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Review Article

Bortezomib, a proteasome inhibitor, in cancer therapy: from concept to clinic*

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Abstract

The proteasome is a multiprotease complex that degrades the majority of cellular proteins in a highly regulated manner. The elimination of many key proteins by the proteasome is required for essential cellular processes, including cell-cycle progression, cell survival and cellular homeostasis. Conversely, inhibition of the proteasome results in cell-cycle arrest or programmed cell death. The observation that malignant cells were more susceptible to the effects of proteasome inhibition than normal cells raised the notion of proteasome inhibition as a novel approach in cancer therapy. The present review will focus on the potential for proteasome inhibitor therapy in cancer with bortezomib (Velcade™; formerly known as PS-341; Millennium Pharmaceuticals, Inc., Cambridge, MA, USA). Bortezomib is an extremely potent and selective proteasome inhibitor. In cell culture and animal models of cancer, it has potent tumoricidal effects and sensitizes cancer cells to conventional anticancer agents. Bortezomib is the only proteasome inhibitor that has entered clinical trials in patients with cancer. With approximately 200 patients treated in phase I trials to date, bortezomib has been generally well tolerated at doses that achieve a desired degree of proteasome inhibition. Encouraging antitumor activity has been observed. These data served as the basis for phase II clinical trials of bortezomib in patients with a broad range of tumors and also for clinical studies of bortezomib plus chemotherapy. The early results of combination trials show that bortezomib was generally well tolerated at doses that resulted in a good level of proteasome inhibition when combined with chemotherapy in patients in these trials. No major overlapping toxicities have been observed to date and there was evidence of antitumor activity by many of the combinations tested in chemorefractory patients. Phase II or definitive phase III studies of bortezomib and chemotherapy will be considered after the completion of these initial trials and should serve to contribute to a further understanding of the potential role of bortezomib in the treatment of human malignancies in the near future.

Introduction

The proteasome is a multiprotease complex found in the nucleus and cytoplasm of eukaryotic cells that degrades nearly all cellular proteins in a highly regulated manner (1). It has long been known that the proteasome is important for cellular housekeeping since it is responsible for the degradation of mutant, damaged and misfolded proteins. However, it has more recently become clear that the proteasome is also involved in the targeted elimination of regulatory proteins such as transcription factors, signaling molecules and cell-cycle inhibitors (2). The rapid and timely elimination of such key proteins by the proteasome is required for the regulation of many cellular processes, including cell-cycle progression, cell survival and the maintenance of cellular homeostasis. Therefore, the proteasome is a fundamental component of the cellular machinery (3).

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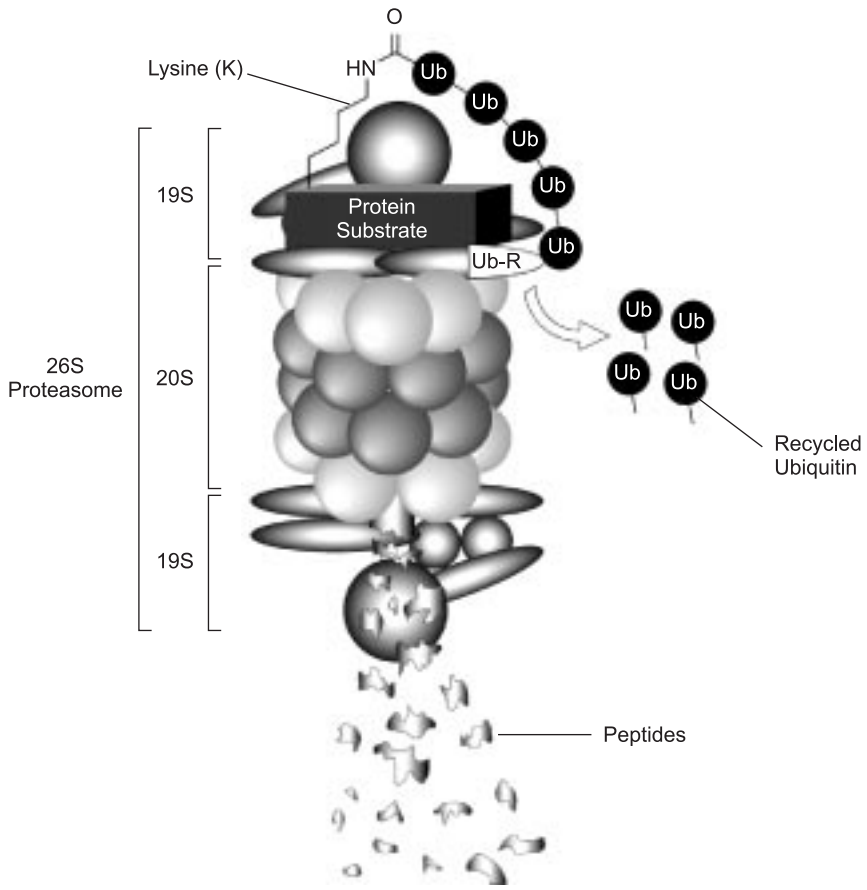

Fig. 1. Protein degradation by the ubiquitin-proteasome parhway. The ubiquitin-proteasone pathway comprises two main steps which allow a selective degradation of proteins. A first step is the identification and tagging of the proteins by poliubiquitinization. The second step consists of the identification of the ubiquitinated protein by the proteasome and its subsequent degradation. The 26S proteasome is a multiprotein complex comprised of a cylindrical 20S core particle and two 19S regulatory particles.

Inhibition of the proteasome results in the abnormal accumulation of many intracellular proteins that under normal conditions should have been degraded. Such aberrant accumulation of proteins disrupts cellular homeostasis and as a result cells undergo cell-cycle arrest or programmed cell death (3, 4). A fundamental observation that has fueled the potential of proteasome inhibition as a novel anticancer strategy is that malignant cells appear to be more susceptible to the effects of proteasome inhibition than normal cells. Furthermore, in studies carried out in cell culture and animal models of cancer, proteasome inhibition has potent tumoricidal effects and sensitizes cancer cells to conventional anticancer agents. Proteasome inhibition, therefore, is a promising new approach to cancer therapy. The present review will focus on the potential for proteasome inhibitor therapy in cancer patients with bortezomib (Velcade™; formerly known as PS-341; Millennium Pharmaceuticals, Inc., Cambridge, MA, USA). Bortezomib is the only proteasome inhibitor that has been extensively studied in *in vitro* and *in vivo* models of cancer and that has entered clinical trials in cancer patients.

The ubiquitin-proteasome pathway

The ubiquitin-proteasome pathway comprises two main steps which allow a selective degradation of proteins that need to be removed from the cells (3-5). The first step is the identification and tagging of the proteins and the second step consists of the identification of the tagged protein by the proteasome and its subsequent degradation (Fig. 1).

The first step (substrate recognition and tagging) is the ubiquitination of the protein to be degraded. This is a highly regulated process that generally is initiated when a single ubiquitin molecule is attached to a lysine side chain on the substrate protein. The recognition of the substrate is a specific event whose selectivity is conferred by the enzymes involved in this reaction (6). Further ubiquitin molecules are then sequentially added to form the polyubiquitin chain and this allows the protein targets to be recognized by the proteasome (6).

The second main step requires a functional 26S proteasome. The 26S proteasome is a multiprotein complex comprised of two functional subunits: a 20S proteolytic

Table I: Partial list of proteasome-dependent substrates regulating the cell cycle (from ref. 11).

Protein substrate	Function
Cyclins A, B, D, E	Cell-cycle progression
p53	Tumor suppressor
p27	CDK inhibitor
p21	CDK inhibitor
INK family	Cyclin D CDK inhibitor
IκB	Transcription factor
c-fos/c-jun	Transcription factor
β-Catenin	Transription factor
E2F-1	Transcription factor
Topo-I	DNA metabolism

core particle and a 19S regulatory subunit that caps both ends of the 20S particle. The 19S regulatory subunit has a receptor that binds to the polyubiquitin chain of the target protein (7) and then cleaves it from the substrate. It is then thought to unfold the protein substrate, allowing entry to the 20S core particle (2, 8, 9). The 20S catalytic chamber is a barrel-shaped structure made up of four stacked rings of seven subunits each. The two outer rings associate with the 19S regulatory complexes, while the two inner rings each contain three active sites (7). Protein hydrolysis is mediated by the N-terminal threonine residue of each proteolytic active site; therefore, the proteasome is classified as an N-terminal nucleophilic hydrolase (10). The three major proteolytic activities of the 20S core particle have been identified as chymotrypsin-like, trypsin-like and post-glutamyl peptide hydrolase-like (10). Proteins entering the core particle are cleaved to generate small polypeptides (10).

Table I lists critical proteins whose degradation is regulated by the proteasome (11). These proteins generally have a short-half life and include cyclins, cyclin-dependent kinase inhibitors (12, 13), tumor suppressors (14-16) and transcription factors (17, 18).

Proteasome inhibition as a novel anticancer strategy

The concept of targeting the proteasome as a potentially useful anticancer strategy is based on the observation that proteolysis by the 26S proteasome is a fundamental metabolic process, and that inhibition of proteasomal activity results in growth arrest and programmed cell death (4). There are two important additional observations when considering proteasome inhibition for cancer therapy. First, in laboratory studies tumor cells are considerably more sensitive to the proapoptotic effects of proteasome inhibition than normal cells, thus providing a therapeutic window (19-24), and second, complete blockade of proteasome function is not required for antitumor activity (25).

There are several possible reasons for the increased sensitivity of cancer cells to proteasome inhibition, although the precise mechanism(s) remain unclear, and include a major susceptibility to proteasome inhibition in actively dividing cells compared to quiescent or differentiated cells, an increased rate and dependence of protein degradation in tumor cells and effects of proteasome inhibitors on regulatory molecules that act as key growth-promoting or antiapoptotic factors in specific tumor types. Tumor cells would be highly dependent on these factors while normal cells would be less dependent and have alternative factors allowing their survival.

Regarding the possible role of proliferation rates on proteasome inhibition sensitivity, several studies have shown that proliferating cells had higher sensitivity to proteasome inhibition than nonproliferating cells (4, 26, 27). However, it is unlikely that proliferation rates play a major role in the response to proteasome inhibition.

Several studies indicate that human tumors commonly have high levels of proteasome expression and it is possible that this could confer relative selectivity of tumor cells compared to normal cells when they are treated with proteasome inhibitors. For example, in renal cell carcinoma, an increased expression of proteasome subunits was commonly seen compared to normal kidney tissues, suggesting a role for the proteasome system in this malignancy (28). Aberrant proteasome expression has also been reported in the cancer cells and bone marrow of patients with a range of hematologic malignancies, as compared with cells from healthy volunteers (29). These *in vivo* studies are also supported by findings in tumor cell lines. For instance, MCF-7 human breast cancer cells express elevated levels of one of the highly conserved proteasome subunits (30). More recently, an analysis of transcriptional profiles from approximately 200 solid tumors including colon, prostate, breast and ovarian cancers indicated that mRNAs encoding proteasome subunits were highly coregulated in these cancers (31). Additionally, in certain tumor types, proteasome gene expression was elevated in a subset of tumors when compared to normal samples of the same tissue type. Consistent with the observation of altered RNA levels, in a subset of tumors proteasome subunit expression was elevated in tumor cells when compared to normal adjacent epithelial cells, as assayed by immunohistochemistry (31). Taken together, these studies point to increased proteasome levels as a common finding in human malignancies. However, it is not known whether the proteasome contributes directly to tumorigenesis or whether the elevated expression of this enzyme is in response to the cancer cell's higher metabolism.

The effects of proteasome inhibition on a variety of cellular regulatory proteins, including cyclins, cyclin-dependent kinase inhibitors (12, 13), tumor suppressors (14-16) and transcription factors (17, 18) have been clearly demonstrated (Table I). The abnormal accumulation of such proteins and the resulting disruption of cellular homeostasis of the cell might be particularly intolerable for certain tumor cells. However, the precise mechanisms by which proteasome inhibition induces growth arrest and apoptosis in cancer cells are not clear. Indeed, it is likely that protein stabilization results in the

accumulation of multiple conflicting signals within the cell, and that no one protein is directly responsible for cell death (4).

An additional consequence of proteasome inhibition that might contribute to antitumor effects is inhibition of angiogenesis (27, 32). Angiogenesis is a critical step for *in vivo* tumor growth beyond a size of 2-3 mm and therefore the antiangiogenic effect of proteasome inhibition might further limit tumor growth.

Proteasome inhibitors

To date, a wide range of proteasome inhibitors have been developed, including synthetic inhibitors such as peptide aldehydes (33, 34), peptide vinyl sulfones (35) and the dipeptidyl inhibitor CEP-1612 (36), natural proteasome inhibitors such as lactacystin (37), the TMC-95 cyclic peptides (38) and epoxyketone compounds (39, 40). These compounds have been useful as research tools in the laboratory, but they are far from ideal for *in vivo* or clinical use due to their lack of specificity (33, 34), irreversibility (35, 38-42) or relative instability *in vivo* (33, 34).

To consider the use of proteasome inhibition for clinical purposes, the inhibition must be potent, specific and reversible, so that proteasome function may be restored when treatment ends. Reversibility is critical to exploit the higher sensitivity of tumor cells compared to nonmalignant cells to the suppression of proteasome activity. Considering these premises, the proteasome inhibitors with perhaps the greatest clinical potential are the peptide boronic acids. These compounds reversibly inhibit the proteasome in a manner similar to the peptide aldehydes, but they are up to 100-fold more potent than the latter and are highly selective for the proteasome over other cellular proteases (33). Furthermore, the slow binding and slow dissociation of these compounds from the proteasome confers stable inhibition (34). Of particular interest is the small, water-soluble dipeptide boronic acid bortezomib. Bortezomib is an extremely potent and selective proteasome inhibitor that binds to the proteasome in a stable but reversible manner (33) and has no known activity against any other cellular protease (34) (Fig. 2). This drug has been extensively tested as a tumoricidal agent in laboratory models and is already in clinical trials.

Bortezomib as a single agent

Preclinical studies

1) In vitro *studies*

Bortezomib is a low molecular weight, water-soluble dipeptide boronic acid (K_i = 0.6 nM) that was selected for development in cancer therapy (25). In a seminal study, bortezomib exhibited substantial cytotoxicity against a broad range of human tumor cells, as determined by the National Cancer Institute *in vitro* screen of 60 cancer cell lines derived from a range of human tumors (25). At very low concentrations, bortezomib was shown to penetrate cancer cells and inhibit both intracellular proteolysis and cell growth (25). The average growth inhibition value of 50% (GI_{50}) for bortezomib across the entire NCI cell panel was 7 nM. Interestingly, bortezomib was demonstrated to be cytotoxic independent of p53 status. The mechanistic relationship between proteasome inhibition and antitumor activity was supported by a strong correlation noted between K_i *versus* GI_{50} values when 13 dipeptide proteasome inhibitors from the boronate series were examined. The pattern of bortezomib-induced cytotoxicity, when compared with the historical file of 60,000 compounds of the NCI, was unique, with little correlation to other "standard" or investigational agents. This view of bortezomib as representing a novel and unique mechanism of potential anticancer action is further supported by another study showing that bortezomib, unlike most other known anticancer cytotoxic drugs, was as effective in killing tumor cells grown in the form of multicell spheroids as in killing tumor cells grown in monolayer cell culture. This cytotoxicity in multicell spheroids is a measure of the potential of bortezomib to circumvent multicellular drug resistance (43).

In the NCI *in vitro* screen the prostate tumor PC-3 cell line (25) was shown to be sensitive to the antiproliferative effects of bortezomib and was selected for further studies to examine potential mechanisms of bortezomib-induced cytotoxicity. Exposure of such cells to bortezomib caused them to accumulate in the G_2-M phase of the cell cycle and subsequently underwent apoptosis. The molecular mechanism of the bortezomib-induced G_2-M block probably involves the dysregulated processing and degradation of cell-cycle regulatory proteins by the proteasome. The cyclin-dependent kinase inhibitor p21, which normally is ubiquitinated and degraded by the proteasome (44), accumulated along with an increase in cells in S and G_2-M upon bortezomib exposure (25).

Following this study, the antitumor activity of bortezomib in cultured tumor cells was reported against a broad range of tumors and mechanistic effects were further addressed. In human MCF-7 breast cancer cells, a

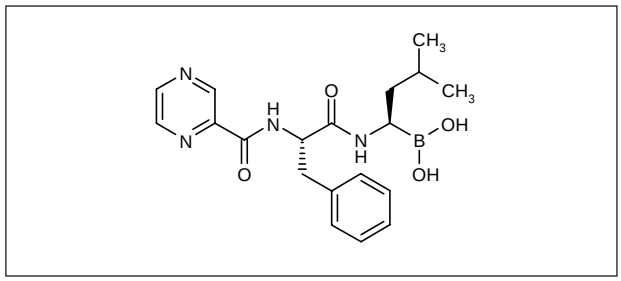

Fig. 2. The chemical structure of PS-341. Bortezomib (formerly known as PS-341) is a dipeptidyl boronic acid compound that inhibits the proteasome in a potent and specific manner. Copyright Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts.

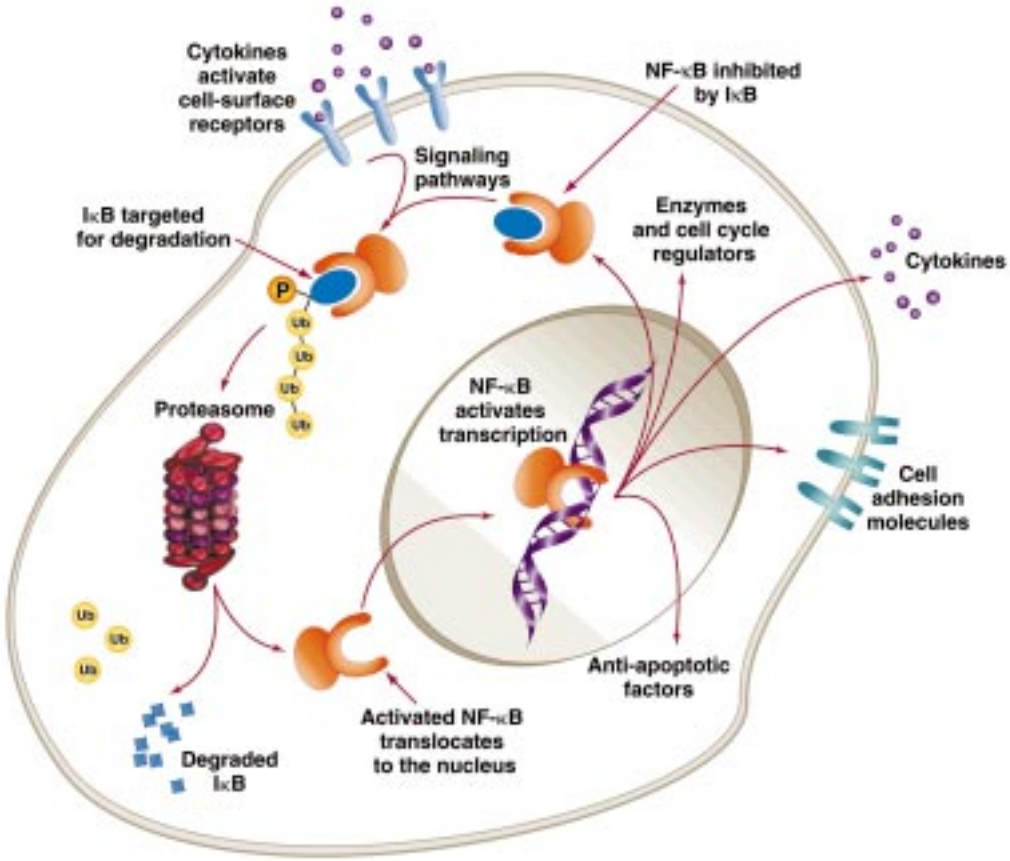

Fig. 3. The activation of NF-κB. NF-κB appears to be constitutively active in breast cancer, and it may also be induced by chemotherapy and radiotherapy. The induction of NF-κB-dependent gene expression promotes cell survival and contributes to the resistance of some cancer cells to conventional tumoricidal agents. Copyright Millennium Pharmaceuticals, Inc., Cambridge, Massachussetts.

low dose of bortezomib killed 99% of these cells within 48 h following exposure (45). In head and neck cancer cells, bortezomib also resulted in accumulation of p21 and cells in the S and G_2-M phase (46). Interestingly, bortezomib did not result in detectable changes in p27 protein levels, in contrast to what is reported in other cells treated with proteasome inhibitors (13, 36, 46-48). These head and neck cancer cells underwent apoptosis and the commitment to apoptosis caused by bortezomib treatment appeared to occur as early as 6 h (46). In lung cancer cells, bortezomib also induced a G_2-M phase arrest that was accompanied by an accumulation of p53 protein and inhibition of the degradation of several cell cycle-related regulators (49).

The levels of p27, another cyclin-dependent kinase inhibitor, are also regulated by the ubiquitin-proteasome pathway (50), and proteasome inhibition is known to lead to the stabilization of p27 (13, 36). p27 is a multifunctional protein which, in addition to its cell-cycle regulatory role, is a putative tumor suppressor (a proapoptotic protein) (50, 51). In certain cancers, the stabilization of p27 by bortezomib may play an important role in the induction of apoptosis (13, 36, 46-48).

Another important target of proteasome inhibition is the nuclear factor NF-κB, a transcription factor involved in many cellular processes. Several studies have confirmed that bortezomib is a potent inhibitor of NF-κB activation in a range of tumor cell lines (46, 47, 52). This effect on NF-κB is important since this transcription factor appears to be involved in cell survival and in the progression of some cancers. NF-κB is normally sequestered in the cytoplasm and rendered inactive by the inhibitor protein IκB (Fig. 3). However, a wide range of stimuli induce the phosphorylation of IκB and its subsequent degradation by the proteasome (53-55). NF-κB is thereby released, and it translocates to the nucleus where it drives the expression of genes associated with cell survival. Genes known to be regulated by NF-κB include those encoding proinflammatory cytokines such as interleukin-1 (IL-1) or IL-6, cell-adhesion molecules, stress-response enzymes, angiogenesis regulators and antiapoptotic proteins (56-61). These *in vitro* studies are complemented by a series of reports in clinical human tumors supporting a role of NF-κB in oncogenesis (62). NF-κB is constitutively active in certain tumors, including myeloma (63), leukemias (63, 64) and breast cancer (65-68).

The activation of NF-κB in these tumors commonly correlates with a more aggressive phenotype and less responsiveness to treatments. Consistent with its important role in cell survival, genetic and pharmacological approaches have shown that stabilization of the IκB protein and blockade of NF-kB activity makes cells more susceptible to apoptosis (69, 70). Bortezomib represents a novel potential way to prevent NF-κB activation. The mechanism by which bortezomib prevents NF-κB activation is by its ability to prevent the degradation by the proteasome of the NF-κB inhibitory protein IκBα, which then allows NF-κB to remain sequestered in the cytoplasm (47). However, considering that inhibition of the proteasome affects a wide variety of regulatory proteins, the inhibition of NF-κB activity is probably only one of a number of factors contributing to cell death.

The inhibition of growth and induction of apoptosis by bortezomib *in vitro* has been also shown in hematological malignancies (10, 63, 71). In particular, very promising results were seen in multiple myeloma (MM) cell lines and in freshly isolated patient MM cells (52, 63). Bortezomib inhibited mitogen-activated protein kinase (MAPK) growth signaling in MM cells, induced apoptosis in both p53 wild-type and p53 mutant MM cells, overcame drug resistance, enhanced the antitumor activity of dexamethasone, a commonly used drug for the treatment of MM, and overcame the resistance to apoptosis in MM cells conferred by the MM growth factor IL-6. Bortezomib also inhibited the paracrine growth of human MM cells by decreasing their adherence to bone marrow stromal cells (BMSCs) and related NF-κB-dependent induction of IL-6 secretion. These results showed that bortezomib acted directly on MM cells and altered cellular interactions and cytokine secretion in the bone marrow millieu to inhibit tumor cell growth, induce apoptosis and overcome drug resistance. The critical role of NF-κB-dependent transcription and secretion of the MM growth factor IL-6 may be of fundamental importance in these observations.

Collectively, the current findings indicate that the effects of bortezomib reflect more than one proteasome-dependent mechanism. In-depth analysis of cellular response to proteasome inhibition is being studied by the use and integration of novel technologies such as transcript profiling and population genomics (72, 73). These studies will likely contribute to an understanding of which critical molecules are responsible for sensitivity to bortezomib in tumor cell lines. In addition, the genomic changes that occur at different timepoints following bortezomib treatment are being elucidated, thus providing evidence of the pharmacodynamic events that result from proteasome inhibition at the transcriptional level. The final goal of these studies is to provide potential markers of response *versus* resistance to bortezomib and to comprehensively characterize the pharmacodynamic effects of the drug. Currently, the reasons for differente degrees of sensitivity to bortezomib among different cell lines even from the same cancer are as yet unknown (74, 75). Hopefully, these studies would then be applied to a rational selection of patients with a high likelihood to respond to proteasome inhibition based on the molecular portrait of their tumors and to help to characterize *in vivo* the pharmacodynamic effects of bortezomib in cancer patients.

2) In vivo *studies*

Bortezomib has been extensively studied in murine models of cancer (25, 45). In the initial study using the PC-3 prostate cancer model, weekly intravenous treatment of mice bearing the PC-3 tumor with bortezomib resulted in a significant decrease in tumor burden (25). In addition to its antitumor activity, this study also revealed that i.v. administration of bortezomib resulted in a rapid and widespread distribution of bortezomib, with highest levels identified in the liver and gastrointestinal tract and lowest levels in the skin and muscle. Modest levels were found in the prostate, whereas there was no apparent penetration of the central nervous system. An assay to follow the biological activity of bortezomib was established and used to determine temporal drug activity as well as its ability to penetrate tissues. As such, bortezomib was shown to penetrate PC-3 tumors and inhibit intracellular proteasome activity 1 h after dosing. These data illustrate that bortezomib not only reached its biological target but also had a direct effect on its biochemical target, the proteasome. Importantly, the data show that inhibition of this target site by bortezomib resulted in reduced tumor growth in murine tumor models. Taken together, these results highlighted the antitumor potential of bortezomib (25).

The ability of bortezomib to inhibit tumor growth has been also demonstrated in mice bearing a range of subcutaneously grafted cancers, including pancreatic (12, 74, 76), colorectal (47, 77), breast (45, 78), bladder (79), head and neck (46, 75) and prostate tumors (80, 81). The studies conducted *in vivo* may also help to characterize the effects of bortezomib on angiogenesis. In tumor xenografts from head and neck cancer, bortezomib treatment resulted in an antiangiogenic effect, as observed by a reduced vessel density in tumors from treated animals (46). In agreement with this effect, bortezomib treatment in cultured cells inhibited the expression of NF-κB-dependent proangiogenic cytokines. Furthermore, in addition to the inhibition of angiogenesis due to the effects on tumor cells and the resulting suppression of neoangiogenesis mechanisms, bortezomib may also be directly cytotoxic to endothelial cells.

Clinical trials of bortezomib as a single agent in cancer patients

1) Phase I trials

In July 1998, a series of phase I trials were initiated to evaluate the safety profile, tolerance, pharmacokinetics and preliminary activity of escalating doses of bortezomib

Table II:Phase I trials of bortezomib as single agent.

Center	Tumor type	Schedule
Millennium phase I		
MDACC	Solid tumors	1x/wk x 4 wks/6 wks
MSKCC	Solid tumors	2x/wk x 2 wks, 10-day rest
Univ. North Carolina/MSKCC	Hematological	2x/wk x 4 wks, 17-day rest
NCI phase I trials		
Dana Farber/NY University	Solid tumors	2x/wk qow
Mayo/Wisconsin	Solid tumors	2x/wk x 4 wks
MDACC	Leukemia	2x/wk x 4 wks

given as a single agent in patients with solid and hematological malignancies (Table II) (82-86). These studies incorporated a pharmacodynamic *ex vivo* assay developed to study the degree and kinetics of proteasome inhibition achieved in treated patients (87). The assay rapidly and reliably measures *ex vivo* proteasome activity in blood and tissues biopsies in the presence or absence of bortezomib. This bioassay has been crucial for the clinical development of bortezomib at least for two reasons. First, more than 90% of bortezomib is cleared from the plasma compartment within 15 min of i.v. administration (88). This rapid clearance makes bortezomib unsuitable for pharmacokinetic assays. Therefore, the measure of bortezomib effects on the targeted proteasome was envisioned as an additional and novel way to develop the drug. A second reason that highlights the need to measure proteasome inhibition is that pharmacological and toxicological studies suggested that twice-weekly regimens resulting in proteasome inhibition (as measured in blood) approaching but not exceeding 80% would be optimal. Proteasome inhibition of 80% or above resulted in reduced gastrointestinal toxicity in animal models. The bioassay has shown that the inhibition of proteasome activity by bortezomib is dose-dependent (85) and that there is very little difference in the degree of baseline proteasome activity among subjects or over time. These two observations support the notion that once a recommended dose/schedule of bortezomib is achieved for clinical use, it would not be necessary in routine clinical practice to use the bioassay to confirm proteasome inhibition in patients treated with bortezomib.

The initial phase I and pharmacodynamic study tested a schedule of bortezomib given as 4 once-weekly doses followed by a 14-day rest period (85). The *ex vivo* assay served as a guide for dose escalation. The maximum pharmacodynamic effect of bortezomib on proteasome activity was seen at 1-h postdose. Consistent with the reversible inhibition of the proteasome caused by bortezomib, the baseline proteasome activity in whole blood completely returned to original levels in 48-72 h following bortezomib dosing (88). This reversibility is important to have a therapeutic index for bortezomib since tumor cells are more sensitive than normal cells to proteasome inhibition but chronic inhibition would be intolerable to the host. In this trial, mean proteasome inhibition of 75% was achieved at the 1.45 and 1.6 mg/m^2 weekly dose levels, while no dose-limiting toxicities occurred. On repeated dosing, no evidence of tolerance to blood proteasome inhibition or tachyphylaxis was observed. Adverse events commonly reported in this trial, related or not to bortezomib, included fatigue, constipation, nausea, vomiting, fever without neutropenia, but were not dose-limiting.

While the once-weekly bortezomib schedule might be of interest for further trials, particularly in combination with weekly chemotherapy schedules, this study was quickly followed by other phase I trials testing a more frequent (twice-weekly) administration of bortezomib that, consistent with the preclinical data (Table II), was thought to potentially result in improved activity. In these trials, twice-weekly administration of bortezomib was feasible and tolerable in humans at doses that achieved a high degree of proteasome inhibition (~60-70%) (82-84, 86). When the results of proteasome inhibition obtained in the *ex vivo* proteasome assay from the various phase I studies were combined, the dose that resulted in proteasome inhibition of 80% was estimated to be 1.96 mg/m^2 (89). This corresponds to the highest inhibition that could be safely achieved in animal models (25). In tumor biopsies performed in a limited number of patients, the degree of proteasome inhibition measured was consistent with the findings in matched blood samples (84).

At doses of 1.0 mg/m^2 and higher, some patients experienced low-grade fever and/or fatigue after several cycles. At a recommended dose of 1.3 mg/m^2 twice weekly in a heavily pretreated solid tumor population, the mean proteasome inhibition was 65% (82). However, it is possible that in a less pretreated population a higher dose might be tolerated. In fact, doses in the range of 1.0-1.5 mg/m^2 twice weekly are planned in ongoing phase I/II studies in less heavily pretreated patients. Other adverse events that have been reported are thrombocytopenia (not dose-limiting) and diarrhea. Self-limited diarrhea is a side effect predicted from preclinical studies and can be prevented by prophylactic loperamide treatment. Dose-limiting toxicity seen in the phase I setting has been peripheral neuropathy. However, most of the patients experiencing this event previously had a neuropathy due to pretreatment with neurotoxic chemotherapeutic agents (mainly platinum analogs). The possibility of this side

Table III: Current or planned bortezomib trials (adapted from ref. 90).

Single-agent studies		
Phase I	Advanced malignancies or B-cell lymphoproliferative disorders	
	Glioma	
	Leukemia, myelodysplastic disorders, myelogenous leukemia	
	Pediatric solid tumors	
Phase II	Chronic lymphocytic leukemia	
	Chronic myelogenous leukemia	
	Low-grade lymphoproliferative disorders	
	Malignant melanoma	
	Metastatic breast cancer	
	Metastatic neuroendocrine tumors	
	Metastatic renal cell carcinoma	
	Multiple myeloma	
	Ovarian cancer	
	Sarcoma	
Phase III	Multiple myeloma	
Combination studies		
Phase I	Advanced solid tumors	+carboplatin and etoposide
		+irinotecan
		+doxorubicin
		+gemcitabine
		+leucovorin and 5-flourouracil
		+liposomal doxorubicin
		+paclitaxel and carboplatin
	Breast cancer	+docetaxel
	Head and neck squamous cell carcinoma	+radiation
	Non-small cell lung cancer	+docetaxel
	Ovarian cancer	+carboplatin
Phase I/II	Androgen-independent prostate carcinoma	+docetaxel
Phase III	Pancreatic cancer	

effect occurring is being closely monitored in the ongoing trials but. However, to date, in the absence of extensive prior chemotherapy or known clinical peripheral neuropathy, neurotoxicity does not seem to be particularly troublesome. Skin rashes have been reported, although infrequently. In general, the primary adverse effects associated with bortezomib treatment are similar to those predicted from toxicological studies in rodents and primates (25). Interestingly, hematological toxicities have not been dose-limiting, therefore indicating good prospects for the combination of bortezomib with myelosuppressive chemotherapeutic agents.

Although the assessment of clinical activity was not a major endpoint in these phase I trials, evidence of antitumor activity was seen against a variety of tumor types, including myeloma, melanoma, lymphoma, as well as prostate, kidney, head and neck and lung cancers. In conclusion, approximately 200 patients have been treated in phase I trials with bortezomib as a single agent, and treatment has generally been well tolerated at doses and schedules sufficient to achieve a desired degree of proteasome inhibition. Encouraging activity has been observed in both solid tumors and hematological malignancies. These data served as the basis for the design and conducting of disease-directed and phase II clinical trials of bortezomib and also to conduct studies of bortezomib in combination with chemotherapy.

2) Phase II trials

A series of clinical trials were initiated in 2001 to study the activity of bortezomib as a single agent in a variety of human malignancies (90, 91) (Table III). The preliminary results of some of these studies are reviewed below.

As mentioned earlier, multiple myeloma (MM) represents an especially attractive target for bortezomib based mainly on the role of NF-κB and IL-6 in this malignancy and the evidence of potent activity of bortezomib in preclinical myeloma models and in early phase I trials. In a phase II study in patients with relapsed/refractory MM, patients received bortezomib at 1.3 mg/m^2 on days 1, 4, 8 and 11 of a 21-day cycle (92). This study accrued 200 patients in two cohorts. In a preliminary analysis of cohort 1 that consisted of a heavily pretreated population (median number of prior therapies 5; range 2-14), with progression to latest therapy, 85% either responded or were stabilized after only 2 cycles of bortezomib. Complete responses were also observed. The promising activity of bortezomib against MM in this trial has led to the design

of a large-scale multinational phase III trial of bortezomib in this disease.

Another disease-specific study was conducted in prostate cancer, based partly on the role of NF-κB in the progression of prostate cancer (93). Since bortezomib inhibits NF-κB activation which is required for IL-6 production, this study, in addition to proteasome inhibition assays, incorporated serum IL-6 measurements as a surrogate marker of NF-κB inhibition. In this dose escalation trial, 41 patients with metastatic androgen-independent prostate cancer were treated with bortezomib administered weekly x 4 every 6 wks over 14 dose levels (0.13-1.6 mg/m^2). Some patients in this study experienced a dose-dependent decline in serum IL-6 concentration and PSA slope. The degree of decline in IL-6 was greater in patients who achieved a level of proteasome inhibition of at least 70% in peripheral blood (dose levels 1.32-16 mg/m^2) compared to those with a level of proteasome inhibition of 45-55% (dose levels 0.75-1.21 mg/m^2). These results further supported bortezomib as a potentially active agent in prostate cancer and reinforced the hypothesis that its action may be mediated, at least in part, through the inhibition of NF-κB.

Bortezomib has been also evaluated in patients with metastatic neuroendocrine tumors that are characterized by the lack of active therapies (94). Bortezomib was given at a dose of 1.5 mg/m^2 twice a week for 2 weeks followed by 1 week of rest. At the time of reporting, 8 of the 14 patients enrolled were evaluable, 62% of whom had stable disease at 12 weeks. In this trial, the reported grade 3 adverse events were peripheral neuropathy (n=3), transient thrombocytopenia (n=3), reversible ileus (n=3), neutropenia (n=2), fatigue (n=1), conjunctivitis (n=1) and hypertension/atrial flutter (n=1). While most of these adverse events were expected, ileus was not. The 3 patients who experienced ileus had prior laparotomies for bowel carcinoid. Ileus resolved within 24 h without surgery. This observation emphasizes the need to evaluate this potential side effect and the need for caution in future clinical trials combining bortezomib with chemotherapeutic agents with similar gastrointestinal toxicity.

Additional trials are ongoing in many other malignancies, such as low-grade lymphoproliferative diseases, chronic myelogenous leukemia, mantle cell lymphoma, breast cancer, ovarian cancer, non-small cell lung cancer, kidney cancer, sarcomas, gliomas and pediatric tumors (Table III) (80, 90, 91, 95). The results of these trials are eagerly awaited.

Bortezomib in combination with chemotherapy

Preclinical studies

Given the novel mechanisms by which proteasome inhibitors induce apoptosis, it was hoped that they would prove particularly effective when used in combination with standard chemotherapy or radiation. Indeed, proteasome inhibitors appeared to enhance the effects of conventional tumoricidal agents in a number of cancer models. The use of bortezomib in combination with 5-fluorouracil, cisplatin, paclitaxel, docetaxel, doxorubicin, CPT-11 and gemcitabine has been investigated, where it appeared to have an enhanced antiproliferative effect and to inhibit the formation of metastases (96). The ability of bortezomib to act synergistically with other tumoricidal agents has been demonstrated in mice bearing a number of s.c. grafted cancers, including pancreatic (12, 76), colorectal (47, 77), ovarian (96), breast (78) and prostate tumors (80, 81).

A major mechanism underlying the potentiation of chemotherapy-induced cytotoxicity by bortezomib seems to be mediated by NF-κB. NF-κB expression in cancer cells may be activated by standard chemotherapeutic agents (97). This activation would result in antiapoptotic effects that would limit the cytotoxicity of the chemotherapeutic agents, since chemotherapy-induced NF-κB activation is viewed as a defensive, antiapoptotic response to the drugs. In fact, inhibition of NF-κB has been shown to sensitize transformed cells to tumor necrosis factor-α and various chemotherapeutic agents (98). A direct role of NF-κB in this phenomenon has been strongly supported by using a gene therapy approach with recombinant adenovirus-mediated transfer of a modified form of IκBα (99). This resulted in significant augmentation of chemosensitivity and enhanced induction of apoptosis in a xenograft tumor model in response to chemotherapy treatment. While this study suggested that NF-κB may represent an important molecular target for the purpose of enhancing the sensitivity of certain cancer cells to apoptotic stimuli, the use of gene therapy to deliver NF-κB inhibitors systemically is limited. The use of a systemically active proteasome inhibitor (*i.e.*, bortezomib) may overcome this limitation and provide effective NF-κB blockade (47).

Several preclinical studies indicated that bortezomib can prevent chemotherapy-induced NF-κB activation and result in enhanced apoptosis both *in vitro* and *in vivo* when a combined treatment of bortezomib and chemotherapy is given in comparison with each agent alone. For instance, bortezomib blocked the activation of NF-κB in human breast cancer cells and enhanced the tumoricidal effect of doxorubicin in mice bearing human breast cancer xenografts (78). Similarly, in human colorectal cancer cells bortezomib inhibited activation of NF-κB induced by SN-38 (the active metabolite of the topoisomerase I inhibitor, CPT-11) and resulted in a significantly higher level of growth inhibition compared to treatment with bortezomib alone or SN-38 alone (47). Combination therapy resulted in a dramatic decrease in tumor size and the level of apoptosis was 80-90% compared with the control groups. These and similar results from other studies suggest that the enhanced anticancer effects observed when bortezomib is combined with chemotherapy are attributable, at least in part, to inhibition of inducible NF-κB activation.

However, potentially other cell-cycle regulatory processes and apoptotic response mechanisms impacted by proteasome inhibition play a contributing role. For

instance, p21, p27 and p53 are stabilized by chemotherapy or bortezomib treatment, suggesting that stabilization of these key cell-cycle regulatory proteins may be involved in the antitumor effects of these molecules when used alone or in combination. For instance, a sequential treatment of docetaxel followed by bortezomib resulted in enhanced accumulation of p27 and absence of the anti-apoptotic bcl-2 protein in lung cancer cells (48). It is also possible that proteasome inhibitors and some standard chemotherapeutic agents result in enhanced tumoricidal effects simply by acting against different cellular targets. In fact, it has been shown for a variety of standard chemotherapeutic agents that they do not affect proteasome activity (96).

In addition to the potentiation of chemotherapy activity, bortezomib has been found to be capable of improving the sensitivity of tumor cells to several other anticancer approaches, including external beam radiation (100), radioimmunotherapy (100), the experimental agent TNF-related apoptosis-inducing ligand (TRAIL) (100, 101) and the Hsp90 antagonist geldanamycin (102). It is anticipated that data with many other biological anticancer agents plus bortezomib will soon be available.

Clinical trials of bortezomib in combination with chemotherapy

As reviewed above, there is compelling preclinical evidence to support the development of bortezomib in combination with standard chemotherapeutic agents. Furthermore, in the phase I trials of bortezomib as a single agent, the adverse events did not generally overlap with the majority of chemotherapeutic agents, especially with regard to myelotoxicity. These studies also showed that bortezomib had antitumor activity against many human malignancies. As a result, many trials of bortezomib and chemotherapy are ongoing or planned (Table III). Preliminary results of some of these studies were presented at the American Society of Oncology annual meeting in May 2002 and are discussed below (79, 103, 104). These phase I studies had as their primary objective to explore the feasibility of combined therapies using standard chemotherapeutic agents and bortezomib, and specifically, to determine the dose-limiting toxicity (DLT) and the maximum tolerated dose (MTD) of the combinations tested.

In a phase I dose-escalation trial of combined therapy with bortezomib and gemcitabine (104), bortezomib was administered on days 1, 4, 8 and 11 at either 1.0 or 1.3 mg/m^2, and gemcitabine (500, 800 or 1000 mg/m^2) on days 1 and 8 every 21 days in sequential cohorts of patients. In patients dosed with bortezomib 1.0 mg/m^2 and escalating doses of gemcitabine, the MTD was established at 1000 mg/m^2 of gemcitabine, with no DLTs. Disease stabilization was observed in pancreatic cancer patients. Based on these results and in the preclinical synergy between gemcitabine and bortezomib in pancreatic cancer, a phase III trial using bortezomib in patients with advanced pancreatic cancer is being planned.

Another phase I dose-escalation study combined bortezomib and irinotecan (103). Bortezomib was administered on days 1, 4, 8 and 11 at either 1.0 or 1.3 mg/m^2 as in the above study, and irinotecan was administered at escalating doses (50, 75, 100 and 125 mg/m^2). At the time of reporting, 11 patients were enrolled and no DLTs occurred at the first 3 dose levels. A patient previously treated with vinorelbine and paclitaxel experienced grade 3 neuropathy. The trial continues to accrue patients.

A combination of doxorubicin and bortezomib is also being tested in the clinic. In this phase I trial, bortezomib is administered on days 1, 4, 8 and 11 every 21 days, and doxorubicin is administered after bortezomib on days 1 and 8 (79). Dose levels evaluated include 1.0 mg/m^2 bortezomib/15 mg/m^2 doxorubicin; 1.3 mg/m^2 bortezomib/15 mg/m^2 doxorubicin and 1.3 mg/m^2 bortezomib/20 mg/m^2 doxorubicin. At the time of reporting, no grade 3 or 4 drug-related adverse events occurred. A response in lung metastasis was seen in a patient with androgen-independent prostate cancer.

Another trial combined bortezomib with 5-fluorouracil/leucovorin (5-FU/LV). In this study escalating doses of bortezomib were given twice weekly, followed by a fixed dose of 5-FU 500 mg/m^2/LV 20 mg/m^2 weekly for 4 weeks with 2 weeks of rest (105). This trial included heavily pretreated patients who were refractory to 5-FU. Out of 3 patients treated at the highest dose level tested, 2 had dose-limiting toxicities (1 grade 3 diarrhea and 1 grade 3 abdominal pain). This cohort was being expanded to better assess the tolerance of the combination. The anticipated MTD of bortezomib was 1.0 mg/m^2. Notably, of 10 patients evaluable for response, 6 had stable disease and 1 a partial response to the combination.

These initial phase I combination trials are complemented by many other ongoing clinical studies (Table III). Among them, at Hospital Clinic we are participating in a multicenter dose-escalation phase I trial sponsored by Millennium Pharmaceuticals of bortezomib in combination with docetaxel in advanced breast cancer. There is strong rationale to target breast cancer with bortezomib (see ref. 95 for a recent review) as well as for the combination of bortezomib with docetaxel (48). A similarly designed trial of bortezomib plus docetaxel is ongoing in lung cancer. As yet, no data on these trials are available. Combination trials in other cancers and using other drug combinations are also under way (91). Finally, a trial of bortezomib plus radiation therapy is also in progress in patients with recurrent or metastatic squamous cell carcinoma of the head and neck (91).

In summary, the early results of combination trials of bortezomib with standard chemotherapeutic agents are promising. In clinical trials, bortezomib has been administered at doses capable of achieving a good level of proteasome inhibition when combined with chemotherapy. No clear overlapping toxicities have been observed to date and there has been preliminary evidence of antitumor activity by many of the combinations tested in

chemorefractory patients. Disease-directed phase II and III studies of bortezomib and chemotherapy will follow after the completion of these initial trials and should serve to define the role of bortezomib in the treatment of malignancies in the near future.

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