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Proteasome Inhibitors in the Treatment of Multiple Myeloma

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1. Introduction

The ubiquitin proteasome system is responsible for the degradation of proteins involved in a wide range of cellular processes such as the cell cycle, apoptosis, transcription, cell signalling, immune response and antigen presentation. Protein homeostasis is essential for normal cell growth and inhibition of proteasome function has emerged as a viable strategy for anti-cancer treatment. The first proteasome inhibitor to enter clinical practice, bortezomib, was approved by the Food and Drug Administration as a single agent to treat relapsed/refractory Multiple Myeloma in 2003 and expanded to first-line treatment in combination with melphalan and prednisone in 2008. It is now a routine component of Multiple Myeloma therapy and has had a major impact on expanding treatment options in the last few years. Bortezomib exhibits novel action against Multiple Myeloma by targeting both intracellular mechanisms and interactions within the bone marrow environment. Although it demonstrates significant anti-Myeloma activity when used alone, it has been shown to have even greater benefits when used in combination with conventional and novel chemotherapeutic agents. There are currently over 200 clinical trials ongoing or recently completed examining bortezomib alone and in combination in various stages of disease and treatment. The clinical success of bortezomib has prompted the development of a number of second generation proteasome inhibitors with improved pharmacological properties. In this chapter, we review the development of bortezomib as a novel therapeutic agent in Multiple Myeloma and summarize the key observations from recently completed and ongoing studies on the effect of bortezomib both as a single agent and in combination therapies in the setting of newly diagnosed Multiple Myeloma and for relapsed disease. We also discuss the progress of next generation proteasome inhibitors in the clinic.

2. The ubiquitin proteasome system

The ubiquitin proteasome pathway represents the major pathway for intracellular protein degradation. It is responsible for the degradation of approximately 80% of cellular proteins, including misfolded and mutated proteins as well as those involved in the regulation of development, differentiation, cell proliferation, signal transduction, apoptosis and antigen presentation. Proteins are degraded by the ubiquitin proteasome pathway via two distinct and successive steps: the covalent attachment of multiple monomers of ubiquitin molecules to a protein substrate and degradation of the tagged protein by the 26S proteasome. Tagging

of a protein by ubiquitin requires the action of three classes of enzymes – ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). A single E1 enzyme activates ubiquitin by forming a thiol ester bond between E1 and ubiquitin in an ATP-dependent step. Following activation, ubiquitin is then transferred to an active site residue within an E2 enzyme which shuttles ubiquitin either directly or in concert with an E3 enzyme to a lysine residue in the target protein. There are more than 30 different E2 and over 500 E3 enzymes, which work in cooperation to confer exquisite substrate specificity to the ubiquitin proteasome pathway. The successive conjugation of ubiquitin moieties generates a polyubiquitin chain that acts as a signal to target the protein for degradation by the 26S proteasome (Figure 1a).

The 26S or constitutive proteasome is found in the nucleus and cytoplasm of all eukaryotic cells. It is composed of a core 20S particle capped with a 19S structure at each end. The 20S catalytic core is made up of 28 subunits arranged into four stacked rings, creating a central chamber where proteolysis occurs. The two outer rings are composed of 7 different α subunits, which are predominantly structural and the two inner rings are composed of 7 different β subunits, at least three of which contain catalytic sites (Groll et al., 1997). Catalytic activities of the proteasome are classified into three major categories, based upon preference to cleave a peptide bond after a particular amino acid residue. These activities are referred to as chymotrypsin-like, trypsin-like and caspase-like and are associated with β5, β2 and β1 subunits respectively. The chymotrypsin-like activity cleaves after hydrophobic residues, the trypsin-like activity cleaves after basic residues and the caspase-like activity cleaves after acidic residues (Groll et al., 1999; Heinemeyer et al., 1997). Substrates gain access to the proteolytic chamber by binding to the 19S regulatory particle at either end of the 20S proteasome. Polyubiquitin-tagged proteins are recognised by the 19S particle, where ubiquitin is cleaved off and recycled and the target protein is unfolded and fed into the 20S catalytic chamber (Groll et al., 2000; Navon & Goldberg, 2001). An alternative proteasome isoform known as the immunoproteasome can be formed in response to cytokine signalling. Interferon-γ and tumour necrosis factor – α induce the expression of a different set of catalytic β-subunits and regulatory cap to form the immunoproteasome. Subunits β1i (LMP2), β2i (MECL1) and β5i (LMP7) replace constitutive subunits β1, β2 and β5 and the 19S regulatory cap is replaced with an 11S regulatory structure (Figure 1b). These modifications allow the immunoproteasome to generate antigenic peptides for presentation by the major histocompatability (MHC) class 1 mediated immune response (Rock & Goldberg, 1999). The expression of the immunoproteasome appears to be tissue specific and is particularly abundant in immune-related cells. Immunoproteasomes are highly expressed in haemopoietic tumours such as Multiple Myeloma.

3. Proteasome inhibitors as drug candidates

As the ubiquitin proteasome pathway plays a critical role in regulating many cellular processes, it is not surprising that defects within this pathway have been associated with a number of pathologies, including neurodegenerative diseases and cancer. Proteasome inhibitors were initially synthesized as *in vitro* probes to investigate the function of the proteasome's catalytic activity. However, as the essential role of the proteasome in cell function was established, the proteasome emerged as an attractive target for cancer therapy. Early studies showed that proteasome inhibitors induced apoptosis in leukaemic cell lines
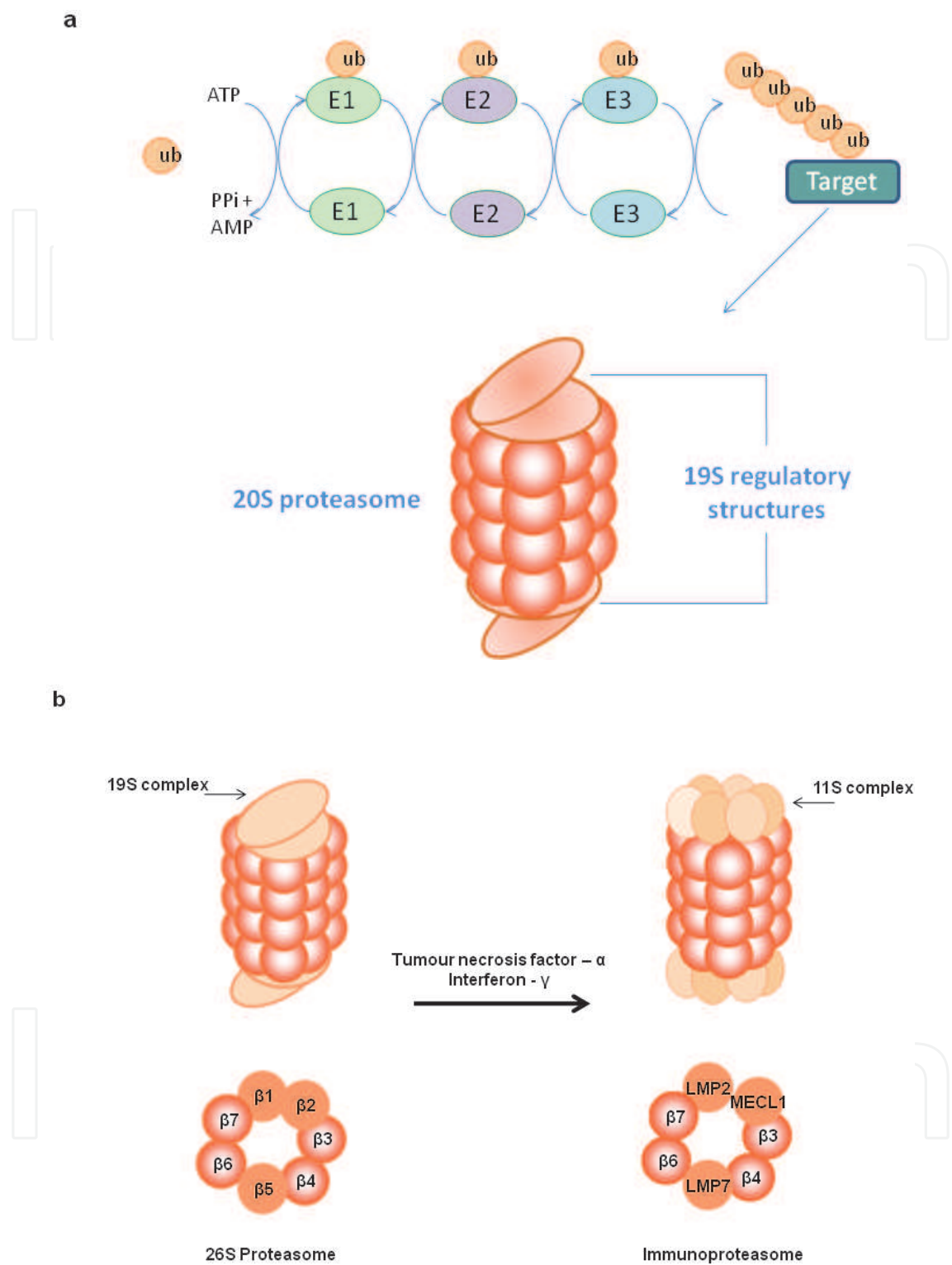
Fig. 1. a. Ubiquitin proteasome pathway mediated degradation. b. Proteasome composition.

(Imajoh-Ohmi et al., 1995; Shinohara et al., 1996; Drexler, 1997) and were active in an *in vivo* model of Burkitt's lymphoma (Orlowski et al., 1998). Further *in vitro* investigations demonstrated that proteasome inhibitors displayed a broad spectrum anti-proliferative and pro-apoptotic activity against haematological and solid tumours. While these studies

established the potential of proteasome inhibitors as anti-cancer agents, many of the compounds available were limited to laboratory studies due to a relative lack of potency, specificity or stability. This led to the development of a series of dipeptide boronic acids, which were more potent and selective than many previously available inhibitors. These inhibitors were screened *in vitro* against the National Cancer Institute's panel of cancer cell lines and on the basis of its cytotoxicity, the compound bortezomib (PS-341, Velcade®) was brought forward for further testing.

4. Mechanisms of action of bortezomib in multiple myeloma

Bortezomib is a reversible proteasome inhibitor, primarily of the chymotrypsin-like activity of both the constitutive (β5) and immunoproteasome (LMP7) (Lightcap et al., 2000; Crawford et al., 2006). Initial *in vitro* evaluation of bortezomib demonstrated that it induced an accumulation of intracellular proteins, leading to G2-M arrest and then apoptosis through dual activation of caspase – 8 and caspase - 9 (Adams et al., 1999, Mitsiades et al., 2002). Importantly, bortezomib was also demonstrated to be significantly more toxic to Multiple Myeloma tumour cells than to normal counterparts. Hideshima et al. (2001) demonstrated that Multiple Myeloma cell lines and primary patient cells were 20-40 times more sensitive to bortezomib-induced apoptosis than bone marrow or peripheral blood mononuclear cells from healthy donors. Another novel aspect for bortezomib in Multiple Myeloma was that it was found to act not only on the Multiple Myeloma cells themselves but also on the protective bone marrow microenvironment. In addition, inhibition of proteasome function was found to both sensitize tumour cells to conventional chemotherapy and to overcome chemotherapy resistance. Finally, studies in murine xenograft models demonstrated that bortezomib significantly inhibited Multiple Myeloma cell growth and angiogenesis and prolonged survival (Leblanc et al., 2002). The main mechanisms attributed to bortezomib-induced apoptosis in Multiple Myeloma are outlined below.

4.1 NFκB

One of the first mechanisms of action attributed to bortezomib in Multiple Myeloma was inhibition of the inflammation associated transcription factor NFκB. NFκB, is constitutively activated in Multiple Myeloma and plays an important role in cell survival, proliferation and resistance to cytotoxic agents. NFκB is bound to its inhibitor IκB in the cytoplasm and is activated by proteasomal degradation of IκB. When activated, this transcription factor induces the expression of cell adhesion molecules (e.g. vascular cell adhesion molecule) and anti-apoptotic proteins (e.g. Bcl-2 and XIAP) and increases interleukin-6 production in bone marrow stromal cells. There are two pathways which activate NFκB, known as the canonical (or classical) pathway and the alternative non-canonical pathway (Gilmore, 2006). Inhibition of proteasome activity was demonstrated to prevent degradation of IκB and subsequent activation and translocation of NFκB to the nucleus to activate downstream pathways (Hideshima et al., 2001; Russo et al., 2001; Sunwoo et al., 2001). However, recent studies are challenging the concept that proteasome inhibitors inhibit NFκB activation and suggest that bortezomib may actually activate upstream NFκB activating kinases via the canonical pathway and increase NFκB activity (Markovina et al., 2008; Hideshima et al., 2009). In contrast, Chauhan et al. (2011) recently assessed the action of the second generation proteasome inhibitor MLN2238 on NFκB and report that this compound inhibits both the

canonical and non-canonical pathways of activation. As MLN2238 is structurally distinct from bortezomib, this suggests that different proteasome inhibitors may exert differential effects on the NFκB pathway by blocking either one or both pathways of activation.

4.2 Apoptosis

Apoptosis is regulated by the opposing activities of pro-apoptotic and anti-apoptotic molecules. Cancer cells often have disregulated apoptotic signalling pathways with increased levels of anti-apoptotic proteins which provide a survival advantage and confer resistance to chemotherapeutic agents. Inhibition of proteasome activity by bortezomib is associated with an upregulation of pro-apoptotic factors such as p53, Bik, BIM and NOXA and a related decrease in anti-apoptotic proteins such as Bcl-$_{XL}$ and Bcl-2. Induction of NOXA has been reported to be a key mechanism in bortezomib-mediated apoptosis which is independent of p53 status but dependent on c-Myc (Qin et al., 2005; Gomez-Bougie et al., 2007; Nikiforov et al., 2007; Fennell et al., 2008). Bortezomib-mediated apoptosis is accompanied by induction of c-Jun-NH2 terminal kinase, generation of reactive oxygen species, release of cytochrome c, second mitochondria-derived activator of caspases and apoptosis-inducing factor and activation of the intrinsic caspase-8 pathway and extrinsic caspase-9 pathway.

4.3 Unfolded protein response

The endoplasmic reticulum plays a central role in protein homeostasis. Proteins are processed and folded in the lumen of the endoplasmic reticulum and misfolded proteins are returned to the cytosol and degraded in the proteasome. Multiple Myeloma cells have a high rate of protein synthesis and this is inherently associated with a high level of misfolded proteins. Accumulation of misfolded proteins in the endoplasmic reticulum triggers the Unfolded Protein Response. This process is mediated through three endoplasmic reticulum transmembrane receptors: ATF6, IRE1 and PERK. In resting cells the endoplasmic reticulum chaperone BiP (GRP 78) maintains these receptors in a resting state; BiP becomes dissociated from the endoplasmic reticulum receptors when unfolded proteins accumulate and triggers the Unfolded Protein Response.

It has been recognised for some time that bortezomib can induce the Unfolded Protein Response in Multiple Myeloma cells and that this contributes to its pro-apoptotic activity (Obeng et al., 2006; Meister et al., 2007). Numerous studies have now shown that treatment of Multiple Myeloma cell lines *in vitro* with bortezomib triggers activation of ATF6, IRE 1 and PERK (Davenport et al., 2007; Gu et al., 2008; Dong et al., 2009). Caspase 2 is believed to act upstream of mitochondrial signalling in this bortezomib ER stress- induced apoptosis (Gu et al., 2008). Similar mechanisms have been implicated in mantle cell lymphoma cell lines (Rao et al., 2010; Roue et al., 2011). It is clear that a greater understanding of the Unfolded Protein Response is fundamental to allow the rational development of combination therapies (Kawaguchi et al., 2011).

4.4 Bone marrow microenvironment

Interactions between Multiple Myeloma cells and bone marrow stroma regulate the growth and survival of Myeloma cells and play a critical role in angiogenesis, bone disease and

drug resistance. The success of bortezomib in Multiple Myeloma has been attributed not only to direct effects on Myeloma cells but also its effect on the bone marrow microenvironment. Vascular cell adhesion molecule-1 is a major ligand on bone marrow stromal cells that mediates binding of Multiple Myeloma cells via the cell surface molecule very late antigen-4. Early studies on proteasome inhibitors demonstrated that they downregulate cytokine-induced expression of vascular cell adhesion molecule-1 (Read et al., 1995). Hideshima et al., (2001) subsequently reported that treatment with bortezomib decreased binding of Myeloma cells to bone marrow stromal cells by 50% and consequently inhibited the related upregulation of interleukin-6 secretion and paracrine tumour growth.

Bortezomib has also been demonstrated to have both direct and indirect effects on angiogenesis. Initial studies found that bortezomib treatment decreased the secretion of vascular endothelial growth factor from Myeloma cells, thereby decreasing vasculogenesis and angiogenesis (Nawrocki et al., 2002). More recent studies using functional assays including chemotaxis, adhesion to fibronectin and capillary formation demonstrated that bortezomib has direct anti-proliferative effects on vascular endothelial cells. Tamura et al. (2010) demonstrated that bortezomib potently inhibits cell growth of vascular endothelial cells by suppressing the G2/M transition of the cell cycle and increasing permeability, thus acting as a vascular targeting drug.

A critical role of the bone marrow microenvironment in the efficacy of bortezomib in Multiple Myeloma was further established by Edwards et al. (2009). *In vivo* studies demonstrated that bortezomib had a greater effect on tumour burden when Myeloma cells were grown in the bone marrow of mice than when they were grown at sub-cutaneous sites.

4.5 Bortezomib and bone formation

Osteolytic lesions characterised with activated osteoclast activity accompanied with a reduction in osteoblast activity are a major feature of Multiple Myeloma. Bortezomib exhibits important effects on the development and progression of Myeloma-associated bone disease by reducing osteoclast activity and increasing osteoblast function, therefore reducing bone resorption and stimulating new bone formation. Both preclinical and clinical analysis have demonstrated that bortezomib exerts these effects in part by inhibiting dickkopf-1 and receptor activator of nuclear factor-kappa B ligand and increasing levels of alkaline phosphatase and osteocalcin (Terpos et al., 2006; Heider et al., 2006; Giuliani et al., 2007). However, a recent study by Lund and colleagues (2010) found that the combination of a glucocorticoid such as dexamethasone with bortezomib could inhibit the positive effects of bortezomib on osteoblast proliferation and differentiation, suggesting that bortezomib may result in better healing of osteolytic lesions when used without a glucocorticoid.

4.6 Gene expression studies

While a number of mechanisms of action of bortezomib have been outlined above, the full mechanism of bortezomib-induced cytotoxicity remains to be elucidated. Gene expression studies have been employed to try and increase our understanding of the cytotoxic action of this compound in Multiple Myeloma. Mitsiades et al., (2002) performed gene expression profiling in a Multiple Myeloma cell line and demonstrated that bortezomib resulted in a downregulation of growth and survival signalling pathways and upregulation of molecules

implicated in pro-apoptotic cascades, as well as upregulation of heat shock proteins and ubiquitin proteasome pathway members. Chen et al. (2010) performed a genome-wide siRNA screen in malignant cell lines to evaluate the genetic determinants that confer sensitivity to bortezomib. They found that bortezomib promotes apoptosis primarily by disregulating Myc and polyamines, interfering with protein translation and disrupting DNA damage repair pathways. More recently, Takeda and colleagues (2011) investigated genes affecting the toxicity of bortezomib in the fission yeast *S. pombe* and identified factors involved in the ubiquitin proteasome pathway, chromatin silencing, nuclear/cytoplasmic transportation, amino acid metabolism and vesicular trafficking. Gene expression profiling of Multiple Myeloma patients found that treatment with bortezomib resulted in an upregulation of proteasome genes and that high levels of the proteasome subunit PSMD4 was associated with an inferior prognosis (Shaughnessy et al., 2011). Further investigation into fully understanding the mechanism of action of bortezomib will help to identify therapeutic strategies to overcome resistance to bortezomib and to identify agents to enhance its efficacy.

5. Clinical use of bortezomib in Multiple Myeloma

5.1 Bortezomib therapy for relapsed or refractory Multiple Myeloma

Following encouraging preclinical results bortezomib was introduced into clinical trials to test for safety and efficacy in relapsed and refractory Multiple Myeloma. These studies established that bortezomib was effective and well-tolerated in Multiple Myeloma and led to approval of bortezomib in patients that had undergone at least two prior therapies. The incorporation of bortezomib into the treatment options for Myeloma represented a significant milestone as being the first proteasome inhibitor to be implemented into clinical use and also as the first novel therapy for Multiple Myeloma in over a decade. The main findings of the trials are outlined below.

Phase 1

Orlowski et al. (2002) conducted a Phase 1 trial evaluating the pharmacodynamics of bortezomib, along with toxicity and clinical responses in 27 patients with advanced refractory haematological malignancies. This study demonstrated that bortezomib could be safely administered, with a tolerable side-effect profile. There was significant evidence of anti-tumour activity in patients with Multiple Myeloma, with all 9 evaluable Multiple Myeloma patients showing some evidence of clinical benefit, including one complete response. Taken together with preclinical data this provided the rationale for Phase 2 clinical trials with bortezomib for the treatment of relapsed, refractory Myeloma.

Phase 2

The activity of bortezomib in relapsed and refractory Multiple Myeloma was confirmed with two Phase 2 trials, SUMMIT and CREST. SUMMIT (Study of Uncontrolled Multiple Myeloma managed with proteasome Inhibition Therapy) was a large multi-centre trial that enrolled 202 heavily pre-treated patients (Richardson et al., 2003). An overall response rate of 35% was achieved, including 10% of patients who achieved a complete or near complete response. Median time to progression was 7 months compared with 3 months on previous therapy. Grade 3 toxicities included cyclical thrombocytopenia, fatigue, peripheral

neuropathy and neutropenia. Of these, the most clinically significant was peripheral neuropathy. The CREST (Clinical Response and Efficacy Study of PS-341 in the Treatment of relapsing Multiple Myeloma) trial was a smaller multicentre study that enrolled 54 patients with only one prior treatment (Jagannath et al., 2004). Patients were randomized to receive 1.0 or 1.3 mg/m² bortezomib. The overall response rates were 30% for patients receiving 1.0 mg/m² and 38% for patients receiving 1.3 mg/m². Adverse effects were similar to those seen in SUMMIT, however, less peripheral neuropathy was seen with reduced dose used in CREST. These findings led to the approval of bortezomib by the Food and Drug Administration and European Medicines Agency for relapsed/refractory Multiple Myeloma patients that had at least 2 prior therapies (Kane et al., 2006). Bortezomib was the first new therapy approved for Multiple Myeloma for over a decade.

Phase 3

APEX (Assessment of Proteasome inhibition for EXtending remissions) was a Phase 3 study of 668 patients with relapsed and refractory Multiple Myeloma after one to three prior treatments, who were randomized to receive either bortezomib or high-dose dexamethasone (Richardson et al., 2005). Bortezomib induced a better overall response rate than dexamethasone (38% vs. 18%), including a 13% vs. 2% complete or near complete response. Median time to progression for bortezomib was 6.22 months vs. 3.49 months for dexamethasone and overall survival was 29.3 months vs. 23.7 months. The adverse events were similar to those observed previously, however the rates of adverse events were higher for bortezomib.

5.2 Bortezomib-based combination therapy in relapsed or refractory Multiple Myeloma

Early preclinical work demonstrated that bortezomib sensitized Myeloma cells to other chemotherapeutic agents and this prompted clinical investigation of bortezomib-based combination therapies in relapsed or refractory Multiple Myeloma. Dexamethasone was the first agent to be combined with bortezomib in the clinic and is the most common agent to be used in bortezomib-based combinations. Both preclinical data and clinical trials showed that the combination increased anti-Myeloma activity. Data from the SUMMIT and CREST trials demonstrated additional responses in 18% and 33% of patients who received both drugs, including patients who had previously been refractory to dexamethasone. Bortezomib has since been demonstrated to enhance the activity of many chemotherapeutic agents in Multiple Myeloma, demonstrating promising response rates in early clinical trials (summarized in Table 1). Larger Phase 3 trials will be required to confirm response and survival to these combinations.

5.3 Bortezomib–based combinations with novel therapies

The increased understanding of intracellular pathways that are involved in the proliferation and survival of Myeloma cells has led to the identification of novel targets for therapeutic intervention. Numerous small molecule inhibitors have been developed in recent years, targeted against key cellular proteins or signalling pathways that may enhance the anti-tumour effect of bortezomib, or overcome resistance to bortezomib. These novel small molecule compounds include heat shock protein 90 inhibitors, histone deacetylase inhibitors, farnesyltransferase inhibitors, Bcl-2 inhibitors, monoclonal antibodies and a number of different kinase inhibitors. Many of these novel agents have demonstrated

Combination	Study	Overall Response	Reference
Bortezomib & alvocidib	Phase 1	44%	Holkova et al., 2011
Bortezomib & tanespimycin	Phase 1	27%	Richardson et al., 2011
Bortezomib, doxorubicin & intermediate dose dexamethasone	Phase 1		Takamatsu et al., 2010
Bortezomib, cyclophosphamide, thalidomide & dexamethasone	Phase 1	88%	Kim et al., 2010
Bortezomib & vorinostat	Phase 1	42%	Badros et al., 2009
Bortezomib & samarium lexidronam	Phase 1	21%	Berenson et al., 2009a
Bortezomib & temsirolimus	Phase 1/2	33%	Ghobrial et al., 2011
Bortezomib, low dose melphalan & dexamethasone	Phase 1/2	76%	Popat et al., 2009
Bortezomib, arsenic trioxide & ascorbic acid	Phase 1/2	27%	Berenson et al., 2007
Bortezomib, melphalan, prednisone & thalidomide	Phase 1/2	67%	Palumbo et al., 2007
Bortezomib & melphalan	Phase 1/2	68%	Berenson et al., 2006
Bortezomib, pegylated liposomal doxorubicin & thalidomide	Phase 2	55%	Chanan-Khan et al., 2009
Bortezomib, thalidomide & dexamethasone	Phase 2		Pineda-Roman et al., 2008
Bortezomib, melphalan, dexamethasone & intermittent thalidomide	Phase 2	66%	Terpos et al., 2008
Bortezomib, dexamethasone & cyclophosphamide	Phase 2	90%	Kropff et al., 2007

Table 1. Bortezomib-based combination therapy for relapsed/refractory Multiple Myeloma.

synergistic activity with bortezomib in preclinical studies and are under evaluation in early clinical trials in combination with bortezomib.

5.3.1 Heat shock protein 90 inhibitors

Heat shock protein 90 is a chaperone that stabilizes numerous proteins that contribute to tumour cell survival and proliferation. Inhibition of heat shock protein 90 in Myeloma cells results in decreased expression of insulin-like growth factor-1 and interleukin-6 receptors, with a related decrease in the PI3K/Akt signalling pathway. Preclinical studies with the heat shock protein 90 inhibitor tanespimycin in combination with bortezomib demonstrated a synergistic effect and resulted in enhanced accumulation of ubiquitinated proteins (Mitsiades et al., 2006). A Phase 1 clinical trial of bortezomib in combination with tanespimycin demonstrated significant and durable responses (Richardson et al., 2011) and a study of bortezomib in combination with KW-2478 is underway (NCT01063907).

5.3.2 Histone deactylase inhibitors

Ubiquitinated and misfolded proteins are degraded not only by proteasomes but also by aggresomes. Aggresome formation, which is dependent on the histone deacetylase HDAC6, is increased in response to inhibition of proteasome function. Histone deacetylase inhibitors are a class of compounds that regulate gene expression by interfering with the function of histone deacetylases. Preclinical studies demonstrated that the combination of bortezomib with a HDAC inhibitor resulted in significant cytotoxicity and show a marked accumulation of polyubiquitinated proteins (Catley et al., 2006; Nawrocki et al., 2008). A Phase 1 trial of bortezomib and vorinostat in relapsed/refractory myeloma demonstrated encouraging results, with an overall response rate of 42%, including 3 responses among 9 bortezomib refractory patients (Badros et al., 2009). Further trials of bortezomib along with HDAC inhibitors vorinostat and panobinostat are currently being investigated.

5.3.3 Farnesyltransferase inhibitors

Farnesyltransferase inhibitors block activation of the Ras dependent MAPK signalling pathway to regulate signal transduction and proliferation. Combination of the farnesyltranserase inhibitors lonafarnib and tipifarnib with bortezomib induced synergistic cell death and overcame cell adhesion-mediated drug resistance in Multiple Myeloma cell lines and primary cells (David et al., 2005; Yanamandra et al., 2006). David and colleagues (2005) observed that this combination resulted in a down-regulation of Akt signalling, an effect which was absent when either drug was used alone. Early phase clinical trials evaluating bortezomib and tipifarnib combination therapy are ongoing (NCT00243035; NCT00972712).

5.3.4 Bcl-2 inhibitors

Bcl-2 family members play a critical role in mediating tumour cell survival and chemoresistance in Multiple Myeloma. There are a number of small molecule inhibitors available that interfere with the function of Bcl-2 proteins and induce apoptosis in Multiple Myeloma cells. In preclinical studies, three Bcl-2 inhibitors obatoclax, ABT-737 and ABT-263 have shown synergistic activity with bortezomib (Chauhan et al., 2007; Trudel et al., 2007; Ackler et al., 2010). The combination of bortezomib with a Bcl-2 inhibitor resulted in enhanced NOXA-mediated activation of Bak and increased activation of the mitochondrial apoptotic pathways. Obatoclax is being investigated in combination with bortezomib in early clinical trials (NCT00719901).

5.3.5 Monoclonal antibodies

Monoclonal antibody therapy can selectively target specific molecules, proteins or receptors involved in disease processes. There are a number of antigens currently under investigation as potential targets in Multiple Myeloma in combination with bortezomib. Bevacizumab is a monoclonal antibody that is targeted towards vascular endothelial growth factor to disrupt angiogenensis (Brekken et al., 2000). The combination of bevacizumab and bortezomib is being evaluated in Phase 2 studies for relapsed Myeloma (NCT00464178). Interleukin-6, a key intermediate in Multiple Myeloma signalling pathways, is targeted by the chimeric

antibody siltuximab. Preclinical evaluation of siltuximab and bortezomib demonstrated enhanced cytotoxicity of bortezomib in Myeloma cell lines and primary cells in the presence of bone marrow stromal cells (Voorhees et al., 2007). A Phase 2 trial is evaluating this combination in relapsed Myeloma (NCT00401843). Elotuzumab is directed towards CS1, a cell surface glycoprotein expressed at high levels on Multiple Myeloma cells. This anti-CS1 antibody demonstrated significantly enhanced anti-tumour activity in combination with bortezomib in *in vitro* and *in vivo* models of Myeloma (van Rhee et al., 2009) and Phase 1/2 trials are underway (NCT00726869). AVE1642 is an anti-insulin-like growth factor 1 antibody that demonstrated synergistic apoptosis in combination with bortezomib in preclinical studies (Descamps et al., 2009), however, response rates from a Phase 1 study were insufficient to warrant further investigation (Moreau et al., 2011). Early phase clinical trials combining bortezomib with anti-chemokine receptor 4 and anti-CD40 antibodies are also underway (NCT01359657 and NCT00664898).

5.3.6 Mammalian target of rapamycin inhibitor

Mammalian target of rapamycin (mTOR) inhibitors inhibit the mTOR kinase and related signalling pathways resulting in decreased expression of cyclins and c-Myc, increased expression of p27 and G_1 arrest. *In vitro* studies have demonstrated synergistic action of the mTOR inhibitors NVP-BEZ235 and pp242 with bortezomib (Baumann et al., 2009; Hoang et al., 2010). A Phase 1/2 study of bortezomib in combination with mTOR inhibitor temsirolimus demonstrated a partial response rate of 33% in heavily pre-treated refractory Myeloma (Ghobrial et al., 2011).

5.3.7 Cyclin-dependent kinase inhibitors

Cyclin-dependent kinase inhibitors are small molecule inhibitors that induce cell cycle arrest. Cyclin dependent kinase inhibitors (seliciclib and alvocidib) were shown to be synergistic with proteasome inhibitors in leukaemic cell lines (Dai et al., 2003, 2004). A subsequent study demonstrated that the cyclin dependent kinase inhibitor PD0332991 sensitizes an *in vivo* Multiple Myeloma model to bortezomib through enhanced induction of mitochondrial depolarization (Menu et al., 2008). A combination of bortezomib along with the cyclin dependent kinase inhibitor alvocidib (flavopiridol) was recently assessed in a Phase 1 trial for refractory B-cell malignancies and demonstrated an overall response rate of 44% with manageable toxicities (Holkova et al., 2011).

5.3.8 Akt inhibitors

Perifosine is an alkylphospholipid that inhibits Akt activation and associated growth and drug resistance in Multiple Myeloma. As a single agent, perifosine demonstrated significant toxicity both *in vivo* and *in vitro* and it has also been shown to inhibit bortezomib-induced upregulation of survivin resulting in enhanced bortezomib cytotoxicity (Hideshima et al., 2007). Perifosine is currently being evaluated in a Phase 1/2 study in combination with bortezomib with or without dexamethasone in relapsed Myeloma (NCT00401011) and a Phase 3 study of perifosine in combination with bortezomib and dexamethasone is currently recruiting (NCT01002248).

5.3.9 Multi-kinase inhibitors

Sorafenib and dasatinib are multi-kinase inhibitors that have been shown to enhance anti-Myeloma activity with bortezomib. Sorafenib inhibits RAF kinase, VEGF receptors, platelet-derived growth factor β, Flt-3, c-Kit and RET receptor tyrosine kinases. The combination of sorafenib and bortezomib produced synergistic apoptosis in a number of malignant cell lines and was dependent on Akt inhibition (Yu et al., 2006). This combination is currently being investigated in a Phase 1/2 trial in relapsed Myeloma (NCT00536575). Dasatinib is an inhibitor of c-abl, src family proteins, EphA2 and btk. The triple combination of dasatinib along with bortezomib and dexamethasone produced greater synergistic effects compared to single agents or double combinations in Multiple Myeloma cell line models and primary cells (de Queiroz Crusoe et al., 2011). A Phase 1 study combining all three agents in relapsed or refractory Myeloma has recently been completed (NCT00560352).

5.3.10 Other combinations

The combination of bortezomib with second generation immunomodulatory drug pomalidomide (NCT01212952), telomerase inhibitor GRN163L (NCT00718601), aurora A kinase inhibitor MLN8237 (Gorgun et al., 2010; NCT01034553), p38 mitogen-activated kinase inhibitor SCIO-469 (Navas et al., 2006; NCT00095680) and protease inhibitor nelfinavir mesylate (NCT01164709) are all being evaluated in early clinical trials. In addition there are numerous more novel targeted therapies under preclinical assessment in combination with proteasome inhibitors.

5.4 Bortezomib in front-line therapy

For over 40 years melphalan and prednisone was the standard therapy for patients with newly diagnosed Multiple Myeloma that were ineligible for high-dose therapy and autologous stem cell transplantation. Following encouraging activity of bortezomib combined with melphalan in patients with relapsed or refractory Myeloma, bortezomib plus melphalan and prednisone was evaluated in a Phase 1/2 trial for newly diagnosed Myeloma patients who were at least 65 years of age. The combination gave a response rate of 89% and a median time to progression of 27 months. This led to the Phase 3 trial VISTA (Velcade as Initial Standard Therapy in Multiple Myeloma), which compared bortezomib, melphalan and prednisone with melphalan and prednisone in newly diagnosed Myeloma patients who were ineligible for high-dose therapy. Results of this trial demonstrated that when bortezomib was included in the regimen the overall response rate increased from 30% to 71% and the time to progression was 24 months compared with 16.6 months (San Miguel et al., 2008). There was also fewer bone disease events, improvement in bone remodelling and evidence of bone healing. These results suggested a benefit for bortezomib at earlier use and provided the framework for approval of bortezomib for use as front-line therapy.

In newly diagnosed patients who were candidates for high-dose therapy with autologous stem cell transplantation, the combination of vincristine, doxorubicin and dexamethasone was the standard induction therapy. Four randomized trials evaluated the role of bortezomib–based combinations for induction therapy in transplant candidates. Bortezomib was combined with dexamethasone (Harousseau et al., 2010), with adriamycin and dexamethasone (Popat et al., 2008), with thalidomide and dexamethasone (Cavo et al., 2010)

and with thalidomide, dexamethasone and chemotherapy (Barlogie et al., 2007). The bortezomib-based combinations all demonstrated superior response rates than the regimens without bortezomib. A number of other combinations incorporating bortezomib for both transplant eligible and ineligible patients are in clinical trials and are achieving overall response rates of up to 100% (Table 2).

Combination	Study Phase	Overall Response	Reference
Bortezomib, thalidomide & chemotherapy	Phase 1	83%	Badros et al., 2006
Bortezomib, dexamethasone, cyclophosphamide & lenalidomide	Phase 1	96%	Kumar et al., 2010
Bortezomib, doxorubicin & dexamethasone	Phase 1/2	89/95%	Popat et al., 2008
Bortezomib, lenalidomide & dexamethasone	Phase 1/2	100%	Richardson et al., 2010
Bortezomib, melphalan & prednisone	Phase 1/2	95%	Gasparetto et al., 2010
Bortezomib & melphelan	Phase 1/2	87%	Lonial et al., 2010
Bortezomib, lenanlidomide, pegylated liposomal doxorubicin & dexamethasone	Phase 1/2	96%	Jakubowiak et al., 2011
Bortezomib & dexamethasone	Phase 2	66%	Harousseau et al., 2006
Alternating bortezomib & dexamethasone	Phase 2	68%	Rosinol et al., 2007
Bortezomib, cyclophosphamide & dexamethasone	Phase 2	88%	Reeder et al., 2009
Bortezomib, ascorbic acid & melphalan	Phase 2	74%	Berenson et al., 2009b
Bortezomib, pegylated liposomal doxorubicin & dexamethasone	Phase 2	85%	Jakubowaik et al., 2009
Bortezomib & high dose melphalan	Phase 2	70%	Roussel et al., 2010
Bortezomib, cyclophosphamide & dexamethasone	Phase 2	95%	Besinger et al., 2010
Bortezomib & dexamethasone	Phase 2	86%	Corso et al., 2010
Vincristine, adriamycin & dexamethasone followed by bortezomib, thalidomide & dexamethasone	Phase 2	75%	Kim et al., 2011
Bortezomib & thalidomide	Phase 2	82%	Ghosh et al., 2011
Bortezomib, pegylated liposomal doxorubicin & thalidomide	Phase 2	78%	Sher et al., 2011
Bortezomib, thalidomide & dexamethasone	Phase 3	31%	Cavo et al., 2010
Bortezomib & dexamethasone	Phase 3	79%	Harousseau et al., 2010

Table 2. Bortezomib-based combinations for induction and front-line therapy.

6. Resistance to bortezomib

Despite the clinical success of bortezomib, many patients with Multiple Myeloma are unresponsive and drug resistance can also develop (Dispenzieri et al., 2010). The mechanisms underlying this drug resistance, both intrinsic and acquired, are as yet poorly understood.

Resistance to proteasome inhibitors may occur either at the level of the proteasome complex or in the downstream signalling pathways. Several researchers have approached this problem by growing cell lines in increasing concentration of bortezomib. Ri et al. (2010) found a unique point mutation in the proteasome β5 subunit (PSMB5) in bortezomib resistant Multiple Myeloma cell lines. Using overexpression studies they demonstrated that the mutation may act by interfering with the Unfolded Protein Response pathway. Shaughnessy and colleagues have recently applied gene expression studies to a group of 142 Multiple Myeloma patients and identified PSMD4 as associated with adverse response to bortezomib; PSMD4 is one of the non-ATPase subunits of the proteasome 19S regulator (Shaughnessy et al., 2011).

The anti-tumour effects of bortezomib have been mainly attributed to its' actions on the NFκB and Bcl-2 regulatory protein pathways. It is not therefore surprising that polymorphisms of the NFκB family genes have been associated with treatment outcome in Multiple Myeloma patients. Studies with lymphoid cell lines have recently shown Noxa/Bcl-2 interactions contribute to bortezomib resistance (Smith et al., 2011) and there have been similar reports in Mantle Cell Lymphoma cell lines (Weniger et al., 2011); there is no supporting clinical evidence as yet. Overexpression of apoptosis regulators REDD1 and survivin have also been associated with bortezomib resistance in cell line models (Decaux et al., 2010; Ling et al., 2010).

In cases where drug resistance is directly associated with the proteasome enzymatic complex it may be possible to overcome resistance by using second generation inhibitors which act through a different mechanism to bortezomib (Ruschak et al., 2011; Arastu-Kapur et al., 2011; Chauhan et al., 2011). Knowledge of the resistance mechanism may also allow rational design of future combination therapies.

7. Second generation inhibitors

The success of bortezomib in the clinic prompted the development of a new generation of structurally distinct proteasome inhibitors. In addition to bortezomib, there are currently five proteasome inhibitors in clinical development, representing three different structural classes - peptide boronic acids, peptide epoxyketones and β-lactones (Figure 2). These inhibitors bind either reversibly or irreversibly to catalytic sites within the proteasome.

7.1 Carfilzomib

Epoxomicin, a member of the epoxyketone family of natural peptide proteasome inhibitors, inhibits proteasome activity through a unique mechanism, by binding to both the hydroxyl and amino groups of the catalytic site threonine residue (Groll & Huber, 2004). Carfilzomib (formerly PR-171) is an epoxomicin-based proteasome inhibitor, with improved pharmaceutical properties. Unlike bortezomib, carfilzomib binds irreversibly to the

chymotrypsin-like (β5 and LMP7) subunit, leading to more sustained proteasome inhibition. In preclinical studies carfilzomib was shown to exhibit equal potency but greater selectivity than bortezomib for the chymotrypsin-like activity. *In vitro* and *in vivo* studies demonstrated anti-tumour activity, tolerability and dosing flexibility in several xenograft models (Kuhn et al., 2007; Demo et al., 2007). Carfilzomib has also been shown to act synergistically with histone deacetylase inhibitors *in vitro* in lymphoma and leukaemia (Fuchs et al., 2009; Dasmahapatra et al., 2010; 2011). Results from Phase 1 studies in patients with haematological malignancies demonstrated that carfilzomib was well tolerated and may exhibit less peripheral neuropathy than bortezomib (O'Connor et al., 2009). Phase 2 trials of carfilzomib as a single agent in relapsed and refractory Multiple Myeloma demonstrated an overall response rate of 35.5% including patients with bortezomib-refractory disease. (Zangari et al., 2011) The main toxicities were fatigue and nausea, with limited peripheral neuropathy seen in less than 10% of patients. Carfilzomib is currently progressing in a number of trials for relapsed and newly diagnosed Multiple Myeloma and as both a single agent and in combination.

7.2 Marizomib (NPI-0052)

Marizomib, also known as Salinosporamide A, is a β-lactone compound derived from the marine bacterium *Salinospora tropica* (Macherla et al., 2005) and is structurally related to the lactacystin-derived proteasome inhibitor Omuralide. In contrast to bortezomib which is a slowly reversible inhibitor of chymotrypsin-like activity, marizomib binds irreversibly to all three catalytic activities of the proteasome. While bortezomib is administered intravenously, marizomib has the advantage of being orally bioactive. Initial *in vitro* studies established the effectiveness of this compound in Multiple Myeloma cell lines, including those that were resistant to bortezomib (Chauhan et al., 2005). Animal tumour model studies demonstrated reduced tumour growth without significant toxicity (Chauhan et al., 2005; Singh et al., 2010). Preclinical studies demonstrated synergistic results when marizomib was combined with bortezomib or lenalidomide (Chauhan et al., 2008; 2010a). Phase 1 trials of marizomib in Myeloma are currently ongoing. Marizomib displays a broader, faster acting and more durable proteasome inhibition than bortezomib and treatment does not appear to induce the limiting toxicities associated with bortezomib, such as peripheral neuropathy and thrombocytopenia.

7.3 MLN9708/MLN2238

MLN9708 like bortezomib is also a boron containing peptide proteasome inhibitor and was selected from a panel of inhibitors based on having a biochemical profile distinct from that of bortezomib. MLN9708 hydrolyses immediately in plasma to its biologically active form MLN2238. MLN2238 displays similar potency and selectivity for the chymotrypsin-like proteasome subunit, however, it has a substantially shorter half-life than bortezomib which may improve tissue distribution. Cell viability studies revealed a strong anti-proliferative effect on a variety of tumour cell lines and *in vivo* studies have demonstrated efficacy in human prostate xenograft, colon cancer and lymphoma models where both intravenous and oral dosing were effective (Kupperman et al., 2010). MLN2238 has been demonstrated to induce apoptosis in cells resistant to both conventional therapies and to bortezomib. Synergistic activity is seen by combining this compound with lenelidomide, HDAC

inhibitors and dexamethasone *in vitro*. It is well tolerated in plasmacytoma xenograft mouse models and demonstrates significantly longer survival time than mice treated with bortezomib (Chauhan et al., 2011). This compound is currently being evaluated in Phase 1 studies in patients with lymphoma and non-haematological malignancies and in Phase 1/2 trials for Multiple Myeloma.

7.4 CEP-18770

CEP-18770 is a next-generation boronic acid-based proteasome inhibitor and in common with bortezomib it is a reversible inhibitor, primarily of the chymotrypsin-like activity. CEP-18770 was demonstrated to induce apoptosis in Multiple Myeloma cell lines and primary Myeloma cells, while displaying a favourable cytotoxicity profile towards normal cells (Piva et al., 2008; Dorsey et al., 2008). Its anti-tumour activity was demonstrated in several animal tumour models and it has been shown to demonstrate marked anti-Myeloma effects in combination with bortezomib and melphalan (Sanchez et al., 2010). CEP-18770 has completed early Phase 1 trials for solid tumours and non-Hodgkin's lymphoma and is currently being evaluated in Phase 1/2 trials for Multiple Myeloma.

7.5 ONX0912

ONX0912 (formerly PR-047) is a novel orally available analogue of the proteasome inhibitor carfilzomib. Carfilzomib, in common with bortezomib, is administered intravenously, however, proteasome inhibitor therapy requires twice weekly dosing and therefore an orally available inhibitor would be more advantageous. ONX0912 has demonstrated similar anti-tumour activity to carfilzomib *in vitro* in cell lines and primary cells and enhanced the anti-Myeloma activity of bortezomib, lenolidomide and histone deacetylase inhibitors; animal models of Multiple Myeloma, non-Hodgkin's lymphoma and colorectal cancer demonstrated reduced tumour progression and prolonged survival (Zhou et al., 2009; Roccaro et al., 2010; Chauhan et al., 2010b). A Phase 1 trial of ONX0912 in advanced solid tumours is currently recruiting.

7.6 Immunoproteasome Inhibitors

A novel approach that is looking promising is the use of proteasome inhibitors that specifically inhibit catalytic activities of the immunoproteasome. Immunoproteasomes are constitutively expressed in immune tissues and expressed at a much lower level in other cell types. Thus targeting immunoproteasomes confers a certain amount of specificity and provides an opportunity to overcome toxicities associated with proteasome inhibition, such as peripheral neuropathy and gastrointestinal effects. A number of immunoproteasome specific inhibitors have recently been described and exhibit encouraging preclinical activity in haematological malignancies. PR-924 is a tripeptide epoxyketone related to carfilzomib. It exhibits 100-fold greater selectivity for the LMP7 subunit than carfilzomib and was demonstrated to inhibit the growth of Multiple Myeloma cell lines and primary tumour cells and inhibited tumour growth in animal models without significant toxicity (Singh et al., 2010). The immunoproteasome inhibitor IPSI-101 is a peptide aldehyde which preferentially inhibits the LMP2 subunit. IPSI-101 induced accumulation of polyubiquitinated proteins and pro-apoptotic protein and inhibited proliferation in *in vitro* models of haematological

malignancies (Kuhn et al., 2009). At the time of writing this review there were no clinical trials of immunoproteasome inhibitors in progress, however, it is likely that the encouraging preclinical data on PR-924 and ISPS-101 will form the basis for future clinical evaluation of these compounds.
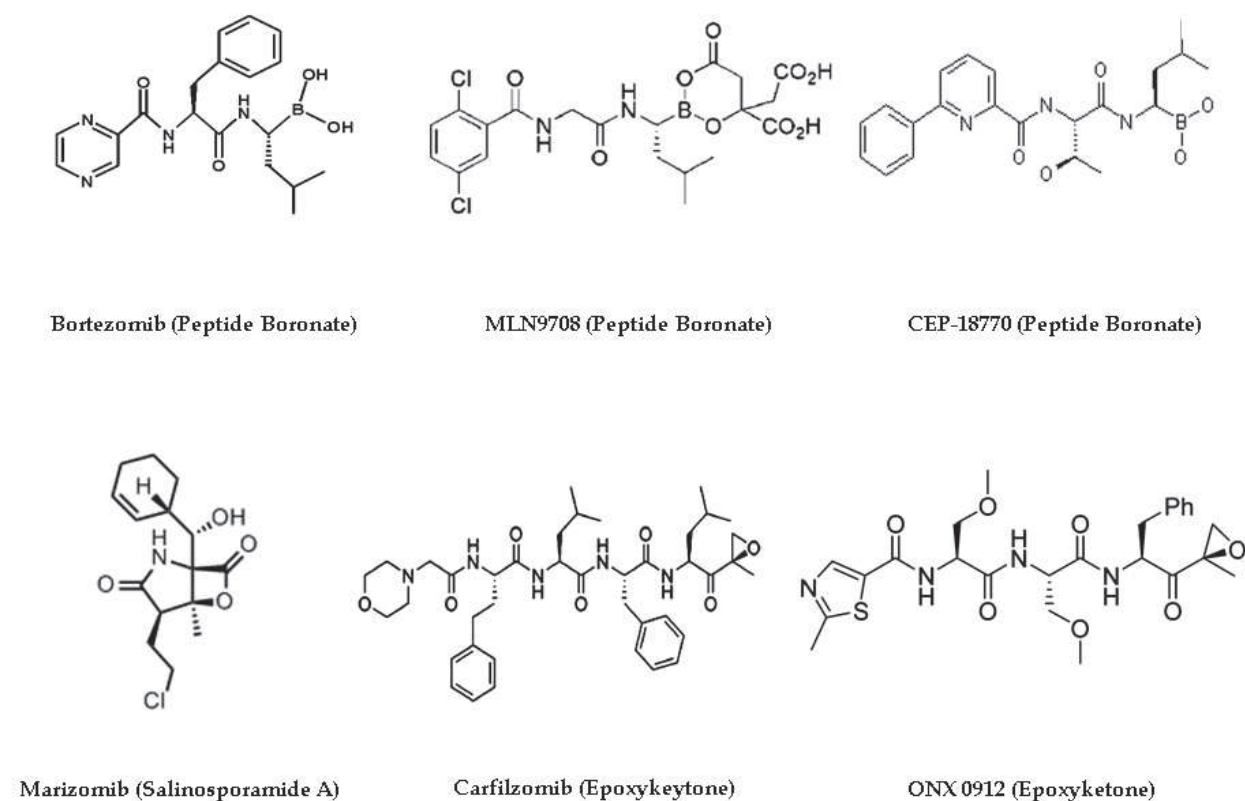

Fig. 2. Structure and class of proteasome inhibitors in clinical trials.

8. Conclusion

Proteasome Inhibitors have provided a major new therapeutic strategy for the treatment of Multiple Myeloma. Bortezomib, the first-in-class of these inhibitors, has shown remarkable success since its introduction almost ten years ago. Second generation compounds are already demonstrating increased selectivity with a more acceptable therapeutic window. Researchers are turning to other parts of the Ubiquitin Proteasome Pathway to look for potential druggable targets which would confer greater specificity. The E3 ligases play a key role in substrate selection and the Pharma already have agents in their pipeline which show promise in modifying their action. Modulation of the Ubiquitin Proteasome Pathway with novel inhibitors offers a powerful approach to Myeloma therapy.

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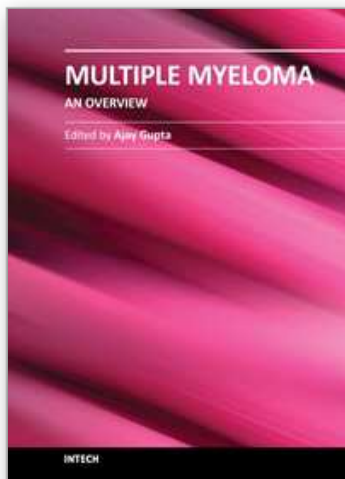
Multiple Myeloma - An Overview

Edited by Dr. Ajay Gupta

Multiple myeloma is a malignant disorder characterized by the proliferation of plasma cells. Much insight has been gained into the molecular pathways that lead to myeloma and indeed much more remains to be done. The understanding of these pathways is closely linked to their therapeutic implications and is stressed upon in the initial chapters. Recently, the introduction of newer agents such as bortezomib, lenalidomide, thalidomide, liposomal doxorubicin, etc. has led to a flurry of trials aimed at testing various combinations in order to improve survival. Higher response rates observed with these agents have led to their integration into induction therapies. The role of various new therapies vis a vis transplantation has also been examined. Recent advances in the management of plasmacytomas , renal dysfunction, dentistry as well as mobilization of stem cells in the context of myeloma have also found exclusive mention. Since brevity is the soul of wit our attempt has been to present before the reader a comprehensive yet brief text on this important subject.

How to reference

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