Effects of PS-341 on the Activity and Composition of Proteasomes in Multiple Myeloma Cells

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Abstract

Multiple myeloma is a B-cell malignancy for which no curative therapies exist to date, despite enormous research efforts. The remarkable activity of the proteasome inhibitor bortezomib (PS-341, Velcade) observed in clinical trials of patients with relapsed refractory myeloma has led to investigations of the role of the ubiquitin-proteasome pathway in the pathogenesis of myeloma. Here we report a biochemical analysis of proteasome activity and composition in myeloma cells exposed to PS-341 in the presence or absence of cytokines present in the bone marrow milieu. We observed that the myeloma cell lines MM1.S, RPMI8226, and U266 contain active immunoproteasomes, the amount of which is enhanced by IFN-γ and tumor necrosis factor-α. Using a radiolabeled active site–directed probe specific for proteasome catalytic subunits, we show that PS-341 targets the β5 and β1 subunits in a concentration-dependent manner. Furthermore, PS-341 also targeted the corresponding catalytic subunits of the immunoproteasome, β5i and β1i, respectively. These data suggest that PS-341 targets both normal and immunoproteasome species to a similar extent in myeloma cells. (Cancer Res 2005; 65(17): 7896-901)

Introduction

Multiple myeloma is a monoclonal plasma cell malignancy that, despite intensive investigation, remains universally fatal. Preclinical data with the dipeptide boronic acid proteasome inhibitor bortezomib (PS-341, Velcade) shows potent in vitro and in vivo cytotoxicity against many malignant cell lines, most notably those derived from multiple myeloma (1). These promising early results were followed by clinical trials showing striking activity, including some complete responses, in heavily pretreated patients with multiple myeloma (2). Bortezomib is now among the most promising novel therapies for multiple myeloma (3, 4).

The core proteasome (20S) is a large cytosolic and nuclear protease complex composed of 28 subunits, assembled into four stacked rings of seven α and β subunits each. The β1, β2, and β5 subunits are catalytically active and mediate the caspase-like, trypsin-like, and chymotrypsin-like activity, respectively (5). The predominant in vivo proteasome consists of the 20S core adored at each end with a 19S regulatory particle and is known as the 26S proteasome. After exposure to IFN-γ or tumor necrosis factor-α (TNF-α), the catalytic subunits are replaced with β1i (LMP2), β2i (MECL1), and β5i (LMP7) to form what is termed the immunoproteasome (6, 7). Incorporation of these IFN-inducible subunits changes the catalytic activity of the proteasome, enhancing the generation of antigenic peptides presented by class I MHC molecules (MHC-I; ref. 8). Inhibition of proteasome function using small molecule compounds has revealed a central role of the proteasome in many biological processes (9). To date, bortezomib has been shown to efficiently target the proteasome and have specificity for the chymotrypsin-like activity (10). However, the ubiquitous nature of the ubiquitin-proteasome pathway raises the question as to how specific cell types are affected more than others. In particular, the reason why myeloma cells are more sensitive than nontransformed lymphocytes, remains unclear (1). Given the potential in variability, proteasome composition might be a critical determinant for the effectiveness of newly developed proteasome inhibitors in clinical responses (11).

Elevated levels of many cytokines, including interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), insulin-like growth factor-I (IGF-I), CD40L, IFN-γ, and TNF-α, have been reported in the bone marrow microenvironment where multiple myeloma cells replicate in vivo (12–15). In some cases, they provide critical signals required for multiple myeloma cell growth and survival. This suggests that immunoproteasomes may be formed in multiple myeloma cells residing in their native bone marrow microenvironment. Therefore, it is of great interest to understand the specificity of bortezomib towards the different species of proteasomes.

We have employed an extended peptide-based proteasome inhibitor Ada-Y-[125I]-Ahx3L3VS as a probe of active proteasome composition in multiple myeloma cells treated with a panel of cytokines to mimic those seen in the bone marrow microenvironment. When the multiple myeloma cell lines MM1.S, RPMI8226, and U266 were incubated with IFN-γ and TNF-α, we observed the formation of the active immunoproteasome including the subunits β1i (LMP2), β2i (MECL1), and β5i (LMP7). Furthermore, using two-dimensional electrophoresis, we have determined that bortezomib is able to inhibit the activity of the constitutive proteasome subunits β5 and β1 as well as their IFN-γ inducible counterparts β5i and β1i.

Materials and Methods

Cell lines, antibodies, and reagents. The human multiple myeloma cell lines MM1.S (16), RPMI8226, U266 and the mouse thymoma cell line EL-4...
were cultured in RPMI 1640 and the human cervix carcinoma HeLa cell line in DMEM, both supplemented with 10% FCS, 1% glucose, and 1% penicillin/streptomycin. All cells were cultured at 37°C and 5% CO₂.

Preparation of crude cell extracts. Preparation of cell lysates was performed as previously described (17, 18). In brief, cells were harvested and washed with cold PBS, pelleted, and lysed using one volume glass beads (106 μm, acid washed Sigma, St. Louis, MO) and two volumes of homogenization buffer (pH 7.4; 50 mmol/L Tris base, 5 mmol/L MgCl₂, 250 mmol/L sucrose, and fresh 2 mmol/L ATP and 1 mmol/L DTT). Beads, membrane fractions, nuclei, and cell debris were removed by centrifugation at 13,000 rpm for 15 minutes and an additional ultra centrifugation of 72,200 × g for 1 hour at 4°C (Beckman, Fullerton, CA). The protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA), using a UV-spectrophotometer (Beckman) at 595 nm and bovine serum albumin as a standard.

Proteasome activity profiling and analysis by one-dimensional and two-dimensional gel electrophoresis. Labeling of the active proteasomal β subunits using the active site–directed probe was done essentially as described previously (17). In brief, the radiolabeled active site labeling probe Ada-Y-[¹²⁵I]-Ahx₃L₃VS (prepared as described in ref. 17) was added to 25 μg of cell lysates and incubated for 2 hours at 37°C. For the analysis by one-dimensional the samples were denatured by adding SDS-sample buffer and incubated at 95°C for 5 minutes. Proteins were then separated on a 12.5% SDS-PAGE gel. For two-dimensional analysis (nonequilibrium pH gradient and SDS-PAGE; NEPHGE), the samples were prepared by adding urea (8 mol/L final concentration) and NEPHGE sample buffer with Pharmacia ampholyte (pH 3.5-9.5). Two-dimensional NEPHGE SDS-PAGE analysis was done as described previously (17). All gels were analyzed by autoradiography on Kodak XOMAT-AR film. Films were scanned as 8-bit tiff images and quantified by densitometry using the ImageJ software (http://rsb.info.nih.gov/ij/).

Cytokine and PS-341 treatment. MM1S, RPMI8226, and HeLa cells (5 × 10⁶) were cultured in the presence of medium alone, IFN-γ (100 units/ml, Roche, Nutley, NJ), TNF-α (100 units/ml, Roche), IL-6 (100 ng/ml, R&D Systems, Minneapolis, MN), VEGF (100 ng/ml, R&D Systems), IGFI (200 ng/ml, R&D Systems), or CD40L (1 mg/ml, R&D Systems) for 48 hours. Cells were then treated with or without 20 nmol/L of PS-341 for the last 4 hours. Cells were harvested, lysed, and analyzed using active site labeling probes, and subunits were separated using one-dimensional and two-dimensional SDS-PAGE as described above.

Western blot analysis. Cells were harvested and lysed in radioimmunoprecipitation assay buffer (Boston Bioproducts, Worcester, MA) supplemented with 1 mmol/L sodium fluoride, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Equal amounts of protein from cell lysates were separated by 12% SDS-PAGE and subjected to Western blotting as described previously (19). Antibodies detecting the following proteins were used: β5i (LMP7) and β5i (Affinity UK) and tubulin (Sigma).

Proteasome activity assays. Chymotrypsin-like activity of the 20S proteasome was measured in freshly prepared cell lysates as described previously (20). Briefly, treated or untreated cells were lysed in 1 mmol/L DTT and the lysates were cleared by centrifugation. The cell lysates (50 μg protein) were incubated with fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (100 μmol/l), in a buffer containing 50 mmol/L Tris-HCl (pH 7.8), 20 mmol/L KCl, 5 mmol/L MgOAc, and 0.5 mmol/L DTT for 2 hours at 37°C. The proteolysis reaction was terminated by addition of an equal volume of ice-cold ethanol and 10 volumes of 0.1 mol/L sodium borate (pH 9). Release of 7-amido-4-methylcoumarin was monitored using fluorescence at an emission wavelength of 450 nm (excitation, 360 nm) in a fluorescence plate reader (SpectraMax Gemini XS, Molecular Devices Corp., Sunnyvale, CA).

Results

PS-341 targets different catalytic β subunits of the proteasome in a concentration-dependent manner. Initial biochemical studies were done on the MM1S cell line, the growth of which shows very high sensitivity to PS-341, in the low nanomolar range (2-5 nmol/L; ref. 1). Crude extracts prepared from cells exposed to 20 nmol/L PS-341 were incubated with Ada-Y-[¹²⁵I]-Ahx₃L₃VS to visualize the unoccupied catalytic β subunits, as described (17). We observed that MM1S cells express both the constitutive proteasome, as well as a low level of active immunoproteasomes (Fig. 1). The β5 subunit, responsible for the chymotryptic activity of the proteasome (5), is modified by PS-341 to 40% in 1 hour and to ≥70% after 2 hours (Fig. 1A), consistent with the inhibition of the chymotryptic activity as...
reported in other studies (10, 21, 22). The β1 subunit and also its immunoproteasome counterpart, β1i, were likewise targeted by PS-341 and inhibited over 30% at 3 and 6 hours, respectively (Fig. 1A). In contrast, the β2 and β2i subunits were affected only after 8 hours, a time point where MM1.S cells start to undergo apoptosis at 20 nmol/L of PS-341 (1). Next, we examined the ability of PS-341 to target different protosomal β subunits in a concentration-dependent manner. For this purpose, MM1.S extracts were incubated with 5 to 320 nmol/L PS-341 for 2 to 8 hours followed by incubation with the proteasome active site probe Ada-Y-[125I]-Ahx3L3VS as above (Fig. 1B). Consistent with previous reports (10, 23, 24), 5 nmol/L bortezomib modified predominantly the β5/β5i subunits. At concentrations of ≥20 nmol/L, the β1 subunit was also targeted, and almost completely modified at 80 nmol/L. The β2 and β2i subunits were not affected at a concentration of 320 nmol/L (Fig. 1B). Peak serum concentrations of bortezomib are reported to reach the low micromolar range (Millennium, Velcade prescriptive information). We therefore conclude that PS-341 targets β5/β5i as well as β1/β1i at clinically relevant concentrations.

**Multiple myeloma cells have increased expression of active immunoproteasome upon stimulation with IFN-γ.** We next examined how PS-341 affects β subunits of the immunoproteasome induced by cytokines such as IFN-γ and TNF-α. For this purpose, MM1.S cells were exposed to IFN-γ for 48 hours and 20 nmol/L PS-341 for the last 4 hours. Extracts prepared from cells treated or not with IFNγ and/or PS-341 were labeled with the proteasome-specific active site probe Ada-Y-[125I]-Ahx3L3VS and analyzed by SDS-PAGE and autoradiography (Fig. 2). As expected, IFN-γ treatment increased expression of active immunoproteasomes, as reflected by increased labeling of the corresponding subunits β1 and β2i (Fig. 2A). IFN-γ treatment increased expression of active immunoproteasomes, as reflected by increased labeling of the corresponding subunits β1 and β2i (Fig. 2A). As observed previously, 20 nmol/L PS-341 modified the β5/β5i subunits almost completely, whereas the β1 and β1i subunits were affected partially. Labeling of the β2/β2i subunits is slightly elevated in presence of PS-341, indicating an increase in their activity. This observation is consistent with previous reports that the trypsin-like activity of the proteasome is increased in proteasomes derived from multiple myeloma patients treated with PS-341, as measured by fluorogenic assays (21, 24). Targeting of the β5/β5i and β1/β1i subunits by PS-341 is accompanied by a dramatic decrease in chymotrypsin-like activity in MM1.S extracts, independent of whether the cells have been exposed previously to IFN-γ or TNF-α (Fig. 2D). We conclude that multiple myeloma cells express low levels of active immunoproteasome and that its expression is enhanced upon exposure to IFN-γ or TNF-α.

**PS-341 targets individual catalytic β subunits of the normal and immunoproteasome.** To further examine the ability of PS-341 to target individual β subunits of the different proteasome species in more detail, we did two-dimensional gel electrophoresis to more clearly separate the proteasomal subunits, in particular β5 and β5i polypeptides (Fig. 3). Labeled extracts prepared from MM1.S cells contained predominantly the

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**Figure 2.** Multiple myeloma cells express active immunoproteasome upon stimulation with IFN-γ. **A,** MM1.S cells were cultured either without or in presence of 10 and 100 units/mL IFN-γ for 48 hours. PS-341 (20 nmol/L) was added in the final 4 hours of the incubation. Crude extracts were prepared and the labeling of individual catalytic β subunits of the proteasome was assessed using Ada-Y-[125I]-Ahx3L3VS and analysis by SDS-PAGE and autoradiography as described for Fig. 1. Individual labeled β subunits were identified based on the labeling profile of EL-4 cell extracts (16, 27). **B,** MM1.S, RPMI8226, and HeLa cells were cultured without or in presence of IFN-γ (10 and 100 units/mL) for 48 hours. One half of each sample was added 20 nmol/L of PS-341 for the last 4 hours. Crude extracts were prepared and labeling of the individual catalytic β subunits of the proteasome was assessed with Ada-Y-[125I]-Ahx3L3VS and analyzed by SDS-PAGE and autoradiography. The β5 and β5i subunits can be separated upon prolonged migration of SDS-PAGE gels. Individual bands were identified based on the HeLa extract labeling profile as described (17) and the two-dimensional gel profile presented in Fig. 3. **C,** IFN-γ-induced expression of immunoproteasome subunits. Western blot analysis of MM1.S cells treated with IFN-γ or TNF-α (100 units/mL) for 48 hours. Crude cell extracts were blotted for β5i (LMP7, top), β5 (X, middle), and tubulin (bottom). **D,** MM1.S cells cultured with IFN-γ and TNF-α for 48 hours and treated or not with 20 nmol/L PS-341 for the last 4 hours were lysed and 20 μg extracts were assayed for the chymotryptic-like activity of the proteasome using 20 μg of the fluorogenic substrate Suc-LLVY-AMC as described in Materials and Methods.
constitutive proteasome subunits, as examined at the protein level (Fig. 2C) and activity profiles indicated the presence of a low level of active immunoproteasome (Fig. 3A). In addition to the labeled \( \beta 1/\beta 2/\beta 5 \) subunits, the probe Ada-Y-[\( ^{125}I \)]-Ahx3L3VS also modified the \( \beta 1 i, \beta 2 i, \) and \( \beta 5 i \) subunits. The labeling intensity of \( \beta 1 i \) and \( \beta 5 i \) was stronger than that of their counterparts, \( \beta 1 \) and \( \beta 5 \), respectively, but immunoblot experiments suggested that the major form expressed in MM1S cells is the normal proteasome (Fig. 2C). Upon treatment with IFN-\( \gamma \) for 24 hours, labeling of the immunoproteasome \( \beta 5 i \) subunit is increased 9-fold, 6-fold for \( \beta 2 i \), and 10\% for \( \beta 1 i \) subunits as expected, but also efficiently targeted the \( \beta 5 i \) subunit (Fig. 3B and D). Consistent with previous experiments, labeling of the \( \beta 2 \) and \( \beta 2 i \) subunits is reduced only slightly, 40\% and 10\%, respectively. We conclude that the IFN-\( \gamma \) inducible \( \beta \) subunits \( \beta 5 i \) and \( \beta 1 i \) are targeted with similar efficiency by PS-341 compared with their counterparts \( \beta 5 \) and \( \beta 1 \).

Effects of cytokines present in the bone marrow milieu on immunoproteasome formation and activity in multiple myeloma cells. In previous studies, 20S proteasome activity is usually measured using fluorogenic substrates (21). However, these assays do not provide information on proteasome content or composition and little is known about the species of proteasome expressed in multiple myeloma cells, particularly in the context of their in vivo microenvironment. To approach this scenario in an in vitro model system, we exposed MM1S, RPMI8226, and U266 cells to different cytokines such as IL-6, VEGF, IGF-I, CD40L, IFN-\( \gamma \), or TNF-\( \alpha \), which are cytokines reported to be present in the bone marrow milieu (13). Crude extracts were prepared from these cells and incubated with the proteasome active site probe Ada-Y-[\( ^{125}I \)]-Ahx3L3VS followed by SDS-PAGE analysis and autoradiography (Fig. 4). RPMI 8226 and U266 cells contained a higher content of immunoproteasome subunits compared with MM1S cells (Fig. 4; data not shown). Because both cell lines are sensitive to PS-341 to a similar extent (1), it seems that the level of active immunoproteasomes does not contribute to resistance to this proteasome inhibitor. These cell lines were exposed to a panel of different cytokines including IL-6, VEGF, IGF-I, CD40L, IFN-\( \gamma \), and TNF-\( \alpha \) for 48 hours followed by a treatment of PS-341 or not (Table 1). Crude cell extracts were then prepared and proteasome activity assessed using the active site probe Ada-Y-[\( ^{125}I \)]-Ahx3L3VS as described above. The level of active immunoproteasome was evaluated based on the intensity of the labeled polypeptide corresponding to the \( \beta 2 i \) subunit. As expected, IFN-\( \gamma \) and TNF-\( \alpha \) were the only cytokines capable of inducing measurable amounts of active immunoproteasome species (Fig. 4, top, lanes 3, 4, and 7, 8 and bottom, lanes 3, 4, and 7, 8). As observed previously, PS-341 mainly affected labeling of the \( \beta 5 i/\beta 5 i \) subunits but not \( \beta 2 \) and \( \beta 2 i \), indicating that the corresponding activities are not modulated in the presence of these cytokines. We conclude that multiple myeloma cells that reside in the bone marrow stroma may express active immunoproteasome in vivo, but the mixture of proteasome species present in these cells do not provide them with an elevated level of resistance to PS-341.

**Figure 3.** PS-341 targets catalytic \( \beta \) subunits of the normal and immunoproteasome. A-D. MM1S cells were cultured in presence of IFN-\( \gamma \) (100 units/mL) for 48 hours, and half of the samples were incubated with 20 nmol/L PS-341 for the last 4 hours: untreated (A), +PS-341 (B), +IFN-\( \gamma \) (C), +PS-341 and IFN-\( \gamma \) (D). Crude extracts were prepared and analyzed by two-dimensional gel electrophoresis (NEPHGE) as described (see Materials and Methods and ref. 17). Individual spots were identified as labeled catalytic \( \beta \) subunits based on molecular weight and isoelectric point (Swissprot, http://www.expasy.ch, accession numbers indicated in brackets): \( \beta 1, p f = 3.4 \); \( \beta 1 i (P28072), p f = 4.80 \); \( \beta 1 i (P28065), p f = 4.84 \); \( \beta 2 (Q99436), p f = 5.61 \); \( \beta 2 i (P40306), p f = 6.07 \); \( \beta 5 (P28074), p f = 8.66 \); \( \beta 5 i (P28062), p f = 8.29 \). For the calculations of the theoretical \( pf \), the precursor sequence was removed before the NH\(_2\)-terminal threonine. Bottom, individual spots corresponding to proteasomal \( \beta \) subunits were quantified by densitometry and displayed as bar graphs relative to the value obtained for untreated cells in percent.
is therefore very likely that multiple myeloma cells
reportedly expressed (1). sensitive to this compound regardless of the level of immunoproteasome formation and activity. MM1.S (top) and RPMI8226 cells (bottom) either untreated or cultured in presence of CD40L (1 mg/ml), IFN-γ (100 units/ml, Roche), TNF-α (100 units/ml), for 48 hours. Half of each sample was incubated with 20 nmol/L PS-341 for the final 4 hours. Proteasome activity measured by labeling with the proteasome probe Ada-Y-[125I]-Ahx3L3VS was assessed as described for Fig. 1.

Discussion
Pharmacologic inhibition of the proteasome is a rapidly developing therapeutic modality for multiple myeloma, other hematologic malignancies, and perhaps autoimmune disorders. We report a biochemical analysis of proteasome activity and composition in multiple myeloma. Using a proteasome-specific active site probe Ada-Y-[125I]-Ahx3L3VS and two-dimensional electrophoresis, we visualized the active catalytic subunits present in multiple myeloma cells and monitored the activity of these subunits in response to cytokine stimulation and drug treatment. In the absence of additional cytokine stimulation, the multiple myeloma cell line MM1.S contained active proteasomes predominantly consisting of the constitutive subunits β1, β2, and β5, although a small amount of active immunoproteasome subunits were also detected in these cells. It is generally accepted that models of multiple myeloma pathophysiology must take into account the interactions between multiple myeloma cells and elements of the bone marrow microenvironment (13). Many reports have documented the growth promoting effects of cytokines in the bone marrow microenvironment, such as TNF-α, INF-γ, IL-6, VEGF, IGF-I, and CD40L on multiple myeloma cells. Following the addition of TNF-α or INF-γ to MM1.S cells, we observe a dramatic increase in active immunoproteasome subunits, as reflected by reactivity with Ada-Y-[125I]-Ahx3L3VS. It is therefore very likely that multiple myeloma cells in situ contain both constitutive and immunoproteasome subunits in an active state. The immunoproteasome is unlikely to contribute to PS-341 resistance, because all cell lines tested were shown to be equally sensitive to this compound regardless of the level of immunoproteasome subunits expressed (1).

Activity-based active site labeling with Ada-Y-[125I]-Ahx3L3VS also allowed us to investigate the actions of bortezomib (PS-341) in multiple myeloma cells. We observe a time- and dose-dependent targeting of proteasome subunits in MM1.S cells exposed to bortezomib. At early time points and lower concentrations (5 nmol/L), the β5 subunit is the primary target for bortezomib. However, with increasing time and increased drug concentrations, we observe a profound inhibition of the β1 subunit. Using two-dimensional electrophoresis to separate all active proteasome subunits, we conclusively show that bortezomib inhibits not only the constitutive subunits β1 and β5 but also their immunoproteasome counterparts β1i and β5i. Given that the median peak concentrations of bortezomib in patients undergoing treatment for multiple myeloma at least briefly reach the low micromolar range (Millennium, Velcade prescribing information), it is very likely that the pharmacologic effects of bortezomib reflect inhibition of both the β1 and β5 subunits in myeloma cells as well as the IFN-inducible subunits β1i and β5i.

To date, the inhibitory effects of bortezomib have been evaluated in assays optimized for 20S proteasome activity, using fluorogenic substrates and buffer conditions including SDS (21, 24). Under these conditions, the 19S regulatory components dissociate from the 20S core proteasome complex. However, only the 26S proteasome is capable of degrading ubiquitinated protein targets. A major advantage of the activity-based reagent Ada-Y-[125I]-Ahx3L3VS is its ability to probe the reactivity of individual proteasome catalytic subunits in the presence and absence of drug treatment in crude extracts, in which 26S proteasome complexes are mostly conserved. In addition, the published data on bortezomib’s inhibitory effects consist of the measured catalytic activities of proteasomes isolated from the peripheral blood cells of patients being treated with bortezomib. Given that multiple myeloma is a malignancy restricted to the bone marrow, the measurement of peripheral blood proteasome activity may not accurately reflect the inhibitory status in the targeted malignant myeloma cells. Indeed, wide variation of proteasome inhibition between tumor and blood were recently reported in a murine model using multiple myeloma cells (25). Using drug concentrations well below that of the peak concentrations seen in patients we have documented proteasome inhibition significantly above the 70% level seen in the peripheral blood. This is in agreement with data from monkeys injected with 14C-bortezomib in which the concentration of radioactivity in the bone marrow was more than twice that of the peripheral blood 1 hour following administration of the drug (Millenium, http://www.quintiles.com).

Table 1. Effects of cytokines on immunoproteasome formation

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NOTE: Summary of the cytokines used on MM1.S, RPMI8226 or U266 cells. No or little detection (−) of active immunoproteasome as assayed with the radiolabeled probe Ada-Y-[125I]-Ahx3L3VS as described for Figs. 1-4. Detection of active immunoproteasome at a low (+) and high (+++) level, respectively.
Inhibition of the proteasome by PS-341 has been shown to affect nuclear factor-kB (NF-kB) activation and IL-6 secretion, both important factors in multiple myeloma cell survival (1). However, it is noteworthy that specific NF-kB inhibitors do not elicit the same cytotoxic effects on multiple myeloma cells, suggesting other mechanisms of anti-multiple myeloma activity for PS-341. The observation that bortezomib inhibits the immunoproteasome may have implications for these types of drugs to modulate immune responses in general and to multiple myeloma specifically, in particular MHC-I restricted (CD8+ T-cell responses). This concept is underscored by the recent report of bortezomib's potent inhibition on the development of acute graft-versus-host disease in a mouse model of bone marrow transplantation (26). Preliminary experiments show that low nanomolar concentrations of bortezomib dramatically reduce MHC-I surface expression (HLA-ABC; data not shown). This might suggest a potential role of natural killer (NK) cells in anti-multiple myeloma immune responses. Recently, reports of anti-myeloma NK cell activity have been documented, although the role of this immune response in the progression of multiple myeloma is unclear (27, 28). Additionally, activity of thalidomide and other immunomodulatory drugs against multiple myeloma has been associated with augmented anti-myeloma NK cell activity (29). It is intriguing to postulate that bortezomib treatment may result in an enhanced killing of multiple myeloma cells in vivo by NK cells as a result of decreased surface HLA expression. Specific inhibitors of immunoproteasome subunits may allow the exploitation of this mechanism while sparing the toxicity of inhibition of the constitutive proteasome subunits.

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