A Fluorescent Broad-Spectrum Proteasome Inhibitor for Labeling Proteasomes In Vitro and In Vivo

Martijn Verdoes,1,5 Bogdan I. Florea,1,5 Victoria Menendez-Benito,2 Christa J. Maynard,5 Martin D. Witte,1 Wouter A. van der Linden,1 Adrianus M.C.H. van den Nieuwendijk,1 Tanja Hofmann,1 Celia R. Berkers,3 Fijis W.B. van Leeuwen,3 Tom A. Groothuis,4 Michiel A. Leeuwenburgh,4 Huib Ovaa,3 Jacques J. Neefjes,4 Dmitri V. Filippov,1 Gijs A. van der Marel,1 Nico P. Dantuma,2 and Herman S. Overkleeft1,5

1Bio-organic Synthesis
Leiden Institute of Chemistry
Leiden University
2300 RA Leiden
The Netherlands
2Department of Cell and Molecular Biology
The Medical Nobel Institute
Karolinska Institutet
SE-171 77 Stockholm
Sweden
3Division of Cellular Biochemistry
Division of Tumor Biology
Netherlands Cancer Institute
1066 CX Amsterdam
The Netherlands

Summary

The proteasome is an essential evolutionary conserved protease involved in many regulatory systems. Here, we describe the synthesis and characterization of the activity-based, fluorescent, and cell-permeable inhibitor Bodipy TMR-Ahx3L3VS (MV151), which specifically targets all active subunits of the proteasome and immunoproteasome in living cells, allowing for rapid and sensitive in-gel detection. The inhibition profile of a panel of commonly used proteasome inhibitors could be readily determined by MV151 labeling. Administration of MV151 to mice allowed for in vivo labeling of proteasomes, which correlated with inhibition of proteasomal degradation in the affected tissues. This probe can be used for many applications ranging from clinical profiling of proteasome activity, to biochemical analysis of subunit specificity of inhibitors, and to cell biological analysis of the proteasome function and dynamics in living cells.

Introduction

The 26S proteasome is the central protease in ATP– and ubiquitin-dependent degradation of proteins in the eukaryotic cytoplasm and nucleus and is responsible for the degradation of 80%–90% of all cellular proteins. The proteasome is involved in the degradation of abnormal and damaged proteins, cell-cycle regulators, oncogenes, and tumor suppressors, and it is imperative in the generation of MHC class I antigenic peptides [1]. Eukaryotic proteasomes contain two copies of seven distinct α and β subunits each. These subunits assemble into two types of heterooligomeric rings that are each composed of seven subunits (α1–α7 and β1–β7). The 20S proteasome is formed by two juxtaposed rings of β subunits flanked on top and bottom by a ring of α subunits [2]. When capped by the 19S regulatory complex at both ends, the proteolytically active 26S proteasome is formed and is responsible for ATP-dependent proteolysis of polyubiquitinated target proteins [3].

In the eukaryotic proteasome, three of the seven β subunits are responsible for the proteolytic activities of the proteasome. Characterization of the active β1, β2, and β5 subunits led to the classification of their substrate specificity as peptidylglutamyl peptide hydrolytic, trypsin-like, and chymotrypsin-like, respectively. In immune-competent cells, three additional active β subunits (βii) are expressed upon interferon-γ stimulation. These subunits assemble in a new proteasome particle called the immunoproteasome, which coexists with the constitutive proteasome [2].

The proteolytic subunits β1, β2, and β5 and their immunoproteasomal counterparts, β1i, β2i, and β5i, respectively, act by nucleophilic attack of the γ-hydroxyl of the N-terminal threonine on the carbonyl of the peptide bond destined for cleavage. The α-amine of the threonine acts as a base in the catalytic cycle. The existence and evolutionary development of six different active β subunits, their divergent substrate specificities, and their individual roles in cellular processes constitute a vast research field of interest in both academia and the pharmaceutical industry. This scientific demand can benefit from an activity-based proteasome probe that ideally (1) specifically targets the proteasome, (2) covalently and irreversibly binds to the three active β and βi subunits indiscriminately, (3) facilitates direct, rapid, accurate, and sensitive detection, (4) is cell permeable, and (5) enables monitoring of the proteasome by microscopic techniques in living cells.

To date, none of the available activity-based proteasome probes meet all of these requirements [4, 5]. The compound that comes closest is the radiolabeled proteasome inhibitor AdaY(125I)Ahx3L3VS [6]. In this compound, the leucine vinyl sulfone mimics the peptide carbonyl and acts as a nucleophilic trap that covalently modifies the γ-hydroxyl of the N-terminal threonine through a Michael addition. This inhibitor is selective for the proteasome, labels the β subunits with equal intensity, and enables accurate and sensitive in-gel detection. However, usage of this activity-based probe is restricted to in vitro applications since this compound is not cell permeable. Recently, the weakly fluorescent and cell-permeable proteasome inhibitor dansyl-Ahx3L3VS was developed for profiling proteasome activity in living cells, enabling readout by antidansyl immunoblotting [7]. The low quantum yield and near-UV excitation of the dansyl makes this compound

*Correspondence: h.s.overkleeft@chem.leidenuniv.nl
These authors contributed equally to this work.
unsatisfactory for in-gel detection and standard fluorescence microscopic techniques.

Here, we present the synthesis and characterization of the fluorescent, cell-permeable, and activity-based proteasome probe Bodipy TMR-Ahx$_3$L$_3$VS (MV151). After proteasome labeling and protein separation by SDS-PAGE, the modified proteasome subunits are immediately visualized by in-gel fluorescence readout. Furthermore, this compound enables fast and sensitive labeling of proteasomes in vitro, in cells, and in mice; is compatible with live-cell imaging techniques; and facilitates screening and determination of the subunit specificity of novel proteasome inhibitors.

Results and Discussion

Synthesis of Bodipy TMR-Ahx$_3$L$_3$VS

Bodipy TMR-Ahx$_3$L$_3$VS (MV151, 6) and the inactive, negative control, Bodipy TMR-Ahx$_3$L$_3$ES (MV152, 7), in which the vinyl sulfone moiety is reduced to an ethyl sulfone, were synthesized as depicted in Figure 1. Acidic cleavage of Fmoc-Ahx$_3$-Wang resin (3), synthesized by using standard Fmoc-based solid-phase peptide chemistry, gave the crude Fmoc-Ahx$_3$-OH, which was blocked coupled to TFA-H-Leu$_3$VS (2) [8], to yield Fmoc-protected hexapeptide (4). In situ deprotection of the Fmoc-protecting group with DBU and treatment with Bodipy TMR succinimidyl ester (5) ([9–11, 12]; see the Supplemental Data available with this article online) afforded target compound 6. In order to obtain the inactive control compound 7, hexapeptide 4 was first treated with hydrogen gas and palladium on charcoal in methanol to reduce the vinyl sulfone, followed by Fmoc cleavage and introduction of the Bodipy TMR moiety.

Proteasome Labeling and In-Gel Detection

The potency of MV151 (6) was determined by measuring proteasomal activity by using fluorogenic substrates. EL-4 lysates were incubated with increasing concentrations of MV151, and the cleavage of the substrates Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like activity), Z-Ala-Ala-Arg-AMC (trypsin-like activity), and Z-Leu-Leu-Glu-$\beta$NA (peptidylglutamyl peptide hydrolytic activity) was monitored. At concentrations below 1 μM, MV151 appears to inhibit trypsin-like activity and chymotrypsin-like activity more efficiently than it inhibits PGPH activity (Figure 2A). This might be due to differences in activity between the subunits, to allosteric effects, to minor subunit specificities of the probe, or to nonsaturation kinetics. At concentrations of 1 μM and higher, MV151 completely inhibits all three activities.
Direct in-gel visualization of MV151-labeled proteasome subunits was explored by using a fluorescence scanner. Treatment of purified human 20S proteasome with MV151 showed uniform labeling of the active subunits \(\beta_1, \beta_2,\) and \(\beta_5\) (Figure 2B). To determine the sensitivity of the in-gel detection, we directly compared fluorescence readout of the gel (Figure 2B) with detection of proteasome subunits by silver staining of proteins (Figure 2C). The in-gel detection was shown to be very sensitive since as little as 3 ng proteasome was sufficient to detect individual MV151-labeled proteasome subunits; detection with this method is at least three times more sensitive than silver staining.

We next compared the labeling of the constitutive \(\beta_1,\) \(\beta_2,\) and \(\beta_5\) subunits and the immunoproteasome \(\beta_{1i},\) \(\beta_{2i},\) and \(\beta_{5i}\) subunits. For this purpose, we labeled the proteasomes in lysates of the human cervix carcinoma cell line HeLa (expressing constitutive proteasome) and the murine lymphoid cell line EL-4 (expressing both constitutive and immunoproteasome) with increasing concentrations of MV151. All active constitutive and inducible \(\beta\) proteasome subunits were neatly and uniformly labeled by MV151 (Figures 2D and 2E). All subunits were already detectable at a concentration of 10 nM MV151 and reached saturation in fluorescence signal at 1 \(\mu\)M MV151. At higher concentrations of MV151, an increased nonspecific labeling was observed in the high molecular weight region.

Proteasome Profiling Screen of Known Inhibitors

Next, we performed competition experiments with MV151 to determine the subunit specificity of a panel of known proteasome inhibitors. EL-4 and HeLa cell lysates (10 \(\mu\)g total protein) were first incubated for 1 hr with the inhibitor of interest. After incubation with the proteasome inhibitor, the subunits that were still active were fluorescently labeled by treating the lysates with 100 nM MV151 for 1 hr.

In HeLa lysates, epoxomicin preferentially inhibits the \(\beta_5\) subunit, already visible at a 10 nM concentration. At epoxomicin concentrations over 100 nM, \(\beta_1\) and \(\beta_2\) are also targeted, with a slight preference for \(\beta_2\) (at 5 \(\mu\)M epoxomicin, \(\beta_2\) fluorescence is absent and a faint band of \(\beta_1\) is still visible) (Figure 3A, right panel). This is in accordance with the inhibition profile of epoxomicin determined with purified 20S proteasome [12]. Interestingly, in EL-4 lysates, epoxomicin preferentially inactivates \(\beta_2\) and \(\beta_2i\) and is less active toward constitutive and immunoinduced \(\beta_1\) and \(\beta_5\) subunits (Figure 3A, left panel).

Dansyl-Ahx3L3VS [7] inhibits all active constitutive and immunoinduced subunits in EL-4 lysates from concentrations of 500 nM and greater (Figure 3B, left panel). In HeLa lysates, dansyl-Ahx3L3VS has a preference for the \(\beta_5\) subunit, which is visible at 100 nM, and less of a preference for the \(\beta_1\) and \(\beta_2\) subunits, which are visible at slightly higher concentrations (Figure 3B, right panel).

The dipeptidyl pinanediol boronic ester (pinanediol boronic ester of Bortezomib [13]) shows a strong selectivity for the constitutive \(\beta_1\) and \(\beta_5\) subunits in HeLa lysates (Figure 3C, right panel) and \(\beta_1i,\) \(\beta_{1i},\) and \(\beta_{5i}\) in EL-4 lysates (Figure 3C, left panel). The inhibition profile of the dipeptidyl pinanediol boronic ester is comparable to the labeling profile of Bortezomib [7], with potency in the same order of magnitude.
As previously reported, NLVS [8] shows a predilection for β5 (Figure 3D), whereas ZLVS [8] (Figure 3E) proves to be the least potent compound and shows some preference for constitutive and immunoinduced β1 and β5 subunits.

In EL-4 lysates, ada-Ahx3L3VS [6] first targets the β2 and β2i subunits, and it shows a preference for β2 and β5 in HeLa lysates (Figure 3F).

Altogether, this experiment shows that MV151 can be used for the determination of inhibition profiles of proteasome inhibitors. Exploiting the sensitivity of in-gel detection of MV151, it is possible to demonstrate that the inhibitors tested show subtle differences in the proteasome inhibition profile.

Functional Proteasome Inhibition in Living Cells

We next addressed whether MV151 is able to label proteasome subunits in living cells. EL-4 and HeLa cells were incubated with increasing concentrations of MV151. Specific and sensitive labeling of all proteasome subunits was observed in EL-4 (Figure 4A) and HeLa cells (Figure 4B), although higher concentrations were required than for labeling of subunits in lysates. Labeling of the β1 subunit shows a lower intensity than in lysates, whereas β5 labeling looks more pronounced. This difference in the labeling profile between the proteasome in cell lysates and living cells has been previously reported [7]; however, the reason for this remains unclear. Importantly, incubation of EL-4 and HeLa cells with the inactive control compound MV152 (7), which is almost identical to MV151 but lacks the reactive vinyl sulfone warhead, showed no labeling of the proteasome or any other protein (Figures 4A and 4B).

In vivo functionality of MV151 was determined in HeLa cells stably expressing a green fluorescent protein (GFP) reporter proteasome substrate [14]. The ubiquitinG76V-GFP (UbG76V-GFP) fusion expressed by these cells is normally rapidly degraded by the proteasome. Indeed, untreated UbG76V-GFP HeLa cells emitted only low levels of GFP fluorescence (Figure 4C, left panel). Cells that were exposed to 10 μM of the inactive MV152 for 12 hr did accumulate the control compound, but they did not show increased levels of GFP fluorescence (Figure 4C, middle panel). During 12 hr of exposure to 10 μM MV151, cells accumulated the inhibitor and showed significantly increased levels of GFP fluorescence (Figure 4C, right panel). Strong fluorescence is apparent in the membranous compartments of cells treated with the inactive MV152. This fluorescence, which appears to be stronger than in MV151-treated cells, is likely to accumulate in the lipid bilayers, because it is sequestered by the proteasome active sites. It should be noted that Bodipy dyes fluoresce strongly in hydrophobic environments. There was no visual evidence of cellular toxicity at the dose and exposure time used in this study. These results were confirmed by a study with the human melanoma cell line MelJuSo stably expressing the N-end-rule reporter proteasome substrate Ub-R-GFP [14] (data not shown).

We next set out to determine whether the intracellular staining pattern of MV151 colocalized with the proteasome in living cells. To this end, we used MelJuSo cells

Figure 3. Proteasome Profiling Screen of Known Inhibitors by Using MV151

(A–F) EL-4 and HeLa lysates (10 μg total protein) were incubated with the indicated concentrations of the (A) proteasome inhibitor epoxomicin, (B) dansyl-Ahx3L3VS, (C) dipeptidyl pinanediol boronic ester, (D) NLVS, (E) ZLVS, and (F) Ada-Ahx3L3VS for 1 hr at 37°C. The remaining activity of the β subunits was fluorescently labeled by incubation with 0.1 μM MV151 for 1 hr at 37°C.
that stably express a GFP-tagged βi proteasome subunit, which is efficiently incorporated into the proteasome particles [15]. The GFP-βi fusion construct shows ubiquitous distribution throughout the cytoplasm and nucleus, with exception of nucleoli and the nuclear envelope (Figure 4D). The GFP-βi cells were incubated with 10 μM MV151 and the distribution of proteasomes and inhibitor was compared. The intracellular permeation of MV151 was monitored in time and is characterized by a fast permeation phase (several minutes), followed by a slow distribution phase (several hours, data not shown). During the permeation phase, the compound showed significant association with the plasma membrane, in discrete cytoplasmatic vesicular and membranous fractions and at the nuclear envelope.

After 5 hr of distribution, MV151 is localized throughout the cell, with the exception of the nucleoli, similar to the GFP-βi fusion (Figures 4D–4F). The fact that MV151 is excluded from the nucleoli is in line with the idea that the compound is associated with the proteasome. In some cells, granular accumulation of MV151 was observed in the cytoplasm in close proximity to the nucleus.

To attest whether the in-gel readout could be correlated with the fluorescent microscopy data, MV151 was competed with the proteasome inhibitor MG132 (Figures 4G–4J). MelJuSo Ub-R-GFP cells incubated with MV151 for 1 hr showed labeling of the active proteasome subunits on gel (Figure 4G, lane 1) and, after fixation with formaldehyde, strong fluorescence in the cytoplasm and nucleus, with the exception of nucleoli (Figure 4H). In Figure 4I, cells incubated with MV151 for 1 hr, followed by a 1 hr incubation with MG132, showed labeling of the active proteasome subunits on gel (Figure 4G, lane 2) and a similar cellular localization to that shown in Figure 4H. When the cells where first

**Figure 4. Functional Proteasome Inhibition in Living Cells**

(A and B) Proteasome profiling in living (A) EL-4 and (B) HeLa cells after a 2 hr incubation with the indicated concentrations of MV151. As a control, the cells were incubated with the inactive compound MV152. A purified proteasome labeled with MV151 is also shown.

(C) Representative micrographs of UbG76V-GFP HeLa cells that were untreated (left panel), incubated for 12 hr with 10 μM inactive MV152 (middle panel), and incubated for 12 hr with 10 μM MV151 (right panel). Bodipy TMR and UbG76V-GFP fluorescence are shown.

(D–F) Colocalization of a GFP-labeled proteasome and MV151 in living GFP-βi MelJuSo cells treated for 8 hr with 10 μM MV151. (D) GFP-βi, (E) Bodipy TMR fluorescence, and (F) a merged image are shown.

(G) In-gel visualization of proteasome labeling in living EL-4 cells: lane 1, a 1 hr incubation with MV151 (250 nM); lane 2, a 1 hr incubation with MV151 (250 nM), followed by a 1 hr incubation with MG132 (5 μM); lane 3, a 1 hr incubation with MG132 (5 μM), followed by a 1 hr incubation with MV151 (250 nM).

(H–J) CLSM pictures of Bodipy TMR fluorescence in MelJuSo Ub-R-GFP cells after formaldehyde fixation, gain 700. (H) Confocal picture after a 1 hr incubation with MV151 (500 nM). (I) Confocal picture after a 1 hr incubation with MV151 (500 nM), followed by a 1 hr incubation with MG132 (5 μM). (J) Confocal picture after a 1 hr incubation with MG132 (5 μM), followed by a 1 hr incubation with MV151 (500 nM).
incubated with MG132 for 1 hr, followed by a 1 hr incubation with MV151, in-gel readout proved negative (Figure 4G, lane 3) and the fluorescence in the cells had dramatically decreased (Figure 4J). This competition study proves that the vast majority of the fluorescence observed in cells, after fixation, is due to proteasome labeling.

Monitoring of Proteasome Inhibition in Mice
The results obtained in cell lines prompted us to investigate whether MV151 could be used to label proteasomes in mice. To test the bioavailability of MV151, C57Bl/6 mice were given a single intraperitoneal injection with MV151 (20 μmol/kg body weight) and were sacrificed 24 hr postinjection.

Fluorescence microscopic analysis of mouse tissues revealed the capacity of MV151 to penetrate tissues in vivo. The highest Bodipy TMR fluorescence was detected in the liver (Figure 5A) and in the pancreas (Figure 5B). Interestingly, Bodipy TMR fluorescence was higher in the peripheries of the tissues, indicating that the probe might reach the liver most efficiently by diffusion from the peritoneal cavity rather than being distributed by entering the bloodstream.

To examine the effect of administration of the proteasome probe, we took advantage of a recently developed transgenic mouse model for monitoring the ubiquitin-proteasome system, which is based on the ubiquitous expression of the Ub<sup>G76V</sup>-GFP reporter [16]. We have previously shown that administration of the proteasome inhibitors epoxomicin and MG262 results in a substantial accumulation of the Ub<sup>G76V</sup>-GFP reporter in affected tissues [16]. The accumulation was primarily found in the liver and at higher concentrations in other tissues. In the present experiment, the Ub<sup>G76V</sup>-GFP reporter mice were given a single intraperitoneal injection with

Figure 5. Functional Proteasome Inhibition in Mice
(A and B) Micrographs of (A) liver and (B) pancreas cryosections from C57Bl/6 mice that were treated with vehicle only or with MV151 (20 μmol/kg body weight). Hoechst staining and Bodipy TMR fluorescence are shown. The scale bar represents 40 μm.

(C–G) Micrographs and in-gel fluorescence readout of (C and D) liver and (E and F) pancreas. (C and E) Cryosections from Ub<sup>G76V</sup>-GFP mice that were treated with vehicle only or with MV151 (20 μmol/kg body weight). Hoechst staining, Bodipy TMR fluorescence, Ub<sup>G76V</sup>-GFP fluorescence, and Bodipy TMR and Ub<sup>G76V</sup>-GFP merged images are shown. The scale bar represents 40 μm (upper and middle panels) and 5 μm (lower panels). (D and F) SDS-PAGE analysis and in-gel fluorescence readout of homogenates (10 μg total protein) from liver and pancreas tissues shown in (C) and (E), respectively, and (G) in spleen.
MV151 (20 μmol/kg body weight). A total of 24 hr postinjection, the mice were sacrificed and several tissues were analyzed by fluorescence microscopy. Cells accumulating Ub G76V-GFP were detected in the liver (Figure 5C) and the pancreas (Figure 5E), which also contained the highest Bodipy TMR fluorescence of all of the examined tissues (spleen, intestine, kidney, liver, and pancreas). Importantly, all of the cells that accumulated the UbG76V-GFP reporter contained very high Bodipy TMR fluorescence. The proteasome probe was distributed both in the cytoplasm and nuclei of the cells that accumulated the reporter. Similar to our observations from experiments in cell culture, the affected cells in the mice contained granular accumulations of MV151 in the cytoplasm in close proximity to the nucleus. We verified that accumulation of UbG76V-GFP in the liver and pancreas coincided with proteasomal blockade by MV151. SDS-PAGE followed by in-gel fluorescence analysis of liver (Figure 5D) and pancreas (Figure 5F) homogenates of animals treated with MV151 revealed that the proteasome catalytic subunits were labeled as expected, although higher background labeling compared to in vitro studies was observed. (For Figures 5D and 5F, respectively, the tissues from the images in Figures 5C and 5E were used.) SDS-PAGE followed by in-gel fluorescence analysis of spleen homogenates showed labeling of both constitutive and inducible proteasome catalytic subunits (Figure 5G).

As the final set of experiments, we monitored the biodistribution of MG262 in UbG76V-GFP transgenic mice. Animals were injected subcutaneously with either 5 μmol/kg or 10 μmol/kg body weight of the boronic acid MG262 and were sacrificed 24 hr postinjection. Spleen and pancreas were lysed and treated with MV151. SDS-PAGE analysis revealed significant reduction of labeled bands corresponding to the proteasome catalytic subunits when compared with tissue lysates from untreated animals (Figure 6A, pancreas; Figure 6B, spleen). Fluorescence microscope analysis of the same tissues (Figures 6A and 6B) confirmed the concentration-dependent inhibition of the proteasome in MG262-treated UbG76V-GFP mice, as indicated by increased levels of UbG76V-GFP reporter accumulation. In summary, we described the synthesis of MV151 and characterized it as being a cell-permeable, broad-spectrum proteasome inhibitor. MV151 enables broad-spectrum proteasome profiling, both in cell lysates and in living cells. The Bodipy TMR dye proved to be very useful for in-gel readout of labeled active subunits in that it provided a straightforward method for direct and sensitive proteasome profiling and omitted the need for western blotting, radioactivity, and gel drying. MV151 could be readily detected upon administration to mice and correlated with inhibition of the proteasome in the affected tissues. Finally, MV151-mediated proteasome labeling in combination with UbG76V-GFP transgenic mice is a useful strategy for monitoring the biodistribution of proteasome inhibitors.

**Significance**

The proteasome is a key enzyme in the maintenance of cellular homeostasis. Here, the synthesis and
characterization of the activity-based, fluorescent, and cell-permeable MV151 are presented. MV151 targets the protease specifically and shows broad-spectrum activity by covalent and irreversible binding to the catalytic N-terminal threonine residues of immunoinducible and constitutively active $\beta$ subunits. The bright fluorophore facilitates rapid and sensitive detection of active protease subunits by in-gel detection and fluorescence microscopy in living cells. Potentially, MV151 can find application in diverse fields of protease research: in medical research, for profiling the active protease fractions in a clinically relevant sample; and in chemistry and biochemistry, facilitating rapid determination of potency and subunit specificity of new protease inhibitors.

Experimental Procedures

Synthesis

**General Methods and Materials**

All reagents were commercial grade and were used as received unless indicated otherwise. Toluenesulfonyl chloride, ethyl acetate (EA/rac), and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën and were distilled prior to use. Dichloromethane (DCM), tetrahydrofuran (THF), and dioxane (Biosolve) were stored on 4 Å molecular sieves. Tetra-n-butylammonium p-toluenesulfonate (Tf$_2$N$_4$, a TMS-salt) and DMAP (12 mg, 0.1 mmol, 0.05 equiv.) for 2 hr. The resin was then filtered off, washed with DCM (3 × 10 mL), and was stirred for 30 min before being concentrated in vacuo. Purification by column chromatography (0.1% TEA in DCM → 3% MeOH, 0.1% TEA in DCM) afforded Bodipy TMR-Ahx$_3$VS (6) (0.22 g, 197 mg, 99%). 1H-NMR: calculated for C$_{53}$H$_{82}$N$_6$O$_9$SH, 979.59388, found 979.59276.

**Bodipy TMR-Ahx$_3$VS, 6**

DBU (30 µL, 0.2 mmol, 1 equiv.) was added to a solution of 4 (0.2 g, 0.2 mmol) in DMF. After 5 min of stirring, HOBT (0.12 g, 0.9 mmol, 4.5 equiv.) and DiPEA (0.2 ml, 1.2 mmol, 6 equiv.) were added, and the mixture was stirred for 30 min before being concentrated in vacuo. Purification by column chromatography (0.1% TEA in DCM → 3% MeOH, 0.1% TEA in DCM) afforded Bodipy TMR-Ahx$_3$VS (6) (0.22 g, 197 mg, 99%). 1H-NMR: calculated for C$_{53}$H$_{82}$N$_6$O$_9$SH, 979.59388, found 979.59276.

**Bodipy TMR-Ahx$_3$L$_3$VS, 4**

The tripeptide Fmoc-Ahx$_3$-OH was released from resin (0.45 mmol) by treatment with TFA/DCM (1/1, v/v, 30 min, 3 equiv.). The fractions were collected and coevaporated with toluene (3×). The crude Fmoc-Ahx$_3$-OH was dissolved in DCM/DMF (99/1, v/v) and concentrated under reduced pressure. The residue was dissolved in chloroform and washed with 1 M HCl and sat. aq. NaHCO$_3$. The organic layer was dried over MgSO$_4$ and concentrated. Silica column chromatography (DCM → 4% MeOH in DCM) yielded the title compound, 4 (0.41 g, 0.44 mmol, 98%). 1H-NMR: calculated for C$_{53}$H$_{82}$N$_6$O$_9$SH, 979.59388, found 979.59276.

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Proteasomal Activity Measurement with Fluorogenic Substrates

Protein lysates from EL-4 (1 mg/ml) were incubated with various concentrations of MV151 (100 nM) for 1 hr at 37 °C. For measurement of proteasomal activities, 10 μg labeled lysate was added to 100 μl substrate buffer, containing 20 mM HEPES (pH 8.2), 0.5 mM EDTA, 1% DMSO, 1 mM ATP, and 10 μM Z-Ala-Ala-Arg-AMC (trypsin-like), or 60 μM Suc-Leu-Leu-Val-Tyr-AMC (chymotryptic-like), or 60 μM Z-Leu-Leu-Glu-7-11NA (caspase-like). Fluorescence was measured every minute for 25 min at 37 °C by using a Fluostar Optima 96-well plate reader (BMG Labtechnologies) (λex/λem = 355/450 nm for AMC and 320/405 nm for 11NA). The maximum increase in fluorescence per minute was used to calculate specific activities of each sample. Nonspecific hydrolysis was assessed by preincubation with 1 μM epoxomicin for 1 hr at 37 °C and was subtracted from each measurement.

In-Gel Detection of Labeled Proteasome Subunits

Whole-cell lysates were made in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM DTT, 5 mM MgCl2, 250 mM sucrose, 2 mM ATP. Protein concentration was determined by the colorimetric Bradford method. For the labeling reactions, 10 μg total protein lysates was incubated for 1 hr prior to incubation with MV151 (0.1 μM) for 1 hr at 37 °C. For assessment of background labeling, heat-inactivated lysates (10 μg) were exposed to the known inhibitors for 1 hr at 37 °C and was detected at 500–530 nm. MV151 and MV152 were excited at 490 nm and was detected at 500–530 nm. MV151 and MV152 were excited at λex = 498 nm and was detected at 500–530 nm. MV151 and MV152 were excited at λex = 543 nm and were detected at 560–620 nm. CLSM images were adjusted for brightness and contrast by using Photoshop software.

Mouse Experiments

All animal experiments were approved by the Ethical Committee in Stockholm (ethical permission numbers N-46/04 and N18/05). Mice were housed according to Swedish animal care protocols with a 12 hr day/night cycle, and they were fed standard laboratory chow and tap water ad libitum. Adult C57Bl/6 and UbCzo6-GFP/F1 mice [16], matched for sex and age, were given a single intraperitoneal injection of vehicle (60% DMSO, 40% PBS), MV151 (20 μmol/kg body weight), or MQ262 (Affinity) (5 or 10 μmol/kg body weight) in a total volume of 200 μl. Based on prior experience in our lab, the bortezomib dose was then doubled to 10 μmol/kg, and the experiment was repeated. Further, the two groups showed no apparent toxicity in mice. Mice were euthanized 24 hr postinjection by anesthesia with inhaled isoflurane (4.4% in oxygen), followed by transcardial perfusion with 50 ml PBS for removal of contaminating blood. Tissues collected for immunocytochemical analysis were processed as described previously [16]. Briefly, 12 μm cryosections were fixed for 15 min in 4% paraformaldehyde/PBS and washed in PBS; where mentioned, Hoechst nuclear stain (2 μg/ml in H2O) was applied for 15 min in the dark, followed by washing in PBS. Sections were mounted in a matrix containing 2.5% DABCO (Aldrich). Confocal microscopy was performed on a Zeiss LSM 510 META system. Tissues isolated for in-gel analysis were lysed with a Heidolph tissue homogenizer in 300 μl lysis buffer and were further treated as described above.

Supplemental Data

Supplemental Data include information on the synthesis and analytical data of the Bipyridyl TMR-succinimidyl ester, 5, and are available at http://www.chembiol.com/cgi/content/full/13/11/1217/DC1/.

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