Proteasome inhibitors as therapeutics

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Abstract

The ubiquitin–proteasome pathway is a principle intracellular mechanism for controlled protein degradation and has recently emerged as an attractive target for anticancer therapies, because of the pleiotropic cell-cycle regulators and modulators of apoptosis that are controlled by proteasome function. In this chapter, we review the current state of the field of proteasome inhibitors and their prototypic member, bortezomib, which was recently approved by the U.S. Food and Drug Administration for the treatment of advanced multiple myeloma. Particular emphasis is placed on the pre-clinical research data that became the basis for eventual clinical applications of proteasome inhibitors, an overview of the clinical development of this exciting drug class in multiple myeloma, and an appraisal of possible uses in other haematological malignancies, such non-Hodgkin’s lymphomas.

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Introduction

The elucidation of the role of ubiquitin–proteasome pathway, as a principle intracellular route for controlled protein degradation, represented a major conceptual advancement in our understanding of cell biology [1], reflected by the recent Nobel prize awarded to Drs Ciechanover, Hershko and Rose. Importantly, it also paved the way for therapeutic targeting of protein degradation in human diseases, such as cancer. In this chapter, we review the current state of the field of proteasome inhibitors, with particular emphasis on the bench-to-bedside research studies that led to the development of bortezomib (Velcade™, formerly known as PS-341), as the prototypical proteasome inhibitor for the treatment of human neoplasias, such as multiple myeloma (MM).

Over the years, an extensive list of proteins (previously reviewed in [2–4]) involved in tumour cell proliferation, survival and drug resistance were shown to be regulated by proteasome-mediated degradation. This list includes cell-cycle regulators, e.g. various cyclins; cyclin-dependent kinase inhibitors (i.e. p21WAF1/CIP1 and p27KIP1); regulators of oncogenic transformation, e.g. c-Fos, c-Jun or c-Myc; tumour suppressors, such as p53; pro-apoptotic and anti-apoptotic regulators, including Bax and Bel-2 (B-cell lymphocytic-leukaemia proto-oncogene 2); and the inhibitor of NF-kB (nuclear factor kB), IκBα (inhibitory κB α) [5]. The critical roles of these proteins in tumour cell biology raised the hypothesis that interfering with proteasome function might perturb their intracellular levels in a manner conducive to antitumour effects, e.g. cell-cycle arrest owing to accumulation of cyclin-dependent kinase inhibitors p21WAF1/CIP1 and/or p27KIP1, facilitation of tumour cell apoptosis owing to accumulation of p53 or Bax, as well as accumulation of IκB and inhibition of nuclear translocation of NF-κB, which is known to activate multiple anti-apoptotic genes [6]. However, because proteasomal degradation regulates diverse processes pertinent to both healthy and abnormal cells, there were initially concerns that this pathway cannot be therapeutically manipulated without major perturbations in proteins critical for normal cellular physiology, thereby causing extensive toxicities that would be incompatible with use in a clinical setting.

Despite these concerns, small molecule proteasome inhibitors were developed with the goal at least to use them as chemical probes to interrogate the biological roles of proteasome function. Five main groups of such inhibitors have been synthesized so far: peptide aldehydes, peptide vinyl sulphones, peptide boronates, peptide epoxycarbonates (the less extensively studied group), and β-lactones (lactacystin and its derivatives) [2,7]. Peptide aldehydes, peptide vinyl sulphones and β-lactones were not considered amenable to clinical development, for several reasons, including instability in vivo [8], suboptimal enzyme specificity, and irreversible binding to the proteasome [9]. Nonetheless, these inhibitors were used in informative pre-clinical studies, which provided proof-of-concept of
in vitro and in vivo antitumour effects of proteasome inhibitors, including studies showing their selective cytotoxicity against transformed cells, rather than their normal counterparts [10,11]; and their ability to sensitize tumour cells to other anticancer therapies, including radiotherapy or cytotoxic chemotherapy [12]. These data provided a rationale for more research on peptide boronic acids, which were synthesized as derivatives of peptidyl aldehydes with substitution of the leucine carbon for a boron atom [8]. This was a critical step in proteasome-inhibitor research because peptidyl aldehydes are not only potent inhibitors of proteasome-mediated proteolysis, but are also potent inhibitors of thiol proteases such as cathepsin B and calpains [8]. However, the three-dimensional structure...
of the boronated derivatives of peptide aldehydes could not only allow them to form stable tetrahedral intermediates with the N-terminal threonine residues of the catalytically active proteasome β-subunits, but also prevent similar interactions with common serine proteases (e.g. leucocyte elastase, chymotrypsin or thrombin) or with cysteine proteases (e.g. cathepsin G) [8]. Furthermore, the potent (with even sub-nanomolar $K_i$ values) and selective binding of boronic dipeptides to the β-proteasome subunits is accompanied by other favourable features for possible clinical applications, including reversible activity [9], \textit{in vivo} stability, and potent anti-proliferative effects against diverse types of tumour cells (e.g. non-small-cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast cancers) in the NCI-60 panel of tumour cell lines [13]. Of the peptide boronic acids, bortezomib (originally designated as PS-341) exhibited a unique cytotoxicity profile, in comparison with historical data of the NCI on 60000 compounds tested in the NCI-60 panel [13], and was selected for further pre-clinical studies and clinical development.

\textbf{Spectrum of pre-clinical antitumour activity and mechanism(s) of actions of bortezomib}

Bortezomib (PS-341, Velcade™) is the prototypical small-molecule proteasome inhibitors that has been used clinically, mainly in MM, an incurable neoplasia of malignant plasma cells. Preceding or concomitant pre-clinical studies evaluated the activity of bortezomib against other haematological neoplasias and solid tumours, including MM itself [14–17], mantle cell lymphoma [18] and various histological subtypes of lung [19], ovarian [20], pancreatic [21], prostate [13] and breast [19] cancers.

Bortezomib probably kills tumour cells via multi-factorial mechanisms (Figure 1), which conceivably involve tumour type-specific features. For example, p53 expression is essential for proteasome inhibitor-induced apoptosis in breast epithelial cells [22], but it is dispensable for induction of $G_2/M$ cell-cycle arrest and apoptosis of the p53 null PC-3 prostate cancer cells [13]. Bortezomib triggers cell-cycle arrest attributable to stabilization of p21 and p27 [13], but eventually induces apoptosis even in tumour cells with low proliferative rates [11,14,23]. In MM cells, the prototypic tumour model for antitumour activity of proteasome inhibitors, bortezomib triggers concomitant activation of a dual caspase 8 and caspase 9 pro-apoptotic pathway, which is accompanied by inhibition of anti-apoptotic pathways, such NF-κB transcriptional activity [15]. Indeed, bortezomib leads to intracellular accumulation of c-Jun [which leads to increased transcriptional activity of AP-1 (activator protein 1)] and c-Myc [15], which in turn increase the expression, in MM cells, of Fas and Fas ligand (FasL) respectively [15], resulting in activation of caspase-8. Furthermore, Bax-triggered release of cytochrome $c$ from the mitochondria activates caspase-9-mediated apoptosis, and recent data in MM and other tumour models suggest the activation of caspase 12 by ER (endoplasmic reticulum) stress related to the
accumulation of undegraded proteins ([24] and C.S. Mitsiades, unpublished work).

Bortezomib not only directly triggers pro-apoptotic cascades but also facilitates their activation, by suppressing the function of a series of anti-apoptotic pathways, such as NF-κB transcriptional activity. NF-κB stimulates expression of caspase 8 inhibitors, such as FLIP (FADD-like interleukin-1β-converting enzyme inhibitory protein) and cIAP-2 (cellular inhibitor of apoptosis protein 2) [25,26], caspase 9 inhibitors, such as XIAP (X-linked inhibitor of apoptosis protein) [6,26], or anti-apoptotic Bcl-2 family members [6,25,26], such as A1/Bfl-1 and Bcl-2 itself. Consequently, by causing accumulation of IκB and preventing nuclear translocation of NF-κB, proteasome inhibition facilitates both extrinsic (caspase-8-dependent routes after, for example, Fas/FasL interaction) and intrinsic mitochondrial-dependent (caspase-9-dependent) apoptotic cascades [15].

The antitumour effects of bortezomib cannot be attributed exclusively to just one of the aforementioned pathways. It is more likely that bortezomib’s antitumour activity is due to simultaneous effects on multiple such pathways. These pleiotropic effects can also help to explain the activity of bortezomib, even against tumour cells resistant to conventional or other investigational agents [14]. For instance, bortezomib simultaneously activates three distinct pathways of caspase-mediated apoptosis (caspase 8, 9 and 12), which suggests that it can be active even against cells in which, for example, one of these cascades is not operational. The pleiotropic effects of bortezomib also allow it to sensitize tumour cells to a broad range of antitumour agents. Bortezomib-induced suppression of caspase 8 inhibitors, such as FLIP and cIAP-2, sensitizes MM cells to caspase-8-dependent pro-apoptotic stimuli, including ligands or agonistic antibodies against Fas or TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) receptors [6,15], as well as thalidomide and its immunomodulatory derivatives [16]. Bortezomib-induced suppression of the caspase 9 inhibitor XIAP explains the (at least) additive effect of bortezomib with dexamethasone against MM cells [14,15]. In addition, bortezomib sensitizes MM cells to diverse DNA-damaging chemotherapeutic agents [17,19], which is attributed to inhibition of NF-κB, suppression of caspase inhibitors (which generally increase the resistance of tumour cells to various anticancer agents [26,27]) and, more specifically, suppression of various DNA repair enzymes [17].

Proteasome inhibitors also influence the non-malignant cells of the tumour micro-environment. It is well established that MM cells, which generally reside in the bone marrow of patients, adhere to bone marrow stromal cells (BMSCs), triggering an NF-κB-dependent secretion of IL-6 (interleukin-6) by, mainly, BMSCs [28]. IL-6 is a key proliferation factor for MM cells [29] and its NF-κB-dependent (and proteasome-dependent) up-regulation due to MM–BMSC interaction is also targeted by bortezomib. This allows proteasome inhibition to
target not only the MM cell itself, but also the BMSCs and their ability to support the tumour cell population with paracrine proliferative stimulation [14].

It is still unclear why bortezomib has a therapeutic window in vitro and in vivo. Quiescent HL60 leukaemia cells or contact-inhibited quiescent endothelial cells are less susceptible to proteasome inhibitors than rapidly proliferating HL60 cells or primary endothelial cells respectively [30,31]. However, other types of tumour cells are more sensitive to proteasome inhibitors than their normal counterparts, even when their proliferation rates are not substantially different, e.g. in chronic lymphocytic leukaemia (CLL) [32], acute myelogenous leukaemia (AML) stem cells [23], or primary myeloma tumour cells [14]. In view of these data, an alternative, but less discretely formed, hypothesis is that tumour cells are more susceptible to proteasome inhibition, not necessarily just because of higher proliferation rates, but because of concomitant dysregulation of cell-cycle regulators, such as cyclin-dependent kinases, and the defective cell-cycle check points in tumour cells [2,9,33].

Clinical development of proteasome inhibitors

Bortezomib was first administered as a single agent in four different phase I clinical trials (two in hematologic malignancies [34,35] and two in solid tumours [36,37]) as bolus intravenous injections in various schedules, including 6-week cycles (4 weeks of therapy followed by a 2-week rest) at doses of 0.40, 1.04, 1.20, or 1.38 mg/m² for patients with advanced B-cell malignancies [34], or at doses of 0.75, 1.25, or 1.5 mg/m² for patients with acute leukaemias refractory to or relapsing after prior therapy [35]. In the phase I trials for solid tumours, bortezomib was administered (again by bolus intravenous injections) weekly, at doses ranging from 0.13–2.0 mg/m² per dose for 4 weeks of 5-week cycles, for mostly patients with advanced prostate cancer) [37], or twice weekly (at doses ranging from 0.13–1.56 mg/m² per dose) for 2 weeks, followed by a 1-week recovery period, in patients with advanced solid tumour malignancies [36].

In the phase I trial in advanced prostate-cancer patients, PK (pharmacokinetic) data were obtained from analyses of peripheral blood samples by LC/MS/MS (liquid chromatography with tandem mass spectrometry) detection assay. This PK assessment was performed for patients receiving doses between 1.45 and 2.0 mg/m² per dose. The majority of these plasma profiles are described by a two-compartment PK model with a rapid initial distribution half-life (t_{1/2a}: 0.22–0.46 h), followed by a more sustained terminal elimination half-life (t_{1/2b}>10 h) and a large (>500 litres) volume of distribution [37]. These data, taken together with tissue distribution results from pre-clinical animal models, indicated that after its intravenous administration, bortezomib is rapidly distributed into extravascular tissues, cleared slowly from them, and returns to the systemic circulation to be eliminated by the hepatic and renal routes [37]. In that same phase I trial, the relationship between bortezomib plasma concen-
tration and proteasome inhibition was assessed over a 24-h period from bortezomib injection. The 1-h plasma concentrations of bortezomib had a heterogeneous 8-fold range, in contrast to the rather homogenous response in reduction of 20 S activity of approx. 70% (and a homogeneous pattern of recovery in 20 S proteasome activity in 24-h measurements) [37], which suggest a disconnection between plasma bortezomib levels and suppression of 20 S activity. These results suggested that either the early part of bortezomib administration (e.g. 0 to 1-h interval) is very important for determining the clinical outcome of the drug, or perhaps that the intracellular bioavailability of bortezomib is more informative than its plasma PK. Furthermore, the observed clinically relevant efficacy and toxicity occurred at saturation (plateau) levels of inhibition of 20 S proteasome activity (65–80%), whereas no relationship appears to exist between plasma bortezomib concentration and 20 S activity [37]. Overall, in several trials, bortezomib treatment was associated with a dose-related inhibition of 20 S proteasome activity in peripheral blood samples, suggesting that these measurements can be viewed as a surrogate pharmacodynamic marker. Generally, doses of bortezomib that achieve up to approx. 80% inhibition of chymotryptic 20 S proteasome activity in the peripheral blood can be well tolerated [38]. The fact that no direct correlation has yet been established between the precise percentage inhibition of 20 S proteasome activity in the peripheral blood and the clinical response of patients to bortezomib could perhaps reflect the fact that the sensitivity of tumour cells to proteasome inhibitors depends not only on the degree of inhibition of the target, but also on downstream pathways that regulate how dependent tumour cells are on proteasome function for their proliferation and viability.

In view of the different schedules, dose escalation schemes and patient populations in the various trials of the phase I programme for the clinical development, the recommended phase II doses differed, e.g. 1.56 mg/m² per dose for twice weekly administration (2 weeks on, 1 week off treatment) [36], 1.6 mg/m² per dose for weekly administration (for 4 weeks of a 5-week cycle) [37], 1.04 mg/m² per dose for twice weekly administration (for 4 weeks of a 6-week cycle) [34] and 1.25 mg/m² per dose for twice weekly administration (for 4 weeks of a 6-week cycle) [35].

Two major conclusions emerged from these phase I clinical trials. The first was that bortezomib can be safely administered with acceptable and manageable toxicity (e.g. for thrombocytopenia, diarrhoea, electrolyte imbalances, and peripheral neuropathy [34–37]), in contrast to initial concerns that proteasome inhibition would lead to clinically unacceptable and catastrophic toxicities. The second was that bortezomib achieved very encouraging clinical responses in patients with plasma cell neoplasias, such as MM. Among nine fully assessable patients with heavily pretreated plasma-cell malignancies, one patient had complete response and eight others had reductions in tumour burden (assessed by serum paraprotein levels and/or marrow plasmacytosis). In addition, one patient with mantle cell lymphoma and another with follicular
lymphoma had shrinkage of lymph node involvement of their disease [34]. One major response was observed in a non-small-cell-lung-carcinoma patient [36], yet the activity of bortezomib in MM patients was so favourable that it warranted extensive phase II testing in this disease.

Two phase II clinical trials of single agent bortezomib in advanced MM have been conducted [39,40]. In the SUMMIT (Study of Uncontrolled MM managed with Proteasome Inhibition Therapy) trial, 202 heavily pretreated patients with relapsed and refractory myeloma received bortezomib at 1.3 mg/m² intravenously on days 1, 4, 8, and 11 of a 3-week cycle for up to eight cycles [39]. In 193 patients that could be evaluated, bortezomib achieved a 35% overall response rate, including 4% complete responses (complete clinical remission and undetectable myeloma-related monoclonal protein (M-protein) by electrophoresis and immunofixation), 6% near-complete responses (M-protein detectable only by immunofixation), 18% partial responses (>50% decrease of M-protein levels compared with the baseline), and 7% minimal responses (25–50% reduction of M-protein levels). Response to bortezomib was independent of most prognostic factors, including type of MM or type or number of previous therapies, as well as chromosomal abnormalities of tumour cells (e.g. chromosome 13 deletion) [39]. These clinical responses were not only durable (median time to disease progression of 7 months), but were observed in cases resistant to multiple other therapies [39], which was quite encouraging.

In the CREST (Clinical Response and Efficacy Study of Bortezomib in the Treatment of Relapsing MM) trial, 67 patients with relapsed or refractory MM following front-line therapy were randomized to receive bortezomib at either 1.0 or 1.3 mg/m² [40] on the same schedule used in the SUMMIT trial. Clinical responses were observed at either dose level, indicating that dose reduction to 1.0 mg/m² could still be therapeutic in cases of patients experiencing side effects at 1.3 mg/m². In both the SUMMIT and CREST trials, patients with progressive disease after two cycles or stable disease after the first four cycles could receive 20 mg of oral dexamethasone on the day of, and the day after, bortezomib administration. Additional responses were observed in both trials when dexamethasone was combined with bortezomib, consistent with the prior preclinical data, suggesting that the molecular effects of proteasome inhibition can sensitize MM cells to various treatments [14,15], including dexamethasone.

The profile of side effects of bortezomib-treated patients is generally manageable with routine therapeutic measures. The most frequently reported treatment-emergent side effects are nausea, fatigue, and diarrhoea [40,41]. Thrombocytopenia, fatigue, peripheral neuropathy, neutropaenia, lymphopaenia, and hyponatraemia correspond to the most frequently reported drug-related grade 3/4 adverse events [40,41]. Bortezomib-emergent thrombocytopenia is cyclical, and is characterized by a drop in platelet count during the first 2 weeks of each cycle of treatment and gradual recovery to baseline counts during the third week (i.e. the rest phase of each cycle, according to the schedule of bortezomib administration in the phase II and III clinical trials of
The nadir platelet count within each cycle usually corresponds to approx. 40% of the baseline value, suggesting that the risk of treatment-emergent clinically significant thrombocytopaenia primarily pertains to patients with already low baseline platelet counts [41]. Bortezomib-emergent peripheral neuropathy was the cause for discontinuation of therapy in 9% and 4% of patients in the CREST and SUMMIT trials respectively, which corresponded to the highest proportion of treatment discontinuations among adverse events in these trials [40,41]. It is important to emphasize that symptoms of peripheral neuropathy were present at baseline (prior to initiation of bortezomib therapy) in many patients enrolled in these trials and were largely attributed to previous treatment with neurotoxic agents, incorporated in the therapeutic management of myeloma (e.g. thalidomide, vincristine). Importantly, peripheral neuropathy improved or resolved in the majority of patients after completion or discontinuation of therapy [41].

Based on the results of the pivotal SUMMIT phase II trial, the U.S. Food and Drug Administration approved, in May 2003, bortezomib for the treatment of patients with MM who have received at least two prior therapies and who have demonstrated disease progression on their last therapy. In addition, bortezomib was approved in April 2004 for use in the European Union.

The phase III trial APEX (Assessment of Proteasome Inhibition for Extending Remissions) was a large international study, in which 669 relapsed myeloma patients (who were known not to be refractory to dexamethasone) were randomized to receive either bortezomib (administered for the first eight cycles using the phase II schedule, and thereafter at 1.3 mg/m² on day one of the first 4 weeks of a 5-week cycle for an additional three cycles) or high-dose dexamethasone [41]. A pre-specified interim analysis demonstrated superiority of the bortezomib arm over dexamethasone with regard to the primary end point, median time to disease progression, and median overall survival [41].

At least two phase I trials of bortezomib, one in combination with melphalan and the other in combination with doxorubicin, are ongoing in patients with relapsed or refractory haematological malignancies, based on the pre-clinical observations on the bortezomib-induced chemosensitization of MM cells [17]. Activity reported to date in these trials has been promising, and importantly, approx. 50% of patients with prior resistance to melphalan or doxorubicin responded to the original agent combined with bortezomib.

**Studies of bortezomib in NHL (non-Hodgkin’s lymphoma) and other malignancies**

Although the main emphasis of the clinical development of bortezomib was on MM, clinical responses in the phase I trials were also observed in patients with other types of haematological malignancies, e.g. one patient with Waldenström’s macroglobulinaemia (WM; lymphoplasmacytic lymphoma), one patient with mantle cell lymphoma (MCL) and another with follicular...
lymphoma [34]. These considerations, along with pre-clinical data indicating that bortezomib can be active against tumour cells from WM patients [42] or against MCL cell lines [18], provided sufficient support for the notion that bortezomib warranted further clinical testing in haematological malignancies other than MM. Results of two such phase II trials were recently published [43,44] and showed that bortezomib, which was well tolerated, has significant single-agent activity in patients with certain subtypes of NHL, such as MCL [43,44].

Several phase II clinical trials of bortezomib in patients with advanced solid tumours are ongoing or have been reported recently [45–52]. Many of these trials explore the chemosensitizing properties of bortezomib, using it in combination with other anti-neoplastic agents. So far, no histological type or subtype of solid tumour has been shown to respond clinically to bortezomib to the same degree of clinical response observed in MM. However, there is still potential for bortezomib use in solid tumours, mainly in combination with cytotoxic chemotherapy, although more pre-clinical studies will be needed to define which solid tumours may be more amenable to such combination regimens.

**Conclusion**

Proteasome inhibitors constitute a novel class of drugs for the treatment of human cancer. Bortezomib, the prototypical member of this class, has been approved in the U.S. and Europe for the treatment of advanced myeloma. Clinical trials of bortezomib as first-line treatment of myeloma are underway, and further investigation should be carried out to evaluate the role of bortezomib in other haematological malignancies, including MCL, in view of the encouraging data from phase II studies, which show substantial clinical activity in this subtype of NHL.

**Summary**

- Despite the role of the proteasome in both normal and transformed cells, inhibition of the chymotryptic activity of the 20 S proteasome has more significant functional consequences for malignant cells.
- **Bortezomib is the first-in-class proteasome inhibitor used for treatment of human neoplasias.**
- **Bortezomib kills tumour cells via pleiotropic mechanisms, including both simultaneous activation of multiple caspase-mediated apoptotic cascades and inhibition of anti-apoptotic pathways.**
- **Bortezomib is active in vitro and in vivo against even cells resistant to multiple conventional antitumour therapies.**
- **Bortezomib can be combined with diverse other anticancer therapeutics to achieve enhanced antitumour effect in vitro or in vivo.**

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Ongoing clinical studies are evaluating the role of proteasome inhibitors in diseases other than myeloma (e.g. various subtypes of lymphomas).

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