Probing proteasome activity and function : cancer diagnostics and mechanism of antigen processing

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Chapter 1.1

Drug discovery and assay development in the ubiquitin–proteasome system

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Drug discovery and assay development in the ubiquitin–proteasome system

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The observation that tumour cells are more sensitive to pharmacological inhibition of the proteasome than normal cells has led to the development of the proteasome inhibitor bortezomib. To date, this is the only proteasome inhibitor that has been approved for clinical use. The clinical success of bortezomib, combined with the occurrence of adverse effects and the development of clinical resistance against this compound, has initiated the development of a broad range of second-generation proteasome inhibitors as well as of assays that can be used to establish a relationship between the extent and type of proteasome inhibition and the effectiveness of a particular drug. In the present paper, we discuss new strategies that may be used in the future to overcome drug resistance and to broaden the use of proteasome inhibitors for the treatment of both cancer and infectious and autoimmune disease.

THE UPS (UBIQUITIN–PROTEASOME SYSTEM)

The UPS is responsible for the degradation of redundant and misfolded proteins, as well as for the turnover of many key regulatory proteins that control a wide range of cellular processes such as cell-cycle progression, cell differentiation, apoptosis, stress response, DNA repair and signal transduction.[1,2] Proteins that are destined for destruction are first post-translationally modified with a polyubiquitin chain via the consecutive action of three different types of ubiquitinating enzymes[3] (Figure 1). First, an E1 ubiquitin-activating enzyme activates ubiquitin in an ATP-dependent manner and then transfers it to an E2 ubiquitin-conjugating enzyme. In a final step, ubiquitin is conjugated to the target protein or a previously attached ubiquitin via the combined action of the E2 and an E3 ubiquitin ligase. The latter ligase is capable of interacting with both the E2 and the target protein and thereby confers substrate specificity on the ubiquitination cascade.

Sequential ubiquitination cycles lead to the formation of polyubiquitinated proteins, which are subsequently degraded by the 26S proteasome, a large threonine protease complex. The proteolytic activity of the 26S proteasome resides within its barrel-shaped 20S core, which is formed by four stacked rings of seven subunits each and has an overall architecture of $\alpha(1–7)\beta(1–7)\beta(1–7)\alpha(1–7)$. Within the two inner β-rings, three different catalytically active subunits, termed β1, β2 and β5, are responsible for the proteasome's caspase-like, trypsin-like and chymotrypsin-like activity, respectively. In lymphoid tissues and upon induction with interferon-γ, these constitu-

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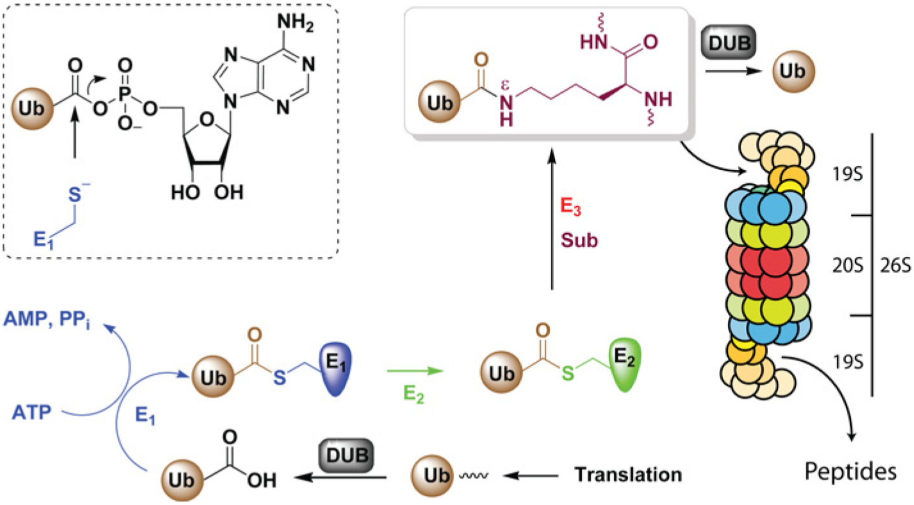

Figure 1 | Ubiquitin conjugation and deconjugation, and proteasomal protein breakdown. DUB, deubiquitinating enzyme; Ub, ubiquitin.

tive subunits can be replaced by their immunoproteasome counterparts, termed β1i, β2i and β5i, to form the immunoproteasome, whereas mixed-type proteasomes have also been reported.[4,5] The two outer α-rings provide stability to the 20S complex and serve as docking stations for the attachment of 19S regulatory complexes, which consist of a base that binds directly to the α-rings and which contain several ATPases. These 19S caps recognize and unfold the substrates and subsequently thread them into the 20S catalytic chamber for degradation.

PROTEASOME INHIBITORS AS ANTICANCER THERAPEUTICS

Proteasome inhibition is more cytotoxic to proliferating malignant cells than to quiescent normal cells, making the proteasome an attractive pharmacological target for the treatment of cancer.[6] Bortezomib[7] (velcade, PS341) (Figure 2) was the first proteasome inhibitor to enter the clinic, and has now been approved as a single agent for the treatment of relapsed/refractory MM (multiple myeloma) and mantle cell lymphoma, whereas many clinical trials in solid and haematological tumours are ongoing. Although the clinical success of bortezomib is evident, bortezomib treatment has been associated with serious adverse effects.[8] In addition, the occurrence of both primary and acquired resistance to bortezomib has been reported and although many haematological malignancies seem to respond well to proteasome inhibitor treatment, results in treatment of solid tumours have been disappointing.[9] This has initiated an ongoing search for novel proteasome inhibitors with distinct subunit specificity, lower toxicity or improved bioavailability.

Several second-generation proteasome inhibitors that vary in their mode of binding are now in clinical trials, including carfilzomib (PR-171),[10,11] NPI-0052 (salinosporamide A)[12] and CEP-18770[13,14] (Figure 2). For all inhibitors, the first step in proteasome inhibition is the deprotonation of the proteasomal Thr1 O^{γ}, which is thought to occur by the N-terminal amino group acting as a base, either di-

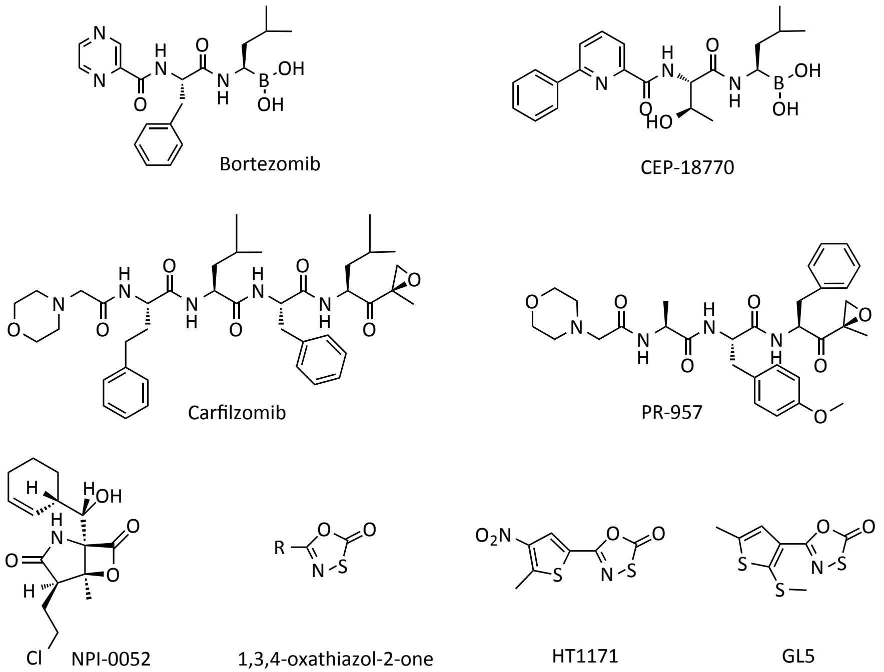

Figure 2 | Structures of (clinically relevant) 20S proteasome inhibitors.

rectly or via a neighbouring water molecule.[15] The structure of bortezomib complexed to the yeast 20S proteasome[16] has shown that for boronic acid-based inhibitors such as bortezomib and CEP-18770, subsequent nucleophilic attack of the Thr1 O$^{\gamma}$ results in a covalent interaction between the boron atom and the Thr1 O$^{\gamma}$ (Figure 3A). The proteasome–inhibitor complex is stabilized further by the Thr1 N, which forms a tight hydrogen bond with one of the acidic boronate hydroxyl groups.[16] In the case of epoxyketone-based inhibitors, such as carfilzomib, nucleophilic attack of Thr1 O$^{\gamma}$ is thought to result in hemiacetal formation, followed by subsequent cyclization of Thr1 N onto the epoxide, resulting in the formation of a morpholino adduct (Figure 3B), as observed in the crystal structure of the 20S proteasome–epoxomicin complex.[17] NPI-0052 is a proteasome inhibitor isolated from the marine actinomycete *Salinispora*. The crystal structure of the 20S proteasome–NPI-0052 complex[18] suggests that nucleophilic attack by the catalytic Thr1 O$^{\gamma}$ on the β-lactone ring results in the formation of an ester between the Thr1 O$^{\gamma}$ and the carbonyl derived from the β-lactone ring (Figure 3C). Subsequent chlorine substitution then gives rise to a cyclic ether,[18] resulting in an adduct resistant to further hydrolysis.

The proteasome inhibitors that are currently in use in the clinic also vary in their subunit specificity, downstream mechanisms of action and bioavailability. Bortezomib and CEP-18770 both reversibly inhibit β1 and β5 subunits,[7,14,19] carfilzomib predominantly targets the β5 subunit,[11] whereas NPI-0052 irreversibly inhibits all constitutive and immunoproteasome subunits.[12] As a consequence, carfilzomib and CEP-18770 induce apoptosis via similar downstream mechanisms to that of bortezomib,[11,14,20] whereas NPI-0052 induces

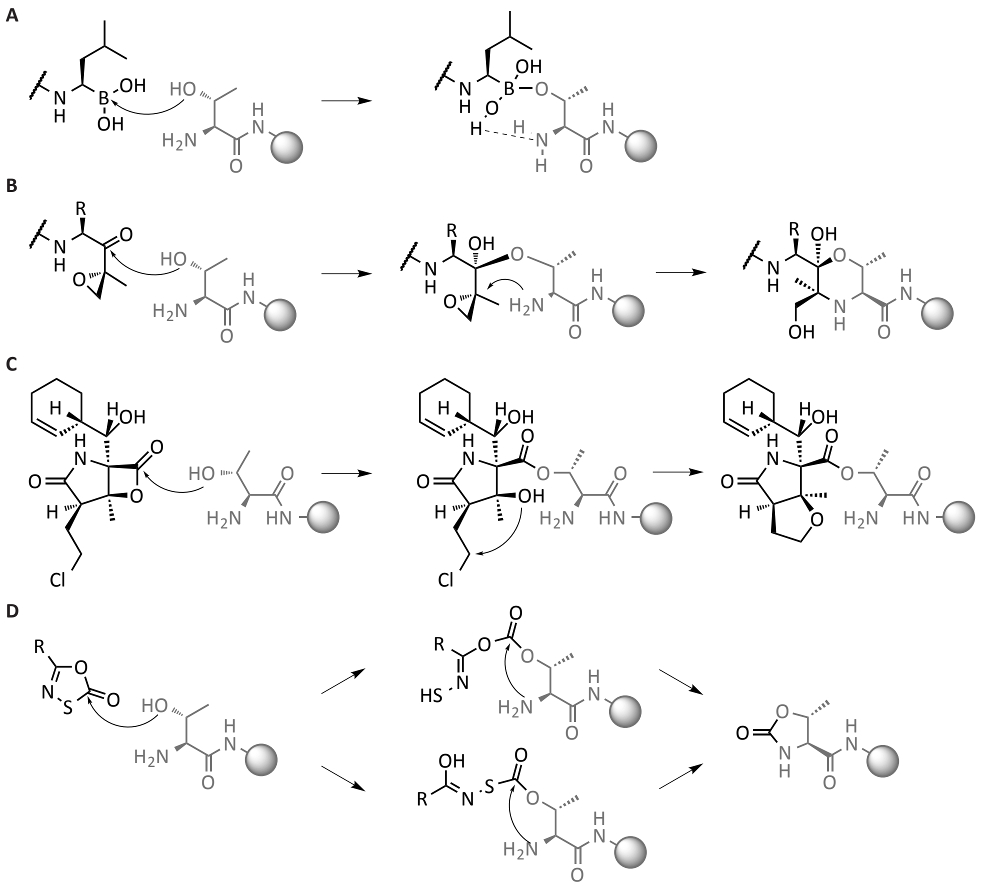

Figure 3 | Proposed mechanisms of proteasome inactivation by (clinically relevant) 20S proteasome inhibitors. Inhibitors are indicated in black, N-terminal threonine residues of active proteasomal β-subunits are in grey. Inactivation mechanism of **A)** boronic acid-based proteasome inhibitors (bortezomib and CEP-18770),[16] **B)** epoxyketone-based proteasome inhibitors (carfilzomib and PR-957),[17] **C)** β-lactone-based NPI-0052[18] and **D)** 1,3,4-oxathiazol-2-one compounds (mycobacterial-specific proteasome inhibitors).[47]

apoptosis also via different pathways.[12] Both carfilzomib and NPI-0052 were shown to be able to overcome bortezomib resistance, both in cell lines and in samples from patients with clinical bortezomib resistance.[11,12] NPI-0052 and CEP-18770 are orally bioavailable.[12,14]

PROTEASOME ACTIVITY ASSAYS

For effective treatment with proteasome inhibitors, it is essential to establish a relationship between the effectiveness of the drug in a particular tumour type and the extent and type of proteasome inhibition.[6] There is therefore an emerging need for proteasome activity assays that can profile the efficacy and specificity of proteasome inhibitors, both *in vitro* and in a cellular context.

Traditionally, different fluorogenic substrates, which typically consist of a short peptide and a C-terminally attached fluorogenic reporter, are used to measure the activity of the different proteasome active sites. The proteasome cleaves these substrates between the last amino acid and the reporter, resulting in the release of a fluorescent molecule.[21] Fluorogenic substrates are widely used to measure effects of proteasome inhibitors,[22] but often only the chymotrypsin-like activity is measured.[21] It should be noted, however, that measuring only the chymotrypsin-like activity may not accurately reflect the degree of proteasome inhibition, and that the activities of all active sites need to be assayed in order to reliably evaluate the efficacy of inhibitors.[21,23] Most fluorogenic substrates cannot be used in cells, and prior cell lysis is required before activity measurements can be performed. This treatment isolates the proteasome from its regulatory environment,[6] and may lead to dissociation of the 19S caps. Efects of the 19S regulatory particle or other associated proteins may thus not be accounted for.[24] Furthermore, fluorogenic substrates cannot distinguish between constitutive and immunoproteasome activity, as immunoproteasome-specific substrates have not been described to date.[6]

To measure proteasome activity *in vivo*, both cell-based assays[25] and transgenic mouse models[24,26] have been developed that are based on recombinant proteasome substrates, typically ubiquitin–GFP (green fluorescent protein) or ubiquitin–luciferase fusion protein. Under normal cellular conditions, these fusion proteins are rapidly degraded by the proteasome, and no fluorescence or luciferase activity can be detected, but when the proteasome is inhibited, degradation is impaired and intracellular fluorescence increases. These methods and models are useful in screening for new inhibitors and for testing bioavailability, but their activity readout depends on the balance between synthesis and degradation of fusion proteins, which involves many cellular factors other than the proteasome, including the rate of fusion protein synthesis. Moreover, because they are based on reporter proteins, their use remains confined to genetically altered cells and organisms and does not allow profiling of patient material.

A third type of assay uses chemical proteasome probes that allow profiling of proteasome activity in cell lysates, cells and *ex vivo*.[11,19,27-30] In general, these probes consist of a reactive group that covalently modifies all active-site N-terminal threonine residues, which is attached to a peptide-recognition motif to target the probe to the proteasome. A tag allows for visualization or isolation of the proteasome–probe complex. An example of such a probe is dansyl-Ahx_3Leu_3vinylsulfone (where Ahx is aminohexanoic acid),[19] which was used successfully to profile bortezomib,[19] NPI-0052[12] and carfilzomib[11] in cells using an antibody against the dansyl moiety. More recently, chemical probes in which the dansyl moiety has been replaced with a fluorescent tag have been developed.[27,30] These probes are more sensitive and allow for easier visualization of proteasome activity either using SDS/PAGE or directly using confocal microscopy. Importantly, the incorporation of a fluorescent tag also enables the use of these probes in flow-cytometry-based assays,[27] which can be applied in a high-throughput fashion to screen for direct and indirect regulators of proteasome activity. Chemical proteasome probes have also been used for patient tumour profiling,[31] thereby confirming that these probes can be used to quantitatively and qualitatively monitor proteasome blockage in patients receiving proteasome inhibitor therapy.[6] Finally, proteasome active-site probes were used for the development of an ELISA-based activity assay.[11,28,29] This assay combines the use of a biotinylated epoxyketone-based proteasome probe with subunit-specific antibodies to measure proteasome activities and has been used to profile the activity of carfilzomib and several recently developed immunoproteasome-specific inhibitors.

NOVEL ANTICANCER STRATEGIES

With the availability of a panel of different proteasome inhibitors, the outstanding clinical challenge will be to define treatment schedules that offer a therapeutic advantage over bortezomib treatment.[32] Different proteasome inhibitors may find use in different therapeutic settings. Specific inhibitors of the $\beta 5$ subunit, such as bortezomib, carfilzomib or CEP-18770, may offer a large therapeutic window in some tumours, whereas pan-proteasome inhibitors that block all three activities may lead to improved proteasome inhibitor efficacy in others,[29,33] depending on subunit activity profile, tissue type and disease.

An interesting strategy to overcome resistance to the current generation of proteasome inhibitors may be the development of inhibitors that do not interfere with the 20S core, but with other elements of the UPS. An obvious strategy would be the development of inhibitors of the 19S regulatory particle, especially inhibitors of the 19S ATPases, which also play non-proteolytic roles.[34] The first compound of this type, RIP-1 (regulatory particle inhibitor peptoid-1) was recently identified and the 19S ATPase Sug2/Rpt4 was identified as its molecular target.[34,35] A more advanced UPS-modulating compound that holds promise for the treatment of cancer is MLN4924,[36] an inhibitor of NEDD8 (neural-precursor-cell-expressed developmentally down-regulated 8)-activating enzyme, an essential component of the NEDD8 conjugation pathway that, in turn, controls the activity of a subset of E3 ubiquitin ligases. MLN4924 treatment induced apoptosis in human tumour cells, and suppressed tumour growth in pre-clinical models.[36] Currently, MLN4924 is being evaluated in clinical trials.

Proteasome inhibitors can be combined with other proteasome inhibitors or chemotherapeutics to increase their therapeutic potential. The combination of bortezomib and NPI-0052 remarkably induced synergistic cytotoxicity,[12,33,37] indicating that combination therapy of proteasome inhibitors might improve patient outcome.[6] It is tempting to speculate that combining proteasome inhibitors that induce apoptosis via distinct mechanisms, such as bortezomib and NPI-0052, may be particularly beneficial.[6] Interesting in this respect is the recent identification of argyrin A,[38] a cyclic peptide derived from the mycobacterium *Archangium gephyra* that inhibits all proteasome activities. Induction of apoptosis by argyrin A was shown to be dependent on $p27^{kip1}$, but not on IκBα (inhibitor of nuclear factor κB α) stabilization, whereas the cytotoxic effects of bortezomib are mediated via IκBα stabilization, but are not dependent on p27.[38] Argyrin A may therefore represent another class of proteasome inhibitors that can be successfully combined with other proteasome inhibitors in anticancer therapy.

Many of the pathways used by tumour cells to survive chemotherapy are blocked by proteasome inhibition, which provided the rationale for combining bortezomib with a variety of chemotherapeutics.[9,39] The combination of bortezomib and PEGylated doxorubicin has been approved for the treatment of relapsed MM,[40,41] and many treatment regimens combining bortezomib and other proteasome inhibitors with conventional or novel anti-MM agents have yielded promising results.[6,9,42,43] Another strategy currently being evaluated is the simultaneous targeting of the proteasomal and lysosomal degradation pathways using a combination of bortezomib and HDAC (histone deacetylase) inhibitors.[6]

NEW FRONTIERS IN UPS DRUG DISCOVERY

Proteasome inhibitor therapy may not remain restricted to the treatment of cancer, but may also be used to treat a variety of other disorders. Recently, PR-957 (Figure 2), an epoxyketone-based inhibitor specific for the immunoproteasomal β5i subunit, was developed.[28] Immunoproteasome is found predominantly in cells of haemopoietic origin. Specific immunoproteasome inhibitors may therefore be used for the treatment of haemopoietic tumours, while causing fewer side effects in tissues that express only low levels of immunoproteasome subunits, such as neurotoxicity or gastrointestinal side effects.[9] Immunoproteasome inhibitors may also be useful for the treatment of other disorders. Increased immunoproteasome expression has been observed in autoimmune diseases such as rheumatoid arthritis[44] and has also been correlated to neurodegenerative disease states, including Huntington's and Alzheimer's disease.[45,46] Consistent with this, Muchamuel et al.[28] showed that PR-957 reversed signs of disease in mouse models of rheumatoid arthritis, providing a rationale for development of immunoproteasome inhibitors for the treatment of autoimmune diseases. Furthermore, various infectious diseases may be treatable with proteasome inhibitors. Lin et al.[47] identified two related 1,3,4-oxathiazol-2-one compounds, HT1171 and GL5 (Figure 2), as selective inhibitors of the proteasome of *Mycobacterium tuberculosis*. Nucleophilic attack of the proteasomal Thr1 O^{γ} on the oxathiazol-2-one is thought to result in the formation of a carbonated or carbonothioated enzyme intermediate on Thr1.[47] Subsequent nucleophilic attack of the Thr1 N results in cyclocarbonylation of the active-site threonine residue to form an oxazolidin-2-one and prevents regeneration of the active protease (Figure 3D), thereby killing *M. tuberculosis*.[47] It is important that these oxathiazol-2-one compounds did not significantly inhibit the human proteasome nor a panel of cysteine, serine or metallo-proteases. The selectivity of these compounds for *M. tuberculosis* over mammalian proteasomes seemed to be conferred by residues distant from the active site, suggesting that a functionally exploitable diversity exists between *M. tuberculosis* and human proteasomes and that compounds that are specific for different types of mycobacteria or a variety of other bacteria could also be developed, although *in vivo* data are required. This opens the way for the treatment of a variety of (myco)bacterial infections with proteasome inhibitors, either alone or in combination with other antibiotic strategies.

CONCLUSION

Proteasome inhibition has emerged as a novel strategy for the treatment of cancer, as is evident from the clinical success of bortezomib, the first proteasome inhibitor to pass clinical trials. The occurrence of side effects, the development of resistance and low success rates in the treatment of solid tumours, however, limits the use of this agent. The recent development of a broad range of second-generation proteasome inhibitors is likely to contribute to a more widespread use of this class of agents for the treatment of both haematological and solid tumours. The development of compounds that interfere with the UPS via different mechanisms and the development of proteasome inhibitors that are specific for proteasome subtypes or other species may broaden the clinical application of UPS-modulating compounds in cancer and infectious and autoimmune diseases.

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