

Comparative Selectivity and Specificity of the Proteasome Inhibitors BzLLCOCHO, PS-341, and MG-132

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Abstract

The 26S proteasome is a multicatalytic protease responsible for regulated intracellular protein degradation. Its function is mediated by three main catalytic activities: (a) chymotrypsin-like (CT-L), (b) trypsin-like, and (c) peptidylglutamyl peptide hydrolysing (PGPH). Proteasome inhibition is an emerging therapy for many cancers and is a novel treatment for multiple myeloma. Here, we profile the contributions of the three catalytic activities in multiple myeloma cell lines and compare the specificity and cytotoxicity of the novel proteasome inhibitor BzLLCOCHO and inhibitors PS-341 (Velcade, bortezomib) and MG-132. Using fluorogenic substrates and an active site-directed probe specific for proteasome catalytic subunits, we show differential subunit specificity for each of the inhibitors. Addition of BzLLCOCHO strongly inhibited all three catalytic activities, treatment with PS-341 completely inhibited CT-L and PGPH activities, and treatment with MG-132 resulted in weak inhibition of the CT-L and PGPH activities. Multiple myeloma cells were more sensitive to induction of apoptosis by PS-341 and MG-132 than BzLLCOCHO. This study emphasizes the need for further investigation of the effects of these compounds on gene and protein expression in the cell to allow for the development of more specific and targeted inhibitors. (Cancer Res 2006; 66(12): 6379-86)

Introduction

The ubiquitin proteasome pathway plays a central role in the regulation of protein degradation and maintenance of normal cell function (1-3). Proteins destined for proteolysis are tagged by the attachment of a polyubiquitin chain and subsequently degraded by the 26S proteasome. The 26S proteasome is a large multi-subunit complex composed of a central 20S catalytic core and two 19S regulatory caps. The 20S core particle is a cylindrical structure consisting of two outer heptameric rings of α subunits and two inner heptameric rings of β subunits. The three main catalytic activities of the proteasome, peptidylglutamyl peptide hydrolysing (PGPH), trypsin-like (T-L), and chymotrypsin-like (CT-L), are mediated by three distinct catalytic β 1, β 2, and β 5 subunits, respectively (4). On exposure to IFN- γ , the activity of the 20S catalytic core can be modified. IFN- γ induces antigen-presenting cells to express β -type subunits β 1i, β 2i, and β 5i, which replace β 1, β 2, and β 5, and the

19S caps are replaced by the 11S regulatory complex to form the immunoproteasome (5). The immunoproteasome is involved in the generation of antigenic peptides for presentation by major histocompatibility complex class I molecules (6).

Disregulation of the ubiquitin proteasome pathway has been implicated in the pathogenesis of many diseases, including cancer (7, 8). The proteasome has recently emerged as an attractive target for anticancer therapy. For example, clinical efficacy of proteasome inhibitors has been shown in multiple myeloma. PS-341 (Velcade, bortezomib), the first proteasome inhibitor to enter clinical trials, has been approved for the treatment of relapsed and refractory multiple myeloma (9) and is undergoing further clinical trials in previously untreated multiple myeloma, both as a single agent and in combination with conventional therapies (10, 11). The majority of proteasome inhibitors, including PS-341, are directed against CT-L activity, traditionally considered the rate-limiting step in protein degradation. Consequently, inhibitor potency has often only been measured against this activity. Despite much research into the substrate specificity of the individual catalytic activities, information about the involvement of the T-L and PGPH activities in the overall functioning of the complex is limited.

We have previously developed a method to extract proteasomes and to measure CT-L activity (12). In this study, we have extended this method to profile the relative contributions of the three catalytic activities of the proteasome in multiple myeloma cell lines. The specificity of the peptidyl α -keto aldehyde proteasome inhibitor BzLLCOCHO (13) and proteasome inhibitors PS-341 and MG-132 on individual proteolytic activities was investigated using conventional fluorogenic substrates. In addition, we used a novel active site-directed probe to label catalytic subunits of both the constitutive and immunoproteasome (14). Differential subunit specificities were found for each of the inhibitors toward subunits of the constitutive and immunoproteasome. Furthermore, inhibition of proteasome activity did not correlate with induction of apoptosis.

Materials and Methods

Cells and culture conditions. Multiple myeloma cell lines U266 and OPM-2 and cervical carcinoma cell line HeLa were purchased from the European Collection of Cell Cultures (Salisbury, United Kingdom). KMS-11 and KMS-18 cells were obtained from Dr. Otsuki (Kawasaki Medical School, Okayama, Japan). PS-341-resistant and PS-341-sensitive lymphoma cell lines SU(DHL-4) and SU(DHL-6) were as previously described (13). All cell lines with the exception of HeLa were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS and 10 μ g/mL penicillin/streptomycin (Life Technologies, Inc., Paisley, United Kingdom). KMS-11 and KMS-18 cells were cultured in the absence of penicillin and streptomycin. HeLa cells were cultured in DMEM supplemented with 10% heat-inactivated FCS and 10 μ g/mL penicillin-streptomycin.

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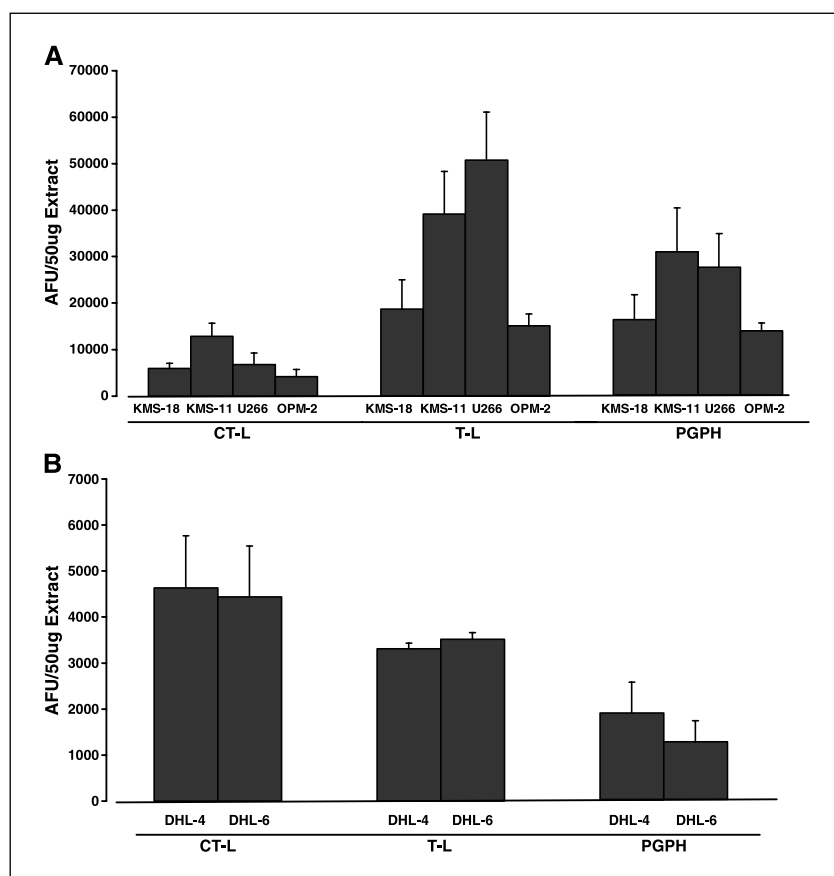


Figure 1. Proteasome activity is higher in multiple myeloma cell lines than in lymphoma cell lines. **A**, proteasome extracts were prepared from KMS-18, KMS-11, U266, and OPM-2 multiple myeloma cell lines. Levels of CT-L, T-L, and PGPH activities were measured using fluorogenic substrates specific for each activity and expressed as arbitrary fluorescence units. **B**, proteasome extracts were prepared from DHL-4 and DHL-6 lymphoma cell lines, and proteasome activity was measured as in (A). Columns, mean of three independent experiments; bars, SD.

Reagents. BzLLCOCO and the active site-directed probe (Dansyl-Ihx₃L₃VS) were synthesized as described previously (14, 15). PS-341 was supplied by Millennium Pharmaceuticals (Cambridge, MA), and MG-132 was obtained from Sigma (Dorset, United Kingdom). Fluorogenic substrates Z-ARR-AMC, Z-LLE-AMC (Calbiochem, Nottingham, United Kingdom), and Succ-LLVY-AMC (Sigma) and caspase-8 and caspase-9 inhibitors (Sigma) were reconstituted in DMSO as a 10 mmol/L stock solution and stored at -20°C .

Proteasome extraction. Cells (1×10^7) were harvested, resuspended in 1 mL ATP/DTT lysis buffer [10 mmol/L Tris-HCl (pH7.8), 5 mmol/L ATP, 0.5 mmol/L DTT, 5 mmol/L MgCl₂], and incubated on ice for 10 minutes, followed by sonication for 15 seconds. The lysates were centrifuged at $400 \times g$ for 10 minutes at 4°C , and the resulting supernatant containing proteasomes was stable at -80°C with the addition of 20% glycerol for at least 1 month (12). Protein concentrations of the samples were measured using the Bradford dye binding assay (Perbio Science, Northumberland, United Kingdom) with bovine serum albumin as a standard.

Enzyme assay. The fluorogenic substrates Succ-LLVY-AMC, Z-ARR-AMC, and Z-LLE-AMC were used to measure CT-L, T-L, and PGPH proteasome activities, respectively. Assays were carried out in a 200 μL reaction volume containing 50 μg proteasome extract, 50 mmol/L EDTA, and 50 $\mu\text{mol/L}$ fluorogenic substrate in ATP/DTT lysis buffer at 37°C . The rate of cleavage of fluorogenic peptide substrates was determined by monitoring the fluorescence of released aminomethylcoumarin using a Cytofluor Series 400 multiwell plate reader (Applied Biosystems, Warrington, United Kingdom) at an excitation wavelength of 395 nm and emission wavelength of 460 nm over a period of 35 minutes.

Western blotting. Multiple myeloma cells were cultured in the presence of each of the proteasome inhibitors as indicated, harvested, washed, and lysed in radioimmunoprecipitation assay lysis buffer (PBS,

1% Igepal CA-630, 0.1% SDS, 10% protease inhibitors). Equal amounts of protein were denatured by boiling in reducing sample buffer, separated by 4% to 12% SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. Immunoblotting was performed using an antibody against dansyl-sulfonamido-hexanoyl (Molecular Probes, Leiden, the Netherlands), caspase-8, or caspase-9 (Calbiochem). Blots were scanned into the AutoChem System (UVP, Cambridge, United Kingdom), and densitometry was performed using LabWorks 4.5 image acquisition and analysis software.

Cell viability and apoptosis assays. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Sigma). Apoptosis was measured using two methods: Hoechst 33342/propidium iodide staining (Calbiochem) and ApoAlert Mitochondrial Membrane Sensor kit (Clontech United Kingdom, Hampshire, United Kingdom). Hoechst 33342 assesses membrane integrity, and propidium iodide assesses nuclear morphology. Cells were incubated with Hoechst 33342 (5 $\mu\text{g/mL}$) in culture medium for 20 minutes at 37°C , pelleted, and resuspended in 1 mL PBS containing 10 μL propidium iodide (10 mg/mL). Apoptosis was assessed using a fluorescent microscope with a 4',6-diamidino-2-phenylindole band-pass filter at $\times 40$ magnification. ApoAlert detects changes in mitochondrial membrane potential using a cationic dye called Mitosensor. The kit was used according to the manufacturer's instructions. Cells were viewed under a fluorescent microscope using a band-pass filter to detect fluorescein and rhodamine at $\times 40$ magnification. For both methods, a minimum of 200 cells were scored for each sample.

Results

Profile of catalytic activities may be cell type dependent. Fluorogenic assays were used to investigate the level of the three

individual activities of the proteasome in multiple myeloma and lymphoma cell lines. Proteasome activity was measured by monitoring the release of the fluorophore aminomethylcoumarin (AMC) from peptide substrates specific for each activity (Suc-LLVY-AMC for CT-L, Z-ARR-AMC for T-L, and Z-LLE-AMC for PGPH). Activity was measured in four multiple myeloma cell lines (U266, OPM-2, KMS-11, and KMS-18), and the T-L and PGPH activities were found to account for a large proportion ($88 \pm 3\%$) of total proteasome activity (Fig. 1A). The rate of T-L activity was highest ($50 \pm 7\%$) followed by PGPH ($35 \pm 3\%$) and CT-L was the lowest ($13 \pm 3\%$). We next measured proteasome activity in the PS-341-resistant and PS-341-sensitive lymphoma cell lines (DHL-4 and DHL-6). The absolute CT-L activity in the lymphoma cell lines (4534 ± 685 AFU/50 μ g) was comparable with that observed in KMS-18, U266, and OPM-2 lines (5625 ± 1310 AFU/50 μ g); however, CT-L activity accounted for $48 \pm 1\%$ of total activity in lymphoma cell lines (Fig. 1B). These results suggest that the balance of catalytic activities may be dependent on cell type.

Subunit specificity of BzLLCOCHO. The fluorogenic assay was initially used to evaluate the effect of the inhibitor BzLLCOCHO on CT-L, T-L, and PGPH activities. Proteasome extracts were incubated with increasing concentrations (1, 5, and 10 μ mol/L) of the inhibitor, and fluorescence was monitored using a fluorescent plate reader to determine activity. BzLLCOCHO inhibited all three activities to a similar extent, with $<75\%$ of total activity achieved using 10 μ mol/L BzLLCOCHO (Fig. 2A). To determine whether BzLLCOCHO is cell permeable, cells were incubated with 1, 5, and 10 μ mol/L BzLLCOCHO for up to 24 hours before extracting proteasome, and fluorescence was monitored. Maximal inhibition was reached within 3 hours, and incubation with 10 μ mol/L BzLLCOCHO inhibited total proteasome activity in U266 cells by an average of $91 \pm 3\%$, indicating that the inhibitor is cell permeable (Fig. 2B).

To investigate the accuracy of the fluorogenic assay measurements, we used an active site-directed probe, DansylAhx₃L₃VS (15),

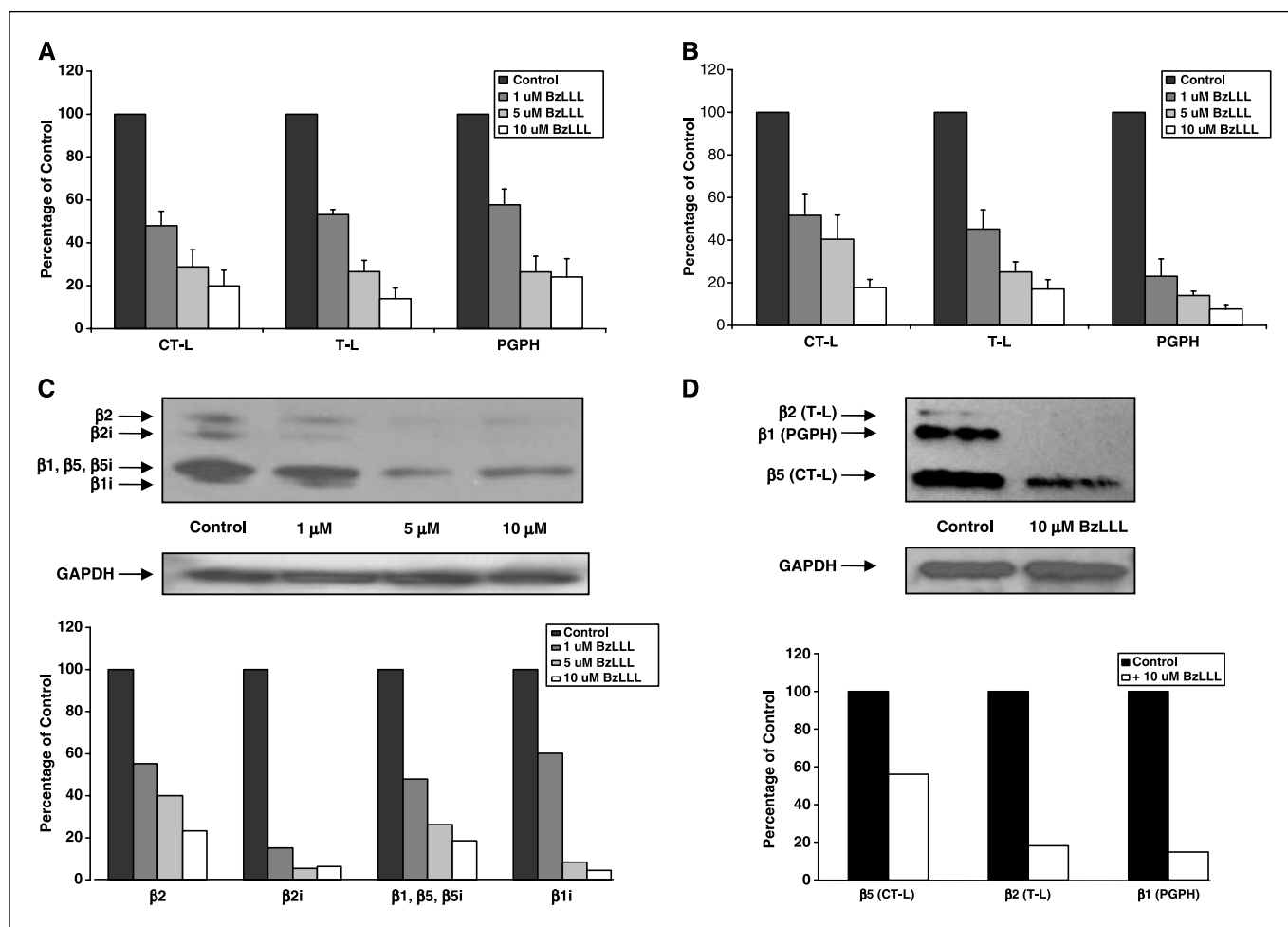


Figure 2. BzLLCOCHO is a cell-permeable inhibitor of catalytic activities of the constitutive and immunoproteasome. **A**, proteasome extracts were prepared from U266 cells, and extract (50 μ g) was assayed for the individual catalytic activities of the proteasome following addition of 1, 5, or 10 μ mol/L BzLLCOCHO. **B**, U266 cells were cultured in the presence of 1, 5, or 10 μ mol/L BzLLCOCHO for 24 hours at 37°C. Cells were harvested, and proteasome extracts were prepared as before. **A** and **B**, percentage change from untreated cells. Columns, mean of three independent experiments; bars, SD. **C**, U266 cells were cultured in the presence of 1, 5, or 10 μ mol/L BzLLCOCHO for 24 hours followed by 2 hours of incubation with DansylAhx₃L₃VS to label remaining active proteasome subunits. Cell lysates were prepared, and labeling of the individual catalytic β subunits was assessed by immunoblotting using an anti-dansyl antibody (1:1,000). **D**, HeLa cells were cultured in the presence of 10 μ mol/L BzLLCOCHO for 24 hours followed by 2 hours of incubation with DansylAhx₃L₃VS to label remaining active proteasome subunits. Cell lysates were prepared and subject to immunoblotting as in (**C**). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels confirm equal loading. Individual bands were quantified by densitometry and expressed as change from untreated controls (in %).

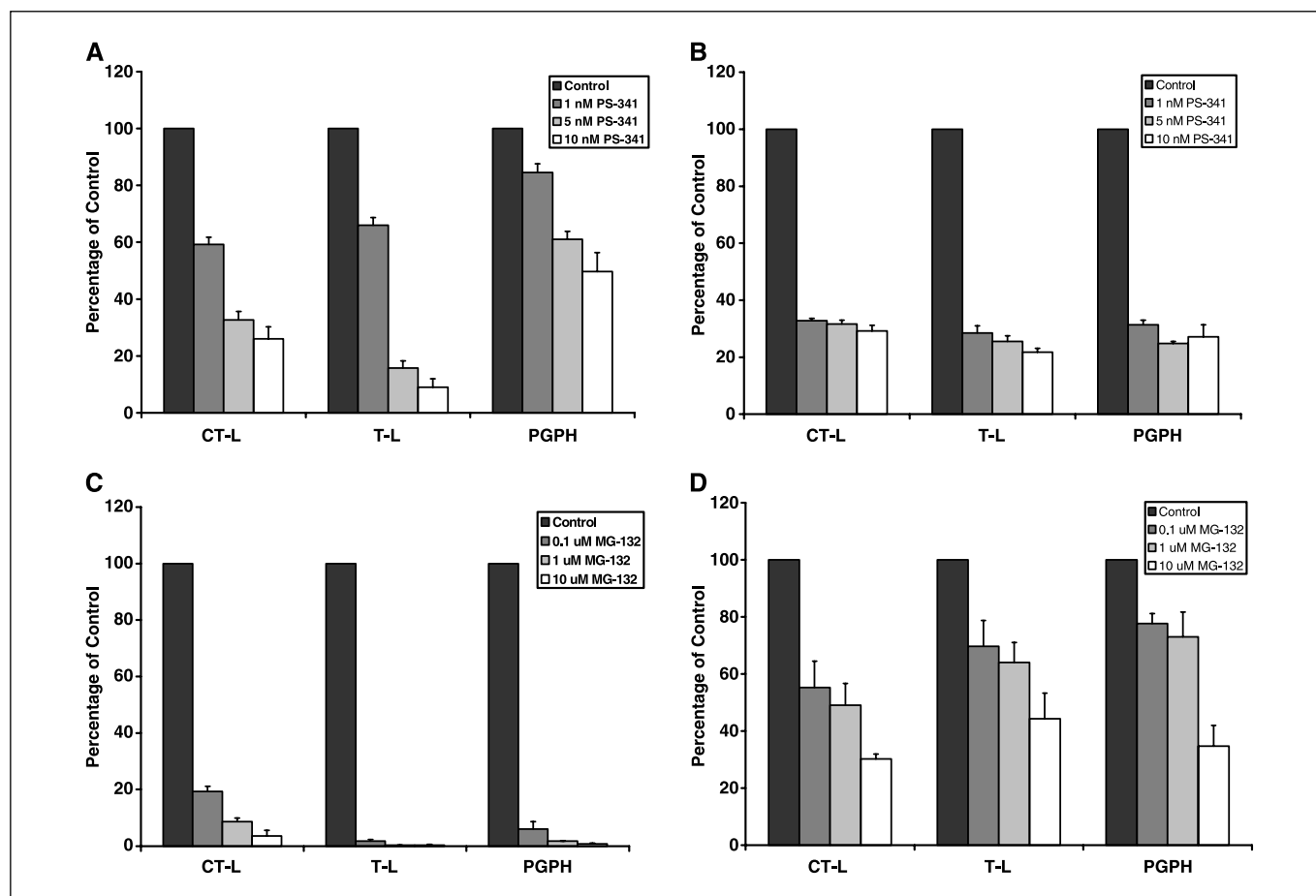


Figure 3. Inhibition of proteasome catalytic activities by PS-341 and MG-132. *A*, proteasome extracts were prepared from U266 cells, and extract (50 μ g) was assayed for the CT-L, T-L, and PGPH activities of the proteasome following addition of 1, 5, or 10 nmol/L PS-341. *B*, U266 cells were cultured in the presence of 1, 5, or 10 nmol/L PS-341 for 24 hours. Cells were harvested, and proteasome extracts were prepared and assayed as in (*A*). *C*, proteasome extracts were prepared from U266 cells and assayed following addition of 0.1, 1, or 10 μ mol/L MG-132 as in (*A*). *D*, U266 cells were cultured in the presence of 0.1, 1, or 10 μ mol/L MG-132 for 24 hours, and proteasome extracts were prepared and assayed as in (*A*). Results are percentage change from untreated cells. Columns, mean of three independent experiments; bars, SD.

to determine the subunit specificities of BzLLCOCHO by its ability to compete for proteasome subunit labeling. DansylAhx₃L₃VS is a cell-permeable inhibitor that can covalently and irreversibly modify all of the exposed catalytic subunits of the proteasome. This probe has a dansyl-sulfonamido-hexanoyl hapten tag attached to label and detect proteasome active sites of both constitutive proteasome and immunoproteasome. U266 cells were cultured in the presence of increasing concentrations of BzLLCOCHO for 24 hours and then incubated with DansylAhx₃L₃VS to label remaining available subunits. Addition of BzLLCOCHO reduced the ability of DansylAhx₃L₃VS to modify all proteasomal subunits of the constitutive and immunoproteasome in a dose-dependent manner. There was almost complete inhibition of the β 1i and β 2i subunits observed using 5 μ mol/L BzLLCOCHO, and labeling of the β 2 band and β 1, β 5, and β 5i bands was reduced by 77% and 82%, respectively, with 10 μ mol/L BzLLCOCHO (Fig. 2C). To further delineate the ability of BzLLCOCHO to target the β 1 and β 5 subunits, HeLa cells expressing only the constitutive proteasome were incubated with 10 μ mol/L BzLLCOCHO and DansylAhx₃L₃VS. We found that labeling of the β 2 (T-L) and β 1 (PGPH) subunits was reduced by <80%, and the β 5 (CT-L) subunit labeling

was reduced by 46% (Fig. 2D). These results correspond well to our findings with the fluorogenic assay, showing that BzLLCOCHO is a cell-permeable proteasome inhibitor targeting all three catalytic activities of the constitutive proteasome. In addition, we have found that BzLLCOCHO is a strong inhibitor of all active subunits of the immunoproteasome.

Subunit specificity of the proteasome inhibitors PS-341 and MG-132. After characterizing BzLLCOCHO, we compared its inhibitory profile to PS-341 (a dipeptide boronic acid analogue) and MG-132 (a peptide aldehyde), both of which are reported to target the CT-L activity. Proteasome activity was first measured using the fluorogenic assay following addition of increasing concentrations of PS-341 or MG-132 to U266 cell extracts. U266 cells were then incubated with either PS-341 or MG-132 for up to 24 hours before proteasome was extracted, and activity was measured to assess the activity of the inhibitors in whole cells. In cell extracts, PS-341 inhibited all three catalytic activities in a dose-dependent manner (Fig. 3A). Addition of 10 nmol/L PS-341 reduced CT-L activity by $74 \pm 4\%$, T-L activity by $91 \pm 3\%$, and PGPH activity by $50 \pm 7\%$. Maximal inhibition was observed following preincubation with PS-341 for 6 to 12 hours, and there

was a comparative reduction of all three activities with $\geq 70\%$ of total proteasome activity achieved with 1 nmol/L PS-341 (Fig. 3B). Addition of MG-132 to cell extracts potently inhibited all catalytic activities with almost complete ($>95\%$) inhibition achieved with 10 $\mu\text{mol/L}$ MG-132 (Fig. 3C). However, when we measured proteasome activity in cells preincubated with MG-132, there was only $62 \pm 7\%$ inhibition of total activity with 10 $\mu\text{mol/L}$ MG-132 (Fig. 3D). The reason for this difference is unclear and may be due to cell permeability or less specificity of MG-132 for the proteasome in whole cells.

The active site-directed probe was also used to profile the subunit specificities of PS-341 and MG-132. U266 cells were cultured for 24 hours in the presence of 10 $\mu\text{mol/L}$ BzLLCOCHO, 5 nmol/L PS-341, or 1 $\mu\text{mol/L}$ MG-132 and then incubated with DansylAhx₃L₃VS (Fig. 4). In comparison with previous observations (15, 16), treatment with PS-341 completely inhibited labeling of the $\beta 1$ (PGPH) and $\beta 5$ (CT-L) subunits along with immunoproteasome counterparts $\beta 1i$ and $\beta 5i$. In addition, there was an increase in labeling of the $\beta 2$ (T-L) and $\beta 2i$ subunits, suggesting an increase in activity in these subunits. This is consistent with an increase in T-L activity on treatment with PS-341 as reported in other studies (16, 17). Treatment with

MG-132 reduced labeling of all bands by $\geq 53\%$, with the exception of $\beta 2$, which is increased 5-fold in response to this inhibitor. This is in keeping with findings by Berkers et al. (15) who also observed that MG-132 inhibited the $\beta 2i$ subunit but not $\beta 2$, suggesting a difference in the active site chemistry between these two subunits. Looking at overall activity levels following treatment with the different inhibitors, BzLLCOCHO reduces the labeling of all active subunits, whereas PS-341 and MG-132 seem to simultaneously activate one subunit while reducing the activity of others. This suggests that BzLLCOCHO is the most effective of the three inhibitors in reducing total proteasome activity.

Proteasome inhibitor-induced apoptosis. The ability of the proteasome inhibitors to induce apoptosis in multiple myeloma cell lines (U266 and OPM-2) was evaluated using Mitosensor and Hoechst/propidium iodide staining. Cell lines were incubated with either 10 $\mu\text{mol/L}$ BzLLCOCHO, 5 nmol/L PS-341, or 1 $\mu\text{mol/L}$ MG-132, and apoptosis was measured at 24-hour intervals. As seen in Fig. 5, cells were significantly more sensitive to the induction of apoptosis by PS-341 and MG-132 than BzLLCOCHO. PS-341 has been shown previously to function through both caspase-8 and caspase-9 apoptotic pathways (18).

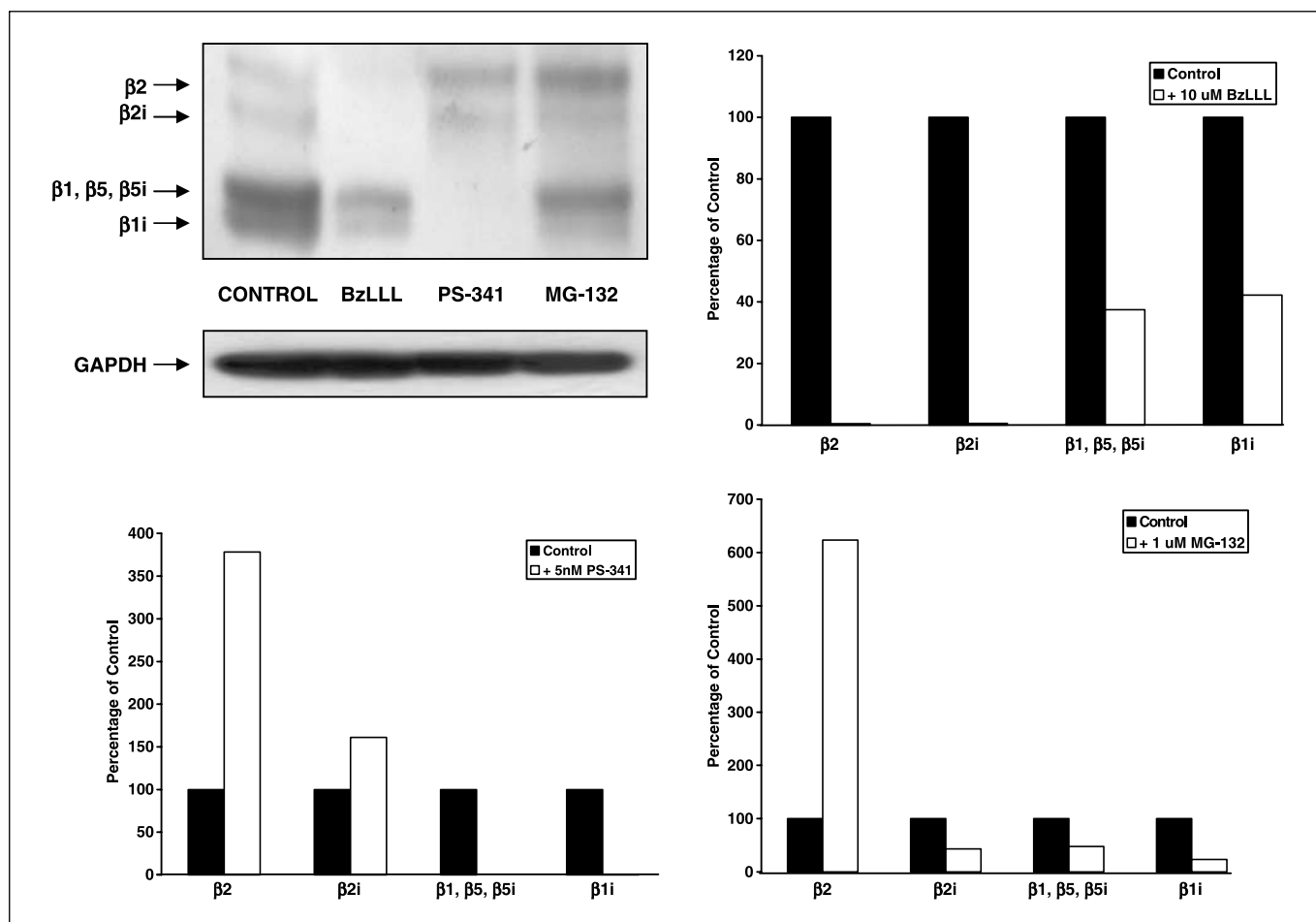


Figure 4. Subunit specificity of PS-341 and MG-132. U266 cells were cultured in the presence of 10 $\mu\text{mol/L}$ BzLLCOCHO, 5 nmol/L PS-341, or 1 $\mu\text{mol/L}$ MG-132 for 24 hours followed by 2 hours of incubation with DansylAhx₃L₃VS to label remaining active proteasome subunits. Cell lysates were prepared, and labeling of catalytic β subunits was assessed by immunoblotting using an anti-dansyl antibody (1:1,000). GAPDH levels confirm equal loading. Individual bands were quantified by densitometry and expressed as change from untreated control (in %).

To determine the requirement for caspase-8-induced versus caspase-9-induced apoptosis with the different proteasome inhibitors, U266 cells were incubated with BzLLCOCHO (10 μ mol/L), PS-341 (5 nmol/L), or MG-132 (1 μ mol/L) for 24 hours, harvested, and subjected to immunoblot analysis. As seen in Fig. 6A, treatment of U266 cells with BzLLCOCHO and MG-132 also induce cleavage of caspase-8 and caspase-9 as is the case with PS-341. These results are confirmed by incubation of U266 cells with caspase-8 or caspase-9 inhibitors. Inhibition of either caspase-8 or caspase-9 leads to a significant decrease in cell death triggered by all three agents (Fig. 6B).

Discussion

Proteasome inhibition has shown great promise in the treatment of multiple myeloma, a malignancy that remains largely incurable with conventional therapies. There are three distinct proteolytic activities associated with the proteasome. The CT-L activity is the primary target of the majority of proteasome inhibitors. The individual roles of the three activities in the functioning of the proteasome are still under investigation, and there is conflicting evidence on how each activity contributes to the overall biological process. Although Jager et al. (19) suggested a hierarchy among the different sites, with the

CT-L activity determining the rate of protein breakdown, they also indicated a degree of redundancy for all active subunits. Kisselev et al. (20) proposed the existence of mutual allosteric regulation between the active sites responsible for the CT-L and PGPH activities. More recently, it has been shown that the contributions of the different activities are dependent on the protein substrate, and Kisselev et al. (21) suggest that all three catalytic activities contribute significantly to protein degradation. In this study, we have profiled the relative contributions of the three activities in proteasome extracts from multiple myeloma and lymphoma cells. Although the rate of CT-L activity was highest in the lymphoma cell lines, the T-L and PGPH activities accounted for a large proportion of total activity in the multiple myeloma cell lines. This suggests that the balance of activities may be cell type dependent and that there may be increased therapeutic benefit by targeting all three catalytic subunits of the proteasome.

Fluorogenic peptide substrates have been widely used to measure the potency of proteasome inhibitors against the proteolytic activities. We have used this approach, along with a novel active site-directed probe, to characterize the subunit specificities of BzLLCOCHO. Initial studies revealed our inhibitor to function as a slow-binding reversible inhibitor of the CT-L activity of the proteasome (14). In this study, we found

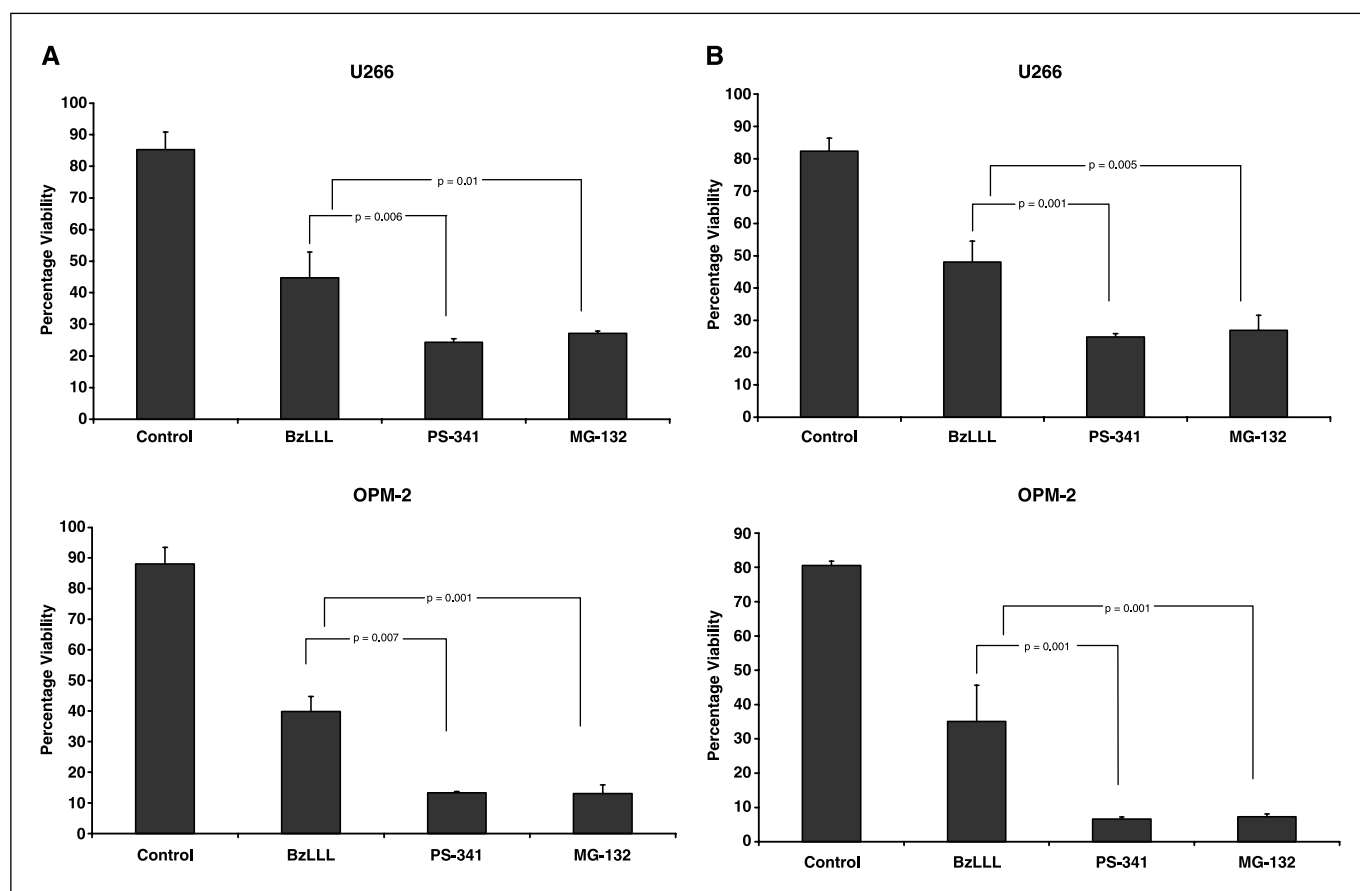


Figure 5. Proteasome inhibitor induced apoptosis in multiple myeloma cell lines. **A**, U266 and OPM-2 cells were cultured in the presence of 10 μ mol/L BzLLCOCHO, 5 nmol/L PS-341, or 1 μ mol/L MG-132, and apoptosis was measured after 48 hours by dual staining with Hoechst and propidium iodide. **B**, U266 and OPM-2 cells were cultured in the presence of 10 μ mol/L BzLLCOCHO, 5 nmol/L PS-341, or 1 μ mol/L MG-132, and apoptosis was measured after 48 hours using ApoAlert Mitochondrial Membrane Sensor kit. Columns, mean of three independent experiments; bars, SD.

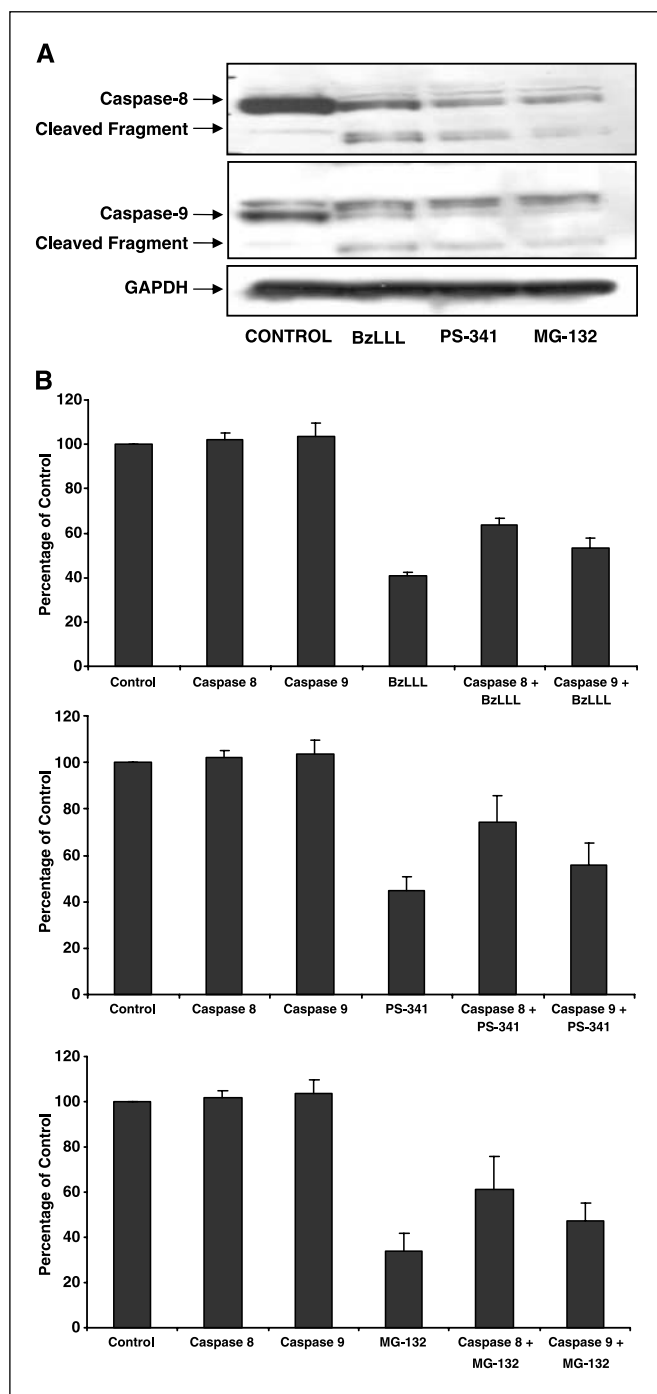


Figure 6. Requirement of caspases in proteasome inhibitor-induced apoptosis. **A**, U266 cells were cultured in the presence of 10 $\mu\text{mol/L}$ BzLLCOCHO, 5 nmol/L PS-341, or 1 $\mu\text{mol/L}$ MG-132 for 24 hours and then harvested, and whole-cell lysates were prepared for immunoblot analysis for activated (cleaved) caspase-8 and caspase-9. GAPDH levels confirm equal loading. **B**, U266 cells were cultured in the presence of 40 $\mu\text{mol/L}$ caspase-8 or caspase-9 inhibitor followed by 24 hours of incubation with 10 $\mu\text{mol/L}$ BzLLCOCHO, 5 nmol/L PS-341, or 1 $\mu\text{mol/L}$ MG-132 BzLLCOCHO. Cell viability was assessed by MTT assay. Columns, mean of four independent experiments; bars, SD.

BzLLCOCHO to be a cell-permeable inhibitor that interacts with all subunits of both constitutive and immunoproteasome. Inhibition profiles of the well-characterized inhibitors PS-341 and MG-132, both of which were reported previously to be specifically targeted toward the $\beta 5$ subunit (17, 22), were also investigated. There were differences in results obtained using fluorogenic assays compared with the active site-directed probe for these two inhibitors. These differences may, at least, in part, be due to the inability of fluorogenic assays to distinguish between the constitutive and immunoproteasome, underscoring the importance of additional tools for the characterization of subunit specificities of proteasome inhibitors. In agreement with Berkers et al. (15), Altun et al. (16) and Chauhan et al. (18) who also used active site-directed probes, we found that $\beta 5$ was not the only subunit inhibited by PS-341 and MG-132. The kinetics of proteasome inhibition may be cell-type specific and occur in time and dose-dependent manner. Differential subunit specificities were observed for each of the inhibitors. Furthermore, all inhibitors were found to target immunoproteasome subunits, highlighting the need to take this proteasome species into account when evaluating the effects of proteasome inhibitors.

The majority of conventional therapies for multiple myeloma induce apoptosis through either death receptor pathway that is mediated by caspase-8 (e.g., γ radiation) or mitochondrial intrinsic pathway that is mediated by caspase-9 (e.g., dexamethasone; ref. 23). PS-341 has been reported to act through both caspase-8 and caspase-9 apoptotic signaling pathways (18, 24). The structurally distinct proteasome inhibitors BzLLCOCHO and MG-132 were also shown to trigger apoptosis through both caspase-8 and caspase-9. When we compared the ability of the proteasome inhibitors to induce apoptosis in multiple myeloma cell lines, we found that induction of apoptosis by BzLLCOCHO was significantly lower than with PS-341 and MG-132. In contrast, BzLLCOCHO was shown to be the strongest inhibitor of proteasome activity both in fluorogenic assay measurements and using the activity-based probe. Many proteasome inhibitors do not specifically target the proteasome, and their biological effects may be partly explained by inhibition of other proteases. Cathepsin B and calpain 1 are additional targets of MG-132 (25). No additional targets for PS-341 have been reported to date. However, it is possible that proteasome inhibition is not the only requirement for PS-341-triggered or indeed BzLLCOCHO-triggered apoptosis.

PS-341 is a first-in-class proteasome inhibitor and has validated the proteasome as a novel and legitimate target (26). Further investigation using genomic and proteomic approaches are required to delineate the effects of these inhibitors on gene and protein expression in the cell, providing the framework for the development of more specific and targeted inhibitors in the future.

Acknowledgments

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References

- Myung J, Kim KB, Crews CM. The ubiquitin-proteasome pathway and proteasome inhibitors. *Med Res Rev* 2001;21:245-73.
- Goldberg AL, Stein R, Adams J. New insights into proteasome function: from archaeobacteria to drug development. *Chem Biol* 1995;2:503-8.
- Naujokat C, Hoffman S. Role and function of the 26S proteasome in proliferation and apoptosis. *Lab Invest* 2002;82:965-80.
- Groll M, Heinemeyer W, Jager S, et al. The catalytic sites of 20S proteasomes and their role in subunit

- maturation: a mutational and crystallographic study. *Proc Natl Acad Sci U S A* 1999;96:10976–83.
5. Strehl B, Seifert U, Kruger E, Heink S, Kuckelcorn U, Kloetzel PM. Interferon- γ , the functional plasticity of the ubiquitin-proteasome system, and MHC class I antigen processing. *Immunol Rev* 2005;207:19–20.
 6. Rock KL, Goldberg AL. Degradation of cell proteins and generation of MHC class I-presented peptides. *Annu Rev Immunol* 1999;17:739–79.
 7. Pagano M, Benmaamar R. When protein destruction runs amok, malignancy is on the loose. *Cancer Cell* 2003;4:251–6.
 8. Schwartz AL, Ciechanover A. The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annu Rev Med* 1999;50:57–74.
 9. Kane RC, Bross PF, Farell AT, Pazdur R. Velcade: U.S. FDA approval for the treatment of multiple myeloma progressing on prior therapy. *Oncology* 2003;8:508–13.
 10. Jaganneth S, Durie BG, Wolf J, et al. Bortezomib therapy alone and in combination with dexamethasone for previously untreated symptomatic multiple myeloma. *Br J Haematol* 2005;129:776–83.
 11. Oakervee HE, Popat R, Curry N, et al. PAD combination therapy (PS-341/bortezomib, doxorubicin, and dexamethasone) for previously untreated patients with multiple myeloma. *Br J Haematol* 2005;129:755–62.
 12. Magill L, Lynas J, Morris TCM, Walker B, Irvine AE. Proteasome proteolytic activity in hematopoietic cells from patients with chronic myeloid leukaemia and multiple myeloma. *Haematologica* 2004;89:1428–33.
 13. Chauhan D, Li G, Shringarpure R, et al. Blockade of Hsp27 overcomes bortezomib/proteasome inhibitor PS-341 resistance in lymphoma cells. *Cancer Res* 2003;63:6174–7.
 14. Lynas JF, Harriott P, Healy A, McKervey MA, Walker B. Inhibitors of the chymotrypsin-like activity of proteasome based on di- and tri-peptidyl α -keto aldehydes (glyoxals). *Bioorg Med Chem Lett* 1998;8:373–8.
 15. Berkers CR, Verdoes M, Lichtman E, et al. Activity probe for *in vivo* profiling of the specificity of proteasome inhibitor bortezomib. *Nat Methods* 2005;2:357–62.
 16. Altun M, Galaray PJ, Shringarpure R, et al. Effects of PS-341 on the activity and composition of proteasomes in multiple myeloma cells. *Cancer Res* 2005;65:7896–900.
 17. Lightcap ES, McCormack TA, Pien CS, Chau V, Adams J, Elliot PJ. Proteasome inhibition measurements: clinical applications. *Clin Chem* 2000;46:673–83.
 18. Chauhan D, Catley L, Li G, et al. A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from bortezomib. *Cancer Cell* 2005;8:407–19.
 19. Jager S, Groll M, Huber R, Wolf DH, Heinemeyer W. Proteasome β -type subunits: unequal roles of propeptides in core particle maturation and a hierarchy of active site function. *J Mol Biol* 1999;291:997–1013.
 20. Kisselev AF, Akopian TN, Castillo V, Goldberg AL. Proteasome active sites allosterically regulate each other suggesting a cyclical bite-chew mechanism for protein breakdown. *Mol Cell* 1999;4:395–402.
 21. Kisselev AF, Callard A, Goldberg AL. Importance of the proteasome's different proteolytic sites and the efficacy of inhibitors varies with protein substrate. *J Biol Chem* 2006;281:8582–90.
 22. Kisselev AF, Goldberg AL. Proteasome inhibitors: from research tools to drug candidates. *Chem Biol* 2001;8:738–58.
 23. Chauhan D, Pandley P, Hideshima T, et al. SHP2 mediates the protective effect of interleukin-6 against dexamethasone-induced apoptosis in multiple myeloma cells. *J Biol Chem* 2000;275:27845–50.
 24. Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. *Nat Rev Cancer* 2002;2:927–36.
 25. Tsubuki S, Saito Y, Tomioka M, Ito H, Kawashima S. Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine. *J Biochem* 1996;119:572–6.
 26. Mitsiades CS, Mitsiades N, Hideshima T, Richardson PG, Anderson KC. Proteasome inhibition as a therapeutic strategy for hematological malignancies. *Expert Rev Anticancer Ther* 2005;5:465–76.

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