Proteasome Inhibitors in Cancer Therapy: Lessons from the First Decade

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Abstract
The ubiquitin-proteasome pathway is involved in intracellular protein turnover, and its function is crucial to cellular homeostasis. First synthesized as probes of proteolytic processes, proteasome inhibitors began to be thought of as potential drug candidates when they were found to induce programmed cell death preferentially in transformed cells. They made their first leap into the clinic to be tested as therapeutic agents 10 years ago, and since then, great strides have been made in defining their mechanisms of action, their clinical efficacy and toxicity, and some of their limitations in the form of resistance pathways. Validation of the ubiquitin-proteasome pathway as a target for cancer therapy has come in the form of approvals of the first such inhibitor, bortezomib, for relapsed/refractory multiple myeloma and mantle cell lymphoma, for which this agent has become a standard of care. Lessons learned from this first-in-class agent are now being applied to the development of a new generation of proteasome inhibitors that hold the promise of efficacy in bortezomib-resistant disease and possibly in a broader spectrum of diseases. This saga provides a salient example of the promise of translational medicine and a paradigm by which other agents may be successfully brought from the bench to the bedside.

Intracellular protein degradation occurs predominantly through the proteasome, which is the final common effector for ubiquitin-dependent and most ubiquitin-independent proteolysis (refs. 1, 2; Fig. 1). In eukaryotic cells, substrate proteins are subjected to polyubiquitination by the ubiquitin-conjugating system. Drs. Aaron Chiechanover, Avram Hershko, and Irwin Rose were awarded the 2004 Nobel Prize in Chemistry for their contributions to the elucidation of this portion of the ubiquitin-proteasome pathway. Polyubiquitinated proteins are then subject to proteolysis through the proteasome, which contains up to five different proteolytic activities (3) promoting digestion of proteins into oligopeptides. Whereas the 26S proteasome is probably the workhorse of intracellular proteolysis, contributions may be made by the immunoproteasome (Fig. 2), and the 20S core may play a role as well, such as in p21 (4, 5) and p27 (6) turnover.

Proteasome inhibitors were first synthesized as tools to probe the function and specificity of this particle's proteolytic activities (7, 8). Most synthetic inhibitors (Fig. 3) rely on a peptide base, which mimics a protein substrate, attached to a COOH terminal “warhead” (Fig. 4). Notable warheads include boronic acids (9), such as bortezomib (10), and epoxyketones (11), such as carfilzomib (12–14). A variety of natural products also inhibit the proteasome that are not peptide-based, most notably lactacystin (15), that is related to NPI-0052, or salinosporamide A, another inhibitor in clinical trials (16, 17).

Antitumor Effects of Proteasome Inhibitors

The possibility that proteasome inhibitors could be drug candidates was considered after studies showed that they induced apoptosis in leukemic cell lines (18, 19), including chemotherapy-resistant and radiation-resistant chronic lymphocytic leukemia cells (20). This was bolstered by findings that proteasome inhibitors induced apoptosis preferentially in transformed cells (20, 21) and were active against an in vivo non–Hodgkin’s lymphoma model (21). One of the early mechanisms of action attributed to proteasome inhibitors was that they repressed nuclear factor-κB (NF-κB) signaling by stabilizing IκB, which binds NF-κB and prevents its nuclear translocation (22). Given the role of NF-κB in angiogenesis, cell invasion, oncogenesis, proliferation, and suppression of apoptosis, NF-κB inhibition was already an attractive approach to cancer therapy. Moreover, NF-κB inhibition induced chemosensitization because many chemotherapeutics activated antiapoptotic NF-κB functions (23–25). An especially strong rationale for targeting NF-κB had been worked out in multiple myeloma (MM). Adhesion of myeloma cells to bone marrow stroma induced NF-κB–dependent production of the antiapoptotic and growth factor interleukin-6 (26). Later studies
documented the efficacy of proteasome inhibition against preclinical models as a single approach (27) and in chemosensitization and overcoming resistance (27–30) with predominantly synergistic effects when bortezomib was combined with other agents.

Proteasome inhibitors are targeted because they are very potent and selective for the proteasome. Due to their effect on proteolysis of a wide array of cellular proteins, however, they share characteristics with general cytotoxic agents, such as vinflunine, satraplatin, aurora kinase inhibitors, and epothilones, as discussed in the accompanying reviews and overview (31–35). In that light, proteasome inhibitors have a number of important mechanisms of action beyond their effects on NF-κB that have been validated preclinically in cell line models (36, 37). By interfering with timely degradation of cyclins and other cell cycle regulatory proteins, proteasome inhibitors induce cell cycle arrest. Through their ability to stabilize proapoptotic proteins, such as p53 and Bax, while reducing levels of some antiapoptotic proteins, such as Bcl-2, they induce a proapoptotic state. Bortezomib-mediated programmed cell death is accompanied by c-Jun-NH2 terminal kinase induction, generation of reactive oxygen species, transmembrane mitochondrial potential gradient dissipation, release of proapoptotic mitochondrial proteins, such as cytochrome c, and activation of intrinsic, caspase-9–mediated and extrinsic, caspase-8–mediated apoptosis. Other mechanisms include induction of aggresome formation, endoplasmic reticulum stress, and the unfolded protein response (38–41), with the latter possibly having special relevance for MM cells given their large basal load of immunoglobulin protein substrates. Readers interested in more detailed coverage of the mechanisms of action of proteasome inhibitors are referred to several excellent reviews (42, 43).

Interestingly, the pleiotropic effects of proteasome inhibitors also result in activation of antiapoptotic pathways that may suppress antitumor activity and could be targets for chemosensitization to bortezomib. Heat shock response proteins have been some of the best characterized, including HSP-27 (44), HSP-70 (45, 46), and HSP-90 (47). Other examples include stress response proteins like mitogen-activated protein kinase phosphatase-1 (48–50) and protein kinase B/Akt (51).

**Proteasome Inhibitors in the Clinic as Single Agents**

Building on this solid preclinical rationale, a number of phase I studies have documented that bortezomib can be safely given on a variety of schedules (52–57). Early indications of activity were seen in non–small cell lung (52) and androgen-independent prostate carcinoma (54), as well as MM and mantle cell and follicular non–Hodgkin’s lymphoma (53). The most dramatic findings were in myeloma, in which all nine patients all showed some clinical benefit, including one durable complete remission. Pharmacodynamic studies showed a dose-dependent 20S proteasome inhibition in peripheral blood mononuclear cells and in limited studies of tumor tissue. However, a correlation between peripheral blood mononuclear cell proteasome inhibition and response could not be established in these small trials, which were not designed with the sample size necessary for such an analysis. Pharmacokinetic studies showed rapid bortezomib plasma clearance and tissue distribution, with an initial t1/2 of 0.22 to 0.46 hours followed by...
by a more gradual terminal elimination half-life, with $t_{1/2\beta}$ of
>10 hours and a large volume of distribution of >500 L. Activity
against MM was confirmed with a phase II trial (58) that
showed a 27% overall response rate (partial response + complete
remission) in heavily pretreated patients, who received
what has become the most common dose and schedule, 1.3
mg/m² as an i.v. bolus on days 1, 4, 8, and 11 of every 21-day
cycle. Further follow-up (59) determined that the median
duration of response was 12.7 months, the median time to
progression was 7 months, and the median overall survival
was 17.0 months. A subsequent phase III randomized trial
(60, 61) comparing dexamethasone with bortezomib showed
that the latter induced a better overall response rate (43% for
bortezomib versus 18% for dexamethasone), a better re-
response quality, as well as a longer median time to progression
(6.22 months versus 3.49 months, respectively) and overall
survival (29.3 months versus 23.7 months, respectively).
Together, these studies led to the approval of bortezomib for
relapsed/refractory myeloma in patients who have progressed
after at least one prior regimen.

In non-Hodgkin’s lymphoma, several phase II studies
(62–64) confirmed activity in follicular, mantle cell, and
marginal zone lymphoma. Most recently, a multicenter pivotal
trial (65) determined that the overall response rate in relapsed
mantle cell lymphoma was 33%, including 8% complete
remission/unconfirmed complete remission, with a median
duration of response of 9.2 months and time to progression
of 6.2 months, leading to approval of bortezomib for this
indication. Activity has also been described in other B-cell pro-
cessions, including Waldenström’s macroglobulinemia (66–69)
and amyloidosis (70).

When bortezomib was being developed as a drug candidate,
there was great concern that, because of the proteasome’s vital
role in cellular homeostasis, it could not be inhibited without
dire consequences. Fortunately, an acceptable therapeutic index
has been documented, but patients do face the risk of some
toxicities. During phase I studies, dose-limiting toxicities in-
cluded diarrhea, fatigue, fluid retention, hypokalemia, hypo-
natremia, hypotension, malaise, nausea, orthostasis, sensory
neuropathy, and thrombocytopenia. In the phase II trial of MM
patients, adverse events reported in at least 10% included
anemia, anorexia, constipation, dehydration, diarrhea, dizzi-
ness, fatigue, headache, limb pain, nausea, neutropenia,
peripheral neuropathy, pyrexia, rash, thrombocytopenia, vomit-
ing, and weakness. Subsequent studies have better character-
ized thrombocytopenia (71) and neuropathy (72), which
probably must limit dosing in the clinic. These have elucidated
some of the risk factors involved in these transient, reversible
effects, but a better understanding of the underlying mecha-
nisms would be of benefit, as would the identification of
biomarkers to predict efficacy or toxicity.

## Proteasome Inhibition as a Means to Overcome Resistance and Induce Chemosensitization

Proteasome inhibition is a rational therapeutic approach
both by itself and as a means to induce chemosensitization and
overcome chemoresistance. As noted earlier, many cytotoxic
agents activate the antiapoptotic NF-$\kappa$B pathway, and blockade
of this induction by proteasome inhibition enhanced their
antitumor efficacy (28, 73). In addition, several strategies by
which tumor cells survive the effects of chemotherapy can be
similarly abrogated. Overexpression of Bcl-2 is one such
mechanism, but proteasome inhibitors induce Bcl-2 phosphor-
ylation and cleavage into proapoptotic fragments (74). Selec-
tion of cells overexpressing P-glycoprotein is another,
but because proteasome function is needed for P-glycopro-
tein maturation when the proteasome is inhibited, inactive
P-glycoprotein isoforms accumulate that cannot remove
chemotherapeutic agents from cancer cells (75, 76).

Using these rationales, bortezomib has been combined with
a variety of chemotherapeutics, including carboplatin (77),
doxetaxel (78), irinotecan (79), melphalan (80), pegylated
liposomal doxorubicin (81), and thalidomide (82), among
others. Bortezomib has also been incorporated into more
complex regimens, such as paclitaxel and carboplatin (83) and
gemcitabine and cisplatin (84). From these studies, it seems
possible to conclude that bortezomib has generally been
successfully combined with other agents without significantly
increased toxicity, and without the need for large dose
adjustments. In several cases, these combinations have shown
evidence of enhanced activity, most notably with the bortezo-
mb/pegylated liposomal doxorubicin regimen, in which
myeloma patients showed a 73% overall response rate (81)
and an excellent response duration and overall survival (85).
These findings led to a randomized trial comparing bortezomib

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**Fig. 2.** Proteasome isoforms. The 26S or constitutive proteasome is found in most
tissues and consists of a 20S core particle with two 19S cap structures. Each
outer ring contains seven nonidentical $\alpha$ subunits (green), which are predominantly
structural, whereas the two inner rings contain seven nonidentical $\beta$ subunits
(red), some of which encode proteases (Fig. 4). Together, the four rings are stacked
one on top of each other around a central channel where proteolysis occurs.
A second proteasome isoform is the immunoproteasome, which is expressed in a
more tissue-specific manner and can also be induced by some cytokines, such as
$\gamma$-IFN. Immunoproteasomes contain two different regulatory cap structures
known as 11S particles at each end. Also, six of the $\beta$ subunits from the 26S
proteasome are replaced by two copies each of three new proteases (purple)
that slightly change the substrate preferences and cleavage patterns of the
immunoproteasome. These modifications may allow the immunoproteasome to
more efficiently generate antigenic peptides for presentation in the context of MHC
class I molecules. Studies of the relative contributions of these two isoforms to
intracellular proteolysis in cells which contain both isoforms have not been done.
Cells may also contain a hybrid proteasome with one 11S and one 19S regulatory
particle around a 20S core. This figure has been modified from Voorhees et al. (36)
with permission.
with bortezomib/pegylated liposomal doxorubicin in relapsed and/or refractory myeloma (86), which found the combination induced a superior time to progression compared with bortezomib (9.3 months versus 6.5 months, respectively). Also, combination therapy improved the duration of response (10.2 months versus 7.0 months, respectively) and progression-free survival (9.0 months versus 6.5 months, respectively) and showed a trend for a superior overall survival. As a result, the bortezomib/pegylated liposomal doxorubicin combination has been approved for bortezomib-naive patients who have received at least one prior regimen and is recommended by the National Comprehensive Cancer Network (87). A number of other combinations are being currently compared with single-agent bortezomib, and it seems likely that several will outperform bortezomib.

Further development of bortezomib in MM is now focusing on its incorporation into therapy of previously untreated patients. Encouraging results have been seen using bortezomib with dexamethasone (88, 89), and early results of an ongoing randomized study comparing this with infusional vincristine, doxorubicin, and dexamethasone suggest the former is superior (90). Another induction regimen combining bortezomib with melphalan and prednisone (MP; ref. 91) showed an impressive overall response rate and durability, even in patients with high-risk cytogenetics. This led to an international randomized study of MP versus bortezomib/MP, which showed a superior overall response rate (82% for bortezomib/MP versus 50% for MP) and a better response quality (35% immunofixation-negative complete remission with bortezomib/MP versus 5% for MP). Most notably, time to progression was improved from 16.6 to 24.0 months by addition of bortezomib to MP, as was overall survival at 2 years, which improved from 69% for MP to 82.6% with bortezomib/MP (92). As a result, it is likely that bortezomib/MP will become one of the standards of care for initial therapy of older patients with myeloma, as well as patients who may not be transplant candidates. Other attractive regimens include bortezomib with thalidomide and dexamethasone (93) and bortezomib with combination chemotherapy (94). Toxicities in the up-front setting have been comparable with those seen in relapsed/refractory patients, and stem cell mobilizations have been possible without significant compromise in stem cell yields.

Fig. 3. Chemical structures of clinically relevant proteasome inhibitors. Structures are presented of the proteasome inhibitors that are currently either approved for clinical use or in clinical trials, including bortezomib (A), carfilzomib (B), and salinosporamide A (C).
Resistance to Proteasome Inhibitors

Bortezomib has documented activity in a number of hematologic malignancies, but despite encouraging preclinical data, studies in solid tumors have yielded disappointing results. This contrast is as yet unexplained, but a number of hypotheses can be advanced to account for the resistance of solid tumors. Most of the preclinical modeling of proteasome inhibitor–based regimens used chemotherapy-naive tumor cells or xenografts. Subsequent trials targeted chemotherapy-exposed populations, including some patients who had already received the chemotherapeutic with which bortezomib was then paired. A more fruitful approach may be to randomize chemotherapy-naive patients to an active drug or the same agent with a proteasome inhibitor. Another issue of trial design may revolve around the appropriate sequencing of bortezomib vis-à-vis the other agent(s) with which it is paired. One recent preclinical study showed that synergy between bortezomib and cytarabine was sequence-dependent (95), whereas another found that bortezomib given before docetaxel inhibited the latter’s activity in a p21-dependent fashion (96), possibly because cells were arrested at G1-S before the point at which docetaxel was maximally active. Because such considerations were not factored into the design of earlier trials, it is possible that dosing schedules prepared with such findings in mind could result in enhanced efficacy.

From a molecular perspective, it is interesting to note that mantle cell (97) and a substantial proportion of myelomas (98) are driven by overexpression of cyclin D isoforms. Because proteasome inhibitors induce p21 and p27 accumulation, which blocks the cell cycle distal to the effects of cyclin D, it may be that bortezomib works best against cyclin D–driven tumors. Whereas a fraction of some solid tumors are cyclin D–dependent, such as breast cancer (99), no clinical trials have specifically targeted patients with these molecular lesions (100). Indeed, there is preclinical evidence that bortezomib may be more active against cyclin D1–expressing breast cancer models (101). Also of interest are recent findings that activation of the noncanonical NF-κB pathway (102, 103) predicted for the highest response rate to bortezomib in myeloma, providing clinical validation of the importance of NF-κB in the mechanism of action of bortezomib. In that the activation status of this pathway in solid tumor patients has not been as well studied, enriching for this population could also be of benefit. Indeed, some solid tumor models have been described in which bortezomib may paradoxically activate NF-κB (104), possibly abrogating one of its major mechanisms of action. Finally, proteasome activity may itself be a determinant of resistance to bortezomib and related agents. Interestingly, chronic lymphocytic leukemia cells have been found to have increased proteasome chymotrypsin-like activity (105), as is the case in a number of tumor types, and this was felt to underlie their sensitivity to proteasome inhibitors. Contrary to this hypothesis, however, chronic lymphocytic leukemia patients did not benefit from bortezomib (106), and recent studies in resistant cell lines showed that they have increased proteasome activity (107), suggesting that tumor types with compromised ubiquitin-proteasome pathway function may be most sensitive. Thus, it may be that with appropriate selection of patients with disease whose molecular determinants predict for responsiveness to bortezomib, more positive findings in solid tumor populations may be obtained.

In addition to primary resistance, secondary or acquired resistance is also emerging as an area of interest. Individual patients can achieve excellent responses on retreatment with bortezomib (108), but in larger studies its ability to reinduce a response in patients with previously sensitive disease is 31% to 60% (109, 110), with the higher rate reflecting the addition of other agents. Acquired resistance to peptide-aldehyde proteasome inhibitors has been ascribed to efflux through P-glycoprotein (111) and metabolism by an aldo-keto reductase (112). The former may have some applicability to bortezomib as P-glycoprotein blockade enhanced bortezomib sensitivity in models of leukemia (113) and Ewing’s sarcoma (114). Another approach to overcome acquired resistance may be to block 3-hydroxy-3-methyl-glutaryl-CoA reductase (115), although the mechanism involved has not been defined. Further studies will be needed with in vitro preclinical models of bortezomib resistance, ideally validated with clinical samples from patients with acquired resistance, to better delineate the relevant mechanisms in vivo and test targeted approaches to abrogate these pathways.

Next Generation Proteasome Inhibitors

With the validation of the proteasome as a target for cancer therapy, interest has focused on the possibility that other
inhibitors could offer some advantages. Two second-generation agents have entered phase I trials: NPI-0052 (salinosporamide A) and carfilzomib (formerly PR-171). Unlike bortezomib, which binds the proteasome in a slowly reversible manner, NPI-0052 and carfilzomib bind irreversibly, abrogating one mechanism of recovery from proteasome inhibition, namely release of the target by the drug. They both induce depolarization of the transmitochondrial membrane potential (Fig. 5) and activate caspase-8–mediated apoptosis, whereas carfilzomib also activates caspase-9. Preclinical studies have shown that both (12, 17) at least partially overcome bortezomib resistance in vitro. Moreover, in a number of models, including MM (12, 17) and chronic lymphocytic leukemia (116), these inhibitors have shown enhanced potency compared with bortezomib, suggesting they may have a broader spectrum of activity. Early results from phase I studies of carfilzomib indicate that it is well tolerated, even on a dose-intense schedule, and may have less neurotoxicity than bortezomib (117, 118). Evidence of antitumor activity is being seen in MM and Waldenström’s macroglobulinemia, including in myeloma patients with previously bortezomib-refractory disease, and phase II studies are planned. Another interesting target may be the immunoproteasome (119), whose expression may be more tissue-restricted than the constitutive proteasome. All of the currently available inhibitors target both the constitutive and immunoproteasome isoforms, but the identification of specific immunoproteasome inhibitors (120, 121) may allow for further improvements in the therapeutic index of these drugs. In that the immunoproteasome is expressed predominantly in hematopoietic tissues, it is possible that such agents could act without incurring neurotoxicity or gastrointestinal effects, among others, because those tissues express much lower levels of immunoproteasome subunits.

Conclusions

The first clinical trials evaluating the therapeutic potential of proteasome inhibition began not quite 10 years ago, and since then, remarkable progress has been made. Bortezomib, the first such inhibitor in the clinic, has been approved for relapsed/refractory myeloma and mantle cell lymphoma. Trials evaluating bortezomib-based combination therapies have supported the possibility that proteasome inhibitors can induce chemosensitization and overcome chemoresistance. Indeed, the first bortezomib-based regimen has just been approved for clinical use against myeloma, and other such combinations are likely to achieve a similar status. Studies of bortezomib’s activity in the up-front setting are showing encouraging results, and it is likely that bortezomib-based therapies will gain additional approvals in these and a number of other indications. In that preclinical studies identified non–Hodgkin’s lymphoma and MM as being particularly susceptible to proteasome inhibitor–based therapies, the validation of the ubiquitin-proteasome pathway as a target clinically represents a triumph of translational medicine.

Fig. 5. Mitochondrial membrane depolarization due to carfilzomib. RPMI 8226 myeloma cells were treated with vehicle (DMSO) or carfilzomib and then stained with the cationic dye JC-1 (123). In cells with intact mitochondria, JC-1 accumulates inside the mitochondria as aggregates and exhibits a fluorescence emission shift from red (600 nm) to green (525 nm). Mitochondrial depolarization causes a collapse of the mitochondrial membrane, allowing JC-1 to diffuse throughout the cell and exhibit a green fluorescence emission. Thus, viable cells will have mitochondria that fluoresce both red and green, whereas dying or dead cells will have a higher red/green ratio. Vehicle-treated RPMI 8226 (top) show dual fluorescence, whereas carfilzomib-treated cells show loss of red fluorescence indicative of mitochondrial depolarization.
Additional studies are needed, however, to better understand the mechanisms of proteasome inhibitor–mediated toxicities, such as peripheral neuropathy, as well as mechanisms of primary and secondary resistance, for this agent to gain wider applicability. A new generation of proteasome inhibitors are now entering the clinic and may initially represent an effective option for patients who are bortezomib intolerant or whose disease is bortezomib refractory. It seems clear at this time that proteasome inhibitors will be part of our current and future armamentarium against a number of diseases and that we are only beginning to appreciate the full therapeutic potential of targeting this pathway.

References
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