The development of proteasome inhibitors as anticancer drugs

Julian Adams*

Infinity Pharmaceuticals, Inc., 780 Memorial Drive, Cambridge, Massachusetts 02139
*Correspondence: julian.adams@ipi.com

The ubiquitin-proteasome pathway plays a central role in the targeted destruction of cellular proteins, including cell cycle regulatory proteins. Because these pathways are critical for the proliferation and survival of all cells, and in particular cancerous cells, proteasome inhibition is a potentially attractive anticancer therapy. Based on encouraging cytotoxic activity, bortezomib was the first proteasome inhibitor to be evaluated in clinical trials. Efficacy and safety results from a phase 2 clinical trial contributed to approval of bortezomib for use in patients with relapsed and refractory multiple myeloma who have received at least 2 prior therapies and have demonstrated disease progression on their last therapy.

Protein homeostasis is critical to biological processes that are fundamental to cancer cell survival. Therefore, targeting the regulation of protein production and destruction, particularly factors that mediate proliferation and other hallmark characteristics of malignancy, has been a major focus of cancer research. Recently, it has become widely appreciated that the orderly destruction of cell cycle regulatory proteins is critical to the control of cellular processes associated with cancer. The ubiquitin-proteasome pathway (UPP), which processes more than 80% of all cellular proteins, is the principal mechanism for degradation of proteins, including those involved in cell cycle regulation (Adams, 2002a). Accordingly, the proteasome has emerged as an attractive target for cancer therapy. This article focuses on proteasome inhibition as an anticancer strategy and on the development of bortezomib, the first-in-class proteasome inhibitor to enter clinical practice.

The ubiquitin-proteasome pathway

Proteins are targeted for recognition and for subsequent degradation by the proteasome via the attachment of multiple ubiquitin molecules. The 26S proteasome is a 2,000 kDa multisubunit cylindrical complex comprised of a 20S core catalytic component (the 20S proteasome) capped at one or both ends by a 19S regulatory component (Figure 1) (Adams, 2003). The 19S subunit recognizes and binds the polyubiquitinated protein and cleaves the ubiquitin chain from the protein substrate. The protein is then unfolded and fed into the 20S core, and the ubiquitin molecules are recycled. The 20S core is composed of 4 stacked rings: 2 outer rings (α rings) and 2 internal rings, termed β rings, in which proteolysis occurs. Each β ring consists of 7 subunits containing 3 active enzymatic sites termed trypsin-like, chymotrypsin-like, and post-glutamyl peptide hydrolase-like (caspase-like), after enzymes that show similar activity or specificity (Almond and Cohen, 2002; Adams, 2003). Within the 20S core, proteins are progressively degraded to small, 3- to 25-amino-acid peptides (Nussbaum et al., 1998).

Research has shown that the UPP is integral to mechanisms underlying carcinogenesis and metastasis, including cell cycle regulation, apoptosis, and angiogenesis (King et al., 1996; Adams 2004). The proteasome has a direct role in allowing the cell to progress through the cell cycle by degrading cell cycle regulatory proteins, and an indirect role by regulating the availability of transcriptional activators, such as nuclear factor (NF)-κB (see Table 1 and discussion below).

Proteasome inhibitors

The most important proteasome inhibitors fall into 5 classes: peptide aldehydes, peptide vinyl sulfones, peptide boronates, peptide epoxyketones (epoxomycin and eponomycin), and β-lactones (lactacystin and its derivatives), based on the pharmacophore that reacts with a threonine residue in the active site of the proteasome (reviewed in Kisselev and Goldberg, 2001). The biological activities of the epoxyketones have not been extensively studied; of the others, all but the peptide boronates exhibit properties that render them unsuitable for clinical development. Reasons to reject a proteasome inhibitor for future clinical development have included metabolic instability (Adams et al., 1998; Adams et al., 2000), lack of enzyme specificity, and irreversible binding to the proteasome (reviewed in Almond and Cohen, 2002).

Nonetheless, the peptide aldehydes and lactacystin have been useful in the study of effects of proteasome inhibition in cancer cell lines and in tumor xenografts. Key findings include: (1) cytotoxicity selective for transformed, as opposed to normal, cells (An et al., 1998; Orlowski et al., 1998; Masdehors et al., 2000); (2) additive effects when a proteasome inhibitor is combined with other anticancer agents (Cusack et al., 2001), including cytotoxicity in transformed cells at doses lower than those

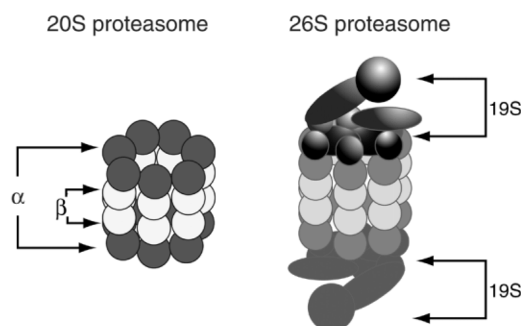

Figure 1. A schematic of the 26S proteasome

The 26S proteasome, a 2,000 kDa multiprotein complex, is comprised of a proteolytically active 20S core particle that is capped by 1 or 2 19S regulatory particles. The 19S regulatory units recognize ubiquitinated proteins and control access to the proteolytic core. Reprinted from Adams (2003) with permission from Cancer Treatment Reviews.

Table 1. Selected processes affected by proteasome activity in normal and cancerous cells

Process	Proteins degraded by the proteasome	Reference
Cell cycle control	Cyclins A, B, D, E; cyclin-dependent kinase inhibitors (p27, $p21^{WAF1/CIP1}$); cdc25 phosphatase	Adams, 2004
Oncogenic transformation	C-fos, C-jun, N-myc	Adams, 2002a; Almond and Cohen, 2002
Tumor suppression	p53	Adams, 2002a; Adams, 2004
Apoptosis	Bax	Li and Dou, 2000
Protein turnover	80% of cellular proteins	Adams, 2002a; Adams, 2004
NF-κB activation	IκB	Karin et al., 2002
Proinflammatory cytokines (TNF-α, IL-1, IL-6)		Karin et al., 2002 ; Adams, 2004
Cell adhesion molecules (ICAM-1, VCAM-1)		Adams, 2002a; Adams, 2004
Stress response enzymes (COX-2, NOS, 5-lipoxygenase)		Karin et al., 2002; Adams, 2004
Antiapoptotic factors (Bcl-2, IAP,TRAIL)		Almond and Cohen, 2002; Adams, 2004; Zhang et al., 2004
Proangiogenesis factors (VEGF, GRO-α)		Sunwoo et al., 2001
ER stress (URP)		Lee et al., 2003

required when the agents are used individually (Guzman et al., 2002); (3) sensitization of cell lines resistant to radiotherapy (Pajonk et al., 2000) or chemotherapy (Ogiso et al., 2000; Desai et al., 2001); and (4) induction of apoptosis in cells overexpressing Bcl-2 (An et al., 1998). In addition, in proliferating, subconfluent, endothelial cell cultures, the peptide aldehyde proteasome inhibitor can induce apoptosis at a concentration 340-fold lower than that required in quiescent cells, grown to confluence and contact-inhibited, suggesting that proteasome inhibition may affect tumor growth by inhibiting angiogenesis (Drexler et al., 2000).

Peptide boronic acid analogs

Because peptide boronic acids inhibit serine proteases such as chymotrypsin by mimicking substrate binding at the active site (Kettner and Shenvi, 1984; Weber et al., 1995), it was postulated that they might inhibit the proteasome by binding to the chymotrypsin-like site in the 20S core (Adams et al., 1998). Dipeptidyl boronates synthesized to test this hypothesis showed high potency, high specificity for the chymotryptic site, and reversible activity (Almond and Cohen, 2002; Adams, 2003). Thirteen boron-containing compounds were screened for anticancer activity in the National Cancer Institute (NCI) panel of 60 cancer cell lines. One compound, PS-341, later designated bortezomib (Figures 2 and 3), was potent, inhibited the enzyme at nM concentration (K_i 0.6 nM), was active against a broad range of cancer cell lines, including non-small cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast cancers, and had a unique cytotoxicity profile, compared with the NCI's historical file of 60,000 compounds (Adams et al., 1999). Thus, it was selected for intensive study.

Bortezomib

Mechanism of action

Numerous proteins are degraded by the proteasome, so multiple cellular processes are affected by proteasome inhibition (see Table 1). Therefore, the activity of bortezomib in different cancers probably involves a variety of molecular mechanisms. Extensive preclinical research has been conducted with bortezomib to elucidate its mechanism of action and to examine its activity, both as a single agent and in combination with other anticancer modalities, in a wide variety of solid tumor and hematologic cancer cell lines and tumor models.

In cell culture, bortezomib induces apoptosis in both hematologic and solid tumor malignancies, including myeloma (Hideshima et al., 2001), mantle cell lymphoma (Pham et al., 2003), and non-small cell lung (Ling et al., 2003), ovarian (Frankel et al., 2000), pancreatic (Shah et al., 2001; Bold et al., 2001), prostate (Adams et al., 1999; Frankel et al., 2000), and head and neck (Sunwoo et al., 2001) cancers. The mechanisms by which bortezomib induces apoptosis are unclear, although the stabilization of pro- and antiapoptotic proteins, including

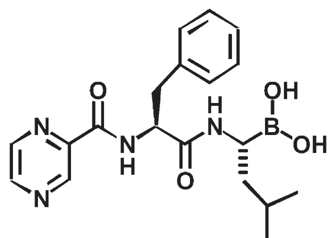

Figure 2. The structure of the dipeptidyl boronic acid proteasome inhibitor bortezomib

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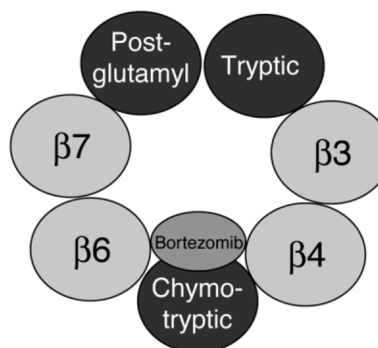

Figure 3. Cross-sectional view of the bortezomib binding site in the proteasome.

Bortezomib interacts with a threonine residue on the β subunit that confers chymotryptic proteolytic activity. Reprinted from Adams (2003) with permission from Cancer Treatment Reviews.

cyclin-dependent kinase inhibitors (e.g., p21 and p27) and tumor suppressors (e.g., p53) has been associated with proteasome inhibition (Shah et al., 2001; An et al., 1998; MacLaren et al., 2001). Proteasome inhibition has also been demonstrated to interfere with the unfolded protein response (UPR), thereby causing endoplasmatic reticulum stress (ER-stress) and increased apoptosis (Lee et al., 2003). Additionally, bortezomib sensitizes resistant solid tumor cells to TNF-like apoptosis, inducing ligand (TRAIL)-induced apoptosis, most likely by increasing the levels of death receptors (DR) DR4 and DR5 (Zhang et al., 2004).

Proteasome inhibition may induce apoptosis or increase sensitivity to apoptosis by shifting the balance between pro- and antiapoptotic signals (see Adams, 2003, for discussion). The involvement of regulatory proteins in apoptosis differs according to cell type. For example, p53 expression was essential for proteasome inhibitor-induced apoptosis in mammary epithelial cells (MacLaren et al., 2001), whereas it was irrelevant in PC-3 prostate cancer cells, which are p53 null (Adams et al., 1999). In the latter, bortezomib caused G2/M cell cycle arrest and apoptosis (Adams et al., 1999). These in vitro cytotoxic effects of bortezomib were affirmed in a murine xenograft model of human prostate cancer in which bortezomib reduced tumor growth by about 60% ($p < 0.05$) compared with vehicle treatment (Adams et al., 1999).

One molecule with a central role in mediating many of the effects of proteasome inhibition is the transcriptional activator NF-κB. Known to be involved in inflammatory and immune responses, NF-κB and its signaling pathways were also recently implicated in tumor development (Karin et al., 2002). Under normal conditions, NF-κB is bound to its inhibitor IκB, and transcriptional activation of genes by NF-κB is suppressed. In response to cellular stresses, IκB is degraded by the proteasome and NF-κB is released, activating transcription of genes for growth factors, stress response enzymes, cell adhesion molecules, and apoptosis inhibitors (reviewed in Karin et al., 2002) (Table 1). Activation of NF-κB may also occur in response to chemotherapy and radiation and confer resistance (Wang et al., 1999). In contrast, bortezomib inhibits NF-κB activation through proteasome inhibition (Hideshima et al., 2001; Cusack et al., 2001; Hideshima et al., 2002), potentially providing a rationale for the use of bortezomib in cells that constitutively express NF-κB or in combination with chemotherapy and radiation.

However, inhibition of NF-κB activation does not completely explain the anticancer activity of bortezomib. Hideshima and colleagues (2002) compared the effects of an IκB kinase inhibitor, PS-1145, and bortezomib in multiple myeloma cells. Although both PS-1145 and bortezomib blocked NF-κB activation, bortezomib completely suppressed cell proliferation, whereas PS-1145 inhibited cell proliferation by only 20% to 50%.

The tumoricidal activity of bortezomib involves other mechanisms as well. Cancer progression and metastasis depend on tumor angiogenesis, as well as on adhesion factors and growth factors. Several preclinical studies report that bortezomib causes inhibition of tumor angiogenesis, consistent with the selective effect of proteasome inhibition on proliferating endothelial cells. Bortezomib markedly decreased microvessel density in murine and human xenograft head and neck tumors and in human prostate tumors implanted in mice (Sunwoo et al., 2001; Williams et al., 2003). Decreased vascular endothelial cell growth factor secretion and high levels of endothelial cell apoptosis were also seen in the prostate tumor model (Williams et al., 2003). Decreased tumor angiogenesis was also observed in tumors from mice implanted with multiple myeloma xenografts (LeBlanc et al., 2002).

In some cancers, such as multiple myeloma, bortezomib alters the microenvironment such that it is less susceptible to tumor cell growth. In multiple myeloma, tumor cells bind to bone marrow stromal cells (BMSCs), triggering them to secrete interleukin (IL)-6 through activation of NF-κB. Bortezomib decreases myeloma cell binding to BMSCs, reducing NF-κB-mediated synthesis of IL-6 by the stroma (Hideshima et al., 2001).

Bortezomib has shown the ability to have additive activity with other therapies through several mechanisms as observed in vitro and in animal models. Moreover, bortezomib has conferred a chemosensitizing effect that allows the use of lower doses of other antineoplastic therapies to achieve a tumoricidal effect at least equivalent to that of higher monotherapy doses. This effect has been observed using bortezomib with:

- doxorubicin, melphalan, or dexamethasone in multiple myeloma cell lines (Mitsiades et al., 2003; Hideshima et al., 2001; Ma et al., 2003)
- irinotecan or radiation therapy in colon cancer xenografts (Cusack et al., 2001; Russo et al., 2001)
- gemcitabine in pancreatic cancer xenografts (Bold et al., 2001)
- daclizumab (an anti-IL-2Rα antibody) in a model of adult T cell leukemia (Tan and Waldmann, 2002)
- radiation therapy, cyclophosphamide, and cisplatin in a mouse mammary tumor model (Teicher et al., 1999).

Bortezomib has also been shown to confer renewed sensitivity to chemotherapeutic agents in cell lines that previously developed resistance to their cytotoxic effects. Specifically, in cell lines previously resistant to doxorubicin, melphalan, mitoxantrone, and dexamethasone, bortezomib was able to restore the therapeutic activity of these agents (Hideshima et al., 2001; Mitsiades et al., 2003).

Differential sensitivity of neoplastic cells to bortezomib

Selective toxicity of proteasome inhibitors toward transformed cells has also been observed with bortezomib (Hideshima et al., 2001). In general, proteasome inhibition tends to induce apoptosis in proliferating cells while being protective in some but not all quiescent cells (Drexler, 1997). One of the more notable exceptions is the potent induction of apoptosis by proteasome inhibitors in malignant B cell chronic lymphocytic leukemia cells, which are largely quiescent (Masdehors et al., 2000). The increased sensitivity of cancer cells to proteasome inhibition may be partially related to the dysregulation of molecules that drive the cell cycle, such as the cyclin-dependent kinases, and high proliferation rates observed in neoplastic cells. However, bortezomib induces cell death in prostate cancer cells with a very low growth fraction (Frankel et al., 2000). Nonetheless, because cancer cells generally are defective in cell cycle check points, they may be particularly susceptible to the stress imposed by proteasome inhibition (see discussion in Almond and Cohen, 2002). This may explain why, in a human plasmacytoma xenograft mouse model, bortezomib significantly inhibited tumor growth with low systemic toxicity, despite the observation that the degree of proteasome inhibition was greater in normal tissues than in tumors (LeBlanc et al., 2002).

Clinical trials

Bortezomib was the first proteasome inhibitor to enter clinical trials. Two phase I trials, one in solid tumors and one in hematologic malignancies, have been published and establish that

bortezomib could be administered with acceptable and manageable toxicity (Aghajanian et al., 2002; Orlowski et al., 2002). In the solid tumor trial, a major response was observed in a patient with refractory non-small cell lung carcinoma, and the investigators suggested that bortezomib should be evaluated further, perhaps in combination with other agents in future trials (Aghajanian et al., 2002). Encouraging activity, especially in multiple myeloma and malignant lymphoma, was seen in the phase I trial in hematologic malignancies (Orlowski et al., 2002).

A pivotal phase II trial of bortezomib was conducted in 202 patients with relapsed and/or refractory multiple myeloma who had progressed after a median of 6 previous therapeutic regimens. Using modified criteria of the European Group for Blood and Marrow Transplantation (Bladé et al., 1998), a 35% response rate was observed in 193 evaluable patients, including 4% with complete response (myeloma protein undetectable by electrophoresis and immunofixation), 6% near-complete response (myeloma protein detectable only by immunofixation), 18% partial response, and 7% minimal response (Richardson et al., 2003). Using criteria of the Southwest Oncology Group, 18% of patients experienced clinical remission (≥75% paraprotein reduction). The incidence of grade 4 adverse events was relatively low, and most could be managed with standard approaches (Richardson et al., 2003). Among all 202 patients, the median time to progression was 7 months while they were receiving bortezomib, as compared with 3 months while receiving the last treatment before entering the study. These results are encouraging, in view of the fact that complete responses are rare in patients with multiple myeloma that has become refractory to prior regimens (Richardson et al., 2003). A positive therapeutic response to bortezomib was not influenced by the type of multiple myeloma or by the type or number of previous therapies (Richardson et al., 2003).

In May 2003, the US Food and Drug Administration approved bortezomib for the treatment of patients with multiple myeloma who have received at least 2 prior therapies and who have demonstrated disease progression on their last therapy. In addition, bortezomib was approved in April 2004 by the CPMP for use in the European Union. Ongoing clinical trials are investigating the use of bortezomib in an earlier myeloma patient population and in combination with other antineoplastic therapies (Adams, 2002b). Promising early clinical results in non-Hodgkin's lymphoma (Goy et al., 2003; O'Connor et al., 2003) and in several solid tumors (Aghajanian et al., 2002; Albanell et al., 2003; Appleman et al., 2003) have been reported. These findings support current investigations that are elucidating the efficacy and safety of bortezomib in the treatment of various types of malignancies and identifying optimal parameters for its use, particularly in combination with other agents.

Preclinical and clinical studies have validated the hypothesis that the proteasome is a viable therapeutic target. It is especially encouraging that after little more than one decade, proteasome inhibition has moved from hypothesis to clinical application in the treatment of cancer.

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