Chemical tools to monitor and control human proteasome activities

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CHAPTER 6

Development of β1i and β5i selective activity-based probes

Introduction

The ubiquitin proteasome system is responsible for the degradation of the majority of proteins inside living cells and is an important target in both cancer and immune diseases.[1] Ubiquitinated proteins are recognized by the 19S regulatory particles of the proteasome, were the ubiquitin chain is removed and the protein is unfolded and translocated to the 20s core particle (CP). The 20S CP is a cylindrical protein complex, consisting out of two outer α-rings and two inner β-rings in which the catalytic activity resides. Proteins are cleaved into 3-12 amino acid peptides, which can be further degraded by aminopeptidases or presented to the immune system on the outside of the cell by MHC-I complexes. The 20S CP exist in two major forms, namely the constitutive proteasome (cCP) and the immunoproteasome (iCP). In the cCP the catalytic active subunits are β1c (caspase-like), β2c (trysin-like) and β5c (chymotrypsin like). In immune cells and in tissues exposed to inflammatory cytokines (for instance interferon-γ) the iCP is expressed in which β1c, β2c and β5c are (partly) replaced by β1i (chymotrypsin-like), β2i (trypsin-like) and β5i (chymotrypsin-like). The changed substrate specificities of the iCP results in the generation of peptides with higher affinity for MHC-I complexes and as such the iCP plays a major role in the immune system.[2] Proteasome inhibitors have been widely used to study the proteolytic system and proteasome inhibition was found to induce cytotoxicity in certain cancers, leading to the development of bortezomib[3] (Velcade, approved for the treatment of multiple myeloma (MM) and mantle cell lymphoma) and carfilzomib[4] (Kyprolis, FDA approved for the treatment of MM). Bortezomib and carfilzomib target both cCPs and iCPs, however, many haematological malignancies predominantly express iCPs. The last years, much research has been directed to the development of selective immunoproteasome inhibitors and selective β5i inhibition has already been shown suppress autoimmunity in various preclinical mouse models.[5] In addition,

it was found that selective inhibition of β5i and β1i is cytotoxic to acute lymphocytic leukemia (ALL) cells.[6]

Fluorescently labelled activity-based probes (ABPs) have been widely used to monitor proteasome activities *in vitro* and *in vivo* and are used for inhibitor screening and functional studies (see chapter 2). Most proteasome ABPs are either broad spectrum (for instance BODIPY-epoxomicin[7] and MV-151[8]) or are in-class selective, targeting subunit pairs, such as Cy5-NC-001[6], BODIPY(FL)-LU-112[9], BODIPY(TMR)-NC-005-VS[7] (see chapter 3). However, given the fact that the iCP is gaining attention and that the iCP subunits are considered to be important drug targets, it would be desirable to have access to iCP subunit selective ABPs. Such probes could facilitate research aiming to unravel the role and functions of iCP subunits. Based on the first generation of β5i (PR-924) and β1i (UK-101) inhibitors (see chapter 5), Kim and co-workers developed ABPs selective for either β5i (LKS01-B650)[10] or β1i (UK101-B660 and UK101-Fluor)[11] (Figure 1). However, given the selectivity windows of PR-924 and especially UK101, which is only 10x selective for β1i over β5c/β5i, the selectivity of these probes is questionable. As described in the previous chapter, a new generation of β5i and β1i inhibitors with improved selectivity profiles has been developed.[12] In this chapter, these optimized iCP subunit selective inhibitors are taken as starting point for the development of fluorescent ABPs that are selective for either β5i or β1i.

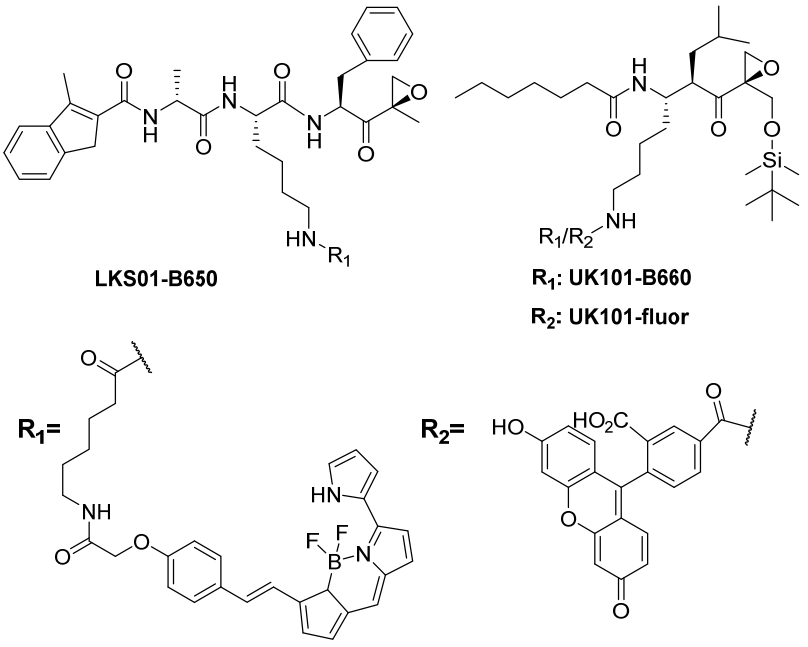


Figure 1. Structures of activity-based probes selective for β5i (LKS01-B650) or β1i (UK101-B660/Fluor).

Results and discussion

β5i selective probes

Substitution of the P1 Phe residue of PR-924 for cyclohexylalanine (Cha) in LU-015i (Figure 2) led to a 4-fold increase in β5i selectivity, making this compound the most selective β5i inhibitor known to date (see chapter 5). Therefore, LU-015i was taken as a lead compound for the development of a β5i selective probe. Many proteasome ABPs bear a fluorescent group at the N-terminus, however, LU-015i does not have a N-terminal group that allows straightforward functionalization with a fluorophore. Therefore, ABP **1** and **2** (Figure 2) were designed in which the 3-methylindene N-cap of LU-015i is replaced by azido-Phe to which BODIPY(FL)-alkyne[13] or Cy5-alkyne can be attached via a copper(I)-catalysed azide-alkyne cycloaddition (CuAAC, or 'click') reaction.

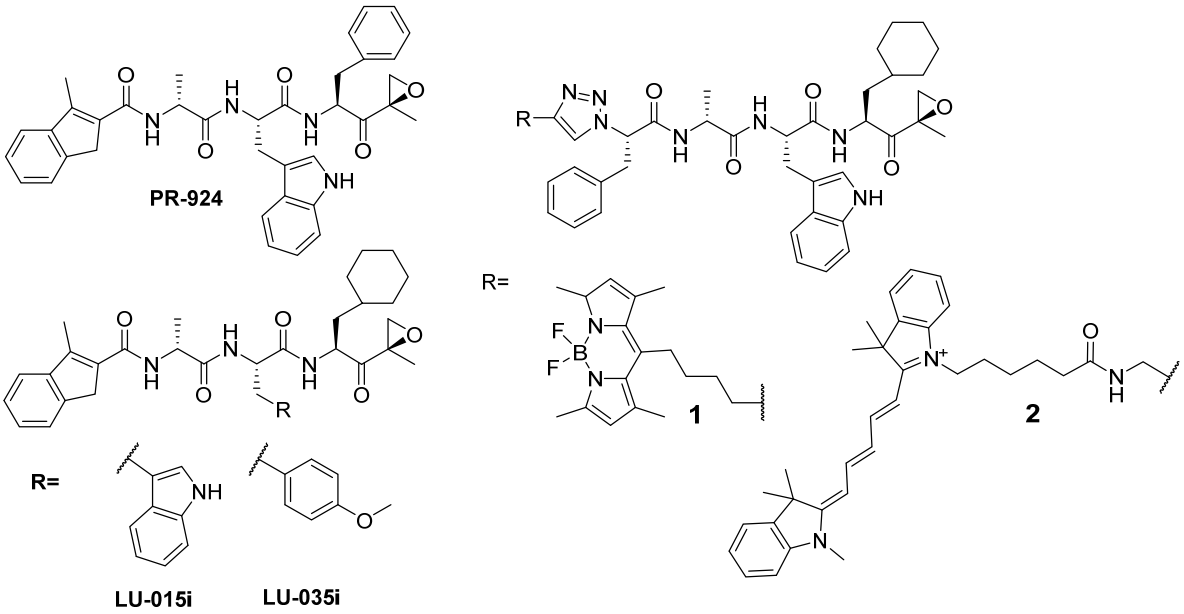
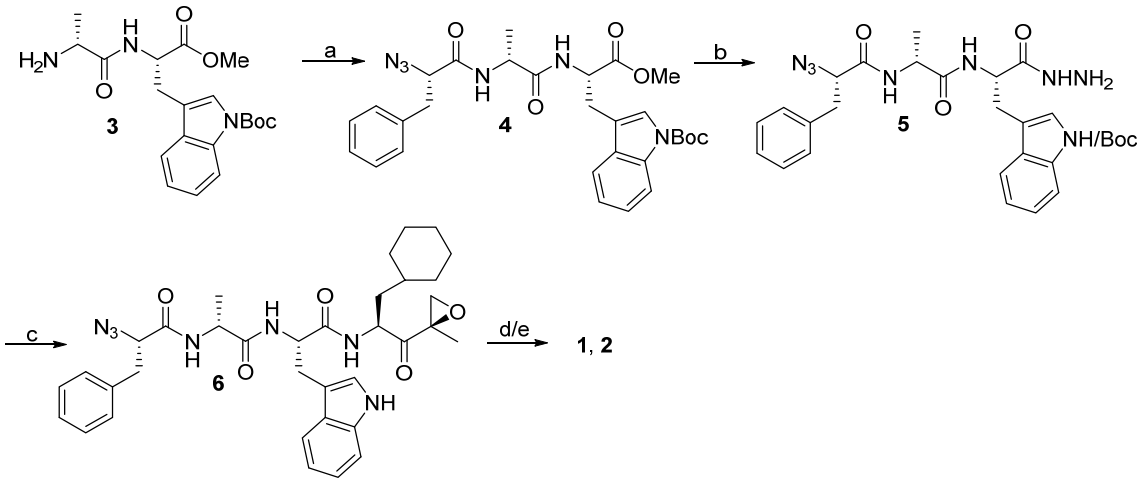


Figure 2. Chemical structures of PR-924, LU-015i, LU-035i and ABPs 1 and 2.

The synthesis of ABPs **1** and **2** commenced with a peptide coupling between dipeptide **3** (chapter 5) and azido-Phe giving tripeptide **4**, which was converted to hydrazide **5** by hydrazinolysis of the methyl ester (Scheme 1). Azide coupling with deprotected Cha epoxyketone provided compound **5**, which was reacted to either BODIPY(FL)-alkyne or Cy5-alkyne yielding ABPs **1** and **2**. Both probes were evaluated for β5i selectivity in Raji cell lysate. Because most proteasome probes do not resolve β5c and β5i on SDS-PAGE, the samples were treated with BODIPY(TMR)-NC-005[7] (chemical structure: see chapter 3) after incubation with ABP **1** or **2** to verify the amount of inhibition for β5c and β5i (Figure 3). Although both probes show some selectivity for β5i, complete labelling of β5i without concomitant modification of β5c could not be achieved.



Scheme 1. Synthesis of ABP 1. Reagents and conditions: a. N_3Phe-OH, HCTU, DiPEA, DCM, 81%; b. $NH_2NH_2 \cdot H_2O$, MeOH, 100%; c. 1. tBuONO, HCl, DMF. 2. H-Cha-EK, DiPEA, 37%; d. BODIPY(FL)-alkyne, $CuSO_4$, NaAsc, DMF/H_2O, 100%; e. Cy5-alkyne, $CuSO_4$, NaAsc, DMF/H_2O, 20%.

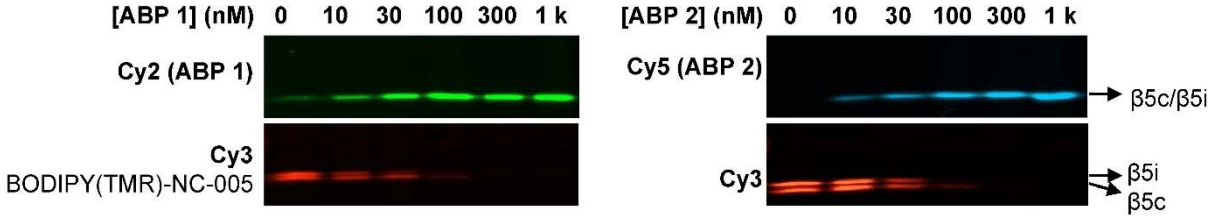
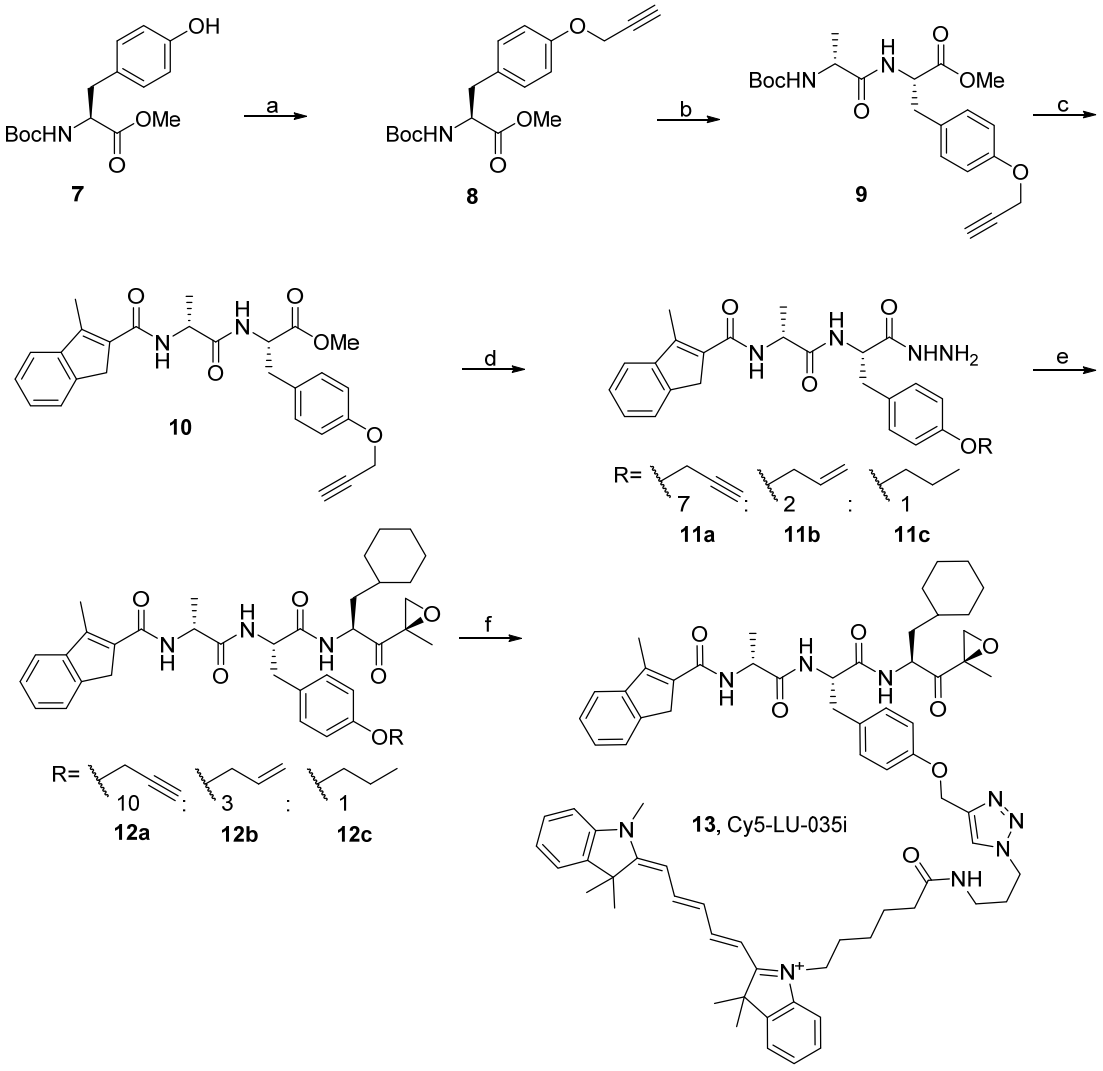


Figure 3. Evaluation of ABPs **1** and **2** in Raji lysate. Lysates were treated with indication concentrations of probe **1** or **2** for 1 h, followed by the addition of 100 nM BODIPY(TMR)-NC-005 (end concentration) for 1 h.

Clearly, the nature of the N-cap of β5i inhibitors is highly important to maintain selectivity (see also chapter 5). Although ABPs **1** and **2** are not selective for β5i over β5c, both probes do not target β1c/β1i and β2c/β2i and can therefore be used as β5c/β5i selective probes, complementary to BODIPY(TMR)-NC-005.

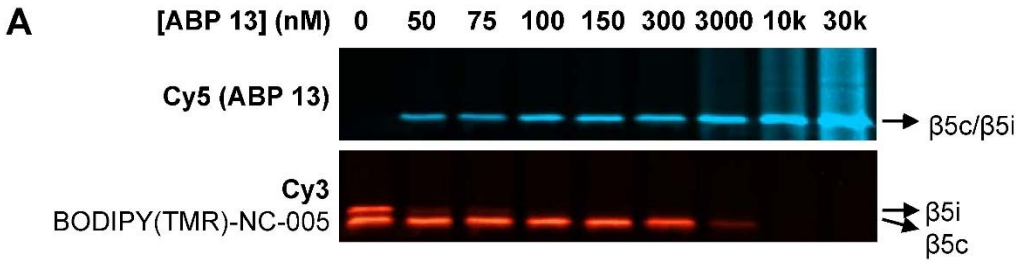
Since attachment of a fluorophore at the P4 position proved to be detrimental for β5i selectivity, a new probe was designed with a fluorophore at the P2 position, similarly to ABP LKS01-B650 (Figure 1). The S2 pockets of all proteasome active β-subunits are solvent exposed and therefore fit large residues.[10, 11, 14] Due to a mutation of Gly48 in β5c to Cys48 in β5i, the S2 pocket of β5i is more shallow than β5c.[14] Residues that can interact with Cys48 are favoured and required for β5i selectivity[15, 16], consequently, all currently known β5i selective inhibitors have aromatic residues at P2 (either O-methyl-Tyr or Trp[12, 17]). Therefore, LU-035i (Figure 2) was chosen as starting point, as it was envisioned that an alkyne functionality could be easily introduced at the 4-OH position of Tyr. This strategy resulted in the design of ABP **13** (Cy5-LU-035i, Scheme 2). The synthesis commenced with the alkylation of Boc-Tyr-OMe **7** with propargyl bromide to provide alkyne **8**. Boc removal followed by a peptide coupling with Boc-

D-Ala-OH provided dipeptide **9**. Boc removal and peptide coupling with 3-methylindene-2-carboxylic acid provided compound **10**, which was converted to hydrazide **11**. During hydrazinolysis, partial reduction of the alkyne functionality resulted in a 7:2:1 mixture of products **11a**:**11b**:**11c**. The synthesis was continued with the mixture and standard azide coupling of hydrazide **11a**/**b**/**c** with deprotected Cha epoxyketone yielded compounds **12a**/**b**/**c**. Since only alkyne **12a** would react in a CuAAC reaction, it was decided to perform the 'click' reaction with the mixture of compounds. For future studies a β5i probe with near infrared properties was desired, therefore Cy5-azide was chosen as fluorophore (see chapter 10) and was reacted to **12a**/**b**/**c** affording pure ABP **13** after HPLC purification.



Scheme 2. Synthesis of ABP 13. Reagents and conditions: a. K_2CO_3, propargylbromide, DMF, 72%; b. 1. TFA; 2. Boc-D-Ala-OH, HCTU, DiPEA, DCM, 100%; c. 1. TFA; 2. 3-methylindene-2-carboxylic acid, HCTU, DiPEA, DCM, 93%; d. $NH_2NH_2 \cdot H_2O$, MeOH, 100%; e. 1. tBuONO, HCl, DMF. 2. H-Cha-EK, DiPEA, 50%; f. Cy5-azide, $CuSO_4$, NaAsc, DMF/H_2O, 28%.

As before, ABP **13** was tested in Raji lysates for β5i selectivity (Figure 4). In contrast to ABPs **1** and **2**, ABP **13** shows both good selectivity for β5i over β5c. At 100 nM, complete labelling of β5i is found and β5c remains untouched up to 300 nM. In addition, at concentrations up to 300 nM no labelling of the β1 or β2 subunits is visible. However, at higher concentrations at which β5i and β5c both are completely labelled by ABP **13** the band in the Cy5 channel shows higher intensities at increasing concentration of **13**, indicating partial labelling of the β1 subunits (compare 10000 and 30000 nM). The selectivity of ABP **13** becomes also apparent form the kinetic constants (Figure 4B). ABP **13** has a ten-fold higher K_{inact} for β5i compared to β5c, meaning that the covalent bond formation of **13** with β5i is 10x faster than with β5c. In addition, the K_i (concentration of inhibitor at 0.5 K_{inact}) of **13** is also 3 times lower for β5i compared to β5c, indicating a higher affinity for β5i. The selectivity of a covalent inhibitor for its target can best be determined by comparing the second order rate constants (K_{inact}/K_i) for the different targets.[18] The K_{inact}/K_i value of ABP **13** is >30x higher for β5i compared to β5c, confirming the good selectivity for β5i.



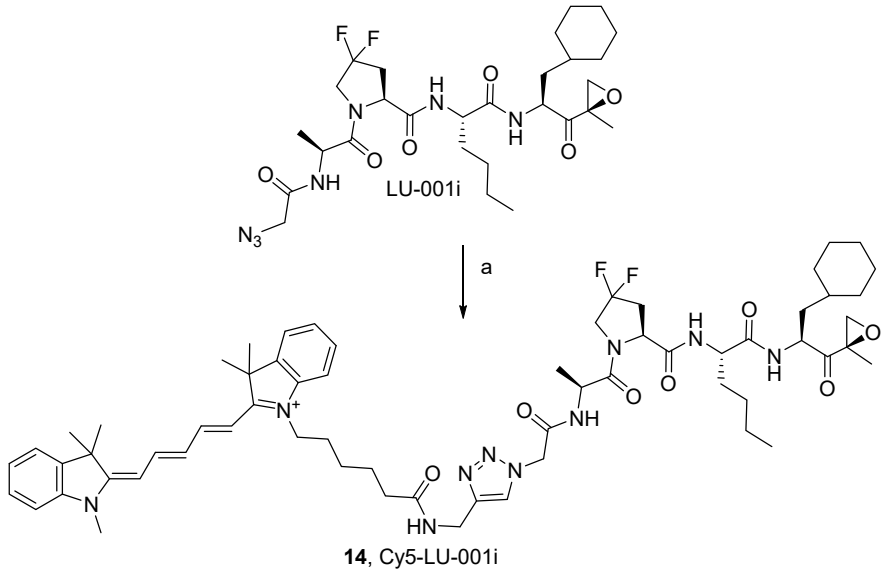
B

ABP 13	β5c	β5i
$K_{(inact)}$ (s^{-1})	$0.0010 \pm 3.2 \times 10^{-5}$	$0.0117 \pm 5.0 \times 10^{-4}$
K_i (nM)	8699 ± 705	3006 ± 316
$K_{(inact)}/K_i$ ($M^{-1}s^{-1}$)	120	3.9×10^3

Figure 4. Evaluation of ABP 13 in Raji lysate. A) Lysates were treated with indicate concentrations of ABP **13** for 1 h, followed by the addition of 100 nM BODIPY(TMR)-NC-005 (end concentration) for 1 h. B) Kinetic constants of ABP **13** as determined in Raji lysates. Log (% activity) for various concentrations is plotted versus time, from which the first order rate constants (K_{obs}) are derived. K_{obs} were plotted versus probe concentration, yielding K_{inact}= max k_{obs} and K_i= [probe] at 0.5 K_{inact}.. K_{inact}/K_i=second order rate constant.

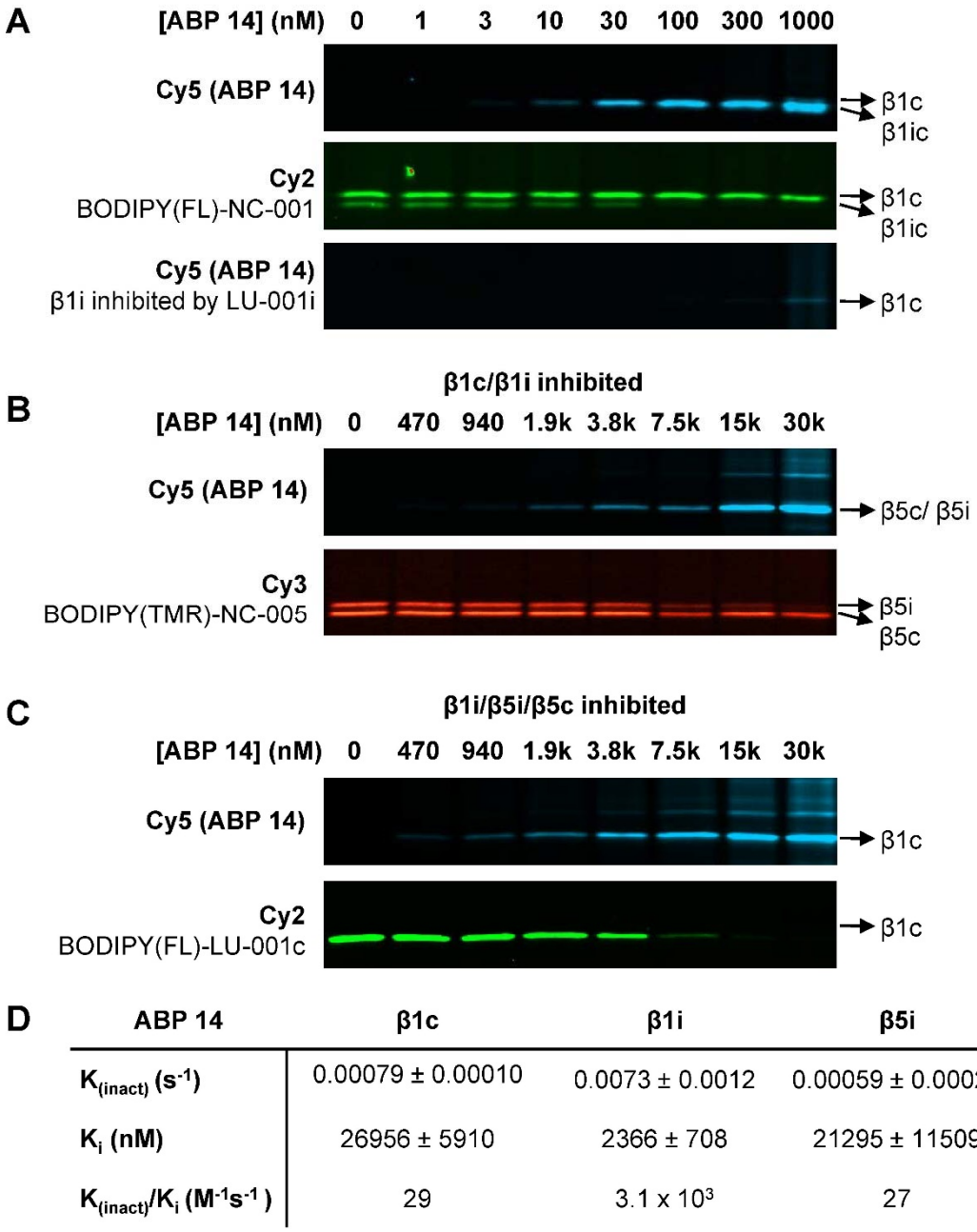
β1i selective probe

As described in chapter 5, the substitution of P1 Leu for Cha and P3 Pro for 4,4-F_2-Pro in NC-001[19] resulted in the highly selective β1i inhibitor LU-001i.[12] Since LU-001i is already equipped with a N-terminal azido-Gly residue, it could straightforwardly be converted to a fluorescent ABP by CuAAC with Cy5 alkyne, providing ABP **14** (Cy5-LU-001i, Scheme 3). Again, for future studies also a β1i probe with near infrared properties was desired.



Scheme 3. Synthesis of ABP 14. Reagents and conditions: a. Cy5-alkyne, $CuSO_4$, NaAsc, DMF/H_2O, 75%.

The potency and selectivity of ABP **14** was also evaluated in Raji lysates. After incubation of lysates with increasing concentrations of ABP **14** for 1 h, the residual activity of β1c and β1i was labelled by β1 selective probe BODIPY(FL)-NC-001 (Figure 5A).[7] At 100 nM β1i is inhibited completely, while very minor labelling of β1c is visible at 1 µM. The inhibition of β5c and β5i by ABP **14** was also examined in more detail (Figure 5B). Because of overlapping bands for the β1 and β5 subunits on SDS-PAGE, β1c and β1i were completely blocked by β1 selective inhibitor NC-001[19], before incubation with ABP **14.** Residual activity of β5c and β5i was labelled by β5 selective probe BODIPY(TMR)-NC-005. At concentrations of >2 µM β5i is significantly inhibited, while β5c is barely inhibited even at concentration up to 30 µM. In addition, inhibition of β1c by ABP **14** was investigated in more detail (Figure 5C). In order to do so, β1i and β5c/ β5i were inhibited by LU-001i and NC-005, prior to incubation with ABP **14**. Residual activity of β1c was labelled by β1c selective ABP BODIPY(FL)-LU-001c (see chapter 8). Labelling of β1c by ABP **14** is already visible at 470 nM, however, complete labelling is only visible at 15 µM. The kinetic constants also prove the high β1i selectivity of ABP **14** (Figure 5D). Compared to β1c and β5i, the K_{inact} is about 10 x larger and the K_i about 10x smaller for β1i. This results in a 100x larger second order rate constant (K_{inact}/K_i) for β1i compared to β1c and β5i, indicating that ABP **14** has excellent selectivity for β1i.



D

ABP 14	β1c	β1i	β5i
$K_{(inact)}$ (s^{-1})	0.00079 ± 0.00010	0.0073 ± 0.0012	0.00059 ± 0.00021
K_i (nM)	26956 ± 5910	2366 ± 708	21295 ± 11509
$K_{(inact)}/K_i$ ($M^{-1}s^{-1}$)	29	3.1×10^3	27

Figure 5. Evaluation of ABP 14 in Raji lysate. A) Lysates were treated with indication concentrations of ABP **13** for 1 h, followed by the addition of BODIPY(FL)-NC-001 (100 nM end concentration) for 1 h. B) Raji lysates were treated with NC-001 (5 μM) for 1 h to inhibit β1c and β1i, followed by incubation with ABP **14** at indicated concentrations for 1h. Subsequently, the samples were treated with BODIPY(TMR)-NC-005 (100 nM end concentration) to label residual β5c or β5i activity. C) Raji lysates were treated with LU-001i (1 μM) and NC-005 (2.5 μM) for 1 h to inhibit β1i, β5c and β5i, followed by incubation with ABP **14** at indicated concentrations for 1h. Subsequently, the samples were treated with BODIPY(FL)-LU-001c (100 nM end concentration) to label residual β1c activity. D) Kinetic constants of ABP **14** as determined in Raji lysates. Log (% activity) for various concentrations is plotted versus time, from which the first order rate constants (K_{obs}) are derived. K_{obs} were plotted versus probe concentration, yielding K_{inact}= max k_{obs} and K_I= [probe] at 0.5 K_{inact}.. K_{inact}/K_i=second order rate constant.

Conclusion

Based on inhibitors selective for β5i or β1i, activity-based probes selective for either β5i (ABP **13**) or β1i (ABP **14**) were developed. Straightforward N-terminal attachment of a Cy5 fluorophore to β1i inhibitor LU-001i provided a potent and selective probe for β1i. In contrast, it proved to be impossible to obtain a selective β5i probe by N-terminal attachment of a Cy5 fluorophore to a β5i selective inhibitor. However, when the fluorophore was positioned at the P2 position a potent and selective β5i probe was obtained. In cell lysate both probes have a good selectivity window in which only β5i (ABP **13**) or β1i (ABP **14**). The probes have not been verified in intact cells, however, the positive charge of the Cy5 dye could cause poor membrane permeability. Therefore, when a selective β5i or β1i probe is desired it might be necessary to replace Cy5 by an uncharged fluorophore, such as a BODIPY dye. Although both probes were not directly compared to the probes published Kim *et al.* (LKS01-B650 and UK-101-B660/Fluor, Figure 1) it is expected that, based on the selectivity of the parent compounds, these new probes are more selective for their respective targets.

Experimental

Synthetic procedures

General procedures

Acetonitrile (ACN), 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, Iris Biotech) 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% sulphuric 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 °C. ^1H and ^{13}C NMR spectra were recorded on a Bruker AV-300 (MHz), AV-400 (400 MHz), AV-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, CD_3OD or $CDCl_3$ 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 dioctylphthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C_{18} 50 × 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The applied buffers were H_2O, ACN and 1.0% aq. TFA. Method: xx→xx% MeCN, 13.0 min (0→0.5 min: 10% MeCN; 0.5→8.5 min: gradient time; 8.5→10.5 min: 90% MeCN; 10.5→13.0 min: 10% MeCN), 15 min (0→0.5 min: 10% MeCN; 0.5→10.5 min: gradient time; 10.5→12.5 min: 90% MeCN; 12.5→15 min: 90% → 10% MeCN). HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5µm 250×10 mm column and a GX281 fraction collector. H-D-Ala-H-Trp(Boc)-OMe, Boc-Cha-EK and LU-001i were synthesized as described in chapter 5.

N_3Phe-D-Ala-Trp-OMe (4)

H-D-Ala-H-Trp(Boc)-OMe **3** (149 mg, 0.38 mmol, 1 equiv.) was dissolved in DCM. HCTU (198 mg, 0.48 mmol, 1.3 equiv.), N_3Phe-OH (92 mg, 0.48 mmol, 1.3 equiv.) and DiPEA (0.24 mL, 1.4 mmol, 3.7 equiv.) were added and the mixture was stirred overnight before being concentrated. The residue was dissolved in EtOAc and washed with 1M HCl (2x), sat aq $NaHCO_3$ (2x), brine and dried over Na_2SO_4, filtered and concentrated. Column chromatography (20→40% EtOAc:pent) yielded the title compound (175 mg, 0.31 mmol, 82%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, *J* = 6.3 Hz, 1H), 7.49 (d, *J* = 7.7 Hz, 1H), 7.42 (s, 1H), 7.34 – 7.14 (m, 7H), 7.10 (d, *J* = 7.7 Hz, 1H), 6.99 (d, *J* = 7.5 Hz, 1H), 4.89 (q, *J* = 6.1 Hz, 1H), 4.54 (p, *J* = 7.0 Hz, 1H), 4.01 (dd, *J* = 9.1, 4.6 Hz, 1H), 3.67 (s, 3H), 3.34 – 3.11 (m, 3H), 2.86 (dd, *J* = 13.9, 9.2 Hz, 1H), 1.64 (s, 9H), 1.27 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.73, 171.43, 168.68, 149.53, 136.30, 130.34, 129.35, 128.72, 127.21, 124.64, 124.28, 122.60, 118.77, 115.34, 114.82, 83.78, 65.27, 53.54, 52.59, 52.45, 48.71, 38.74, 28.19, 27.36, 18.55. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): R_t (min): 10.45 (ESI-MS (m/z): 562.87 ($M+H^+$)).

N_3Phe-D-Ala-Trp(Boc)-$NHNH_2$ (5)

Methyl ester **4** (175 mg, 0.31 mmol, 1 equiv.) was dissolved in MeOH (4 mL). Hydrazine hydrate (425 µl, 9.0 mmol, 30 equiv.) was added and the mixture was stirred for 3 h before being co-evaporated with tol (3x). The residue was used without further purification (isolated as mixture of compounds with or without Boc). ^1H NMR (400 MHz,

MeOD) δ 7.55 (dd, *J* = 7.6, 4.2 Hz, 1H), 7.35 – 7.14 (m, 7H), 7.06 (dt, *J* = 27.1, 7.6 Hz, 2H), 4.69 – 4.59 (m, 1H), 4.22 (p, *J* = 7.0 Hz, 1H), 3.95 (ddd, *J* = 10.3, 8.5, 6.1 Hz, 1H), 3.26 (dt, *J* = 14.6, 5.2 Hz, 1H), 3.13 (dq, *J* = 14.6, 8.1, 7.5 Hz, 2H), 3.04 – 2.82 (m, 1H), 1.43 (s, 5H (partially –Boc), 1.05 (dd, *J* = 10.5, 7.1 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 173.16, 172.16, 170.54, 136.98, 136.64, 129.69, 129.06, 127.79, 127.57, 125.02, 124.41, 123.89, 123.12, 122.05, 119.43, 119.27, 118.57, 116.35, 115.61, 111.88, 109.77, 64.65, 53.35, 52.43, 49.65, 38.65, 28.51, 28.34, 28.21, 27.75, 17.78, 17.64. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): R_t (min): 6.24 (ESI-MS (m/z): 462.93 ($M+H^+$ -Boc) and 7.83 (ESI-MS (m/z): 562.93 ($M+H^+$ +Boc).

N_3Phe-D-Ala-Trp-Cha-EK (6)

Hydrazide **5** (28 mg, 0.05 mmol, 1 equiv.) was dissolved in DMF (1 mL) and cooled to -30 °C. *t*BuONO (8 µL, 1.1 equiv.) and HCl (35 µL, 4M solution in 1,4-dioxane, 2.8 equiv.)) were added, and the mixture was stirred for 3h at -30 °C after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. TFA·H-Cha-EK (1.1 equiv.) was added to the reaction mixture as a solution in DMF (1 mL). DiPEA (5 equiv.) was added to the reaction mixture, and this mixture was allowed to warm to RT slowly overnight. The mixture was diluted with EtOAc and extracted with H_2O (3×). Purification by column chromatography (1→2% MeOH in DCM) provided the title compound (12.01 mg, 37.4%) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 1H), 7.72 (d, *J* = 7.8 Hz, 1H), 7.41 – 7.05 (m, 9H), 6.86 (d, *J* = 7.0 Hz, 1H), 6.71 (d, *J* = 7.4 Hz, 1H), 6.14 (d, *J* = 7.7 Hz, 1H), 4.69 (td, *J* = 7.7, 5.8 Hz, 1H), 4.53 – 4.43 (m, 1H), 4.33 (p, *J* = 7.0 Hz, 1H), 4.04 (dd, *J* = 9.0, 4.6 Hz, 1H), 3.31 (dd, *J* = 14.1, 4.9 Hz, 2H), 3.19 (d, *J* = 5.0 Hz, 1H), 3.11 (dd, *J* = 14.5, 7.9 Hz, 1H), 2.97 – 2.85 (m, 1H), 2.81 (d, *J* = 5.0 Hz, 1H), 1.78 – 1.38 (m, 10H), 1.25 (d, *J* = 6.9 Hz, 3H), 1.20 – 0.97 (m, 4H), 0.94 – 0.68 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 208.24, 171.42, 170.92, 168.99, 136.40, 136.37, 129.49, 128.89, 128.07, 127.44, 127.40, 123.73, 122.48, 120.47, 120.00, 118.97, 111.43, 110.48, 65.44, 59.13, 53.83, 52.57, 50.00, 49.35, 38.89, 38.56, 34.30, 33.99, 32.01, 28.25, 26.42, 26.22, 26.00, 18.25, 16.85. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): R_t (min): 10.16 (ESI-MS (m/z): 642.20 ($M+H^+$)). HRMS: calculated for $C_{35}H_{43}N_7O_5$ 642.33984 $[M+H]^+$; found 642.33984

BODIPY(FL)-Phe-D-Ala-Trp-EK (1)

To a degassed solution of azide **6** (4.3 mg, 6.7 µmol, 1 equiv.) and BODIPY(Fl)-alkyne (3.3 mg, 10.1 mmol, 1.5 equiv.) in DMF (0.8 mL) under an argon atmosphere was added $CuSO_4 \cdot 5H_2O$ (0.5 equiv, 3.4 µmol (100 µL from degassed stock solution of 34 µmol/mL)) and NaAsc (0.75 equiv, 5.0 µmol (100 µL from degassed stock solution of 50 µmol/mL)). After stirring overnight, the reaction solution was diluted with EtOAc and washed with H_2O (1x) and brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated. Purification by column chromatograpy (0→3% MeOH:DCM) provided the title compound in a quantitative yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.20 (s, 1H), 7.66 (d, *J* = 7.9 Hz, 1H), 7.46 (s, 1H), 7.35 (d, *J* = 8.1 Hz, 1H), 7.18 (dd, *J* = 6.1, 2.4 Hz, 4H), 7.13 – 7.06 (m, 2H), 7.03 (d, *J* = 3.3 Hz, 2H), 6.89 (s, 1H), 6.52 (d, *J* = 7.2 Hz, 1H), 6.21 – 6.12 (m, 1H), 6.03 (s, 2H), 5.27 (s, 1H), 4.65 (q, *J* = 7.4 Hz, 1H), 4.48 (dd, *J* = 12.3, 5.3 Hz, 1H), 4.27 – 4.17 (m, 1H), 3.49 (dd, *J* = 13.4, 7.4 Hz, 1H), 3.32 (ddd, *J* = 19.4, 14.1, 6.8 Hz, 2H), 3.19 (d, *J* = 4.9 Hz, 1H), 3.07 (dd, *J* = 14.6, 7.7 Hz, 1H), 2.99 – 2.91 (m, 2H), 2.81 (d, *J* = 5.0 Hz, 1H), 2.74 (t, *J* = 7.3 Hz, 2H), 2.51 (s, 6H), 2.37 (s, 6H), 1.92 – 1.83 (m, 2H), 1.75 – 1.51 (m, 10H), 1.45 (m, 4H), 1.11 (m, 5H), 0.86 – 0.77 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 208.54, 171.00, 167.33, 154.06, 146.07, 140.45, 136.34, 135.18, 131.54, 129.05, 128.95, 127.64, 127.47, 125.67, 123.62, 122.50, 121.81, 119.98, 118.88, 111.49, 110.28, 67.25, 65.93, 59.14, 53.72, 52.57, 49.94, 39.59, 38.52, 34.29, 33.99, 32.01, 31.45, 30.48, 29.63, 28.23, 26.42, 26.20, 26.00, 16.85, 16.59, 14.60. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): R_t (min): 11.45 (ESI-MS (m/z): 970.20 ($M+H^+$)). HRMS: calculated for $C_{54}H_{86}BF_2N_9O_5$ 970.53298 $[M+H]^+$; found 970.53345

Cy5-Phe-D-Ala-Trp-EK (2)

To a degassed solution of azide **6** (1.82 mg, 2.8 µmol) and Cy5-alkyne (3.2 mg, 5.6 µmol, 2 equiv.) in DMF (1 mL) under an argon atmosphere was added $CuSO_4 \cdot 5H_2O$ (0.5 equiv, 1.4 µmol (100 µL from degassed stock solution of

14 µmol/mL)) and NaAsc (0.75 equiv, 2.1 µmol (100 µL from degassed stock solution of 21 µmol/mL)). After stirring overnight, the reaction mixture immediately purified by HPLC (C_{18}, 50-80% MeCN, 0.1% TFA, 10 min gradient) provided the product as a blue powder after lyophilisation (0.66 mg, 0.56 µmol, 20%). ^{1}H NMR (850 MHz, MeOD) δ 8.22 (td, *J* = 13.1, 5.5 Hz, 2H), 8.04 (s, 1H), 7.53 (d, *J* = 7.9 Hz, 1H), 7.48 (d, *J* = 7.0 Hz, 2H), 7.44 – 7.35 (m, 2H), 7.30 (d, *J* = 8.1 Hz, 1H), 7.28 – 7.18 (m, 7H), 7.16 (d, *J* = 7.0 Hz, 2H), 7.06 – 7.02 (m, 1H), 7.00 (s, 1H), 6.98 – 6.94 (m, 1H), 6.58 (t, *J* = 12.4 Hz, 1H), 6.27 (d, *J* = 13.6 Hz, 1H), 6.22 (d, *J* = 13.7 Hz, 1H), 5.51 (t, *J* = 8.0 Hz, 1H), 4.60 (dd, *J* = 9.3, 4.9 Hz, 1H), 4.50 (dd, *J* = 10.5, 3.1 Hz, 1H), 4.47 (d, *J* = 15.2 Hz, 1H), 4.39 (d, *J* = 15.3 Hz, 1H), 4.10 (q, *J* = 7.1 Hz, 1H), 4.05 (t, *J* = 8.0 Hz, 2H), 3.57 (s, 3H), 3.42 (dd, *J* = 13.6, 8.3 Hz, 1H), 3.40 – 3.38 (m, 1H), 3.36 – 3.33 (m, 1H), 3.23 – 3.22 (m, 1H), 3.22 (d, *J* = 5.2 Hz, 1H), 2.98 (dd, *J* = 14.9, 9.2 Hz, 1H), 2.85 (d, *J* = 5.1 Hz, 1H), 2.25 – 2.19 (m, 2H), 1.71 (d, *J* = 2.5 Hz, 6H), 1.71 – 1.70 (m, 6H), , 1.40 (s, 3H), 0.96 (d, *J* = 7.1 Hz, 3H), 1.84 – 0.81 (m, 19H). ^{13}C NMR (214 MHz, MeOD) δ 209.76, 175.59, 174.28, 173.82, 169.21, 155.39, 146.01, 144.25, 143.51, 142.64, 142.50, 138.02, 136.81, 130.25, 129.79, 129.71, 129.63, 128.70, 128.29, 126.65, 126.25, 126.11, 124.49, 123.84, 123.38, 123.25, 122.42, 119.79, 119.35, 112.30, 112.09, 111.80, 111.03, 104.36, 65.80, 60.14, 57.48, 55.00, 53.13, 51.27, 51.15, 50.55, 50.47, 49.48, 49.38, 44.78, 39.93, 38.48, 36.55, 35.71, 35.51, 35.16, 32.94, 31.49, 30.90, 28.41, 28.12, 27.95, 27.82, 27.55, 27.32, 27.06, 26.36, 17.11, 17.08. HRMS: calculated for $C_{70}H_{85}N_{10}O_6$ 1161.66481 $[M]^+$; found 1161.66479 LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 13.0 min):R_t (min): 8.48 (ESI-MS (m/z): 1161.73 (M^+)).

Boc-Tyr(OCH$_2$CCH)-OMe (8)

To a solution of Boc-Tyr-OMe **7** (380 mg, 1.28 mmol, 1 equiv.) in DMF (10 mL) were added K_2CO_3 (353 mg, 2.56 mmol, 2 equiv.) and propargylbromide (193 µL, 2.56 mmol, 2 equiv.). After stirring overnight at rt, the reaction mixture was diluted with EtOAc and washed with 1N HCl (2x) and brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated. Purification by column chromatography (10→20% EtOAc/pent) provided the title compound (309 mg, 0.93 mmol, 72%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.04 (d, *J* = 8.4 Hz, 2H), 6.88 (d, *J* = 8.6 Hz, 2H), 4.99 (d, *J* = 7.9 Hz, 1H), 4.64 (d, *J* = 2.3 Hz, 2H), 4.52 (q, *J* = 6.0 Hz, 1H), 3.69 (s, 3H), 3.12 – 2.84 (m, 2H), 2.51 (t, *J* = 2.3 Hz, 1H), 1.40 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.43, 156.68, 130.36, 129.03, 114.99, 79.93, 78.63, 75.62, 55.83, 54.54, 52.26, 37.50, 28.35.

Boc-D-Ala-Tyr(OCH$_2$CCH)-OMe (9)

Boc-Tyr(OCH$_2$CCH)-OMe **8** (309 mg, 0.93 mmol) was treated with 1:1 TFA/DCM for 30 min, followed by co-evaporation with toluene (2x). The resulting product TFA·H-Tyr(OCH$_2$CCH)-OMe was dissolved in DCM and Boc-D-Ala-OH (211 mg, 0.11 mmol, 1.2 equiv.), HCTU (461 mg, 0.11 mmol, 1.2 equiv.) and DiPEA (566 µl, 3.26 mmol, 3.5 equiv.) were added. After stirring overnight, the reaction mixture was concentrated and redissolved in EtOAc and washed with 1N HCl (2x), sat. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $NaSO_4$, filtered, concentrated providing the title compound in a quantitative yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.02 (d, *J* = 8.7 Hz, 2H), 6.85 (d, *J* = 8.7 Hz, 2H), 6.81 (d, *J* = 7.4 Hz, 1H), 5.16 (d, *J* = 7.5 Hz, 1H), 4.85 – 4.73 (m, 1H), 4.62 (d, *J* = 2.4 Hz, 2H), 4.23 – 4.10 (m, 1H), 3.67 (s, 3H), 3.10 – 2.93 (m, 2H), 2.50 (t, *J* = 2.4 Hz, 1H), 1.40 (s, 9H), 1.25 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.47, 171.97, 156.68, 155.49, 130.32, 128.80, 114.98, 80.04, 78.55, 75.64, 55.79, 53.23, 52.38, 49.96, 37.04, 28.35, 18.54.

3MeIndAc-D-Ala-Tyr(OCH$_2$CCH)-OMe (10)

Boc-D-Ala-Tyr(OCH$_2$CCH)-Me **9** (430 mg, 0.93 mmol) was treated with 1:1 TFA/DCM for 30 min, followed by co-evaporation with toluene (2x). The resulting product TFA·D-Ala-Tyr(OCH$_2$CCH)-OMe was dissolved in DCM and 3-Methylindene-2-carboxylic acid (194 mg, 0.11 mmol, 1.2 equiv.), HCTU (461 mg, 0.11 mmol, 1.2 equiv.) and DiPEA (566 µl, 3.26 mmol, 3.5 equiv.) were added. After stirring overnight, the reaction mixture was concentrated and redissolved in EtOAc and washed with 1N HCl (2x), sat. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $NaSO_4$, filtered and concentrated. Purification by column chromatography (20→75% EtOAc/pent) provided the title compound (396 mg, 0.86 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.39 (m, 2H), 7.32

(dtd, *J* = 15.9, 7.3, 1.2 Hz, 2H), 7.19 (d, *J* = 8.1 Hz, 1H), 7.07 (d, *J* = 8.6 Hz, 2H), 6.83 (d, *J* = 8.7 Hz, 2H), 6.63 (d, *J* = 7.3 Hz, 1H), 4.90 – 4.78 (m, 1H), 4.72 (p, *J* = 7.0 Hz, 1H), 4.53 (d, *J* = 2.4 Hz, 2H), 3.67 (s, 3H), 3.61 – 3.53 (m, 2H), 3.20 – 2.94 (m, 2H), 2.48 (t, *J* = 2.1 Hz, 4H), 1.37 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.44, 171.92, 165.73, 156.64, 147.62, 145.50, 142.17, 131.78, 130.36, 128.86, 127.29, 126.77, 123.85, 120.80, 114.91, 78.52, 75.61, 55.68, 53.32, 52.42, 48.69, 38.30, 36.98, 18.91, 12.32.

3MeIndAc-D-Ala-Tyr(OCH_2CCH)-$NHNH_2$ (11)

To a solution of 3MeIndAc-D-Ala-Tyr(OCH_2CCH)-OMe **10** (396 mg, 0.86 mmol) in MeOH (10 mL) was added hydrazine-hydrate (2.5 mL, 50 mmol, 60 equiv.). After stirring for 3 hours, the reaction mixture was concentrated and co-evaporated with toluene (3x) providing the product in a quantitative yield. Partial reduction of the alkyne functionality resulted in a 7:2:1 mixture of triple:double:single bond, as determined by HPLC/MS analysis (See Figure 6). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) (peaks reported for desired product). δ 7.51 – 7.44 (m, 2H), 7.39 – 7.30 (m, 2H), 7.13 (d, *J* = 8.6 Hz, 2H), 6.83 (d, *J* = 8.6 Hz, 2H), 4.64 – 4.58 (m, 1H), 4.54 (d, *J* = 2.4 Hz, 2H), 4.48 – 4.39 (m, 1H), 3.65 – 3.56 (m, 2H), 3.16 (dt, *J* = 13.4, 6.7 Hz, 1H), 2.88 (dd, *J* = 14.0, 9.0 Hz, 1H), 2.68 (t, *J* = 2.4 Hz, 1H), 2.50 (t, *J* = 2.1 Hz, 3H), 1.26 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) (all peaks reported) δ 174.37, 171.79, 167.57, 157.13, 149.07, 145.85, 142.98, 133.78, 131.83, 130.64, 130.01, 129.21, 127.92, 127.25, 124.29, 121.30, 117.64, 115.35, 115.17, 114.94, 100.61, 78.95, 76.12, 69.15, 56.10, 53.74, 38.49, 37.31, 17.93, 12.46.

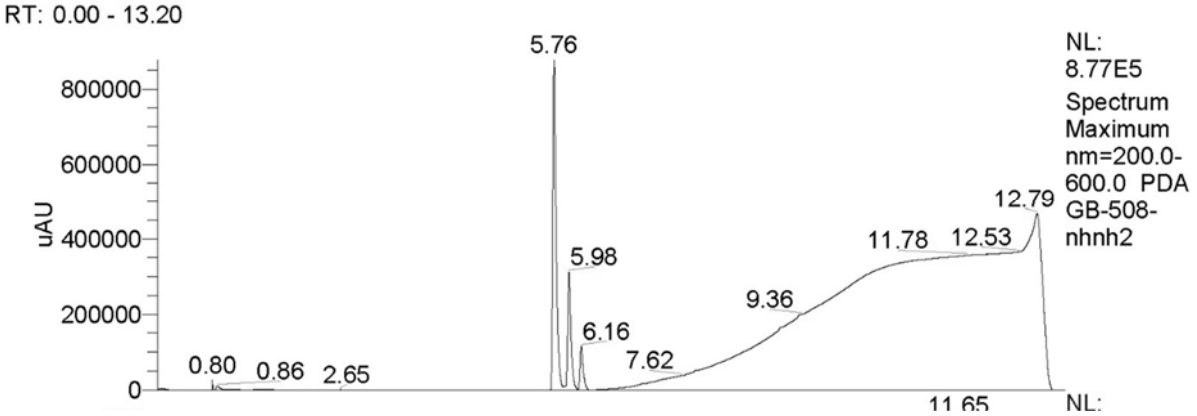


Figure 6. HPLC trace of compound 11. Retention time (min)/(m/z): 5.76/461.07 (alkyne); 5.98/463.07 (alkene); 6.16/465.00 (alkane).

3MeIndAc-D-Ala-Tyr(OCH_2CCH)-Cha-EK (12)

Hydrazide **12** (47 mg, 0.1 mmol, 1 equiv.) was dissolved in DMF (2 mL) and cooled to -30 °C. *t*BuONO (16 µL, 1.1 equiv.) and HCl (70 µL, 4M solution in 1,4-dioxane, 2.8 equiv.)) were added, and the mixture was stirred for 3h at -30 °C after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. TFA·H-Cha-EK was added to the reaction mixture as a solution in DMF (1 mL). DiPEA (5 equiv.) was added to the reaction mixture, and this mixture was allowed to warm to RT slowly overnight. The mixture was diluted with EtOAc and extracted with H_2O (3×). The organic layer was dried over $MgSO_4$, concentrated and purified by column chromatography (0-2% MeOH in DCM) providing the product as 7:2:1 mixture of triple:double:single bond (32 mg, 0.05 mmol, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) (peaks reported for desired product) δ 7.50 – 7.42 (m, 2H), 7.40 – 7.28 (m, 2H), 7.14 (d, *J* = 8.7 Hz, 2H), 6.95 (t, *J* = 8.7 Hz, 1H), 6.85 (d, *J* = 8.7 Hz, 2H), 6.53 – 6.46 (m, 2H), 4.71 – 4.47 (m, 5H), 3.58 (s, 2H), 3.23 (d, *J* = 5.0 Hz, 1H), 3.04 (d, *J* = 6.8 Hz, 2H), 2.83 (d, *J* = 5.0 Hz, 1H), 2.51 (t, *J* = 2.2 Hz, 3H), 2.48 (t, *J* = 2.4 Hz, 1H), 1.92 (s, 1H), 1.80 – 1.44 (m, 5H), 1.43 (s, 3H), 1.36 (d, *J* = 7.0 Hz, 3H), 1.28 – 1.05 (m, 5H), 0.83 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) (all peaks reported) δ 208.31, 172.56, 170.83, 166.09, 156.76, 148.36, 145.57, 142.23, 133.34, 131.48, 130.57, 130.53, 129.41, 128.53, 127.49, 126.91, 123.92, 120.95, 117.71, 115.09, 114.93, 114.70, 76.84, 75.61, 68.81, 59.15, 55.85, 54.35, 52.47, 49.79, 49.10, 38.56, 38.34, 37.02, 34.38, 33.98, 32.02, 26.40, 26.24, 25.98, 18.53, 16.81, 12.50.

Cy5-LU-035i (13)

To a degassed solution of **12** (mix alkyne, alkene and alkane) (10 mg, 16 µmol) and Cy5-alkyne (13 mg, 24 µmol, 1.5 equiv.) in DMF (1 mL) under an argon atmosphere was added $CuSO_4 \cdot 5H_2O$ (0.5 equiv, 8 µmol (100 µL from degassed stock solution of 8 µmol/mL)) and NaAsc (0.75 equiv, 12 µmol (100 µL from degassed stock solution of 120 µmol/mL)). After stirring overnight, the reaction mixture immediately purified by HPLC (C_{18}, 55-65% MeCN, 0.1% TFA, 10 min gradient) provided the product as a blue powder after lyophilisation (5.91 mg, 4.5 µmol, 28%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 (s, 1H), 7.86 (s, 1H), 7.76 (td, *J* = 13.0, 8.2 Hz, 2H), 7.54 (d, *J* = 7.9 Hz, 1H), 7.45 (d, *J* = 7.5 Hz, 2H), 7.38 (t, *J* = 7.7 Hz, 2H), 7.34 (d, *J* = 7.6 Hz, 3H), 7.32 – 7.28 (m, 2H), 7.26 – 7.21 (m, 2H), 7.18 (d, *J* = 6.7 Hz, 1H), 7.12 (d, *J* = 8.0 Hz, 1H), 7.07 (d, *J* = 8.3 Hz, 3H), 7.02 – 6.97 (m, 1H), 6.74 (d, *J* = 8.5 Hz, 2H), 6.62 (t, *J* = 12.5 Hz, 1H), 6.28 (d, *J* = 13.7 Hz, 1H), 6.17 (d, *J* = 13.5 Hz, 1H), 5.13 – 5.03 (m, 2H), 4.62 – 4.49 (m, 3H), 4.42 (t, *J* = 6.9 Hz, 2H), 4.00 (t, *J* = 7.5 Hz, 2H), 3.77 – 3.62 (m, 2H), 3.55 (s, 3H), 3.25 (d, *J* = 5.1 Hz, 1H), 3.22 (d, *J* = 9.4 Hz, 2H), 3.04 (q, *J* = 6.3, 4.0 Hz, 2H), 2.81 (d, *J* = 5.1 Hz, 1H), 2.49 (t, *J* = 2.0 Hz, 3H), 2.28 (t, *J* = 7.6 Hz, 2H), 2.14 – 2.04 (m, 2H), 1.85 – 1.04 (m, 33H), 0.93 – 0.78 (m, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 208.35, 173.32, 172.92, 171.47, 166.20, 157.05, 153.15, 152.55, 148.29, 145.65, 142.82, 142.55, 141.94, 141.05, 140.74, 131.81, 130.45, 129.69, 129.08, 128.91, 127.40, 126.78, 126.00, 125.71, 125.29, 124.22, 124.02, 122.26, 122.19, 120.85, 115.69, 111.16, 110.49, 104.24, 103.54, 67.23, 62.24, 59.19, 55.26, 52.51, 49.97, 49.81, 49.50, 49.15, 48.08, 44.50, 38.39, 38.25, 36.95, 36.32, 36.00, 34.38, 34.30, 33.92, 32.04, 31.26, 30.46, 30.26, 29.83, 29.79, 29.74, 29.59, 29.39, 29.28, 28.17, 27.15, 26.51, 26.48, 26.25, 26.05, 25.31, 25.06, 18.27, 16.95, 12.52. HRMS: calculated for $C_{73}H_{90}N_9O_7$ 1204.69577 $[M]^+$; found 1204.69580 LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 13.0 min):R_t (min): 8.46 (ESI-MS (m/z): 1204.47 (M^+)).

Cy5-LU-001i (14)

To a degassed solution of LU-001i (6.0 mg, 9.8 µmol) and Cy5-alkyne (5.0 mg) in DMF (0.8 mL) under an argon atmosphere was added $CuSO_4 \cdot 5H_2O$ (0.5 equiv, 4.9 µmol (100 µL from degassed stock solution of 49 µmol/mL)) and NaAsc (0.75 equiv. 7.4 µmol (100 µL from degassed stock solution of 74 µmol/mL)). After stirring overnight, the reaction mixture was concentrated and purified by column chromatography (0-3-6-10% MeOH in DCM) providing the product after lyophilisation as a blue powder (8.3 mg, 7.3 µmol, 75%). ^{1}H NMR (850 MHz, $CDCl_3$) δ 7.88 (s, 2H), 7.78 (q, *J* = 12.5 Hz, 2H), 7.31 (ddd, *J* = 10.1, 6.2, 2.8 Hz, 3H), 7.29 (dd, *J* = 7.1, 2.6 Hz, 2H), 7.16 (q, *J* = 7.2 Hz, 2H), 7.10 – 7.06 (m, 1H), 7.04 (d, *J* = 7.9 Hz, 1H), 6.74 (s, 1H), 6.55 (s, 1H), 6.30 (d, *J* = 13.4 Hz, 1H), 6.22 (d, *J* = 13.5 Hz, 1H), 5.14 (s, 2H), 4.76 (s, 1H), 4.50 (td, *J* = 16.8, 13.7, 8.5 Hz, 3H), 4.24 – 4.18 (m, 1H), 4.18 – 4.07 (m, 2H), 3.98 (s, 2H), 3.80 (s, 1H), 3.56 (d, *J* = 11.7 Hz, 3H), 3.23 (d, *J* = 5.0 Hz, 1H), 2.78 (d, *J* = 5.0 Hz, 1H), 2.60 (s, 2H), 2.30 – 2.20 (m, 2H), 1.87 – 0.93 (m, 40H), 0.95 – 0.69 (m, 7H). ^{13}C NMR (214 MHz, $CDCl_3$) δ 208.60, 173.03, 172.99, 172.24, 171.87, 169.56, 153.23, 152.76, 142.86, 142.01, 141.16, 140.88, 129.00, 128.83, 126.45, 126.28, 125.51, 125.21, 125.13, 122.29, 122.21, 111.13, 110.57, 105.98, 104.29, 103.96, 92.00, 66.91, 59.23, 58.32, 54.05, 52.61, 49.73, 49.47, 49.21, 48.07, 44.58, 38.83, 38.35, 34.36, 34.04, 32.06, 31.94, 31.67, 29.83, 29.79, 29.50, 28.30, 28.25, 27.65, 27.29, 26.55, 26.48, 26.32, 26.02, 25.24, 22.83, 22.41, 17.16, 16.92, 14.27, 14.09. HRMS: calculated for $C_{63}H_{85}F_2N_{10}O_7$ 1131.65653$[M]^+$; found 1131.65649. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 13.0 min):R_t (min): 7.96 (ESI-MS (m/z): 1131.67 (M^+)).

Biochemical experiments

General

Lysates of cells were prepared by treating cell pellets with 4 volumes of lysis buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM MgCl2, 10% glycerol, 2 mM ATP, and 0.05% digitonin for 60 min. Protein concentration was determined using Qubit® protein assay kit (Thermofisher). All cell lysate labelling experiments were performed in assay buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol, 2 mM ATP. Cell lysate labelling and competition experiments were performed at 37°C. Prior to fractionation on 12.5% SDS-PAGE

(TRIS/glycine), samples were boiled for 3 min in a reducing gel loading buffer. The 7.5x10 cm (L x W) gels were run for 15 min at 80V followed by 120 min at 130V. In-gel detection of (residual) proteasome activity was performed in the wet gel slabs directly on a ChemiDoc™ MP System using Cy2 setting to detect BODIPY(FL), Cy3 settings to detect BODIPY(TMR) and Cy5 settings to detect Cy5.

Competition experiments in cell lysate

Cell lysates (diluted to 10-15 µg total protein in 9 µL buffer) were exposed to the inhibitors (10 or 20x stock in DMSO) or ABPs (10 or 20x stock in DMSO) at indicated concentrations for 1 h at 37 °C. In case of multiple inhibition/labelling steps, max 20% DMSO is used and incubation conditions are always 1 h at 37 °C. SDS-PAGE analysis is performed as described above.

Determination of kinetic constants

General

All incubations are performed at 37 °C. Raji cell lysates (10 µg/ 9 µL) were incubated with appropriate inhibitor(s) (1µL from 10x stock in DMSO) followed by incubation with increasing concentrations of ABP for different lengths of time. The reaction was stopped by snap-freezing in liquid nitrogen and while still frozen, the denaturing sample buffer is added. Next, SDS-PAGE analysis is performed as described in the general methods. Intensities of bands were measured by fluorescent densitometry and normalized to full labelling. When the Log (% activity) is plotted versus time, a straight line is observed, from which the first order rate constants (K_{obs}) can be derived for each concentration. K_{obs} were plotted versus probe concentration, from which inhibition constants K_I and K_{inact} were calculated using Graphpad Prism software. See chapter 3, supporting figure S3 for example.

*ABP **13***

β5c: Raji cell lysates (10 µg/ 9 µL) were incubated LU-035i (1 µM, 1µL from 10x stock, to inhibit β5i) and NC-001 2.5 µM, 1µL from 10x stock, to inhibit β1c/β1i) for 1 h, followed by incubation with increasing concentrations of probe for 0, 15, 30 or 60 min. Concentrations used: 0.1, 0.3, 1.0, 3.0, 10, 30 µM; Full labelling of β5c is achieved by incubation with 30 µM of probe for 1 h.

β5i: Raji cell lysates (10 µg/ 9 µL) were incubated LU-005c (1 µM, 1µL from 10x stock, to inhibit β5c) and NC-001 2.5 µM, 1µL from 10x stock, to inhibit β1c/β1i) for 1 h, followed by incubation with increasing concentrations of probe for 0, 1, 2 or 5 min. Concentrations used: 80, 160, 320, 640, 1280, 2560, 5120, 10240 nM; Full labelling of β5i is achieved by incubation with 100 nM of probe for 1 h.

*ABP **14***

β1c: Raji cell lysates (10 µg/ 9 µL) were incubated LU-001i (1 µM, 1µL from 10x stock, to inhibit β1i) and NC-005 5.0 µM, 1µL from 10x stock, to inhibit β5c/β5i) for 1 h, followed by incubation with increasing concentrations of probe for 0, 15, 30 or 60 min. Concentrations used: 0.94, 1.89, 3.75, 7.5, 15, 30 µM; Full labelling of β1c is not achieved by incubation with 30 µM of probe for 1 h, however, higher concentrations did show too much background. Therefore the labelling with 30 µM of probe for 1 h was used as a reference and was determined to be 94%.

β1i: Raji cell lysates (10 µg/ 9 µL) were incubated LU-001c (10 µM, 1µL from 10x stock, to inhibit β1c) and NC-005 5.0 µM, 1µL from 10x stock, to inhibit β5c/β5i) for 1 h, followed by incubation with increasing concentrations of probe for 0, 1, 2 or 5 min. Concentrations used: 100, 200, 400, 800, 1600, 3200 nM; Full labelling of β1i is achieved by incubation with 300 nM of probe for 1 h.

β5i: Raji cell lysates (10 µg/ 9 µL) were incubated NC-001 (2.5 µM, 1µL from 10x stock, to inhibit β1c/β1i) for 1 h, followed by incubation with increasing concentrations of probe for 0, 1, 2 or 5 min. Concentrations used: 100, 200, 400, 800, 1600, 3200 nM; Full labelling of β5i is achieved by incubation with 30 µM of probe for 1 h.

References

1. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-79 (1998).
2. Ferrington, D.A. & Gregerson, D.S. Immunoproteasomes: structure, function, and antigen presentation. *Prog. Mol. Biol. Transl. Sci.* **109**, 75-112 (2012).
3. Adams, J. et al. Proteasome inhibitors: A novel class of potent and effective antitumor agents. *Cancer Res.* **59**, 2615-2622 (1999).
4. Demo, S.D. et al. Antitumor Activity of PR-171, a novel irreversible inhibitor of the proteasome. *Cancer Res.* **67**, 6383-6391 (2007).
5. Kisselev, A.F. & Groettrup, M. Subunit specific inhibitors of proteasomes and their potential for immunomodulation. *Curr. Opin. Chem. Biol.* **23**, 16-22 (2014).
6. de Bruin, G. et al. A set of ativity-based probes to visualize human (immuno)proteasome activities. *Ang. Chem. Int. Ed.* **13**, 4199-4203 (2015).
7. Verdoes, M. et al. A panel of subunit-selective activity-based proteasome probes. *Org. Biomol. Chem.* **8**, 2719-2727 (2010).
8. Verdoes, M. et al. A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo. *Chem. Biol.* **13**, 1217-1226 (2006).
9. Geurink, P.P. et al. Incorporation of non-natural amino acids improves cell permeability and potency of specific inhibitors of proteasome trypsin-like sites. *J. Med. Chem.* **56**, 1262-1275 (2013).
10. Sharma, L.K. et al. Activity-based near-infrared fluorescent probe for LMP7: a chemical proteomics tool for the immunoproteasome in living cells. *ChemBioChem* **13**, 1899-1903 (2012).
11. Carmony, K.C. et al. A bright approach to the immunoproteasome: development of LMP2/β1i-specific imaging probes. *Bioorg. Med. Chem. Lett.* **20**, 607-613 (2012).
12. de Bruin, G. et al. Structure-based design of β1i or β5i specific inhibitors of human immunoproteasomes. *J. Med. Chem.* **57**, 6197-6209 (2014).
13. Verdoes, M. et al. Acetylene functionalized BODIPY dyes and their application in the synthesis of activity based proteasome probes. *Bioorg. Med. Chem. Lett.* **17**, 6169-71 (2007).
14. Huber, Eva M. et al. Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. *Cell* **148**, 727-738 (2012).
15. Huber, E.M. et al. Systematic analyses of substrate preferences of 20S proteasomes using peptidic epoxyketone inhibitors. *J. Am. Chem. Soc.* **137**, 7835-7842 (2015).
16. Shenk, K.D. et al. *US/20070293465,* (2007).
17. Muchamuel, T. et al. A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med* **15**, 781-787 (2009).
18. Singh, J., Petter, R.C., Baillie, T.A. & Whitty, A. The resurgence of covalent drugs. *Nat. Rev. Drug. Discov.* **10**, 307-317 (2011).
19. Britton, M. et al. Selective inhibitor of proteasome's caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. *Chem. Biol.* **16**, 1278-1289 (2009).