Activity-based proteasome profiling

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Copper-catalyzed Huisgen's azide-alkyne cycloaddition and Staudinger-Bertozzi reaction in a two-step activity-based proteasome profiling experiment,

a comparative study

4.1 Introduction

Activity-based protein profiling (ABPP) is a chemically promoted method that allows researchers to label active enzymes selectively in a complex biological system, such as full cell protein extracts, living cells, or even animals(*1*). To perform an ABPP experiment, chemical tools, named activity-based probes (ABP), which react specifically with the active sites of the targeted enzymes are necessary. A typical ABP consists of a reactive group (warhead), a recognition site (linker), and a reporter group (tag). Normally, the warhead is an electrophilic trap, which reacts with the active site amino acid of the enzyme resulting in a covalent and irreversible bond. The linker is usually a chemical motif which is recognized by the targeted enzymes as their endogenous substrate. The tag can either be a fluorophore or a biotin group, to visualize the enzymes on gel or on immunoblots(*2*).

In some cases, the reporter group can influence the properties of the probe, such as the potency and selectivity of the probe or its cell permeability. To avoid these effects, a two-step ABPP strategy can be applied, which entails incorporation of a bioorthogonal ligation handle into the ABP (*3, 4*). By the use of this strategy, the tags are installed after labeling of the enzymes. There were several successful two-step ABPP examples published, since the pioneering reports using Staudinger-Bertozzi reaction and copper-catalyzed Huisgen's azide-alkyne cycloaddition (also known as click chemistry) for ABPP of proteasome and glutathione S-transferases, respectively(*5, 6*).

The azide group is commonly used for two step ABPP experiments. However, when a secondary azide is installed in a proteasome ABP, inefficient bioorthogonal ligation by biotin-phosphine **5** was observed in native cell lysate, possibly due to steric effects. To address this problem, comparison of click chemistry and Staudinger-Bertozzi reaction to install the biotin tag on the protein-probe complexes was performed, using the proteasome as the model enzyme on both primary and secondary azide groups, under both native and denatured lysate conditions.

4.2 Results and discussion

The chemical tools used in this study are shown in Figure 1. To compare the efficiency of bioorthogonal ligations on primary and secondary azides, comparative ABPP experiments were performed using proteasome probes equipped with both types of azide, respectively. ABP **1** has a primary azide, while **2** has a more sterically hindered secondary azide on the N terminus. A competitive ABPP experiment was performed in HEK293T lysate. Active proteasome subunits were first labeled with either ABP**1**, **2** or **3**, and the residual proteasome activity was labeled with fluorescent proteasome probe **4**(*7, 8*). The results are shown in Figure 2. It shows that, both **1** and **2** prefer β5 and β2 over β1. Both inhibitors completely block β5 and β2 at the concentration of about 100μM, however, β1 is still partially active. As expected, **3** shows great potency of inhibition. At 5 μM concentration of **3**, no residual activity could be labeled by **4**.

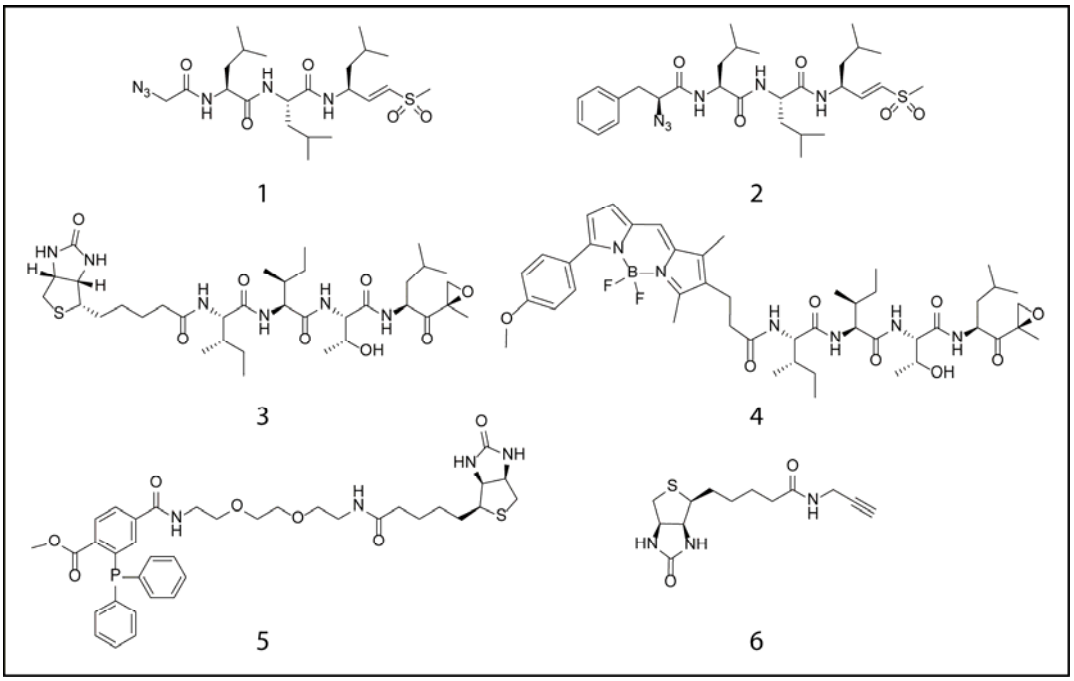

Figure 1: Structures of the chemical tools in this study.

According to these results, 100 μM of **1** and **2** were used to compare the bioorthogonal ligation efficiency. Alongside, 10 μM of **3** was chosen as a positive control to show the fully biotin labeling of the active proteasome subunits. However, the free **1** and **2** probes might label more active proteasome subunits during the time necessary for the bioorthogonal ligations. This extra labeling with **1** and **2** could change the results of the two-step ABPP experiments. To avoid that, all the residual active proteasome subunits were blocked by the potent fluorescent probe **4** after proteasome labeling by **1**, **2** and **3**, before the bioorthogonal ligations (Fig 3). This can also be seen as a control step showing the amount of active proteasome subunits that were labeled by **1** and **2**. While **4** shows the

same amount of competitive proteasome labeling against **1** and **2**, the difference shown in the anti-biotin western blot is due to the different efficiency of the bioorthogonal ligations. Following the control step, the biotin tag was installed via either click chemistry or Staudinger-Bertozzi reaction on the azide labeled proteins, with or without denaturing the proteins. Proteins were separated on SDS-PAGE, in-gel fluorescent imaging and anti-biotin chemiluminescent imaging were performed subsequently.

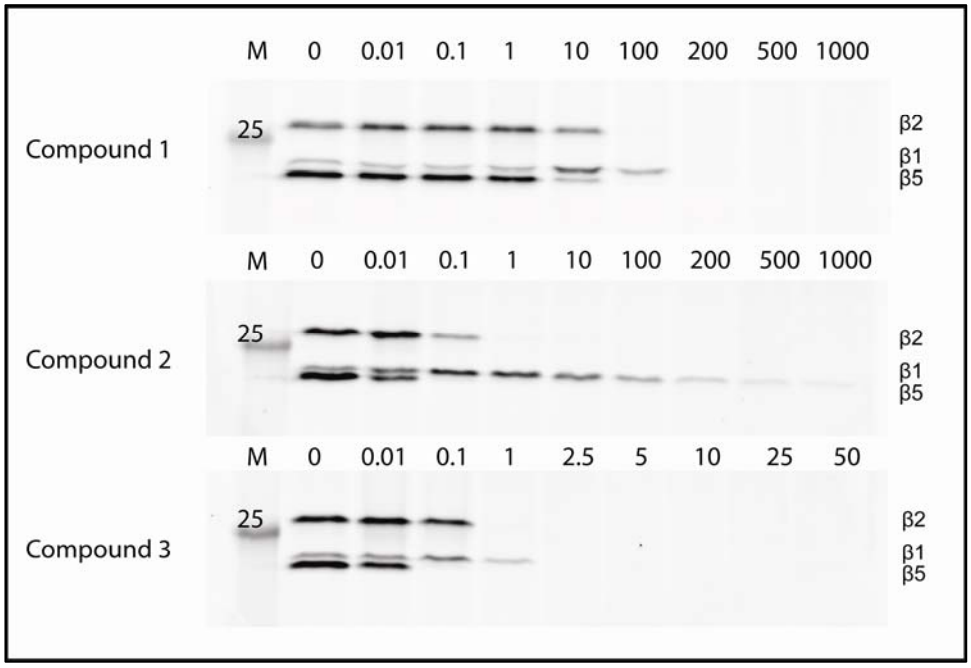

Figure 2: Determination of the inhibitor concentrations for the later experiments, by Competitive ABPP of proteasome inhibitors **1**, **2**, **3** and ABP **4**. HEK293T lysate was first treated with different concentrations of **1**, **2**, or **3**, and then labeled by **4**. Proteins were separated by SDS-PAGE and visualized by fluorescent scanning.

Fluorescent imaging in Figure 4 shows that both N_3-Leu_3-VS probes **2** (lanes B and E) and **1** (lanes C and F) blocked the proteasome activity almost completely displayed by disappearance of the bands of **4**. This indicates the difference shown on the anti-biotin blot is due to the different efficiency of bioorthogonal ligations. In the native condition labeling experiment (upper panel, Fig 4), both biotin-alkyne **6** (lane C) and biotin-phosphine **5** (lane F) could label the primary azide of **1**. With respect to the secondary azide **2**, neither **6** (lane B) nor **5** (lane E) showed very promising labeling. Click chemistry mediated ligation seemed to label the proteasome subunits more efficiently than Staudinger-Bertozzi reaction, comparing lanes B to E and C to F. However, **6** (lane D) showed more background labeling than **5** (lane G), where only bioorthogonal reagents but no azide equipped probes were added. Considering the heavier background labeling by **6**, it is more difficult to access the proteasome labeling, compared to labeling by **5**.

In the denatured condition labeling experiment (lower panel, Fig 4), both biotin-alkyne **6** (lanes B) and biotin-phosphine **5** (lanes E) labeled secondary azide efficiently, which is the most significant difference from the native condition experiment (upper panel, Fig 4). This is perhaps because under native conditions, the secondary azide group attached to the N terminus of the proteasome subunit is confined in the relatively small binding pockets and less accessible for the bioorthogonal ligation reagents. Denaturation of the proteins removed the folding of the proteasome subunits and made the azide groups more accessible. Another unexpected finding is that biotin-alkyne **6** labeled less background proteins under denatured condition (lanes B and C in lower panel) than native condition (lanes B and C in higher panel). A possible explanation is that active cysteins lost their reactivity to alkyne group after protein denaturation(9).

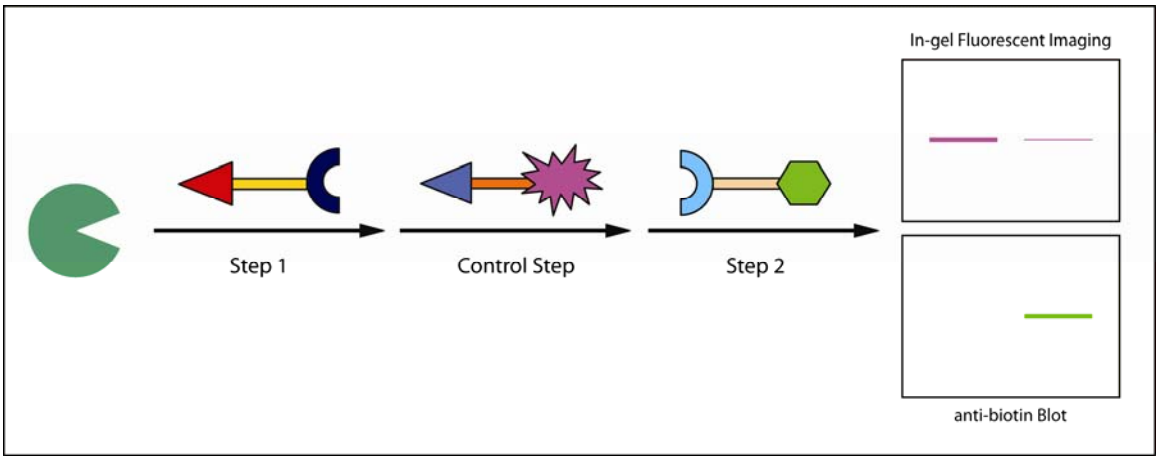

Figure 3: Scheme of the three-step ABPP experiment. Step 1, labeling of the proteasome by azide equipped inhibitors. Control step, labeling the remained proteasome activity by fluorescent probe. Step 2, bioorthorgonal ligations to install the biotin tag. Visualize the differently labeled proteasome by both in-gel fluorescent imaging and anti-biotin blot.

4.3 Conclusion

In conclusion, it might be an additional effect of the steric-effect of the secondary azide and the folded proteasome subunits surrounding the azide with their binding pockets, which will together prevent it from reacting with the bioorthogonal ligation reagents under native condition. This also explains why better labeling efficiency is achieved under denaturing conditions, in which all the proteins have been unfolded by boiling with SDS followed by a chloroform/methanol precipitation. Secondly, labeling of azide proteins seems to be more efficient using biotin-alkyne **6** than biotin-phosphine **5**, with the same concentration of ligation reagents and reaction time. However, it shows more background labeling by performing click chemistry, which might be due to the possible reactions between alkyne group and active cysteins(9). This has to be considered, when one wants to

combine the two-step ABPP with a pull down experiment for affinity purification of the target enzymes, because the background proteins covalently modified by biotin-alkyne **6** will not be washed away after binding to the streptavidin beads. In that case, the Staudinger-Bertozzi reaction, through which it labels less background protein, might be an advisable choice. Finally, bioorthogonal ligations on proteasome probes equipped with secondary azide can be efficiently processed under denatured conditions. For the ligation on primary azide, both conditions are suitable. It suggests that while making azide equipped proteasome probes, one should consider which type of azide is needed for the downstream visualization work.

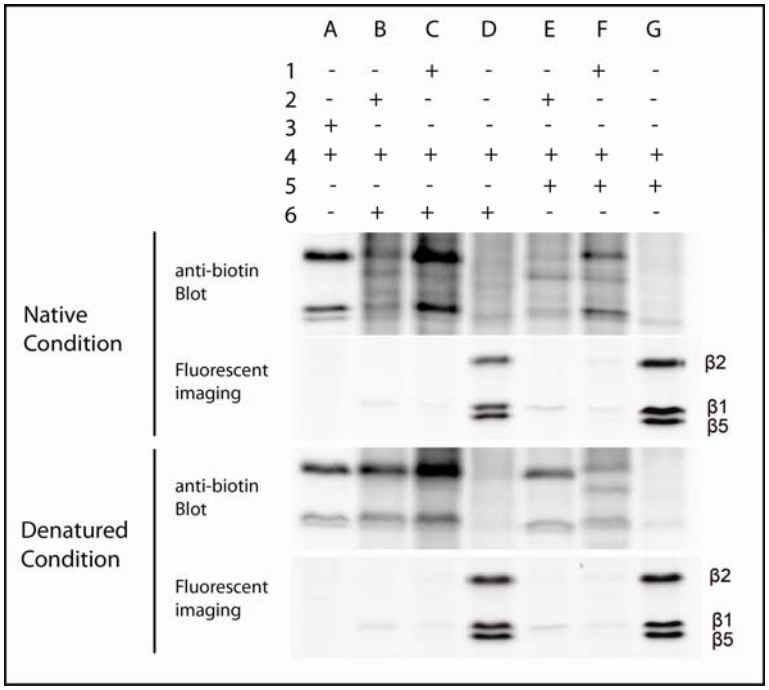

Figure 4: anti-biotin blots and in-gel fluorescent imaging to visualize the proteasome labeling patterns. The bioorthogonal ligations were performed on either native proteins (shown in upper panels) or denatured proteins (lower panels).

4.4 Experimental procedures

4.4.1 Synthesis of the chemical tools

Dichloromethane (DCM), N,N-dimethylformamide (DMF), methanol (MeOH), diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma) were used as received. Column chromatography was performed on Screening Devices b.v. Silica Gel, with a particle size of 40-63 μm and pore diameter of 60 Å. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F254). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in 10% sulfuric acid, a

solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 oC. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to CD_3OD as internal standard. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile 50/50 (v/v) and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at m/z 400 (mass range m/z = 150-2,000) and dioctylpthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). . $BocLeu_3VS$ and Tris-[1-(3-hydroxypropyl)-1H-[1,2,3]triazol-4-yl)methyl]amine (MDW999, ligand for CuAAC) were synthesized according to literature procedures(*10, 11*).

GB123 **1** and WLB426A **2** were synthesized from $BocLeu_3VS$. To synthesize **1**, $BocLeu_3VS$ (110 mg, 213 µmol) was dissolved in 1:1 TFA/DCM (2 mL). After stirring for 30 min., the reaction mixture was concentrated and co-evaporated with toluene (3x), providing the free amine as TFA salt, which was directly used in the next step. To a solution of the TFA salt in DMF was added DiPEA (44.57 µL, 255 µmol) followed by the addition of N_3Gly-OSu (51 mg, 255 µmol). After 3 hours, TLC showed completion of the reaction and the reaction mixture was diluted with EtOAc, washed with 1N HCl (1x), sat. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $NaSO_4$ and concentrated. The product was precipitated from DCM, filtered off and isolated as a white solid (49 mg, 46%). ^{1}H NMR (400 MHz, Methanol-d_4): δ ^{1}H NMR (400 MHz, Methanol-d4) δ 6.77 (dd, J = 15.1, 5.1 Hz, 1H), 6.47 (dd, J = 15.1, 1.6 Hz, 1H), 4.78 – 4.48 (m, 1H), 4.48 – 4.19 (m, 2H), 3.89 (s, 2H), 2.93 (s, 3H), 1.69 – 1.10 (m, 9H), 1.10 – 0.62 (m, 18H). ^{13}C NMR (101 MHz, MeOD) δ 173.07, 172.70, 168.63, 148.05, 129.36, 52.40, 52.37, 48.15, 42.77, 42.65, 41.09, 40.51, 25.10, 25.03, 25.00, 22.96, 21.87, 21.81. LC-MS: Rt (min): 8.08 (linear gradien 10-90% in 15 min, m/z 501.13 [M+H+]. HRMS: calcd. for $C_{22}H_{40}N_6O_5S$ 501.38537 $[M+H]^+$; found 501.28531.

For synthesis of **2**, $BocLeu_3VS$ (46 mg, 89 µmol) was dissolved in 4N HCl in dioxane (1 mL). After stirring for 40 min., the reaction mixture was concentrated and co-evaporated with toluene (3x), providing the free amine as HCl salt, which was directly used in the next step. To a solution of the HCl salt in DMF was added N_3PheOH (288 µL of a 0.34 M sol. in DMF, 98 µL), HBTU (41 mg, 107 µmol) and DiPEA (51 µL, 312 µmol). After 2 hours, TLC showed completion of the reaction. The reaction mixture was diluted with DCM and washed with 1N HCl (2x), sat. $NaHCO_3$ (4x). The organic layer was dried over $NaSO_4$ and concentrated. Purification by column chromatography (0.5—1% MeOH in DCM) provided the product as a white solid.(47 mg, 89%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.35 – 7.21 (m, 5H), 6.80 (dd, J = 15.2, 5.2 Hz, 1H), 6.60 (dd, J = 15.2, 1.3 Hz, 1H), 4.67 (dt, J = 9.2, 5.0 Hz, 1H),

4.44 – 4.34 (m, 2H), 4.16 (dd, J = 8.5, 4.9 Hz, 1H), 3.20 (dd, J = 14.0, 4.9 Hz, 1H), 3.04 – 2.93 (m, 4H), 1.73 – 1.40 (m, 9H), 1.03 – 0.87 (m, 18H). ^{13}C NMR (101 MHz, MeOD) δ 174.26, 174.18, 171.69, 148.47, 137.78, 130.77, 130.41, 130.29, 129.59, 128.04, 65.42, 53.39, 53.34, 49.14, 43.28, 42.80, 41.80, 41.57, 38.62, 25.93, 25.78, 23.43, 23.39, 23.34, 22.12, 22.09, 21.91. HRMS: calcd. for $C_{22}H_{40}N_6O_5S$ 591.33232 $[M+H]^+$; found 591.33246.

Compounds **3, 4, 5** and **6** were synthesized through literature procedures(*5, 7, 12*).

4.4.2 Competitive activity-based proteasome profiling

HEK293T cells (derived from ATCC) were grown in DMEM supplemented with 10% fetal calf serum and 10mg/ml penicillin and streptomycin in a humid CO_2 (5%) incubator at 37°C. At 90% confluence, the cells were harvested by scrapping in pre-chilled PBS. The cell pellet was lysed with a mild lysis buffer containing Tris pH 7.5 (50 mM), sucrose (250 mM), $MgCl_2$ (5 mM), dithiothreitol (DTT; 1 mM), ATP (2mM), digitonin (0.025%)(*13*).

The protein concentration was determined by Qubit protein assay (Invitrogen). 20µg of lysate was first incubated with different concentration of **1, 2** or **3** at 37°C for 1 hour, and then labeled with **4** for 1 hour at 37°C. Subsequently, the samples were boiled at 95°C for 5min after addition of 3x sample buffer (6% SDS, 3% β-mercaptomethanol, 30% v/v Glycerol, 0.1% Bromophenol Blue, 150mM Tris pH 6.8).

The boiled samples were separated on a 12.5% SDS-PAGE, and then imaged with a ChemiDoc MP system (BioRad). The gels were further stained by coomassie blue, and imaged and quantified as loading controls.

4.4.3 Two step activity-based proteasome profiling

100µg of HEK293T lysate was incubated with **1, 2** or **3**, at 37°C for 1 hour. Afterwards, fluorescent probe **4** was added in label the remained proteasome activity. The mixture was again incubated at 37°C for 1 hour. For click chemistry under native condition, the mixture was first diluted by lysis buffer to 50µl, then the same volume of click cocktail (10mM CuSO4, 10mM MDW999, 10mM Sodium Ascorbate and 0.8mM **6** in 50mM Tris pH 8.0) was added. For Staudinger-Bortozzi reaction under native condition, the mixture volume after labeling with **4** was taken up by lysis buffer to 100µl while adding 0.4mM **5**.

For performing click chemistry and Staudinger-Bortozzi reactions under denatured condition, the lysate after **4** labeling was denatured by boiling with 1% SDS at 95°C for 5 min followed by a chloroform/methanol precipitation (C/M (*13*)). The pellet was dissolved by 8M Urea in 50mM Tris pH8.0, and then the protein solutions can be processed as described above in the native reaction protocols.

All of the reaction mixtures were incubated at 37°C for 1 hour. C/M precipitation was performed to quench the reaction and remove the excess biotin. All the samples were dissolved in 3x sample buffer and boiled at 95°C for 5 min. The boiled samples were

separated on SDS-PAGE and transferred to western blot. The labeled proteasome subunits were detected by either fluorescent scanning or chemiluminascent scanning after probing the blot with Streptavidin-HRP.

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