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

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Chapter 1.2

Activity probe for *in vivo* profiling of the specificity of proteasome inhibitor bortezomib

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Activity probe for *in vivo* profiling of the specificity of proteasome inhibitor bortezomib

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Proteasome inhibitors, such as the dipeptide boronic acid bortezomib, are emerging as important tools in the treatment of the fatal hematologic malignancy multiple myeloma. Despite the recent US Food and Drug Administration approval of bortezomib (PS341, Velcade) for the treatment of refractory multiple myeloma, many of the basic pharmacologic parameters of bortezomib and its mode of action on myeloma cells remain to be determined. We describe the synthesis and use of a cell-permeant active site–directed probe, which allows profiling of proteasomal activities in living cells. When we compared proteasome activity patterns in cultured cells and crude cell extracts with this probe, we observed substantial differences, stressing the importance for bioassays compatible with live cells to ensure accuracy of such measurements. Using this probe, we investigated the *in vivo* subunit specificities of bortezomib and another inhibitor, MG132.

INTRODUCTION

The proteasome inhibitor bortezomib (**2**) (PS341, Velcade; Figure 1) is among the most promising pharmaceuticals developed for the treatment of multiple myeloma.[1] The ability to target the proteasome pharmacologically without prohibitive toxicity is in itself remarkable: the proteasome is a large proteolytic complex, which is abundant in the cytosol and the nucleus, and it is considered central to many different aspects of cellular physiology, as polypeptides are continuously synthesized and destroyed to maintain steady-state protein levels.[2] Protein turnover by the proteasome has many critical regulatory roles, including quality control by removing abnormal proteins that arise by mutation, metabolic damage or misfolding.

Eukaryotic proteasomes consist of a 20S core, which is most often the central part of higher-order complexes, of which the 26S proteasome is the major active species (Figure 2). In 26S proteasomes, the 20S core particle is complexed at one or both ends with a 19S regulatory complex that modulates the entrance of substrates to the core and is required for the recognition of ubiquitin-tagged substrates and the unfolding of substrates prior to their threading into the proteolytic chamber.[3] Hy-

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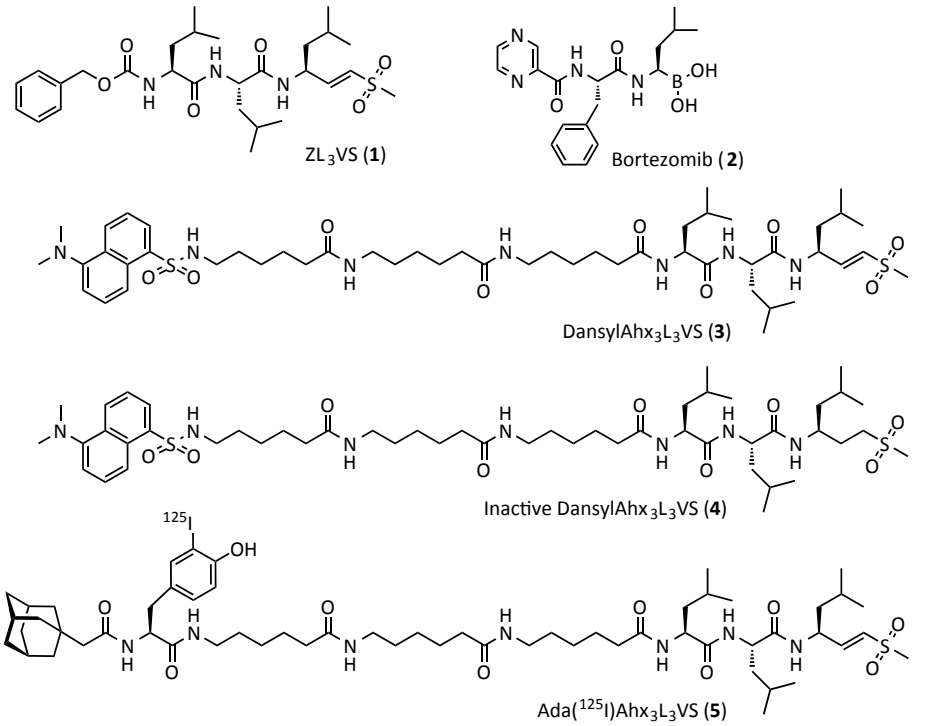

Figure 1 | Representative structures of bortezomib and vinyl sulfone–based proteasome probes.

brid proteasomes, in which different regulatory caps are attached to the 20S core, also exist. The 20S core is a hollow, barrel-shaped protein complex[4] composed of 14 α and 14 β subunits arranged in four stacked rings with an overall architecture of α(1–7)β(1–7)β(1–7)α(1–7). The two outer α rings form gated channels through which substrates enter and products exit the catalytic core. In addition, the outer α rings provide binding sites for regulatory complexes such as the 19S caps. The proteolytic activity of the 20S particle resides in the two inner β rings. The proteasome has three distinct catalytic activities, caspase-like, tryptic-like and chymotryptic-like, each of which can be roughly correlated to the action of an individual catalytic β subunit. These are termed β1, β2 and β5, respectively, and can be replaced by three interferon-inducible subunits, termed β1i, β2i and β5i to form immunoproteasomes—proteasomes with altered catalytic specificity favoring production of antigenic peptides. The secondary alcohol of the N-terminal threonine of the active β subunits acts as the nucleophilic species.

The dipeptide boronic acid bortezomib is a potent inhibitor of the proteasome's chymotryptic-like activity.[5] But variations in proteasomal composition alter the proteolytic specificities of the proteasome[6–8] and may therefore affect the sensitivity of the proteasome to the action of bortezomib. Little is known about the composition of active proteasomes in living multiple myeloma cells. Furthermore, most investigators measure proteasomal activity in cell extracts using fluorogenic substrates, and as a result, it has been difficult to assess the extent of inhibition of individual

catalytic activities *in vivo* after treatment with bortezomib. This underscores the need for improved tools that allow the accurate assessment of proteasomal activity *in vivo*.

To facilitate proteomic research, activity-based chemical probes have been developed for the study of serine, cysteine and threonine proteases.[9–11] These probes form covalent adducts with active proteases of the class that is targeted, leading to a convenient readout of enzyme activity. Most of these probes, however, can not be used in intact living cells.[12,13] Tags that are incorporated to provide an enzymatic activity readout, such as [^{125}I]iodotyrosine, often render probes impermeant to cell membranes, thereby restricting their use to cell extracts. Furthermore, they are nonspecific and target many proteases at the same time. Thus, such probes do not provide information on *in vivo* activities of specific proteases.

Peptide vinyl sulfone–based proteasome inhibitors[9,14] such as ZL_3VS (**1**) (Figure 1) modify catalytically active threonine residues through a Michael addition reaction, resulting in the formation of a covalent β-sulfonyl ether linkage. When a detectable tag is attached to such inhibitors, they can be used to label and detect proteasome active sites. Recently we set out to profile proteasome activity in multiple biological samples. A previously developed peptide vinyl sulfone–based inhibitor proved to be cell-permeant,[15] but the need for secondary chemical labeling procedures made this method unsuitable as a rapid screen of proteasome inhibition in multiple samples. In need for fast, reproducible activity readouts we reasoned that the use of small haptens, which can be recognized by antibodies, may allow retention of cell permeability of probes and allow detection of labeled enzymes directly by SDS-PAGE and Western blot analysis. Therefore we synthesized an inhibitor with a dansyl-sulfonamidohexanoyl hapten as a fast and easy method to monitor proteasome activity in living cells. Using this probe, we could profile active proteasome subunits in cell extracts and cultured cells. Furthermore, we investigated the *in vivo* subunit specificities of the proteasome inhibitors bortezomib and MG132. We found that the β1 and β1i subunits are additional targets for bortezomib and that MG132 inhibits all subunits.

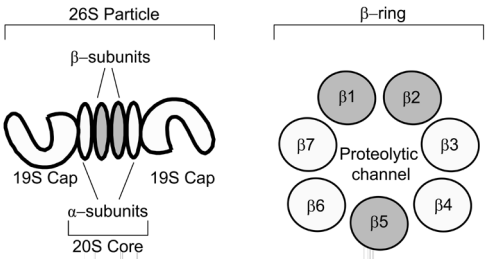

Figure 2 | Schematic of the 26S proteasome.

RESULTS

Synthesis and characterization of proteasome probes

We synthesized compound **3** (Figure 1) containing a dansyl-sulfonamidohexanoyl hapten, as well as control **4** in which the reactive vinyl sulfone moiety is reduced by hydrogenation, rendering the overall probe unreactive. Using a cell-based assay,[16] we tested proteasome inhibition by compounds **3** and **4**, and compared the inhibition to that of other known inhibitors. We exposed cells expressing a substrate fusion protein comprising ubiquitin, the amino acid arginine and green fluorescent protein (ubiquitin-Arg-GFP) to proteasome inhibitors. Efficient proteasome inhibition results in the accumulation of GFP, which can be quantitated by fluorescence activated cell sorting (FACS; Figure 3A,B). Whereas treatment with increasing amounts of **3** or $Ada\text{-}Ahx_3Leu_3VS$, the parent compound, resulted in a substantial increase in fluorescence, treatment with

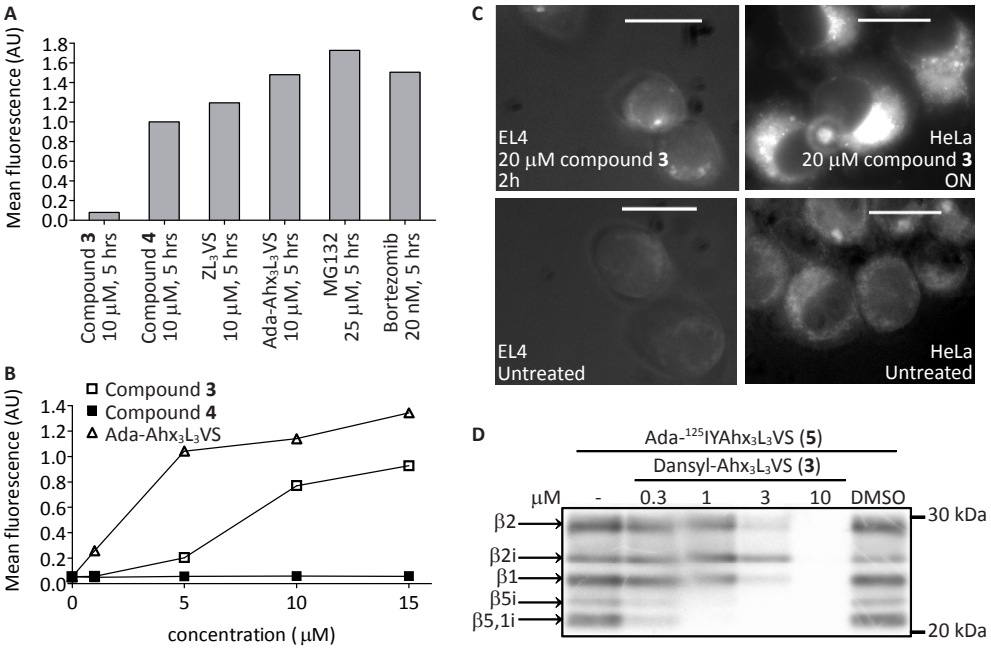

Figure 3 | Characterization of compounds 3 and 4. A,B) FACS analysis results of ubiquitin-Arg-GFP–expressing U373[12] cells upon incubation with proteasome probes and proteasome inhibitors. Incubation with compound **3** resulted in an increase in fluorescence, comparable to that of known proteasome inhibitors, whereas incubation with inactive compound **4** did not **(A)**. **B)** Graph shows the increase in fluorescence after a 5-h incubation with the indicated concentrations of compound **3** and the parent compound Ada-Ahx_3L_3VS, from which an approximate IC_{50} value could be estimated. Graphs are of representative results obtained in two independent experiments. **C)** Visualization by fluorescence microscopy of the distribution of compound **3** in EL4 and HeLa cells. Bar, 10 μm. **D)** Competition experiment between inhibitor **3** and inhibitor **5**. Autoradiograph showing the proteasome subunits that have reacted with **5**.

the control inhibitor **4** was ineffective. The approximate half-maximal inhibitory concentration (IC_{50}) values for Ada-Ahx_3Leu_3VS and **3** were 3 μM and 8 μM, respectively (Figure 3B). Thus, although the potency and/or bioavailability of compound **3** is somewhat lower than that of the parent compound, it is by far sufficient as a probe for activity measurements. By observing the relatively weak fluorescence emitted by the dansyl moiety of inhibitor **3**, we confirmed cell permeability of **3** by fluorescence microscopy (Figure 3C). After incubation with 20 μM of **3**, we visualized fluorescence throughout the cytosol and nucleus of cultured HeLa and EL4 cells. The microscopy results conclusively demonstrate the cell permeability of inhibitor **3**. We next determined the subunit specificity of inhibitor **3** by its ability to compete for proteasome subunit labeling with the fully characterized radiolabeled inhibitor Ada-^{125}IYAhx_3Leu_3VS (**5**) (Figures 1 and 3D), that we have previously shown to react with all active subunits of the proteasome.[14,15] In a dose-dependent manner inhibitor **3** could compete with Ada-^{125}IY-Ahx_3Leu_3VS for labeling of all active protea-

some subunits in extracts from the multiple myeloma cell line MM.1S. We therefore conclude that compound **3** is a cell-permeant proteasome inhibitor that can be used to label all active proteasome subunits.

Probing proteasome activity in cell extracts

To directly visualize the proteins labeled by **3** in EL4 cell extracts, we took advantage of the probe's dansyl-sulfonamidohexanoyl tag. We incubated cell extracts with increasing concentrations of **3** followed by SDS-PAGE and immunoblotting for the dansyl-sulfonamidohexanoyl moiety (Figure 4A). We observed a clear labeling profile, devoid of spurious cross-reactivity attributable to the antibody used. At a concentration of 300 nM, inhibitor **3** labeled all active proteasome subunits to a similar extent, despite weaker reactivity of the probe towards the β2i subunit. In the presence of increasing concentrations of inhibitor, the proteasomal active sites were rapidly saturated, and we observed a nonspecific highmolecular-weight labeling profile at the higher concentrations. This underscores the need for a careful titration of directed activity-based probes. Notably, subunits migrate slightly differently on the gels (Figure 4A compared to Figure 3D); the migration of a particular subunit-probe complex on gel is probe-dependent, which explains the subtle differences. To validate the accuracy of the method, we performed proteasome activity measurements with fluorogenic substrates in parallel. We incubated extracts with increasing concentrations of inhibitor **3** and then monitored the cleavage of SucLeuLeuVal-TyrAMC (chymotryptic-like activity), ZAlaArgArgAMC (tryptic-like activity) and ZLeuLeuGluβNA (caspase-like activity) to determine the remaining activities (Figure 4B). The activity measurements with fluorogenic substrates corresponded well to the immunoblot profile. At a concentration of 10 μM of **3**, labeling of subunits with this inhibitor reached saturation, and activity against fluorogenic substrates was abolished almost completely, thus confirming the accuracy of the use of inhibitor **3** as a probe to measure proteasome activity *in vitro*.

Probing proteasome activity in living cells

We next probed proteasomal activity in intact cells. We incubated cultured EL4 cells with increasing concentrations of **3**, collected the cells, and immunoblotted for the dansyl group as described above (Figure 4C). We observed a dose-dependent labeling of active proteasome subunits from EL4 cells, which confirms that probe **3** permeates cell membranes and modifies all active proteasome subunits. When we compared the labeling pattern obtained in experiments with EL4 extracts to that obtained in experiments with cultured cells, we found that elevated concentrations of probe are needed to efficiently label active proteasome subunits, probably owing to kinetics of membrane permeation. Furthermore, the *in vivo* labeling profile differed in comparison to the labeling pattern in cell extracts. In EL4 cell lysates, the β1 and β1i subunits appear to be quite active. But *in vivo* the β1 subunit was labeled only in the presence of high concentrations of inhibitor **3**, whereas the β1i subunit was not labeled at all. In contrast, the β5 subunits seem to be more active and therefore the target for the majority of the observed *in vivo* active-site labeling. The exact reason for the observed activity difference is not clear. Possible explanations include partial dissociation of associated (19S) regulatory proteasomal cofactors upon preparation of extracts, as well as disruption of transient associations of active proteasomes with cell membranes in detergent extracts. In addition, when we compared *in vivo* labeling experiments to experiments in lysates, virtually no highmolecular-weight adducts were observed despite

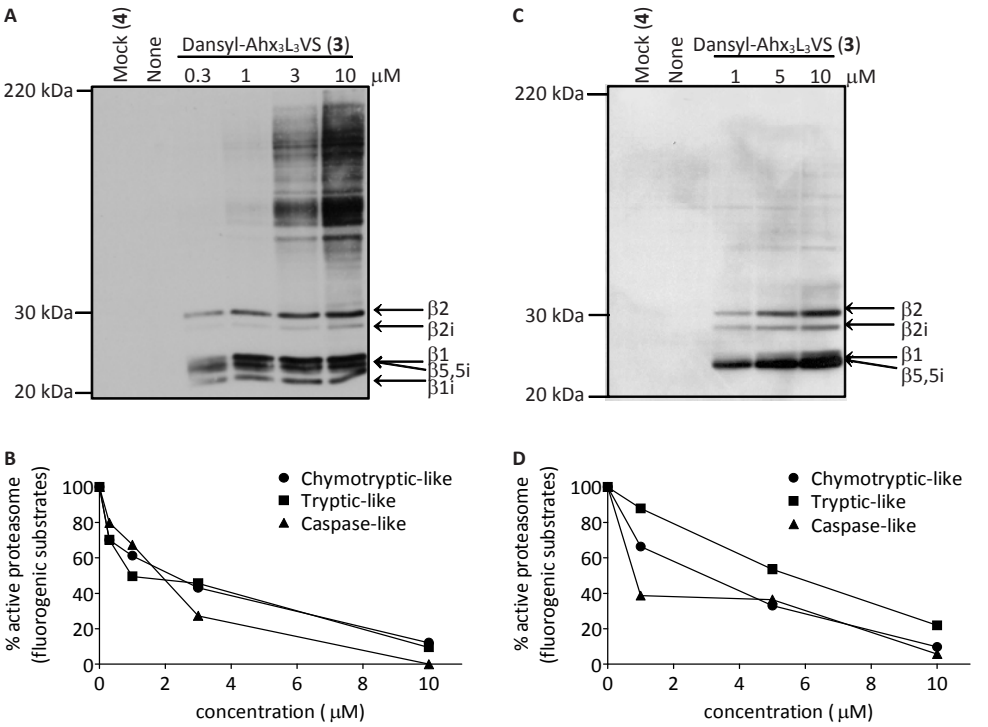

Figure 4 | Profiling of proteasome activity in EL4 cell extracts and cells. A,C) Immunoblots originating from the same gel showing results of a 1-h labeling experiment in EL4 cell extracts **(A)** or a 2-h labeling experiment in living EL4 cells **(C)** with the indicated concentrations of inhibitor **3** or 10 μM inactive control **4**. **B,D)** Measurement with fluorogenic substrates of residual proteasome activity in EL4 cell extracts **(B)** or living EL4 cells **(D)** after a 1-h **(B)** or 2-h **(D)** treatment with the indicated concentrations of inhibitor **3**. Results are representative of two independent experiments.

the presence of equal concentrations of inhibitor. Thus, inhibitor **3** labels proteasomes with greater selectivity *in vivo*. Next, we validated the *in vivo* activity pattern obtained with probe **3** by performing activity measurements with fluorogenic substrates in extracts prepared from identical samples (Figure 4D). *In vivo*, the saturation of subunit labeling corresponded to the inhibition of proteasome activity. At 10 μM, compound **3** almost completely inhibited proteasome activity, as was the case *in vitro*, confirming that it is unlikely that an altered bioavailability of compound **3** *in vivo* is responsible for the altered labeling pattern. Next, we used inhibitor **3** to profile the proteasome activity in HeLa cells *in vivo*. Again, the β5 subunits seemed to be most active and therefore the targets for the majority of the *in vivo* active-site labeling (Supplementary Figure 1). We conclude that proteasome activity profiles obtained by performing measurements in cell extracts are not necessarily representative of the *in vivo* activity patterns, stressing the need for live cell–based assays.

In vivo profiling of bortezomib and MG132

Next, we determined whether the probe could be used to accurately measure the remaining proteasome activity after inhibition by other proteasome inhibitors. We cultured EL4 cells

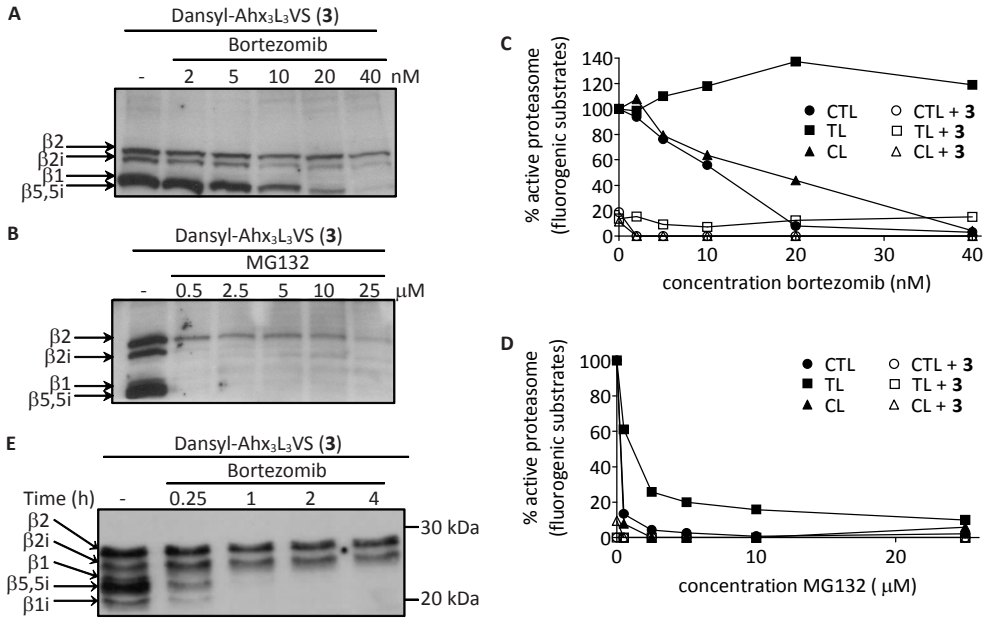

Figure 5 | *In vivo* profiling of proteasome inhibitors. A,B) Competition experiment between inhibitor **3** and bortezomib **(A)** or MG132 **(B)** in EL4 cells. **C,D)** Measurement of residual proteasome activity with fluorogenic substrates after treatment of EL4 cells with bortezomib or a combination of bortezomib and inhibitor **3 (C)** or MG132 or a combination of MG132 and inhibitor **3 (D)**. Results are representative of two independent experiments. CTL: chymotryptic-like, TL, tryptic-like; CL, caspase-like. **E)** Competition experiment between bortezomib and inhibitor **3** in MM.1S cells.

in the presence of bortezomib or MG132, then incubated with inhibitor **3** and immunoblotted as described above. We performed activity measurements with fluorogenic substrates in parallel before and after addition of inhibitor **3**. The addition of bortezomib resulted in a very specific reduction in the ability of compound **3** to modify the proteasomal β5, β5i and β1 subunits, with maximum inhibition of these subunits by bortezomib reached at 40 nM (Figure 5A). The β2 and β2i subunits remained fully available for modification with compound **3** at all time points, indicating the lack of a substantial interaction of bortezomib with these subunits *in vivo*. Bortezomib was previously reported to be a specific inhibitor for the chymotryptic-like activity largely provided by the β5 subunits. Here, we identified the β1 subunits as additional targets for bortezomib.

After incubation of EL4 cells with MG132, compound **3** only labeled the β2 subunit suggesting that all other subunits are inhibited by MG132 at high-nM concentrations (Figure 5B). Notably, MG132 completely inhibited the β2i subunit, which suggests a difference in the active-site chemistry between the β2 and β2i subunits. The results we obtained with fluorogenic substrates correlated in both cases to the immunoblot experiments using inhibitor **3** (Figure 5C,D). Inhibitor **3**, however, can be used to distinguish between constitutive and immunoproteasome subunit activity, whereas fluorogenic substrates cannot. Therefore, we did not observe the difference in labeling between the β2i subunit and the

β2 subunit using fluorogenic substrates. Combined, these results validate inhibitor **3** as a probe for measuring proteasome activity as well as for characterization of subunit specificity of proteasome inhibitors *in vivo*. Using inhibitor **3**, we next determined the composition of the active proteasome in the multiple myeloma cell line MM.1S, as well as the proteasomal targets of bortezomib in these cells. We cultured MM.1S cells in the presence or absence of 20 nM bortezomib, a concentration previously demonstrated to be toxic to this cell line.[17] In this cell line, we observed that all constitutive and immunoproteasome catalytic subunits were active in the absence of interferon γ (Figure 5E). The labeling pattern in MM.1S cells resembled the profile that was observed in EL4 cells. A notable difference was the activity of the β1i subunit in living MM1.S cells: we detected this subunit in extracts of EL4 cells, but not when we performed the assay in living cells. As quickly as in 15 min, 20 nM bortezomib substantially inhibited the activity of all β5 and β1 subunits with maximum inhibition reached within 2 h (Figure 5E).

DISCUSSION

Of the proteasome inhibitors described to date, each has a varying ability to inhibit each of the three known catalytic activities.[14,18,19] With proteasome inhibition becoming widely used in the treatment of multiple myeloma, the composition of proteasomes may be an important determinant of clinical response to treatment with such agents. Most methodologies in common practice for measuring proteasome activity use fluorogenic peptidyl substrates and often involve procedures to reduce potentially cross-reacting protease activities.[5,20,21] More importantly, procedures that rely on the hydrolysis of fluorogenic substrates do not distinguish between constitutive and immunoproteasome activity and therefore do not reflect the relative activity of different proteasome subunits accurately.

Recently, cell-based assays[16,22] as well as mouse models[23,24] have been developed for *in vivo* monitoring of proteasome activity. These methods may be useful in screening for new inhibitors and testing bioavailability, respectively, but their activity readout depends on the balance between synthesis and degradation of fusion proteins, which involves many cellular factors other than the proteasome, including the rate of fusion-protein synthesis. Therefore these methods may not accurately reflect true proteasomal activity. Moreover, the use of expressed fusion proteins makes these methods unfit for monitoring human samples. In addition, they do not provide insight in the constitutive/immunoproteasome activity ratio and cannot be used to characterize the specific activity of proteasome inhibitors *in vivo*. The small molecule–based assay described here overcomes these problems.

We have devised proteasome activity–profiling probe **3** that freely reaches cellular targets in intact cells and that can modify covalently and irreversibly all of the proteasome's exposed catalytic subunits. Use of antibodies directed against the dansylsulfonamidohexanoyl hapten present in **3** allows direct measurement of activity of all proteasome subunits in living cells without the need for prior lysis or purification steps. This ensures not only a fast but also accurate activity readout, as under these conditions proteasomes remain in their native state. Using this probe, we characterized the anti-neoplastic agent bortezomib and revealed that the β1 and β1i subunits are targets for this drug, in addition to the previously identified β5 subunits.[20] We also characterized the widely used proteasome inhibitor MG132 and found that it inhibited all subunits, although the β2 subunit was inhibited to a lesser extent. Notably,

MG132 was found to fully inhibit the immunoproteasome subunit β2i.

We have described an easy method for characterizing the specificity of (new) proteasome inhibitors that may be helpful in a clinical setting and could be used to study resistance to this new class of drugs. Differences in labeling profiles observed in experiments with extracts and living cells, which can not be detected using methodology now used to measure proteasome activity, underscores the need for reliable *in vivo* labeling techniques. The technique described here could be adapted for use with other proteases and optimized. Switching to a more chemoselective reactive group could improve both probe reactivity and selectivity, and improving the fluorescence yield may allow for *in vivo* localization studies of active proteasomes and a truly high-throughput, in-gel readout.

MATERIALS AND METHODS

Materials

Unless indicated otherwise, chemicals were obtained commercially of the highest available grade. Cell culture. The cell lines EL4 (mouse, thymoma) and MM.1S (human, multiple myeloma) were cultured in RPMI 1640 medium. HeLa cells (human, cervix epitheloid carcinoma) and U373 cells (human, astrocytoma) were cultured in DMEM. Both media were supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin.

Synthesis and characterization of proteasome probes

Detailed information on synthetic procedures and characterization can be found in the Supplementary Methods and Supplementary Figures 2–4 online. Compounds **3** and **4** were tested and compared to other inhibitors as described previously.[16] U373 cells expressing an ubiquitin-Arg-GFP fusion protein were exposed to various concentrations of compounds **3**, **4**, Ada-Ahx$_3$Leu$_3$VS and various other proteasome inhibitors. After 5 h, we quantitated the accumulation of GFP by FACS. The specificity for proteasomal targets was determined by assaying competition for proteasome inhibition with compound **5**. We incubated equal amounts of protein, obtained by lysis of EL4 cells by glass beads (see below), with the indicated amounts (Figure 3D) of inhibitor **3** or DMSO for 1 h at 37 °C, and then incubated with compound **5** for 2 h at 37 °C. Proteins were denatured by boiling in reducing sample buffer, separated by 12.5% SDS-PAGE and autoradiographed.

Probing proteasome activity in cell extracts

To obtain lysates for proteasome activity assays, we lysed cells with glass beads as previously described.[18] In brief, cells were washed twice with cold phosphate-buffered saline, pelleted and lysed with one volume of glass beads (<106 microns, acid-washed; Sigma) and an equal volume of homogenization buffer (50 mM Tris-HCl pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP and 250 mM sucrose) by vortexing at high speed for 15–30 min at 4 °C. Beads, membrane fractions, nuclei and cell debris were then removed from the supernatant by centrifugation at 16,000g for 5 min. We quantitated the protein content of extracts using the Bradford assay (BioRad) and then incubated equal amounts of protein with the indicated amounts (Figure 4A) of probe **3**, 10 μM control probe **4** or buffer for 1 h at 37 °C. Proteasome activity was assayed as described below.

Probing proteasome activity in living cells

We incubated 10 x 10^6 EL4 cells in 10 mL RPMI 1640 medium supplemented with 10% FCS and penicillin and streptomycin with the indicated amounts (Figure 4C) of probe **3** or 10 μM probe **4**, at 37 °C for 2 h. HeLa cells were incubated with 10 μM probe **3** for the indicated time periods (Supplementary Figure 1). Cells were collected by centrifugation. We lysed cells using glass beads and assayed protein content using the Bradford assay. Proteasome activity was assayed as described below.

In vivo profiling of bortezomib and MG132

We incubated 10 x 10^6 EL4 cells in 10 mL RPMI 1640 medium supplemented with 10% FCS and penicillin and streptomycin with the indicated amounts of bortezomib (Figure 5A) or MG132 (Figure 5B) for 1 h at 37 °C. When indicated, this was followed by a 2-h incubation at 37 °C with 10

μM probe **3**. We incubated 5 x 10^6 MM.1S cells in 5 mL RPMI 1640 medium supplemented with 10% FCS and penicillin and streptomycin with 20 nM bortezomib for the indicated time periods at 37 °C, followed by a 1-h incubation with 10 μM probe **3** at 37 °C. We lysed cells using glass beads or by incubating them for 30 min in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40])and centrifuged the lysates for 5 min to remove membrane fractions, nuclei and cell debris. Proteasome activity was assayed as described below.

Assaying proteasome activity by immunoblotting

Equal amounts (typically 60 μg) of protein were denatured by boiling in reducing sample buffer, separated by 12.5% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. Immunoblotting was performed using a dansyl-sulfonamidohexanoyl polyclonal antibody (1:7,500, rabbit; Molecular Probes) and horseradish peroxidase–coupled goat or swine anti-rabbit secondary antibody (Southern Biotech) followed by enhanced chemiluminescence (Western Lightning, Perkin-Elmer).

Assaying proteasome activity with fluorogenic substrates

Assays were performed as previously described[25] with minor changes. 10 μg of lysate was added to 100 μL of substrate buffer, containing 20 mM HEPES (pH 8.2), 0.5 mM EDTA, 1% DMSO, 1 mM ATP and 30 μM ZAlaArgArg-AMC (tryptic-like), 60 μM ZGlyGlyLeuAMC (chymotryptic-like), 60 μM SucLeuLeuValTyr-AMC (chymotryptic-like) or 60 μM ZLeuLeuGlu-βNA (caspase-like). SDS was omitted from the substrate buffer. Fluorescence was measured every minute for 25 min at 37 °C using a Fluostar Optima 96-well plate reader (BMG lab technologies) (λ ex/em = 355/450 nm for AMC and 320/405 nm for βNA) and the increase in fluorescence per minute was used to calculate specific activities of each sample. Cells were incubated with 1 μM epoxomicin (Affiniti) at 37 °C for 1 h to determine the nonspecific activity, which was subtracted from each measurement.

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Supplementary information

SUPPLEMENTARY METHODS

Inhibitors: Bortezomib was synthesized as previously described[1]. Dansyl-Ahx$_3$L$_2$-OH (**5**) was synthesized (Supplementary Scheme 1) on Wang resin using standard Fmoc-based solid-phase peptide synthesis protocols and purified by silica gel chromatography using a gradient of 10% to 20% of MeOH in CH_2Cl_2. **5** was coupled by *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI)-mediated condensation with (S,*E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-amine (leucine vinyl sulfone, LeuVS) **7** to afford **3**. MS(ESI): calculated 990.5 $(M+H)^+$ 1012.5 $(M+Na)^+$. Observed 990.5 $(M+H)^+$ 1012.5 $(M+Na)^+$. Hydrogenation and deprotection of **6** followed by condensation of the resulting (S)-5-methyl-1-(methylsulfonyl)hexan-3-amine **9** with **5** afforded the negative control **4**. MS(ESI): calculated 992.5 $(M+H)^+$ 1014.5 $(M+Na)^+$. Observed 992.5 $(M+H)^+$ 1014.5 $(M+Na)^+$.

SUPPLEMENTARY INFORMATION ONLINE

Detailed information on the characterization of compound **3** can be found in Supplementary Figures 2–4. This material is available via the Internet at http://www.nature.com/nmeth, as supplementary information of *Nature Methods* **2**, 357-362 (2005).

SUPPLEMENTARY REFERENCES

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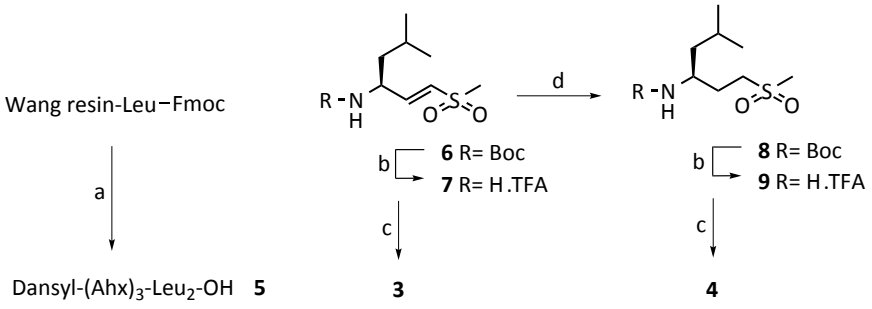
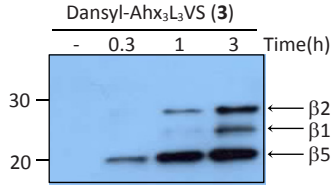


Supplementary Scheme 1 | a) Fmoc based SPPS then TFA, (78%). b) TFA quant. c) EDCI, DIPEA, CH_2Cl_2/DMF (**3**: 64%, **4**: 84%). d) 10% Pd/C, H_2, MeOH (98%).

Supplementary figures



Supplementary Figure 1 | Incubation of HeLa cells with inhibitor 3.