity for MCL1 inhibitors compared to human and other vertebrate species

With the new class of MCL1 inhibitors developed, different groups tested them on several organismal cells to test if the inhibitors had species specificity. The Fesik used sequence alignments and structures of human MCL1/inhibitor complexes to identify key differences in the

amino acid sequences. It was discovered that all MCL1 inhibitors made against human MCL1 are poor murine Mcl1 inhibitors due to amino acid differences[39]. The human L246 residue in the binding pocket of MCL1 is important in interacting with MCL1 inhibitors, whereas the amino acid residue F226 in rat and F227 in mouse have lower affinity towards current developed MCL1 inhibitors due to reduced compound complementarity (Figure 5)[39]. With this data supporting the fact that murine Mcl1 will not be affected by MCL1 inhibitors, I tested whether my mutant MCL1 cells will be sensitive to MCL1 inhibitors with the presence of murine Mcl1.

S63845-induced cell death is attenuated in cells expression MCL1^{L267V}

As mentioned previously, no inhibitor developed against human MCL1 is as effective an inhibitor of murine Mcl1. Therefore, we were able to test our MCL1 mutants with current MCL1 inhibitors. S63845 is a novel small molecular inhibitor that targets the BH3 binding pocket of MCL1. We treated EV, WT, and the four mutant MCL1 expressing cells with increasing concentrations of the MCL1 inhibitor S63845 and measured cell death (Annexin V/PI staining) at 24 hours. As expected, empty vector cells were highly resistant to S63845-induced cell death (less than 20% at 1000 nM) while cells expressing the human WT MCL1 were significantly more susceptible to the MCL1 inhibitor at 300 and 1000 nM. The V249L, N223S and R214Q mutations mimicked the sensitivity of the WT MCL1 suggesting they did not alter MCL1 function in the cells. In contrast, the L267V mutation resulted in a dose curve that was more similar to the empty vector control, suggesting this mutation was either resulted in loss of function or in an MCL1 molecule that could not be inhibited by the drug (Figure 6).

MCL1 is stabilized by occupying the BH3-binding pocket

Because MCL1 has a degradative PEST domain that subjects MCL1 to rapid ubiquitin-mediated degradation, it is a labile protein with a quick turnover rate. It was previously described that binding interaction with other BH3 proteins such as BIM could stabilize MCL1 protein level[40]. By knocking down BIM with siRNA, MCL1 protein was destabilized in RPCI-WM1 cell line. Nevertheless, proteasome inhibitor bortezomib delayed MCL1 degradation and thus maintained normal MCL1 levels despite the absence of BIM (Figure 7A). In addition, a functional BH3 domain is important in establishing stabilizing interaction with MCL1. When a BIM construct with mutant BH3 domain was expressed, it did not recapitulate the stabilization seen with its BIM counterpart with a WT BH3 domain (Figure 7B). Moreover, knockdown of BIM via siRNA destabilized MCL1 protein level across different multiple myeloma lines (Figure 7C)[41]. These results illustrated the stabilization phenotype in MCL1 when bound to functional substrates. Since drug binding can stabilize MCL1 by competing for E3 ligase binding, we could determine effective binding of S63845 if MCL1 protein level escalates. We found that within all the mutants, S63845 dramatically increased human MCL1 protein expression ruling out lack of drug binding (Figure 8).

$MCL1^{L267V}$ is stabilized by S63845 similar to $MCL1^{WT}$ but does not induce the release of BIM bound to the mutant

After ruling out the possibility that drug binding accounts for decreased death in L267V, we wanted to investigate if the mutation affected MCL1 protein-protein interaction (PPI). We next performed MCL1 co-immunoprecipitation assays and discovered that BIM release was correlated with S63845 sensitivity. In WT MCL1 expressing cells, treatment of S63845 released high levels of BIM from MCL1. On the other hand, the L267V did not effectively release BIM, NOXA and

BAK after S63845 treatment (Figure 9). Taken together, the L267V mutation did not prevent the binding of S63845 to free MCL1, rather it blocked the ability of drug to displace pro-apoptotic proteins required to induce cell death. Therefore, the inefficient cell death induction observed in L267V was due to the inability to release pro-apoptotic proteins from MCL1.

AZD5991 occupies a larger area within the MCL1 BH3 binding domain when compared to S63845

Since the functional reasoning behind increased S63845 resistance in L267V was determined, we were interested to see if different cell death phenotype among the four mutants could be extrapolated based on protein structure. We utilized the crystal structures to generate structures of MCL1 bound with MCL1 inhibitor S63845. The blue residues indicated wild-type amino acids, while yellow residues in the figures were indicative of mutant amino residues. From the structure, S63845 bound closely into the BH1 binding grove. The wild type leucine267 was right behind the binding pocket when S63845 interacts, whereas the mutant valine from L267V protruded more into the binding pocket and could potentially impede drug interaction. In addition to S63845, we were able to analyze another novel MCL1 inhibitor AZD5991. Structurally speaking, AZD5991 had a larger cyclic structure that can potentially occupy a larger surface area compared to S63845[42,43]. Thus, we also created a docking model of AZD5991 with MCL1. Unlike S63845, AZD5991 appeared to be in closer proximity to more mutant amino acid residues such as L249V and L186F (Figure 10). Therefore, we hypothesized that different amino acid residues other than L267V can affect AZD5991 sensitivity.

AZD5991 activity is impacted by additional MCL1 mutations

We treated the cells with increasing concentrations of the other MCL1 inhibitor AZD5991 and measured cell death (Annexin V/PI) at 24 hours. Similar to the viability curve conducted with S63845, WT cells were highly sensitive to MCL1 inhibition, and L267V mutation was completely resistant to AZD5991-induced apoptosis despite evidence of drug binding (Figure 12A). Interestingly, the other 3 mutations also resulted in diminished killing activity when compared to cells expressing the WT MCL1, suggesting that these mutations might influence drug function (Figure 12B). As hypothesized previously, we predicted AZD5991 might have effects on more MCL1 mutations since it occupied a larger surface area than S63845 (Figure 11). Together, these data suggested that myeloma-derived mutations in MCL1 might not necessarily influence MCL1 function, however they could alter responses to an emerging class of inhibitors.

BCL2 inhibition with venetoclax sensitizes MCL1 inhibition

Since multiple anti-apoptotic proteins can protect a cell from apoptosis, we decided to test if inhibition of BCL2, a commonly targeted anti-apoptotic protein, would change the cells' sensitivity to S63845. We conducted a viability assay with Annexin-V/PI staining and found that the B-ALL cells were resistant to venetoclax even at high dosage that was therapeutically irrelevant. We used 1 μM as a dose to conduct combination treatment since it did not kill any cell. We treated WT and L267V cells with 0 or 1 μM of venetoclax then combined with an increasing dose of S63845. The results indicated that BCL2 inhibition by venetoclax sensitized both WT and L267V mutants to S63845 (Figure 13A). Moreover, we conducted a co-immunoprecipitation assay on MCL1 before and after venetoclax treatment, and discovered that BCL2 inhibition increased MCL1 binding with BIM (Figure 13B). This increased binding pattern also slightly elevated MCL1 expression level since increased BIM binding stabilized MCL1 protein. This shift of

binding towards MCL1 could explain the sensitization for MCL1 inhibition since more MCL1 would be saturated with BIM.

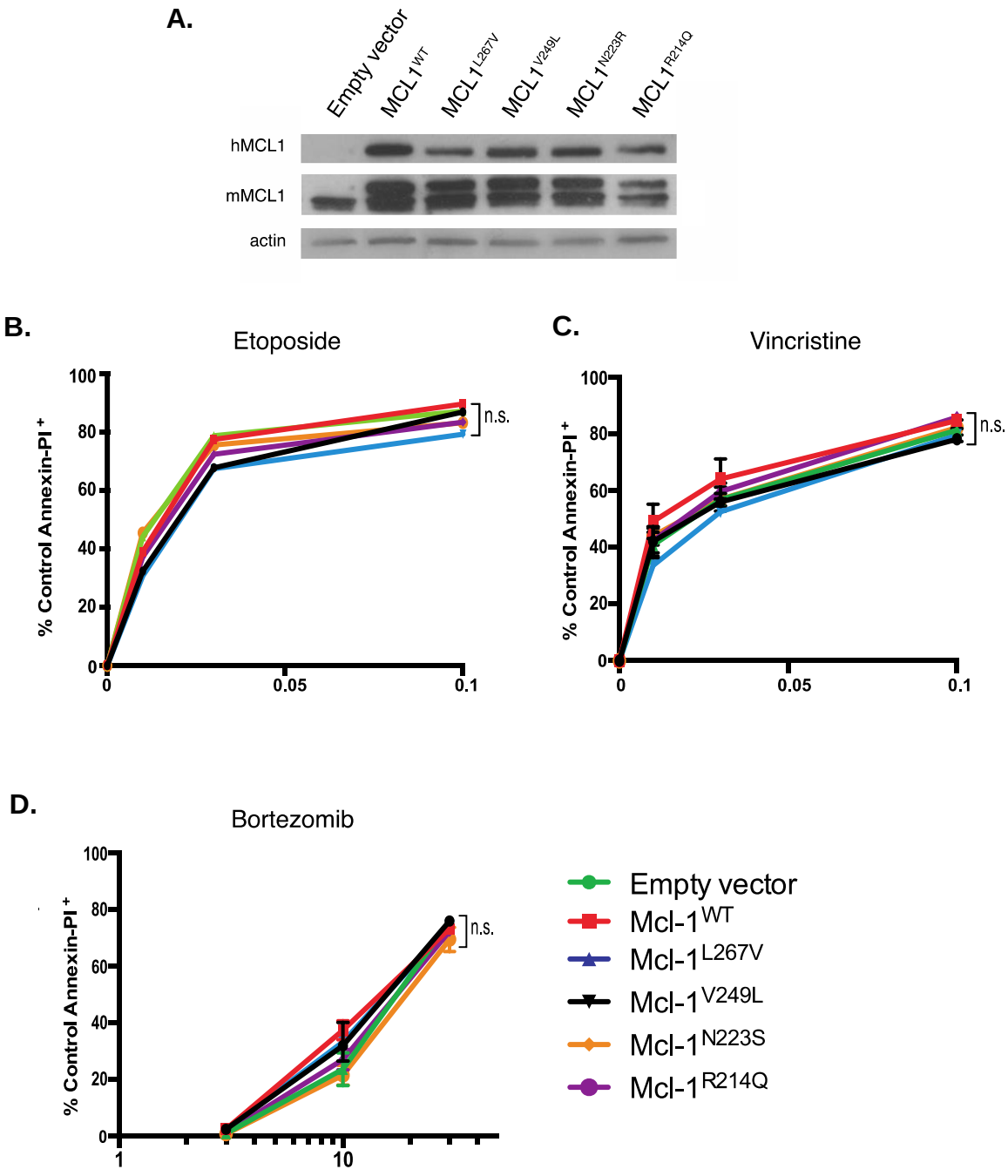


Figure 4. Cytotoxicity-inducing agents cannot differentiate between murine, wild type and mutant MCL1. (A) Western blot analysis of MCL1 protein expression. B-ALL cells were treated with increasing concentration of (B) Etoposide, (C) Vincristine, and (D) Bortezomib. Cell death

was measured by Annexin-V/PI 24hr post treatment. Data shown are mean of 3 independent experiments. Statistical significance was assessed using two-tailed t-test using GraphPad (NS $p>0.05$).

Figure 5. Rodent MCL1 has decreased binding affinity for MCL1 inhibitors compared to human and other vertebrate species. Amino acid sequence alignment of MCL1 across various vertebrate species (human, mouse, rat, canine, rhesus, ferret, rabbit). The amino acid difference in F226 (rat) or F227 (mouse) leads to an inability for all the current MCL1 inhibitors to bind efficiently to the BH3 binding pocket due to the large side aromatic group on phenylalanine. Figure derived from Zhao et al, *Biochemistry*, 2018.

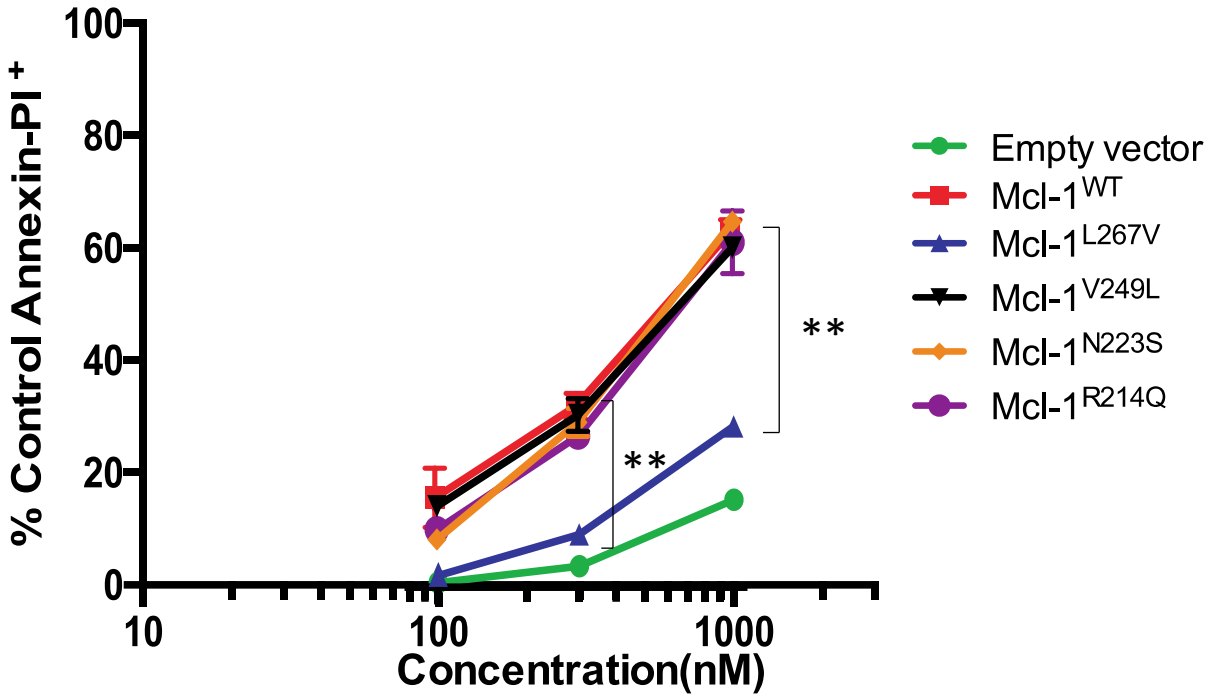


Figure 6. S63845-induced cell death is attenuated in cells expression MCL1^{L267V}. B-ALL cells with empty vector and different MCL1 constructs got subjected to 100, 300, and 1000 nM of S63856. Cell viability was measured through Annexin-V/PI staining 24 hours post treatment. *** indicates P value < 0.001. **** indicates P value < 0.0001. Data shown are mean of 3 independent experiments. Statistical significance was assessed using two-tailed t-test using GraphPad.

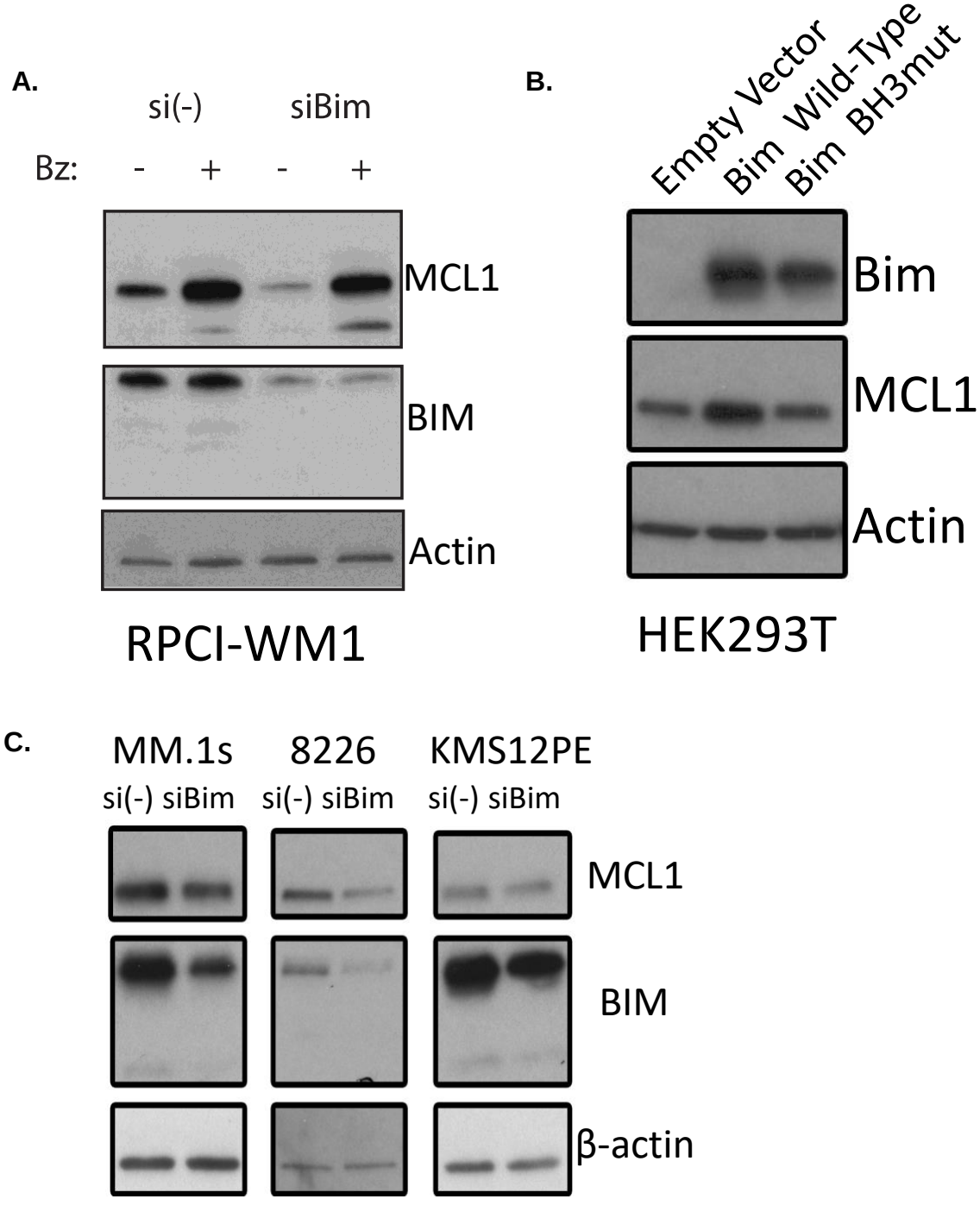


Figure 7. MCL1 is stabilized by occupying the BH3-binding pocket. (A) BIM knockdown led to destabilization but was rescued in the presence of proteasome inhibitors. (B) wild-type but not mutant BH3 domain of BIM is essential to stabilize MCL1. (C) Knockdown of BIM in three different multiple myeloma lines destabilizes MCL1 protein expression. Figured derived from Conage-Pough and Boise, *FEBS*, 2018.

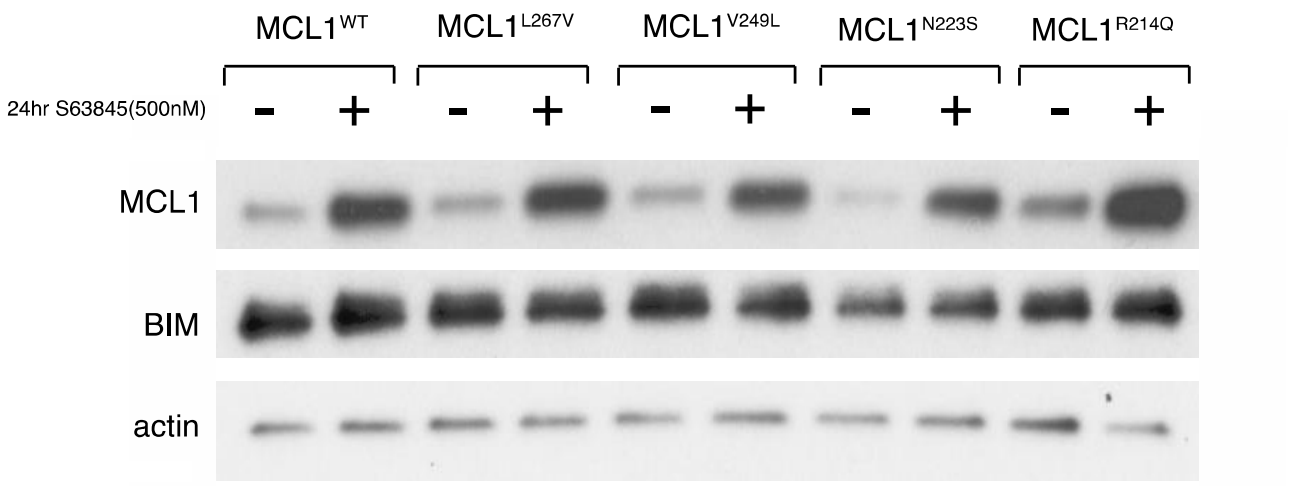


Figure 8. MCL1^{L267V} is stabilized by S63845 similar to MCL1^{WT}. We collected protein lysate from B-ALL cells expressing different MCL1 constructs after 24 hours of S63845 treatment. MCL1, BIM, and actin protein expression levels were probed with respective antibodies. We observed a stable increase of MCL1 protein level across all experimental groups upon S63845 treatment.

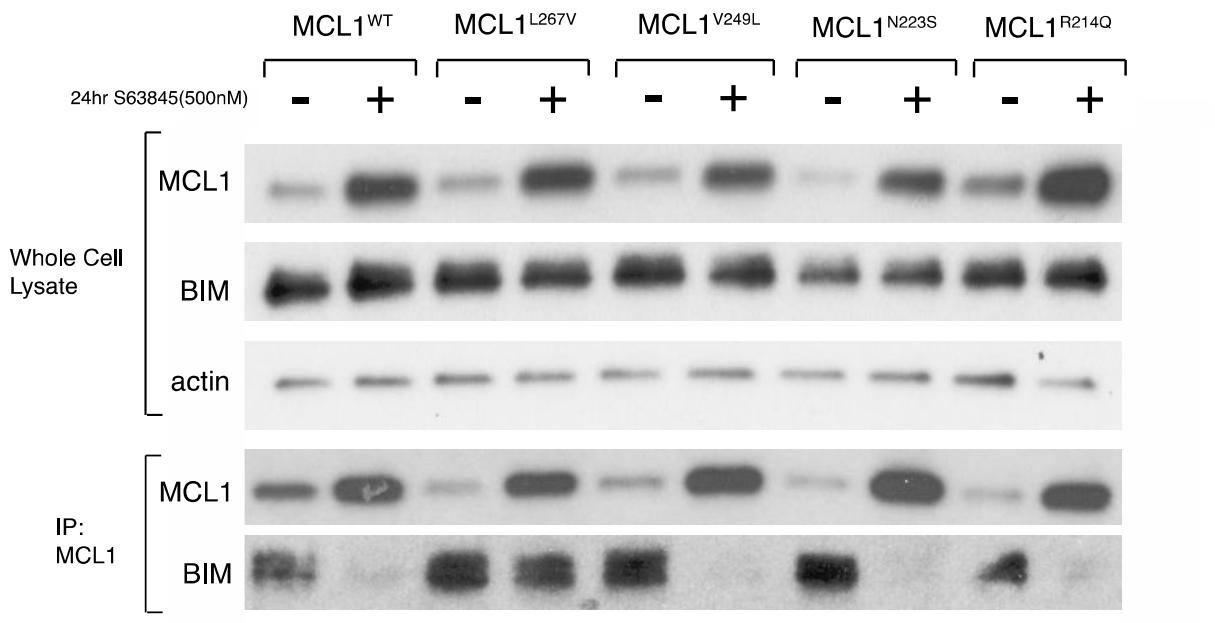
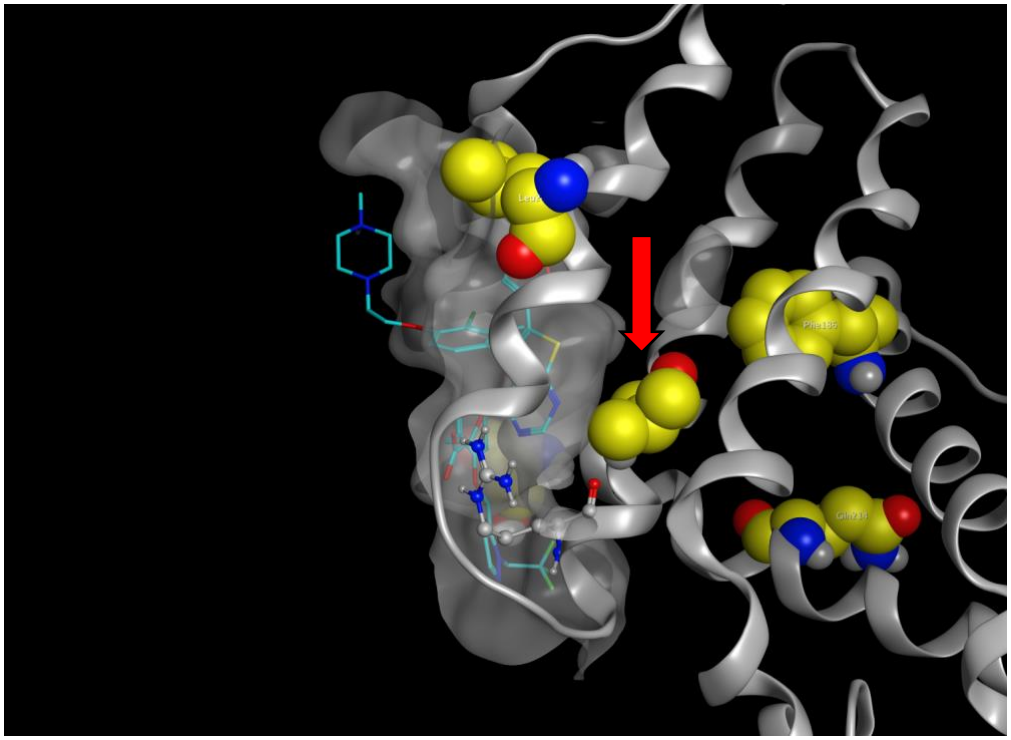


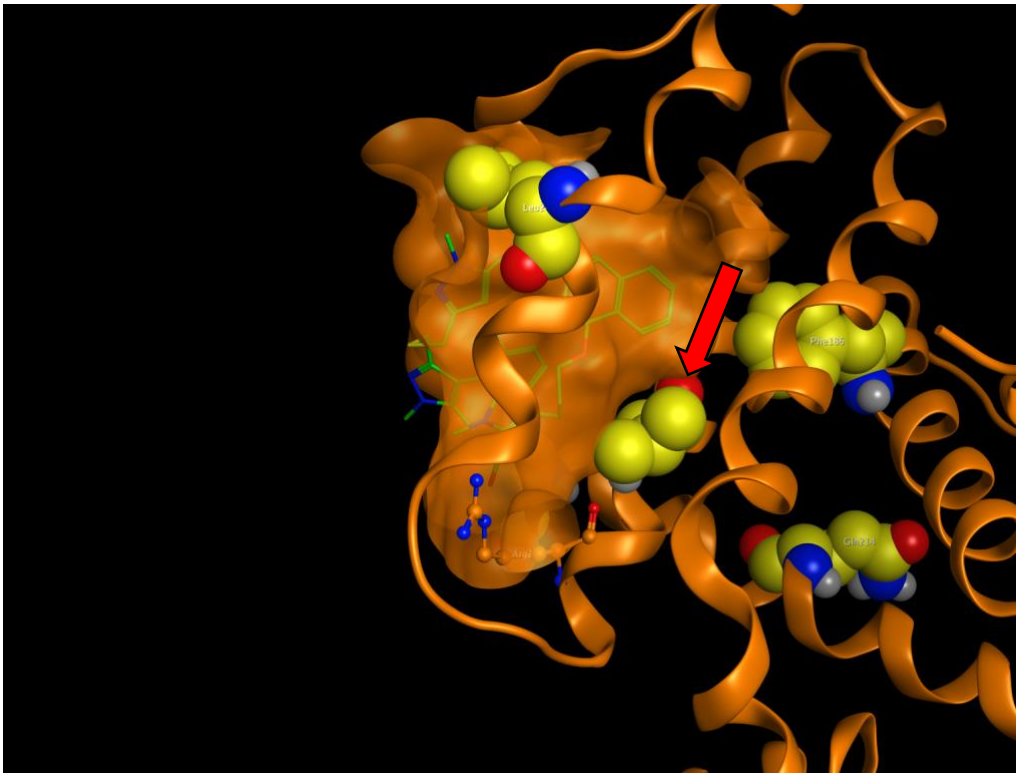
Figure 9. MCL1^{L267V} is stabilized by S63845 similar to MCL1^{WT} but does not induce the release of BIM bound to the mutant. We collected protein lysate from B-ALL cells expressing different MCL1 constructs after 24 hours of S63845 treatment. We subjected the protein lysate to co-immunoprecipitation of MCL1 and kept the whole cell lysate as control. MCL1, BIM, and actin protein expression levels were probed with respective antibodies. The IP results indicated the L267V mutation disabled MCL1 to release BIM when treated with S63845 unlike wild-type and other mutants.

A.



S63845

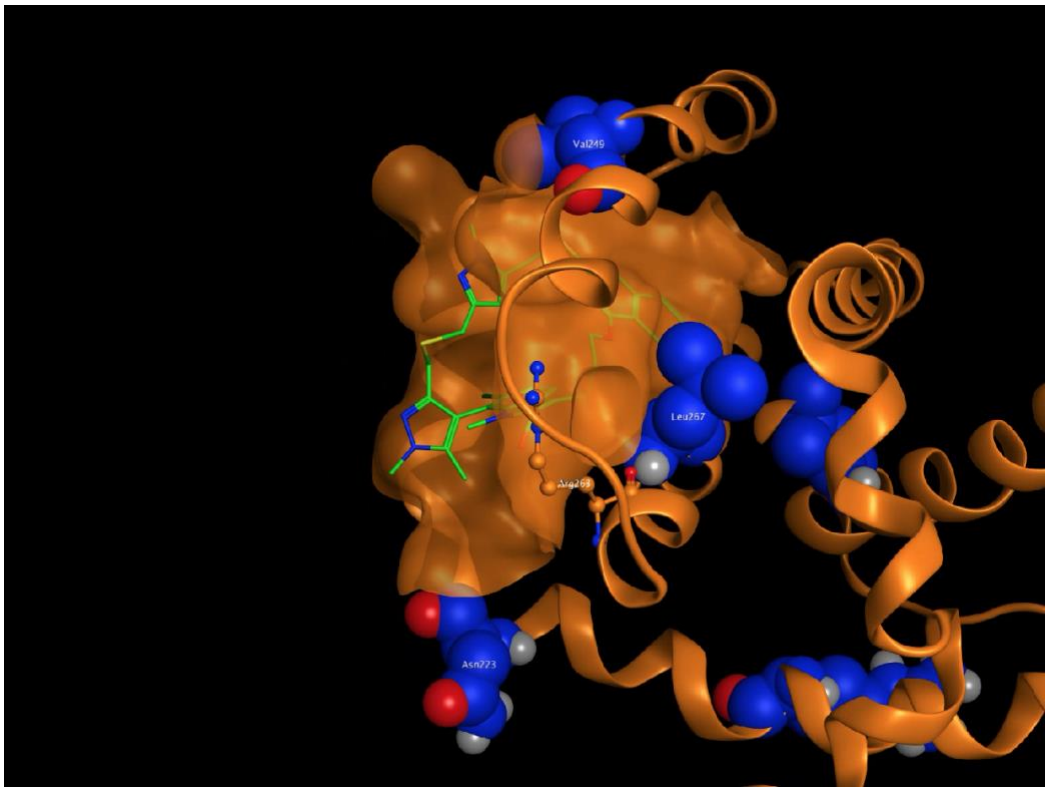
B.



AZD5991

Figure 10. AZD5991 occupies a larger area within the MCL1 BH3 binding domain when compared to S63845. From the docking model of MCL1 and MCL1 inhibitors: (A) S63845 (B) AZD5991, we observed that AZD5991 occupied a larger area in the BH3 binding grove than S63845 indicated by the stick figures. The yellow structure indicated the mutant amino acid residues in both (A) and (B). The red arrows pinpointed the L267V mutation that can impinge on both inhibitors. Models performed by Dr. Michelle Lamb, AstraZeneca.

A.



B.

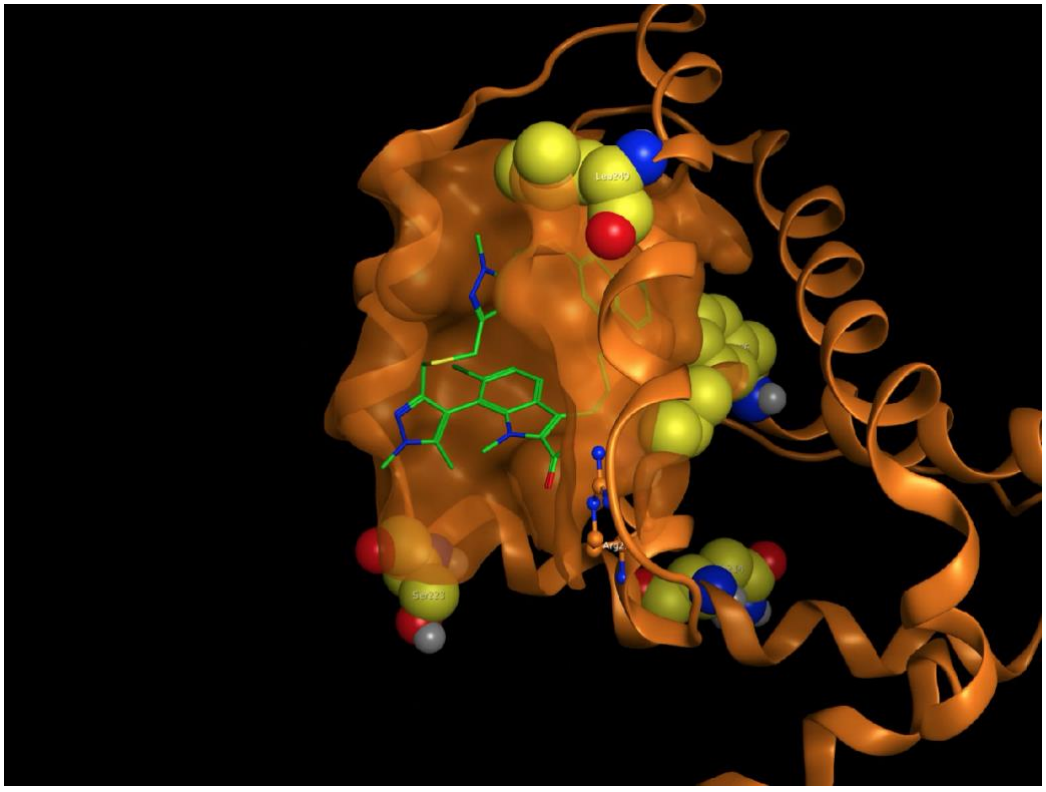


Figure 11. AZD5991 is a novel MCL1 inhibitor. The blue residues in (A) are the wild-type amino acids, whereas (B) illustrates the mutations in yellow. Due to the structural differences between wild-type and mutant amino acids, each mutation will potentially have varying effects on MCL inhibitors. Since AZD5991 occupies a larger area, it might be affected by more mutations. Models performed by Dr. Michelle Lamb, AstraZeneca.

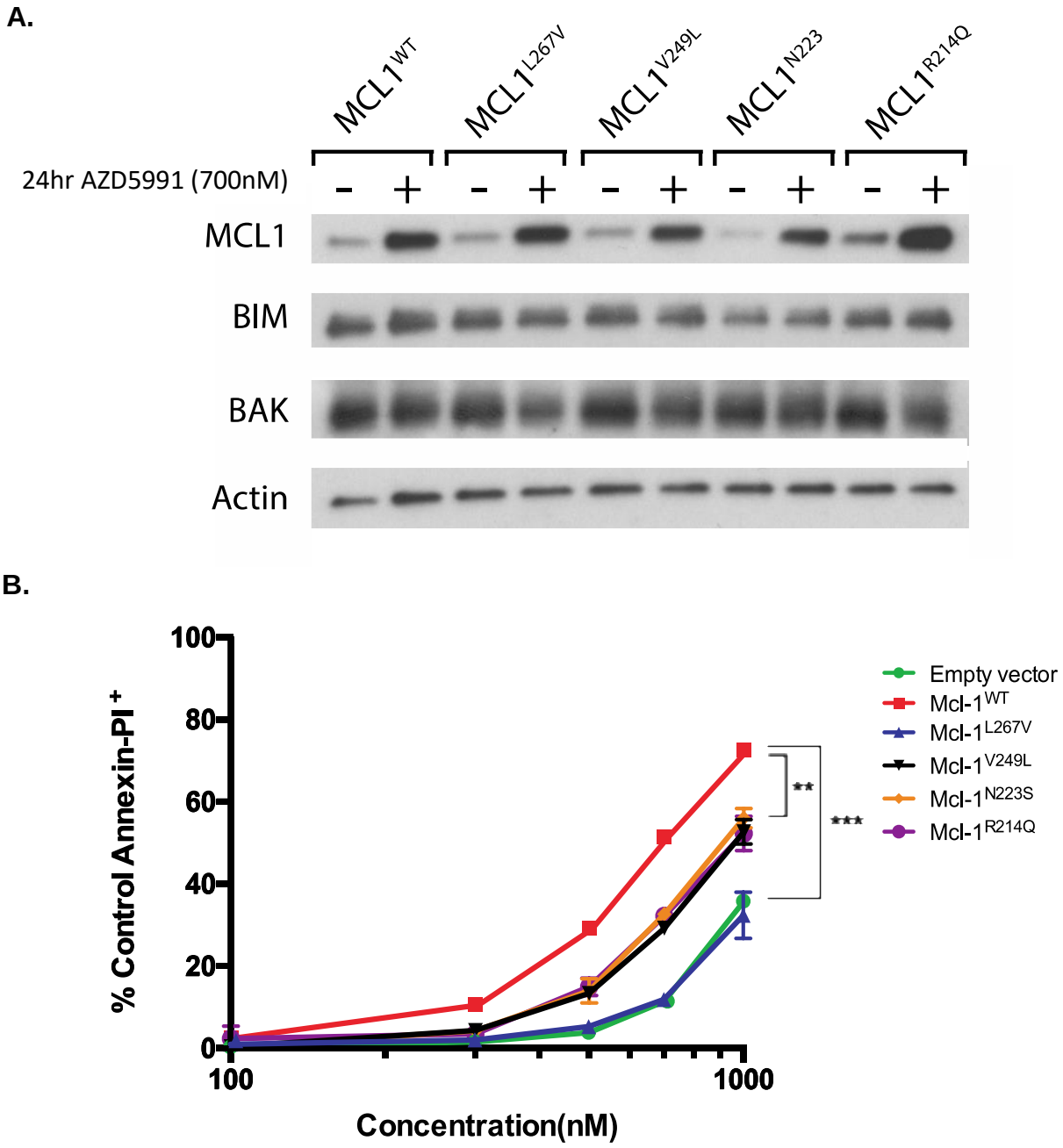


Figure 12. AZD5991 activity is impacted by additional MCL1 mutations. (A) AZD5991 stabilized MCL1 level similarly to S63845. (B) WT cells were highly sensitive to MCL1 inhibition and L267V mutation was completely resistant to AZD5991-induced apoptosis. The other 3 mutations also resulted in diminished killing activity when compared to cells expressing the WT MCL1. Data shown are mean of 3 independent experiments. Statistical significance was assessed using two-tailed t-test using GraphPad. ** indicates P value < 0.01. *** indicates P value < 0.001.

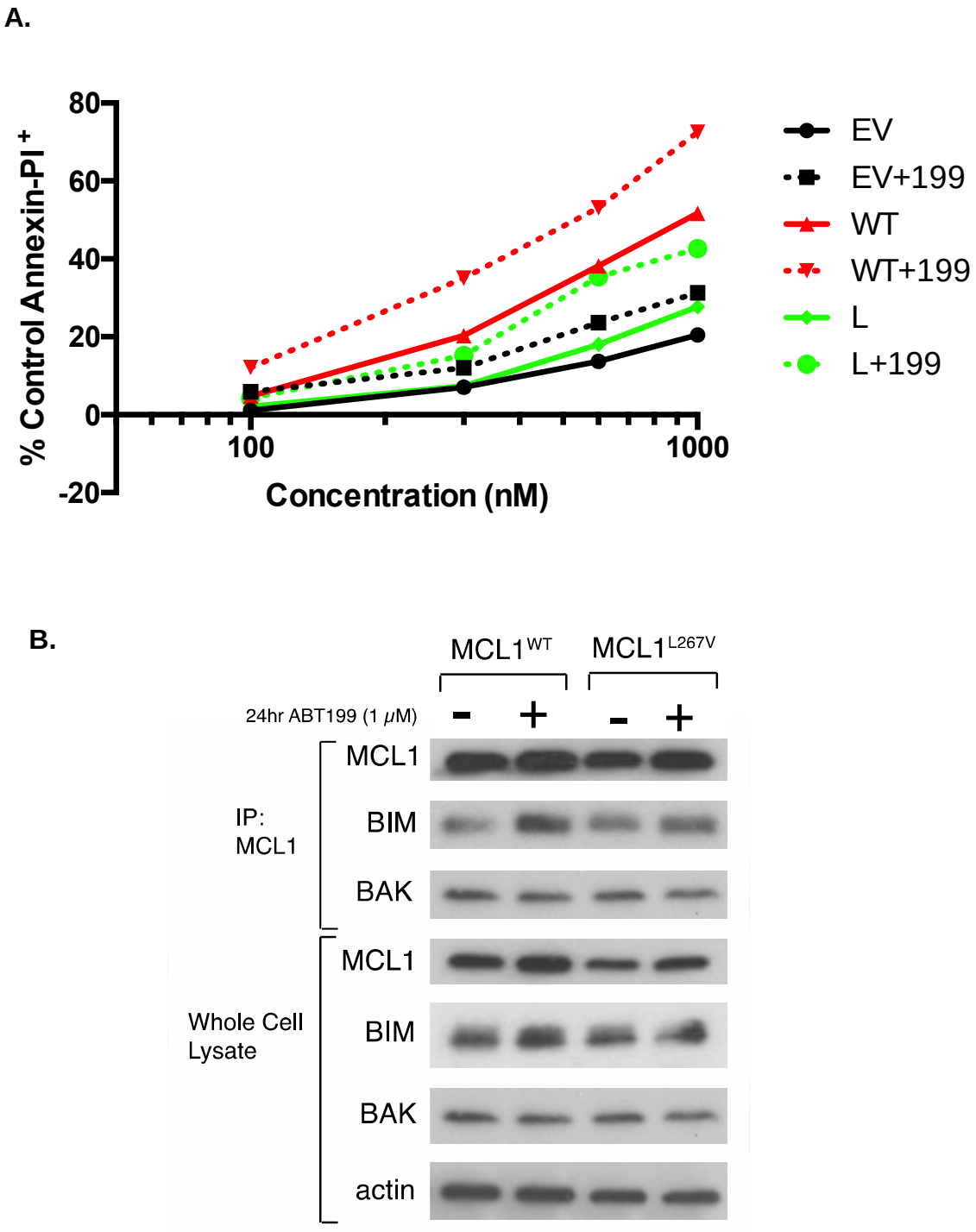


Figure 13. BCL2 inhibition with venetoclax sensitizes MCL1 inhibition. (A) Both WT and L267V MCL1 expressing B-ALL cells were sensitized to MCL inhibitors with non-lethal dose of venetoclax (1 μM). (B) BCL2 inhibition led to increase binding of BIM to MCL1. Data shown are mean of 3 independent experiments.

Discussion

Our study here described novel myeloma-derived point mutations in MCL1 that could potentially impede the mechanism of action for different MCL1 inhibitors. We focused on the mutations situated in proximity to the functional domain of MCL1 to investigate whether they could alter a cell's apoptotic threshold. We discovered that L267V point mutation led to decreased sensitivity towards MCL1 inhibitor S63845 and AZD5991 compared to wild type MCL1. The decreased cell death was attributed to the inability to release pro-apoptotic proteins such as BIM, BAK, and NOXA, whereas the WT and three other MCL1 mutations did efficiently release them.

This study of MCL1 described the first patient-derived MCL1 mutation recorded to display potential resistance pattern towards this new class of MCL1 inhibitors. Since S63845, AZD5991, and AMG176 are all on phase I clinical trial for multiple hematological malignancies, it is critical to understand potential resistance mechanisms. Since MCL1 inhibitors are still early in their trial stages, we are not able to study resistance phenotype from MCL1 inhibitor relapsed myeloma patients. Nevertheless, a similar study conducted by Blombery et. al, discovered a similar phenomenon in ventoclax relapsed chronic lymphocytic leukemia (CLL) patients[44]. As mentioned earlier, venetoclax is a potent and selective inhibitor of the anti-apoptotic protein BCL2. It is FDA approved for the treatment of previously treated CLL as monotherapy or can be combined with rituximab, a CD20 targeting monoclonal antibody[45]. When whole genome sequencing (WGS) was conducted in venetoclax relapsed CLL patient cells, a common G101V mutation in BCL2 was discovered as an acquired mutation that appeared only after venetoclax therapy. Gly101Val reduced the affinity of BCL2 for venetoclax by approximately 180-fold, thus preventing the drug

from displacing proapoptotic activators from BCL2 and conferring acquired resistance in cell lines and patient cells. From this study, we knew that a potential resistance to MCL1 inhibitors could be developed by acquiring mutations that impedes drug binding similarly to the G101V mutation in BCL2. This is also similar to the L267V mutation we found and characterized from the CoMMpass database. The only difference is that G101V mutation in BCL2 is acquired after venetoclax treatment, while L267V mutation in MCL1 is a naïve mutation. Therefore, understanding how MCL1 mutations affect protein-protein interaction and binding with MCL1 inhibitors can potentially give us insight into alternative treatments for MCL1 inhibitor resistant cancers in the future. Perhaps this L267V will appear in myeloma patients if they are resistant to MCL1 inhibitors.

In order to fully understand the effects of our mutant our B-ALL cells, we still have to complete the removal of endogenous Mcl1 via Cre recombinase expression. The Cre recombinase construct we obtained from the Opferman lab has a GFP tag, so we can utilize fluorescent activated cell sorting (FACS) to sort GFP^+ cells after viral transduction. With a cleaner background that does not have murine Mcl1, we can get a better resolution of the full effects of MCL1 mutations. In addition, I will like to conduct an *in vitro* binding assay between L267V mutant MCL1 and various MCL1 inhibitors to investigate whether the mutation directly inhibits drug binding. Since the G101V mutation discovered in BCL2 led to a 180-fold decrease in venetoclax binding affinity, I hypothesized a similar result can be observed in L267V mutant MCL1 and S63845. Currently, we are testing another mutation, L186F, that is closer to the BH1 domain of MCL1. From the docking model with S63845 and AZD5991, it indicated this L186F mutant phenylalanine could protrude into the alpha helix behind the BH3 binding pocket and impede its MCL1 structure integrity. On

the other hand, we are in the process of generating an S63845 resistant myeloma cell line. We decided to use KMS12-PE cell line that is highly sensitive to S63845 inhibition with an IC_{50} approximately 50 nM. After corroborating increased IC_{50}, I will subject these cells to whole exome sequencing (WES) to identify whether there is acquired L267V or other SNVs in MCL1.

Ever since MCL1 inhibitors have been developed and undergone phase I clinical trials, researchers started to research the potential application of combination treatment for multiple cancers[46]. From our experiment that combined non-lethal dose of venetoclax and S63845, we discovered that BCL2 inhibition shifted the anti-apoptotic dependency towards MCL1 and sensitized the cells to MCL1 inhibition. Additionally, pre-clinical data conducted on different multiple myeloma cell lines and patient samples indicated varying sensitivities to venetoclax and MCL1 inhibitor AZD5991[47]. This outcome suggested myeloma cells encompass variable heterogeneity for MCL1/BCL2 dependencies, and alluded to a potential combination treatment in the future. In addition to finding synergistic effects by targeting other anti-apoptotic BCL2 protein family, other groups have characterized potential synergistic combinations with drugs targeting the Mitogen Activated Protein Kinase (MAPK) and AKT/mechanistic Target of Rapamycin (mTOR) pathways[48-50]. Both MAPK and PI3K/AKT/mTOR signaling cascades govern a multitude of pro-survival signaling that drives carcinogenesis[50]. For example, MAPK can overcome cell cycle by interacting with the cyclin/cyclin-dependent kinases (CDKs)[51] and AKT phosphorylates pro-apoptotic protein BAD to inactivate its function[52]. From the examples given above, we could conclude that multiple pro-survival cell signaling pathways can cross-talk with one another to maintain cancer cell survival. Not only can we utilize MCL1 inhibitors to induce apoptosis in

MCL1-dependent myeloma cells, but we might be able to combine other therapeutic agents we currently have to broaden the function of this new class of drugs.

In conclusion, our study described a unique subset of multiple myeloma patient harboring different point mutations in the functional domain of MCL1. Although the SNVs only accounted for 1% of the total patient sample and did not show significant effect with progression-free and overall survival, we discovered some mutations could alter MCL1 function in the presence of MCL1 inhibitors. The L267V mutation in the BH1 domain had the most drastic effects since it gave cells expressing this mutant MCL1 resistance to S63856 and AZD5991. We subsequently figured out that the mutation incapacitated the inhibitors' functions to release pro-apoptotic proteins such as BIM and BAK. Moreover, we discovered that different mutations in MCL1 responded differently to S63845 and AZD5991. These differences observed between the two MCL1 inhibitors could be explained by structural variances in the inhibitors and also the locations of amino acid substitutions in MCL1. Together these data suggested that myeloma-derived mutations in MCL1 might not necessarily influence MCL1 function, however they could have potential to alter responses to an emerging class of MCL1 inhibitors where 3 drugs are currently in clinical trials.

Materials and Methods

Cell Culture

Mouse $p185^{+}$ $Arf^{-/-}$ B-cell acute lymphoblastic leukemia (B-ALL) were grown in RPMI (Life Technologies, CA) with 10% fetal bovine serum, 55 μM 2-mercaptoethanol, 2 mM glutamine, penicillin, and streptomycin. were cultured in Dulbecco's Modification of Eagle's Medium supplemented with 10% Fetal Bovine Serum, 100 unit/mL penicillin/streptomycin, 2 mM-glutamate, 1% non-essential amino acids, 1 mM sodium pyruvate, 1% HEPES buffer, and 55 μM 2-mercaptoethanol. Cells were cultured at 37 ℃ in 5% carbon dioxide. B-ALL stable cell lines were cultured in the presence of 0.50 μg/mL puromycin.

Polymerase Chain Reaction (PCR)

PCR was used to amplify Mcl-1 coding region insert from pcDNA-MCL1-1. 1X Taq Reaction Buffer, 200 μM dNTPs, 0.2 μM forward primer, 0.2 μM reverse primer, 35 ng or 50 ng of template DNA, and 1.25 unit/50 μL Taq Polymerase were used. The forward primer had the sequence 5' GGG GAA TTC CTA TCT TAT TAG ATA TGC CAA ACC 3' and the reverse primer had the sequence 5' GGG GGA TCC CAC CAT GTT TGG CCT CAA AAG AAA C 3'. Thermocycling conditions were as follows: 1) 95℃ for 30 seconds, 2)30 cycles of 95℃ for 30 seconds, 60 ℃ for 30 seconds, 68 ℃ for 2 minutes and 30 seconds and 3)72 ℃ for 5 minutes. PCR products were visualized using gel electrophoresis on 1% agarose gel dissolved in 1X TAE buffer with 1μg/mL of ethidium bromide.

Generation of pBABE-MCL1 Constructs

MCL1 coding region sequence was cloned into pBABE-puro using the recognition sites of BamHI and EcoRI. pBABE-puro was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid # 1764). The amino acid substitutions L267V, V249L, N223S, R214Q in MCL1 were generated by site-directed mutagenesis using QuikChange II XL Site- Directed Mutagenesis Kit from Agilent, following the manufacturer's protocol. Mutagenesis primers were designed using the QuikChange® Primer Design Program. The mutagenesis primers' sequences are as follows: L267V—5' ACT GGG GCA GGA TTG TGA CTG TCA TTT CTT TTG G 3' and 5' CCA AAA GAA ATG ACA GTC ACA ATC CTG CCC CAG T 3' ; V249L—5' CTG AAA ACA TGG ATC ATC AAT CGA GAC AAC GAT TTC ACA TCG 3' and 5' CGA TGT GAA ATC GTT GTC TCG ATT GAT GAT CCA TGT TTT CAG 3'; N223S—5' CCG TCT CGT GGC TGC GCT GCA CGC C 3' and 5' GGC GTG CAG CGC AGC CAC GAG ACG G 3'; R214Q—5' GCG CTG GAG ACC TTA CAA CGG GTT GGG G 3'. Site-directed mutagenesis products were sent for sequencing to confirm the incorporation of the intended mutations. Generation of mutant MCL1 was conducted by Pamela Wong, BS.

Retroviral Transduction to Generate Stable Cell Lines

Phoenix-Ecotropic (Phoenix) cell line was transfected by a plasmid (pBABE-puro, pBABE-MCL1^{WT}, pBABE-MCL1^{L267V}, pBABE-MCL1^{V249L}, pBABE-MCL1^{N223S}, pBABE-MCL1^{R214Q}, pBABE-MCL1^{L186F}) using lipofectamine 2000. 2,000,000 B-ALL cells were seeded onto a 6-well plate. Viruses generated by the Phoenix cells were used to infect B-ALL cells. The virus-containing supernatant of the Phoenix cells was filtered through 0.45-μm syringe filters at 36, 46, and 60 hours after transfection and was applied to the B-ALL cells with Polybrene Infection

Reagent. 24 hours after the last infection, the viral supernatant was replaced with fresh media for 24 hours. B-ALL cells were then selected with 0.50 μg/mL puromycin.

Western Blot Analysis

Cells were washed in PBS and then lysed in desirable volume of RIPA buffer supplemented with Protease Inhibitor Cocktail and PMSF protease inhibitor. A BCA Protein Assay kit was used to determine total protein concentration. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-page) was performed on 30 μg of proteins and transferred to nitrocellulose membranes. The membrane was blocked with 5% milk at room temperature for 1 hour, and then incubated with a primary antibody overnight. The membrane was then washed with 1X TBST 3 times for 5 minutes each and incubated with a secondary antibody for 1 hour. After the secondary incubation, 3 more 5 minutes TBST washes. The membranes were exposed to enhanced chemiluminescence solution (ECL) and developed on high performance chemiluminescence films () with a developer.

Co-immunoprecipitation

Immunoprecipitation (IP)-antibody matrixes were formed with 3 μg mouse anti-Mcl-1 monoclonal antibody and 25 μL of Protein G, incubated on rocker at 4 °C overnight. Wild-type and mutant B-ALL cells were treated with vehicle or 1 mM S63845 to prior to lysate collection. Cells were collected and lysed in CHAPS lysis buffer (10 mM HEPES, pH 7.2, 150 mM NaCl, 2% CHAPS) supplemented with Protease Inhibitor Cocktail and PMSF protease inhibitor. Total protein concentration was measured by BCA Protein Assay Kit. Next, 200-300 μg of protein was incubated with 50 μL of Protein G for 1 hour on rocker at 4 °C for preclearing. The precleared

lysate was then incubated with the Immunoprecipitation (IP)-antibody matrixes on rocker at 4 °C overnight. The matrix was pelleted and washed twice with PBS, and the proteins were eluted using a reducing electrophoresis sample buffer with RIPA for western blot analysis as described above.

Viability Assay

Cells were collected at indicated time points, washed with 3 mL of PBS, and stained with 2 μg/mL of Propidium Iodide in 500 μL of 1X Annexin V Binding Buffer. Flow cytometry on a BD FACSCanto II system with FACSDiva software was used to measure percent viability. FlowJo software was used to analyze the obtained data.

Drug Treatment

In 24 well-plates, 250,000 B-ALL cells were resuspended in 1 mL of B-ALL media and treated with 0.01, 0.03, and 0.1 μg/mL of Vincristine, 0.01, 0.03, and 0.1 μg/mL of Etoposide, 3, 10, 30 μg/mL of Bortezomib, 100, 300, 1000 nM of S63845, 100, 300, 500, 700, 1000 nM of AZD5991. Cells were collected at 0 and 24 hours after drug treatment for cell death analysis as described above.

Statistical Analysis

Statistical analysis was performed on experiments with at least 3 independent replicates. To determine the statistical significance of difference in cell death between two sets of samples, 2-sample-2-tailed-t-tests were used.

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