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REVIEW

The proteasome: a novel target for cancer chemotherapy

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The ubiquitin-proteasome system is an important regulator of cell growth and apoptosis. The potential of specific proteasome inhibitors to act as novel anti-cancer agents is currently under intensive investigation. Several proteasome inhibitors exert anti-tumour activity *in vivo* and potently induce apoptosis in tumour cells *in vitro*, including those resistant to conventional chemotherapeutic agents. By inhibiting NF-κB transcriptional activity, proteasome inhibitors may also prevent angiogenesis and metastasis *in vivo* and further increase the sensitivity of cancer cells to apoptosis. Proteasome inhibitors also exhibit some level of selective cytotoxicity to cancer cells by preferentially inducing apoptosis in proliferating or transformed cells or by overcoming deficiencies in growth-inhibitory or pro-apoptotic molecules. High expression of oncogene products like c-Myc also makes cancer cells more susceptible to proteasome inhibitor-induced apoptosis. The induction of apoptosis by proteasome inhibitors varies between cell types but often occurs following an initial accumulation of short-lived proteins such as p53, p27, pro-apoptotic Bcl-2 family members or activation of the stress kinase JNK. These initial events often result in a perturbation of mitochondria with concomitant release of cytochrome c and activation of the Apaf-1 containing apoptosome complex. This results in activation of the apical caspase-9 followed by activation of effector caspases-3 and -7, which are responsible for the biochemical and morphological changes associated with apoptosis.
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Introduction

Cancer is characterized either by uncontrolled cellular proliferation or by a failure of cells to undergo apoptosis. As the proteasome is an important regulator of both these processes, therapeutic regimes to manipulate proteasomal activity could potentially restore normal cellular homeostasis in some cancer patients. Drug resistance and lack of tumour specificity frequently hinder the treatment of neoplastic disease, creating a need for new classes of potent and specific anticancer drugs. Recent studies have shown that proteasome inhibitors potently induce apoptosis in many types of cancer cells, with reduced cytotoxicity in normal cells. In this review we will discuss recent evidence that the proteasome represents a novel therapeutic target for the treatment of cancer.

The proteasome

Appropriate, targeted proteolysis is essential for cellular function and homeostasis. In eukaryotic cells, the ubiquitin-proteasome pathway is the central non-lysosomal pathway for protein degradation. Under normal conditions, the lysosomal pathway degrades extracellular proteins imported into the cell by endocytosis or pinocytosis, whereas the proteasome controls degradation of intracellular proteins.[1] Proteins are initially targeted for proteolysis by the attachment of a polyubiquitin chain, and then rapidly degraded to small peptides by the proteasome and the ubiquitin is released and recycled (Figure 1).[1] This co-ordinated proteolytic pathway is dependent upon the synergistic activity of the ubiquitin-conjugating system and the 26S proteasome.

The 26S proteasome is a large (1500–2000 kDa) multi-subunit complex present in the nucleus and cytoplasm of eukaryotes.[2] The catalytic core of this complex, referred to as the 20S proteasome, is a cylindrical structure consisting of four heptameric rings containing α- and β-subunits (Figure 1). The two outer rings are composed of α-subunits, whereas β-subunits containing the proteolytically active sites form the inner rings. The proteasome is a threonine protease, the N-terminal threonine of the β-subunit providing the nucleophile that attacks the carbonyl group of the peptide bond in target proteins. At least three distinct proteolytic activities are associated with the proteasome: chymotryptic, tryptic and peptidylglutamyl. The ability to recognize and bind polyubiquitinated substrates is conferred by 19S (PA700) subunits, which bind to each end of the 20S proteasome (Figure 1). These accessory subunits unfold substrates and feed them into the 20S catalytic complex, whilst removing the attached ubiquitin molecules. Both the assembly of the 26S proteasome and the degradation of protein substrates are ATP-dependent.[3–5]

The targeting of proteins for proteolysis by ubiquitination is controlled by groups of enzymes collectively referred to as the ubiquitin system. Ubiquitin molecules are covalently attached to potential proteasome substrates via an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ϵ-amino group of a lysine residue in the target protein. Ubiquitin monomers are first activated for conjugation to other proteins by an ubiquitin-activating enzyme (E1). The E1 linked ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2 or UBC), and ultimately to the target protein. The final step requires an ubiquitin ligase (E3), which is involved in selecting a protein for ubiquitination.[6,7]

The activity of the 20S catalytic core of the proteasome can be modulated by the incorporation of different catalytically active-β-subunits and by association with various regulatory complexes. For example, interferon-γ induces the synthesis of three β-type subunits, LMP2, LMP7 and MECL-1, which replace constitutive subunits of the 20S proteasome to form the 'immunoproteasome' This modified catalytic core associates with 11S/REG/PA28 accessory subunits, causing a change in the proteolytic activity, favouring the generation of peptide antigens for presentation by MHC class I molecules.[5,8] The combination of specific targeting of substrates for degradation by the ubiquitin-system and modulation of the 20S multicatalytic protease by remodelling of β-subunits and association

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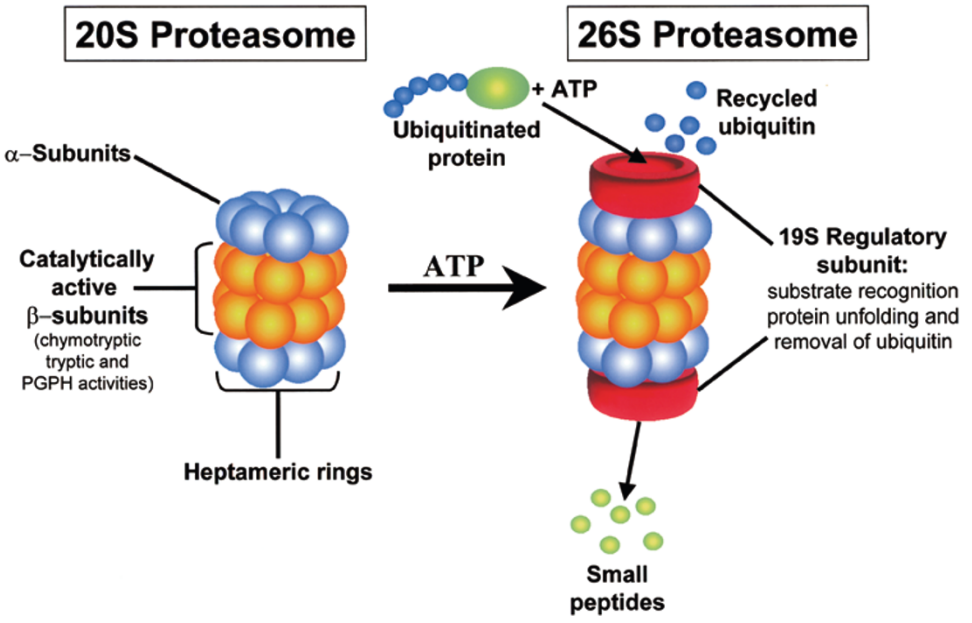

Figure 1 The ubiquitin proteasome pathway for protein degradation. Protein substrates are first conjugated to multiple molecules of ubiquitin. The ubiquitinated substrate is rapidly degraded by the 26S proteasome, which is an ATP-dependent complex containing the core 20S proteasome together with two 19S regulatory subunits.

with regulatory complexes, constitute a high level of regulation of proteasomal proteolysis. The specificity of the ubiquitin-proteasome system is such that the degradation of individual substrates can be upregulated, without affecting proteolysis of other substrates, thereby enabling the proteasome to function as a regulator of metabolic pathways. As discussed later, different classes of proteasome inhibitors (Table 1, Figure 2) can differentially affect the degradation of various proteasome substrates so controlling the cellular levels of certain proteins that are involved in oncogenicity and tumour progression, without necessarily having a generalized systemic effect.

Functions of the ubiquitin-proteasome system

By the coordinated and temporal degradation of short-lived proteins, the ubiquitin-proteasome system regulates many important cellular processes. The synchronized proteolysis of cyclins and cyclin-dependent kinase inhibitors is critical for cell cycle progression.[9–11] Disruption of this process in proliferating cells leads to cell cycle arrest at G_1/S, G_2/M, or both, depending on the cell type.[12] The proteasome controls gene expression by degrading transcription factors such as NF-κB, p53, c-Jun, c-Myc, c-Fos, HIF1α, sterol-regulated element-binding proteins and MATα2.[1,8,13] Perhaps the best charac-

Table 1 Proteasome inhibitors

Class of compound	*Inhibitor*	*Specificity*	*Mechanism*	*Refs*
Peptide aldehydes	MG132, LLnV, PSI, ALLN, ALLnM, CEP1612, Z-LLF	Also inhibit Calpain I and Cathepsin B	Potent transition state analogues. Aldehyde moiety interacts reversibly with the catalytic threonine residue of the proteasome	26
Streptomyces metabolite (natural product)	Lactacystin	Relatively specific but weak inhibitor of the proteasome (also inhibits cathepsin A)	Lactacystin (pro-drug metabolized to clasto-lactacystin β-lactone which is the active inhibitor). Irreversibly forms a covalent ester with the amino-terminal threonine of the catalytic β-subunit of proteasome	26, 28–30
Dipeptidyl boronic acids	PS-341	Selective and potent inhibitors of the proteasome	Boron atom interacts reversibly with the catalytic threonine residue of the proteasome	31, 32, 40
Vinyl sulfone tripeptides	NLVS	Also inhibit cathepsins	Function as irreversible adducts with the threonine hydroxyl of the catalytic β-subunits of the proteasome	26, 27
Natural products	Eponomycin Epoxomycin	Selective inhibitors of the proteasome	Covalently bind to catalytic β-subunits of the proteasome	27, 82

These compounds primarily inhibit the chymotrypsin-like activity of the proteasome, which is sufficient to inhibit the proteolysis of ubiquitinated proteins.[27] The structures of some of the major inhibitors are given in Figure 2.
MG132, z-leu-leu-leucinal; LLnV, z-leu-leu-norvalinal or MG115; PSI, cbz-ile-glu-(O-t-Bu)-ala-leucinal; z-IE(OtBu)AL-CHO; ALLN, N-acetyl-leu-leu-norleucinal; ALLnM, acetyl-leu-leu-normethional; Z-LLF, z-leu-leu-phenylalaninal; NLVS, z-leu-leu-leu-vinyl sulfone.

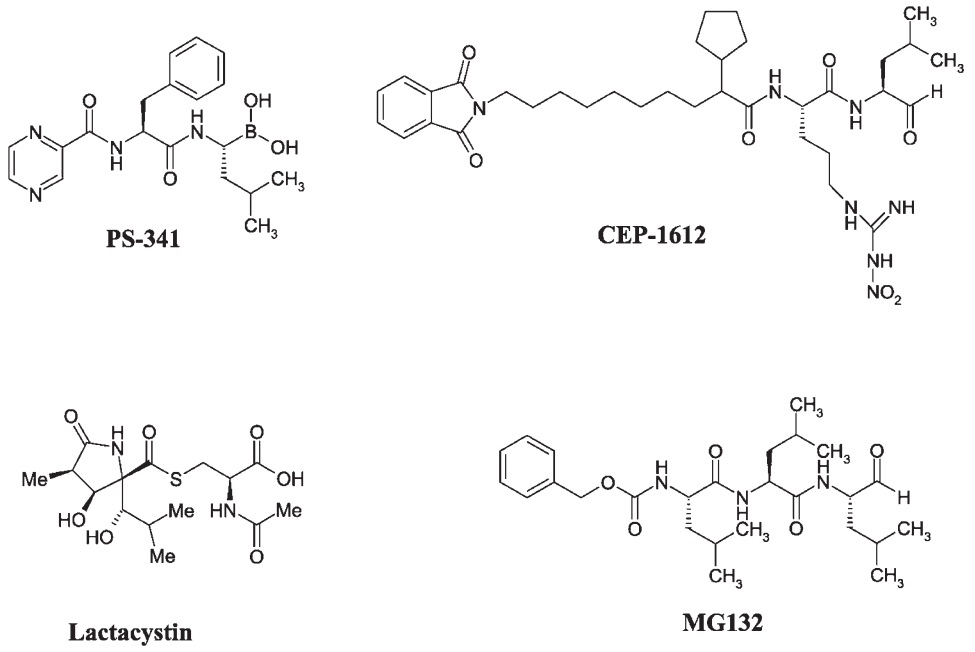

Figure 2 Structures of some commonly used proteasome inhibitors.

terized of these is NF-κB, the collective name for inducible dimeric transcription factors composed of members of the Rel family of DNA-binding proteins that recognize a common sequence motif.[14] Activated NF-κB is present in the nucleus as a heterodimer of p50 and p65 subunits. Proteasomal proteolysis is required both for the production of the active subunits from their precursors and for regulation of NF-κB activity. Inactive NF-κB is sequestered in the cytoplasm bound to its inhibitory regulator IκB. Following activation of NF-κB by stimuli such as tumour necrosis factor (TNF)-α and interleukin (IL)-1, IκB is first phosphorylated then ubiquitinated and degraded so revealing the nuclear localization sequence of NF-κB, which translocates to the nucleus and initiates transcription.[14] NF-κB is a regulator of cell proliferation, apoptosis, immune and inflammatory responses, and controls the expression of genes encoding cytokines, chemokines, growth factors, cell adhesion molecules and cell surface receptors.[1,8,13] In malignant disease, NF-κB-inducible factors can contribute to drug resistance, angiogenesis and metastasis. In addition, many of the transcription factors post-translationally regulated by the ubiquitin-proteasome system are involved in oncogenesis.[13,15] Thus by controlling levels of many key cellular proteins, the proteasome acts as a regulator of cell growth and apoptosis and disruption of its activity may have profound effects on both the aetiology and treatment of cancer.

Apoptosis and cancer

Apoptosis is a major form of cell death, which is important both in controlling cell numbers during development and in the removal of damaged cells. Defective apoptosis is involved in the pathogenesis of several diseases including certain cancers, such as B cell chronic lymphocytic leukaemia (B-CLL), where there is an accumulation of quiescent tumour cells. Apoptosis is generally induced either by ligation of membrane-associated death receptors of the tumour necrosis factor (TNF) receptor superfamily or by mitochondrial perturbation resulting in cytochrome c release and activation of the Apaf-1 containing caspase-activating apoptosome complex[16,17] (Figure 3). Both pathways generally involve the activation of a family of aspartate-specific cysteine proteases, caspases, which dismantle the cell by cleaving key structural and regulatory molecules.[18–20] Death receptor-induced apoptosis involves initial formation of a death-inducing signalling complex (DISC) and subsequent activation of caspase-8 as the apical caspase. Less is known about the events that lead to mitochondrial perturbation and cytochrome c release during stress-induced apoptosis, although cytochrome c release is controlled partly by members of the Bcl-2 family. The ubiquitin-proteasome system is an important regulator of cellular sensitivity to apoptosis as it regulates the levels of many proteins involved in the control of apoptosis including some members of the Bcl-2 family, inhibitor of apoptosis proteins (IAPs) and some caspases.[12,21–25] Many currently used anticancer agents exert their anticancer effects by inducing apoptosis, and resistance to apoptosis contributes to drug resistance and tumour progression. Therefore, clinical manipulation of the proteasome could potentially be useful in the treatment of cancer.

Proteasome inhibitors are cytotoxic to cancer cells and cytoprotective to some but not all quiescent cells

Proteasome inhibitors have helped to elucidate cellular functions of the proteasome (Table 1).[26] Probably the most widely used of these are peptide aldehydes, such as MG132 (Figure 2), however, caution should be used in the interpretation of results from studies using these agents as they also inhibit cathepsins and calpains.[26,27] The natural product lactacystin (Figure 2) and its active metabolite *clasto*-lactacystin β-lactone have a much higher specificity for the proteasome but can also inhibit cathepsin A.[26,28–30] More recently, new boronic acid peptide inhibitors, including the dipeptidyl

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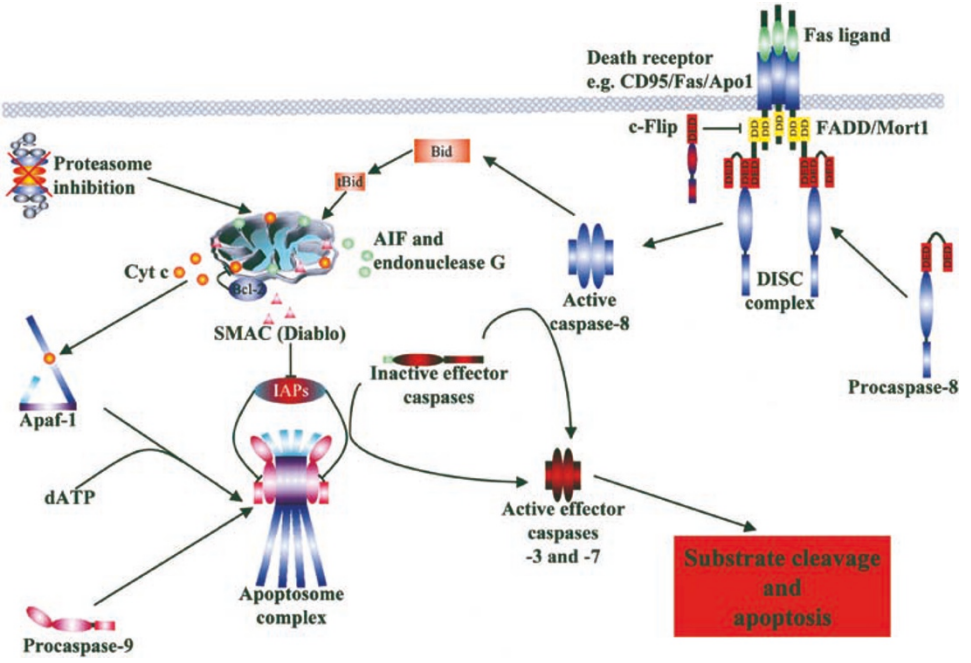

Figure 3 Mechanisms of induction of apoptosis. There are two major pathways for induction of apoptosis. One involves cell surface death receptors, such as CD95, which activate caspase-8 as the apical caspase. The other pathway involves initial perturbation of mitochondria with release of pro-apoptotic molecules, such as cytochrome c, Smac or endonuclease G, from the inter mitochondrial membrane space. Cytochrome c binds to Apaf-1 causing a conformational change leading to formation of an Apaf-1 containing apoptosome complex. This recruits and activates caspase-9 as the apical caspase, which in turn recruits and activates effector caspases-3 and -7. Inhibitors of apoptosis (IAPs) may modulate both initiator and effector caspase activity. There can be cross-talk between the mitochondrial and death receptor pathways of apoptosis. Proteasome inhibitors appear to induce apoptosis primarily by perturbation of mitochondria.

boronic acid, PS-341 (Figure 2), have been developed which are very potent and specific inhibitors of the proteasome.[31,32] PS-341 is currently under investigation in clinical trials as an anticancer agent.

Proteasome inhibitors induce apoptosis in some cells and seem to protect in others (Tables 2 and 3). As a generalization they appear to induce apoptosis in proliferating cells and are protective in quiescent or terminally differentiated cells.[33] However, there are notable exceptions to this generalization as proteasome inhibitors are potent inducers of apoptosis in malignant B cell chronic lymphocytic leukaemia (B-CLL) cells, which are predominantly quiescent.[34–39]

Proteasome inhibitors induce apoptosis and overcome drug resistance in many different types of cancer cell lines, and in primary leukaemic cells from patients with B-CLL (Table 2). In the National Cancer Institute (NCI) pre-clinical screen, the proteasome inhibitor PS-341 (Table 1, Figure 2) is cytotoxic to a broad range of the 60 human tumour cell lines used, and interestingly, its mechanism of cytotoxicity differs from the other 60 000 compounds in the NCI database.[40] These results clearly highlight the potential of proteasome inhibitors to act as novel anticancer agents.

Proteasome inhibitors protect thymocytes and some other cells from apoptosis induced by some but not all stimuli (Table 3). Precisely where they inhibit apoptosis is not clear but it is most likely upstream of mitochondrial perturbation[41] and effector caspase activation as caspase-3 processing and activity are partially blocked as evidenced by inhibition of processing of caspase-3 and cleavage of poly (ADP-ribose) polymerase (PARP).[42–45] In cell death pathways like these, where the proteasome has an important role in initiating apoptosis, it may act by degrading anti-apoptotic regulatory proteins or other proteins necessary for cell survival. In some cells proteasome inhibitors appear to induce both pro- and anti-apoptotic signals, the predominant signal determining the fate of the cell.[46] For example, activation of the stress kinase JNK (c-Jun N-terminal kinase) was critical for induction of apoptosis whereas the concomitant accumulation of Hsp72 protected against apoptosis (Table 3). Prolonged exposure to proteasome inhibitors results in cell death even in cells where they are initially protective, most likely due to a disruption of cellular metabolism following deregulation of proteolysis. Regardless of mechanism, the reduced toxicity of proteasome inhibitors to normal cells such as thymocytes compared to many tumour cell lines could be valuable clinically, as it may signify that they have a lower toxicity to non-malignant cells. In addition, different classes of proteasome inhibitors, with differing effects on the various proteasomal activities, may also preferentially inhibit the degradation of certain groups of proteasome substrates. Therefore, in a clinical setting, careful selection of proteasome inhibitors and their doses may allow selective induction of growth arrest and apoptosis in cancer cells.

Mechanism(s) of proteasome inhibitor-induced apoptosis

We have highlighted the potential of proteasome inhibitors to act as novel anticancer agents. Their ability to induce apoptosis of cancer cells is of key importance for this activity. The mechanism(s) through which these agents induce apoptosis is unclear and different mechanisms seem to be important in different cells. We will now discuss some of the major mechanisms that have been implicated in various tumour cells (Figure 4).

Table 2 Proteasome inhibitors induce apoptosis *in vitro*

Cell type	*Inhibitor*	*Observed effects/mechanism after treatment*	*Refs*
U937 human monoblast leukaemia	Lactacystin	Condensed nuclei and DNA fragmentation	83
MOLT-4 human T cell leukaemia	MG132	↑ p53	84
HL60 human leukaemia Proliferating primary endothelial cells	LLnV PSI	↑ p27 and induce apoptosis in proliferating cells Less potent inducers of apoptosis in quiescent/differentiated cells	33, 55
Rat-1 fibroblasts PC12 pheochromocytoma cells	PSI LLnV	↑ p53 (with p21 and Mdm-2 in Rat-1 cells) Dominant negative p53 protects and wild-type p53 induces apoptosis. Overexpression of Bcl-2 inhibits apoptosis. Quiescent Rat-1 cells insensitive to apoptosis, whereas quiescent differentiated PC-12 cells sensitive	51
Activated/proliferating T cells and Jurkat T cell leukaemia Bcl-2 overexpressing Jurkat cells	Lactacystin LLnV	Inhibits mitogen-induced T cell proliferation Do not induce apoptosis in quiescent T cells Activation of JNK and p38 (independent of caspase activation) ↑ Bax prior to cytochrome c release	11, 24, 59
Jurkat and HL-60 human leukaemia cell lines	CEP1612	Induces apoptosis in Jurkat cells overexpressing Bcl-2	52, 54, 78
Human prostate, breast, lung, tongue and brain tumour cell lines Normal and SV40 transformed human fibroblasts	LLnV	Resistant to etoposide MDA-MB-231 breast cancer cells – ↑p21 and p27 Induced apoptosis and ↑p27 selectively in SV40 transformed cells.	
K562 human chronic myelogenous leukaemia	LLnV Lactacystin	↓Bcr-Abl expression and activity Cells have multiple drug resistance	
Human prostate carcinoma (LNCaP, PC-3)	ALLN ALLnM	Independent of p53 Not inhibited by overexpression of Bcl-2 Stabilization of c-jun, no activation of JNK	53
Human prostate, colorectal, breast, squamous cell carcinoma and multiple myeloma cancer cells NCI screen – 60 human tumour cell lines	PS-341	Cytotoxic to many human tumour cells Cell cycle arrest and ↑ p21 and ↑ p27 Inhibits NF-κB activity, pro-angiogenic cytokines and adherence to bone marrow stromal cells Potentiates SN-38- growth-inhibitory effects by inhibiting NF-κB	40, 68, 69, 71, 77
REF, REF-ras/myc fibroblasts CB33, CB33-myc lymphoblasts Ramos human Burkitt's lymphoma	Z-LLF	Transformed REF-ras/myc, CB33-myc, and Ramos cells are much more sensitive to induction of apoptosis	58
B cell chronic lymphocytic leukaemia – patient samples	Lactacystin ALLN MG132	Overcome drug and radio resistance Sensitize-B-CLL cells to TNF-α induced apoptosis Aberrant ubiquitin-proteasome system in B-CLL cells. Inhibit NF-κB activity Lactacystin specifically induces apoptosis in B-CLL cells but not normal lymphocytes Induce activation of caspases-9, -3, -7, -8 and -2	34–39

SPOTLIGHT

Proteasome inhibitors increase p53 activity

Mutations in the p53 gene are common in many types of cancer resulting in failure to express p53 or inactivation of its transcriptional activity.[47] These mutations contribute to both tumour progression and drug resistance. The tumour suppressor p53 is a short-lived transcription factor that is post-translationally regulated by the ubiquitin-proteasome pathway.[48] In response to cellular stress or DNA damage, wild-type p53 protein is activated by phosphorylation and other signals that cause it to dissociate from its inhibitor MDM2. Once activated, p53 is no longer targeted for proteasomal degradation by MDM2 and therefore accumulates and binds to specific sequences in DNA, initiating transcription of genes that induce growth arrest, DNA repair or apoptosis. Growth arrest is induced via expression of genes such as the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$. Several genes including Bax, NOXA, a pro-apoptotic BH3-only containing protein, P53AIP1, PIDD a new death domain-containing protein, and the death receptors CD95 (Fas/Apo-1) and DR5 (TRAIL-R2, KILLER), are upregulated by p53 and could contribute to its proapoptotic effects.[49,50] In malignancies, such as human papilloma virus (HPV)-related cancers, when p53 is inactivated due to increased degradation by the ubiquitin-proteasome pathway,[13] restoration of p53 status by proteasome inhibitors could have therapeutic potential. In some cancer cells proteasome inhibitors cause the stabilization and accumulation of p53 (Table 2, Figure 4). For example, treatment of Rat-1 fibroblasts and PC12 cells with proteasome inhibitors causes an accumulation of transcriptionally active p53, which is required for apoptosis as overexpression of wild-type or dominant negative p53 induces or protects

Table 3 Cytoprotective effects of proteasome inhibitors *in vitro*

Cell type	*Inhibitor*	*Observed effects/mechanism after treatment*	*Refs*
Thymocytes (murine and rat)	Lactacystin MG132 ALLN ALLnM	Protect against apoptosis induced by ionizing radiation, glucocorticoids or etoposide but not by staurosporine or phorbol ester and calcium ionophore Prevent disruption of mitochondrial transmembrane potential Prolonged exposure to proteasome inhibitors induce cell death ↑ p53 with both etoposide and proteasome inhibitors	41–43, 85
U937 and HL60 human leukaemia	MG132 ALLN	Protect against etoposide-induced apoptosis Prevent NFκB activation Proteasome inhibitors alone induce apoptosis in leukaemia blasts from patients with acute leukaemia	45
Sympathetic neurons (rat)	Lactacystin PSI	Protect against NGF-withdrawal induced apoptosis Prolonged exposure to proteasome inhibitors results in cell death	44
U937 leukaemia and 293 kidney human tumour	MG132 Lactacystin ALLN	↑ JNK activity is a pro-apoptotic signal ↑ Hsp72 protects	46
AT3	MG132 Lactacystin	High concentrations induce apoptosis Low concentrations protect against Sindbis virus-induced apoptosis by inhibiting NFκB activation	86

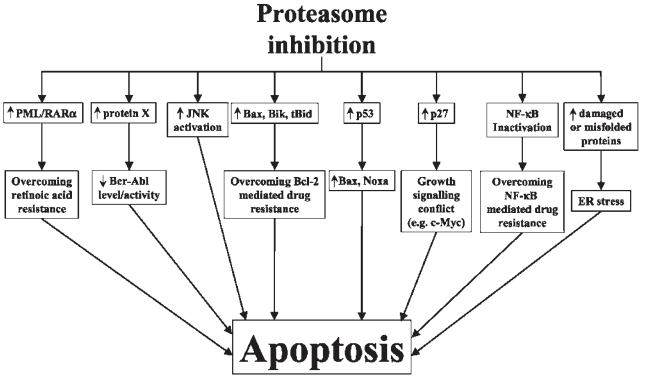

Figure 4 Mechanisms of induction of apoptosis by proteasome inhibitors. Proteasome inhibitors may induce apoptosis in different cells by many different mechanisms.

against apoptosis, respectively.[51] However, not all proteasome inhibitor-induced apoptosis is p53 dependent as these agents potently induce apoptosis in various tumour cell lines bearing p53 mutations including PC-3, Jurkat, HL-60, K562 and MDA-MB-231 cells (Table 2).[52–54] Thus, proteasome inhibitors induce p53-dependent apoptosis in some but not all cells.

Accumulation of the growth inhibitory molecules p27 and p21

Proteasome inhibitors generally induce apoptosis in proliferating but not in quiescent cells (Tables 2 and 3). HL60 human leukaemia cells undergoing apoptosis in response to treatment with proteasome inhibitors are predominantly in the G_1 phase of the cell cycle and accumulate the cyclin-dependent kinase inhibitor $p27^{Kip1}$ as do proliferating endothelial cells after proteasomal inhibition.[33,55] The growth inhibitory molecules $p27^{KIP1}$ and $p21^{WAF1}$ are short-lived proteins that induce growth arrest by inhibiting cyclin-dependent kinases that drive progression through the cell cycle. Cellular levels of $p27^{KIP1}$ are subject to control by the ubiquitin-proteasome system.[56] Correlations are observed between a poor prognosis in colorectal carcinomas and breast cancer and low levels of $p27^{KIP1}$ possibly due to increased proteasomal degradation.[13,57] Apoptosis in some cells such as MDA-MB-231 cells induced by the dipeptidyl proteasome inhibitor CEP1612 is accompanied by an accumulation of the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$, together with the possible appearance of their ubiquitinated forms.[52] In this study proteasome inhibitors selectively induce apoptosis in SV40 transformed but not normal fibroblasts although accumulation of $p21^{WAF1}$ occurs in both cells. Thus accumulation of $p21^{WAF1}$ may be involved in, but is not sufficient for, induction of apoptosis. However, after treatment $p27^{KIP1}$ levels increase to a greater extent in the transformed cells than in the parental cells, consistent with induction of apoptosis, indicating that accumulation of $p27^{KIP1}$ above a threshold level may be sufficient to induce apoptosis.[52] The accumulation of growth inhibitory molecules such as $p27^{KIP1}$, in the context of proliferative signals like c-Myc, might result in a signalling conflict for the cell that is resolved by apoptosis[33] (Figure 4). This could explain the marked sensitivity to proteasome inhibitor-induced apoptosis of cells with high expression of c-Myc.[58] However, cell cycle status is not the sole determinant of the cell's response to proteasome inhibition, as non-proliferating differentiated PC12 cells remained sensitive.[51]

Proteasome inhibitors cause accumulation of pro-apoptotic Bcl-2 family proteins

In cancer cells there is often a defect in the apoptotic signalling pathways giving the malignant cells a survival advantage and conferring resistance to conventional anticancer agents. In several human cancer cell lines, including prostate carcinoma and the Jurkat T cell leukaemia cells, proteasome inhibitors can overcome Bcl-2 protection to induce apoptosis in

otherwise resistant cells (Table 2).[52,53] Bcl-2 overexpression protected against other cell death-inducing agents, demonstrating that it was functionally active. In Jurkat cells, the proteasome inhibitors overcome the protective effects of Bcl-2 overexpression by inhibiting degradation of Bax, resulting in an accumulation of this pro-apoptotic molecule prior to cytochrome c release and PARP cleavage.[24] This study also demonstrated that the degradation of Bax was increased in aggressive human prostate cancer, correlating with decreased levels of Bax protein detected in these malignant cells. These results are in agreement with another study showing that Bax but not Bcl-2 is subject to proteasomal degradation.[22] As other pro-apoptotic Bcl-2 family members, such as Bik[25] and tBid[23] are also proteasome substrates, inhibitors of the proteasome could possibly induce apoptosis by causing an accumulation of pro-apoptotic Bcl-2 family members (Figure 4). However, overexpression of Bcl-2 protects Rat-1 fibroblasts against apoptosis induced by peptide aldehydes, suggesting the importance of alternative mechanisms.[51]

Proteasome inhibitors can activate stress-activated protein kinases (SAPK)

In some cancer cell lines, treatment with proteasome inhibitors induced the activation of the stress-activated protein kinases (SAPK), c-Jun N-terminal kinase (JNK) and p38 (Tables 2 and 3),[46,59] which can have pro- or anti-apoptotic effects depending on the cellular context.[60] The involvement of p38 in proteasome inhibitor-induced apoptosis was ruled out by the use of specific inhibitors of this kinase, however, JNK activity was shown to be important for apoptosis induced by these agents.[46,59] This is in agreement with other studies showing that in some instances JNK translocates to the mitochondria during apoptosis and attenuates the cytoprotective effects of Bcl-X_L[61] and that JNK activity is required for cytochrome c release in response to certain stimuli.[62] JNK functions as the upstream regulator of c-Jun, which is a member of the AP-1 family of transcription factors and is also post-translationally regulated by the ubiquitin-proteasome pathway. In human prostate carcinoma cells, elevated levels of c-jun are detected after treatment with proteasome inhibitors, which induce cell death in these cells.[53] However, despite indications that the up-regulated c-jun protein is phosphorylated and activated, there is no evidence that JNK is phosphorylated and consequently activated in response to proteasome inhibitor treatment, although JNK activity was not directly measured in this study. Nevertheless, these results indicate that activation of the JNK signalling pathway could be an important event in the induction of apoptosis by proteasome inhibitors in some cells (Figure 4).

Proteasome inhibitors overcome the anti-apoptotic effects of NF-κB

NF-κB controls the expression of cytokines and cell adhesion molecules involved in immune and inflammatory responses. The activation of NF-κB can also suppress apoptosis by increasing the expression of survival signals including IAPs, TRAF1, TRAF2, and the Bcl-2 family homologue Bfl-1/A1.[27,63–67] This may present a problem in cancer chemotherapy, as some anticancer agents and irradiation promote the activation of NF-κB possibly resulting in drug resistance. Proteasome inhibitors can inhibit NF-κB transcriptional activity by preventing IκB degradation so possibly overcoming drug resistance by sensitizing the cells to apoptosis. Proteasome inhibitors prevent IκB degradation at concentrations that do not affect the breakdown of the majority of short-lived proteins,[46] so reducing the probability of side-effects. Thus a combination of a proteasome inhibitor and certain chemotherapeutic agents should result in increased cytotoxicity. Such effects are observed in human colorectal cancer cells and xenografts with the DNA topoisomerase I inhibitor SN-38, the active metabolite of CPT-11 (irinotecan), and the proteasome inhibitor PS-341, when combination therapy produces a significantly higher growth inhibition than is observed with either agent alone (Tables 2 and 4).[68]

NF-κB activity also controls the expression of genes that are involved in tumour metastasis and angiogenesis.[8] Therefore, proteasome inhibitors have the potential to inhibit some of the systemic changes that occur during cancer. Evidence of this was observed in squamous cell carcinoma cells where proteasome inhibitors prevent the expression of the NF-κB-dependent, pro-angiogenic cytokines vascular endothelial growth factor (VEGF) and growth-regulated oncogene-α.[69] Proteasome inhibitors are also potent anti-angiogenic agents *in vivo*.[55,70] They prevent the adherence of multiple myeloma cells to bone marrow stromal cells and the NF-κB-dependent production of IL-6 in these cells, both of which promote tumour survival in multiple myeloma.[71] These results indicate that in addition to their effects on malignant cells, proteasome inhibitors could aid in the treatment of cancer by targeting some of the accessory cells that contribute to malignant disease. Therefore the ability of proteasome inhibitors to inhibit NF-κB may be an important property in their effectiveness against angiogenesis and metastasis, and their potentiation of other cytotoxic agents observed *in vivo* (Table 4, Figure 4).

Proteasome inhibitors circumvent mechanisms of drug resistance

Proteasome inhibitors can overcome various types of drug resistance in cancer cells. They potently induced apoptosis in B-CLL cells that are resistant to conventional anticancer therapies[34,35,39] and caused a reduction in the cellular levels and activity of Bcr-Abl in K562 chronic myelogenous leukaemia cells resulting in cell death (Table 2, Figure 4).[54] There is also evidence that they can overcome resistance to retinoic acid (RA) in acute promyelocytic leukaemia (PML) cells, possibly by inhibiting the proteasomal degradation of the PML/RARα fusion protein[72] (Figure 4). A recent study indicated that variations in proteasomal activity could in fact alter the sensitivity of human cells to cytotoxic treatments. The *pad1*$^+$ gene confers resistance to staurosporine and other drugs when overexpressed in *Schizosaccharomyces pombe (S. pombe)*. Identification of POH1, the functional human homologue of pad1, revealed that this protein was a previously unidentified component of the 19S regulatory cap of the 26S proteasome, expressed in many human tissues with highest levels in heart and skeletal muscle. Overexpression of POH1 in mammalian cells resulted in resistance to chemotherapeutic drugs and UV light, via a mechanism that possibly involved modulation of proteasomal activity and proteolysis of AP-1 transcription factors.[73] In such a situation proteasome inhibitors could possibly be used to restore sensitivity to conventional chemotherapeutic agents.

Table 4 Anticancer and anti-angiogenic activity of proteasome inhibitors

Cell type	*Inhibitor*	*Observed effects/side-effects after treatment*	*Refs*
Ramos human Burkitt's lymphoma in mice	Z-LLF	Single interscapular injections well tolerated. Decrease in tumour mass and 42% delay in tumour growth. Induction of apoptosis in tumour sections	58
PC-3 human prostate carcinoma in mice	PS-341	60% decrease in tumour burden after weekly intravenous treatment – penetrates tumours and inhibits proteasome activity Direct injection into tumours most effective	40
Toxicity in primates and rodents	PS-341	Gastrointestinal toxicity main side-effect without other signs of toxicity	40
Squamous cell carcinoma in mice	PS-341	Inhibits tumour growth and angiogenesis without toxicity	69
EMT-6 mammary carcinoma Lewis lung carcinoma in mice	PS-341	Cytotoxic to tumour cells when administered orally or intraperitoneally Antitumour effects against primary and metastatic disease. Some toxicity to bone marrow colony-forming unit granulocyte–macrophage Combination therapy did not sensitize drug-resistant EMT-6 tumours	77
LOVO human colorectal cancer in mice	PS-341	Combination therapy with the DNA topoisomerase-I inhibitor CPT-11 results in a 94% decrease in tumour size in comparison to controls	68
A-549 human lung adenocarcinoma in mice	CEP1612	Daily intraperitoneal treatment at tolerated doses for 31 days induce apoptosis and inhibit tumour growth ↑21 and ↑27	78
Embryonic chick chorioallantoic membrane (CAM)	PSI Lactacystin	Induces apoptosis in endothelial and other cells causing collapse of capillaries and first order vessels forming of areas devoid of blood flow Prevents neovascularization Prevents *in vitro* production of plasminogen activator by endothelial cells	55, 70

SPOTLIGHT

Proteasome inhibitors could induce an 'unfolded protein response'

An additional function of the 26S proteasome is to degrade improperly folded or damaged cellular proteins. Proteasome inhibitors could induce an accumulation of such damaged proteins, which may cause a toxic 'unfolded protein response',[8] resulting in activation of endoplasmic reticulum (ER) stress-induced signalling pathways and induction of apoptosis[49] (Figure 4).

Proteasome inhibitors induce activation of caspases

Initial cellular exposure to proteasome inhibitors appears to result in a perturbation of mitochondria with concomitant release of cytochrome c. Cytochrome c binds to Apaf-1, causing a conformational change, which then allows Apaf-1 to oligomerize in the presence of dATP/ATP so forming the Apaf-1 containing apoptosome complex.[16,17,74,75] The apoptosome then recruits and activates the apical caspase-9 followed by activation of effector caspases-3 and -7, which are responsible for the biochemical and morphological changes associated with apoptosis.[17,76] In B-CLL cells, a broad-spectrum caspase inhibitor completely blocks activation of caspases and other associated biochemical changes further supporting the involvement of caspases in proteasome inhibitor-induced apoptosis.[34]

Antitumour activity of proteasome inhibitors *in vivo*

Over the last few years several studies have also investigated the anticancer potential of proteasome inhibitors *in vivo*. They exhibit marked growth inhibitory and antitumour effects *in vivo* against both primary and metastatic disease (Table 4). In an initial study, Z-LLF-CHO induced early tumour regression and a delay in tumour progression in a murine model of Burkitt's lymphoma without any unacceptable toxicity.[58] More recent studies have focussed on the dipeptidyl boronic acid PS-341 (Table 1, Figure 2), due to its potent anticancer effects and unique toxicity profile observed in the NCI preclinical assay.[40] PS-341 inhibited proteasomal activity in subcutaneously implanted tumours after intravenous administration, demonstrating that it exerts its predicted pharmacological activity at its site of action *in vivo*.[40] Importantly, the effects of some conventional anticancer therapies are also potentiated when used in combination with proteasome inhibitors,[68,77] most probably due to the ability of these agents to inhibit NFκB survival signals and sensitize the cells to apoptosis as discussed earlier. Accumulation of the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ is observed in cancer cells *in vivo*,[78] further demonstrating the bioavailability of the proteasome inhibitors *in vivo*. Proteasome inhibitors are also potent anti-angiogenic agents *in vivo* (Table 4), which could contribute to their overall effectiveness as potential anticancer agents. From a toxicological perspective, the observed effects of proteasome inhibitors *in vivo* are achieved at doses that are well tolerated by the animals used in the

studies. A full toxicological evaluation of PS-341 was performed in primates and rodents, revealing that gastrointestinal toxicity was the main adverse side-effect without other overt signs of toxicity.[40] However, it may be toxic to the bone marrow colony-forming unit granulocyte–macrophage (CFU-GM).[77] In addition, the proteasome processes MHC class-I restricted antigens and has an important role in antigen presentation. Antigen presentation appears altered in malignant cells possibly due to modified proteasomal activity and this aids them in escape from immune surveillance. Reduced levels of proteasome subunits have been reported in small cell lung carcinoma, mouse T cell lymphoma lines, a primary renal cancer and in a lymph node metastasis from the same patient in comparison with normal kidney.[13] Thus interference in normal proteasome function of antigen presentation may lead to unwanted immunological toxicity.

Recently, Ritonavir, an HIV-1 protease inhibitor used in the treatment of HIV and AIDS, was reported to selectively inhibit the chymotrypsin-like activity of the 20S proteasome at clinically relevant doses.[79] The antitumour drug vinblastine also inhibits proteasomal activity at concentrations close to those achieved clinically.[80] Taken together, these results indicate that some level of proteasome inhibition is permissible in humans, at clinically achievable concentrations. Due to the relative success of these early studies, proteasome inhibitors are currently under investigation in phase II clinical trials. In the next few years, we shall begin to see whether the early promise of proteasome inhibitors as potential novel anticancer agents can be realized in the clinic.

Are proteasome inhibitors selectively toxic to cancer cells?

Although proteasome inhibitors can clearly kill cancer cells both *in vitro* and *in vivo*, do they exert any selectivity in their toxicity? Some studies have proposed that they may be selectively toxic to tumour cells. The increased proliferation rate of some tumour cells may make them more susceptible to proteasome inhibitor-induced apoptosis and S phase cells are often more susceptible than growth-arrested tumour cells. Whilst this may be true in some cells, the selectivity is not solely due to proliferative status, as both transformed and normal fibroblasts have similar growth rates although the proteasome inhibitors are selectivity toxic to SV40-transformed but not normal fibroblasts.[52] Their potency is increased in cells with high expression of c-Myc, possibly leading to a conflict of survival and death signals as discussed earlier. This is clinically relevant as the c-myc gene is mutated in many tumours including breast, colorectal and gynaecological carcinomas and Burkitt's lymphomas.[58] Alternatively the tumour-specific killing by proteasome inhibitors may be due to expression of oncogenes, such as c-myc, that deregulate cell proliferation and also induce apoptosis. Oncogene expression might generate a pro-apoptotic signal that is present only in transformed and tumour cells but not in normal cells. This pro-apoptotic signal could be regulated by the proteasome. In this regard the Apaf-1, caspase-9 containing apoptosome complex is known to regulate oncogene-dependent apoptosis.[81] Alternatively, in some cancer cells there may be either deficiencies in, or excessive proteasomal degradation of proteins that inhibit cell growth, safeguard genetic integrity or induce apoptosis, such as $p27^{KIP1}$, p53 and Bax.[13,24,57] Such abnormalities could be crucial for tumour cell survival and proteasome inhibitors could elevate the levels of these short-lived proteins so restoring apoptosis selectively in these cells.

Studies particularly by Delic and co-workers[35] have suggested that proteasome inhibitors may be selectively toxic to B-CLL cells compared to normal lymphocytes. Although initially proposed to involve an inhibition of NF-κB, this was subsequently shown not to be involved.[36] More recently, this group have also demonstrated that only specific proteasomal inhibitors, lactacystin and its active metabolite *clasto*-lactacystin β-lactone, induce selective apoptotic death in B-CLL cells and not in normal lymphocytes whereas non-specific inhibitors such as MG132 and LLnL (which also inhibits calpains) induce death equally in both cell types.[37] B-CLL cells have a constitutively higher chymotrypsin-like proteasomal activity compared to normal human lymphocytes and an altered proteolytic regulation of p53.[37] p53 is degraded primarily by calpains in normal lymphocytes and by the proteasome in B-CLL cells. Thus the constitutively higher chymotryptic activity observed in B-CLL cells might indicate increased degradation of p53. Consistent with this, lactacystin caused accumulation of p53 and apoptosis in B-CLL cells but not in normal lymphocytes.[37] Other related studies have shown that proteasome inhibitors induce apoptosis even in B-CLL cells, which are resistant to conventional chemotherapeutic agents, such as chlorambucil and prednisolone.[34,39]

As well as causing an accumulation of growth inhibitory or cell death signals, proteasome inhibitors also inhibit survival signals such as Bcr-Abl[54] and NF-κB.[69] A further possibility that cannot be excluded is that alterations in proteasomal activity are important in the onset of malignant disease, and may make cancer cells more susceptible to proteasome inhibitor-induced apoptosis.

It is also well known that cell cycle check point and DNA repair control systems are defective in cancer cells, and when placed under stress by a proteasome inhibitor, cancer cells but not normal cells may be unable to correct the cell cycle transition blockade and are driven into apoptosis. Further understanding of the molecular basis for the selectivity of proteasome inhibitors will aid in their development as anticancer agents.

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