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Review

# Latest advances in the multiple myeloma drug research: from molecular signaling pathways to small molecules development

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## ABSTRACT

Multiple myeloma (MM) is an uncontrolled differentiation of plasma B cells and the deposits in the bone marrow. MM accounts for 10% of blood cancers and nearly more than 22,000 new cases are diagnosed yearly in the United States. MM is associated with aggressive clinical manifestations including hematological and metabolic diseases. In addition, bone complications are common in more than 60% of cases such as osteoporosis, bone lesions and fractures. Complicated molecular signaling pathogenesis pathways are involved in the MM disease which accounts for the difficult disease control. MM remains an incurable disease despite the recent discovery of novel proteasome inhibitor (Bortezomib). This review addresses the molecular signaling pathways associated with MM progression and survival, and further demonstrates the mechanisms, molecular targets, and the development of current anti-MM agents. Also, we address the recent discoveries in MM molecular targeting as well as the recent discoveries and development of small molecules in patents and/or clinical trials.

**KEYWORDS:** multiple myeloma, molecular mechanism, pomalidomide, Bortezomib, immunomodulatory drugs, proteasome inhibitors, p62, sequestosome 1, cannabinoid receptor $CB_2$

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## 1. INTRODUCTION

Multiple myeloma (MM) is the malignant proliferation of plasma B cells in the bone marrow. It is associated with high levels of monoclonal proteins in the blood and/or urine, decreased immunoglobulin (Ig) levels and lytic bone diseases [1, 2]. MM is the second most common blood cancer and accounts for 10% of hematological malignancies [3]. In 2013, nearly 22,000 new cases were diagnosed and more than 10,000 deaths occurred from the disease according to the American Cancer Society [4]. The incidence is higher with increasing age (between 60 and 70 years old) and only 3% of all patients are younger than 40 years old [5, 6].

The clinical manifestations of MM patients range from bone disease, hypercalcemia, hematological abnormalities, renal impairment and peripheral neuropathy. Bone diseases are presented in 60% of MM patients. These may include: lytic bone lesions, vertebral fractures and/or osteoporosis. Hypercalcemia is considered the most common metabolic abnormality in MM (accounts for more than one-third of patients) [7]. Hypercalcemia results from osteolysis and bone resorption caused by secreted cytokines in the bone marrow microenvironment, such as receptor activator of nuclear factor-κB ligand (RANKL), macrophage inflammatory protein (MIP)-1α, and tumor necrosis factors (TNFs). Bone resorption leads to the efflux of calcium into the extracellular fluid [8]. The hematological abnormalities associated with MM include anemia, leukopenia and thrombocytopenia. Renal impairment affects more than 20% of

MM patients. The mechanism of renal impairment in MM is related to the excess accumulation of light chains within the distal tubule leading to a condition known as myeloma kidney. Peripheral neuropathy represents a significant manifestation in MM patients during diagnosis and as a toxicity limitation of various agents used in the treatment of MM [7].

MM remains an incurable disease worldwide associated with complicated treatment regimens and modalities. Many complex molecular signaling pathways are involved in the MM pathogenesis, which accounts for the difficulty in managing the disease. The most important pathogenic contributor to the MM pathogenesis is the bone marrow microenvironment (BMME) in addition to other signaling pathways that have shown to be crucial in the MM pathogenesis. The advances in the understanding of the MM pathogenesis mechanisms aided the discovery of new chemical agents. Immunomodulatory drugs (IMiDs) such as thalidomide, lenalidomide and recently pomalidomide are used in the clinic to treat MM patients [9]. The Ubiquitin Proteasome System (UPS) plays a critical role in the MM disease progression. Many cell survival and cell growth proteins are regulated via the proteasome pathway. Bortezomib is the first discovered proteasome inhibitor used in MM and is considered the first line treatment. Although the disease remains incurable, significant advances in the treatment and the development of chemical agents have aided in the understanding of the disease pathogenesis with improved life expectancy of patients with median overall survival prolonged to 7–8 years [10].

## 2. Multiple myeloma (MM) pathogenesis

### 2.1. Bone marrow microenvironment

The bone marrow microenvironment plays a crucial role in the progression of MM disease. Indeed, it promotes cell survival, tumor progression, migration and drug resistance. Cytokines and growth factors are secreted by MM cells within the bone marrow microenvironment. Cytokines within the BMME play essential roles in MM cell growth and survival. MM growth is mediated by insulin-like-growth factor I (IGF-I), Interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) while MM resistance to apoptosis is mediated by IL-6, IGF-I and IL-21 [11, 12]. Further, MM cell migration is mediated by VEGF and stromal cell derived factor-1α (SDF-1α) [13, 14]. Cytokines also mediate the adhesion of MM cells to the bone marrow stromal cells (BMSCs) thus facilitating MM growth and migration. For instance, TNF-α induces upregulation of cell-surface adhesion molecules such as intercellular adhesion molecule -1 (ICAM1) and vascular cell adhesion molecule-1 (VCAM1) through NF-κB signaling pathway, leading to increased secretion of cytokines (IL-6 and VEGF) within the BMSCs [15]. Cytokines further stimulate MM cell growth and survival within the MM cell via different cell survival and growth pathways including PI3K/AKT, NF-κB, JAK and Raf/Ras signaling pathways.

### 2.2. Signaling pathways within MM cell

#### 2.2.1. The PI3K/AKT pathway

Within the MM cell, the phosphatidylinositide 3-kinases/protein kinase B (*PI3K*/AKT) plays a crucial role in the proliferation of MM cells. PI3K is a member of a large family of PI3K-related kinases (PIKK), which includes mammalian target of rapamycin (mTOR) kinase, ataxia telangiectasia mutated (ATM) kinase, ATM and RAD3 related (ATR) kinase, and DNA-dependent protein kinase (DNA-PK). Based on the sequences homology, the PI3K family is divided into three classes. Class I is further subdivided based on its mechanism of activation into class Ia and class Ib. Class Ia forms a heterodimeric complex with the regulatory subunit p85. Both PDGFR (platelet-derived growth factor receptor) and Insulin receptor activate class Ia and is essential for cell proliferation [16]. Class Ib is activated by βγ subunits of the heterotrimeric G proteins. Class II family are mainly membrane bound and are activated by membrane receptors such as RTK and integrins. Three members of class II have been identified including PI3KCα, β and γ. On the other hand, class II is responsible for protein trafficking through lysosome (for review of PI3K biochemistry see [17, 18]). Many of the downstream effects of PI3K are mediated through the serine-threonine protein kinase AKT. Activation of PI3K stimulates the translocation of AKT to the plasma membrane and binds to phosphoinositide where conformation and phosphorylation events occur [19, 20]. AKT plays essentials roles in a variety of cellular functions

including cell growth, cell survival as well as protein translation. Further, knockout studies demonstrated that AKT indeed is essential for the growth of cells. *Akt* $^{-/-}$ mice have shorter life span. In addition, knockout mice showed insulin resistance and diabetes mellitus syndrome thus highlighting the complexity and importance of these targets [21, 22].

PI3K/AKT pathway plays a pivotal role in MM resistance to apoptosis. Cytokines such as IL-6, IGF-I, VEGF and integrins trigger the resistance mechanisms of MM to apoptosis. IL-6 and IGF-I are capable of activating PI3K in MM cells as shown by Tu *et al.* Activation of the PI3K activates its downstream targets BAD and NF-κB as well as the inactivation of caspase-9 leading to its anti-apoptotic mechanism (Figure 1) [23].

PI3K also activates the protein kinase C (PKC) signaling pathway which is involved in MM-cell migration and progression. Podar *et al.* [24] reported the effects of the activation of the PKC pathway in MM progression. They used a selective and orally bioavailable PKC inhibitor (enzastaurin) to study the effect of modulating PKC in MM cell-lines. Enzastaurin showed strong growth inhibition in a broad range of MM cell-lines. Also they explained that these inhibitory effects are due to the inhibition of PKC pathway.

### 2.2.2. Ras/MEK/MAPK pathway

The Ras/MEK/MAPK signaling pathway plays an essential role in cell proliferation, transformation, differentiation and apoptosis. This pathway is activated by extracellular activators as well as stress stimuli which activate the Ras proteins by inducing an exchange of the guanine diphosphate (GDP) with guanine triphosphate (GTP). Activated Ras phosphorylates and activates the mitogen-activated protein kinase (MAPK) which in turn phosphorylates extracellular signal regulated kinase (ERK). Activated ERK stimulates transcriptional factors that control cell survival and growth (for further review see [25]).

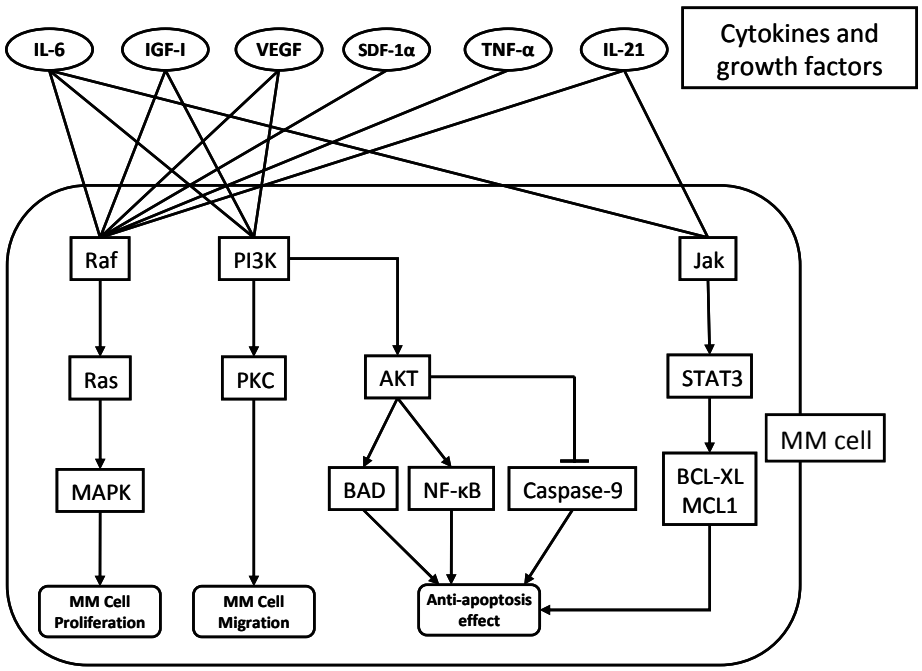



**Figure 1. MM signaling pathways involved in growth, resistance and migration.** IL-6, IGF-I, VEGF, SDF-1α, TNF-α and IL-21 mediate MM cell proliferation through the Ras/MAPK, IL-6, IGF-I and VEGF mediate MM cell resistance (anti-apoptosis) through the PI3K/AKT pathway which activates BAD and NF-kB as well as the inhibition of caspase-9. Protein kinase C (PKC) is induced via the PI3K pathway thus mediating the migration of MM cells. IL-21 and IL-6 mediate MM cell resistance via the Janus kinase.

Specific cytokines such as IL-6, IGF-I, VEGF, TNF-α, SDF-1α, and IL-21 mediate the proliferation of MM cells through the Ras/MEK/MAPK signaling pathway (Figure 1). Several attempts target this pathway in MM treatment. For instance, Bay 43-9006/sorafinib/Nexavar (Bayer Pharmaceuticals), an oral multi-kinase inhibitor of Raf kinase cascade, showed remarkable anti-MM activity [26].

**2.2.3. NF-κB signaling pathway**

NF-κB was first described in 1986 by Ranjan Sen and David Baltimore as a nuclear transcription factor that is necessary for immunoglobulin kappa light chain transcription in B cells [27]. It has been proven that NF-κB exists in the cytoplasm of most cells in its inactive form bound to its inhibitor IκB (Inhibitor of kappa B). Upon activation, IκB kinase (IKK) phosphorylates IκB thus releasing the free form of NF-κB which translocate to the nucleus. Five members of NF-κB family have been identified in mammals including c-Rel, Rel A (p65), Rel B, p50/p105 and p52/p100. The most common heterodimer of the NF-κB is the p50/p65 heterodimer which is present in many cancer cell types [28, 29]. NF-κB activation has been related to tumor growth, survival and proliferation thus promoting it as a hot topic for cancer research. NF-κB induces anti-apoptotic factors thus stimulating tumor growth and progression. It induces the cellular inhibitors of apoptosis (cIAPs) including c-IAP1, c-IAP2, and XIAP (X-linked inhibitor of apoptosis). The cIAPs suppress apoptosis by direct inhibition of caspases-3, -6, -7, and -9 [30]. NF-κB further exerts its anti-apoptotic mechanisms by up-regulating anti-apoptotic members of the BCL-2 family including A1/BFL1 and BCL-$X_L$ [31-34]. In addition, NF-κB induces the FLICE-like inhibitory protein FLIP ((FAS-associated death domain)-like-IL-1β-converting enzyme inhibitory protein) which competes with caspase-8 to the death-inducing signaling complex (DISC) thus inhibiting apoptosis [35, 36].

NF-κB has been proven to play essential roles in cell cycle progression by the regulation of important genes involved in cell cycle such as cyclin D1, D2, D3 and E [37]. Further, it induces the expression of cell adhesion molecules such as ICAM-1 and stimulates angiogenesis by inducing the expression of IL-8 and VEGF [38]. Ni *et al*. studied the expression of NF-κB levels in multiple myeloma and its relation to disease suppression and stimulation. They reported that high levels of NF-κB were detected in MM cell lines. In addition, NF-κB inhibition or stabilization by preventing its inhibitor (IκB) from degradation via the proteasome complex induces apoptosis in MM cell lines and primary MM cells [39]. Thus, NF-κB plays an important role in the pathogenesis of MM disease and its inhibition is considered a major target for MM drug development.

**2.2.4. Jak signaling pathway**

Janus kinases (Jak) are non-receptor tyrosine kinases comprising of four family members Jak1, Jak2, Jak3, and Tyk2 (Tyrosine kinase 2). Upon cytokine binding, phosphorylation of the cytoplasmic domains of the cytokines is activated during Jak kinases activation. It is believed that Jak kinases, in addition to their interferon and cytokine mediated signal transduction, act as mediators of multiple signaling pathways that are essential for normal cellular function. The first discovery of the Jak kinases occurred when a variety of experiments were performed to identify novel protein kinases. The full length cDNA of Jak1 and Jak2 were cloned using partial cDNA fragments as probes [40]. Further studies revealed that Jak represents a new family of protein tyrosine kinases which have an additional kinase domain rendering them a unique type of protein tyrosine kinase [41]. Jak kinases are involved in a variety of signaling pathways induced by interleukins and other signaling pathways which have been shown to be essential in regulating cell growth. Jak kinases are involved in the phosphorylation of specific Signal Transducer and Activation of Transcription protein (STAT). As a result, the phosphorylated STAT proteins dimerize and translocate into the nucleus leading to DNA transcription. Jak mutants have been shown to contribute in the pathogenesis of leukemia as well as myeloproliferative disorders [42]. Nevertheless, Jaks are activated by IL-6, interferon-α, and epidermal growth factor in MM suggesting its important role in cell survival and signaling (Figure 1) [43-45].

## 3. Anti-multiple myeloma agents

### 3.1. Immunomodulatory Drugs (IMiDs)

Immunomodulatory drugs represent a class of chemicals that possess immunomodulatory, anti-inflammatory and anti-angiogenesis properties. The major representative drug is thalidomide (Thalomid®), which was first introduced as a sedative drug for pregnant women (discussed in detail below). Other IMiDs that are known in the treatment of MM are lenalidomide (Revlimid®) and the most recently approved drug, pomalidomide (Pomalyst®).

#### 3.1.1. Mechanism of IMiDs

The enantiomeric inter-conversion and the spontaneous metabolism of thalidomide attributes to the limited understanding of its precise molecular mechanisms. Nevertheless, it is thought that the agent requires metabolic activation which is apparent due to the limited correlation of the *in vitro* and *in vivo* data. However, extensive studies about the mechanism of thalidomide as an anti-neoplastic agent (especially multiple myeloma) highlighted four main mechanisms: i) anti-angiogenic effects; ii) direct inhibition of tumor growth and survival; iii) inhibition of cell adhesion and cytokine interactions; and iv) immunomodulatory effects on T-cell activation and natural killer cell mediated responses [46-48].

##### 3.1.1.1. Effect on angiogenesis

Angiogenesis is the formation of new blood vessels from existing blood vessels during normal physiological growth, tissue healing and regeneration. Abnormal increased angiogenesis is related to tumor spread and metastasis [47]. Studies to identify the teratogenic effects of thalidomide facilitated the understanding of its effect on angiogenesis. D'Amato *et al.* showed that thalidomide is a potent inhibitor of angiogenesis in rabbit corneal assays [49]. As confirmed by Bertolini *et al.*, thalidomide exerts its activity against angiogenesis by inhibiting the two main pro-angiogenic cytokines: basic-fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [50]. It has been shown that thalidomide metabolism is essential for its activity against angiogenesis. A study conducted by Kenyon and colleagues showed that thalidomide and the thalidomide metabolite, 2-phthalimido glutaramic acid (PG acid), exhibited significant inhibition of bFGF-induced corneal neovascularization after i.p. administration. Active metabolite is produced when given *in vivo* due to the effect of liver microsomes. VEGF is considered an important mediator of angiogenesis. It acts as a potent inducer of microvascular permeability as well as a specific endothelial cell mitogen. Bellamy and colleagues have shown high expression levels of VEGF as well as bFGF in human hematopoietic tumor cell lines, including multiple myeloma cell-lines. Recombinant human VEGF is capable to induce a significant increase in the expression of IL-6 (a potent growth factor for myeloma cells and an inhibitor of plasma cell apoptosis) in human vascular endothelial cell-lines (Figure 2) [51].

Lenalidomide has an activity profile against angiogenesis similar to thalidomide. Lenalidomide suppresses angiogenesis by inhibiting the pro-angiogenic cytokines VEGF and bFGF. Lenalidomide has been shown to be 50 to 2000 times more potent than thalidomide in inhibiting angiogenic cytokines as explained by *in vitro* studies [52].

##### 3.1.1.2. Direct inhibition of tumor growth and survival

IMiDs have direct effect on multiple myeloma cells by inducing apoptosis or growth arrest in the $G_1$ phase of the cell cycle. This was demonstrated *in vitro* [53] and *in vivo* [54] in several multiple myeloma cells and in patients' multiple myeloma cells that are resistant to conventional agents, such as melphalan, doxorubicin and dexamethasone [53]. The apoptotic mechanisms of IMiDs in multiple myeloma include activation of caspase-8, downregulation of NF-κB transcriptional activity and sensitizing MM cells to Fas-induced apoptosis [55, 56].

Insulin-like growth factor I (IGF-I) plays an important role in embryonic development as reported by Stephens *et al.* IGF-I is involved in proliferation of early mesenchyme, lens cell growth and development as well as angiogenesis. Nevertheless, IGF-I stimulates chondrogenesis and limb development. Also, IGF-I stimulates VEGF synthesis leading to the activation of NF-κB. Further, fibroblast growth factor (FGF) has been shown to play a significant role in the limb development and limb initiation. Thalidomide has been shown to inhibit the activities of IGF-I and FGFs, thus demonstrating the teratogenic mechanism of thalidomide as well as its anti-MM activity [57].

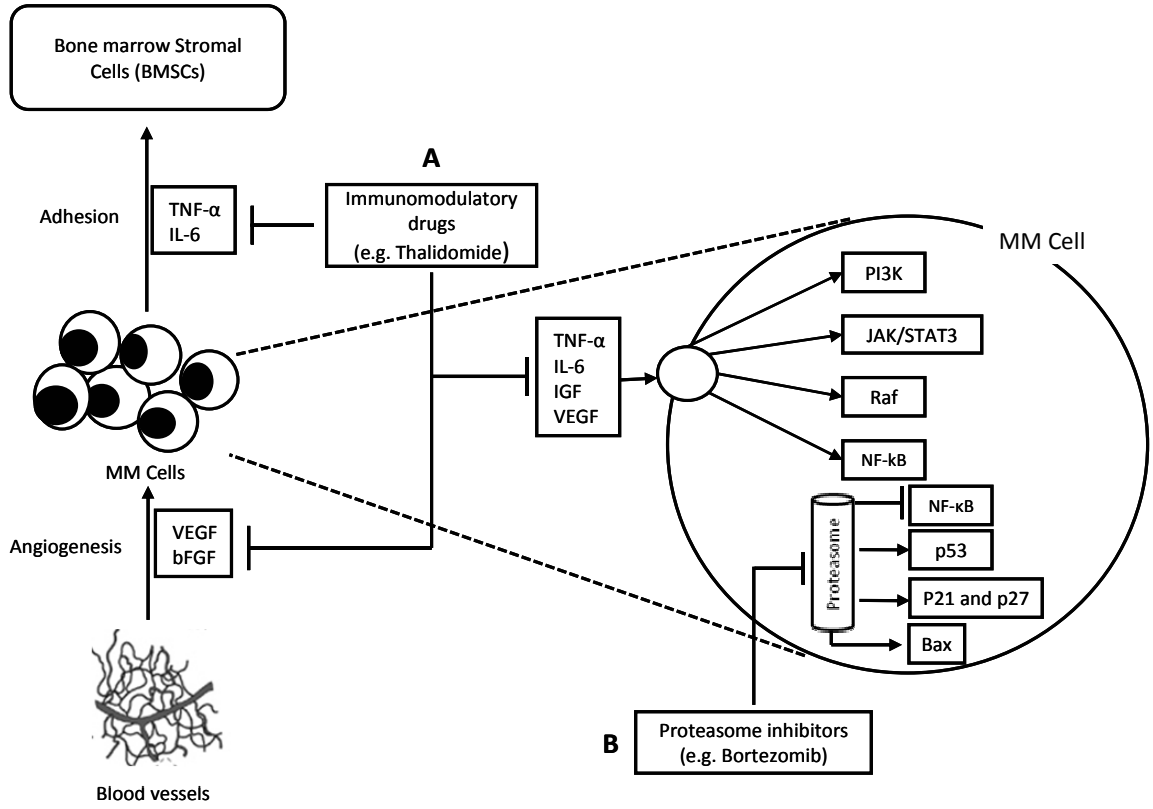



**Figure 2. Anti-MM molecular targets and mechanisms. A)** IMiDs inhibit angiogenesis, MM cell adhesion to BMSCs as well as direct tumor suppression. **B)** Proteasome inhibitors suppress the activation of NF-κB by inhibiting the degradation of its inhibitor IκB, accumulation of the pro-apoptotic factor p53, accumulation of cyclin-dependent kinase inhibitors (p21 and p27), and the accumulation of the pro-apoptotic Bcl-2 member Bax.

Another mechanism of IMiDs is the ability to block transcription factor nuclear factor κ-B (NF-κB). Keifer and colleagues had examined the molecular mechanism for NF-κB blockade. They reported that thalidomide (and other IMiDs) could suppress NF-κB by inhibiting TNF-α transcriptional factor. In addition, suppression of NF-κB will inhibit the ability of NF-κB to activate other gene expression targets. Further, they proved that thalidomide can inhibit the phosphorylation of IκBα by altering IKK activity resulting in the inhibition of NF-κB [56, 58]. As previously discussed, inhibition of NF-κB stimulates apoptosis via release of cytochrome c from the mitochondria to the cytoplasm. Also, caspase-3, -9 and PARP, but not caspase-8, are activated upon NF-κB inhibition. Furthermore, NF-κB inhibition downregulates the anti-apoptotic proteins Bcl-2, A1, XIAP and cIAP-2 and up-regulates the expression of the pro-apoptotic Bax protein. Finally, NF-κB inhibition sensitizes MM cells to TNF-α and doxorubicin as well as inducing a synergistic pro-apoptotic effect with a p38 inhibitor [59].

#### 3.1.1.3. Inhibition of cell adhesion and cytokine interactions

As previously discussed, MM cell adhesion to the bone marrow stromal cells (BMSCs) increases the survival, proliferation and resistance of MM cells to chemotherapeutic agents. Thalidomide and its analogs exert their activity on cell adhesion by modulating the expression of cell surface adhesion molecules such as induced ICAM-I, VCAM-I, E-selectin and L-selectin. The effect of thalidomide on these cell surface adhesion molecules can be explained by the downregulation of TNF-α. Cytokines such as interleukin-6 (IL-6), IGF-I, VEGF are affected by the downregulation of TNF-α resulting in MM cell death (Figure 2) [46, 56, 60].

#### 3.1.1.4. Immunomodulatory effects on T-cell activation and natural killer cell mediated responses

Thalidomide and other IMiDs stimulate natural killer cell (NK) and T lymphocytes. The mechanism was explained by Hayashi *et al*.; thalidomide enhances the anti-MM cytotoxicity of NK cells *in vivo*, inducing the production of IL-2 from T cells and enhancing the IL-2 transcription by nuclear translocation of nuclear factor of activated T cells [61, 62].

### 3.1.2. Thalidomide (Thalomid®)

Thalidomide was initially used in the clinic as a non-barbiturate sedative hypnotic in the late 1956. It was prescribed to pregnant women to treat morning sickness. It was widely marketed in several countries in Europe, Asia and America except the United States. Four years later, several birth defects began to appear linking thalidomide to teratogenic effects [63]. Several malformations have been reported such as absence of the ears, hypoplasia of the arms (phocomelia), defects of the lower limbs in addition to malformations in the internal organs such as heart, bowel, uterus and gallbladder [64]. In 1961, the drug was withdrawn from the market after affecting 10,000 children with birth defects.

Despite its teratogenic effects, thalidomide was approved in the US for the treatment of erythema nodosum leprosm (ENL) in 1998. In addition, several studies concluded its effectiveness against HIV wasting syndrome, aphthous ulcers and chronic graft versus host disease [65]. The European Medicines Agency approved thalidomide for the treatment of erythema nodosum leprosm (ENL), graft-versus-host disease and multiple myeloma [66].

Thalidomide (α-*N*-phthalidoglutarimide) is a glutamic acid derivative with a single chiral center, which exists in a racemic mixture, (*S*) and (*R*) enantiomers (Figure 3). Thalidomide can rapidly interconvert to the *S* and *R* enantiomers at physiological pH. *In vitro* studies demonstrated that the *S* isomer is responsible for the immunological effects and the *R* isomer for the sedative effects. Eriksson *et al*. however, have shown that the racemic mixture of thalidomide cannot be separated due to the *in vivo* inversion [67]. The poor water solubility of thalidomide hampers the formulation of an intravenous administration of the molecule. Therefore, thalidomide exhibits absorption rate limited pharmacokinetics (flip-flop phenomenon). *In vivo* studies demonstrated that after an oral single dose of thalidomide 100 mg for a 70 kg healthy volunteer, the maximum plasma concentration ($C_{max}$) is 0.6 μg/mL for the *R* enantiomer and 0.4 μg/mL for the *S* enantiomer at 4 h ($t_{max}$). In addition, thalidomide is eliminated mainly by spontaneous hydrolysis while hepatic metabolism and renal excretion have minor roles. Furthermore, thalidomide has an elimination half-life ($t½$) of 5 h with an apparent mean clearance for the *R* and *S* enantiomers of 10 L/h and 21 L/h, respectively [68]. Pharmacokinetic parameters of thalidomide are not affected by age, sex and smoking. Food effects are minimal and the parameters for the renal and hepatic dysfunction are not well established [60].

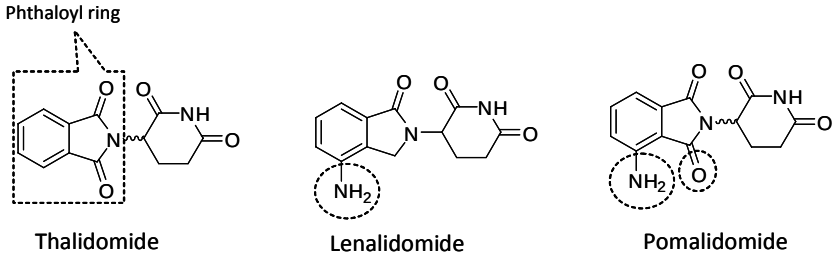



**Figure 3. Chemical structures of immunomodulatory Drugs (IMiDs) currently in the market.** Thalidomide (Thalomid®), Lenalidomide (Revlimid®, CC-5013) and Pomalidomide (Pomalyst®, CC-4047). Thalidomide was the first IMiD introduced in the market. Chemical modifications of the phthaloyl ring were performed by adding an amino group and removing one of the carbonyl groups to generate lenalidomide. Pomalidomide was derived from thalidomide with the addition of an amino group to thalidomide.

### 3.1.3. Lenalidomide (Revlimib®, CC-5013)

Lenalidomide is considered a second generation IMiD that has been developed by Celgene pharmaceuticals. The chemical analog lenalidomide was originally developed to enhance the TNF-α inhibition ability of thalidomide. A series of amino-phthaloyl substituted analogs of thalidomide and its isoindole-1-one analogs were prepared for structure-activity relationship. *In vitro*, thalidomide has an $IC_{50}$ of ~ 200 μM, while the 4-amino analog (amino group at carbon-4 of the phthaloyl ring) was found to be more potent with a TNF-α $IC_{50}$ of about 13 nM (Figure 3) [69].

Lenalidomide has shown a rapid absorption pharmacokinetic profile with maximum plasma concentration ($t_{max}$) reaching at a median of 60–90 minutes after oral administration. Maximum plasma concentration ($C_{max}$) declined in a monophasic profile. The half-life of lenalidomide was 3–4 hours. The mean accumulation ratio in plasma was 0.7–1.0, with a $C_{max}$ and area under the curve (AUC) 0.8–1.2 on day 28 compared to day 1. AUC and $C_{max}$ values demonstrated low to moderate inter-subject variability with values of 10.6–51.8% and 3–33%, respectively [70]. Approximately two-third of lenalidomide is excreted unchanged renally within 24 hours [71]. Incubations of human liver microsomes with lenalidomide demonstrated that it is not extensively metabolized by the liver [72].

### 3.1.4. Pomalidomide (Pomalyst®, CC-4047)

Pomalidomide ((RS)-4-amino-2-(2,6-dioxo-piperidin-3-*yl)*-isoindoline-l,3-dione) is a synthetic derivative of thalidomide with the addition of an animo group at carbon-4 in the phthaloyl ring (Figure 3). It is present as a racemic mixture of S/R enantiomers that undergoes stereoisomer inter-conversion *in vivo*. Pomalidomide exhibits a rapid absorption after oral administration reaching $C_{max}$ within 2.5–4 hours. Pomalidomide is metabolized primarily by CYP3A4 and 1A2 (enzymatic metabolism). Further, pomalidomide undergoes hydrolysis (25%) as a non-enzymatic metabolism. Pomalidomide is mostly excreted through urine with an elimination half-life ($t½$) of 8–12 hours in healthy individuals and 6–8 hours in MM patients [73].

In February 2013, pomalidomide was granted an accelerated approval by the U.S Food and Drug Authority (FDA) for the treatment of multiple myeloma.

## 3.2. Proteasome Inhibitors

Ubiquitin is a cellular 9 kDa protein that plays an essential role in protein homeostasis in the cytosol and nucleus of all eukaryotic cells [74]. Two major proteolytic pathways have been identified: lysosomal pathway, which is responsible for the degradation of extracellular proteins, and the ubiquitin-proteasome pathway (UPP), which is responsible for the degradation of intracellular proteins tagged by ubiquitin for destruction [75]. The UPP plays an important role in various cellular processes, such as cell differentiation, apoptosis, transcriptional regulation, cell cycle progression as well as immune and inflammatory responses [75, 76].

The 26S proteasome system (2.4 MDa) is a hollow cylindrical-shaped non-lysosomal multifunctional proteolytic complex present in the cytosol and nucleus of all eukaryotes [74, 77]. It is composed of a proteolytic core, namely the 20S (700 kDa), and two regulatory particles, namely the 19S (700 kDa). The 19S regulatory particles contain binding sites specific for ubiquitin and the cores are ATP dependent (Figure 4) [78].

The 20S proteolytic core is the major proteolytic complex of the 26S proteasome system and it is composed of four symmetric rings: two identical α rings and two identical β rings. These rings are stacked with the β rings and surrounded by the α rings giving the cylindrical shape of the proteasome (Figure 4). The β rings are composed of seven different subunits (β1 - β7). Among these subunits, three are involved in the proteolytic process: β1 (caspase-like), β2 (trypsin-like) and β5 (chymotrypsin-like) activities [78]. Specifically, the caspase-like (C-L) activity of the β1 subunit preferably cleaves after acidic residues, such as aspartate, glutamate or other acidic residues. The trypsin-like (T-L) activity of the β2 subunit cleaves preferably after basic residues, such as arginine or lysine. The chymotrypsin-like (CT-L) activity of the β5 subunit has been shown to cleave after hydrophobic residues, such as tyrosine and phenylalanine [79]. An amino residue from the amino acid threonine in the β subunits has been explained to play an important role of proteasome catalytic mechanism. In fact, a

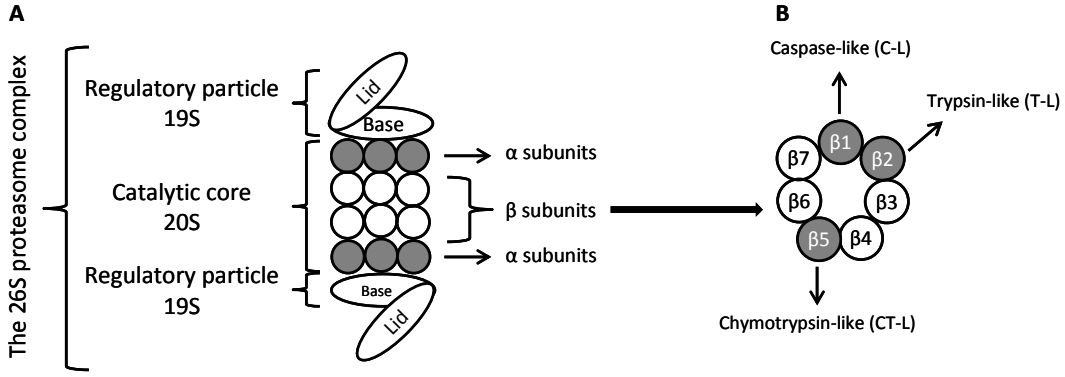



**Figure 4. The 26S Proteasome complex. (A)** The 26S proteasome complex is composed of a proteolytic core (the 20S core), and two regulatory particles (the 19S particle). The proteolytic core is composed of four symmetric rings; two identical α rings and two identical β rings. These rings are stacked with the β rings and are surrounded by the α rings giving the cylindrical hollow shape of the proteasome. **(B)** The β rings are composed of seven different subunits (β1 - β7). Two β1 (caspase-like), two β2 (trypsin-like) and two β5 (chymotrypsin-like) activities.

study on the X-ray of the 20S proteasome in complex with the inhibitor ALLN (N-acetyl-leucyl-leucyl-norleucinal) demonstrated that the formation of a hemi-acetal intermediate is essential for the proteasome inhibition. In addition, another X-ray crystallography analysis of the 20S proteasome in complex with the inhibitor bortezomib highlighted the importance of the N terminal of the threonine residue in the mechanism of the proteasome inhibition (see details in the following sections) [80].

Proteasomes are distributed within the nucleus and the cytoplasm of all eukaryotic cells where they play crucial role in maintaining normal cellular homeostasis. The 19S regulatory complex is a 700 kDa protein composed of 2 substructures: a lid and a base. The lid contains nine ATPases which remove polyubiquitin chain from the substrate in a high selective manner. On the other hand, the base substructure contains six ATPases that are required for protein substrate unfolding and processing the substrate to enter the β chamber for catalysis [74, 79]. Proteins marked for degradation via the 26S proteasome are tagged with polyubiquitin chain with the aid of three enzymes: ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-ligase enzyme (E3). The E1 ubiquitin-activating enzyme forms a thiol ester bond between the COOH-terminal glycine on the ubiquitin and an internal cysteine residue in the E1 enzyme. E2 enzyme transfers the activated ubiquitin moiety. E3 mediates the formation of an isopeptide bond with a lysine residue on the targeted protein (Figure 5) [78, 81].

### 3.2.1. Mechanism of anti-MM proteasome inhibitors

The 26S proteasome is involved in the regulation of many proteins that are known to control cell cycle progression, apoptosis, angiogenesis and expression of genes that regulate other processes. Among those targets are nuclear factor-kappa B (NF-κB), caspases, pro-apoptotic targets and other cell-surface and chemokine receptors. These targets and other related substrates of the 26S proteasome involved in cancer therapy are further discussed below.

#### 3.2.1.1. Nuclear factor-kappa B (NF-κB)

The NF-κB is involved in many cellular processes and tumorigenesis. NF-κB has been shown to induce cell proliferation, migration and angiogenesis as well as suppress apoptosis. In addition, NF-κB is known to be the "central mediator of the immune response" due to its involvement in the regulation of the expression of inflammatory cytokines, chemokines, immunoreceptors, growth factors, cell adhesion molecules and cell surface receptors (Pahl *et al*. [82] provided a concise review of NF-κB activator and target genes).

As previously discussed, NF-κB is a heterodimer of p50 and p65 and it is bound to its inhibitor IκB in the cytoplasm confining it in the inactive state.

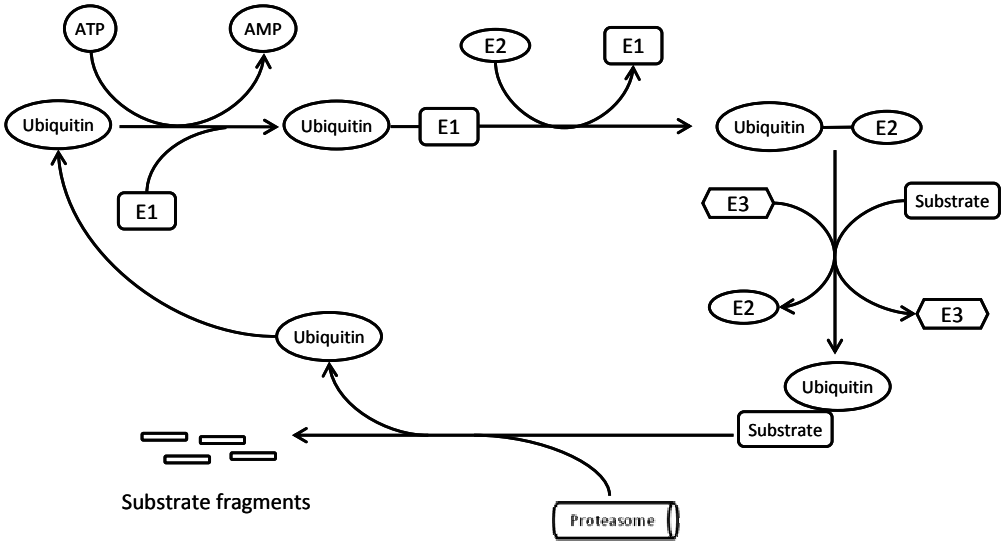



**Figure 5. The ubiquitin-proteasome pathway.** Proteins marked for degradation are tagged with polyubiquitin chain with the aid of three enzymes; ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-ligase enzyme (E3). The E1 ubiquitin-activating enzyme activates the ubiquitin by forming a thiol ester bond between the COOH-terminal glycine on the ubiquitin and an internal cysteine residue in the E1 enzyme. E2 enzyme transfers the activated ubiquitin moiety. The carboxyl terminus of the ubiquitin forms an isopeptide bond with a K residue on the targeted protein with the aid of E3 enzyme and the subsequent degradation of the targeted substrate.

Phosphorylation of IκB by external stimuli, such as irradiation, stress, pathogen etc., triggers the polyubiquitination of IκB marking it for degradation by the proteasome. Degradation of IκB releases the free NF-κB complex and activates the transcription of genes regulating cell-cycle progression and growth, such as growth factors, cell adhesion molecules, angiogenesis factors and anti-apoptotic factors. Proteasome inhibitors block the degradation of IκB by the 26S proteasome complex rendering NF-κB in the inactive state (Figure 2). Therefore, the 26S proteasome complex and the proteasome inhibitors play a pivotal role in cancer therapy. The role of NF-κB activation and inhibition in multiple myeloma patients as well as multiple myeloma cell-lines has been studied by Ni *et al.* [39]. They analyzed the expression levels of NF-κB in 13 primary samples from multiple myeloma patients and four samples in MM cell lines. High expression levels of NF-κB were found in all samples taken from MM patients and MM cell-lines. Furthermore, they reported that proteasome inhibitors, such as Bortezomib, and IκB phosphorylation inhibitors, such as Bay 117082, induced apoptosis, further underlining the important role of proteasome inhibitors in cancer therapy including multiple myeloma.

#### 3.2.1.2. Cyclin-Dependent kinase inhibitors

Cyclin-dependent kinases (CDKs) are serine/ threonine kinases that strongly regulate cell cycle transition between the cell cycle phases [83]. CDKs are regulated through two processes: positive regulation depends on the regulatory subunits including cyclins, while the negative regulation depends on the naturally occurring CDK inhibitors (CDKIs) [83, 84]. It has been reported that in tumors CDK machineries are deregulated and many genes involved in cell cycle progression are mutated. In fact, more than 80% of human tumors are related to the CDK-cyclinD/INK4/retinoblastoma-E2F abnormalities [85]. Five different CDKs are involved in the cell cycle process: CDK1, CDK2, CDK3, CDK4, and CDK6. These CDKs are associated with specific cyclins which restrict the kinases to specific substrates. Cyclin D is activated during mid to late $G_1$ which phosphorylates the cell cycle inhibitor pRb. The phosphorylation of the inhibitor pRb inactivates their regulatory functions which in turn allow the cell cycle to progress into the S phase. During cell cycle progression, cyclin A is accumulated at the $G_1$/S phase. Cyclin A activates CDK1 and CDK2 thus promoting the cell cycle to the $G_2$ interval of the cell cycle. Cyclin B drives the cell cycle into mitosis [83].

Two important CDKIs have been shown to be affected by the proteasome inhibition: p21 and p27 (p21 and p27 members of Cip/Kip family of CDKIs). p21 is a potent CDKI that inhibits the activity of CDK1, CDK2, CDK4 and CDK6, whereas p27 is an inhibitor of CDK2 and CDK4. p21 and p27 are degraded by the proteasome to release active CDKs for cell cycle progression. Proteasome inhibitors inhibit the degradation of p21 and p27 resulting in the accumulation of p21 and p27 (Figure 2). Accumulation of these CDKIs results in cell cycle arrest and eventually apoptosis [78].

#### 3.2.1.3. P53 pro-apoptotic factor

P53 is a tumor suppressor gene that was identified in 1979 and named as "the guardian of the genome". P53 plays essential roles in tumor suppression via the induction of cell cycle arrest, apoptosis and aging. Further, recent studies have shown other tumor suppression pathways of p53 including regulation of mRNA processing, effects on survival proteins in the mitochondria and involvement in DNA repair pathways [86]. Lim and colleagues have shown that mutations in the tumor suppression gene *tp53* occur frequently in cancer and explain the major roles of p53 in cancer prevention [87]. In fact, p53 function is lost in about 50% of human cancers either by mutations or deletions in the p53 gene. As reported by Soussi *et al*., p53 mutations are commonly observed in solid tumors such as lung (70%), colon (60%), head and neck (60%) as well as in ovarian cancers (60%). On the other hand, only 10% of hematological cancer cases involve p53 mutations [88]. Nevertheless, mutation and/or deletion of the p53 gene are associated with more aggressive disease state and high resistance rates in hematological cancers [89].

The 26S proteasome complex controls the degradation of the tumor suppressor factor p53 as the proteasome inhibitors increase the levels of p53 leading to the activation of p53 downstream genes such as Bax, p21, PUMA, Fas ligand and NOXA (Figure 2). Thus, proteasome inhibitors induce cell cycle arrest and apoptosis as p53 levels increases.

#### 3.2.1.4. Caspases apoptosis

Caspases (name derived from cysteine aspartase) are family of conserved cysteine proteases that cleave after an aspartate residue in the substrate. Caspases are considered the major component of apoptosis. Human caspases are classified into two groups: i) initiator caspases which include caspase-2, -8, -9 and -10, ii) effector caspases which include caspase-3, -6 and -7. Caspases are synthesized as catalytically inactive zymogens which must undergo activation during apoptosis. Among these caspases, caspases-3 and -8 are essential to apoptosis. NF-κB activation protects cells against apoptosis by suppressing caspase-8 (for review see [89]). As previously discussed, proteasome inhibitors suppress the activity of NF-κB by inhibiting the degradation of IκB thus decreasing NF-κB activity, which in turn potentiate caspase activity and induce apoptosis [90-94].

#### 3.2.1.5. Mitochondria

To illustrate the role of mitochondria in apoptosis and the effects of proteasome inhibition in cancer therapy, we have to demonstrate the biochemistry and the mechanism of the Bcl-2 family members as they play a pivotal role in the mitochondria apoptosis pathways. The Bcl-2 family members are divided into three major categories based on their Bcl-2 Homology (BH) domains: i) the anti-apoptotic class, which includes molecules that contain BH domains 1, 2, 3 and 4 such as Bcl-2, Bcl-xl, Bcl-$K_L$, Bcl-W, Mcl-1, Bcl-B and Bcl-2A1; ii) the pro-apoptotic class (promote apoptosis), which include molecules that contain BH domains 1, 2 and 3 such as Bax, Bak and Bok; and iii) Another pro-apoptotic class which only contain the BH domain 3 (BH3-only proteins) such as Bad, Bik, Bid, Hrk, Bim, Bmf, NOXA and PUMA. The Bcl-2 members regulate the mitochondrial outer membrane permeabilization (MOMP). The pro-apoptotic Bcl-2 members, such as Bax, Bok and Bad, regulate the mitochondria outer membrane inducing its permeability and subsequent release of apoptotic molecules from the mitochondria membrane including cytochrome c and DIABLO (direct inhibitor of IAP with low pl). Nevertheless, the anti-apoptotic Bcl-2 members, such as Bcl-2 and Bcl-xl, inhibit the prop-apoptotic Bcl-2 members Bax and Bak [95, 96]. Proteasome activity modulates the levels of anti-apoptotic and pro-apoptotic factors of the Bcl-2 family members, thus controlling apoptosis [97, 98]. Inhibition of the proteasome activity by proteasome inhibitors have shown to increase the levels of the pro-apoptotic Bcl-2 protein members

(Bax, Bim, NOXA and Bik) and reduce the levels of the anti-apoptotic Bcl-2 members (Bcl-2 and IAP) (Figure 2) [98]. Therefore, inhibitors of the proteasome activity in cancer cells promote apoptosis by up-regulating the pro-apoptotic factors and down-regulating the anti-apoptotic factors. In fact, several studies demonstrated that upon proteasome inhibition, apoptosis is induced and the cancer cell dies [97].

### 3.2.2. Major classes of proteasome inhibitors

The main target of proteasome inhibitors is the hydroxyl group of the *N*-terminal threonine (Thr) in the catalytic β-subunit located in the proteolytic core of the 26S proteasome system. Proteasome inhibitors are considered members of the Ntn (*N*-terminal nucleophilic) hydrolases group due to the presence of *N*-terminal threonine residues in the proteolytic core. Mutational and X-ray crystallographic studies of the proteasome catalytic sites showed that the chymotrypsin-like (CL) is responsible for the major breakdown of substrates [99-102]. Proteasome inhibitors can be classified based on their pharmacophore that interact with the threonine residue of the proteolytic subunit including aldehyde, boronic acid, vinyl sulfones and epoxyketones.

#### 3.2.2.1. Peptide aldehydes

The first proteasome inhibitor of the 20S proteasome complex discovered is the peptide aldehyde class and yet the most widely used among the proteasome inhibitors [103]. Peptide aldehydes are composed of numerous peptide sequences connected to a functional aldehyde terminal [104]. They are considered reversible and potent inhibitors of the threonine in the active site of the proteasome. Peptide aldehydes form a reversible covalent hemiacetal intermediate between the aldehyde and the hydroxyl group of the amino acid terminal (Thr) (Figure 6 A) [105]. Calpain inhibitor I (N-Acetyl-Leu-Leu-Norleucinal) was the first peptide aldehyde proteasome inhibitor discovered and has been used extensively to explain the mechanism of the proteasome and other proteases [106, 107]. It shows selectivity towards the chymotrypsin-like activity of the proteasome with an $IC_{50}$ of 2.1 μM. It is 25-fold more potent against cathepain B (0.006 μM) and calpain (0.005 μM), albeit it shows a relatively enhanced selectivity towards the CT-L activity of the proteasome (2.1 μM) [108]. Tyropeptin A, isovaleryl-L-tyrosyl-DL-tyrosinal, is a natural proteasome inhibitor isolated from *Kitasatospora species*. It showed a potent inhibition of the proteasome with $IC_{50}$ of 0.14, 5.0 and 68.0 μM for the chymotrypsin, trypsin and caspase-like activities of the proteasome, respectively [109, 110]. Further, the natural product fellutamide B, isolated from *Penicillium fellutanum*, showed potent inhibition of the chymotrypsin-like activity of the proteasome ($IC_{50}$ 9.4 nM) compared to the trypsin and caspase-like activity inhibition, $IC_{50}$ 2.0 μM and 1.2 μM, respectively [111]. Leupeptin, *N*-acetyl-L-leucyl-L-leucyl-L-argininal, is considered a natural protease inhibitor that has shown to inhibit potently and selectively the trypsin-like activity (Table 1). Efforts were made by Mroczkiewicz and colleagues to generate selective and potent proteasome inhibitors based on tripeptide aldehyde backbone (Cbz-P3-P2-P1-H). They reported the development of the proteasome inhibitor MG-132 where they introduced Leu at positions 1, 2 and 3 (Cbz-Leu-Leu-Leu-al). MG-132 showed a comparable potency to ALLN but enhanced selectivity towards the proteasome complex [112]. Dipeptide aldehydes were designed in order to overcome the lack of selectivity towards the CT-L over T-L activity. Iqbal *et al*. designed and synthesized a series of dipeptide aldehydes by modifying a tetrapeptide aldehyde, N-methoxysuccinyl-Glu-Val-Lys-Phe-H. CEP1612 was shown to inhibit the proteasome selectively and potently with an $IC_{50}$ of 0.002 μM towards the CT-L activity (Table 1) [103, 104]. Lindsten *et al*. reported that aldehydes are oxidized rapidly *in vivo* thus abolishing any systemic activity of peptide aldehydes in mice [113]. To overcome this issue, semicarbazone prodrugs have been synthesized and tested for potency towards the proteasome. These prodrugs showed a remarkable stability against acidic and basic condition as well as good anti-tumor activity [114].

#### 3.2.2.2. Peptide boronates

Due to the lack of specificity and the instability of peptide aldehydes, several studies have been conducted to circumvent these limitations. Peptide boronates, which replaces the aldehyde pharmacophore with a boronic acid moiety, are much more potent inhibitors of the proteasome than the aldehydes [115]. Peptide boronates form tetrahedral adducts with the active site of the proteasome (Thr). The tetrahedral adduct

is further stabilized by the second acidic boronate hydroxyl moiety forming a hydrogen bond with the Thr (Figure 6 B) [80]. These adduct showed slower dissociation rates compared to the hemiacetal bond formed with peptide aldehydes giving peptide boronate its strong inhibition. MG-262 (the boronate derivative of MG-132), Z-Leu-Leu-Leu-boronate, is 100-fold more potent than MG-132 with a $K_i$ of 0.03 nM as well as 200,000-fold selectivity for the proteasome. PS-341 (known as bortezomib, developed by Millennium Pharmaceuticals), pyrazylcarbonyl-Phe-Leu-boronate is a dipeptide boronate with introduction of a heterocycle in one of its terminals. Bortezomib exhibits high selectivity towards the proteasome with a $K_i$ of 0.62 nM and remarkable anti-tumor properties [115]. Bortezomib (Velcade®) was the first proteasome inhibitor approved by the United States Food and Drug Administration (FDA) for the first line treatment of MM in 2008. Bortezomib exhibited frequent side effects including nausea, fatigue, and diarrhea as well as serious side effects, including thrombocytopenia, peripheral neuropathy, neutropenia and lymphopenia that were less frequent [116, 117]. CEP-18770 (known as delanzomib), is a water soluble and orally bioavailable peptide boronate. It showed selectivity to the proteasome with a $K_i$ of 1.5 μM [118]. Studies by Ruggeri *et al.* demonstrated that the peripheral neuropathy toxicity of delanzomib is less common than bortezomib thus giving this drug the potential to overcome such limitation of bortezomib [119]. MLN-2238 is the biologically active form of MLN9708 when exposed to aqueous solution or plasma (*in vivo*). It preferablly inhibits the chymotrypsin-like site with a $K_i$ of 0.93 nM ($IC_{50}$ 3.4 nM) (Table 2). Further, it inhibits the

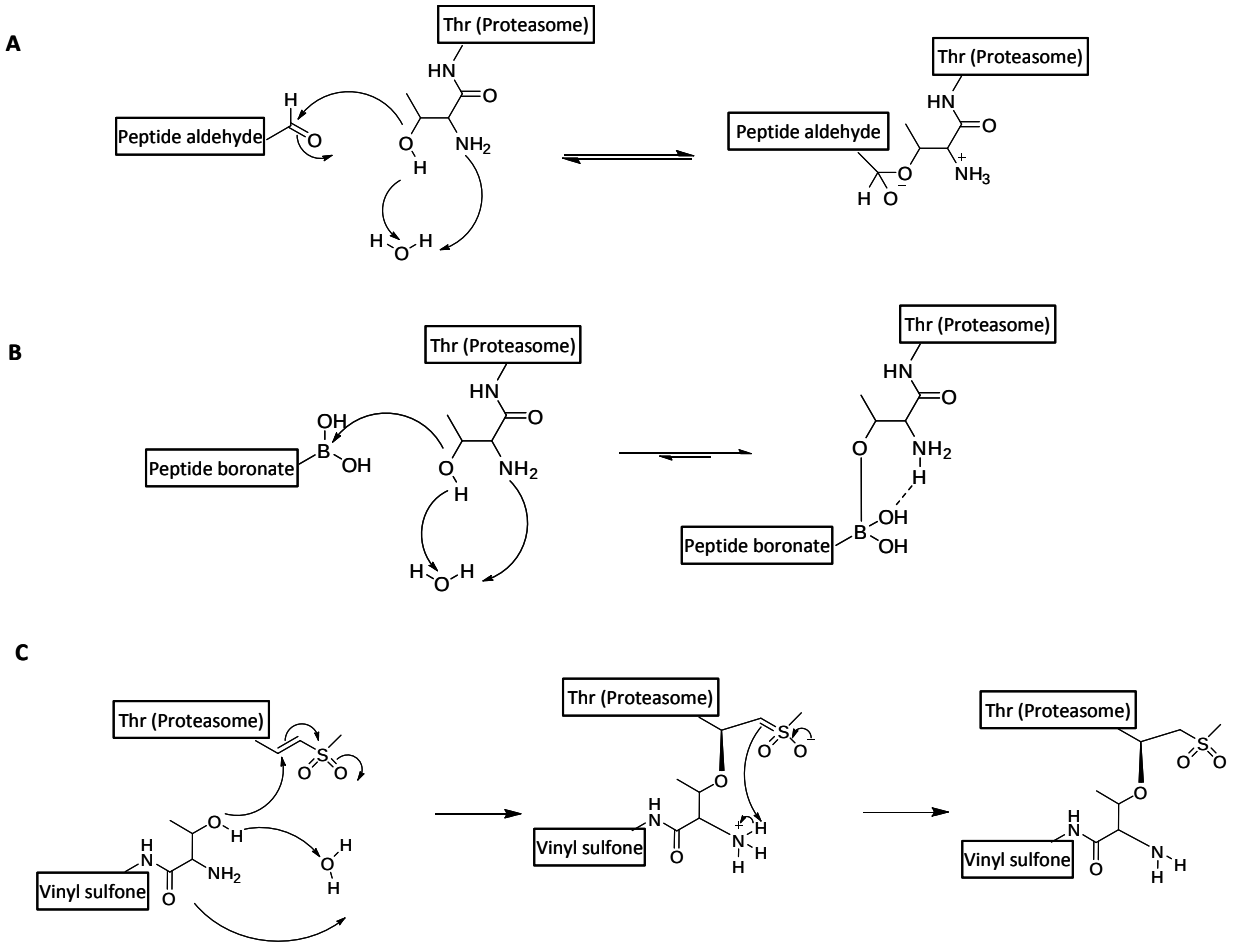



**Figure 6**

Figure 6 contd..

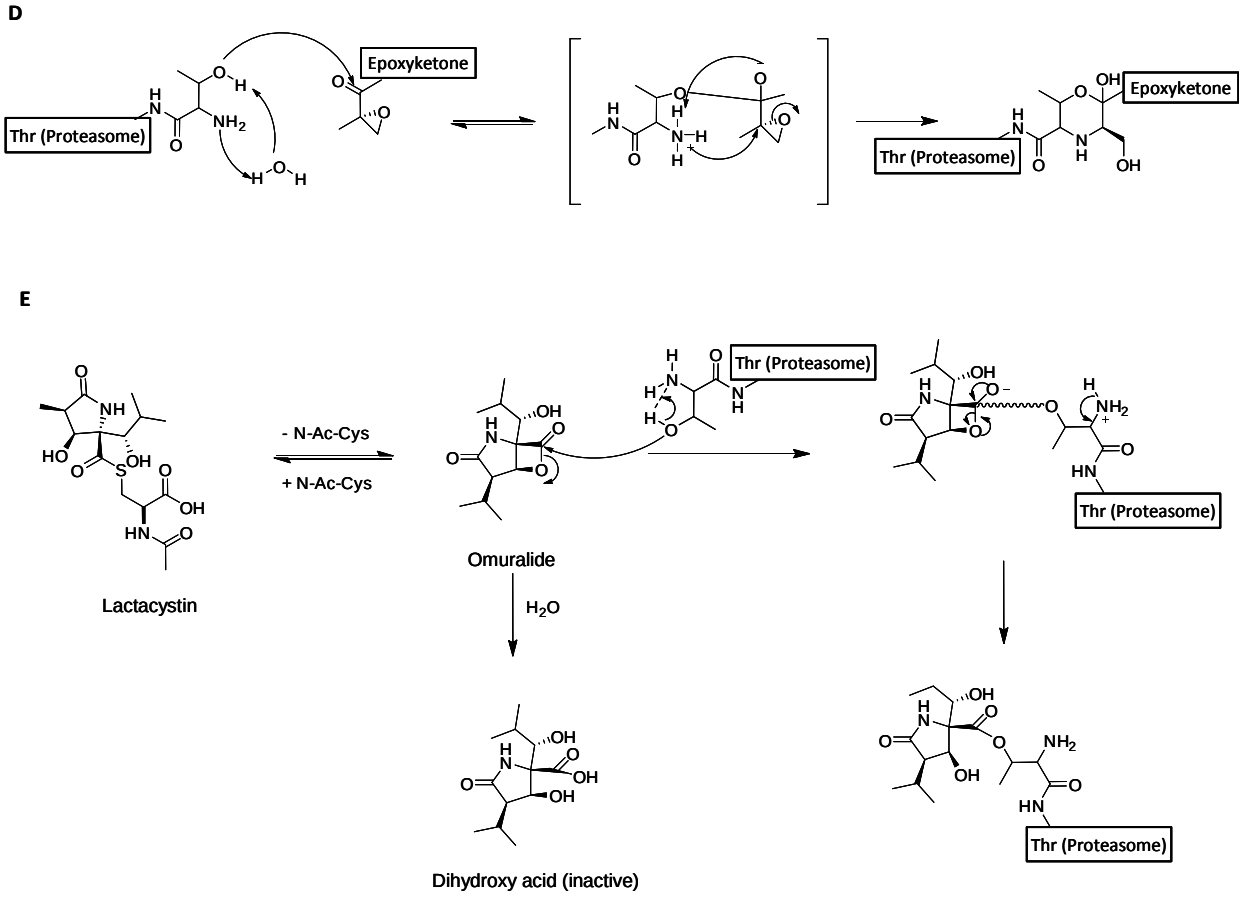



**Figure 6. Mechanism of proteasome inhibitors. A)** Mechanism of peptide aldehyde proteasome inhibition. Peptide aldehydes form a reversible hemiacetal bond between the inhibitor and the threonine residue at the proteolytic core of the 26S proteasome complex. **B)** Mechanism of peptide boronate proteasome inhibition. Peptide boronates form tetrahedral adducts with threonine residue of the proteasome. The tetrahedral adduct is further stabilized by the second acidic boronate hydroxyl moiety forming a hydrogen bond with the threonine residue. **C)** Mechanism of peptide vinyl sulfones proteasome inhibition. The hydroxyl group in the threonine residue of the proteasome proteolytic site form irreversible covalent bond with the vinyl sulfone moiety of the inhibitor. **D)** Mechanism of peptide epoxyketones proteasome inhibition. Epoxyketones form a unique six membered morpholine ring resulting from adduct formation between the α', β'- epoxyketone pharmacophore of the inhibitor and the N-terminal Thr in the proteolytic active site of the 26S proteasome complex. **E)** Mechanism of lactacystin proteasome inhibition the β-lactone intermediate (omuralide) interacts with the active site of the proteasome Thr resulting in ring opening and acylation of the proteasome hydroxyl in the proteolytic site.

caspase-like and trypsin-like sites at higher concentrations with $IC_{50}$ values of 31 nM and 3.5 µM, respectively. Interestingly, both bortezomib and MLN-2238 are peptide boronates having comparable inhibition activities towards the CT-L site but their pharmacokinetic and pharmacodynamic profiles are quite different. *In vivo* studies by Kupperman and colleagues showed that the dissociation half-life (*t½*) for MLN-2238 and bortezomib is 18 and 110 minutes, respectivley. Further, MLN-2238 showed larger volume of distribution which may be attributed to the faster dissociation rate of MLN-2238 compared to bortezomib [120].

#### 3.2.2.3. Peptide vinyl sulfones

Peptide vinyl sulfones were first reported by Bogyo *et al.* as a novel class of proteasome inhibitors that

**Table 1. Peptide aldehyde inhibitors.** 50% inhibitory concentration ($IC_{50}$) of the 26S proteasome complex (chymotrypsin-like, trypsin-like and caspase-like activities) measured in micro-molar (μM), and the chemical structures of the major natural and synthetic peptide aldehydes highlighting the peptide aldehyde pharmacophore.

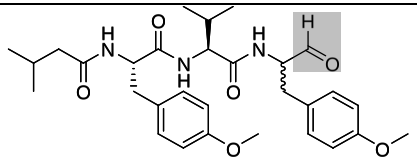
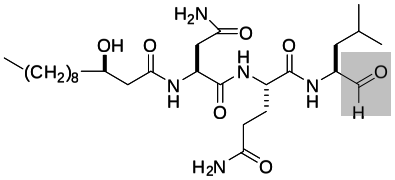
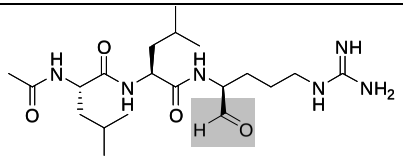
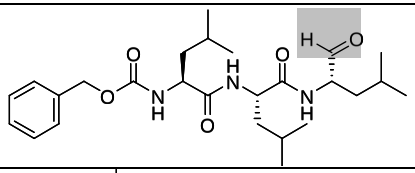
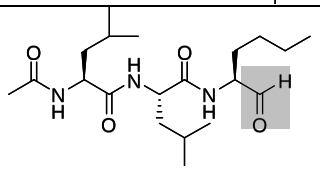
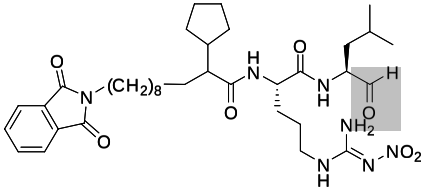
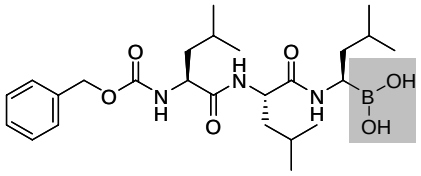
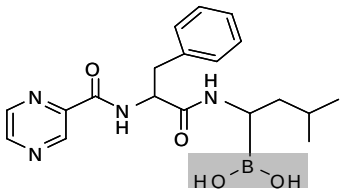
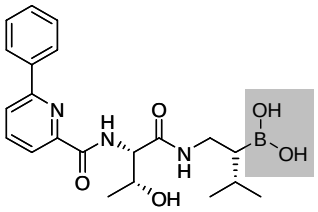
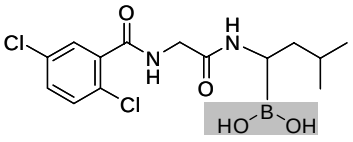
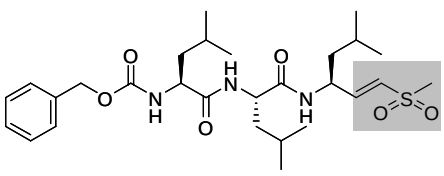
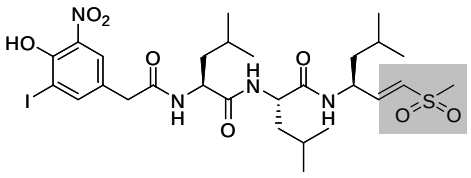
| Peptide Aldehyde Inhibitors | | 26S Proteasome $IC_{50}$ (μM) | | | Structure | Ref. |
|---|---|---|---|---|---|---|
| | | Chymotrypsin-like | Trypsin-like | Caspase-like | | |
| Natural | Tyropeptin A | 0.14 | 5.0 | 68.0 |  | [107, 108] |
| | Fellutamide B | 0.0094 | 2.0 | 1.2 |  | [109] |
| | Leupeptin | - | 0.0035 | - |  | [168] |
| Synthetic | MG-132 | 0.89 | 104.43 | 5.7 |  | [110] |
| | ALLN | 2.1 | > 100 | > 100 |  | [106] |
| | CEP1612 | 0.002 | - | - |  | [101, 102] |

**Table 2. Peptide boronate inhibitors.** binding affinity *($K_i$)* towards the 26S proteasome complex (chymotrypsin-like activity) measured in nano-molar (nM), and the chemical structures of the peptide boronate inhibitors highlighting the peptide boronate pharmacophore.

| Peptide Boronate Inhibitors | 26S Proteasome $K_i$ (nM) | Structure | Ref. |
|---|---|---|---|
| | Chymotrypsin-like | | |
| MG-262 | 0.03 |  | [113] |
| PS-341 (Bortezomib) | 0.62 |  | [113] |
| CEP-18770 (Delanzomib) | 1.5 |  | [114] |
| MLN-2238 | 0.93 |  | [115] |



Z-$L_3$-VS



NL-VS

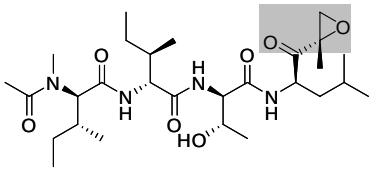
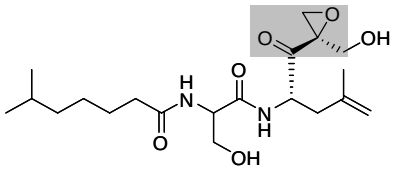
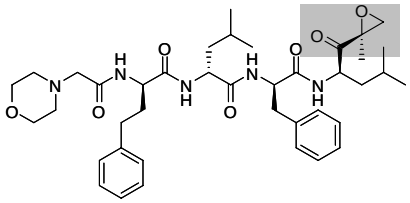
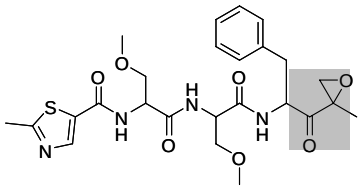
**Figure 7. Chemical structures of peptide vinyl sulfones.** Z-$L_3$-VS is the vinyl sulfone analog of MG-132. Replacement of the benzyloxycarbonyl (Z) group in Z-$L_3$VS by 3-nitro-4-hydroxy-5-indophenylacetate group gives NL-VS peptide vinyl sulfone. The peptide vinyl sulfone moiety is highlighted.

covalently inhibits the proteasome catalytic subunits [121]. Groll *et al.* explained the mechanism of covalent inhibition through X-ray crystallography studies. The hydroxyl group of the threonine residue in the proteasome catalytic site reacts with the double bond of the vinyl sulfone moiety forming an irreversible covalent bond (Figure 6 C) [122]. The vinyl sulfone analog of MG-132, Z-$L_3$VS, potently inhibits the CT-L activity as well as T-L and C-L activities *in vivo* and *in vitro*. Replacement of the benzyloxycarbonyl (Z) group in Z-$L_3$VS by 3-nitro-4-hydroxy-5-indophenylacetate group (known as NLVS) reduces the activity against cathepsin B and S (Figure 7) [118].

**3.2.2.4. Peptide epoxyketones**

Epoxyketones (α', β'-ketoepoxides) are considered the most selective and potent proteasome inhibitors. Epoxomicin and eponemycin (Table 3) are natural epoxyketones proteasome inhibitors isolated from different organisms [123, 124]. These novel antibiotics were first determined to be potent anticancer agents and powerful anti-angiogenic agents [125, 126]. Epoxomicin is a potent inhibitor of the CT-L site of the proteasome complex while eponemycin and its synthetic analog, dihydroeponemycin inhibit CT-L and C-L sites [127]. Epoxyketones are selective to the proteasome and have no inhibition effect on other non-protosomal proteases [128]. These compounds demonstrate a unique mechanism for the proteasome inhibition. The X-ray crystal structure of yeast proteasome in complex with epoxomicin showed a unique six membered morpholine ring resulting from adduct formation between the α', β'- epoxyketone pharmacophore of the inhibitor and the N-terminal Thr in the active site of the proteasome (Figure 6 D) [128]. Pioneer studies done by Meng *et al*. demonstrated the anti-inflammatory and anti-tumor properties of epoxomicin. *In vivo* studies showed that epoxomicin induced p53 accumulation and induce apoptosis. Further, it inhibited the degradation of IκB thus inhibiting NF-κB [127]. Carfilzomib (formerly known as PR-171) developed by Onyx Pharmaceuticals is a second generation peptide epoxyketone that irreversibly inhibits the proteasome complex (Table 3). Earlier studies demonstrated that carfilzomib inhibits the CT-L activity with minimal inhibition towards other catalytic sites. In addition, unlike bortezomib, carfilzomib showed improved selectivity towards the proteasome with reduced off-target inhibition [129]. Further, *in vitro* and *in vivo* studies showed improved activity of carfilzomib on a wide range of cancer types [130]. In 2012, carfilzomib (Kyprolis®) was granted

**Table 3. Chemical structures of the natural and synthetic peptide epoxyketones.** Natural peptide epoxyketones: epoxomicin and eponemycin. Synthetic peptide epoxyketones: carfilzomib and oprozomib. The epoxyketone pharmacophore is highlighted.

| Peptide epoxyketones | | Structure | Ref. |
|---|---|---|---|
| Natural | Epoxomicin |  | [123] |
| | Eponemycin |  | [124] |
| Synthetic | Carfilzomib |  | [129] |
| | Oprozomib |  | [132] |

an accelerated approval by the FDA for the treatment of patients with multiple myeloma [131]. Scientists at Proteolix, Inc. developed an orally administered proteasome inhibitor known as oprozomib (formerly known as PR-047 and ONX-0912). Oprozomib is a chemical derivative of carfilzomib with structure truncation and 2-Me-5-thiazole at the N-cap position (Table 3). As a result, this compound demonstrated improved selectivity to the CT-L activity ($IC_{50}$ 55 nM) with minimal activity towards T-L and C-L sites. Further, oral administration of oprozomib prompted an equivalent response to the parent compound carfilzomib in Non-Hodgkin's lymphoma cell line [132].

#### 3.2.2.5. Lactacystin and β-lactones

A *Streptomyces* metabolite, Lactacystin, has been identified by Omura and colleagues during their studies to address its ability to induce differentiation of neuronal cells [133]. Studies showed that Lactacystin induces neurite outgrowth in the mouse neuroblastoma cell lines (Neuro 2A) [134, 135]. Lactacystin demonstrated inhibitor effect on all proteasome proteolytic sites (CT-L, T-L and C-L) [136]. Dick *et al.* showed that lactacystin undergoes activation at neutral pH to produce the active β-lactone intermediate, *clasto*-lactacystin-β-lactone which reacts with the proteasome [137]. X-ray crystallography analysis demonstrated that the β-lactone intermediate contains an ester bond that interacts with the active site of the proteasome Thr resulting in ring opening and acylation of the proteasome hydroxyl moiety at the proteolytic site (Figure 6 E) [138]. NP-0053 (known as salinosporomide) is a natural compound isolated from *Salinispora tropica* (Figure 8) [139, 140]. NP-0053 showed strong inhibition activity towards all the three proteolytic sites of the proteasome and is currently undergoing phase I clinical trial for various hematologic malignancies (multiple myeloma, leukemia and lymphoma) as well as solid tumors [141].

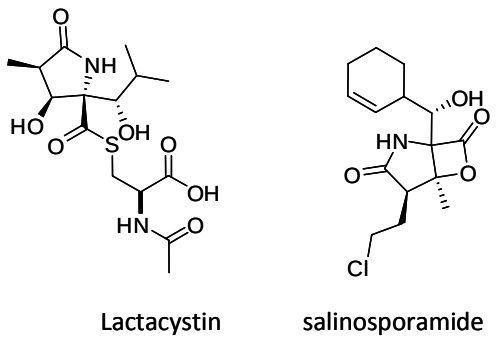



**Figure 8. Chemical structures of lactacystin and β-lactones.** Natural lactacystin and β-lactones: salinosporamide (also known as NP-0053).

## 4. New anti-MM targets and drugs under development

Continuous efforts are undergoing in the field of cancer research and drug discovery to produce new agent and/or to discover new targets to eradicate tumor progression. In the last decade, several new targets and new chemical agents have been filed for patent in the multiple myeloma drug discovery field since, as mentioned before, MM remains an incurable disease with high resistance and relapse rates. In the following section, we summarized the latest discovered targets and/or small molecules that have been filed for patent for the treatment of multiple myeloma.

### 4.1. PI3K/AKT

As mentioned above, the PI3K/Akt signaling cascade has been employed in several cancers since it induces cell survival and growth. Akt phosphorylates BAD thus inducing the expression of pro-apoptotic factors such as Fas. Further, Akt has been shown to have an anti-apoptotic effect by the activation of the transcription factor cyclic AMP element-binding protein (CREB) and IκB kinase (IKK). In addition, Akt plays a key role in cell cycle suppression by inhibiting the phosphorylation of the cytoplasmic signaling molecule β-catenin thus inhibiting the expression of cyclin D1 through GSK3 (glycogen synthase kinase -3) target [142]. Akt phosphorylates p21 as well as p27 thus limiting their anti-proliferative effects by stabilizing p21 and p27 in the cytoplasm [143-146]. Akt phosphorylates Mdm2 inducing a decrease in p53 levels and suppressing its transactivation. As a result, the Mdm-p53 leaves the nucleus and passes to the cytoplasm where they degrade via the ubiquitin/proteasome pathway [147].

LY294002 is a nonselective PI3K inhibitor that has been shown to enhance the destruction of tumor vasculature in endothelial cells [148]. LY294002 inhibits all members of class I PI3K with $IC_{50}$values within 1 μM (Figure 9) [17]. Further, wortmannin

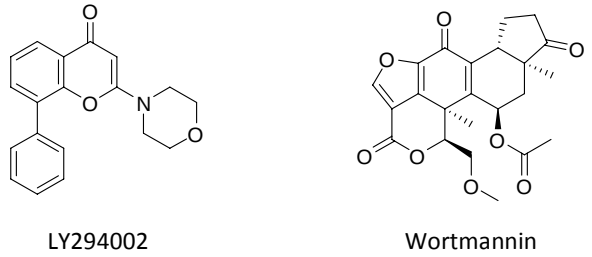



**Figure 9. Chemical structures of nonselective PI3K inhibitors.** LY294002 is a morpholine derivative with potent inhibition of PI3K. Wortmannin is a covalent inhibitor of PI3K.

is another nonselective PI3K inhibitor with $IC_{50}$ values in the range of 1 – 10 nM (Figure 9) [148]. The urge for developing selective PI3K inhibitors is necessary as certain isoforms of PI3K have shown to be effective in certain inflammatory conditions as well as autoimmune disorders.

A recent patent by Evarts *et al.* describes the discovery of novel quinoxaline derivatives as selective PI3K inhibitors with the ability to treat cancers and inflammatory diseases. Five derivatives (Q13, Q14, Q15, Q16, and Q17) showed selectivity for PI3Kδ with some extent to γ as demonstrated by $IC_{50}$ values (Table 4) [149].

Burger and colleagues first discovered a potent, selective and orally bioavailable class I PI3K inhibitor. NVP-BKM120 is a morpholino pyrimidinyl derivative that exhibits high potency and improved pharmacokinetic properties (Figure10, **2**). NVP-BKM120 showed 50-300 nM activity for class I PI3K with lower potency towards class II and class IV. Further, *in vitro* activity of the compound on different cancer cell lines including ovarian (A2780), glioblastoma (U87MG), breast (MCF7) as well as prostate (DU145) demonstrated the high activity of NVP-BKM120 on different types of cancers. As a result, this compound is being investigated in phase I clinical trials for solid tumors [150].

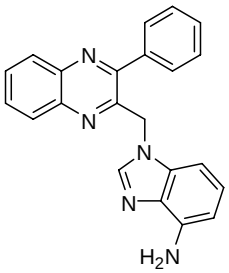
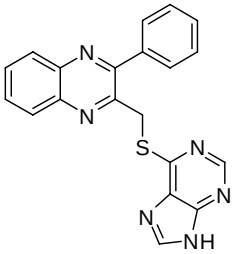
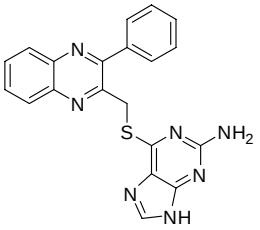
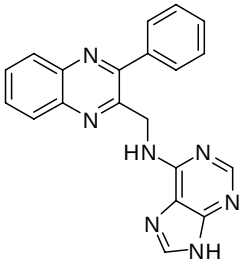
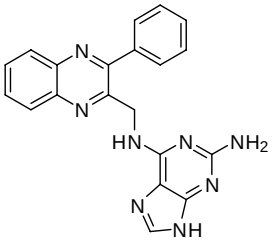
Zheng and colleagues studies the therapeutic efficacy of NVP-BKM120 and its potential use in multiple myeloma. NVP-BKM120 showed cell growth inhibition and induced apoptosis in MM cell-lines (U266, MM.1R, MM.1S, ARK and ARP-1). Furthermore, BKM120 has limited cytotoxicity effects on normal PBMCs. In addition, *in vivo* studies of BKM120 on MM-SCID mouse model demonstrated tumor suppression and prolonged survival [151].

### 4.2. Cannabinoid receptor 2 ($CB_2$) and the endocannabinoid system

The endocannabinoid system is composed of the cannabinoid receptors, endogenous endocannabinoids and the enzymes involved in the synthesis and degradation of endocannabinoids. To date, two distinct cannabinoid receptors have been discovered: cannabinoid receptor 1 ($CB_1$) and cannabinoid receptor 2 ($CB_2$) [152]. The $CB_1$ and the $CB_2$ receptors share 44% receptor homology (68% within the transmembrane domain) [153]. $CB_1$ and $CB_2$ receptors are members of the G-protein coupled receptor (GPCR) which both inhibit adenyl cyclase and activate mitogen protein kinase [154]. $CB_1$ receptors are predominantly expressed in the terminals of the central and peripheral nervous systems while $CB_2$ receptors are mainly expressed in the immune system and periphery. Due to the high expression of $CB_1$ receptors in the CNS, several psychoactive side effects are associated when targeting the $CB_1$ receptors [155]. Rimonabant is a $CB_1$ receptor antagonist that was approved for the treatment of obesity and marketed in the European countries in 2006. In 2008, the drug was withdrawn from the market for its psychoactive side effects and suicidal thoughts [156]. In an attempt to overcome the undesired side effects of $CB_1$, several $CB_2$ ligands have been designed and tested for several physiological and pathological conditions. It has been shown that the cannabinoid receptor 2 is expressed in many cancer tissues/cell lines, for example breast, prostate and bone cancers [157]. Munson and colleagues reported the first anti-tumor activity of cannabinoids in 1975. Since then anti-cancer mechanism of cannabinoids has been extensively studied. Yet, the mechanism by which cannabinoids attenuate tumor growth is not fully understood. However, research shows that cannabinoids exhibit anti-cancer activity through the modulation of cell differentiation, cell signaling, cell migration as well as cell fate [158].

A recent patent by Xie *et al.* studied the expression of $CB_2$ receptors in multiple myeloma cell lines. Unlike $CB_1$ receptors, western blot studies of multiple myeloma cell-lines (RPMI8226, U266, H929 and MM.1S) showed high expression levels of $CB_2$ receptors. This was confirmed by measuring the mRNA levels of $CB_2$ using RT-PCR utilizing $CB_2$ specific primers. As a result, extensive computational pharmacophore/quantitative structure activity relationship (QSAR) and medicinal

**Table 4. Chemical structures and 50% inhibition concentration ($IC_{50}$) of selective quinoxaline PI3K inhibitors.** The $IC_{50}$ of selective quinoxaline PI3K inhibitors: Q13, Q14, Q15, Q16, and Q17 on the four classes of PI3K (PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ). $IC_{50}$ values measured in micro-molar (μM) concentration.

| Compd. | 50% Inhibition Conc. ($IC_{50}$) in μM | | | | Structure |
|---|---|---|---|---|---|
| | PI3Kα | PI3Kβ | PI3Kδ | PI3Kγ | |
| Q 13 | - | - | 1.175 | 46.5 |  |
| Q 14 | - | - | 0.95 | 17.5 |  |
| Q 15 | - | - | 0.25 | 2.375 |  |
| Q 16 | 51 | 14.5 | 0.19 | 20.5 |  |
| Q 17 | 100 | 85 | 0.075 | 0.692 |  |

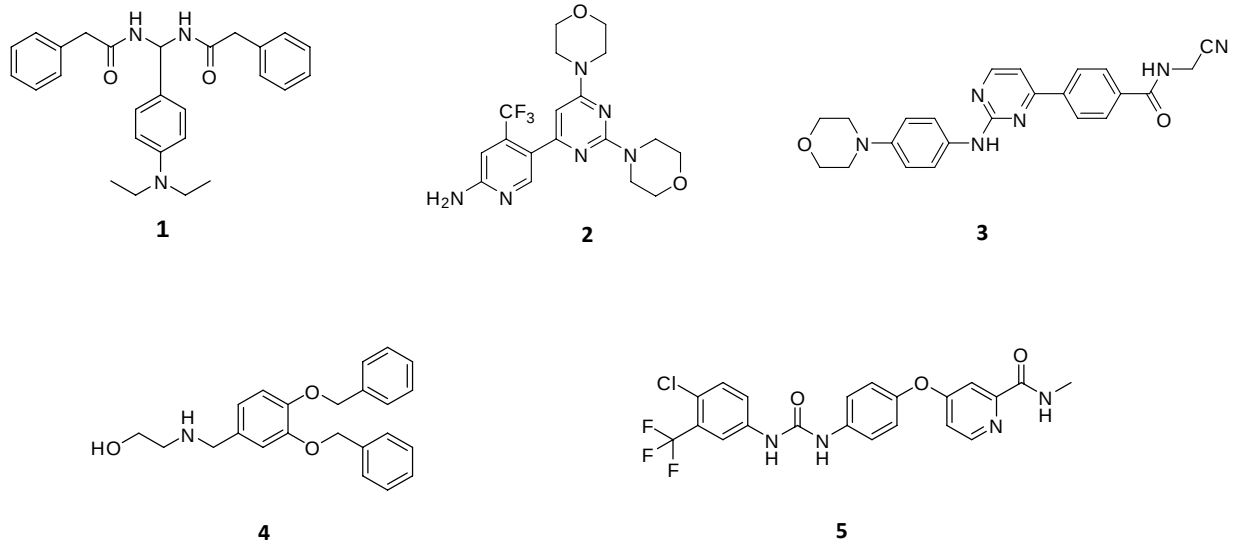



**Figure 10. Chemical structures of anti-MM chemicals reported in patents and/or in clinical trials.** Compound **1** (PAM) is a derivative of phenylacetamide with a di-amide backbone. It showed selectivity towards the cannabinoid receptor ($CB_2$) with a $K_i$ value of 777 nM. PAM was able to kill MM cells *in vitro*. Compound **2** (NVP-BKM120) is a selective inhibitor of PI3K. Compound **3** (CYT387) is a phenylaminopyridine derivative that shows potent and selective JAK inhibition ability. Compound **4** (XRK3) is a derivative of (3,4-bis(benzyloxy) phenyl)methanamine with potent p62-ZZ inhibitor. It showed MM cell growth inhibition with $IC_{50}$ value of 1 μM. Compound **5** (sorafinib) is a bisaryl urea derivative with dual kinase inhibitory effects. It is in phase II clinical trials for MM.

chemistry studies were employed to explore the potential of targeting the $CB_2$ receptors for MM intervention. They developed a series of small molecules that were tested on MM cell lines using different assays. They discovered a small molecule, a Phenylacetamide derivative (PAM), with di-amidebackbone (Figure 10, **1**). PAM showed potent selectivity towards the $CB_2$ receptor ($CB_2K_i$ = 777 nM, $CB_1K_i$ = >20,000 nM selectivity index = >26). Further, PAM was able to kill MM cells as confirmed by *in vitro* studies [159]. These data suggest the involvement of new receptors in the pathogenesis of MM and the possibilities of developing new chemical agents with the novel target.

### 4.3. JAK

Burns *et al.* demonstrated the discovery of a novel Jak inhibitor that targets all types of Jak kinases (Jak1, Jak2, Jak3 and Tyk2). CYT387 is a phenylaminopyridine derivative (Figure10, **3**) with improved selectivity and potency towards the Jak kinases. CYT387 has $IC_{50}$ values of 11 nM, 18 nM, and 155 nM for Jak1, Jak2 and Jak3, respectively. Further, this compound showed improved pharmacokinetic profile after oral and IV administration ($C_{max}$ = 40.4 μM, $t_{max}$ = 4 h, $t½$ = 2.4 h). The improved oral bioavailability may be attributed to the low blood clearance (6.3 mL/min/kg) and thus low susceptibility to hepatic first pass metabolism. *In vitro* cellular proliferation studies demonstrated that CYT387 is a potent inhibitor of Jak2 enzyme mutant and wild type with $IC_{50}$ values of 11 nM and 1430 nM, respectively [160]. In addition, they showed that CYT387 is active in the treatment of MM, in particular IL-6 non-responsive and CD45-phenotype MM cells. Their invention allows for the treatment of MM especially when the MM cells phenotypically shifts from CD45+ to CD45- and/or IL-6 non-responsive cells providing new insights in the targeting and treatment of MM disease [160].

### 4.4. P62-ZZ domain

p62 (also known as sequestosome 1) is an adaptor protein that serves as a modulator for essential cell signaling pathways including cell growth, proliferation, autophagy, controlling oxidative stress response and mitosis [161]. p62 has two essential domains

at the *N* terminus; PB1 domain that binds to the homotypic region in the atypical protein kinase C enzymes (aPKC), and a ZZ zinc finger domain that binds to the receptor interacting protein (RIP), which is a tumor necrosis factor α (TNF-α) signaling adaptor. Further, TBS amino acid sequence harbors the *N* terminus of the p62, which has binding domains to TNF receptor-associated factor 6 (TRAF6).

In several human cancers, levels of p62 were elevated indicating its importance for cell survival and proliferation [161]. p62 activates mTOR pathway leading to the inhibition of autophagy leading to tumor progression. Moreover, defect in autophagy in the presence of p62 causes damaging of mitochondria, elevated oxidative stress, DNA damage response as well as cytotoxic effects. These observations indicate that autophagy plays a crucial role in cancer by inhibiting p62 accumulation [162].

NF-κB is known to be activated upon the interacting of a PKC-interacting protein with receptor-interacting protein (RIP) in TNFα-dependent manner. RIP interacts via its death domain with Tumor necrosis factor receptor type 1 associated death domain (TRADD) that is known to bind to the RING (really interesting new gene) finger containing protein. Moreover, RIP also interacts with TRAF, which mediates the NF-κB signaling. This sequential mechanism of protein interaction suggests that the inhibition or deletion of the ZZ domain of p62 will decrease NF-κB levels leading to cell death. Moreover, Down-regulation of p62 severely inhibits NF-κB activation by interleukin-1 and TRAF6. Such interaction links the atypical PKCs (aPKCs) to NF-κB activation by the TNFα signaling pathway [163].

Dr. Roodman and colleagues studied the p62-ZZ domain inhibitory effects in MM. Different constructions of p62 lacking specific domain (ΔSH2, ΔPB1, ΔZZ, Δp38, ΔTBS and ΔUBA) were generated to identify the domain responsible for MM growth and osteoclast (OCL) formation. These different constructions were transfected into a p62-knockout stromal cell line and tested for their ability to support MM growth mediated by NF-κB and p38 MAPK signaling. These studies showed that the ZZ domain of p62 alone is required for stromal cell support of MM cell growth, increased IL-6, VCAM-1 expression and OCL formation thus illustrating the importance of the ZZ domain in MM progression [164].

In a recent patent by Xie *et al.*, they discovered and developed a series of novel p62-ZZ chemical derivatives from (3,4-bis(benzyloxy)phenyl) methanamine based on computational and experimental approaches (Figure 10, **4**). Specifically, XRK3 showed potent anti-MM effects without toxicity to normal stromal cells. *In vitro* data showed the ability of XRK3 to reduce the proliferation of MM cell lines (MM1.S cells) with $IC_{50}$ as low as 1 μM. Further, XRK3 was able to inhibit p-PKCζ, p-lkB (inhibition of NF-κB) and p-Akt activation thus explaining the mechanism by which these derivatives exert their anti-MM effects [164]. These data revealed new drug discovery opportunity to develop anti-MM chemicals by targeting the ZZ domain of the p62 protein.

### 4.5. Dual kinase inhibitors

Sorafinib (Nexavar®, Bayer Pharmaceutical) is a bisaryl urea targeting the Raf kinase pathway (Figure 10, **5**). The Raf pathway plays an important role in the development and progression of tumors including MM (discussed previously). Sorafinib has shown remarkable *in vitro* and *in vivo* efficacy on a wide range of cancers. In addition, sorafinib exhibited an inhibitory effect on the VEGF which represents an important cytokine in the development of tumors [165].

Ramakrishnan and colleagues explored the *in vitro* efficacy of sorafinib on MM disease as a dual Raf/VEGF inhibitor [166]. Sorafinib showed down-regulation of the phosphorylation of ERK (downstream signaling of the Raf kinase). In addition, sorafinib abrogated STAT3 and AKT phosphorylation as well as inhibition of Bcl-xL. Further, the up-regulation of Mcl-1 levels due to IL-6 and VEGF were diminished after treatment with sorafinib. Finally, sorafinib was able to inhibit the up-regulation of VEGF and IL-6 secretions in MM cell-lines. Collectively, these data prompted the *in vivo* studies of sorafinib [166, 167]. Currently, sorafinib is in Phase II clinical trials for the treatment of MM.

## 5. CONCLUSION

We have shown in this review the complicated pathogenesis involved in MM disease and the

difficulties in treating such a disease. We highlighted the molecular mechanisms of disease development and the strategies as well as key targets for the drugs currently in the market. Despite the discovery of the new IMiDs (Thalidomide and Pomalidomide) and proteasome inhibitors (Bortezomib), MM remains a challenging cancer to cure due to drug resistance and/or disease relapse associated with the current treatment modalities. Furthermore, we have reviewed the promising new targets that are under investigation. Among the new targets that are being investigated and evaluated are the cannabinoid receptor 2, the p62-ZZ domain as well as the introduction of dual (or multiple target) ligands that aim in targeting multiple MM molecular targets in order to overcome resistance and/or disease relapse. These newly discovered targets show a promising new horizon in the treatment of MM. Also, this review shows several novel new inhibitors for previously discovered targets such as Jak as well as PI3K/AKT. The area of MM drug discovery and development is demanding a huge attention worldwide and the discovery of a new small molecule as well as a novel MM target will dramatically benefit the MM patient population.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

## ABBREVIATIONS

AKT, protein kinase B; ATM, ataxia telangiectasia mutated kinase; AUC, area under the curve; bFGF, basic-fibroblast growth factor; BMME, bone marrow microenvironment; BMSCs, bone marrow stromal cells; CB, cannabinoid; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; C-L, caspase like; cIAPs, cellular inhibitors of apoptosis; CT-L, chymotrypsin like; DNA-PK, DNA-dependent protein kinase; ERK extracellular signal regulated kinase; ICAM-1, intercellular adhesion molecule -1; IGF-I, insulin-like-growth factor 1; IL-6, Interleukin-6; IL-21, interleukin-21; IMiDs, Immunomodulatory drugs; Jak, Janus kinases; MAPK, mitogen-activated protein kinase; (MIP)-1α, macrophage inflammatory protein; MM, multiple myeloma; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor of kappa B; OCL, osteoclast; PDGFR, platelet-derived growth factor receptor; PI3K, phosphatidylinositide 3-kinases; PIKK, PI3K-related kinases; PKC, protein kinase C; RANKL, receptor activator of nuclear factor-κB ligand; SDF-1α, stromal cell derived factor-1α; T-L, trypsin like; TNF, tumor necrosis factor; TNF-α tumor necrosis factor-α; UPS, ubiquitin proteasome system; VCAM-1 vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor

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