The validation of the ubiquitin-proteasome pathway as a target for therapy of hematological malignancies stands out as one salient example of the ability to translate laboratory-based findings from the bench to the bedside. Preclinical studies showed that proteasome inhibitors had significant activity against models of non-Hodgkin lymphoma and multiple myeloma, and identified some of the relevant mechanisms of action. These led to phase I through III trials of the first clinically available proteasome inhibitor, bortezomib, which confirmed its activity as a single agent in these diseases. Modulation of proteasome function was then found to be a rational approach to achieve both chemosensitization in vitro and in vivo, as well as to overcome chemotherapy resistance. Based on these findings, first-generation bortezomib-based regimens incorporating traditional chemotherapeutics such as alkylating agents, anthracyclines, immunomodulatory agents, or steroids have been evaluated, and many show promise of enhanced clinical anti-tumor efficacy. Further studies of the pro- and anti-apoptotic actions of proteasome inhibitors, and of their effects on gene and protein expression profiles, suggest that novel agents, such as those targeting the heat shock protein pathways, are exciting candidates for incorporation into these combinations. Phase I trials to test these concepts are just beginning, but have already shown some encouraging results. Finally, novel proteasome inhibitors are being developed with unique properties that may also have therapeutic applications. Taken together, these studies demonstrate the power of rational drug design and development to provide novel, effective therapies for patients with hematological malignancies.

Eukaryotic cells perform the vast majority of their regulated proteolysis through the ubiquitin-proteasome pathway (UPP). Protein targets destined for proteolysis are often first labeled with one or more chains of ubiquitin moieties by the ubiquitin conjugating machinery (Figure 1 see Color Figures, page 551). Polyubiquitinlated proteins are then substrates for degradation through the 26S proteasome (Figure 2; see Color Figures, page 551), which contains up to five distinct catalytic activities in its 20S core that cleave after acidic, basic, branched chain, hydrophobic, and small neutral amino acids, thereby generating oligopeptides. This protein degradation is crucial to many important cellular functions, including timely degradation of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors during mitosis, removal of misfolded or mutated proteins, and processing and turnover of transcription factors and other short-lived proteins. The 20S multicatalytic proteinase complex itself is involved in ubiquitin-independent processes such as proteolysis of oxidized proteins, and also forms the core of other proteolytic particles such as the immunoproteasome, which processes antigens for presentation in association with major histocompatibility class I molecules. Proteasome inhibitors were initially synthesized as in vitro probes of the function of its proteolytic activities, and were substrate-based peptide aldehydes that bound to the unique active site threonine found in proteasome subunits. Given the crucial role of the proteasome to the maintenance of normal cell homeostasis, inhibition of this complex at first glance would seem to represent a dangerous approach to consider in the therapy of hematological malignancies. Through the translation of laboratory-based studies into the clinic, however, proteasome inhibition has been established as a rational strategy against multiple myeloma and non-Hodgkin lymphoma (NHL). Moreover, additional understanding of the mechanisms of action of proteasome inhibitors has led to their incorporation into combination regimens based both on standard chemotherapeutics, as well as novel agents.

Step 1: Translation of Single Agent Proteasome Inhibitors into the Clinic

Many chemotherapeutic agents with activity against malignancies work at least in part by activation of apoptosis, or programmed cell death. During studies of monoblastic...
leukemia cells, lactacystin, a naturally occurring proteasome inhibitor, was the first agent in this class noted to induce apoptosis. Additional impetus for the clinical application of such agents was provided by studies of other hematological malignancies, including NHL. These demonstrated that transformed cells were much more sensitive to the pro-apoptotic effects of proteasome inhibitors than comparable non-transformed counterparts, suggesting the possibility of a reasonable therapeutic index. Also, xenograft studies in an NHL model showed that peptide aldehyde proteasome inhibitors were effective in delaying tumor progression and in inducing apoptosis in vivo without any obvious adverse effects. Despite these encouraging results, it became clear that these types of proteasome inhibitors probably could not be applied to the clinic due to their relative lack of potency and specificity. This problem was overcome initially by the synthesis of peptide boronic acids, which were much more powerful and selective than many previously available inhibitors. The most potent of these, PS-341 (now known as bortezomib or Velcade®), was chosen for further study. It was found to have a unique cytotoxicity profile in the National Cancer Institute’s in vitro screen, and to have pro-apoptotic and anti-tumor activities both in vitro and in vivo. Studies of this agent in multiple myeloma using both cell lines and samples directly isolated from patients indicated a great deal of activity against this malignancy. A major mode of action of bortezomib was its ability to block nuclear translocation of nuclear factor kappa B (NF-κB) through the stabilization of its inhibitor, IκB. Blockade of NF-κB led to decreased expression of myeloma cell adherence factors, and interference with adherence-mediated induction of interleukin-6 production by bone marrow stromal cells. PS-341 also interfered with the p44/42 mitogen-activated protein kinase (MAPK) pathway that communicates proliferative signals, and induced accumulation of cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1.

Given the encouraging results in models of hematological malignancies described above, a phase I trial was begun in that patient population. This study demonstrated that bortezomib could be safely administered with a tolerable side effect profile. Dose-limiting toxicities included malaise, fatigue, thrombocytopenia, and electrolyte abnormalities. There was significant activity in multiple myeloma, with all 9 of 9 evaluable patients having some evidence of a clinical benefit, including 1 durable complete remission (CR). Two patients with NHL also had partial responses (PR), including 1 each with follicular and mantle cell lymphoma. To further explore the activity of bortezomib against multiple myeloma, a multicenter phase II trial studied this agent in patients with relapsed disease, of whom 91% had been refractory to their prior therapy. Grade 3 toxicities included thrombocytopenia, fatigue, peripheral neuropathy, and neutropenia. An overall response rate of 35% was reported, including patients with at least a minor response, and 10% of patients achieved either a CR or near-
initially to increase the efficacy of CPT-11 through blockade of NF-κB in a model of colon cancer. Subsequent work showed that this approach was also feasible in several models of multiple myeloma. Proteasome inhibition with bortezomib in combination with other agents was able to enhance chemosensitivity, overcome chemoresistance, and in some cases induce synergistic anti-myeloma effects in vitro. Modulation of proteasome function may also enhance the therapeutic effects of some chemotherapeutics through other pathways, including by directly inducing phosphorylation and cleavage of Bcl-2, by inhibiting maturation of P-glycoprotein, and by suppressing the cell’s DNA damage repair pathways. These and other studies provided a mechanistic basis for, and raised interest in, the possibility of combining bortezomib with other agents commonly used in multiple myeloma, including doxorubicin, immunomodulatory drugs, melphalan, and steroids. Among these many choices, anthracyclines were shown to have the interesting property of enhancing proteasome inhibitor-mediated programmed cell death by downregulating bortezomib’s ability to induce the anti-apoptotic MAPK phosphatase (MKP)-1, a protein involved in some heat shock and stress response pathways. Thus, an especially strong rationale supports the possibility that combinations of proteasome inhibitors with anthracyclines may have enhanced clinical anti-tumor efficacy (Table 1).

Translation of these first generation combination regimens based on proteasome inhibitors into the clinic has moved forward rapidly. In the initial two phase II trials of patients with relapsed or refractory multiple myeloma, addition of dexamethasone to bortezomib was allowed for those who had either progressive disease, or only stable disease after four cycles. Both documented an improvement in the response quality as a result, with up to 33% of patients benefiting from this intervention. The combination of bortezomib and dexamethasone was also evaluated as an initial therapy for previously untreated patients, yielding an overall response rate in excess of 80% with a significant proportion of CRs. Preliminary reports of a phase I/II study of bortezomib and melphalan in the relapsed/refractory setting indicated an overall response rate of 68%, including patients with at least a minor response. Encouragingly, responses were seen even at dose levels well below what would be considered standard for single-agent bortezomib and melphalan, and patients with prior exposure to this alkylating agent responded as well. Thalidomide with bortezomib, without or with the addition of dexamethasone, is also an active regimen, having an overall response rate of 52%, with 17% CRs or near-CRs. Again, responses were seen in patients who had previously progressed on thalidomide-based therapy, though these patients had an inferior survival compared with those who had not received prior thalidomide. Bortezomib with thalidomide and dexamethasone is another very active regimen in the initial therapy of multiple myeloma, associated with an overall response rate of 80%, and 94% in those patients who received bortezomib at higher levels than the commonly used 1.3 mg/m².

Combination regimens based on bortezomib and doxorubicin have also been encouraging. A phase I trial in patients with advanced hematologic malignancies found that bortezomib with pegylated liposomal doxorubicin (Doxil®) was tolerable. Some increase in grade 1-2 toxicity was observed with the combination, but the incidence of most grade 3-4 toxicities was similar to the historical experience with single agent bortezomib. An overall response rate of 73% in patients with relapsed/refractory multiple myeloma was noted, with a CR + near-CR rate of 36%. Interestingly, in a subset of patients previously treated with doxorubicin-based therapies who were either frankly refractory, or had only brief responses, the CR and overall response rate was comparable to that in patients who had initially responded well to doxorubicin. These results led to an ongoing international phase III trial comparing single-agent bortezomib with the combination of bortezomib and pegylated liposomal doxorubicin in relapsed/refractory myeloma. This regimen is also being evaluated by the Cancer and Leukemia Group B as an induction therapy for patients with previously untreated, symptomatic multiple myeloma. Interim results from the latter trial show an overall response rate of 80%, with no patients suffering progressive disease.

Table 1. Interplay between anthracyclines and proteasome inhibitors.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Anthracyclines</th>
<th>Proteasome Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>How proteasome inhibitors may enhance the activity of anthracyclines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA damage repair</td>
<td>Act in part by damaging DNA, and their activity would be enhanced if repair functions were inactivated</td>
<td>Repress some of the DNA damage repair enzymes, such as the DNA protein kinase c catalytic subunit</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Activate NF-κB, which is anti-apoptotic in part through downstream targets such as Bcl-2 and inhibitors of apoptosis</td>
<td>Inhibit NF-κB by stabilizing its inhibitor IκB, thereby enhancing programmed cell death</td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>Expression selected by prior anthracyclines; acts to reduce intracellular anthracycline levels</td>
<td>Block maturation of P-glycoprotein, leading to accumulation of inactive precursors</td>
</tr>
<tr>
<td>How anthracyclines may enhance the activity of proteasome inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MKP-1</td>
<td>Inhibit MKP-1 through repression of its promoter, augmenting JNK activity and apoptosis</td>
<td>Induce MKP-1, which is anti-apoptotic through its inhibition of JNK phosphorylation and activation</td>
</tr>
</tbody>
</table>
Another trial based on this combination tested the PAD regimen for induction therapy, in which bortezomib was substituted for vincristine in the VAD regimen with infusional doxorubicin and oral dexamethasone. After four cycles of this therapy an overall response rate of 95% was noted among 21 patients, and 57% of the 18 patients who underwent PAD followed by autologous peripheral blood stem cell transplantation ultimately achieved a near-CR or CR. Of the clinical trials described, none have been specifically designed to determine if there was a greater than additive activity of bortezomib-based combination regimens in vivo by, for example, studying the activity of each of the single agents, as well as the combination, in a three-armed trial. Collectively, however, these data are consistent with the notion that bortezomib increases the sensitivity of myeloma to conventional chemotherapy and overcomes chemoresistance. Moreover, the high overall and complete response rates achieved with these regimens suggest that they may eventually become standard treatments for multiple myeloma.

Step 3: Development of Novel Proteasome Inhibitor-Based Combinations

As noted earlier, one of the driving hypotheses for the development of proteasome inhibitor-based regimens with standard chemotherapeutics was the ability of proteasome inhibition to block inducible chemoresistance through NF-kB. It is perhaps ironic, therefore, that many studies have now shown that proteasome inhibitors themselves also induce pathways of chemoresistance. One of the best studied such mechanisms involves the heat shock proteins (HSPs), which for many years have been known to be activated by inhibition of the ubiquitin-proteasome pathway. The induced HSPs play roles as cellular chaperones, and many modulate apoptotic pathways, particularly those involving mitochondria, conferring protection from stressful stimuli including chemotherapeutic agents. In myeloma, for example, bortezomib can induce expression of HSP-90, -27, and -70. Geldanamycin and its family of analogues interact with the HSP-90 ATP binding pocket to inhibit HSP-90, and geldanamycin sensitizes myeloma cells to proteasome inhibition. Direct pharmacologic inhibitors of HSP-27 have not yet been identified, but since HSP-27 is in part activated in a p38 MAPK-dependent fashion, p38 may serve as a surrogate target. Chemical inhibitors of p38 do exist, and here, too, there is encouraging evidence showing that inhibition of p38 enhanced sensitivity to bortezomib in cell line models of multiple myeloma and may even overcome resistance to this agent in cell line models of NHL.

Targeting HSP-70 is also an attractive strategy and the use of anti-sense oligonucleotides can enhance the activity of proteasome inhibitors. While direct pharmacologic inhibitors of HSP-70 have not yet been developed, it may nonetheless be possible to modulate this heat shock protein’s anti-apoptotic influence through other approaches. HSP-70 is induced in part through the action of signal transducer and activator of transcription (STAT)-1, which itself is activated by interleukin-6 (IL-6). This cytokine is known to play a very important role in the pathogenesis of multiple myeloma and is itself a rational target in this disease. Downregulation of IL-6 might result in suppression of HSP-70 and chemosensitization. Consistent with this possibility, we have found that inhibition of IL-6 binding to myeloma cells downregulated activation of STAT-1, decreased bortezomib-mediated induction of HSP-70, and enhanced sensitivity to proteasome inhibitor-mediated apoptosis (unpublished observations).

Recent studies have also suggested that proteasome inhibitors have activity against multiple myeloma in part through interference with the endoplasmic reticulum’s unfolded protein response. This pathway allows plasma cells to ensure the proper folding of immunoglobulin proteins by providing a mechanism through which misfolded molecules can be removed. Bortezomib suppresses this response by blocking the key transcription factor XBP-1, leading to apoptosis of myeloma cells. Preclinical studies have also shown that dual inhibition of the proteasome and the aggresome, an alternative pathway for removal of unfolded and misfolded proteins, results in synergistic anti-myeloma activity.

The hypothesis that modulation of heat shock protein function in combination with proteasome inhibition will enhance anti-tumor efficacy is beginning to be tested clinically. A preliminary report of a phase I trial with the regimen of bortezomib and 17-allylamino-17-demethoxygeldanamycin has indicated that the combination is tolerated by patients and some evidence of clinical activity has been seen. Pharmacologic inhibitors of p38 MAPK and the IL-6 axis are undergoing testing as single agents against multiple myeloma and likely will then proceed onward to combination studies as well.

Step 4: Future Directions for Proteasome Inhibition and Proteasome Inhibitors

With the validation of the proteasome as a target for cancer therapy, there is now an opportunity to rationally design even more efficacious proteasome inhibitors. Bortezomib is a reversible inhibitor of the chymotrypsin-like activity of the proteasome, but inhibitors with other specificities and chemistries have also been described. Lactacystin, one of the first proteasome inhibitors known, is a Streptomyces metabolite that irreversibly inhibits the chymotrypsin-like and trypsin-like proteasome activities and reversibly inhibits peptidyglutamyl-peptide hydrolyzing activities. Inhibitors with a broader specificity and irreversible binding might have a wider range of anti-tumor activities than bortezomib, and might be able to overcome resistance to bortezomib. Support for this concept is found in preliminary results with one such lactacystin-related compound, the novel non-peptide inhibitor NPI-0052. This agent induced apoptosis and overcame resistance to conventional
chemotherapeutics in myeloma cell lines and patient myeloma samples, and acted synergistically with bortezomib itself. These results certainly support translation of this agent into the clinic, though irreversible inhibitors might also have the potential of enhanced toxicity due to the important role of the proteasome in normal cellular homeostasis.

Another approach that may prove promising is to find inhibitors of the proteasome that would be more specific than bortezomib. The proteasome is not a static structure and, under the influence of cytokines such as γ-interferon, three proteolytically active subunits termed X, Y, and Z are replaced by different subunits known as low molecular weight proteins (LMP)-2, -7, and -10. This structure is known as the immunoproteasome, since it may play a role in major histocompatibility complex-class-I-mediated antigen presentation, but the immunoproteasome is also expressed constitutively in some cells of hematopoietic origin. Inhibitors specific for the immunoproteasome might have the ability to induce apoptosis only in hematological malignancies while sparing other tissues. If true, some of the toxicities associated with bortezomib, such as peripheral neuropathy and gastrointestinal effects, might be decreased or abolished, while preserving anti-tumor efficacy, yielding a better therapeutic index. Our group has identified a series of inhibitors that have the ability in vitro to preferentially inhibit the LMP-containing proteasome while sparing the XYZ-containing proteasome, with the most specific of these demonstrating a greater than 100-fold difference in K_i. Incubation of cells containing LMP-based proteasomes with this agent resulted in the induction of apoptosis, while XYZ-containing cell lines were spared. In comparison, bortezomib showed non-specific activity, and induced programmed cell death in all of these cell lines (unpublished observations).

Conclusions
The ubiquitin-proteasome pathway is now firmly established as a therapeutic target for patients with hematological malignancies such as multiple myeloma and non-Hodgkin lymphoma. Development of the first generation proteasome inhibitor, bortezomib, has been guided from its inception by laboratory studies that pointed the way towards the most appropriate clinical application of this drug in these diseases. Laboratory studies have led to a second generation of regimens combining bortezomib with conventional chemotherapy. A third generation of regimens is already beginning to emerge based on detailed analyses of the molecular mechanisms of action of proteasome inhibitors at the gene and protein expression profile levels, which incorporate even newer agents such as HSP-90 inhibitors. Also, a newer generation of proteasome inhibitors is being designed that may have interesting therapeutic applications. From these studies it is clear that agents targeting the proteasome will be firmly entrenched as part of our chemotherapeutic armamentarium in the future. Moreover, their development provides a clear guide to the path by which a multidisciplinary approach to drug development can lead to the successful translation of laboratory findings into novel therapies to improve the outcomes of patients with cancer and other diseases.

References
18. Loo TW, Clarke DM. The human multidrug resistance P-
glycoprotein is inactive when its maturation is inhibited: potential for a role in cancer chemotherapy. FASEB J. 1999;13:1724-1732.


