Fluorescent-based proteasome activity profiling

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Abstract

The proteasome is a large protease complex responsible for the degradation of key regulatory proteins involved in many cellular processes, including cell proliferation and survival. The observation that cancer cells are more sensitive to proteasome inhibition than normal cells has led to the development of several proteasome inhibitors for the treatment of cancer. The proteasome inhibitor bortezomib (the active ingredient of Velcade) is approved for the treatment of multiple myeloma and mantle cell lymphoma, while a number of second-generation proteasome inhibitors, which differ in their mode of inhibition and subunit specificity, are currently in clinical trials. With the proteasome emerging as a therapeutic target for cancer treatment, accurate tools for monitoring proteasome (inhibitor) activity are in demand. In this chapter, the synthesis and use of a fluorescent proteasome activity probe is described that allows for accurate profiling of proteasomal activity in cell lysates, intact cells and murine and patient material, with high sensitivity using SDS-PAGE. The probe allows for direct scanning of the gel for fluorescent emission of the distinct proteasomal subunits and circumvents the use of western-blot analysis. Due to its suitable biochemical and biophysical properties the fluorescent probe can also be used for confocal laser scanning microscopy and flow cytometry-based experiments.

Key Words

Proteasome, inhibiton, activity profiling, fluorescent probe, proteasome activity assay.

1. Introduction

The use of proteasome inhibitors in the clinic for cancer treatment (***1***), has validated the proteasome as therapeutic target. The proteasome is responsible for the degradation of misfolded and redundant proteins and of key regulatory proteins, involved in many cellular processes such as proliferation and survival (***2,3***). Inhibition of the proteasome causes disruption of many of these processes, eventually leading to cell death (***1,4***). The proteasome inhibitor bortezomib (***1,5***) (**Fig. 1,** the active compound of Velcade) is currently used for the treatment of multiple myeloma (***6***) and mantle cell lymphoma (***7***). A number of second-generation proteasome inhibitors, which differ in their mode of inhibition and subunit specificity, are currently in clinical trials.

Eukaryotic 26S proteasomes consist of a 20S core and one or two 19S regulatory caps. The 19S regulatory caps are involved in the recognition and unfolding of polyubiquitinated substrates, while the catalytic activity takes place in the 20S core (***3***). The 20S proteasome is a barrel-shaped protein complex composed of four stacked rings that consist of seven subunits each. The two outer rings are made up of α-subunits and interact with the 19S regulatory caps. The two inner rings consist of β-subunits, from which three constitutive subunits, termed β1, β2 and β5, are responsible for proteolysis. The β1, β2 and β5 subunits each provide a distinct protease activity, the caspase-like (cleavage after acidic residues), tryptic-like (cleavage after basic residues) and chymotryptic-like (cleavage after hydrophobic residues) activity, respectively. Upon interferon-γ stimulation, three additional immunoproteasome subunits are expressed (β1i, β2i and β5i), which replace the constitutive β1, β2 and β5 subunits to form the immunoproteasome. Immunoproteasomes are thought to have altered catalytic activity

favoring production of antigenic peptides and are mainly expressed in lymphoid tissues, e.g. spleen, thymus and lymph nodes (***8***).

Different cells can express different ratios of constitutive and immunoproteasome subunits (***9,10***). Variations in proteasomal composition affect substrate specificity and sensitivity to proteasome inhibition. To predict the sensitivity of patients to proteasome inhibitors, accurate tools are required that can correlate proteasome composition and the extent of proteasome inhibition to treatment response. As such, reagents that can be used to profile proteasome activity are valuable research tools and hold promise as diagnostic reagents.

Techniques that are commonly used to monitor proteasome activity include the application of fluorogenic substrates (***11,12***), small molecule-based activity assays (***13-18***) and models based on recombinant reporter proteins (***19-21***). Traditionally, fluorogenic substrates are used to measure the activity of the different proteasome active sites, but most fluorogenic substrates cannot be used in cells, and prior cell lysis is required before activity measurements can be performed. Reporter proteins can be used in living cells, but their use is limited to genetically altered cells or organisms. In addition, 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.

The first small molecule-based activity assay in its class that could be used to profile the specificity of proteasome inhibitors in living cells was reported in 2005 (***13***). This dansylated vinylsulfone based proteasome probe (**Fig. 1**) contains an α,β-unsaturated sulfone part that reacts through a Michael addition reaction with the γ-hydroxyl of the N-

terminal threonine residue of catalytic β-subunits of the proteasome (***22***), resulting in the formation of a β-sulfonyl ether linkage. Antibodies against the dansyl moiety were used for detection of labeled active subunits by SDS-PAGE and Western blot analysis (***13***). Subsequent replacement of the dansyl group by high quantum yield fluorophores allowed for direct scanning of the SDS-PAGE gel for fluorescence emission of fluorescently labeled subunits (***14,18***). Due to their favorable biochemical and biophysical properties Bodipy-based proteasome activity probes can be used to monitor proteasome activity in cell extracts, living cells and murine tissues using a range of techniques including SDS-PAGE (***14***), confocal laser scanning microscopy (***14,18***) and flow cytometry (***14***).

Here, we describe the facile synthesis of the Bodipy-based fluorophore Me_4BodipyFL-*N*-hydroxy-succinimidyl (NHS) ester from readily available starting materials in 6 steps. This fluorophore with an excitation maximum of 515 nm and an emission maximum of 519 nm can be detected using common fluorescein/GFP filter sets. In addition, we describe the synthesis of the proteasome targeting moiety Ahx_3Leu_3VS and subsequent coupling with Me_4BodipyFL-NHS ester yielding fluorescent probe Me_4BodipyFL-Ahx_3Leu_3VS (**Fig. 1**). Furthermore, optimized procedures are outlined for both SDS-PAGE and FACS based assays using this fluorescent probe.

2. Materials

2.1. General Materials

1. Fmoc peptide building blocks (Novabiochem).

2. All solvents were purchased from Biosolve at the highest grade available.

3. All other chemicals were purchased from Aldrich at the highest available purity. All solvents and chemicals were used as received.

2.2. Synthesis of the fluorescent probe Me_4BodipyFL-Ahx_3Leu_3VS

2.2.1 Synthesis of Me_4BodipyFL-NHS ester

1. *Tert*-butyl acetoacetate, acetic acid, sodium nitrite.

2. Methyl 4-acetyl-5-oxohexanoate, zinc dust, sodium acetate, ethyl acetate (EtOAc), magnesium sulphate ($MgSO_4$).

3. Trifluoroacetic acid (TFA), triethylorthoformate, toluene, EtOAc.

4. (Absolute) ethanol, 2,4-dimethylpyrrole, HCl in dioxane (4N), diethyl ether.

5. 1,2-dichlorobenzene, triethylamine, boron trifluoride etherate, hexanes, EtOAc, toluene.

6. (Absolute) ethanol, 1 M lithium hydroxide solution, ethyl acetate (EtOAc), 0.1 M HCl, magnesium sulphate ($MgSO_4$), acetic acid.

7. Dichloromethane (CH_2Cl_2), N-hydroxysuccinimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), 4-dimethylaminopyridine (DMAP), hexanes, EtOAc.

2.2.2 Synthesis of Me_4BodipyFL-Ahx_3Leu_3VS

1. Fmoc-L-leucine-PEG-polystyrene resin (Applied Biosystems), Fmoc-leucine-OH (Fmoc-Leu-OH), Fmoc-aminohexanoic acid (Fmoc-Ahx-OH), piperidine, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop, coupling reagent), N,N-diisopropylethylamine (DIPEA), N-methyl-2-pyrrolidone (NMP).

2. TFA.

3. Di-*tert*-butyl dicarbonate, DIPEA, dimethylformamide (DMF), CH_2Cl_2, MeOH, acetic acid.

4. (S,*E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-amine (leucinyl vinyl sulfone, LeuVS, EDCI, DIPEA, DMF, CH_2Cl_2, MeOH.

5. TFA, toluene, diethyl ether.

6. Me_4-BodipyFL NHS-ester (**7**), DIPEA, DMF.

2.3. Profiling of proteasome activity using SDS-PAGE based assays

2.3.1. In vitro profiling of proteasome activity in cell lysates.

1. Cell line of choice.

2. Appropriate medium, e.g. DMEM (Dulbecco's Modified Eagle's Medium) for adherent cell lines and RPMI 1640 (Roswell Park Memorial Institute) medium for suspension cell lines (Both from Invitrogen).

3. Fetal calf serum (Greiner Bio-one).

4. Antibiotics: stock solutions of penicillin (5000 U/mL) and streptomycin (5000 μg/mL) (both from GIBCO). Add 10 mL of each stock solution to 500 mL medium.

5. Phosphate buffered saline (PBS).

6. Trypsin solution (0.25%; Invitrogen). Only needed when adherent cell lines are used.

7. HR lysis buffer (*see* **Note 1**): 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT (added fresh from a 1M stock solution before use), 2 mM ATP (added fresh from a 0.5 M stock solution before use). Prepare HR buffer lacking DTT and ATP, filter over a 0.22 μm filter (e.g. MILLEX®GS 0.22 μm Filter unit, Millipore), and store at 4°C. Supplement the amount of HR buffer needed for a single experiment (typically 1 mL) with ATP and DTT before use (s*ee* **Note 2**).

8. 5X Bradford reagent (Biorad). Dilute with water to obtain a 1X working solution before use.

9. Dimethylsulfoxide (DMSO) (Optional).

10. 50X stock solutions of proteasome inhibitors in DMSO (Optional). Store at -20°C.

11. 50 μM stock solution of Me_4BodipyFL-Ahx_3Leu_3VS in DMSO. Store at -20°C.

2.3.2. Labeling of active proteasome subunits in living cells.

1. Cell line of choice.

2. Appropriate medium, e.g. DMEM (Dulbecco's Modified Eagle's Medium) for adherent cell lines and RPMI 1640 (Roswell Park Memorial Institute) medium for suspension cell lines (Both from Invitrogen).

3. Fetal calf serum (Greiner Bio-one).

4. Antibiotics: stock solutions of penicillin (5000 U/mL) and streptomycin (5000 μg/mL) (both from GIBCO). Add 10 mL of each stock solution to 500 mL medium.

5. Dimethylsulfoxide (DMSO) (Optional).

6. 500X stock solutions of proteasome inhibitors in DMSO (Optional). Store at -20°C.

7. 50 μM stock solution of Me_4BodipyFL-Ahx$_3$Leu$_3$VS in DMSO. Store at -20°C.

8. 5 mM stock solution of MG132 (Sigma) in DMSO. Store at -20°C.

9. Phosphate buffered saline (PBS).

10. Trypsin solution (0.25%; Invitrogen). Only needed when adherent cell lines are used.

11. NP40 lysis buffer (Note 1): 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40. Prepare NP40 lysis buffer, filter over a 0.22 μm filter (e.g. MILLEX®GS 0.22 μm Filter unit, Millipore), and store at 4°C.

12. 5X Bradford reagent (Biorad). Dilute with water to obtain a 1X working solution before use.

2.3.3. Ex vivo profiling of proteasome subunit activity in murine tissues.

1. PBS.

2. HR lysis buffer (*see* **Note 1**), see section 2.3.1 point 7 for the recipe.

3. Glass beads (≤ 106 μm, acid washed, Sigma).

4. 5X Bradford reagent (Biorad). Dilute with water to obtain a 1X working solution before use.

5. 50 μM stock solution of Me_4BodipyFL-Ahx$_3$Leu$_3$VS in DMSO. Store at -20°C.

2.3.4. Gel electrophoresis and in-gel fluorescence readout.

1. NuPAGE Pre-cast gel system (Invitrogen).

2. 3X reducing sample buffer (*see* **Note 1**). For 1.2 mL: 900 μL 4X NuPAGE® LDS Sample Buffer (Invitrogen), 90 μL β-mercaptoethanol (Sigma), 210 μL water. Store at room temperature.

3. NuPAGE® Novex 12% Bis-Tris Gel, 1.0 mm (Invitrogen).

4. NuPAGE® MOPS SDS Running Buffer (Invitrogen).

5. NuPAGE® Antioxidant (Invitrogen).

6. SeeBlue® Plus2 Pre-Stained Standard (Invitrogen).

7. Fluorescence imager containing an appropriate filter set (e.g. excitation at 480 nm, emission at 530 nm), e.g. the ProXPRESS 2D Proteomic imaging system (Perkin Elmer).

8. Analysis software to quantify fluorescence intensities, e.g. TotalLab.

2.4 Flow cytometry experiments

1. MelJuso cells (human melanoma).

2. DMEM (Dulbecco's Modified Eagle's Medium) (Invitrogen).

3. Fetal calf serum (Greiner Bio-one).

4. Antibiotics: stock solutions of penicillin (5000 U/mL) and streptomycin (5000 μg/mL) (both from GIBCO). Add 10 mL of each stock solution to 500 mL medium.

5. 96-well flat bottom tissue culture plates (BD Falcon).

6. Dimethylsulfoxide (DMSO).

7. 2.5 mM stock solutions of the desired compounds in DMSO. Store at -20°C.

8. 0.4 mM stock solution of MG132 (Sigma) in DMSO. Store at -20°C.

9. 50 μM stock solution of Me_4BodipyFL-Ahx$_3$Leu$_3$VS in DMSO. Store at -20°C.

10. Phosphate buffered saline (PBS).

11. Trypsin solution (0.25%; Invitrogen).

12. FACS buffer: PBS containing 2% FCS. Store at 4°C.

13. Fixation buffer: PBS containing 2% formaldehyde. Store at room temperature.

14. Flow cytometer capable of measuring in the Fl1 channel (measure emission at 530 nm) e.g. the FACSCalibur (BD Biosciences, 488 nm laser.

3. Methods

In this section the synthesis of the fluorescent proteasome probe Me_4BodipyFL-Ahx_3Leu_3VS (**Fig. 1**) is described as well as the use of this probe in SDS-PAGE and flow cytometry based experiments.

3.1 General Methods

1. L-Leucinyl vinyl sulfone was prepared as reported (***22-25***).

2. Flash column chromatography (***26***) refers to purification using the indicated eluent and Acros silica gel (0.030-0.075 mm).

3. Solution phase reactions were monitored using thin-layer chromatography (TLC) on silica coated plastic sheets (Merck silica gel F_{254}).

4. Nuclear magnetic resonance (NMR) spectra were recorded in the indicated solvent using a Bruker Avance 300 (^{1}H: 300 MHz; ^{13}C: 75 MHz) spectrometer.

5. LC-MS analyses were carried out on a WATERS LCT mass spectrometer in line with a WATERS 2795 HPLC system and a WATERS 2996 photodiode array detector. Reversed phase runs were performed on a 3 μm Atlantis T3, C18 RP, 2.1 x 100 mm column (Waters) using gradient elution with H_2O/0.1% formic acid as solvent A and acetonitrile/0.1% formic acid as solvent B at a flow rate of 0.4 mL/min.

6. Reversed phase HPLC runs were performed on a Waters 1525 EF HPLC system in line with a Waters 2487 dual λ absorbance detector using gradient elution with H_2O/0.05% TFA as solvent A and acetonitrile/0.05% TFA as solvent B. Analytical runs were performed on a 10 μm 4.6x150 mm Atlantis dC18 column (Waters) at a flow rate of 1.4

ml/min and preparative runs were performed on a 10 μm 19x250 mm Atlantis dC18 column (Waters) at a flow rate of 18 ml/min.

7. Fluorescence spectra were measured on a customized fluorimeter set-up using a mercury vapor lamp at 72 W, appropriate gratings and a photomultiplier at 1000 V (Photon Technology International). Excitation spectra were recorded at 100 nM in water at 550 nm emission, while emission spectra were recorded in water at 480 nm excitation.

8. In-gel fluorescence intensity of SDS-PAGE gels was measured using a ProXPRESS 2D Proteomic imaging system (Perkin Elmer).

9. Flow cytometry experiments were performed using a FACSCalibur (BD Biosciences, 488 nm laser).

10. Cell lysates or samples containing protease activity were kept on ice until incubation with the probe.

11. Sonication of cells was performed on a Bioruptor.

3.2. Synthesis of the fluorescent proteasome probe Me_4BodipyFL-Ahx_3Leu_3VS

The Bodipy fluorescent dye described here was chosen for its ease of synthesis from commercially available reagents on a large scale and for its favorable spectral properties (fluorescein-like excitation and emission maxima), which make it widely applicable to common fluorescence based methodologies (e.g. fluorescence scanning, FACS).

3.2.1 Synthesis Me_4BodipyFL-NHS ester (Scheme 1)

1. To a mixture of *tert*-butyl acetoacetate (2.80 g, 17.4 mmol) in acetic acid (4 mL), stirred on an ice bath, add dropwise a solution of sodium nitrite (1.40 g, 20.3 mmol) in deionized water over a 5 minute period. Stir for an additional 16 hours at 4 ºC (e.g. in a cold room) (***27***).

2. Slowly, add the resulting solution containing oxime **1** in 5 portions to a solution of methyl 4-acetyl-5-oxohexanoate (5 mL, 28.6 mmol) in acetic acid (15 mL) kept at 65 ºC. Simultaneously, add a mixture of zinc dust (3 g, 45.9 mmol) and sodium acetate (3 g, 36.6 mmol) in 5 portions. Stir the mixture for 2 more hours at 65 ºC, then pour it into ice water (250 mL) and leave slowly stirring for 16 hours, allowing the mixture to warm up to room temperature. Filter off the precipitate, including product and zinc residues. Take up the residue in ethyl acetate (100 mL) and dry the organic layer with magnesium sulfate ($MgSO_4$). Filter off the $MgSO_4$ and concentrate the filtrate *in vacuo* to dryness to yield tetrasubstituted pyrrole **2** as a light brown solid (2.87 g, 10.2 mmol, 59 % yield). The crude product can be used without further purification in the next step.

3. Dissolve pyrrole **2** (1 g, 3.55 mmol) in TFA (20 mL) and stir for 20 minutes on ice. Slowly, add triethylorthoformate (2 mL, 12 mmol) and stir the solution gently at 0 ºC for 15 minutes. Add water (2 mL) and stir for 10 minutes at 0 ºC. Coevaporate the solution with toluene (60 mL) *in vacuo* to dryness at room temperature. Purify the crude product by flash column chromatography using ethyl acetate as the eluent to yield aldehyde **3** as a brown solid (469 mg, 2.25 mmol, 63 % yield).

4. Dissolve aldehyde **3** (469 mg, 2.25 mmol) in absolute ethanol (8 mL) and cool to 0 ºC. Add 2,4-dimethylpyrrole (256 mg, 2.69 mmol) under an argon atmosphere and add 4 mL of cold 4N HCl in dioxane. Stir for 15 minutes at 0 ºC and then transfer the solution to a 50 mL Falcon tube and centrifuge at 1500g for 10 minutes at 4 ºC. Discard the supernatant, resuspend the resulting orange solid in cold diethylether (40 mL) and centrifuge again. Decant the supernatant and dry the residue under a stream of nitrogen to yield crude dipyrrole **4** as an orange solid, which is used in the next step without further purification.

5. Suspend dipyrrole **4** in 1,2-dichlorobenzene (20 mL) under an argon atmosphere and add triethylamine (1 mL, 7.16 mmol) and boron trifluoride diethyl etherate (1 mL, 8.12 mmol) in concert. Stir the resulting metallic solution for 30 minutes at 100 ^{o}C and then allow the mixture to cool to room temperature. Dilute the solution with dry hexanes (25 mL) and apply directly onto a silica gel column for purification by flash column chromatography using 25% EtOAc in toluene as the eluent to afford Me_4BodipyFL-methyl ester **5** as an orange solid (481 mg, 1.44 mmol, 64 % yield starting from aldehyde **3**).

6. Dissolve Me_4BodipyFL-methyl ester **5** (400 mg, 1.24 mmol) in ethanol (6 mL) and add a solution of 1 M lithium hydroxide in water (1.3 mL). Stir the solution until TLC (eluent: EtOAc containing 0.1% acetic acid) reveals complete conversion. Add ethyl acetate (40 mL) and 0.1 M HCl (30 mL) and separate the two layers. Dry the organic layer with $MgSO_4$ and concentrate *in vacuo* to dryness to obtain an orange solid. Purify by flash column chromatograpy using EtOAc containing 0.1% acetic acid as the eluent to yield Me_4BodipyFL-OH **6** as an orange solid (306 mg, 0.95 mmol, 77 % yield). ^{1}H NMR

($CDCl_3$): δ 7.09 (s, 1H), 6.10 (s, 1H), 2.66 (dd, *J*=8.2 Hz, *J*=7.1 Hz, 2H), 2.59 (s, 6H), 2.60-2.53 (m, 2H), 2.31 (s, 3H), 2.27 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 178.2 (CO), 156.5 (C_q), 155.3 (C_q), 141.0 (C_q), 138.1 (C_q), 133.3 (C_q), 132.6 (C_q), 127.8 (C_q), 119.7 (CH), 118.9 (CH), 33.9 (CH_2), 19.2 (CH_2), 14.6 (CH_3), 12.7 (CH_3), 11.2 (CH_3), 9.6 (CH_3). Fluorescence data: λ_{ex} = 515 nm, λ_{em} = 519, *see* **Fig.2**.

7. Dissolve Me_4BodipyFL-OH **6** (306 mg, 0.95 mmol) in dichloromethane (60 mL) and add N-hydroxy succinimide (120 mg, 1.05 mmol), coupling reagent EDCI (201 mg, 1.05 mmol) and coupling catalyst DMAP (11 mg, 0.1 mmol). Stir the mixture for 16 hours at room temperature and add 0.05 M HCl (40 mL) and separate the two layers. Dry the organic layer with $MgSO_4$ and concentrate *in vacuo* to dryness to obtain an orange solid. Purify by flash column chromatography using EtOAc : hexanes (2:3 v/v) as the eluent to obtain Me_4BodipyFL-NHS ester **7** as a dark orange solid (242 mg, 0.85 mmol) in 61 % yield.

^{1}H NMR ($CDCl_3$): δ 7.02 (s, 1H), 6.02 (s, 1H), 2.85-2.78 (m, 6H), 2.75-2.70 (m, 2H), 2.50 (s, 6H), 2.22 (s, 3H), 2.19 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 169.0 (2xCO), 167.7 (CO), 156.9 (C_q), 154.8 (C_q), 141.3 (C_q), 138.0 (C_q), 133.4 (C_q), 132.5 (C_q), 126.7 (C_q), 119.9 (CH), 119.0 (CH), 31.1 (CH_2), 25.6 (2xCH_2), 19.2 (CH_2), 14.6 (CH_3), 12.6 (CH_3), 11.2 (CH_3), 9.6 (CH_3). Fluorescence data: λ_{ex} = 513 nm, λ_{em} = 517.

3.2.2 Synthesis of Me_4BodipyFL-Ahx_3Leu_3VS (see Note 3)

The synthesis of H_2N-Ahx_3Leu_2-OH involving standard Fmoc-based solid-phase peptide synthesis (SPPS) protocols and the subsequent coupling of fluorescent dye and vinyl sulfone electrophile to yield the final proteasome probe are depicted in **Scheme 2**.

1. Fmoc-L-Leu-PEG-polystyrene Wang resin (1 g, 0.17 mmol equivalents) (*see* **Note 4**) is subjected to four coupling cycles, in which deprotection of the Fmoc-group with piperidine/NMP (1:4 v/v; 10 mL/g of dry resin), is followed by a coupling cycle with 3 equivalents of Fmoc-protected amino acid, 3 equivalents of DIPEA and 3 equivalents of PyBop coupling reagent in NMP (10 mL/g of dry resin). After each step the resin is thoroughly washed with NMP (5x10 mL). Coupling steps are performed with Fmoc-Leu-OH (1x) and Fmoc-Ahx-OH (3x), sequentially (*see* **Note 5**) (***14***).

2. After the final coupling step, remove the Fmoc group with piperidine/NMP (1:4 v/v), to afford solid supported **8**, and subsequently cleave the peptide from the resin by treating the resin with 100% TFA (10 mL) for 30 min. Coevaporate the cleavage mixture with toluene (40 mL) to obtain H_2N-Ahx_3Leu_2-OH **9** (100 mg, 0.17 mmol) in > 90% purity as judged by LC/MS analysis. The product is used in the next step without further purification.

3. Dissolve H_2N-Ahx_3Leu_2-OH **9** (100 mg, 0.17 mmol) in DMF (2 mL) and add DIPEA (148 µL, 0.85 mmol) and di-*tert*-butyl-dicarbonate (44 mg, 0.20 mmol). Stir the resulting suspension for 16 hours at room temperature and then concentrate *in vacuo* to dryness to yield an off-white solid. Purify by flash column chromatography using a gradient of 10 % to 20% MeOH in CH_2Cl_2 containing 0.1 % acetic acid as the eluent to obtain Boc-Ahx_3Leu_2OH **10** as a white solid (116 mg, 0.17 mmol) in > 98 % yield.

4. Dissolve L-leucinyl vinyl sulfone (LeuVS, 50 mg, 0.26 mmol) in DMF and add Boc-Ahx_3Leu_2OH **10** (116 mg, 0.17 mmol), DIPEA (89 µL, 0.51 mmol) and coupling reagent EDCI (50 mg, 0.26 mmol). Stir the solution for 3 hours at room temperature and then concentrate *in vacuo* to dryness to yield an off-white solid. Purify by flash column

chromatography using 10 % MeOH in CH_2Cl_2 as the eluent to obtain Boc-Ahx_3Leu_3VS **11** as a white solid (103 mg, 0.12 mmol) in 71 % yield.

5. In a 50 mL Falcon tube, dissolve BocAhx_3Leu_3VS **11** (12 mg, 14 μmol) in TFA (1 mL) and leave at room temperature for 30 minutes. Precipitate the product by adding cold diethylether (40 mL). Isolate the product by centrifugation at 1000g (low brake speed) for 5 minutes at 4 ˚C to afford H_2N-Ahx_3Leu_3VS **12** as a white solid (10.6 mg, 14 μmol) in > 98 % yield.

6. Dissolve H_2N-Ahx_3Leu_3VS **12** (10.6 mg, 14 μmol) in DMF (1 mL) and add Me_4BodipyFL-NHS ester **7** (6.4 mg, 15.5 μmol) and DIPEA (24 μL, 0.14 mmol) (*see* **Note 6**). Stir the resulting solution at room temperature under an argon atmosphere for 16 hours. Concentrate the solution *in vacuo* to dryness and purify by reversed phase HPLC to obtain the fluorescent proteasome probe Me_4BodipyFL-Ahx_3Leu_3VS (6 mg, 5.7 μmol) in 40% yield (see **Fig. 3** for LC/MS data). Fluorescence data: λ_{ex} = 515 nm, λ_{em} = 519.

3.3. Profiling of proteasome activity using SDS-PAGE based assays

Activity-based proteasome probes can be used to profile the effects of proteasome inhibitors in a broad range of sample types. Profiling experiments have been described in cell lysates and living cells (***14***) and *ex vivo* in both rodents (***14***) and patients *(**28**)*. The probe described here (Me_4BodipyFL-Ahx_3Leu_3VS, **Scheme 2**) consists of a fluorescent tag, a proteasome-targeting motif and a vinyl sulfone (VS) reactive group that covalently reacts with the N-terminal threonine of all active subunits. The fluorescent tag allows for visualization of proteasome subunit labeling on SDS-PAGE by scanning the gel for fluorescence emission. Upon incubation of proteasome with probe, all active proteasome

subunits become fluorescently labeled. Prior inhibition of a subunit with a proteasome inhibitor prevents probe binding, resulting in the disappearance of a fluorescent band on the gel. Therefore, the measured fluorescence intensity directly correlates to the activity of the labeled β-subunit.

Figure 4 shows a typical profiling experiment in both MelJuso (**Fig. 4A**) and THP-1 (**Fig. 4B**) cell lysates. In this experiment, MelJuso and THP-1 lysates were incubated with the proteasome inhibitors MG132 (25 μM), bortezomib (0.1 μM and 1 μM), epoxomicin (1 μM) or a DMSO control. Subsequently, samples were incubated with Me_4BodipyFL-Ahx_3Leu_3VS to label proteasome subunits, proteins were separated by SDS-PAGE and the resulting gel was scanned for fluorescence emission (see below). In the DMSO controls, all active subunits are labeled, and the composition of proteasome in these cells was visualized. MelJuso cells mainly expressed constitutive proteasome, resulting in the labeling of all three constitutive subunits (β1, β2, β5) in these cells (**Fig. 4A**). THP-1 cells expressed both constitutive and immunoproteasome, as evidenced by the labeling of both the constitutive β2 and β5 proteasome subunits and the immunoproteasomal β2i and β1i subunits (**Fig. 4B**). Incubation with different proteasome inhibitors resulted in the disappearance of particular bands on the gels, indicative of the subunit specificity of these inhibitors. Bortezomib inhibited only the β5(i) and β1(i) subunits, whereas both epoxomicin and MG132 inhibited all subunits. Epoxomicin completely inhibited the β2 and β2i and β1i subunits at 1 μM, while some residual activity of the β5/β5i subunits was still visible. On the other hand, 25 μM MG132 completely inhibited both the β5/β5i and β1/β1i activities but not the β2 and β2i subunits. These results are in accord with the published subunit specificities of these inhibitors. Bortezomib is a known inhibitor of the

β5(i) and β1(i) activities, while both epoxomicin and MG132 are pan-proteasome inhibitors. A typical procedure is described below.

3.3.1 In vitro profiling of proteasome activity in cell lysates.

1. Grow the cell line of choice at 37º C and 5% CO_2 in a humidified incubator in the appropriate medium supplemented with 10% fetal calf serum (FCS) until log-phase (suspension cells) or until 80% confluency is reached (adherent cells). Medium may be supplemented with antibiotics.

2. Harvest suspension cells by centrifugation. To this end, transfer cells to a falcon tube and centrifuge at 1200g for 2 minutes at 4°C. Discard the supernatant and resuspend the cell pellet in 10 to 20 pellet volumes of PBS. Transfer cells to an eppendorf tube and pellet cells by centrifugation at 1200g for 2 minutes at 4°C. Discard the supernatant. Harvest adherent cells by trypsinization. To this end, aspirate the medium, wash cells with PBS and aspirate. Add just enough trypsin to cover the cells. As soon as cells detach, add medium containing 10% FCS to the cells to inactivate the trypsin and transfer the cells to a falcon tube. Pellet cells by centrifugation at 1200g for 2 minutes at 4°C. Discard the supernatant and resuspend the cell pellet in 10 to 20 pellet volumes of PBS. Transfer cells to an eppendorf tube and pellet cells by centrifugation at 1200g for 2 minutes at 4°C. Discard the supernatant.

3. Resuspend cells in 2 pellet volumes of cold HR buffer.

4. Lyse cells mechanically (e.g. by sonication using the Bioruptor according to the manufacturer's instructions). **Critical:** For proper labelling, do not add detergent to the lysis buffer. If detergent is absolutely required for proper cell lysis, keep the detergent concentration as low as possible.

5. Centrifuge for at 14,000g for 3 minutes at 4°C to remove membrane fractions and cell debris and transfer the supernatant to a fresh eppendorf tube.

6. Determine protein concentrations using a Bradford assay. For incubation with proteasome inhibitors prior to proteasome labeling, proceed to step 7. To label the proteasome directly, proceed to step 9. **Critical:** Proceed with labeling directly and do not store non-incubated lysates. The quality of labeling will decrease if lysates are freeze-thawed.

Option 1: incubation with proteasome inhibitors followed by labeling with probe

7. To study the effects of proteasome inhibitors, transfer 25 μg of lysate to a fresh eppendorf tube and adjust the volume to 24.5 μL with HR buffer. Add 0.5 μL of a 50X stock solution of the desired proteasome inhibitor in DMSO to obtain the desired 1X concentration of proteasome inhibitor and a final protein concentration of 1μg/μL. Include a reference sample to which 0.5 μL DMSO but no proteasome inhibitor is added. Vortex and incubate the samples for the desired time period at 37 °C.

8. Add 0.5 μL of a 50 μM Me_4BodipyFL-Ahx$_3$Leu$_3$VS stock solution in DMSO to the samples to obtain a final concentration of 1 μM Me_4BodipyFL-Ahx$_3$Leu$_3$VS. Vortex and incubate for 1 hour at 37°C. Proceed to 3.3.4.

Pause point: At this point, incubated lysates can be snap-frozen in liquid N_2 and stored at -20°C until further use.

Option 2: incubation with probe only

9. To label proteasome subunits directly, transfer 25 µg lysate to a fresh eppendorf tube and adjust the volume to 24.5 µL with HR buffer. Add 0.5 µL of a 50 µM Me_4BodipyFL-Ahx_3Leu_3VS stock solution in DMSO to obtain a final probe concentration of 1 µM and a final protein concentration of 1µg/µL. Vortex and incubate the sample for 1 hour at 37°C. Proceed to 3.3.4.

Pause point: At this point, incubated lysates can be snap-frozen in liquid N_2 and stored at -20°C until further use.

3.3.2. Labeling of active proteasome subunits in living cells

Labeling of active proteasome subunits can be performed in suspension cells (proceed to step 1) or adherent cells (proceed to step 5).

Suspension cells

1. Grow the cell line of choice in appropriate medium containing 10% FCS at 37 ºC and 5% CO_2 in a humidified incubator until log phase is reached. Medium may be supplemented with antibiotics.

2. Count cells, transfer cells to a falcon tube and pellet cells by spinning at 1200 g for 2 minutes at room temperature. Discard the supernatant.

3. Resuspend cells in fresh medium at 0.5 x 10^6 to 1.0 x 10^6 cells/mL.

4. Add 0.5 mL of cell suspension (0.25 x 10^6 to 0.5 x 10^6 cells) to each well of a 24-well plate (or as many wells as needed). For incubation with proteasome inhibitors prior to proteasome labeling, proceed to step 7. To label proteasome only, directly proceed to step 8.

Adherent cells

5. Grow the cell line of choice in a 24 well plate in appropriate medium supplemented with 10% FCS at 37 ºC and 5% CO_2 in a humidified incubator until 80% confluency is reached. Medium may be supplemented with antibiotics.

6. Aspirate the medium and add 0.5 mL of fresh medium to the cells. For incubation with proteasome inhibitors prior to proteasome labeling, proceed to step 7. To label proteasome only, directly proceed to step 8.

7. To study the effects of proteasome inhibitors, add 1 μL of a 500X stock solution of the desired proteasome inhibitor in DMSO to each well to obtain the desired 1X concentration of proteasome inhibitor. Include a reference sample to which 1 μL DMSO but no proteasome inhibitor is added. Incubate the cells for the desired time period in an incubator.

8. Add 5 μL of a 50 μM Me_4BodipyFL-Ahx_3Leu_3VS stock solution in DMSO to each well to obtain a final probe concentration of 500 nM. Incubate the cells for 1 hour in an incubator. If cells do not tolerate 1% DMSO, 1 μL of a 250 μM Me_4BodipyFL-Ahx_3Leu_3VS stock solution in DMSO can be added alternatively.

9. To block all remaining proteasome activity and prevent post-lysis labeling events, add 1 μL of a 5 mM MG132 stock solution in DMSO to each well to obtain a final MG132 concentration of 10 μM. Incubate the cells for 1 hour in an incubator. Step 9 can be

omitted if no differences in labeling are observed between samples that are incubated with MG132 before cell harvest and samples that are harvested directly after step 8.

10. To harvest suspension cells: transfer the cells in each well to an eppendorf tube and pellet cells by centrifugation at 1200g for 2 minutes at 4°C. Discard the supernatant and resuspend the cell pellet in 1 mL PBS. Pellet cells by centrifugation at 1200g for 2 minutes at 4°C. Discard the supernatant. To harvest adherent cells: aspirate the medium, wash the cells by adding 1 mL of PBS to each well and aspirating the PBS. Add 50 µL trypsin to each well. As soon as cells detach, add 1 mL of fresh medium containing 10% FCS to the cells to inactivate the trypsin. Transfer the cells in each well to an eppendorf tube and pellet cells by centrifugation at 1200g for 2 minutes at 4°C. Discard the supernatant and resuspend the cell pellet in 1 mL PBS. Pellet the cells by centrifugation at 1200g for 2 minutes at 4 °C. Discard the supernatant.

Pause point: At this point, incubated cell pellets can be snap-frozen in liquid N_2 and stored at -20°C until further use.

11. Resuspend cells in 1 to 2 pellet volumes of cold NP40 lysis buffer and lyse for 30 minutes at 4 °C.

12. Centrifuge at 14,000 g for 3 minutes at 4°C to remove membrane fractions and cell debris. Transfer the supernatant to fresh eppendorf tube.

13. Determine protein concentrations using a Bradford assay.

14. Transfer 25 µg lysate to a fresh eppendorf tube and adjust the final volume to 25 µL with NP40 lysis buffer to obtain a final protein concentration of 1 µg/µL. Proceed to 3.3.4.

Pause point: At this point, cell lysates can be snap-frozen in liquid N_2 and stored at -80°C until further use.

3.3.3. Ex vivo profiling of proteasome subunit activity in murine tissues.

1. Remove the tissue types to be analyzed and rinse with PBS.

2. Grind the tissue, transfer to an eppendorf tube and add 1 to 2 volumes of cold HR buffer.

3. Add 1 volume of glass beads (≤ 106 microns, acid washed, Sigma) to the tissue. Lyse cells mechanically by vortexing at high speed for 45 minutes at 4°C. **Critical:** For proper labelling, do not add detergent to the lysis buffer. If detergent is absolutely required for proper cell lysis, keep the detergent concentration as low as possible.

4. Remove beads, membrane fractions and cell debris by centrifugation at 14,000 g for 5 minutes at 4°C and transfer the supernatant to a fresh eppendorf tube.

5. Determine protein concentrations using a Bradford assay. **Critical:** Proceed with labeling directly and do not store non-incubated lysates. The quality of labeling will decrease if lysates are freeze-thawed.

6. Transfer 25 μg lysate to a fresh eppendorf tube and adjust the volume to 24.5 μL with HR buffer. Add 0.5 μL of a 50 μM Me_4BodipyFL-Ahx$_3$Leu$_3$VS stock solution in DMSO to obtain a final probe concentration of 1 μM and a final protein concentration of 1μg/μL. Vortex and incubate the samples for 1 hour at 37°C. Proceed to 3.3.4.

Pause point: At this point, incubated lysates can be snap-frozen in liquid N_2 and stored at -20°C until further use.

3.3.4. Gel electrophoresis and in-gel fluorescence readout

1. These instructions assume the use of the NuPAGE precast gel system (Invitrogen) and precast mini gels to separate proteins on SDS-PAGE. If a mini gel does not separate the individual proteasome subunits sufficiently, a larger gel system can be used, e.g. the PROTEAN II xi Cell system from Biorad (16x20 cm glass plates, 12.5% separating gel, 4% stacking gel.) When using this system, increase the sample volume to 50 µL per sample. Do not change the final concentrations of proteasome inhibitors, protein and probe in the samples. Load 20 to 30 µL sample per well and run at 10 mA for 16h. Subsequently, increase the current to 35 mA, wait until the blue front has run off the gel and run for another hour before removing the gel from the system. The protocol described below can also be adapted to other gel systems. Use common protocols for denatured samples. Separate proteins using a 12 or 12.5% separating gel and a 4% stacking gel.

2. Add 12.5 µL 3X reducing sample buffer (see 2.3.4 for buffer recipe) to each sample obtained in 3.3.1, 3.3.2 or 3.3.3. The volume of these samples should be 25 µL. Vortex and denature by boiling the sample for 10 minutes at 70 °C. Centrifuge at 14,000g for 1 minute at room temperature. Denatured samples can be stored at -20°C for later use. **Critical:** when using the NuPAGE gel system also use the NuPAGE LDS sample buffer to prepare the 3X reducing sample buffer. The use of a different reducing sample buffer leads to improper running of the gel, resulting in fuzzy and unfocussed bands.

3. Assemble the NuPAGE gel unit using a precast NuPAGE 12% Bis-Tris gel according to the manufacturer's instructions. Add 1X MOPS buffer to both the inner and outer chamber of the gel unit. Add 125 µL anti-oxidant to the inner gel chamber only. Load 10

μL of denatured sample per well. Keep one well free and load this well with 6.5 μL prestained molecular weight marker (e.g. SeeBlue® Plus2 Pre-Stained Standard from Invitrogen). Load 3 μL of 3X reducing sample buffer to any remaining wells.

4. Run the gel at 170 to 180V. **Critical:** For proper separation of the β1i and β5 subunits, run the gel until the15 kDa protein in the molecular weight marker (Lysozyme in the SeeBlue® Plus2 Pre-Stained Standard) is at the bottom of the gel.

5. Remove the gel from the cassette and image the wet gel slab for 10 to 120s using a fluorescence imager containing an appropriate filter set (excitation at 480 nm, emission at 530 nm), e.g. the ProXPRESS 2D Proteomic imaging system (Perkin Elmer).

6. Analyze images using appropriate software (e.g. Totallab) to quantify fluorescence intensities.

3.4. Flow cytometry experiments

Fluorescent proteasome activity probes can be used to profile proteasome activity in cells using a fluorescence assisted cell sorting (FACS) assay. The advantage of this assay is that it can be used to measure both proteasome activation and proteasome inhibition in large numbers of samples. In this way, compounds can be screened for their effects on proteasome activity. Information on the activity of distinct subunits however is not obtained in flow cytometry based assays. Results of a typical FACS experiment are shown in Figure 6. MelJuso cells (human melanoma) were incubated with 1 μM MG132, followed by incubation with probe (**Fig. 5**, blue curve). As controls, cells were incubated with Me_4BodipyFL-Ahx$_3$Leu$_3$VS only (**Fig. 5**, red curve) or not incubated (**Fig. 5**, black curve). Figure 5 shows a histogram in which the fluorescence intensity is plotted versus

the cell count. In non-incubated cells, no fluorescence can be detected, resulting in a peak at low fluorescence intensity. When only probe is added, cells are fluorescently labeled, resulting in a shift of the cell population towards higher fluorescence intensity. Upon pre-incubation with MG132, the peak shifts back to lower fluorescence intensity. As some probe always aspecifically sticks to cell membranes, the signal in the MG132 treated population never returns to baseline values, although all proteasome is inhibited in these samples. Therefore, the signal intensity at 1 μM MG132 is taken as 100% inhibition, whereas the signal intensity in probe only-treated cells is taken as 0% inhibition. A typical procedure is described below.

1. These instructions describe a high-throughput FACS-based proteasome activity assay in a 96-well format using MelJuso cells. The protocol can easily be adapted to 384-well or 24-well format by changing the amounts of cells and volumes described below. Do not change the final concentrations of compounds and probe. The protocol can be performed with other adherent cells and can be adapted to suspensions cells. When using suspension cells, perform wash steps by centrifugation (1200g for 5 min, then discard the supernatant and resuspend cells).

2. Seed MelJuso cells in a 96-well flat bottom tissue culture plate in DMEM medium supplemented with 10% FCS and antibiotics. Add 10,000 cells in a total volume of 100 μL per well. Place cells at 37 ºC and 5% CO_2 in a humidified incubator and let cells attach for 16 to 24 hours.

3. To screen compounds for their effects on proteasome activity, add 1 μL of 2.5 mM stock solutions of the desired compounds in DMSO to 99 μL medium to obtain 5X

compound solutions. To obtain 5X control solutions, add 1 μL of a 0.4 mM stock solution of MG132 in DMSO (positive control, 100% inhibition) or 1 μL DMSO only (negative control, 0% inhibition) to 99 μL medium.

4. Remove medium from the cells, add 80 μL of fresh medium to each well and 20 μL of the 5X solutions of compounds and controls obtained in step 3 (final concentration of compound in each well then is 5 μM, final concentration of MG132 is 800 nM). Incubate cells for 16 hours in an incubator.

5. Make a 42X dilution in DMEM of a 50 μM Me_4BodipyFL-Ahx_3Leu_3VS DMSO stock solution to obtain a 1.2 μM probe solution (e.g. for one 96-well plate add 48 μL of a 50 μM Me_4BodipyFL-Ahx_3Leu_3VS DMSO stock solution to 1952 μL DMEM). Add 20 μL of the 1.2 μM probe solution to each well to obtain a final probe concentration of 200 nM. Incubate cells for two hours in an incubator.

6. Discard the supernatant, add 200 μL PBS to each well to wash the cells and discard the PBS.

7. Harvest cells. Add 20 μL trypsin to each well. As soon as cells detach, add 55 μL FACS buffer and 25 μL fixation buffer to each well to obtain a final formaldehyde concentration of 0.5% (see section 2.4 for buffer recipes). Fix cells by shaking the plate on a shaker for at least 20 min.

Pause point: At this point, fixed cells can stored at 4°C until further use.

8. Measure intracellular fluorescence in the cells by flow cytometry. Fluorescence is measured in the Fl1 channel (530/30 filter).

4. Notes

1. All buffers are prepared in water that has a resistivity of 18.2 MΩ-cm (MilliQ water).

2. Store stock solutions of ATP and DTT in water in aliquots at -20°C.

3. A shorter synthetic route to the synthesis of Me_4BodipyFL-Ahx_3Leu_3VS involves the use of a hyper acid-labile resin and introduction of the Me_4BodipyFL fluorophore on solid phase. This route however requires larger amounts of fluorophore NHS ester. Synthesize the (peptide) as described in step 1, starting from pre-loaded hyper acid-labile resin. After the final coupling step, remove the Fmoc group and wash the resin thoroughly. Subsequently, resuspend the resin in NMP (10 mL/g of resin) and add 5 equivalents of Me_4BodipyFL-NHS ester and 5 equivalents of DIPEA and allow the reaction to proceed for 16 hours. Wash the resin thoroughly and cleave the peptide from the resin using 2% TFA in dichloromethane or 25% HFIP (or according to protocol of the manufacturer of the hyper acid-labile resin) in dichloromethane and allow the cleavage to take place for 20 minutes. Precipitate the product by adding 10 volume equivalents of cold diethylether/pentane (3:1 v/v) and isolate by centrifugation for 5 minutes at 1000g (low brake speed). Wash the pellet 3 times by adding cold diethylether/pentane (3:1 v/v) and centrifuging at 800g (high brake speed). Continue with step 4 to obtain the final probe. Steps 5 and 6 are omitted in this procedure.

4. The use of low capacity-and pre-loaded PEG polystyrene resin is recommended.

5. To circumvent the introduction of a Boc group on the N-terminus in solution as described in step 3, it is also possible to use Boc-6-aminohexanoic acid (Boc-Ahx-OH) instead of Fmoc-Ahx-OH, as a building block in the last coupling cycle. Synthesize the (peptide) as described in step 1, starting from pre-loaded hyper acid-labile resin. Couple Boc-Ahx-OH to the resin in the final coupling step. Wash the resin thoroughly and cleave the peptide from the resin using 2% TFA in dichloromethane. Concentrate the compound under reduced pressure. Purify the compound as described in step 3 and continue with step 4.

6. Other commercially available NHS-activated dyes may also be used here, circumventing the synthesis of the Me_4BodipyFL-NHS ester. We recommend the use of BodipyFL-NHS ester or BodipyTMR-NHS ester (Invitrogen) for optimal labeling results in both cell lysates and intact cells (***14***).

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Figure Legends

Figure 1: Structures of Bortezomib, Dansyl-Ahx_3Leu_3VS and Me_4BodipyFL-Ahx_3Leu_3VS.

Figure 2: Fluorescence spectrum of Me_4BodipyFL-OH measured in water, displaying λ_{exc} = 515 nm and λ_{em} = 519 nm. Derivatives of this dye display similar fluorescence spectra.

Figure 3: Reversed phase chromatogram (**A**) and MS spectrum (**B**) of Me_4BodipyFL-Ahx_3Leu_3VS. MS (ESI): 1058.66 $(M+H)^+$; 520.27 $(M+H-F)^{2+}$; 1080.91 $(M+Na)^+$

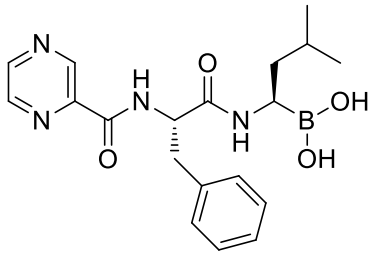
Figure 4: Gel images showing the active proteasome subunit labeling in MelJuso (**A**) and THP-1 (**B**) cell lysates that were pre-incubated with the indicated concentrations of MG132, epoxomicin or bortezomib, followed by incubation with 1 μM Me_4BodipyFL-Ahx_3Leu_3VS.

Figure 5: Flow cytometry histograms showing the fluorescence intensity in MelJuso cells that were non-incubated (black), incubated with 200 nM Me_4BodipyFL-Ahx_3Leu_3VS (red), or pre-incubated with 1 μM MG132, followed by incubation 200 nM Me_4BodipyFL-Ahx_3Leu_3VS (blue).

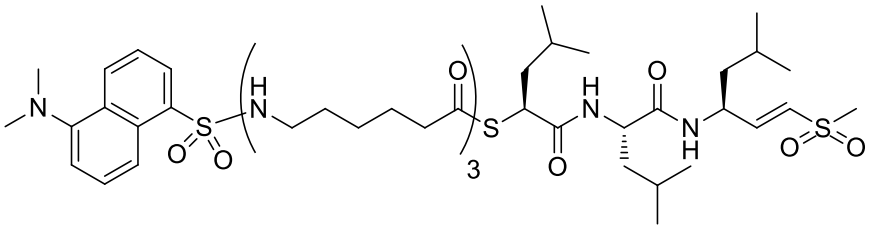
Scheme 1: Synthesis of Me_4BodipyFL-NHS ester **7**. Step numbers correspond to step numbers outlined in paragraph 3.2.1.

Scheme 2: Synthesis of Me_4BodipyFL-Ahx_3Leu_3VS. Step numbers correspond to step numbers outlined in paragraph 3.2.2.

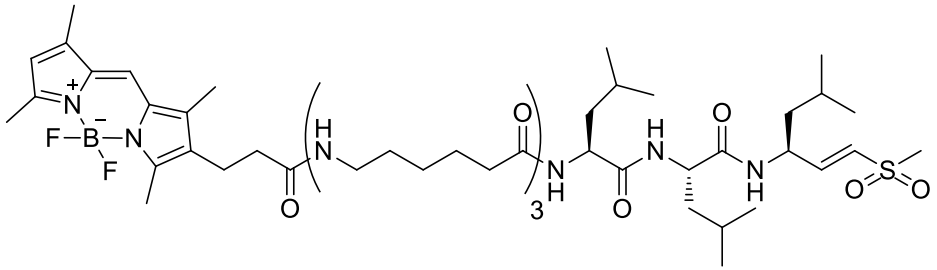
Figure 1



Bortezomib



Dansyl-Ahx$_3$Leu$_3$VS



Me$_4$BodipyFL-Ahx$_3$Leu$_3$VS

Figure 2

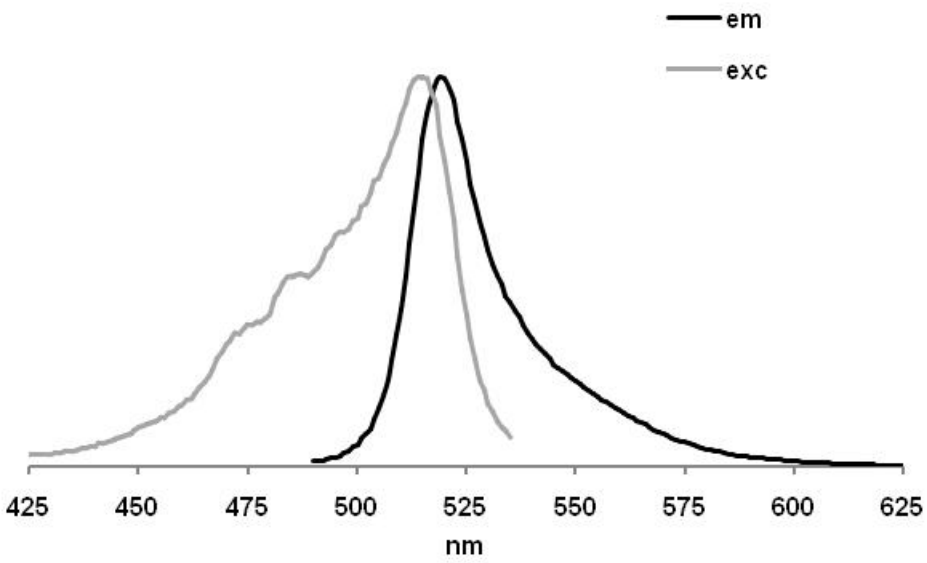
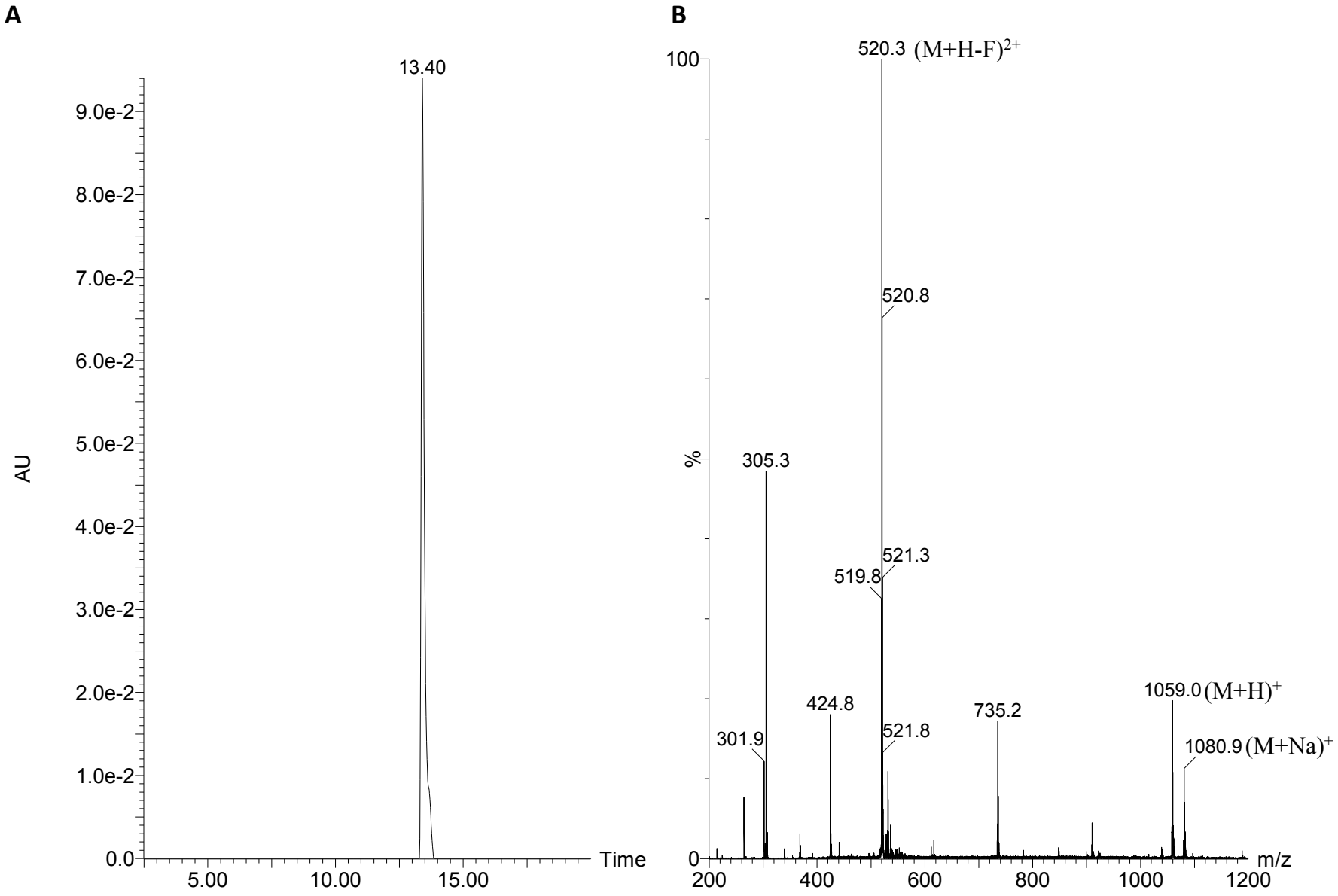


Figure 3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1.0e-2
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%
0
520.3 (M+H-F)2+
520.8
305.3
521.3
519.8
424.8
735.2
1059.0 (M+H)+
301.9
521.8
1080.9 (M+Na)+
200
400
600
800
1000
1200
m/z

Figure 4

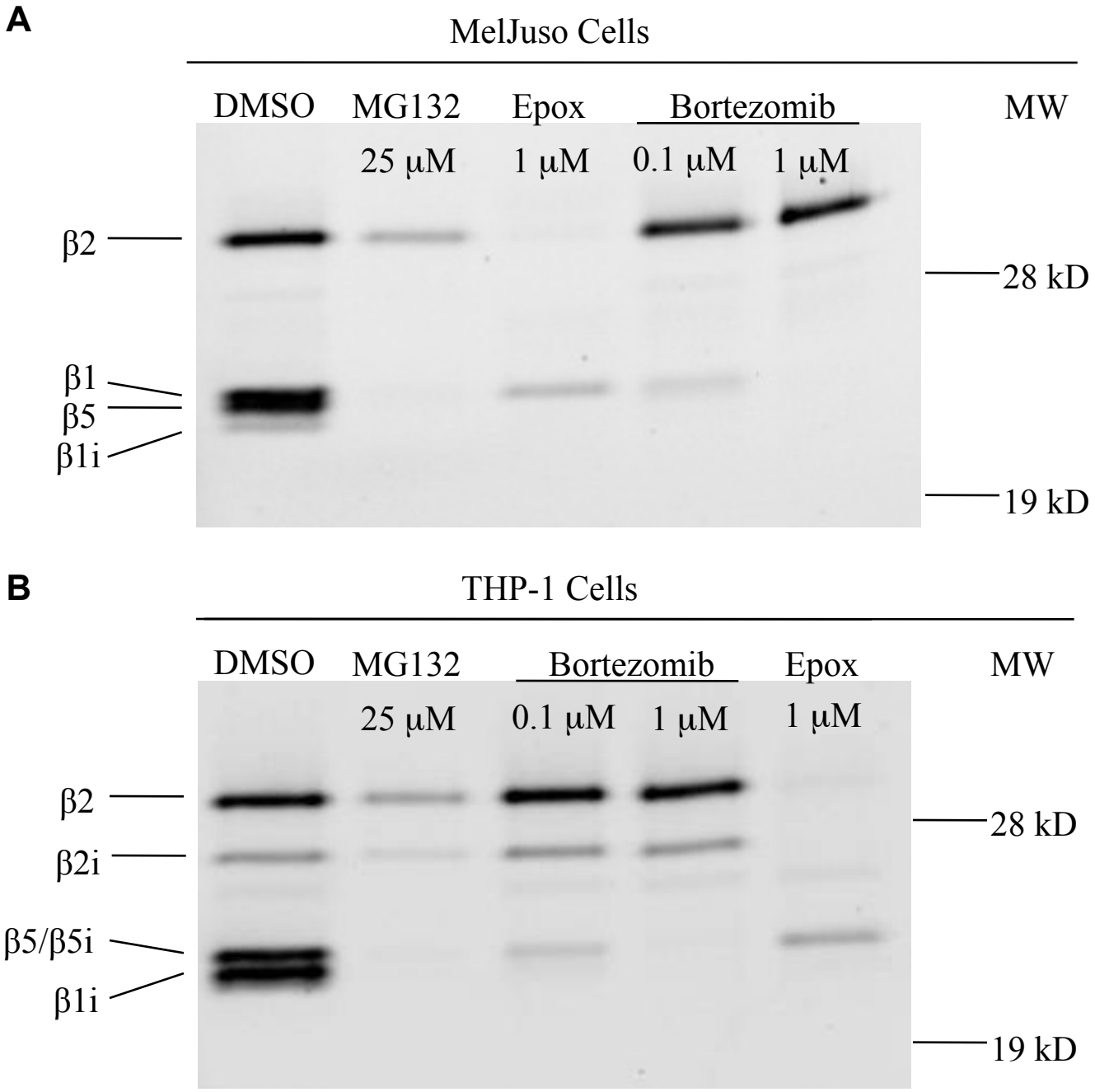
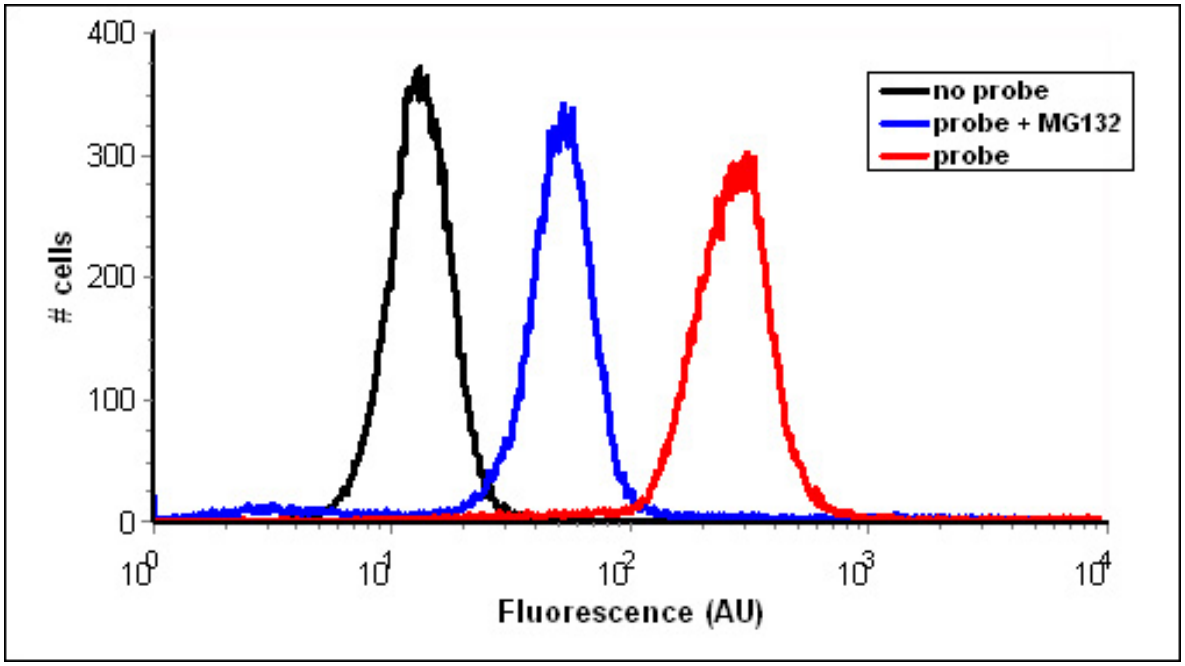

A
MelJuso Cells
DMSO
MG132
Epox
Bortezomib
MW
25 μM
1 μM
0.1 μM
1 μM
β2
β1
β5
β1i
28 kD
19 kD
B
THP-1 Cells
DMSO
MG132
Bortezomib
Epox
MW
25 μM
0.1 μM
1 μM
1 μM
β2
β2i
β5/β5i
β1i
28 kD
19 kD

Figure 5


400
300
200
100
0
cells
10^0
10^1
10^2
10^3
10^4
Fluorescence (AU)
no probe
probe + MG132
probe

Scheme 1