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SELECTIVE SMALL MOLECULE TARGETING OF MCL-1 IN MULTIPLE MYELOMA

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SELECTIVE SMALL MOLECULE TARGETING OF MCL-1 IN MULTIPLE MYELOMA

by

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A Thesis

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Dedications

I would like to dedicate this work to my father Dr. Sami Faleh Al-Odat. Thank you for all your support; without you, I would not be here today. You are my source of inspiration.

To my mom Mrs. Manal. Thank you for your continuous love, support, and constant motivation all the time.

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Abstract

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Multiple Myeloma (MM) is a deadly blood malignancy, characterized by the uncontrolled proliferation of aberrantly differentiated plasma cells. MM is challenging to diagnose and treat, accounting for approximately 12% of hematologic malignancies. The overexpression of anti-apoptotic group of Bcl-2 family proteins, particularly Myeloid cell leukemia 1 (Mcl-1), play a critical role in the pathogenesis of MM. The overexpression of Mcl-1 is associated with drug resistance and overall poor prognosis. Thus, inhibition of the Mcl-1 protein is an attractive therapeutic strategy against myeloma cells. Over the last decade, the development of selective Mcl-1 inhibitors has seen remarkable advancement. In this project, we investigated the effect of the novel Mcl-1 inhibiting agent KS18 on MM cells. We demonstrated the molecules *in vitro* efficacy as well superior potency towards MM. However, Mcl-1 inhibition by KS18 was associated with a significant reduction of MM cell viability. Moreover, we observed that KS18 was able to induce apoptosis in MM cells in a caspase-dependent manner. Our results propose that targeting Mcl-1 by KS18 may represent a new viable strategy for MM treatment. Furthermore, the present study uncovers the mechanism of action of KS18 and provides the foundation for *in vivo* assessment of this novel molecule.

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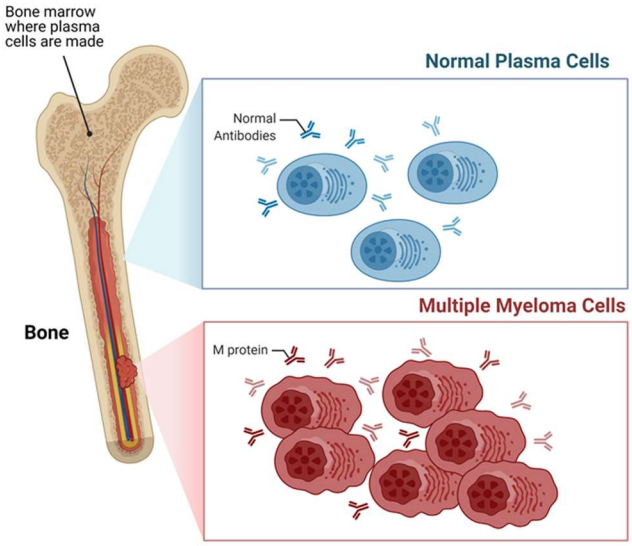
Mcl-1 Inhibition: Managing Malignancy in Multiple Myeloma

Introduction

The plasma cells are a type of unique B cells that reside in the bone marrow (BM) and secrete an antibody corresponding to the antigen. When these plasma cells begin proliferating out of control, they can build up within the BM and form numerous tumors (Figure 1). This type of neoplasms is called Multiple Myeloma (MM) and is considered the second most common hematologic malignancy, accounting for around 12% of hematological malignancies [1]. MM is slightly more common among older men, with a median age of 65 years, and it is rarely diagnosed in younger people [2-4]. Depending on the stage of the disease, symptoms of MM begin with abnormalities in the bone and calcium homeostasis, low blood cell counts, renal insufficiency, and multiple infections. Because the symptoms are so generalized, MM is a challenging disease to diagnose. Furthermore, the protective role of the BM on the proliferating plasma cells makes it even more challenging to treat.

Figure 1

Multiple Myeloma (MM)



Note. MM is a type of blood cancer that initiates from the bone marrow (BM), arising from the aberrant proliferation of plasma cells.

In the last several decades, the treatment options for MM have dramatically improved. Unfortunately, the survival rate is marginal [5]. According to the American Cancer Society 2021 estimation, approximately 34,920 new MM cases will be diagnosed (19,320 men and 15,600 women), approximately 12,410 cancer deaths (6,840 men and 5,570 women) from MM alone in the United States [6]. Table 1 illustrates the common drugs that have been used to treat MM patients. Most therapeutic approaches to date for MM patients, especially in relapsed/refractory (R/R) cases have been based on combined formulations of available therapies. In spite of the efficacy and diversity of therapeutic

approaches, drug resistance is a major challenge as MM continues to show high rates of relapse and quickly acquired resistance to therapies [7]. There are several unanswered questions regarding MM including: what are the causes of progression of MM from its precursor state? Why MM patients instigate to relapse? How MM clones resistant to drugs persist in the presence of effective therapies?

Table 1

Mechanism of Action and Side Effects of Common Therapy in MM

Drug	Mechanism of Action	Side Effects	Ref.
Melphalan	Chemotherapy drug	Bone marrow damage and chemotherapy side effects.	[8]
Thalidomide (Thalomid).	Immunomodulating agent	Drowsiness, fatigue, constipation, and painful nerve damage as well as severe birth defects when taken during pregnancy.	[9]
Bortezomib (Velcade)	Proteasome inhibitor	Vomiting, tiredness, diarrhea, constipation, decreased appetite, fever, lowered blood counts and nerve damage.	[10]
Lenalidomide (Revlimid)	Small molecule analogue of thalidomide	Drowsiness, fatigue, constipation, and painful nerve damage as well as severe birth defects when taken during pregnancy.	[11-13]
Carfilzomib (Kyprolis)	Proteasome inhibitor	Tiredness, nausea, vomiting, diarrhea, shortness of breath, fever and low blood counts and occasionally more serious problems such as pneumonia, heart problems, and kidney or liver failure.	[14]
Pomalidomide (Pomalyst)	Small molecule analogue of thalidomide	Same thalidomide side effects with a less risk of nerve damage side effect.	[15]
Panobinostat (Farydak)	Oral Histone deacetylase (HDAC) inhibitor	Feeling tired, weakness, nausea, diarrhea vomiting, loss of appetite, fever, swelling in the arms or legs, and occasionally altered blood	[16]

Drug	Mechanism of Action	Side Effects	Ref.
		cell counts and blood electrolytes. Rare cases of internal bleeding, liver damage, and changes in heart rhythm which can sometimes be life threatening.	
Ixazomib (Ninlaro)	Oral Proteasome inhibitor	Nausea, vomiting, diarrhea, constipation, swelling in the hands or feet, back pain, lowered blood platelet count and nerve damage.	[17]
Daratumumab (Darzalex)	Intravenous monoclonal antibody	Coughing, wheezing, trouble breathing, throat tightness, runny nose, nasal congestion, feeling dizzy or lightheaded, headache, rash, nausea, fatigue, back pain, fever, and lower blood cell counts.	[18]
Elotuzumab (Empliciti)	Intravenous monoclonal antibody	Chills, feeling dizzy or lightheaded, wheezing, trouble breathing, cough, tightness in the throat, runny nose, nasal congestion, upper respiratory tract infections and pneumonia, rash, fatigue, loss of appetite, diarrhea, constipation, fever, and nerve damage	[19]
Selinexor (Xpovio)	Oral Nuclear export inhibitor of XPO1	Diarrhea, nausea, vomiting, loss of appetite, weight loss, low blood sodium levels susceptibility to infection, low platelet counts, and low white blood cell counts,	[20]

Mcl-1 Protein as a Potential Target for Multiple Myeloma (MM)

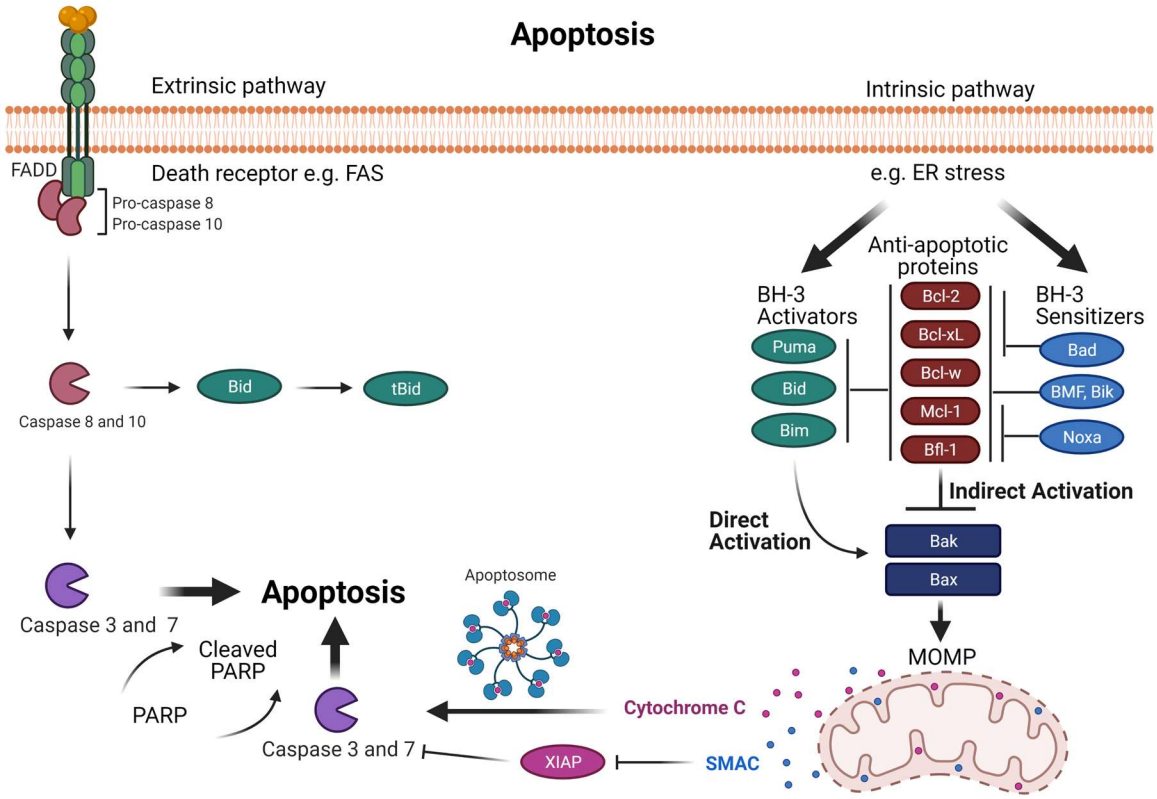
Apoptosis is a vital procedure for regular development and maintaining tissue homeostasis. Mammalian apoptosis occurs via one of two distinct pathways, either the intrinsic or extrinsic pathways (Figure 2). Both the intrinsic and extrinsic pathways end with the activation of a certain group of protease enzymes called Caspase proteins. The intrinsic pathway entails mitochondrial outer membrane permeabilization (MOMP) that regulated directly by interactions between B cell lymphoma 2 (Bcl-2) family proteins. The Bcl-2 family proteins are critical regulators of apoptosis. The members of this family proteins are divided into three groups according to function: anti-apoptotic proteins (Bcl-2, Mcl-1, Bcl-xL, Bcl-W, and Bfl-1); pro-apoptotic BH3-only proteins (Noxa, Puma, Bim, Bid, Bad, BMF, and Bik); and multi-domain pro-apoptotic proteins (Bax, Bak, and Bok). Intrinsic pathways like cytokine deprivation or DNA damage promote overexpression and activation of BH3-only proteins, which stimulate apoptosis in two different ways. First, the BH3-only proteins behave as inhibitors of anti-apoptotic proteins by competing for their binding with Bax and Bak proteins [21] (Figure 2). This is accomplished via the amphipathic α-helix of the BH3 domain that contains four hydrophobic residues (h1-h4) that bind four hydrophobic pockets (P1−P4) within the anti-apoptotic proteins in their BH3 binding groove [22-24]. For example, Noxa selectively inhibits Mcl-1 with high affinity binding thereby indirectly activating the Bax/Bak pathway [25, 26]. Simultaneously, BH3-only proteins can also result in the direct activation of multi-domain pro-apoptotic proteins Bax and Bak, which cause

MOMP, leading to release of Cytochrome C and SMAC proteins into the cytosol resulting in downstream Caspase activation and ultimately activation of apoptosis [27].

The extrinsic pathway is promoted by death receptors activation. This leads to activation of initiator Caspases 8 and 10, which can directly induce the downstream executioner Caspase such as Caspase 3 and 7 to drive full commitment to apoptosis [28]. Moreover, Caspases 8 and Caspase 10 can activate Bid, which in turn activates Bak and Bax to induce MOMP, which is the connecting link between the extrinsic and intrinsic pathways [28] (Figure 2).

Figure 2

The Programmed Cell Death Via Intrinsic and Extrinsic Pathways in Normal Mammalian Cells



Note. Intrinsic and extrinsic pathways result in the activation of a family of protease enzymes called Caspase proteins. The intrinsic pathway is promoted by cellular stresses that modulate Bcl-2 family proteins and activate Bak and Bax. In the indirect activation, upregulation of BH3-only proteins will act as inhibitors of anti-apoptotic proteins by competing for their binding with Bax and Bak proteins, leading Bax and Bak to oligomerize. In the direct activation, upregulation of BH3 activators proteins directly activates Bax and Bak. This activation leads to mitochondrial outer membrane

permeabilization (MOMP), subsequently Cytochrome C and SMAC proteins release into the cytosol, causing the downstream of Caspase activation that ends with apoptosis. The extrinsic pathway is promoted by death receptors activation. This leads to activation of initiator Caspases 8 and 10, which can regulate the downstream executioner Caspase such as Caspase 3 and 7 to drive full commitment to apoptosis. Moreover, Caspases 8 and 10 can activate Bid, which in turn activates Bak and Bax to induce MOMP which establishes the link between the extrinsic and intrinsic pathways.

The mitochondrial membrane engages Mcl-1 with other Bcl-2 family partners for the initiation of apoptosis. The interaction between the family members determines the outcome [26]. Mcl-1 has a diverse localization within human cells. It is primarily found within the mitochondrial outer and inner membranes [29]. However, studies have reported its localization in the nucleus and cytoplasm of polymorphonuclear leukocytes (PMNs) [30]. How different localization affects the function and its stability is not known.

The studies of Kozopas et al. first proved a high Mcl-1 expression in a differentiating human myeloid leukemia ML-1 cell line [31]. Subsequently, it was shown to be expressed in several different cells as well. The MM cells exhibit imbalances in their anti-apoptotic proteins expression levels, especially Mcl-1 that leads to defects in the mitochondrial intrinsic pathway [32, 33]. In order to prevent apoptosis and allow continued cell growth, Mcl-1 forms a heterodimer protein-protein interaction with multi-domain pro-apoptotic proteins Bax and Bak [34, 35]. Mcl-1 is known to be highly

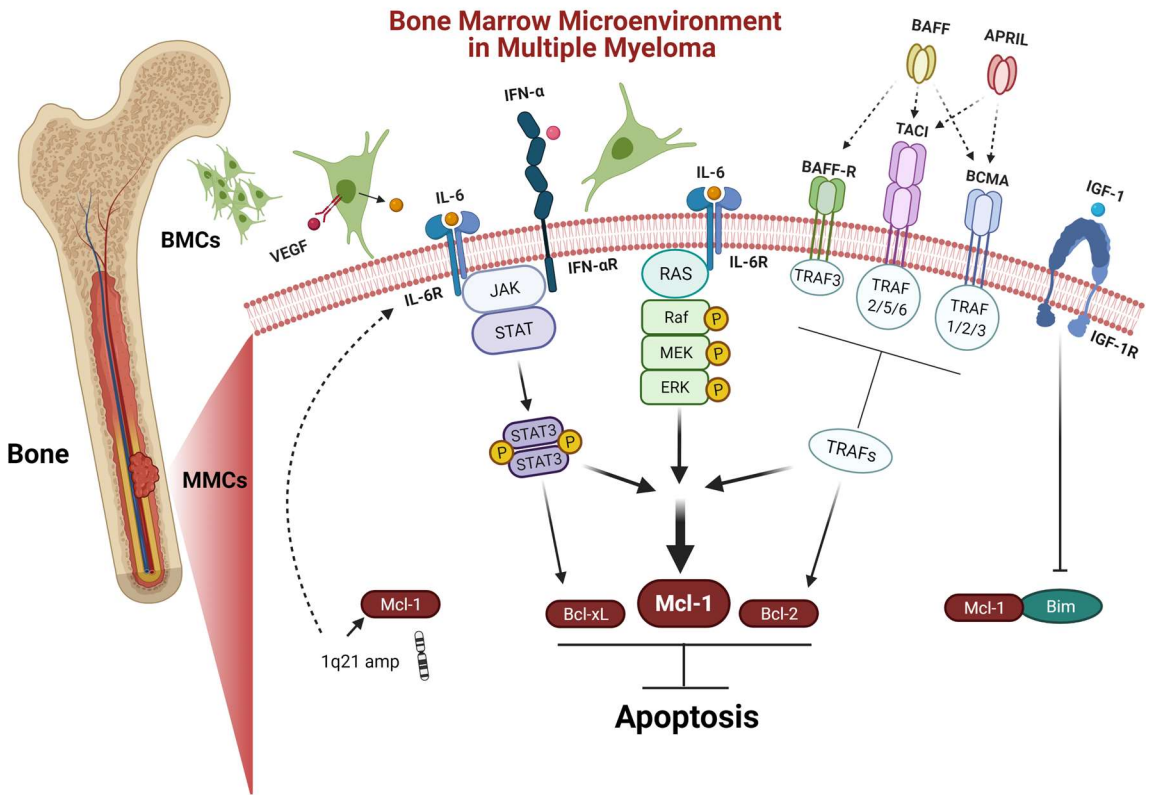
expressed in MM cells and plays a pivotal role in MM initiation, progression, and apoptosis resistance [32, 33]. Newly diagnosed cases of MM have continued to show increasing Mcl-1 protein expression, which predicts a higher relapse and poor patient survival rate [36]. Thus, Mcl-1 is an attractive therapeutic target for MM.

Regulation of Mcl-1 Protein

The interaction of myeloma cells to BM microenvironment (BMM) is the hall mark of MM (Figure 3). Additionally, MM cells receive crucial signals from the BMM that help them to evade apoptosis in order to maintain their long-term survival. The BM stromal cells (BMSCs) regulate the anti-apoptotic Bcl-2 family proteins by secreting a group of signaling cues. Mcl-1 is regulated through several extracellular signaling molecules including interleukins (IL-3, IL-5, and IL-6) [37-39]; growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF)[40, 41]; granulocyte macrophage colony stimulating factors (GM-CSF) [42]; and interferon *alpha* (INF-α) [39]. Combined, these stimuli trigger and modulate multiple signaling pathways including Janus kinase/signal transducer and activator of transcription (JAK/STAT), rat sarcoma/mitogen-activated protein kinase (Ras/MAPK), MEK/extracellular signal-related kinase (ERK) as well as phosphatidylinositol-3 kinase (PI3-K)/Akt (Figure 3).

Figure 3

Bone Marrow Microenvironment (BMM) in MM



Note. BMM facilitates the long-term survival of MM. Stromal cells in BM regulate anti-apoptotic proteins by secreting a variety of signaling molecules including IL-6 and IFN-α that trigger JAK/STAT pathway, leading to the upregulation of Mcl-1, Bcl-xL, and VEGF. VEGF promotes IL-6 induction in neighboring BMCs. Furthermore, IL-6 induces survival of MM cells via Ras/MAPK pathway, which modulates the expression of Mcl-1 The tumor necrosis factor (TNF) family including BAFF and APRIL are other stimuli from the BMM that induce expression of both Mcl-1 and Bcl-2 via tumor necrosis factor receptor-associated factors (TRAFs) including BAFF-R, BCMA, and TACI. IGF-1

is another stimulus that acts by downregulating Bim, leading to release Mcl-1. MM cases have shown chromosomal amplification of 1q21 region, where the gene coding for Mcl-1 and IL-6R is located.

The cytokine IL-6 is the main survival factor for MM cells [43]. IL-6 triggers the upregulation of Mcl-1, Bcl-xL, and VEGF via stimulation of the JAK/STAT-3 signaling pathway [44, 45]. In turn, VEGF promotes IL-6 induction in neighboring BM cells (BMCs) [45]. IL-6 also induces survival of MM cells via stimulating the Ras/MAPK pathway, which engages in Mcl-1 overexpression [46]. Additionally, *IFN-α* induces Mcl-1 in a STAT-3 dependent manner [39]. Furthermore, the tumor necrosis factor (TNF) family, including B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), prevent apoptosis by inducing the expression of Mcl-1 and Bcl-2 [47]. Insulin like growth factor 1 (IGF-1) affects the cell survival, by downregulating pro-apoptotic protein Bim [48]. The imbalance between Bim and Mcl-1 expression plays an important role in MM cell survival [49]. The transcription factors such as B lymphocyte induced maturation protein 1 (Blimp-1), X-box binding protein 1 (XBP-1), and interferon regulatory factor 4 (IRF4) are critical for myeloma cells differentiation and development [50]. The Blimp-1 downregulates the expression of pro-apoptotic protein Bim [51].

Mcl-1 and other anti-apoptotic proteins contain four Bcl-2 homology (BH) domains (BH1-3 domains interact to form a hydrophobic cleft termed "BH3-binding groove"), and a C-terminal tail of hydrophobic transmembrane domain (TM) that permeates into the mitochondrial membrane [31]. Interestingly, compared to the other

anti-apoptotic proteins, Mcl-1 has several unique properties including binding site, size, half-life, and localization. Mcl-1 has a shallow, relatively inflexible and more electropositive binding site abundant in lysine and histidine residues [52]. Bcl-2 and Bcl-xL proteins contain 233 amino acids, whereas Mcl-1 protein contains 350 amino acids. This size difference is due to the presence of a large N-terminal domain of four PEST sequences (amino acids sequence extensive in proline (P), glutamic acid (E), serine (S), and threonine (T)) [31, 53], which can target Mcl-1 for degradation through the ubiquitin-proteasome system (UPS) and renders it short half-life (usually less than three hours depending on the cellular conditions) [29, 31, 54].

In MM, the Mcl-1 gene is the most important and selective of the survival genes [55]. Gene coding of Mcl-1 is located on chromosome 1q21 region. Approximately 40% of MM cases have shown chromosomal amplification of 1q21, hence increased Mcl-1 expression [56, 57]. Additionally, the gene coding of cytokine interleukin 6 receptor (IL-6R) is located on the same chromosome region (1q21) [58]. The coding region of Mcl-1 contains three exons and two introns that undergoes alternative splicing to produce mature RNA (mRNA) isoforms. The Mcl-1L (Mcl-1 long) splice variant joins the three exons, has a full length of 350 amino acids and acts as an anti-apoptotic. On the other hand, Mcl-1S (Mcl-1 short) joins only the first and the third exons without the central exon, with the length of 271 amino acids, shows increased cytosolic localization and lacks the BH1, BH2 and TM domains but has the BH3 domain which plays a critical pro-apoptotic role [59, 60]. Interestingly, Kim et al. (2009), found a new alternative splicing variant detected in the mitochondrion termed Mcl-1ES (Mcl-1 extra short) with a shorter

length of 197 amino acids due to an absence of PEST sequences [61]. Mcl-1ES forms an interaction with Mcl-1L in order to induce apoptosis [61].

The post transcriptional regulation of Mcl-1 is complex and controlled by multiple RNA binding proteins (RPBs) and microRNAs (miRNAs). For example, Mcl-1 has been shown to be downregulated in MM by miR-29b, miR-137, and miR-197 that leads to apoptosis [62-64]. Additionally, at the post-translational level, the large N-terminal domain PEST allows for non-proteasomal degradation via cleavage [65], proteasomal degradation via phosphorylation [53], and ubiquitination [66], which further impact Mcl-1 expression, stability, localization, and function. Mcl-1 PEST undergoes Caspase cleavage at two different sites, located at Asp127 that produce Mcl-$1^{1\text{-}127}$ associated with Mcl-$1^{128\text{-}350}$. At Asp158 that produces Mcl-$1^{1\text{-}157}$ associated with Mcl-$1^{158\text{-}350}$ [65]. Interestingly, not all Mcl-1 cleavage fragments revoke anti-apoptotic function. Mcl-1Δ127 fragment has anti-apoptotic function same as Mcl-1 and exists mainly in the cytoplasm and sequester BH3-only or Bak in order to prevent apoptosis [67].

The Mcl-1 phosphorylation plays a critical role in controlling Mcl-1 function as well. Mcl-1 phosphorylation occurs by several protein kinases including; c-Jun N-terminal kinase (JNK) [68], glycogen synthase kinase 3 (GSK-3) [69, 70], and extracellular signal-regulated kinase (ERK-1) [71, 72]. The phosphorylated Mcl-1 proteins have been reported to result in different functions according to phosphorylation sites [53, 73]. Furthermore, a reversible form of post-translational ubiquitination controls several aspects of Mcl-1 including stability and proteasomal degradation and allows for rapid response to environmental signals in order to change cell state from survival to

apoptosis. The Mcl-1 ubiquitin-proteasome system is mediated by five different E3 ubiquitin-ligases including Mcl-1 ubiquitin ligase E3 (Mule) [74], SCF beta-transducin repeats containing protein ($SCF^{\beta\text{-}TrCP}$) [70], SCF F-box and WD repeat domain containing 7 (SCF^{Fbw7}) [75], anaphase-promoting complex/cyclosome (APC/CC^{dc20}) [76], and tripartite motif containing 17 (Trim17) [77]. Furthermore, the ubiquitin-proteasome system contains an additional deubiquitinase called ubiquitin specific peptidase 9, X-linked (USP9X) that removes poly-ubiquitin chains leading to stabilize Mcl-1 and prevent apoptosis [78]. The degree of ubiquitination is also subject to variation based upon the variable phosphorylation of residues of Mcl-1 [69, 70]. Our understanding of Mcl-1 regulations has been greatly expanded by the findings that have developed over the years and provide deep critical insights into exactly how Mcl-1 protein plays such a key role in cellular apoptosis as well as how it can be modulated to provide new options of potential therapeutic approach in MM and other Mcl-1 dependent cancers.

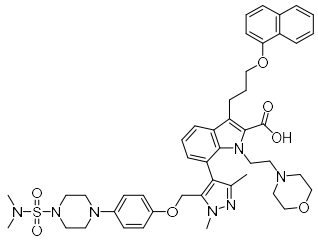
Development of Selective Mcl-1 Inhibitors

Studies have demonstrated that MM depends on Mcl-1 proteins for survival, prognosis, and chemo resistance. Thus, inhibition of Mcl-1 offers an attractive target and a promising strategy for myeloma treatment. Nonetheless, the targeting of Mcl-1 has been challenging because of its complex regulation. So far, two approaches have been adopted to inhibit Mcl-1, one is direct inhibition and the second is indirect targeting. Indirect targeting is a less selective method that inhibits other anti-apoptotic proteins, may have more serious side effects. Whereas direct Mcl-1 inhibitors target the hydrophobic cleft BH3-binding groove of BH3-only proteins interactions domain.

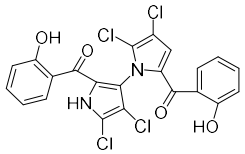
Therefore, these inhibitors are very specific to Mcl-1. Here we will review BH3-mimetic inhibitors that selectively bind Mcl-1. The structures of these inhibitors are shown in Figure 4. The current status of development of these agents are summarized in Table 2.

Figure 4

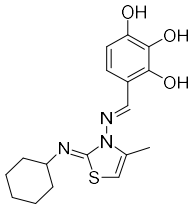
Chemical Structures of Selective Mcl-1 Inhibitors



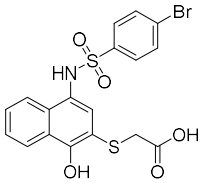
A-1210477



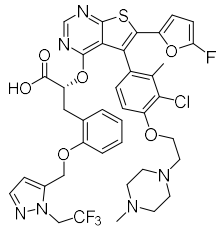
Maritoclax



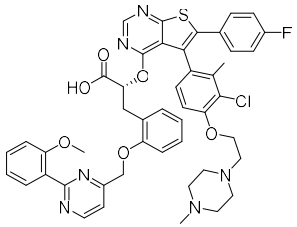
MIM1



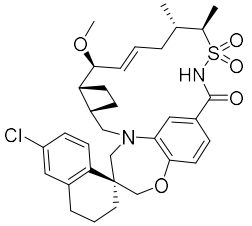
UMI-77



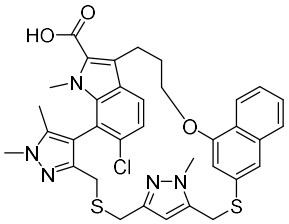
S63845



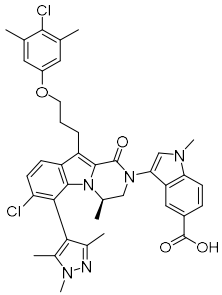
S64315 or MIK666



AMG-176



AZD5991



VU661013

Note. The most prominent Mcl-1 inhibitors including A-1210477, Maritoclax, MIM1, UMI-77, S63845, S64315/MIK666, AMG-176, AZD5991, and VU661013.

Table 2

Direct Mcl-1 Inhibitors BH3 Mimetic and Semi BH3 Mimetic Agents

Mcl-1 Inhibitor	Company	Affinity	Clinical Trial Status
A-1210477	Abbive	Ki = 0.45 nM	Preclinical
Maritoclax	Hong-Gang Wang's group at Pennsylvania State University	IC_{50} 10 μM	Preclinical
MIM1	Cohen and co-workers	Only at very high concentration	Failed *in vivo*
UMI-77	Zaneta Nikolovska-Coleska's group at University of Michigan	Ki = 490 nM	Preclinical
S63845	Servier & Vernalis	Kd = 0.19 nM	Preclinical
S64315/MIK666	Servier & Vernalis and Novartis	Undisclosed	Phase I by Novartis, in R/R lymphoma or R/R MM patients (NCT02992483). Phase I by Servier, in AML and MDS patients (NCT02979366). Phase I by Servier as a combination of S64315/MIK666 plus Venetoclax in AML patients (NCT03672695).

Mcl-1 Inhibitor	Company	Affinity	Clinical Trial Status
AMG-176	Amgen	Ki = 0.06 nM	Phase I in R/R MM and R/R AML patients (NCT02675452). Phase I as a combination of AMG-176 plus Venetoclax in different R/R hematologic malignancies including AML, NHL, and DLBCL (NCT03797261).
AMG-397	Amgen	Undisclosed	Phase I clinical trial is evaluating the safety, tolerability, pharmacokinetics, and efficacy of AMG 397 in MM, AML, DLBCL, and NHL patients (NCT03465540).
AZD5991	AstraZeneca	Ki = 0.2 nM	Phase I as a monotherapy in different R/R hematologic malignancies including NHL, ALL, RS, SLL, T-cell lymphoma, CTCL, CLL, AML/ MDS, and MM patients (NCT03218683). Phase II is sequential, dose-escalation study of combination AZD5991 plus Venetoclax in R/R AML/MDS patients (NCT03218683).
VU661013	Stephen Fesik's group at Vanderbilt University	Ki = 0.097 nM	Have partnership with Boehringer Ingelheim Company for clinical trials but no plan

Mcl-1 Inhibitor	Company	Affinity	Clinical Trial Status
			disclosed yet. (https://www.boehringer-ingelheim.us/press-release/boehringer-ingelheim-and-vanderbilt-university-expand-partnership-develop-novel)

Indole-2-Carboxylic Acids Analog (A-1210477)

This was developed by AbbVie in 2008. A-1210477 induces intrinsic apoptosis pathway by selectively inhibiting Mcl-1 with high binding affinity (Ki = 0.454 nM) [79]. Upon binding, BH3 mimetic A-1210477 results in an accumulation of Mcl-1 protein by preventing its degradation. A-1210477 disrupts the Mcl-1:Bim and Mcl-1:Noxa complexes in order to induce Bax/Bak- dependent MOMP, leading to Cytochrome C release and Caspase activation [79]. The treatment of A-1210477 decreased the association of Mcl-1: Bak complex within an hour, however the complex was totally disrupted after three hours of treatment [80]. Interestingly, the studies of Mallick et al. (2019) showed that A-1210477 induces rapid apoptosis within 0.5–1 hour of treatment, without inducing Noxa [81]. A-1210477 as a monotherapy or in a combination with Navitoclax resulted in the death of different cell lines including MM, melanoma, and non-small cell lung cancer cell lines that were found to be Mcl-1 dependent by BH3 profiling or siRNA rescue experiments [79, 82]. This finding was reinforced by the efficacy of A-1210477 as a combination with Venetoclax against acute myeloid leukemia (AML) [83]. A-1210477 inhibited triple negative breast cancer cell line growth activity *in vitro* which is also considered a Mcl-1 dependent cells type [84]. However, a reference showed A-1210477 induced apoptosis in Bcl-2 dependent cells at higher concentration when compared with Mcl-1 inhibition concentration [85]. Unfortunately, no *in vivo* activity was associated with A-1210477, even with the most sensitive cell lines. This was attributed to cell penetration issues and reduced bioavailability due to the high levels of serum protein binding.

Marinopyrrole A (Maritoclax)

This natural agent was first discovered by Hong-Gang Wang's group at Pennsylvania State University in 2012 [86]. The BH3 mimetic drug Maritoclax induces degradation of Mcl-1 proteins and disrupts Mcl-1: Bim complex. Maritoclax effectively binds the site of the BH3-only proteins p4 binding site and leads to apoptosis. Further, it has been reported that this natural agent is effective against Mcl-1 overexpressing cancer cells [86]. Blocking BH3 binding site is related with increased amounts of Mcl-1 protein, followed by its ubiquitination and degradation by the E3 ligase (ubiquitin ligase) [87]. Moreover, the treatment of Maritoclax did not result in Noxa upregulation [86]. We found that Maritoclax potentiates the apoptotic response of ABT-737 in human melanoma cells [88].

Mcl-1 Inhibitor Molecule 1 (MIM1)

Developed in 2012 by Cohen and co-workers, polyphenol compound MIM1 acts as a semi BH3 mimetic which induces Noxa [89]. MIM1 seems very similar to BH3 mimetic Mcl-1 inhibitors [81]. This Mcl-1 inhibitor exhibited an ability to induce apoptosis in Mcl-1 dependent cells through upregulation of proapoptotic protein Noxa, which selectively inhibits Mcl-1 with high affinity binding [25, 26, 81]. Also, induction of Noxa dissociates Mcl-1:Bim association complex. Unfortunately, MIM1 was only able to induce Bak dependent apoptosis at high concentrations (more than 10 μM). MIM1 failed to induce apoptosis in anti-apoptotic proteins dependent cell lines [90].

UMI-77

Developed in 2013 by Zaneta Nikolovska-Coleska's group at University of Michigan, naphthol derivative UMI-77 is another semi BH3 mimetic Mcl-1 inhibitor with a high binding affinity (Ki = 490 nM) [91, 92]. In order to induce apoptosis, UMI-77 was found to upregulate pro-apoptotic protein Noxa thereby selectively inhibiting Mcl-1 [81]. UMI-77 and Noxa competing for Mcl-1 binding with Bax and Bak proteins ultimately disrupt the Mcl-1: Bak and Mcl-1: Bak complexes, which results in Cytochrome C release and Caspase 3 activation [92]. The *in vitro* and *in vivo* preclinical studies demonstrated that UMI-77 potently inhibits tumor growth and induces apoptosis in MM cells [91], and pancreatic cancer cells lines [92], both of which rely on the Mcl-1 protein as a survival factor [93-95]. In addition to pancreatic cancer cell line BxPC-3 xenograft mouse model and MM animal xenografts, UMI-77 significantly delayed growth activity in breast cancer cell line MDA-MB-468 xenograft mouse model as well [84].

S63845

Developed in 2015 by a Servier and Vernalis partnership, atropisomers thienopyrimidine scaffold molecule S63845 is a selective BH3 mimetic Mcl-1 inhibitor that can activates the Bax/Bak dependent mitochondrial apoptotic pathway [96]. S63845 is a selective and potent BH3 mimetic. It binds with high affinity to the BH3-binding groove of Mcl-1 (Kd = 0.19 nM) without any detectable binding to Bcl-2 or Bcl-xL proteins. S63845 showed effective anti-cancer activity in its *in vitro* and *in vivo* preclinical studies [96]. The IV infusion of S63845 once daily for five consecutive days resulted in 100% tumor regression in MM subcutaneous tumor models and lymphoma

disseminated mouse model Eμ-Myc [96, 97]. The same tumor regression was related to AML as well [96]. This inhibitor had a therapeutic effect without significant weight loss apparent side effects in normal mice tissues [96]. Along with A-1210477 and UMI-77, S63845 also inhibited the growth activity of TN breast cancer cell line [84].

After S63845 proved its eligibility as a selective Mcl-1 antagonist, impressive studies have continued coming up. Recently in 2019, S63845 showed activity both *in vitro* and *in vivo* by killing human T cell acute lymphoblastic leukemia cells (T-ALL) [98]. It was even more potent in inducing apoptosis as a combination therapy with Venetoclax without any appreciable toxicity [98]. In 2020, *in vitro*, *ex vivo,* and *in vivo* preclinical evaluations investigated the combination of S63845 plus Venetoclax. *In vitro* study tested the sensitivities of five MM cell lines to the drug while the *in vivo* study used an aggressive disseminated model of MM. The combined finding came clearly with increasing apoptotic cell death, reduced cell survival as well as delayed tumor growth *in vivo* [99]. Furthermore, S63845 was evaluated in a triple combination with Venetoclax plus dexamethasone. Clearly, *in vitro* and *in vivo* studies showed that dexamethasone increased the effectiveness of both S63845 and Venetoclax. Furthermore, *in vitro* studies illustrated that triple therapy is a stronger synergism than the S63845 plus Venetoclax in resistant MM cell line (MM.1S) [99]. In addition, the combination of S63845 and Venetoclax, enhanced the Venetoclax sensitivity and overcome resistance to Venetoclax in human myeloma cell lines (HMCLs) [100].

Servier & Vernalis and Novartis have created another S-derivative called S64315 or MIK666. S64315/MIK666 is in clinical trial as a single agent in R/R lymphoma or R/R

MM (NCT02992483). Furthermore, this molecule is being tested in AML and myelodysplastic syndrome (MDS) patients (NCT02979366). Another clinical trial is undergoing by Servier & Vernalis in a combination with Venetoclax in AML patients (NCT03672695).

AMG-176

Developed in 2016 by Amgen, chirality macrocyclic acylsulfonamide (spiromacrocyclic) AMG-176 is an orally selective Mcl-1 inhibitor with high binding affinity (Ki = 0.06 nM), induces rapid apoptosis in different hematologic malignancies. The treatment of AMG-176 disrupts the interactions of the Mcl-1: Bak complex [101] [102]. Preclinical studies have demonstrated that AMG-176 is non-toxic and efficacious in both MM subcutaneous xenograft models and disseminated models, inhibiting 100% tumor growth [101]. In preclinical studies, AMG-176 has been shown to eradicate CLL cells as a single agent or in a combination with a low dose of Venetoclax [103]. Interestingly, AMG-176 was the first selective Mcl-1 inhibitor to be studied in humans. Currently, AMG-176 is in phase I clinical trials via IV administrations in patients with R/R MM and patients with R/R AML (NCT02675452). AMG-176 monotherapy has potent anti-myeloma and unique hematologic activity resulting in marked survival improvement. Furthermore, phase I clinical trials have also evaluated AMG-176 as a combination therapy with Venetoclax which presents as an interesting therapy for different R/R hematologic malignancies including AML, diffuse large B cell lymphoma (DLBCL), and Non-Hodgkin's lymphoma (NHL) (NCT03797261). Furthermore, as a

combination with MEK inhibitor (Trametinib), AMG-176 increased the tumor regression effect in murine models of solid tumor cell lines [104].

Amgen has developed another potent and selective analog AM-8621, nonetheless, this molecule has poor oral bioavailability and a short half-life [101]. Interestingly, MM cells showed sensitivity to AM-8621 as a monotherapy and as a combination therapy with dexamethasone [101]. Caenepeel et al. (2019) investigated the activities of AMG 176 and AM-8621 in combination with Cytarabine, Doxorubicin, and Decitabine in a preclinical model of AML [105]. The other analog AMG-397 is evaluated orally in the clinic. A phase I clinical trial evaluating its safety, tolerability, pharmacokinetics, and efficacy in MM, AML, DLBCL, and NHL patients by administrating AMG-397 in a weekly cycle consisting of two consecutive days of one oral dose followed by five days off at a weekly interval (NCT03465540).

AZD5991

Developed in 2017 by AstraZeneca, indole-2-carboxylic acids analog AZD5991, is a potent and selective macrocyclic Mcl-1 inhibitor that rapidly activates Caspase proteins, which leads to apoptosis in MM cell lines (GI_{50} = 10 nM) [106, 107]. AZD5991 is a BH3 mimetic with high binding affinity (Ki = 0.2 nM) disrupts the Mcl-1: Bak complex [106, 107]. Most notably, in a number of MM and AML mouse and rat xenograft models, AZD5991 exhibits a potent activity with the preclinical *in vivo* studies showing 100% tumor regression after a single IV dose in both monotherapy and in combination with Venetoclax or Bortezomib [107]. The preclinical efficacy of AZD5991 is emphasized by the apoptosis and survival improvements in MM models resistant to

Venetoclax [106]. The remarkable *in vitro* and *in vivo* anti-tumor activities of AZD5991 in both MM and AML models support its consideration as a strong clinical candidate in different Mcl-1 dependent hematologic malignancies. The number of clinical trials are ongoing with AZD5991 as a single agent or in combinations. For example, phase 1 as a monotherapy dose escalation study in several R/R hematologic malignancies including NHL, ALL, Richter syndrome (RS), small lymphocytic lymphoma (SLL), T-cell lymphoma and cutaneous T-cell lymphoma (CTCL) (NCT03218683); phase 1 as a monotherapy in expansion groups of R/R CLL, AML/ MDS, and MM patients; and Phase 2 sequential, dose-escalation study in combination with Venetoclax in R/R AML/MDS patients (NCT03218683).

VU661013

Developed in 2017 by Stephen Fesik's group at Vanderbilt University, indole-2-carboxylic acids analog VU661013 is a potent and selective BH3 mimetic Mcl-1 inhibitor with a high binding affinity (Ki=0.097 nM) [108]. VU661013 destabilizes the Mcl-1: Bim complex in order to initiate MOMP [109]. VU661013 proved potency in Mcl-1 inhibition in both *in vitro* and *in vivo* studies through its induction of apoptosis in a variety of Mcl-1 dependent tumors. Furthermore, it demonstrated efficacy in combination with Venetoclax in Venetoclax resistant cells, patient derived xenografts, and murine models of AML [109]. Further modifications of this molecule are being made to improve the efficacy and bioavailability. Another analog has been made (compound 42), which is bound to Mcl-1 with picomolar affinity (Ki= 70-300 pM) in order to displace Bim [110].

Compound 42 showed *in vivo* growth inhibition in xenograft models of MM and AML [110].

Chapter 2

Methods and Materials

Antibodies and Compounds

Antibodies were obtained from the following sources: mouse anti-Bcl-2 (Cell Signaling, 3498); rabbit anti-Mcl-1 (Cell Signaling, 94296); rabbit anti-Bcl-xL (Cell Signaling 2762); rabbit polyclonal anti-caspase-3 (Cell signaling, 9662); rabbit polyclonal cleaved caspase-3 (Cell Signaling, 9664); rabbit polyclonal anti-PARP (Cell Signaling, 9532); rabbit anti-GAPDH (Sigma-Aldrich, G9545). Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Sigma-Aldrich.

Cell Culture

All MM cell lines (U266, MM.1S, MM.1R, and RPMI 8226) were obtained from American Type Culture Collection (ATCC) and maintained per the manufacturer's recommendations in complete medium with 1% HyClone™ antibiotic/antimycotic solution 100X (10,000 U/mL Penicillin G, 10,000 µg/mL Streptomycin, 25 µg/mL Amphotericin B) at 37°C and 5% Carbon dioxide (CO_2). MM.1S, MM.1R, and RPMI 8226 cell lines maintained in RPMI 1640 medium 10% fetal bovine serum (FBS). While U266 cells were maintained in RPMI 1640 medium supplemented with 15% FBS.

The cell lines were stored in liquid nitrogen. Then every cell line was allowed to thaw at 25°C room temperature before being diluted in a centrifuge tube with 5 mL of media and centrifuged for 5 minutes at 900 RPM. Next, the supernatant fluid was discarded and the pellet was resuspended in an appropriate volume of fresh media by slowly pipetting up and down. Lastly, the mixture was transferred to a T25 cell culture

flask and placed in the incubator. The cells were allowed to grow and were screened under the microscope; afterward, the media and flask were exchanged accordingly (Figure 5).

Figure 5

The Placement of Flasks in CO_2 Incubator

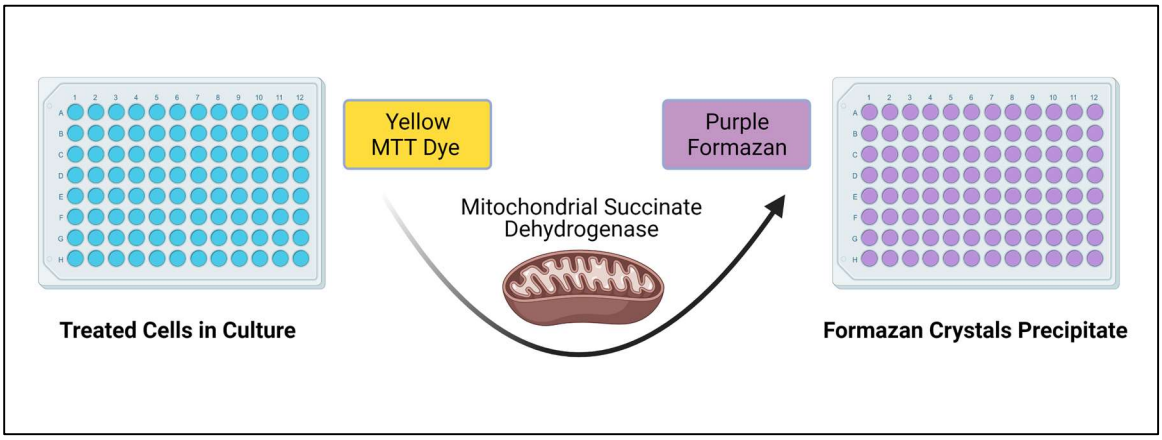
Cell Viability Assay

The effect on cell viability of KS18 was determined by MTT Assay. First, 5000 cells of each cell line were incubated with (0.1-25 µM) concentrations of KS18 in triplicates using a 96-well plate in a final volume of 200 µL for 96 hours at 37°C. Afterward, 20 µL of MTT dye (5 mg/mL in PBS) was added to each well and incubated for 3 hours at 37 °C. After 3 hours of incubation, the 96-well plate was centrifuged for 7 minutes at 1000 rpm, and then the supernatant fluid was removed and 50 µL of DMSO was added to each well to dissolve the resulting formazan crystals (Figure 6). The optical

density of the resulting solution was measured at delta value (570-630 nm) using a 96-well multi-mode microplate reader (BioTek Technologies, Synergy H1; Winooski, VT, USA). The optical density is directly correlated to the number of viable cells remaining in the solution. The percentage of cell viability was calculated by comparing the concentration of formazan crystals formed in each well with the negative control, which has no molecules add.

Figure 6

MTT Assay Mechanism



Western Blot Analysis

The effect of KS18 on Mcl-1 and associated apoptotic pathways was determined by Western Blot. Whole-cell extracts were prepared by subjecting nontreated (negative control) and treated cells to 50 µL of lysis buffer (643 µL RIPA buffer supplemented with 7 µL of 100 X protease/phosphatase inhibitor) in microcentrifuge tubes. After one

hour, lysates were vortexed vigorously three times then spun down at 13,000 rpm for 10 minutes to remove insoluble material. The supernatant was collected into a new microcentrifuge tube and kept at -80°C. BCA protein estimation was employed using to the extracts and the BCA Protein assay kit (Pierce^{TM} BCA Protein Assay Kit; Thermo scientific. Rockford, IL, USA). The protein of each sample was diluted with the proper amount of distilled water, NuPAGE LDS Sample Buffer dye (4X) (Thermo scientific, 89901; Rockford, IL, USA), and DTT (10X), so that 30 ug of protein was loaded per well into a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDSPAGE) gel. The gel was subjected to electrophoresis with added SDS running buffer to the center of the chamber at 110 V at room temperature until the dye reached about half a centimeter above the three grooves of the chamber. After electrophoresis, the proteins were electro-transferred to PVDF membranes at 34 V for 90 minutes with added transfer buffer to the center of the chamber and ice and cold water to the back part of the chamber. The membrane was blocked with 5% nonfat dry milk in 1X PBST (phosphate-buffered saline) (VWR, 20A0756203; Ohio, USA) with 0.05% Tween-20 (Sigma Chemical Co., St. Louis, MO, USA) for 35-45 minutes, subsequently washed with PBST, then blotted with the relevant antibodies (5 µL 1°Ab: 5 mL 5% nonfat dry milk in PBST) overnight in the cold room with shaking. After that, the blot was washed with PBST three times for 5 minutes each, then exposed to horseradish peroxidase (HRP)–conjugated secondary antibodies (1 µL 2°Ab: 5 mL 5% nonfat dry milk in PBST) for two hours at room temperature with shaking. Finally, the blot had another three washes with PBST for 5 minutes each, followed by exposure to an enhanced chemiluminescent substrate for

detection of HRP (Pierce™ ECL Western; Thermo scientific. Rockford, IL, USA). The bands obtained were quantitated using the Imaging system (BIO-RAD, ChemiDoc™ MP; Hercules, CA, USA). To save time and precious samples, sequential detections of different proteins were performed on the same membrane using Gentle ReView™ Stripping Buffer (VWR, 19G0856497; Ohio, USA). All critical blots experiments were repeated at least two to three times.

Chapter 3

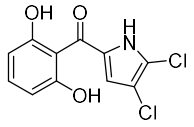
Results and Discussion

KS18 and Mcl-1 Anti-Apoptotic Protein

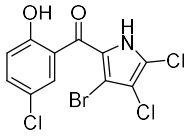
KS18 is a potent pyoluteorin derivative that inhibits Mcl-1 protein selectively in order to induce Mcl-1 dependent apoptosis (Figure 7) [111]. Pyoluteorin is a natural antibiotic, first isolated in the 1950s from cultures of Pseudomonas aeruginosa strains [112]. Pyoluteorin is a small molecule produced by fluorescent Pseudomonas [113]. Remarkably, according to NMR, pyoluteorin derivatives were shown to induce a conformational change in Mcl-1 [111]. Pyoluteorin derivatives are now being studied as a selective Mcl-1 inhibitor in order to target Mcl-1 dependent cancers [111].

Figure 7

Chemical Structures of Pyoluteorin and KS18



Pyoluteorin



KS18

This project was conducted to establish the hypothesis that the small molecule KS18 induces apoptosis in MM by targeting Mcl-1 selectively. The goal of this project aimed to evaluate KS18 selectivity towards Mcl-1 and potency in inducing Mcl-1

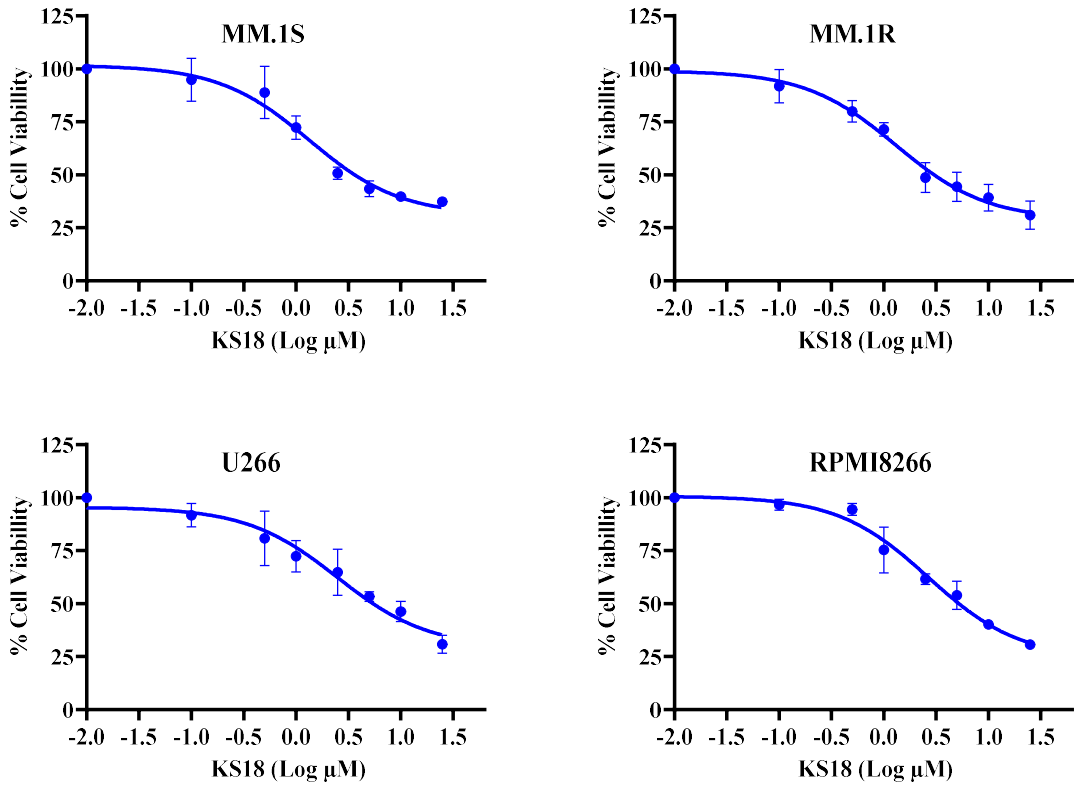
dependent apoptosis in MM. The research model of *in vitro* studies was four different MM cell lines.

KS18 Molecule Reduces Cell Viability of MM

We further demonstrated the effects of KS18, a novel selective Mcl-1 inhibitor, on the cell viability of MM cells by using MTT assay. Figure 9 illustrates that Mcl-1 inhibition by KS18 reduced the cell viability of MM cells. Thereby, KS18 induced apoptosis in MM cells by inhibiting Mcl-1.

Figure 8

Effect of KS18 on Cell Viability of MM Cells



Note. MM cells were treated with increasing concentrations of KS18 (0.1–25 µM) for 96 hrs.

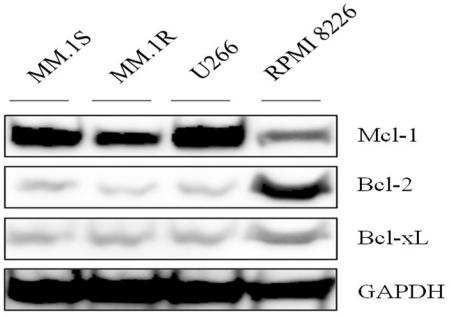
Anti-Apoptotic Proteins Expression in MM Cells

We first investigated the expression levels of anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-xL, and Mcl-1) in MM cells including MM.1S, MM.1R, U266, and RPMI8226. Remarkably, U266 cells have shown the highest expression of Mcl-1 protein

whereas the expression of Bcl-2 was found to be highest in RPMI8226 cells (Figure 8). These findings confirmed that Mcl-1 plays a critical role in MM cells. Our results indicate that U266 cells have the highest expression of Mcl-1. Accordingly, the U266 cell line has been selected for further investigation.

Figure 9

Anti-Apoptotic Proteins Expression in MM Cell Lines



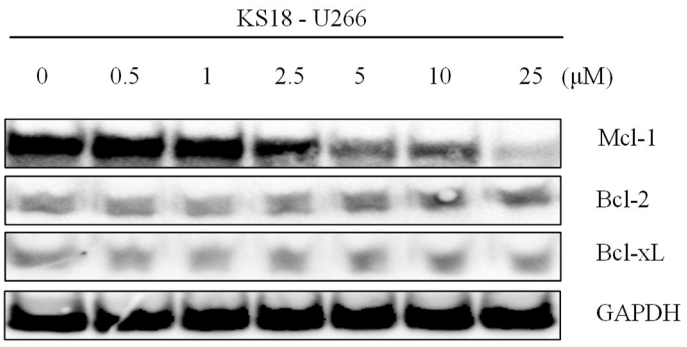
KS18 Selectively Inhibits Mcl-1 Expression in U266 Cells

Mcl-1 is known to be highly expressed in MM cells and plays a crucial role in MM survival. Since KS18 is a novel selective Mcl-1 inhibitor, we investigated the effects of KS18 on Mcl-1 expression in U266 cells. As expected, KS18 provided sufficient inhibition of Mcl-1 protein, whereas no effect on Bcl-2 and Bcl-xL expression. Figure 10 A illustrated that dose-dependent of KS18 inhibits Mcl-1 selectively. Furthermore, 5µM concentration of KS18 showed a significant inhibition of Mcl-1. Thus, 5µM

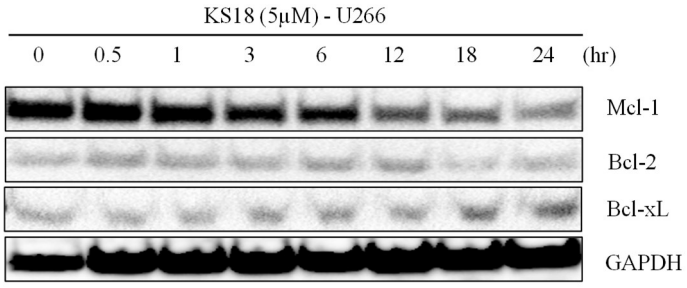
concentration was selected for time-dependent experiment. Figure 10 B illustrated that time-dependent of KS18 (5μM) inhibits Mcl-1 selectively.

Figure 8

Down-Regulation of Mcl-1 by KS18 in U266 Cells



A)



B)

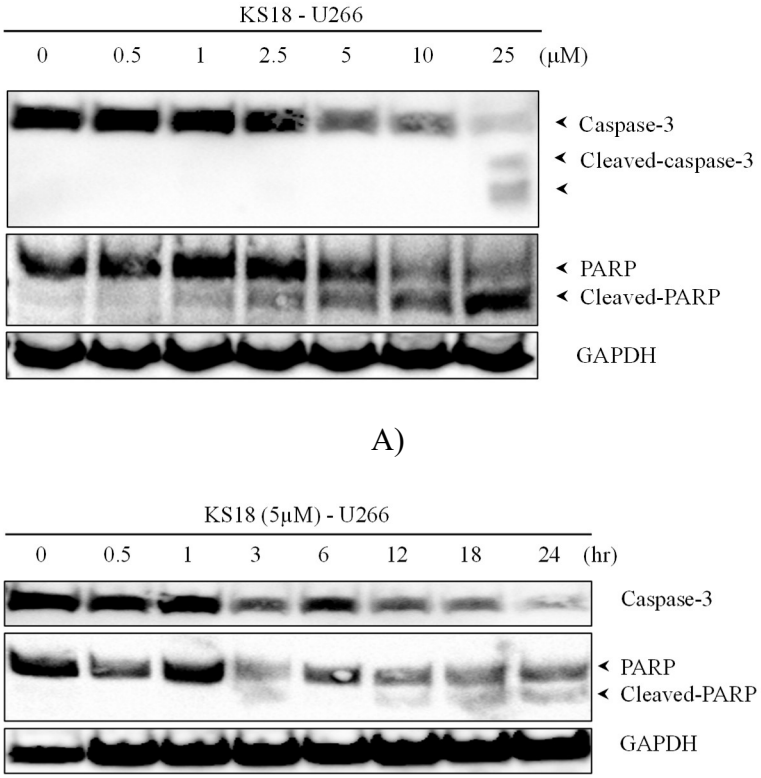
Note. **A,** Dose-dependent effect of KS18 on Mcl-1 and other anti-apoptotic proteins in U266 cells. **B,** Time-dependent effect of KS18 (5μM) on Mcl-1 and other anti-apoptotic proteins in U266 cells.

KS18 Induces Apoptosis in Caspase-Dependent Manner

Since our results have proven Mcl-1 inhibition by KS18, we were interested in expanding our experiments to investigate the effect of KS18 on associated apoptotic pathway in MM cells. Remarkably, our results confirmed that KS18 induces caspase-3 activation in U266, simultaneously inducing caspase-3 and PARP cleavage dose and time-dependently (Figure 11 A & B).

Figure 9

Inhibition of Mcl-1 Induces Apoptosis in U266 Cells



Note. **A,** Dose-dependent effect of KS18 on apoptotic proteins in U266 cells. **B,** Time-dependent effect of KS18 (5μM) on apoptotic proteins in U266 cells.

Chapter 4

Conclusion and Future Direction

MM evades apoptosis by exhibiting an imbalance in anti-apoptotic proteins expression, especially Mcl-1, leading to a defective intrinsic pathway [32, 33]. Mcl-1 protein is essential for the survival and uncontrolled growth of malignant plasma cells. Therefore, Mcl-1 overexpression in MM plays a crucial role in tumor initiation, progression, and relapse [36]. Small molecule targeting of Mcl-1 protein forms an attractive strategy for MM therapy. Thus, the recent research efforts have been focused on discovering a selective Mcl-1 inhibitor with the ambition of developing a promising new treatment for MM.

The anti-apoptotic protein Mcl-1 is critical in the survival and drug resistance of several malignancies including MM [36] [93, 117-122]. Mcl-1 overexpression is one of the main factors in the development of the resistance to current selective Bcl-2/Bcl-xL inhibitors [114, 115]. ABT-737, a Bcl-2 and Bcl-xL inhibitor, is highly potent against Bcl-2 dependent cancers but has no efficacy towards Mcl-1 dependent cancers [116].

KS18 is a pyoluteorin analogue that behaves as a selective Mcl-1 inhibitor with high cytotoxic potency in MM. This project demonstrated that KS18 induces apoptosis in MM cells by inhibiting Mcl-1 protein specifically (Figure 10 A & B). Furthermore, our results suggest activation of caspase-dependent apoptosis, where we confirmed caspase-3 and PARP cleavage after dose and time-dependent Mcl-1 down regulation (Figure 11 A & B).

Taken together, our results support that Mcl-1 selective inhibitors are associated with achieving an extreme therapeutic efficacy and may also overcome drug resistance of Bcl-2/Bcl-xL inhibitors in MM. Combinations strategies are shown to be especially valuable if the combination consists of a selective Mcl-1 inhibitor plus a molecule that inhibits other anti-apoptotic proteins including Bcl-2 and Bcl-xL, or a molecule that induces pro-apoptotic proteins expression.

The last decade or so has seen tremendous development in novel molecules and various Mcl-1 inhibitors have been developed. These new inhibitors may help in overcoming drug resistance and improve the treatment of MM and other hematological malignancies where Mcl-1 is an important survival factor. Interestingly, KS18 may form a very potent molecule in managing malignancy of MM and enhances the efficacy of MM treatment in order to overcome resistance. Therefore, our results suggest that KS18 by targeting Mcl-1 may represent a new viable treatment for MM. The future studies uncover the mechanism of action of KS18 and provide the foundation for *in vivo* assessment of this novel small molecule. The *in vivo* studies utilizing MM mouse model are needed to understand how KS18 downregulates Mcl-1 protein and whether this molecule potentiates the response of approved therapeutic agents in MM. Based on this information, the small molecule targeting of Mcl-1 represents one of the most promising approaches for MM treatment. This places priority on the rational design of novel molecules that bind extremely tightly and selectively to Mcl-1 for the better outcome of the treatment.

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