Activity-based proteasome profiling

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Relative quantification of proteasome activity by activity-based protein profiling and LC-MS/MS

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3.1 Introduction

3.1.1 Focusing on proteasome activities

The proteasome is an evolutionarily conserved proteolytic complex that is responsible for the degradation of most proteins in eukaryotic cells ranging from yeast to human. It is essential for protein homeostasis and production of MHC class I restricted epitopes. Protein degradation is necessary for the turnover of damaged or misfolded proteins and regulation of biochemical pathways by lowering enzyme activity or messenger concentration. Obviously, the proteasome is a central protease in various cellular processes, including transcription, translation, DNA repair, cell division and antigen presentation(*1, 2*). In the last decade, the proteasome has become an attractive clinical target since the approval of the proteasome inhibitor bortezomib (Velcade®) by the US Food and Drug Administration (FDA) for the therapy of multiple myeloma(*3*). Encouraged by the clinical success of bortezomib, series of new generation proteasome inhibitors are being investigated as therapeutics of various diseases thus both (pre)clinical and fundamental knowledge on the activity of proteasomes is required.

The challenge of determining the proteasome activity by a robust and high-throughput method is significant. The proteasome is not a single protease, but a multi-subunit protease cluster which in eukaryotes contains active subunits with different cleavage preferences. Mammalian 30S proteasomes contain the catalytic 20S barrel-shaped core particle (CP) capped on both sides by 19S regulatory particles (RP) (Fig 1a). The 20S core particle consists of four heptameric rings assembled from α or β subunits (α1-7,β1-7,β1-7,α1-7) and harbors three different peptidase activities on each β ring(*4, 5*). Crystallographic and substrate specificity studies show that the active-site pockets of the β5 subunits can accommodate and cut at the C-terminus of bulky, hydrophobic amino acid residues in a manner that resembles chymotrypsin activity(*6*). β2 subunits prefer cleaving after basic residues and are referred to as bearing the "trypsin-like" activities, whereas β1

cuts after acidic residues and is known as the "caspase-like" activity.

This constitutive 20S proteasome core particle is present in all eukaryotic cells. In immune-competent tissues, three additional catalytically active β subunits are expressed: β1i (Low Molecular weight Protein 2, LMP2), β2i (multicatalytic endopeptidase complex-like-1, MECL1) and β5i (LMP7)2. The immuno β subunits show comparable substrate cleavage preference, share around 50% protein sequence identity and have different functional roles compared to the constitutive β1, β2 and β5 when replacing them in newly assembled 20S CP yielding the so-called immuno-proteasomes(*1*). Recently, the β5t subunit was identified that is exclusively expressed in cortical thymus epithelial cells where it is incorporated in immuno-proteasomes instead of β5i, yielding the thymo-proteasome(*7*). Despite this diversity, the activity of the β subunits is conveyed by the same mechanism of nucleophilic attack of the N-terminal threonine (Thr1) γ-hydroxyl on the peptide backbone(*8*).

A commonly used technique to determine the proteasome activity is by means of fluorogenic substrates; short peptides that optically quench the amino coumarin at their C-terminus which upon cleavage by the proteasome is released and starts to fluoresce in solution(*9*). Excellent subunit specific fluorogenic substrates for each of the three constitutive β subunit activities are commercially available, however, these can not discriminate between constitutive and immuno-proteasome activities when present in the same cellular system. This problem is encountered during immunological studies of antigen presentation or (pre)clinical research in immune cells, such as leukemia and myeloma cells.

3.1.2 Activity-based Protein profiling

ABPP is a chemical proteomics technology, which allows capturing, visualization, identification and quantification of the target enzymatic activities either in a test tube or in living systems(*10*). It requires no protein purification step or specific labeled substrates and enables simultaneous labeling of multiple activities providing a robust and high-throughput platform to observe the activity of target enzyme (family). In ABPP workflows, organic compounds are used, the so called activity-based probes (ABP), which are mechanism-based covalent and irreversible inhibitors of the target enzymes, equipped with a reporter group. A typical ABP consists of three parts, a reactive group (electrophilic trap or warhead) that targets the catalytic site of the enzyme, a spacer (linker) that serves as the recognition element for the enzyme binding pockets, and a reporter group (tag) for detection (Fig 1b).

Application of ABPP to proteasome activity assays embraces a rich history, from the early reports of natural products inspired tritium-labeled lactacystin and biotin labeled epoxomicin, to more rational design synthetic probes like the ^{125}I-labeled nitrophenol derivative of peptide vinyl sulfone L3VS (^{125}I-NIP-L3VS) and the commercially available N-terminal extended AdaAhx3L3VS inhibitor and its biotinylated AdaK(bio)Ahx3L3VS

derivative(*11-14*). Advances in fluorescence detection methods allowed the development of fluorescent probes to replace the radioactivity-based probes which are more technically challenging to handle. Berkers et al synthesized DansylAhx3L3VS that could be used for dual readout both via (weak) dansyl fluorescence or western blot analysis with antibodies against dansyl(*15*). The bright, pan-reactive MV151 and Bodipy-TMR-epoxomicin (MVB003, probe **3**) probes were followed by subunit specific probes for the β5 and β1 activities, and several generations of ABPs equipped with different warheads, linkers and tags have been developed in our lab(*16-18*). For low tech but high-throughput detection and quantification DansylAhx3L3VS was used to determine proteasome activity in hematologic malignancies, MV151 was instrumental to determine the proteasome activity in bortezomib adapted cells and MVB003 highlighted the β5t activity(*17, 19, 20*).

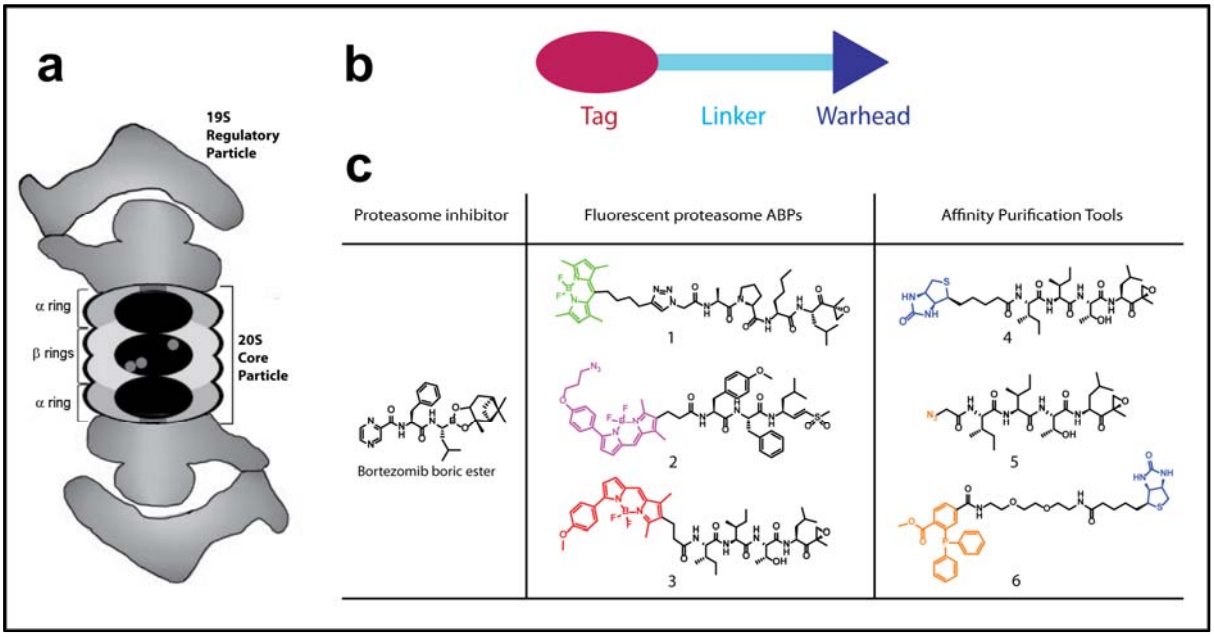

Figure 1:Proteasome ABPP. Model of the 30S proteasome where active β subunits are represented by grey spheres (a); schematics of a typical activity-based probe (b); Activity-based proteasome profiling tool box, LW124 (1) is a β1/β1i specific probe, MVB127 (2) is a β5/β5i specific probe, MVB003 (3) is a pan reactive probe, Biotin-Epoxomicin (4) is a pan reactive probe, N3-epoxomicin (5) is a two-step pan reactive probe, Biotin-Phosphine (6) is a bio-orthogonal ligation compound for installing a biotin tag (c). Synthesis of the compounds is described in refs 17-18, 21-23.

MVB003 contains the epoxomicin sequence (Ile-Ile-Thr) and electrophilic trap (Leu-epoxyketone), which makes the compound pan-reactive to all active proteasome subunits, it binds covalently, essentially labeling them with a fluorescent tag, and after SDS-PAGE separation and fluorescence scanning of the wet gel slab, a characteristic banding pattern appears (Figure 3, MCF7 cells) in which the β2 subunit runs higher than the doublet

consisting of β1 and β5. However, a general shortcoming of the pan-reactive probes is that in the case of immuno-proteasomes the molecular weights and thus the electrophoretic gel shift of the β1/β1i/β5/β5i subunits on a standard 8 cm tall 1D SDS-PAGE gel is limited thus their signals will overlap, show poor resolution and cannot be individually quantified (Figure 3, AMO1 cells). To overcome this problem, a rational design and screening program have been initiated to synthesize the β1/β1i selective sequence (Ala-Pro-dNorLeu) equipped with a Leu-epoxyketone warhead for probe **1** and the β5/β5i selective sequence (metTyr-Phe) with the Leu-vinylsuplhone electrophilic trap for probe **2**(*18*). The subunit specific probes **1** and **2** (Figure 1c) proved to be a remarkable technical improvement that could solve this problem, allowing for separation of β1i from β1, and β5i from β5 on SDS-PAGE with superior resolution, although the β subunits have a difference of some 500 Da in molecular weight. Furthermore, relative quantification of an individual activity between different samples can be done by fluorescent signal analysis in gel. The schematics of this experiment are shown in Figure 2 (lower route).

Alternatively, proteasome ABPP can be done with direct one-step biotinylated ABP (**4**) or two-step bioorthogonal tools (**5** and **6**)(*17, 21*). For one-step ABPP, proteasome active subunits in cell extracts are labeled with biotinylated probe **4** followed by affinity purification with streptavidin coated paramagnetic beads. Because biotin hampers probe permeability in living cells, the two-step approach is used where the azide equipped probe **5** labels the active proteasome in living cells and after cell lysis the reporter or affinity tag of choice is installed via a bio-orthogonal reaction(*22, 23*). After the pull down step, enriched proteins are identified by on-bead tryptic digest and LC/MS-based proteomics analysis (Figure 2, upper route). For relative quantification of proteasome activity, the stable isotope dimethyl labeling method has been combined with the affinity purification ABPP platform because it is a general labeling method that can be applied to peptide samples of various origin ranging from tissue cultures to biopsies taken from patients(*24, 25*). After on-bead digest, tryptic peptides were loaded on a C18 solid support on which both the di-methyl labeling and the desalting were performed, and after elution were mixed and analyzed by LC/MS. The open source MaxQuant software was then used for peptide identification and quantification(*26, 27*). In addition, active site peptides can also be analyzed by LC/MS after elution from the streptavidin beads.

Bortezomib shows much higher affinity for the β5/β5i and β1/β1i subunits because it is stabilized by hydrogen bonding in these active sites and the potency and the subunit specificity of bortezomib can be quantified accurately(*6*). This platform can find other applications for instance the screening for novel proteasome inhibitors. With this combination of methods and using the MVB003 probe it has been demonstrated that the newly discovered β5t subunit, expressed only in the thymus, is indeed catalytically active(*17*).

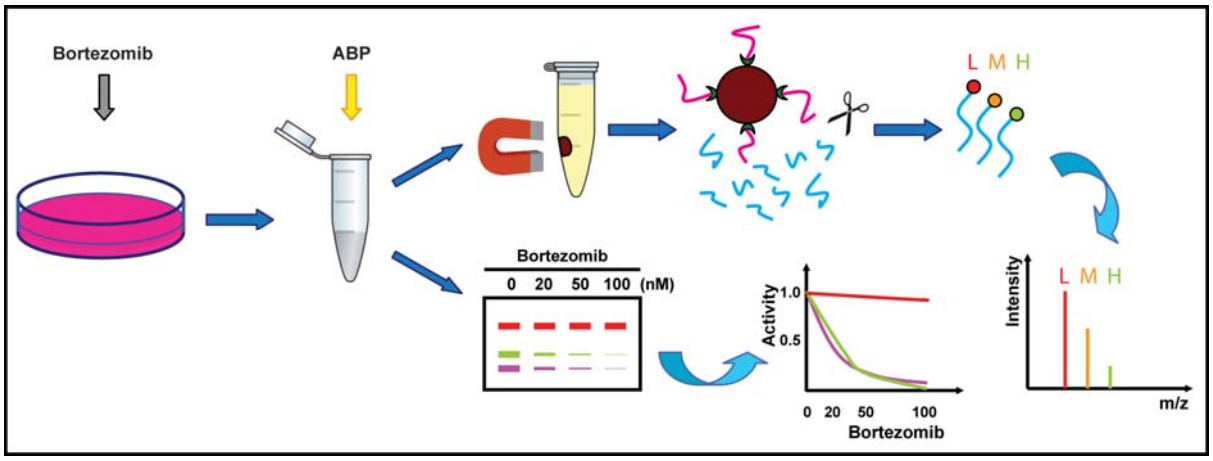

Figure 2: Schematic overview of the quantitative activity-based proteasome profiling experiments. Upper pathway describes the one or two step affinity purification protocol using the biotin/Streptavidin system, followed by stable isotope labeling, LC/MS-based proteomics analysis and quantification. Active site peptides (in pink) are analyzed after elution from the beads. Lower pathway shows the SDS-PAGE analysis and quantification of a competitive ABPP experiment.

3.1.3 Advantages and limitations with respect to other methods

Study of the individual function of active β-subunits by genetic knockdown in yeast proved to be difficult because they are essential for survival of the cell and for that reason chemical modulation and readout of activity is preferred(*28*). Compared to the popular proteasome activity determination by fluorogenic substrate assay, the ABPP method is high-throughput (no need for protein purification), robust and simple (no specific substrates needed and little off-target labeling), of superior resolution (capability to distinguish constitutive and immuno activities) and living cell compatible (good cell-permeability shown by ABPs).

There are some drawbacks due to the chemical and mechanism based nature of the method. It is possible that the chemicals used as probes can also hit other targets than the proteasome that might limit their use in living cells. Because the probes bind covalently to the catalytic sites, the enzymatic activity is consumed during the measurement necessitating the analysis of time dependency when enzyme kinetics needs to be assayed. For accurate kinetic measurements it is necessary to determine enzyme activity by taking increasing concentration of the probe and incubate for increasing amount of time. A rate constant (kobs) for each probe concentration is extracted from the semi-logarithmic plot of enzyme activity versus time and plotted against the probe concentration to determine the affinity of the probe for the enzyme (Ki value). For an excellent review on this methodology see Singh et al(*29*). However, these extensive studies are not necessary for global screens using a standard incubation time and probe concentration which should make the inter-

experiment results comparable. The ABPP assays presented here are not easily scalable to high throughput 96 well format and require relatively high (5-10x10^6) cell numbers. An ELISA based, high throughput method for the analysis and quantification of active site peptides of the proteasome β-subunits is described by Muchamuel and coworkers(*30*).

Despite the few drawbacks, the ABPP platform shows to be a powerful method that facilitates quantitative comparison between different treatments, e.g. increasing concentrations of bortezomib, allows analysis of all proteasome activities in one experiment in living cells, can be used for identification of the active site peptides and is applicable to clinical samples. Although it has been described here for the analysis of proteasome activity, this protocol can be used as guideline for setting up comparable ABPP workflows(*10*). Recently, this method has been adapted to determine the activity of the membrane bound cerebrosidase GBA1 and GBA2 enzymes that are expressed at very low abundance (1:300,000)(*31*). The fluorescent probes can also find application in fluorescence microscopy and flow cytometry analysis as exemplified by asymmetric T lymphocyte division caused by unequal proteasome segregation between daughter cells(*32*). For background information on the use of Click chemistry (TOP-ABPP) for performing two-step labeling and the use of the stable isotope in cell culture labeling (ABPP-SILAC) approach the work from the Cravatt lab can be read(*33, 34*). As future outlook, the proteasome can be an interesting target for rational drug design of novel antibiotics35 and the ABPP method might find application in this field. In this manuscript a step by step description of a competitive ABPP protocol is given, in which the β subunits activity of the proteasome is determined after treatment with bortezomib and in non-treated cells.

3.1.4 Experimental Design

High resolution fluorescent ABPP This method can be easily implemented in any laboratory that has basic cell culture and protein analysis equipment. The method has been used successfully in many different mammalian cell culture systems ranging from immortal cell lines to primary cells and from adhering to suspension cultures. Typical applications are for drug discovery programs of novel proteasome inhibitors, determination of the effect of a drug on the proteasome activity, antibiotic discovery or basic understanding of proteasome biology in plants(*35, 36*). In this protocol, human and mouse cell lines are incubated with increasing concentrations of bortezomib and the remaining activity of individual β-subunits of both the constitutive as the immuno proteasome in the respective cell lysates is determined. The advantage of post-lysis ABPP is that the same lysate can be interrogated with different probes and compared. Alternatively, because probes **1, 2** and **3** are cell permeable they can be added in parallel to cells after bortezomib treatment to study competition in the context of living cells. This assay can be performed in 6 or 12 well format but not higher because protein isolation from small cell numbers would prove too

difficult. The experiments should be performed at least in duplicate and incubation with 0 nM bortezomib serves as positive control for determination of the maximum probe binding signal. Incubation with a potent and pan-reactive inhibitor to all proteasome β-subunits (e.g. N3-epoxomicin, **5**) or boiling the cell lysate with 1% SDS prior to labeling with the activity-based probes can serve as negative controls. Commercially available protease inhibitor cocktails are tolerated during lysis. The protease inhibitor cocktail tablets (Roche) and PMSF (Sigma) have been tested and found out that they do not compete for the covalent binding between ABP and proteasome subunits.

For quantification purposes it is important that the cell lysis buffer is compatible with the protein determination method used after cell lysis as it can be sensitive to some detergents (SDS, Triton X100) or reducing agents (e.g. DTT) that can skew the measured protein concentration. SDS-PAGE analysis is best performed in 12.5% acrylamide gels followed by imaging the ABP labeled β-subunits simply by scanning on a fluorescence bed scanner. Active β-subunits of the constitutive proteasome show a typical banding pattern of β2 above the 25kDa marker and β1 and β5 subunits as a doublet below the 25 kDa. Commercial dual or multicolor protein markers contain a red band at 25 kDa that appears in the "red" channel used for probes **2** and **3** however up to 40x dilution of the marker might be necessary to prevent signal saturation. Mixed proteasomes (constitutive and immuno proteasome) show an additional β2i band between the β2 and β1 band and because the molecular weights of β1, β1i, β5, β5i subunits are in close proximity they are poorly separated on gel. High resolution separation can though be achieved by using the subunit specific probes **1** for β1β1i and **2** for β5β5i allowing for quantification of the individual band signals. Coomassie blue staining is a facile and reliable method to correct and normalize for pipetting errors and should be performed after the fluorescence scanning.

The outcome of a typical competitive ABPP experiment is that the signal of one or several β-subunit bands decreases with increasing competitor concentrations, which highlights the superior sensitivity and resolution of the ABPP method compared with fluorogenic assays because the detected signal provides both the identity as well as the intensity of the active β-subunit. By plotting the signal intensity against the concentration of the competitor compound, one can estimate the potency and selectivity of the competitor for the proteasome subunits.

When exploring organisms with poorly annotated genomes, ABPP can reveal the identity of the active proteasome genes, which is particularly interesting for profiling pathogenic organisms that cause disease in humans and animals. The ABPP method can be used with virtually all proteasome inhibitors given that they display an interaction strong enough to outcompete the covalent ABP from binding. The method is also adaptable to other organisms than mammals, it works in all types of cell lysates, however, probe optimization and identification of gel bands by affinity purification and LC/MS-based

proteomics is necessary for organisms that display cell walls or show extensive efflux pump activities like pathogens, yeasts or plants(*36*).

Quantitative LC/MS based ABPP Protein identification by LC/MS is very sensitive to background pollutants like keratins from dust, plasticizers from plastics or tubing and polymeric detergents (e.g. Triton, Tween). For this work it is advisable to create a dedicated lab space with a laminar flow cabinet, dedicated pipette sets, tubes, tips and very importantly dedicated solutions. Protect your work from dust and wear gloves that should be rinsed regularly to avoid electrostatic dust accumulation. Keep organic solvents in glass containers and use chemically pure water (18 MΩ) either freshly tapped from the de-ionizing apparatus or commercially available (Biosolve). Distrust autoclaved water because it is full of plasticizers or PEG's that pollute the LC/MS. The use of low-binding tips and tubes (e.g. Sarstedt, Eppendorf) is advisable to prevent loss of proteins/peptides due to adsorption to plastics.

In order to determine the proteasome activity in living cells or cell lysates by LC/MS based ABPP, it is necessary to first capture the active subunits by covalent binding to the activity-based probe, followed by affinity purification of the active subunits. Activity-based probes equipped with a biotin tag are the most popular because they facilitate the use of robust biotin-streptavidin affinity purification work flows. However, presence of biotin reduces the cell permeability of ABP's making it unsuitable for use in living cells. A solution to this problem is presented in this protocol where epoxomicin equipped with an N-terminal azide (N_3) ligation handle (compound **5**) that retains perfect cell permeability, was used to capture residual proteasome activity after bortezomib treatment in living cells, followed by post-lysis introduction of biotin via a bio-orthogonal reaction with biotin-phosphine (**6**) in the aqueous environment. Several other bio-orthogonal ligation procedures have been explored like the Cu+ catalyzed Huisgen 1,3-dipolar cycloaddition (Click chemistry) or the copper free approaches and empirically discovered that the azide-phosphine ligation pair under native (non-denaturing) conditions proved to be the most robust for the proteasome activity determination, because the phosphine reagent is simply added to the lysate, there is not need for a catalyst and we have experienced less protein precipitation(*34*, *37*).

High concentrations of biotin-phosphine (250 µM) and removal of this excess after the reaction by precipitating the proteins is necessary for the protocol. Protein precipitation with the chloroform/methanol method has several advantages compared with acetone precipitation or size exclusion cleanup: speed, it takes only 10-15 min, allows choice in partition of chemical impurities either to the aqueous or organic phase, adaptable for a wide range of protein concentrations from 10 µg to several mg and low losses of material(*38*). Drawback of the method is that protein pellets should be dried not longer than 5 minutes and the use of 1-4% SDS containing buffers is necessary for dissolving the

proteins. SDS concentration needs to be decreased before the pull down because it can interfere with and lower the biotin-streptavidin interaction. Several strategies can be adopted: dispersion of protein pellets in a low volume (25µl) of 2% SDS buffer followed by stepwise addition of buffer increasing the volume of solvation but slowly diluting out the SDS to keep its solvation power, as described in this protocol or dissolving the pellet in a higher volume (500 µl) of 2-4% SDS followed by dilution to 0.05-0.1% SDS in several tens of milliliter buffer, use of larger amount of beads (1mg or higher) and increased pull down time like overnight at 4°C as described in Kallemeijn et al(*31*).

Several sources and types of paramagnetic streptavidin coated beads have been tested and for this protocol mono-disperse, high binding capacity, 1 µm diameter beads showed superior pull-down efficiency. The advantage of paramagnetic beads compared to agarose beads is the ease of liquid handling because the beads stick to the tube wall when a magnetic field is applied. The high binding affinity of biotin for streptavidin facilitates stringent washing that decreases protein background but cannot discriminate for endogenously biotinylated proteins. Elution of biotinylated proteins is not trivial and two possibilities are presented: elution by boiling SDS-PAGE sample buffer containing SDS under denaturing conditions and in the presence of an excess of free biotin to displace the bound proteins from the streptavidin matrix, or on bead digest with trypsin for LC/MS proteomics analysis. Elution in sample buffer affords the possibility of SDS-PAGE analysis that yields valuable information about protein molecular weight and after in-gel tryptic digest, protein identity can be determined by LC/MS analysis(*39*). For on-bead tryptic digest an estimation of the trypsin quantity should be made as most protocols use a 1:100 ratio of trypsin:protein. Empirical evaluation showed that when starting with 1 mg of protein and 500 µg of beads, using some 500 ng trypsin yields reproducible results.

For comparison of treated versus non treated samples by relative quantification using LC/MS-based proteomics the "light" "medium" and "heavy" labels with stable isotope by reductive amination of primary amines at the N-termini and ε-position of lysine residues using formaldehyde have been chosen(*24*, *25*). This robust technique, applicable to virtually all types of protein specimens ranging from clinical samples to cell culture lysates, has been adapted and optimized in this protocol for small protein amounts that yield after affinity purification, by using the stage tip method(*40*). Finally, after on-bead tryptic digest, the active site peptides bound to the biotinylated probe can be eluted with acetonitrile/formic acid mixtures (containing free biotin) and used for unraveling the binding mechanism of the probe to the protein by MS/MS or MS/MS/MS analysis of the ion fragments.

3.2 Materials

3.2.1 Reagents

<CRITICAL> The chemical compounds from Fig 1 are available upon request, the primary

papers that describe their synthesis are given in the references(*17, 18, 21, 22*).

MCF7 (HTB-22) and EL4 (TIB-39) cells are commercially available from ATCC (USA). See Box 1 for information regarding cell culture and protein extraction.

AMO-1 (ACC-538) was a kind gift from the Driessen lab and it is commercially available from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). See Box 1 for information regarding cell culture and protein extraction(*19, 20*).

ACN (Acetonitrile, Sigma-Aldrich cat.no. 14261)

ATP (Adenosine triphosphate, Sigma-Aldrich cat.no. A2383)

NH_4HCO_3 (Ammonium Bicarbonate, Sigma-Aldrich cat.no. 09830)

APS (Ammonium Persulfate, Sigma-Aldrich cat.no. A3678)

β-mercaptoethanol (Sigma-Aldrich cat.no. M3148)

BSA (Bovine serum albumin, Sigma-Aldrich cat.no. A7906)

$CaCl_2$ (Calcium Chloride, Sigma-Aldrich, cat.no. 223506)

$CHCl_3$ (Chloroform, Sigma-Aldrich, cat.no. 650498)

Colloidal Blue Staining Kit (Invitrogen, cat.no. LC6025)

Digitonin (Sigma-Aldrich, cat.no. D141)

Na_2HPO_4 (Di-sodium hydrogen phosphate, Sigma-Aldrich, cat.no. S3264)

KH_2PO_4 (Potassium phosphate monobasic, Sigma-Aldrich, cat no. P5655-500G)

K_2HPO_4 (Potassium phosphate dibasic, Sigma-Aldrich, cat no. P2222-500G)

DTT (Dithiothreitol, Sigma-Aldrich, cat.no. 43815)

DMSO (Dimethyl Sulfoxide, Sigma-Aldrich, cat.no. D8418)

ECL Plus Western Blotting System (GE Healthcare, cat.no. RPN2132)

CH_2O (Light Formaldehyde, Sigma-Aldrich, cat.no. F8775)

CD_2O (Intermediate Formaldehyde, Sigma-Aldrich, cat.no. 492620)

$^{13}CD_2O$ (Heavy Formaldehyde, Sigma-Aldrich, cat.no. 596388)

! CAUTION work with formaldehyde in a fume hood, because the solutions and vapours are toxic.

FA (Formic acid, Sigma-Aldrich, cat.no. F0507)

Glycerol (Sigma-Aldrich, cat.no. G5516)

Glycine (Sigma-Aldrich, cat.no. G8898)

HCl (Hydrochloric acid, Sigma-Aldrich, cat.no. 84415)

IAA (Iodoacetamide, Sigma-Aldrich, cat.no. I1149)

$MgCl_2$ (Magnesium Chloride, Sigma-Aldrich, cat.no. M8266)

Tergitol NP-40 (Sigma-Aldrich, cat.no. NP40)

KCl (Potassium Chloride, Sigma-Aldrich, cat.no. P9541)

Trypsin (Promaga, cat.no. V5111)

SDS (Sodium dodecyl sulfate, Sigma-Aldrich, cat.no. L4390)

SilverQuest Staining Kit (Invitrogen, cat.no. LC6070)
Bradford assay reagent (BioRad, cat.no. 500-0205)
NaCl (Sodium Chloride, Sigma-Aldrich, cat.no. S3014)
$NaBH_3CN$ (Sodium cyanoborohydride, Sigma-Aldrich, cat.no. 156159)
$NaBD_3CN$ (Sodium cyanoborodeuteride, Sigma-Aldrich, cat.no. 190020)
NaH_2PO_4 (Sodium dihydrogen phosphate, Sigma-Aldrich, cat.no. S3139)
Sucrose (Sigma-Aldrich, cat.no. 84097)
TEMED (N,N,N′,N′-Tetramethylethylenediamine, Sigma-Aldrich, cat.no. T9281)
Tris (Sigma-Aldrich, cat.no. T6066)
Tween 20 (Sigma-Aldrich, cat.no. P9416)
Urea (Sigma-Aldrich, cat.no. U5378)
Biotin (Sigma-Aldrich, cat.no. B4639)
Dynabeads MyOne Streptavidin C1 (Invitrogen, cat.no. 650-01)
EDTA (Ethylenediaminetetraacetic acid, Sigma-Aldrich, cat.no. E6758)
Ethanol (VWR, cat.no. 20816.298)
Methanol (VWR, cat no. 83638.290)
Bortezomib ester (Fig. 1, S1013, Selleckchem.com, Houston, USA)
LW124 (**1**, Fig. 1)[18]
MVB127 (**2**, Fig. 1)[18]
MVB003 (**3**, Fig. 1)[17]
Biotin-Epoxomicin (**4**, Fig. 1)[17]
N_3-epoxomicin (**5**, Fig. 1)[17]
Biotin-Phosphine (**6**, Fig. 1)[21,22]
DMEM (Dulbecco's modified eagle's medium, PAA, cat.no.E15-883)
RPMI-1640 (PAA, E15-041)
FCS (Fetal calf serum, PAA, cat.no. A15-104)
Stable L-Glutamine (PAA, cat no. M11-006)
Penicillin (Duchefa Biochemie, cat no. P0142.0100)
Streptomicin (Duchefa Biochemie, cat no. S0148.0100)
BPB (Bromophenol Blue, Sigma-Aldrich, cat no. B0126-25G)
DC Marker (Precision Plus Protein standards, Bio-Rad cat.no. 161-0374)

3.2.2 Equipment

Cell culture

Galaxy 170R CO_2 Incubater (New Brunswick)
Clean air cabinet (Thermo)
37°C Water bath (Grant)
Fridge Freezer (Bosch)

Ependorf centrifuge 5702 (Ependorf, cat.no. 5702 000.019)
Ependorf centrifuge 5424 (Ependorf, cat.no. 5424 000.410)
Cell culture Flasks (PAA, cat.no. PAA70325X and PAA70375X)
Cell culture Dishes (PAA, cat.no. PAA20035X, PAA20060X, PAA20101X and PAA20151X)
Ependorf pipettes (Ependorf, cat.no. 3120 000.011, 3120 000.038, 3120 000.054 and 3120 000.062)
Accu-jet pro Pipette Controller (BrandTech, cat.no. 2026330)
Cell lifter (Corning, cat.no. 3008)
Pipette tips (Sarstedt, cat.no. 70.1130, 70.760.002, and 70.762)
Serological pipettes (Sarstedt, cat.no. 86.1252.025, 86.1253.025, 86.1254.025 and 86.1685.020)

Protein extraction

Ependorf cool centrifuge 5402 (Ependorf)
Vortex Genie 2 mixer (Scientific industries, cat no. SI-0256)
Sonicator (Sonics, cat no. VC 505)
GENios plate reader (Tecan)
Ice maker (Hoshizaki)
Forma -86°C ULT Freezer (Thermo)
10-100µl Multichannel pipette (Eppendorf, cat no. 3120 000.046)
96 well plate (Greiner Bio One, cat no. 655101-ORT)

Affinity Purification

Thermomixer compact (Ependorf)
Test tube rotator (Labinco, cat no. 29000)
QBD2 metal heating block (Fisher, cat no. BLD-560-020X)
BioRad mini protean 3 gel running system (with power supply and blot transfer unit)
SSM4 Rocker (Stuart)
DynaMag-2 (invitrogen, cat no. 123.21D)

Imaging

BioRad ChemDoc system (with GS-800 scanner)
Typhoon 9400 scanner (GE Healthcare)

LC/MS system

LTQ-Orbitrap mass-spectrometer (thermo-fisher scientific)

3.2.3 Reagent setup

TBS, 10x Dissolve 12.1g Tris and 88g NaCl in 800ml H_2O, and then adjust the pH to 7.5, add H_2O up to 1 liter and sterile filter it. This can be stored for several months.

TBST Dilute 100ml 10x TBS to 1 liter in H_2O, add 1ml Tween 20, mix well. Make fresh every week.

PBS, 10x Dissolve 6.8g KH_2PO_4 in 50ml H_2O and 26.1g K_2HPO4 in 150ml H2O, mix the solution, dilute it to 800ml, and use it to dissolve 87.7g NaCl, adjust the pH to 7.6, add H_2O up to 1 liter and sterile filter it. This can be stored for several months.

Lysis buffer Mix 50 mM TrisHCl pH 7.5, 250 mM sucrose, 5mM $MgCl_2$, 1mM DTT, 2mM ATP, 0.025% digitonin, 0.1% NP40. Make fresh. Additionally, protease inhibitor cocktails (Roche) can be added because they do not interfere with the proteasome activity.

Assay buffer Mix 50 mM TrisHCl pH 7.5, 250 mM sucrose, 5mM $MgCl_2$, 1mM DTT, 2mM ATP, 50mM KCl. Make fresh.

PD buffer Mix 50 mM TrisHCl pH 7.5 and 150 mM NaCl. This can be stored at 4°C for several weeks.

PD wash buffer I Dissolve 4 M urea in 50 mM NH_4HCO_3. This should be made freshly.

PD wash buffer II Mix 50 mM TrisHCl pH 7.5 and 10 mM NaCl. This can be stored at 4°C for several weeks.

On bead digest buffer Mix 100 mM TrisHCl pH 7.8, 100 mM NaCl, 1mM $CaCl_2$, 2% ACN and 10ng/µl Trypsin. This should be made freshly.

Active site elution solution Add 100 µM biotin in a mixture of 5% formic acid, 25% acetonitrile and 70% H_2O. This should be made freshly.

Sample buffer, 4x Mix 1ml 20% SDS, 1ml 0.625M TrisHCL pH6.8, 2.1ml 87% glycerol, 0.4ml BME, 0.1ml 10%BPB and 0.4ml H_2O. This can be stored at -20°C for several months.

Running buffer, 5x Mix 42.6g Tris, 214g Glycine and 300ml 10%SDS in 4 liters of H_2O, when dissolved well add H_2O up to 5 liters. This can be stored for several weeks.

In gel digest buffer Mix 10 mM NH_4HCO_3, pH 8, 5% ACN, 1 mM $CaCl_2$ and 10ng/µl Trypsin. This should be made freshly.

Gel block extraction solution 2% FA in 67% ACN/H_2O. Make fresh.

Stage tip solution A 0.5% FA in H_2O. Make fresh.

Stage tip solution B 0.5% FA in 80 % ACN/H_2O. Make fresh.

LC/MS sample solution Mix 95ml H_2O, 3ml ACN, and 0.1ml FA. This can be stored for several weeks.

PB 7.5 (Phosphate buffer) Mix 2ml 50µM Na_2HPO_4 and 7ml 50µM NaH_2PO_4. This can be stored for several weeks.

[L] Light Dimethyl labeling buffer (100µl)

Composition	Stock	Volume
PB 7.5		90µl
CH_2O	4% (v/v)	5µl
$NaBH_3CN$	0.6M	5µl

<CRITICAL> Prepare fresh.

[M] Medium Dimethyl labeling buffer (100µl)

Composition	Stock	Volume
PB 7.5		90µl
CD_2O	4% (v/v)	5µl
$NaBH_3CN$	0.6M	5µl

<CRITICAL> Prepare fresh.

[H] Heavy Dimethyl labeling buffer (100µl)

Composition	Stock	Volume
PB 7.5		90µl
$^{13}CD_2O$	4% (v/v)	5µl
$NaBD_3CN$	0.6M	5µl

<CRITICAL> Prepare fresh.

Proteasome inhibitors: While the use of bortezomib ester is described here, this ABPP method is also compatible with other proteasome inhibitors. It is used in the Biosyn lab as a screening technique to determine the activity, potency and subunit specificity of novel and potential proteasome inhibitors. Most compounds are soluble in DMSO and 500x stock dilutions is used prior to addition to the biological sample (like cell culture) to keep the DMSO concentration at <1% of the sample.

3.2.4 Equipment setup

Typhoon settings

The Typhoon 9400 scanner was used to scan the gel with fluorescent ABP labeled proteins. The scanning setting for MVB003 (Probe **3**) and MVB127 (Probe **2**) is Fluorescence Cy3/TAMRA, 600 PMT at 50 microns pixel resolution. The setting for LW124 (Probe **1**) is Fluorescence Cy2 (with Blue Laser), 600 PMT and 50 microns pixel resolution. The ImageJ software is used for quantifying the intensity of the bands after subtracting the background.

ChemDoc settings

The ChemDoc system for imaging the western blots and the GS800 scanner for imaging the commassie stained SDS-PAGE gels. The program Quanty One was used to operate the imaging systems, and subsequently used for quantifying the intensity of the bands and/or lanes. Setting for imaging the western blots is chemiluminescence.

Orbitrap settings

General settings of the mass spectrometer were: an electrospray voltage of 1.5 kV was

applied to the emitter, no sheath and auxiliary gas flow, ion transfer tube temperature 150°C, capillary voltage 15V, tube lens voltage 150V. Internal mass calibration was performed with air-borne protonated polydimethylcyclosiloxane (m/z=445.12002) and the plasticizer protonated dioctyl phthalate ions (m/z= 391.28429) as lock mass(*41*). For shotgun proteomics analysis, 10 µl sample was pressure loaded on the trap column at 10 µl/min flow for 5 min followed by peptide separation with a gradient of 35 min 5-30% B, 15 min 30-60% B and 5 min 100% A at a flow of 300 µl/min split to 250 nl/min by the LTQ divert valve. For each data dependent cycle, one full MS scan (300-2000 m/z) acquired at high mass resolution (60,000 at 400 m/z, AGC target $1x10^6$, maximum injection time 1,000 ms) in the Orbitrap was followed by 3 MS/MS fragmentations in the LTQ linear ion trap (AGC target $5x10^3$, maximum injection time 120 ms) from the three most abundant ions. MS^2 settings were: collision gas pressure 1.3 mT, normalized collision energy 35%, ion selection threshold of 500 counts, activation q = 0.25 and activation time of 30 ms. Fragmented precursor ions that were measured twice within 10 s were dynamically excluded for 60s and ions with z<2 or unassigned were not analyzed(*17*).

BOX1 | Cell culture and protein extraction ● **Timing 1 d**

In this study we have used adherent and suspension growing cells from human and mouse to show that the activity-based protein profiling method can be used in virtually all in vitro cultured mammalian cell types including primary cells. In (micro)organisms bearing a cell wall (e.g. yeast, plants) the method works well in cell lysates but should be optimized (mostly by using higher probe concentrations) for living cells.

1 | Cell culture MCF7(Human breast carcinoma), and EL4 (Murine T-lymphocyte) are grown in DMEM medium with 10% fetal calf serum (FCS), 0.1mg/ml penicillin and 0.1mg/ml streptomycin, in a 37°C, 7% CO_2 incubator. AMO1 (plasmacytoma) cells are grown in RPMI 1640 medium with 10% fetal calf serum (FCS), 0.1mg/ml penicillin, 0.1mg/ml streptomycin, and 2mM stable L-Glutamine and in 37°C, 5% CO_2 incubator.

2 | Harvesting Harvest MCF7 cells by scrapping them in cold PBS. Harvest EL4 and AMO1 cells by centrifugation directly at 1200rpm for 5 min. Wash the cells with ice cold PBS for three times to remove serum and free inhibitor molecules.

■ **PAUSE POINT**: The cells can be stored in -80°C freezer for a couple of months.

3 | Protein extraction Thaw cell pellets from -80°C on ice, add 4 volumes of lysis buffer to the cell pellet and leave on ice for 15-30 min. Sonicate the cells on ice for 3x10 seconds at 12 watts, with 5 second pulse/pause. Centrifuge the lysate for 10 minutes at top speed (13Krpm) at 4°C

! CAUTION; Check by eye whether the supernatant is clearly transparent.

4 | Lysate storage Transfer the supernatant to new Eppendorf tubes. Determine the protein concentration by Bradford Protein Assay, and freeze the lysate at -80°C for future use.

■ **PAUSE POINT**: The lysate can be kept in -80°C for a couple of weeks.

! CAUTION: Aliquot the lysate in small portions to avoid freezing and thawing for multiple times.

3.3 Procedure

There are two options depending on whether you want to do high-resolution fluorescent ABPP on cell lysates (option A) or quantitative LC/MS based ABPP (option B). For option A, three different fluorescent probes for the ABPP are recommended, LW124 (**1**) to detect β1 (β1i), MV127 (**2**) to detect β5 (β5i), MV003 (**3**) to detect all the subunits (Fig. 1)(*17, 18*). This is done in order to get perfect separation between the constitutive and immunoproteasome subunits on SDS-PAGE for quantification purposes. For option B, to quantify proteasome in living cells, N_3-epoxomicin (**5**) is recommended because it is cell permeable, and biotin-phosphine (**6**) for the bioorthorgonal ligation to install the biotin tag; for in vitro experiments biotin-epoxomicin (**4**) can be used directly (Fig. 1)(*17, 21-23*).

Option A High resolution fluorescent ABPP ● Timing 3 d

(i) Make 10 mM stocks of all probes and inhibitors in DMSO and use DMSO as negative control for your experiments.

(ii) Seed $2.5x10^6$ MCF7 cells in 10 cm petri dishes, culture to 70-80% confluency and treat with 0, 20, 50 and 100 nM bortezomib for 16 hours. From logarithmic growing AMO1 cultures use $10x10^6$ cells and treat with 0, 10, 20, 50 nM bortezomib for 16 hrs(*20*).

! CAUTION: The number of cells, concentration of bortezomib and time of treatment should be determined according to the sensitivity of the cells to the proteasome inhibitor.

(iii) Lyse the cells and determine the protein concentration as described in BOX1. Typical cell pellets of 10-20 µl are lysed in 40-80 µl lysis buffer and the protein concentration is in the range of 10-20 µg/µl.

(iv) Prepare 20 µg of total protein for each sample; bring the total volume up to 9 µl by adding assay buffer. Then add 1µl 10x working stock of the ABP, yielding final ABP concentrations of 0.5 µM LW124 (**1**), 0.5 µM MVB127 (**2**) or 2 µM MVB003 (**3**). Incubate the mixture at 37°C for 1 hour. Add 3 µl 4x sample buffer, and boil the sample for 5min at 100°C.

(v) The lanes with 0 nM bortezomib were used as positive controls, as shown in Figure 3, there is clear proteasome inhibition by the treatment. If any other proteasome inhibitor is used in a similar experiment, as negative controls deactivated lysate by boiling with 1% SDS or blocking the proteasome with 10 µM N3-epoxomicin (**5**) prior to incubation with the activity-based probe were suggested. In these lanes no clear proteasome bands were expected to appear.

(vi) Separate the proteins on 12.5% SDS-PAGE.

(vii) Scan the gels on a typhoon scanner with correct settings (as described in the Equipment Setup).

(viii) As loading control, coomassie stain the gels, scan on the BioRad GS-800 scanner and determine the signal intensity for the total amount of protein in each lane using the QuantityOne program. Normalize all lanes to the highest intensity value to

correct for pipetting errors.

(viii) Quantify the fluorescence image with the ImageJ software by drawing a region of interest (ROI) around individual bands followed by background subtraction prior to calculating the proteasome activity and correction for pipetting errors. The fluorescent gel image and the quantification graphs from MCF7 and AMO1 are shown in Figure 3.

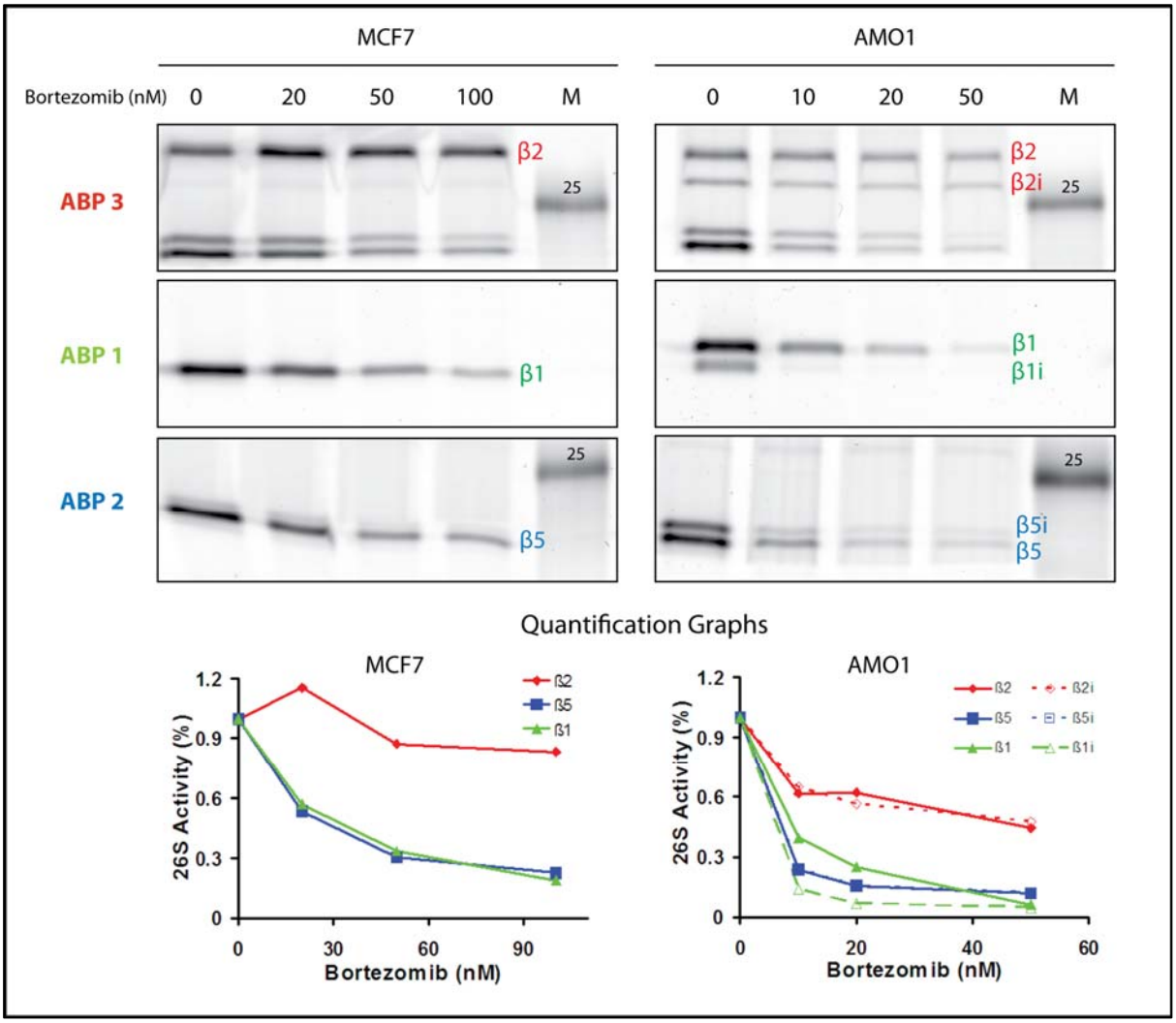

Figure 3: Results of fluorescence based quantitative proteasome ABPP in adherent growing breast carcinoma cell line MCF7 expressing constitutive proteasome and suspension growing cells AMO1 expressing both constitutive and immuno proteasomes incubated with increasing bortezomib concentrations. Top panel global proteasome assay with MVB003 (**3**), used for quantification of β2/ β2i activities, middle panel β1/β1i quantification with LW124 (**1**), lower panel β5/β5i quantification with MVB127 (**2**). The graphs show the proteasome activity, quantified from the gel bands, after treatment with increasing concentrations bortezomib.

Option B Quantitative LC/MS based ABPP ● Timing 8 d

(i) Cell treatment Treat 1 x 10^8 EL4 cells with the proteasome inhibitor bortezomib at following concentrations: 0, 10nM, 100nM, 1μM, and 10μM. DMSO can be used as negative control and the concentration of DMSO is kept below 1%. After 2 hours of incubation, add 10μM N_3-epoxomicin (**5**) to each sample, and incubate again for 2 hours. Check the cells by light microscopy to observe signs of necrosis, shrinking, apoptosis or other toxicities. Lyse the cells as described in BOX1.

(ii) **ABP reaction** For direct labeling, cell lysate (1-2 mg protein in total) plus 10 μM biotin-epoxomicin (**4**) is incubated at 37°C for 1 hour. For two step labeling, add 250 μM biotin-phosphine (**6**) to cell lysates from N_3-epoxomicin (**5**) treated living cells and incubate at 37°C for 1 hour. Empirically, the 2-step labeling of the proteasome in native (non-denatured) lysates with a high excess of biotin-phosphine (**6**) shows the best reaction and pull down efficiency compared to the Cu(I) catalyzed Huisgen 1,3-dipolar cycloaddition (click chemistry) or the copper free click(*34*, *37*).

! CAUTION: Use lysis buffer to adjust total volume to 180 μl.

(iii) **Denaturation** Add 20μl 10% SDS to the reaction mixture and vortex slightly, and then boil it at 100°C for 5 minutes for denaturation. Vortex the solution homogeneously, and centrifuge 5 minutes at 12 krpm to pellet any aggregates. Transfer the clear supernatant into a new 2 ml low-binding eppendorf tube.

(iv) **Chloroform/Methanol precipitation** (steps B iv-ix) (*38*) Add 800 μl Methanol to the 200 μl protein solution, vortex vigorously.

(v) Add 200 μl Chloroform, vortex vigorously.

(vi) Add 600 μl H_2O (to get phase separation), vortex vigorously and centrifuge (2 min, 9000 g). A 3-layer separation is derived, upper layer of water, a film of protein in the middle, and the lower layer of chloroform.

(vii) Carefully remove and discard upper layer.

(viii) Add 600 μl methanol, vortex at low speed, thoroughly and centrifuge (2 min, 9000 g).

! CAUTION: Avoid disrupting the protein membrane in too many small pieces because they will not sediment well during the next centrifugation step.

(ix) Carefully discard supernatant by pippetting and dry the pellet for exactly 5 minutes to the air in the flow air cabinet, tube up side down.

▲ **CRITICAL STEP**. Both too wet or too dry pellets do not hydrate well. The protein pellet should be dried for exactly 5 min. if it is dryer or wetter, it will be hard to dissolve in the buffer for further steps.

(x)Rehydrate the protein pellet in 180 μl urea buffer (8 M Urea/100 mM NH_4HCO_3) for 15 min at room temperature.

(xi) **Reduction and alkylation** (to disable cysteine bridge formation)(Steps B xi-xiii)

Add 10 µl fresh 90 mM DTT (in 8 M Urea/100 mM NH_4HCO_3) to the 180 µl sample and incubate this for 30 min at 37°C. Note that 1%SDS or the acid labile surfactant RapiGest® (Waters) can be used instead of 8 M Urea.

! CAUTION: Temperatures > 37°C can give carbamylation of proteins by urea.

(xii) Add 15 µl 200 mM Iodoacetamide (in 8 M Urea/100 mM NH_4HCO_3) solution to the 190 µl sample and incubate for 30 min at room temperature in the dark.

! CAUTION: The reaction is light sensitive.

(xiii) Centrifuge 5 min at top speed and transfer the supernatant to a new 2 ml eppendorf tube.

(xiv) **Chloroform/Methanol precipitation** Repeat steps B (iv-ix). This extra precipitation step is suggested in order to remove the excess free ABP or free tag in the case of the 2-step labelling protocol.

(xv)**Stepwise dilution of SDS** (steps B xv-xviii) Add 25 µl of 2% SDS in PD buffer to the pellet and vortex to help dissolve. Dilute the SDS with normal PD buffer step by step. Heating to 70°C can also enhance protein solubility

(xvi) Dilute 3 times with 25 µl PD buffer, vortex after each addition of PD buffer, and make sure the solution becomes clear.

(xvii) Add the rest in 9 x100 µl steps of PD buffer, to make the SDS end concentration 0.05% and obtain a clear solution.

! CAUTION: Be patient here, it may take a long time to homogeneously disperse the entire pellet. But it should be dissolved as much as possible. If necessary, use a heated water bath and/or sonic bath to help dissolve. It is important to avoid lumps of protein here because they will a-specifically stick to the beads, yielding high background

▲ **CRITICAL STEP**. This is the hardest step in the whole protocol. This tiny volume might not be enough to solve your protein completely but more is not possible otherwise it will be ended up with too high SDS concentration in the pull down step and lose efficiency there. Disperse the protein in this volume by vortexing vigorously. Do not pipette up and down, because lumps of protein can stick in the tips, plus there is a potential for foam formation. The proteins will dissolve once the PD buffer is stepwise added. This increases the volume while keeping the concentration of SDS relatively high.

(xviii)Centrifuge at top speed for 5 min, and transfer the supernatant to a new 2 ml Eppendorf tube.

▲ **CRITICAL STEP**. After taking up the volume of the PD mixture to 1 ml by adding PD buffer, centrifuge the mixture at top speed for 5 min to remove all the insoluble particles. Or, there might be non-specific protein binding to the beads, and increase of background during analysis.

(xix) **Pull down with paramagnetic beads** Add 50µl streptavidin magnetic beads (pre-washed with water, PD buffer and 0.05% SDS PD buffer). Pull down for 1 hour at room

temperature with vigorous shaking.

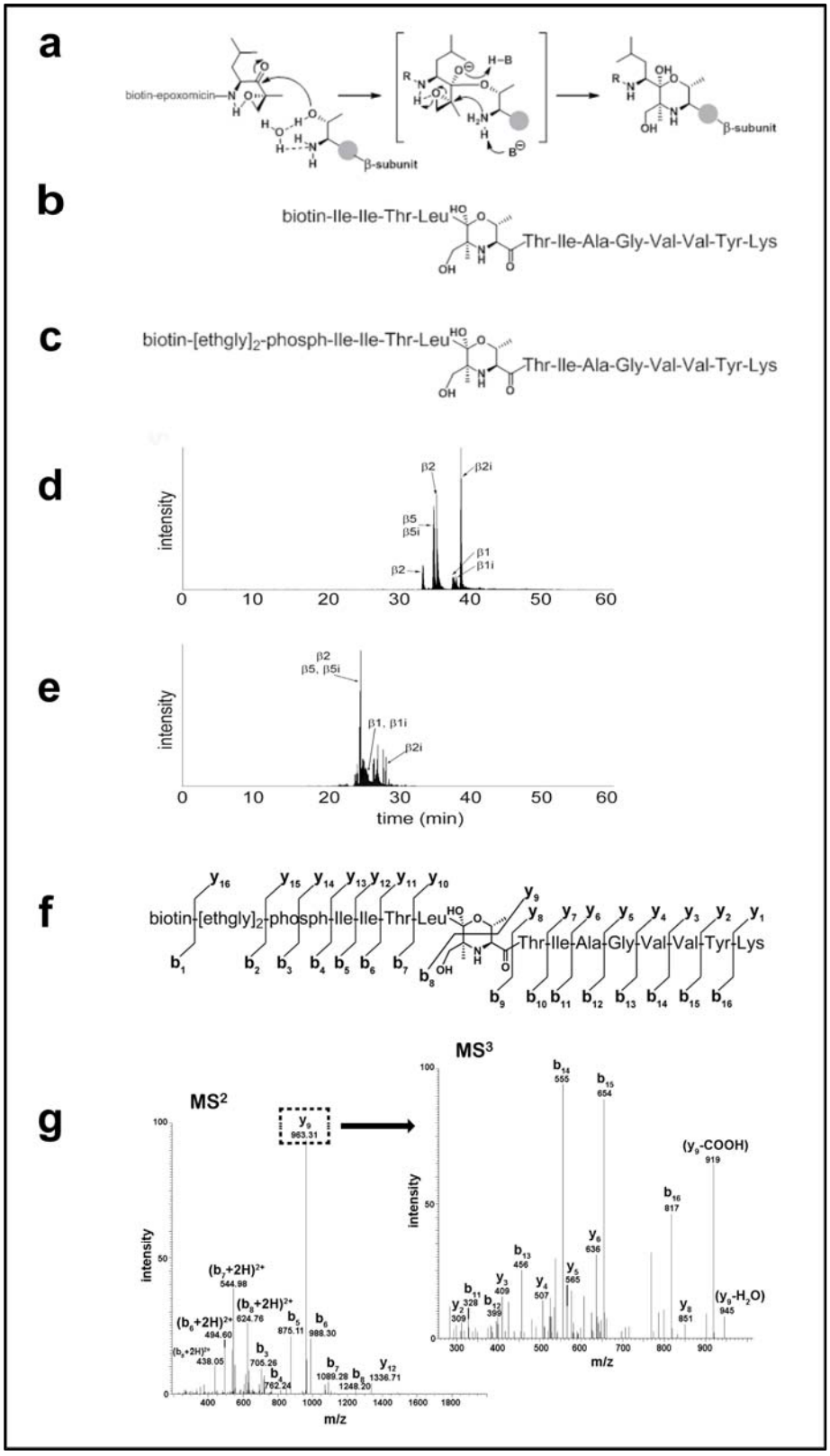

Figure4: Active site identification of the murine β2 subunit by one step and two-step ABPP with LC-MS analysis. Reaction mechanism of biotin-epoxomicin (**4**) with the catalytic T1

residue (**a**); schematic representation of the direct [biotin-epoxomicin]- [β2 active-site peptide] (**b**) and the two-step [biotin-phosphine-epoxomicin]- [β2 active-site peptide] (**c**) construct obtained after on-bead tryptic digest; LC/MS analysis and identification of the [biotin-epoxomicin]-[active site peptides] (**d**) and two-step [biotin-phosphine-epoxomicin]-[active-site peptides] (**e**) from constitutive and immuno proteasomes; theoretical MS/MS fragmentation pattern for the two-step [biotin-phosphine-epoxomicin]- [β2 active-site peptide] construct (**f**), experimental data for f showing the diagnostic ions b6, b7, b8, b3, b4 after MS/MS fragmentation and subsequently the MS^3 fragmentation of the y9 ion revealing the amino acid sequence of the [β2 active-site peptide] part of the construct (**g**).

(xx) Leave tubes with pull down sample on the DynaMag, wait for 3 minutes, and pipette the supernatant to another tube.

(xxi) Run a sample of the supernatant on a SDS-PAGE gel to assess the pull down efficiency (Fig 6).

(xxii) **Washing** Wash the beads as shown in the in-text table, then divide them: 2/3 for in gel detection, and 1/3 for on bead digest.

Once	300µl PD buffer (containing 0.1% SDS)
Twice	300µl PD buffer
Once	300µl wash buffer I
Once	300µl wash buffer II
Twice	300µl water

An interesting observation is that after the pull-down, the beads might behave slightly different in solution because their surface properties have changed by the binding of the biotinylated proteins.

(xxiii) **In-gel detection** (steps B xxiii-xxxiii) Elute the proteins from the beads with 100µl 1x sample buffer containing 10µM biotin, by boiling for 5 min at 100°C.

(xxiv) Put samples on DynaMag for 3 min, transfer supernatant while still warm to another tube.

▲ **CRITICAL STEP**. Keep the boiled sample tubes on the DynaMag, when loading the samples onto the gel, to prevent loading the beads. If the beads are also loaded, background can be increased.

(xxv) Resolve samples on 12.5% SDS-PAGE and silver stain. After staining, scan the stained gel on the GS-800 scanner, excise protein bands and perform in-gel digestion steps(*39*). A typical silver stained gel image of biotin-epoxomicin (**4**) pull down in EL4 cells is shown in Figure 6a. Cut desired gel bands into 1 mm^3 blocks with a surgery knife, and keep them in a 1.5ml Eppendorf tube.

▲ **CRITICAL STEP**. In case coomassie stain is used, destaining is necessary, because the blue dye is not removed by stage tip desalting and it is unwanted in the LC/MS system.

■ **PAUSE POINT**: The gel blocks can be kept in -20°C for a couple of weeks.

(xxvi) Wash the gel blocks with 100µl Milli-Q water.

(xxvii) Discard water and add 500µl ACN to shrink the gel blocks, vortex slightly (the gel blocks will shrink, become white and sticky).

(xxviii) Discard ACN, dry gel blocks for 5 minutes in the air flow.

(xxix) Swell gel blocks with 25µl in gel digest buffer on ice for at least 2 hr.

(xxx) Digest in 37°C incubator (with shaker) overnight.

(xxxi) Add 100µl gel block extraction solution and incubate at 37°C for 15 min.

(xxxii) Spin down and transfer the supernatant to new Eppendorf tubes and evaporate the ACN from the samples at 50°C in a SpeedVac until volume is <30µl.

(xxxiii) Add 50µl stage tip solution A to adjust pH <5.

! CAUTION: The peptides should be desalted by stage tips before LC/MS analysis.

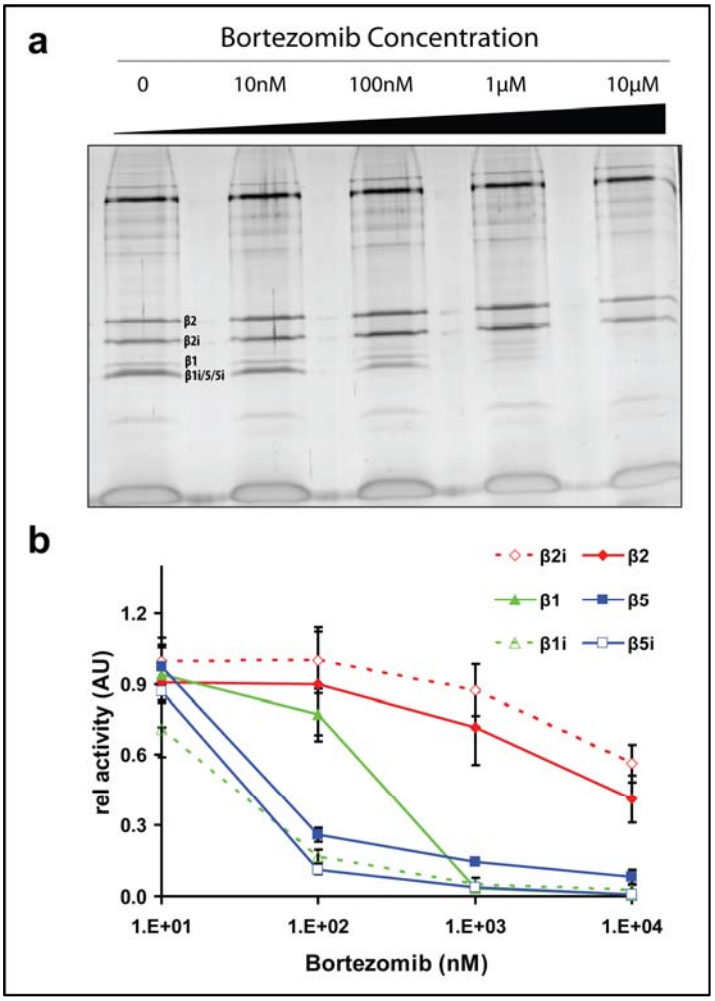

Figure 5: Competitive ABPP assay in EL4 cells (constitutive and immuno proteasome) incubated with increasing bortezomib concentrations for 2 hrs and residual β-subunit activity captured with 10 µM N_3-epoxomicin (**5**) and biotin phosphine (**6**).

Qualitative analysis by SDS-PAGE and silver stained gel after pulldown with Streptavidin beads (**a**); quantification of the β-subunits activity by on-bead tryptic digest, dimethyl stable isotope labeling and LC/MS based proteomics (**b**).

(xxxiv) **On-bead digestion** (steps B xxxiv-xxxv) Add 100µl on-bead digest buffer to the beads and digest proteins at 37°C with shaking overnight.

(xxxv) Place tubes on DynaMag for 3 min, transfer supernatants to new Eppendorf tubes and add 5µl FA for adjusting the pH <4 to inhibit trypsin.

! CAUTION: The peptides should be desalted by stage tips before LC/MS analysis.

(xxxvi) Eluting the active site peptides After on bead digest, wash the beads with 100 µl water, incubate with 100 µl active-site elution solution for 30 min at 37°C to release the active site peptide from the beads, evaporate the ACN in speedvac and add 50ul stage tip solution A. The active site peptides from murine proteasome obtained after tryptic digest of EL4 cell lysates are shown in the in-text table. The reaction mechanism and typical results for the active site identification are shown in Figure 4.

Murine proteasome subunit	Active site peptide sequence after tryptic digest
β1	TTIMAVQFNGGVVLGADSR
β2	TTIAGVVYK
β5	TTTLAFK
β1i	TTIMAVEFDGGVVVGSDSR
β2i	TTIAGLVFR
β5i	TTTLAFK

! CAUTION: The peptides should be desalted by stage tips before LC/MS analysis.

▲ **CRITICAL STEP**.All the tryptic digested peptides should be desalted by Stage Tips before loaded on the LC/MS for analysis. For the protein identification samples (like in gel digest and active site peptide identification samples), a single Stage Tip desalting is enough for the LC/MS sample preparation, which is described in step B (xxxvii). For the samples that need to be quantified, for instance the on bead digest samples treated with different bortezomib concentrations, should be both dimethyl labeled by stable isotopes and desalted on Stage Tip before LC/MS analysis, go directly to step B (xl).

(xxxvii) **Desalting using stage tips** (steps B xxxvii-xl) The Stage Tips are made of C18 material inserted in 200µl pipette tips(*40*). Desalt the peptide samples as shown in the in-text table.

Conditioning	50µl methanol
Conditioning	50µl Stage Tip solution B
Conditioning	50µl Stage Tip solution A
Loading	Load samples on Stage Tips
Washing	100µl Stage Tip solution A
Elution	100µl Stage Tip solution B

! CAUTION: Run the solutions through the stage tips by low speed centrifugation (400-800 g).

(xxxviii) Evaporate the ACN from samples until the volume is < 20µl.

(xxxix) Add 50µl LC/MS sample solution, and then the samples are ready for LC/MS analysis.

(xl) **On Stage Tip dimethyl labeling** (steps B xl-xlvi) Follow the protocol step B (xxxvii) till washing. After washing, load 20µl of L, M, or H reagents into each of the assigned stage tips.

(xli) Centrifuge at 100 g for 5 min in an eppendorf centrifuge (adjust the speed such that 20µl of reagent flows through the stage tip in around 5 min). Prior to this step you can determine the optimal centrifuge speed by using a conditioned stage tip and loaded with 20µl PB7.5.

(xlii) Repeat step B (xli) four times.

▲ **CRITICAL STEP**. Keep the dimethyl labeling buffer on stage tips for longer than 10 min is important, or there might be insufficient labeling.

(xliii) Wash with 100µl Stage Tip solution A.

(xliv) Elute peptides with 100µl Stage Tip solution B.

(xlv) Mix the three samples 1:1:1 in a new Eppendorf tube and evaporate ACN in Speedvac at 50°C until the volume is <60µl.

(xlvi) Add 100 ul LC/MS sample solution and perform LC/MS analysis.

! CAUTION: It is important to keep solutions and reagents on ice to prevent degradation. The isotopic reagents are light sensitive; use brown tubes to store the solutions.

■ **PAUSE POINT**: The prepared LC/MS samples can be kept at 4°C for several days.

(xlvii) **LC/MS analysis, Mascot search and MaxQuant processing** Extract peak file lists from the .raw data files of the ABPP quantification results using the DTA supercharge module from MSQuant and submitted to automated peptide identification by the Mascot search engine using a false discovery rate (FDR) of 1% and ion cutoff scores of >25. Alternatively, the MaxQuant software can be used for the relative quantification of the proteomics data. The competitive inhibition profiles of bortezomib were normalized compared to the negative control and presented in Figure 5b. For an elaborate protocol of the search engines details please see references of Boersema et al. and Cox et al.(*24*, *26*, *27*).

● **Timing**

Option A High resolution fluorescent ABPP (including Box I): 3 d

Option B Quantitative LC/MS based ABPP:

Step B (i) (including Box I), protein extraction: 2 d

Step B (ii-xxii), affinity purification: 1 d

Step B (xxiii-xxxiii), in gel digestion: 2 d

Step B (xxxiv-xlvi), on bead digestion and isotopic labeling: 2 d

Step B (xlvii), LC/MS analysis and data processing: 1 d

3.4 Anticipated results

Figure 3 shows the results for a fluorescence, gel-based ABPP quantification experiment where the β-subunit activities were assayed after treatment of constitutive and immuno proteasome expressing cell lines with bortezomib. Proteins were resolved on a 12.5% SDS-PAGE gel and the separation between the β2 and β2i subunits is sufficient to use the pan-reactive probe MVB003 (**3**) to determine their activities. When only constitutive proteasome is present MVB003 can also be used to quantify the closer running β1 and β5 bands. However, in cells expressing both constitutive and immuno proteasomes the resolution between the β1, β1i, β5, β5i is poor and MVB003 cannot be used for reliable quantification. In this case we use the subunit specific probes LW124 (**1**) for β1/β1i and MVB127 (**2**) for β5/β5i separation and quantification. The results show that adherent MCF7 cells are less sensitive to bortezomib compared to the myeloma cell line AMO1. Interestingly, bortezomib lowers also the β2/β2i activity in AMO1 cells, which is not observed in MCF7 cells. A possible explanation might be that the immuno proteasomes expressed by the AMO1 but not by MCF7 cells, are more active and are thus more efficiently blocked by bortezomib. This method is considered to be a fast, robust and medium throughput (2-8 assays/day) screen for the proteasome activity that can be easily implemented in any biochemical laboratory.

Figure 4 shows a study for the active site identification of the murine β2 subunit using ABPP and LC/MS analysis. The epoxyketone electrophilic trap reacts with the catalytic Thr1 residue yielding a stable morpholine ring. For a two step ABPP protocol, biotin-phosphine (**6**) is then ligated to N_3-epoxomicin (**5**) followed by affinity purification with Streptavidin beads, on-bead tryptic digest and elution of ABP-active-site constructs. Figure 4b and 4c show the one step and two-step constructs for the β2 subunit, respectively. Figure 4d and 4e show chromatograms of the active site constructs of all β subunits after one step and two step ABPP, respectively. Figure 4f shows the theoretical fragmentation of the two-step ABP-active site construct of the β2 subunit after collision induced dissociation (CID) of the peptide bonds in b and y ions. Figure 4g shows the experimental data of 4f. The N-terminus of the molecule is labile and yields a typical fragmentation pattern of b ions and the prominent appearance of the intact y9 ion containing the peptide sequence originating from the β2 protein. Subsequently, the y9 ion was isolated in the LTQ iontrap and subdued to a new round of fragmentation (MS^3) revealing the β2 peptide sequence. Analysis of active-site peptides is interesting from a toxicological perspective because covalent binding inhibitors might cause idiosyncratic drug related toxicity. Expression of an epitope of the active site peptide attached to a xenobiotic compound on MHC class I molecules might induce an adverse immunological response and lead to allergy against the compound which will exclude it from therapy. The method presented here can be used to determine epitope expression on MHC class I

molecules.

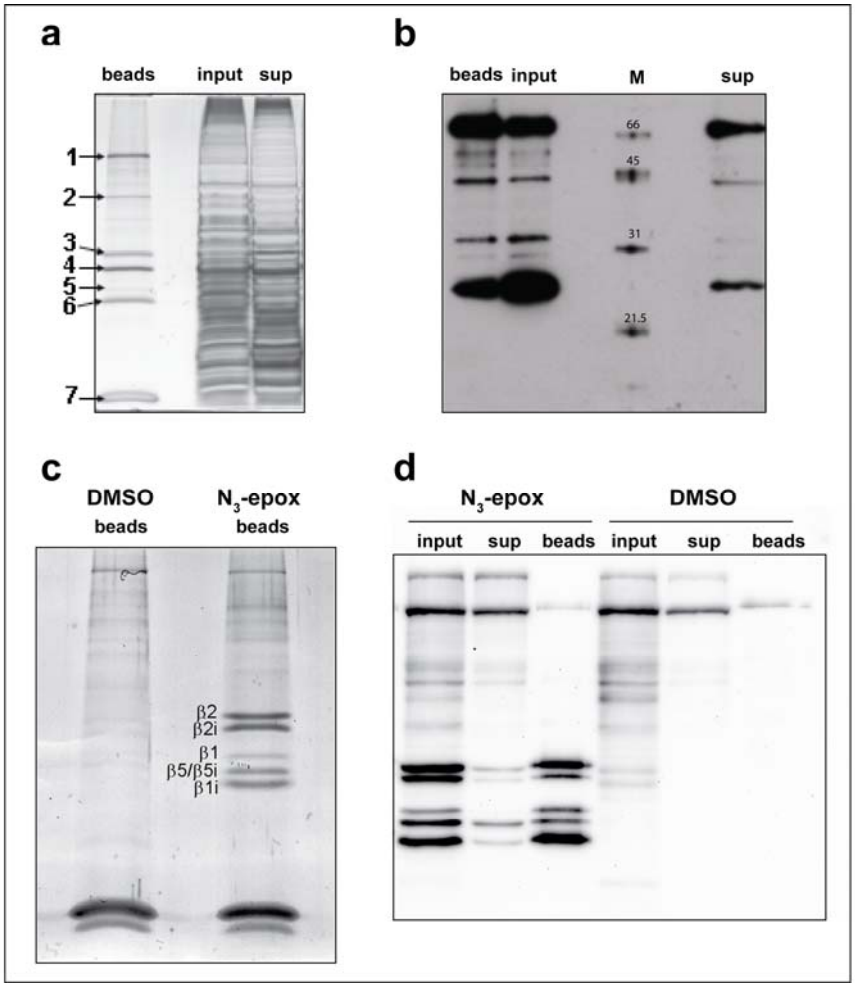

Figure 6: Direct and two-step activity-based affinity purification of the proteasome β-subunits with Streptavidin beads from EL4 lysate. SDS-PAGE/silver stain analysis after treatment with biotin-epoxomicin (**4**) showing proteins eluted from the beads, input and supernatant, bands indicated by arrows were identified by LC/MS (1: Propionyl-CoA carboxylase, 2: 60 S ribosomal protein, 3:β2, 4:β2i, 5:β1, 6:β5β5iβ1i, 7: Streptavidin) (**a**); western blot probed with Streptavidin HRP of the same samples from **a** (M, molecular marker) (**b**); SDS-PAGE/silver stain analysis after treatment with DMSO or N_3-epoxomicin (**5**) and ligation with biotin-phosphine (**6**) showing proteins eluted from the beads (**c**); western blot probed with Streptavidin HRP of the samples from **c** next to the input and supernatant samples (**d**).

The experiment of Figure 5 entails a competitive ABPP experiment between bortezomib and N_3-epoxomicin (**5**) in living EL4 cells that express both constitutive and immuno proteasomes. Cells were treated with 0, 10, 10^2, 10^3 and 10^4 nM bortezomib concentrations in parallel, the residual proteasome activity was captured by two-step ABPP using the N_3-epoxomicin/biotin-phosphine couple, proteins were affinity purified with

Streptavidin beads and analysed qualitatively by SDS-PAGE or subdued to on-bead digest with trypsin and the resulting peptides were captured on stage tips. The first group contains peptides from the 0, 10 and 10^2 nM bortezomib treatment that were labelled light, medium and heavy respectively, eluted from stage tips, mixed 1:1:1 and analysed by LC/MS. The second group contains peptides from the 0, 10^3 and 10^4 nM bortezomib treatment that were labelled light, medium and heavy respectively, eluted, mixed 1:1:1 and analysed by LC/MS. The 0 nM bortezomib condition was used as benchmark to combine the two groups resulting in a quantification report on the proteasome activity. The relative quantitative analysis shows that bortezomib first hits the β1i β5 and β5i subunits and then the β1 activity. At higher concentrations (>500 nM) also the β2 and β2i subunits are inhibited. This method is suitable for quantitative determination of proteasome activity in living cells and it is more accurate than the fluorescence gel-based method described previously because all active subunits are determined in the same LC/MS run.

Figure 6 is an illustration of the efficacy and robustness of the affinity purification protocol used for qualitative or quantitative determination of the proteasome activity. The results show typical pull-down efficiencies for one-step and two-step ABPP work flows followed by Streptavidin purification, SDS-PAGE separation of proteins and western blot analysis of biotinylated β-subunits visualised with Streptavidin-HRP. This method is routinely used to determine the pull-down efficiency.

References and notes

1. Rock, K. L., and Goldberg, A. L. (1999) Degradation of cell proteins and the generation of MHC class I-presented peptides, *Annu Rev Immunol 17*, 739-779.

2. Kloetzel, P. M., and Ossendorp, F. (2004) Proteasome and peptidase function in MHC-class-I-mediated antigen presentation, *Curr Opin Immunol 16*, 76-81.

3. Kane, R. C., Bross, P. F., Farrell, A. T., and Pazdur, R. (2003) Velcade: U.S. FDA approval for the treatment of multiple myeloma progressing on prior therapy, *Oncologist 8*, 508-513.

4. Jung, T., Catalgol, B., and Grune, T. (2009) The proteasomal system, *Mol Aspects Med 30*, 191-296.

5. Marques, A. J., Palanimurugan, R., Matias, A. C., Ramos, P. C., and Dohmen, R. J. (2009) Catalytic mechanism and assembly of the proteasome, *Chem Rev 109*, 1509-1536.

6. Borissenko, L., and Groll, M. (2007) 20S proteasome and its inhibitors: crystallographic knowledge for drug development, *Chem Rev 107*, 687-717.

7. Murata, S., Sasaki, K., Kishimoto, T., Niwa, S., Hayashi, H., Takahama, Y., and Tanaka, K. (2007) Regulation of CD8+ T cell development by thymus-specific proteasomes, *Science 316*, 1349-1353.

8. Verdoes, M., Florea, B. I., van der Marel, G. A., and Overkleeft, H. S. (2009) Chemical Tools To Study the Proteasome, *Eur J Org Chem*, 3301-3313.

9. Kisselev, A. F., and Goldberg, A. L. (2005) Monitoring activity and inhibition of 26S proteasomes with fluorogenic peptide substrates, *Methods Enzymol 398*, 364-378.

10. Li, N., Overkleeft, H. S., and Florea, B. I. (2012) Activity-based protein profiling: an enabling technology in chemical biology research, *Curr Opin Chem Biol 16*, 227-233.

11. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin, *Science 268*, 726-731.

12. Meng, L., Mohan, R., Kwok, B., Elofsson, M., Sin, N., and Crews, C. (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity, *Proceedings of the National Academy of Sciences of the United States of America 96*, 10403-10411.

13. Bogyo, M., McMaster, J. S., Gaczynska, M., Tortorella, D., Goldberg, A. L., and Ploegh, H. (1997) Covalent modification of the active site threonine of proteasomal beta subunits and the Escherichia coli homolog HslV by a new class of inhibitors, *Proceedings of the National Academy of Sciences of the United States of America 94*, 6629-6634.

14. Kessler, B. M., Tortorella, D., Altun, M., Kisselev, A. F., Fiebiger, E., Hekking, B. G., Ploegh, H. L., and Overkleeft, H. S. (2001) Extended peptide-based inhibitors efficiently target the proteasome and reveal overlapping specificities of the catalytic beta-subunits, *Chem Biol 8*, 913-929.

15. Berkers, C. R., Verdoes, M., Lichtman, E., Fiebiger, E., Kessler, B. M., Anderson, K. C., Ploegh, H. L., Ovaa, H., and Galardy, P. J. (2005) Activity probe for in vivo profiling of the specificity of proteasome inhibitor bortezomib, *Nat Methods 2*, 357-362.

16. Verdoes, M., Florea, B. I., Menendez-Benito, V., Maynard, C. J., Witte, M. D., van der Linden, W. A., van den Nieuwendijk, A. M., Hofmann, T., Berkers, C. R., van Leeuwen, F. W., Groothuis, T. A., Leeuwenburgh, M. A., Ovaa, H., Neefjes, J. J., Filippov, D. V., van der Marel, G. A., Dantuma, N. P., and Overkleeft, H. S. (2006) A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo, *Chem Biol 13*, 1217-1226.

17. Florea, B. I., Verdoes, M., Li, N., van der Linden, W. A., Geurink, P. P., van den Elst, H., Hofmann, T., de Ru, A., van Veelen, P. A., Tanaka, K., Sasaki, K., Murata, S., den Dulk, H., Brouwer, J., Ossendorp, F. A., Kisselev, A. F., and Overkleeft, H. S. (2010) Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit beta5t, *Chem Biol 17*, 795-801.

18. Verdoes, M., Willems, L. I., van der Linden, W. A., Duivenvoorden, B. A., van der Marel, G. A., Florea, B. I., Kisselev, A. F., and Overkleeft, H. S. (2010) A panel of subunit-selective activity-based proteasome probes, *Org Biomol Chem 8*, 2719-2727.

19. Kraus, M., Ruckrich, T., Reich, M., Gogel, J., Beck, A., Kammer, W., Berkers, C. R., Burg, D., Overkleeft, H., Ovaa, H., and Driessen, C. (2007) Activity patterns of proteasome subunits reflect bortezomib sensitivity of hematologic malignancies and are variable in primary human leukemia cells, *Leukemia 21*, 84-92.

20. Ruckrich, T., Kraus, M., Gogel, J., Beck, A., Ovaa, H., Verdoes, M., Overkleeft, H. S., Kalbacher, H., and Driessen, C. (2009) Characterization of the ubiquitin-proteasome system in bortezomib-adapted cells, *Leukemia 23*, 1098-1105.

21. Saxon, E., and Bertozzi, C. (2000) Cell surface engineering by a modified Staudinger reaction, *Science (New York, N.Y.) 287*, 2007-2017.

22. Ovaa, H., van Swieten, P. F., Kessler, B. M., Leeuwenburgh, M. A., Fiebiger, E., van den Nieuwendijk, A. M., Galardy, P. J., van der Marel, G. A., Ploegh, H. L., and Overkleeft, H. S. (2003) Chemistry in living cells: detection of active proteasomes by a two-step labeling strategy, *Angew Chem Int Ed Engl 42*, 3626-3629.

23. Willems, L. I., Li, N., Florea, B. I., Ruben, M., van der Marel, G. A., and Overkleeft, H. S. (2012) Triple bioorthogonal ligation strategy for simultaneous labeling of multiple enzymatic activities, *Angew Chem Int Ed Engl 51*, 4431-4434.

24. Boersema, P. J., Raijmakers, R., Lemeer, S., Mohammed, S., and Heck, A. J. (2009) Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics, *Nat Protoc 4*, 484-494.

25. Raijmakers, R., Dadvar, P., Pelletier, S., Gouw, J., Rumpel, K., and Heck, A. J. R. (2010) Target Profiling of a Small Library of Phosphodiesterase 5 (PDE5) Inhibitors using Chemical Proteomics, *Chemmedchem 5*, 1927-1936.

26. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification, *Nat Biotechnol 26*, 1367-1372.

27. Cox, J., Matic, I., Hilger, M., Nagaraj, N., Selbach, M., Olsen, J. V., and Mann, M. (2009) A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics, *Nat Protoc 4*, 698-705.

28. Kisselev, A. F., Songyang, Z., and Goldberg, A. L. (2000) Why does threonine, and not serine, function as the active site nucleophile in proteasomes?, *J Biol Chem 275*, 14831-14837.

29. Singh, J., Petter, R. C., Baillie, T. A., and Whitty, A. (2011) The resurgence of covalent drugs, *Nat Rev Drug Discov 10*, 307-317.

30. Muchamuel, T., Basler, M., Aujay, M. A., Suzuki, E., Kalim, K. W., Lauer, C., Sylvain, C., Ring, E. R., Shields, J., Jiang, J., Shwonek, P., Parlati, F., Demo, S. D., Bennett, M. K., Kirk, C. J., and Groettrup, M. (2009) A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis, *Nat Med 15*, 781-787.

31. Kallemeijn, W. W., Li, K. Y., Witte, M. D., Marques, A. R., Aten, J., Scheij, S., Jiang, J., Willems, L. I., Voorn-Brouwer, T. M., van Roomen, C. P., Ottenhoff, R., Boot, R. G., van den Elst, H., Walvoort, M. T., Florea, B. I., Codee, J. D., van der Marel, G. A., Aerts, J. M., and Overkleeft, H. S. (2012) Novel activity-based probes for broad-spectrum profiling of retaining beta-exoglucosidases in situ and in vivo, *Angew Chem Int Ed Engl 51*, 12529-12533.

32. Chang, J. T., Ciocca, M. L., Kinjyo, I., Palanivel, V. R., McClurkin, C. E., De Jong, C. S., Mooney, E. C., Kim, J. S., Steinel, N. C., Oliaro, J., Yin, C. C., Florea, B. I., Overkleeft, H. S.,

Berg, L. J., Russell, S. M., Koretzky, G. A., Jordan, M. S., and Reiner, S. L. (2011) Asymmetric Proteasome Segregation as a Mechanism for Unequal Partitioning of the Transcription Factor T-bet during T Lymphocyte Division, *Immunity 34*, 492-504.

33. Adibekian, A., Martin, B. R., Wang, C., Hsu, K. L., Bachovchin, D. A., Niessen, S., Hoover, H., and Cravatt, B. F. (2011) Click-generated triazole ureas as ultrapotent in vivo-active serine hydrolase inhibitors, *Nat Chem Biol 7*, 469-478.

34. Weerapana, E., Speers, A. E., and Cravatt, B. F. (2007) Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP) - a general method for mapping sites of probe modification in proteomes, *Nat Protoc 2*, 1414-1425.

35. Lin, G., Li, D., de Carvalho, L. P., Deng, H., Tao, H., Vogt, G., Wu, K., Schneider, J., Chidawanyika, T., Warren, J. D., Li, H., and Nathan, C. (2009) Inhibitors selective for mycobacterial versus human proteasomes, *Nature 461*, 621-626.

36. Gu, C., Kolodziejek, I., Misas-Villamil, J., Shindo, T., Colby, T., Verdoes, M., Richau, K. H., Schmidt, J., Overkleeft, H. S., and van der Hoorn, R. A. (2010) Proteasome activity profiling: a simple, robust and versatile method revealing subunit-selective inhibitors and cytoplasmic, defense-induced proteasome activities, *Plant J 62*, 160-170.

37. van der Linden, W. A., Li, N., Hoogendoorn, S., Ruben, M., Verdoes, M., Guo, J., Boons, G. J., van der Marel, G. A., Florea, B. I., and Overkleeft, H. S. (2012) Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: A comparative study, *Bioorgan Med Chem 20*, 662-666.

38. Wessel, D., and Flugge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids, *Anal Biochem 138*, 141-143.

39. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes, *Nat Protoc 1*, 2856-2860.

40. Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips, *Nat Protoc 2*, 1896-1906.

41. Olsen, J. V., de Godoy, L. M., Li, G., Macek, B., Mortensen, P., Pesch, R., Makarov, A., Lange, O., Horning, S., and Mann, M. (2005) Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap, *Mol Cell Proteomics 4*, 2010-2021.