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Chemical tools to monitor and control human proteasome activities

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CHAPTER 6

Development of β 1i and β 5i 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, where 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 β 1c (caspase-like), β 2c (trypsin-like) and β 5c (chymotrypsin like). In immune cells and in tissues exposed to inflammatory cytokines (for instance interferon- γ) the iCP is expressed in which β 1c, β 2c and β 5c are (partly) replaced by β 1i (chymotrypsin-like), β 2i (trypsin-like) and β 5i (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 bortezomib³ (Velcade, approved for the treatment of multiple myeloma (MM) and mantle cell lymphoma) and carfilzomib⁴ (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 β 5i inhibition has already been shown suppress autoimmunity in various preclinical mouse models.⁵ In addition,

it was found that selective inhibition of $\beta 5i$ and $\beta 1i$ 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-epoxomicin⁷ and MV-151⁸) or are in-class selective, targeting subunit pairs, such as Cy5-NC-001⁶, BODIPY(FL)-LU-112⁹, BODIPY(TMR)-NC-005-VS⁷ (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 $\beta 5i$ (PR-924) and $\beta 1i$ (UK-101) inhibitors (see chapter 5), Kim and co-workers developed ABPs selective for either $\beta 5i$ (LKS01-B650)¹⁰ or $\beta 1i$ (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 $\beta 1i$ over $\beta 5c/\beta 5i$, the selectivity of these probes is questionable. As described in the previous chapter, a new generation of $\beta 5i$ and $\beta 1i$ 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 $\beta 5i$ or $\beta 1i$.

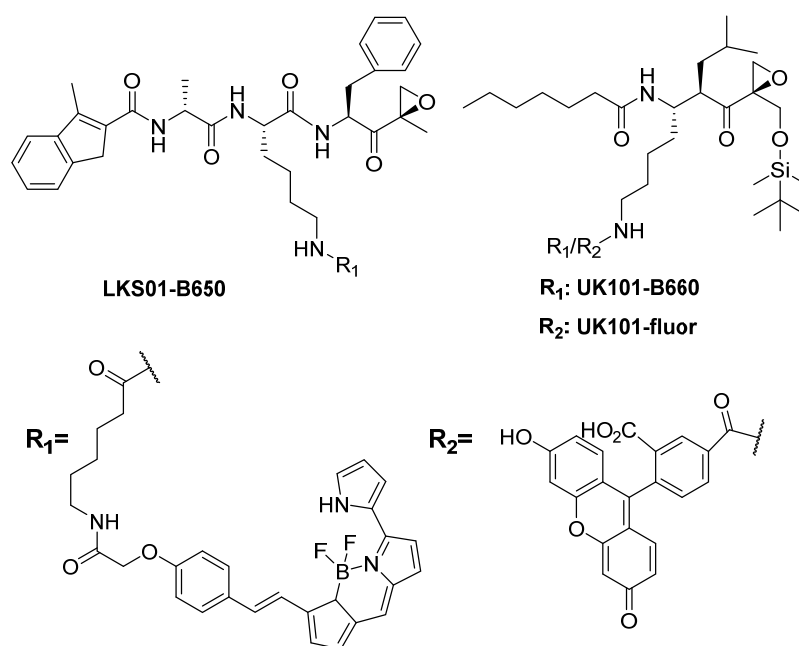


Figure 1. Structures of activity-based probes selective for $\beta 5i$ (LKS01-B650) or $\beta 1i$ (UK101-B660/Fluor).

Results and discussion

β 5i 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 β 5i selectivity, making this compound the most selective β 5i inhibitor known to date (see chapter 5). Therefore, LU-015i was taken as a lead compound for the development of a β 5i 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)-alkyne¹³ or Cy5-alkyne can be attached via a copper(I)-catalysed azide-alkyne cycloaddition (CuAAC, or 'click') reaction.

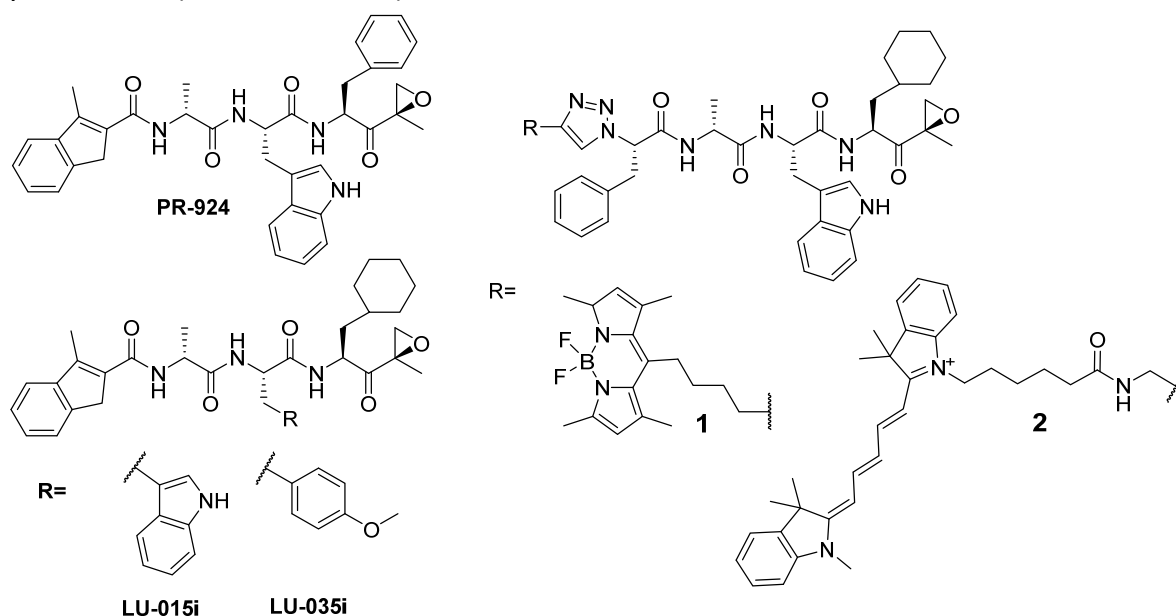
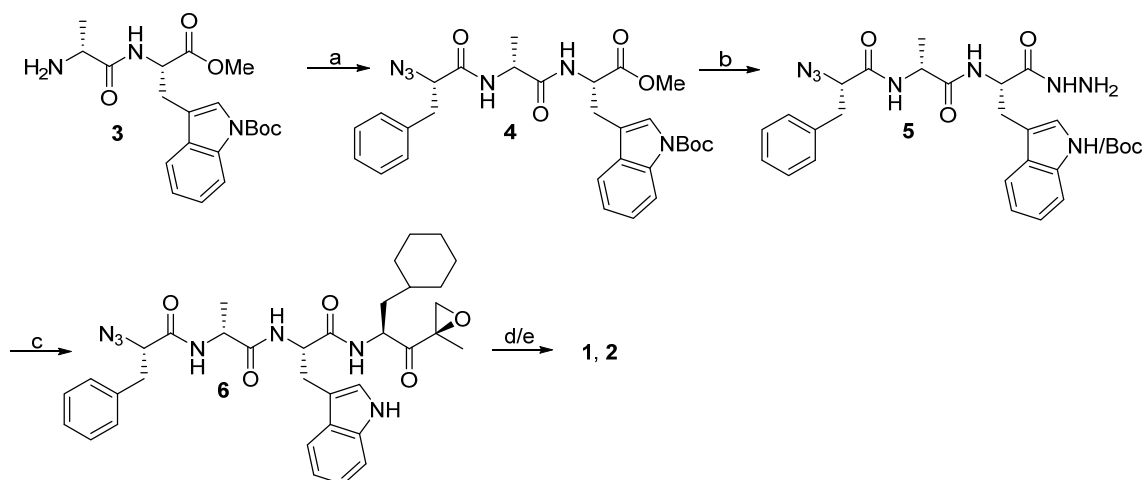


Figure 2. Chemical structures of PR-924, LU-015i, LU-035i and ABPs **1** and **2**.

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 β 5i selectivity in Raji cell lysate. Because most proteasome probes do not resolve β 5c and β 5i on SDS-PAGE, the samples were treated with BODIPY(TMR)-NC-005⁷ (chemical structure: see chapter 3) after incubation with ABP **1** or **2** to verify the amount of inhibition for β 5c and β 5i (Figure 3). Although both probes show some selectivity for β 5i, complete labelling of β 5i without concomitant modification of β 5c could not be achieved.



Scheme 1. Synthesis of ABP 1. Reagents and conditions: a. $\text{N}_3\text{Phe-OH}$, HCTU, DiPEA, DCM, 81%; b. $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$, 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%.

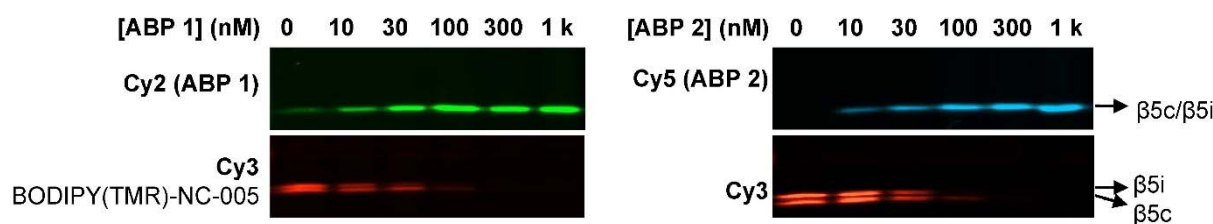
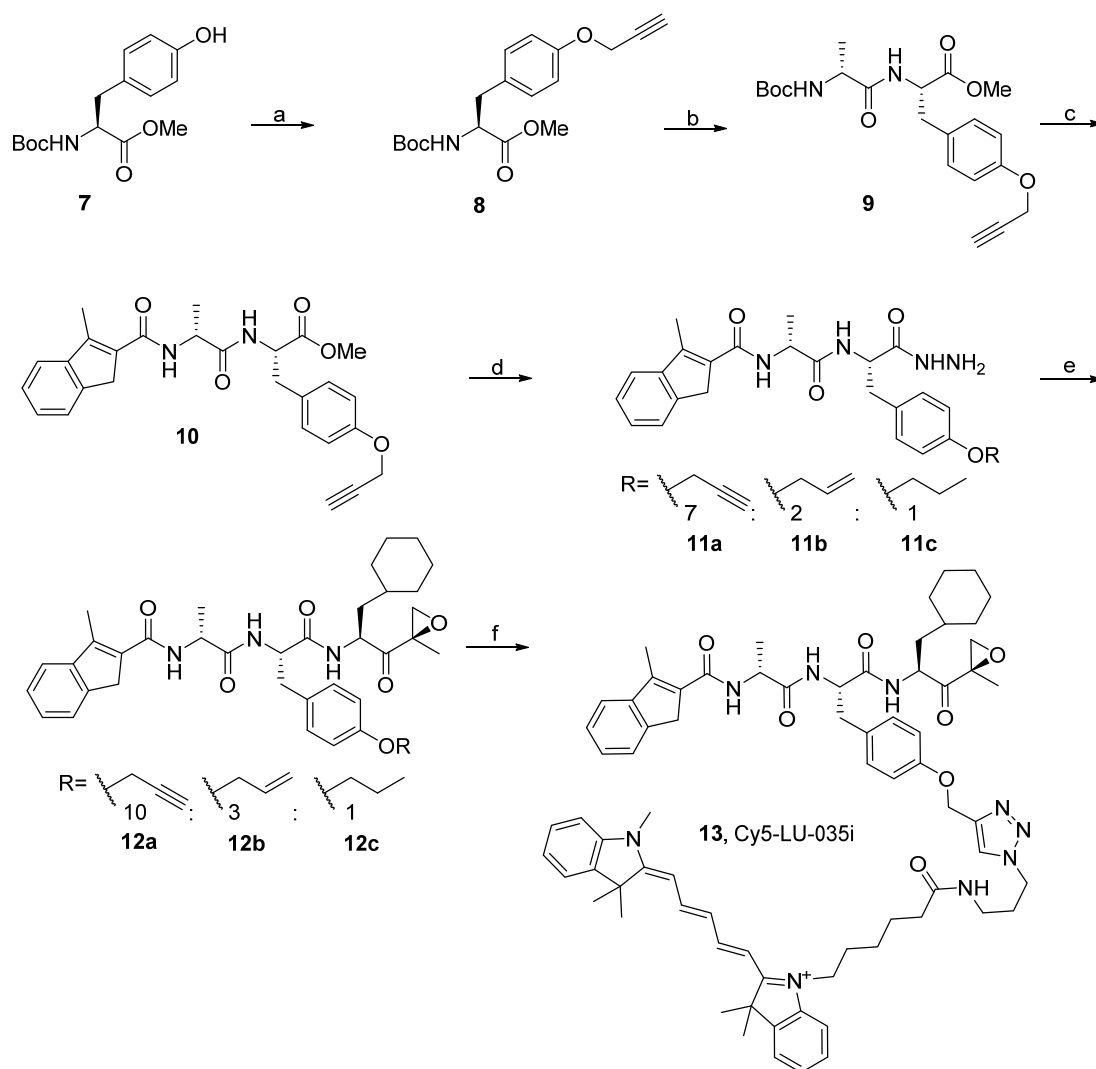


Figure 3. Evaluation of ABPs **1** and **2** in Raji lysate. Lysates were treated with indication concentrations of probe **1** or **2** for 1 h, followed by the addition of 100 nM BODIPY(TMR)-NC-005 (end concentration) for 1 h.

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

Since attachment of a fluorophore at the P4 position proved to be detrimental for $\beta 5i$ 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 $\beta 5c$ to Cys48 in $\beta 5i$, the S2 pocket of $\beta 5i$ is more shallow than $\beta 5c$.¹⁴ Residues that can interact with Cys48 are favoured and required for $\beta 5i$ selectivity^{15, 16}, consequently, all currently known $\beta 5i$ 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 β 5i 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 $\beta 5i$ selectivity (Figure 4). In contrast to ABPs **1** and **2**, ABP **13** shows both good selectivity for $\beta 5i$ over $\beta 5c$. At 100 nM, complete labelling of $\beta 5i$ is found and $\beta 5c$ remains untouched up to 300 nM. In addition, at concentrations up to 300 nM no labelling of the $\beta 1$ or $\beta 2$ subunits is visible. However, at higher concentrations at which $\beta 5i$ and $\beta 5c$ 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 $\beta 1$ subunits (compare 10000 and 30000 nM). The selectivity of ABP **13** becomes also apparent from the kinetic constants (Figure 4B). ABP **13** has a ten-fold higher K_{inact} for $\beta 5i$ compared to $\beta 5c$, meaning that the covalent bond formation of **13** with $\beta 5i$ is 10x faster than with $\beta 5c$. In addition, the K_i (concentration of inhibitor at 0.5 K_{inact}) of **13** is also 3 times lower for $\beta 5i$ compared to $\beta 5c$, indicating a higher affinity for $\beta 5i$. 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 $\beta 5i$ compared to $\beta 5c$, confirming the good selectivity for $\beta 5i$.

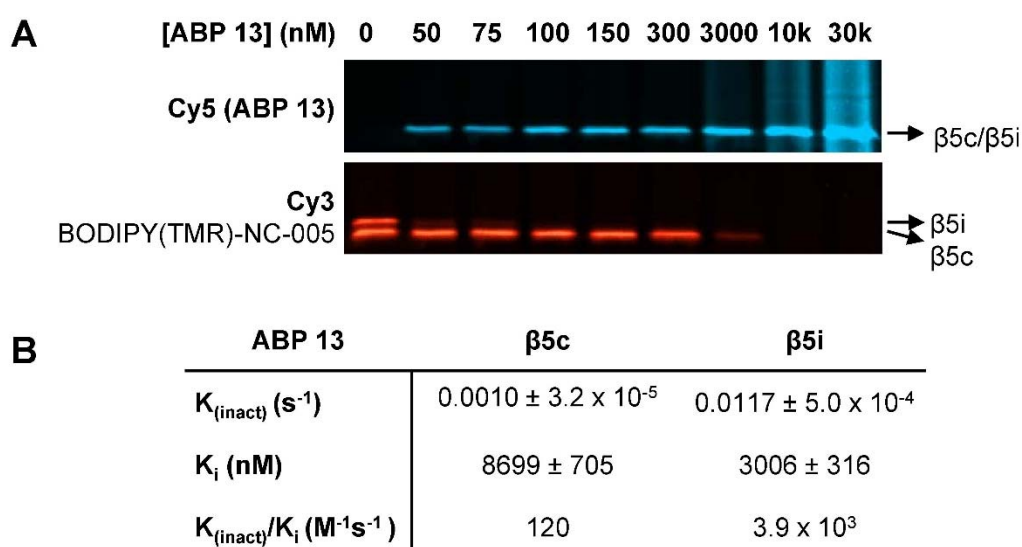
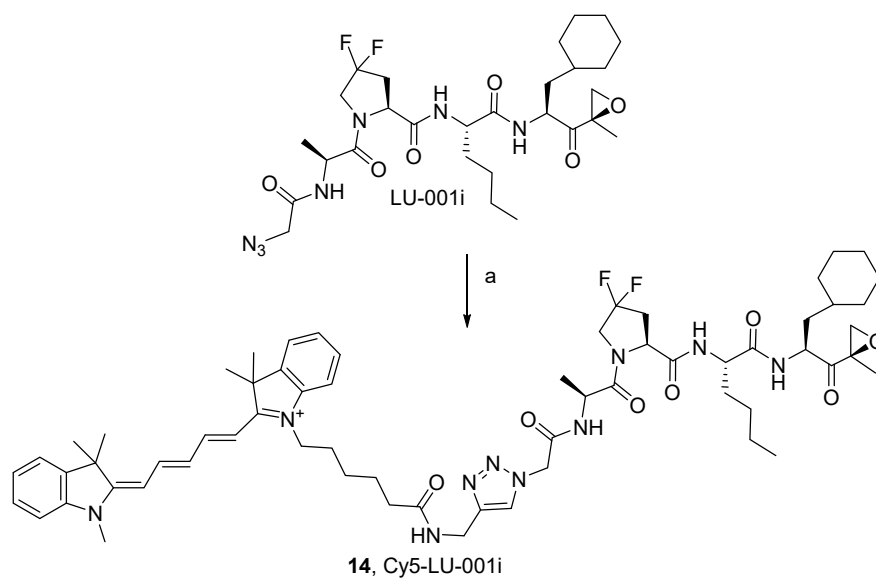


Figure 4. Evaluation of ABP 13 in Raji lysate. A) Lysates were treated with indicate concentrations of ABP **13** for 1 h, followed by the addition of 100 nM BODIPY(TMR)-NC-005 (end concentration) for 1 h. B) Kinetic constants of ABP **13** as determined in Raji lysates. Log (% activity) for various 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 $K_i = [\text{probe}]$ at 0.5 K_{inact} . K_{inact}/K_i = second order rate constant.

β 1i selective probe

As described in chapter 5, the substitution of P1 Leu for Cha and P3 Pro for 4,4-F₂-Pro in NC-001¹⁹ resulted in the highly selective β 1i 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 β 1i probe with near infrared properties was desired.



Scheme 3. Synthesis of ABP 14. Reagents and conditions: a. Cy5-alkyne, CuSO₄, NaAsc, DMF/H₂O, 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 β 1c and β 1i was labelled by β 1 selective probe BODIPY(FL)-NC-001 (Figure 5A).⁷ At 100 nM β 1i is inhibited completely, while very minor labelling of β 1c is visible at 1 μ M. The inhibition of β 5c and β 5i by ABP **14** was also examined in more detail (Figure 5B). Because of overlapping bands for the β 1 and β 5 subunits on SDS-PAGE, β 1c and β 1i were completely blocked by β 1 selective inhibitor NC-001¹⁹, before incubation with ABP **14**. Residual activity of β 5c and β 5i was labelled by β 5 selective probe BODIPY(TMR)-NC-005. At concentrations of >2 μ M β 5i is significantly inhibited, while β 5c is barely inhibited even at concentration up to 30 μ M. In addition, inhibition of β 1c by ABP **14** was investigated in more detail (Figure 5C). In order to do so, β 1i and β 5c/ β 5i were inhibited by LU-001i and NC-005, prior to incubation with ABP **14**. Residual activity of β 1c was labelled by β 1c selective ABP BODIPY(FL)-LU-001c (see chapter 8). Labelling of β 1c by ABP **14** is already visible at 470 nM, however, complete labelling is only visible at 15 μ M. The kinetic constants also prove the high β 1i selectivity of ABP **14** (Figure 5D). Compared to β 1c and β 5i, the K_{inact} is about 10 x larger and the K_i about 10x smaller for β 1i. This results in a 100x larger second order rate constant (K_{inact}/K_i) for β 1i compared to β 1c and β 5i, indicating that ABP **14** has excellent selectivity for β 1i.

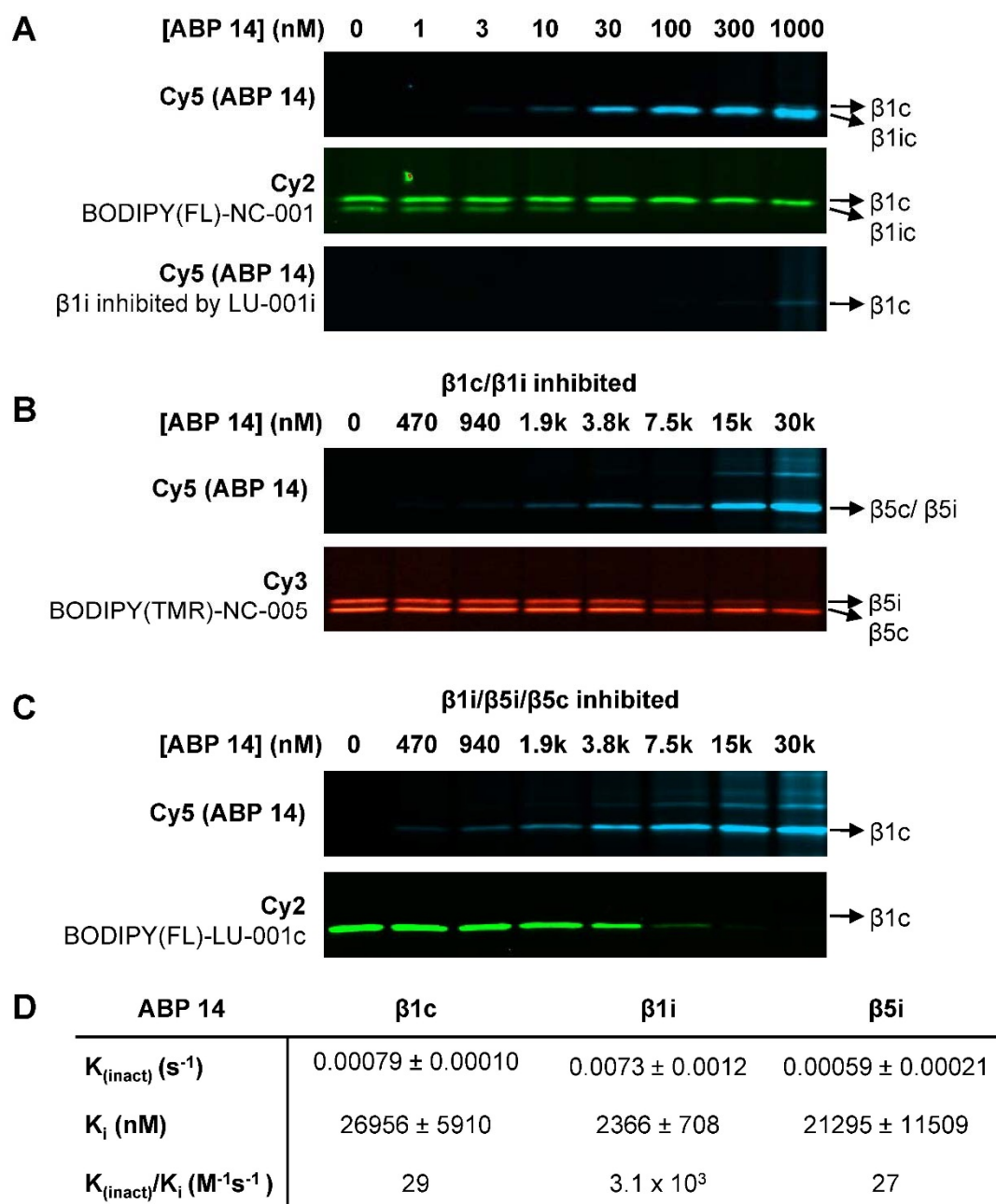


Figure 5. Evaluation of ABP 14 in Raji lysate. A) Lysates were treated with indication concentrations of ABP 13 for 1 h, followed by the addition of BODIPY(FL)-NC-001 (100 nM end concentration) for 1 h. B) Raji lysates were treated with NC-001 (5 μ M) for 1 h to inhibit β 1c and β 1i, followed by incubation 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 β 5c or β 5i activity. C) Raji lysates were treated with LU-001i (1 μ M) and NC-005 (2.5 μ M) for 1 h to inhibit β 1i, β 5c and β 5i, 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 β 1c 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 β 5i or β 1i, activity-based probes selective for either β 5i (ABP **13**) or β 1i (ABP **14**) were developed. Straightforward N-terminal attachment of a Cy5 fluorophore to β 1i inhibitor LU-001i provided a potent and selective probe for β 1i. In contrast, it proved to be impossible to obtain a selective β 5i probe by N-terminal attachment of a Cy5 fluorophore to a β 5i selective inhibitor. However, when the fluorophore was positioned at the P2 position a potent and selective β 5i probe was obtained. In cell lysate both probes have a good selectivity window in which only β 5i (ABP **13**) or β 1i (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 β 5i or β 1i 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 $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ (25 g/L) and $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4\cdot 2\text{H}_2\text{O}$ (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 $\times$ 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 $\rightarrow$ xx% MeCN, 13.0 min (0 $\rightarrow$ 0.5 min: 10% MeCN; 0.5 $\rightarrow$ 8.5 min: gradient time; 8.5 $\rightarrow$ 10.5 min: 90% MeCN; 10.5 $\rightarrow$ 13.0 min: 10% MeCN), 15 min (0 $\rightarrow$ 0.5 min: 10% MeCN; 0.5 $\rightarrow$ 10.5 min: gradient time; 10.5 $\rightarrow$ 12.5 min: 90% MeCN; 12.5 $\rightarrow$ 15 min: 90% $\rightarrow$ 10% MeCN). HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μm 250 $\times$ 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.

N₃Phe-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.), N₃Phe-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 $\rightarrow$ 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 $\rightarrow$ 90% MeCN, 0.1% TFA, 15 min): R_t (min): 10.45 (ESI-MS (m/z): 562.87 ($\text{M}+\text{H}^+$)).

N₃Phe-D-Ala-Trp(Boc)-NHNH₂ (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 $\rightarrow$ 90% MeCN, 0.1% TFA, 15 min): R_t (min): 6.24 (ESI-MS (m/z): 462.93 ($\text{M}+\text{H}^+$ -Boc) and 7.83 (ESI-MS (m/z): 562.93 ($\text{M}+\text{H}^+$ +Boc).

N₃Phe-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 H₂O (3 $\times$). Purification by column chromatography (1 $\rightarrow$ 2% MeOH in DCM) provided the title compound (12.01 mg, 37.4%) as a white powder after lyophilisation. ^1H NMR (400 MHz, CDCl₃) 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, CDCl₃) 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 $\rightarrow$ 90% MeCN, 0.1% TFA, 15 min): R_t (min): 10.16 (ESI-MS (m/z): 642.20 ($\text{M}+\text{H}^+$)). HRMS: calculated for C₃₅H₄₃N₇O₅ 642.33984 [$\text{M}+\text{H}$]⁺; 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 μmol , 1.5 equiv.) in DMF (0.8 mL) under an argon atmosphere was added CuSO₄·5H₂O (0.5 equiv, 3.4 μmol (100 μL from degassed stock solution of 34 $\mu\text{mol}/\text{mL}$)) and NaAsc (0.75 equiv, 5.0 μmol (100 μL from degassed stock solution of 50 $\mu\text{mol}/\text{mL}$)). After stirring overnight, the reaction solution was diluted with EtOAc and washed with H₂O (1 $\times$) and brine (1 $\times$). The organic layer was dried over Na₂SO₄, filtered and concentrated. Purification by column chromatography (0 $\rightarrow$ 3% MeOH:DCM) provided the title compound in a quantitative yield. ^1H NMR (600 MHz, CDCl₃) 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, CDCl₃) 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 $\rightarrow$ 90% MeCN, 0.1% TFA, 15 min): R_t (min): 11.45 (ESI-MS (m/z): 970.20 ($\text{M}+\text{H}^+$)). HRMS: calculated for C₅₄H₈₆BF₂N₉O₅ 970.53298 [$\text{M}+\text{H}$]⁺; 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 CuSO₄·5H₂O (0.5 equiv, 1.4 μmol (100 μL from degassed stock solution of

14 $\mu\text{mol/mL}$) and NaAsc (0.75 equiv, 2.1 μmol (100 μL from degassed stock solution of 21 $\mu\text{mol/mL}$)). 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%). ^1H 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 $\text{C}_{70}\text{H}_{85}\text{N}_{10}\text{O}_6$ 1161.66481 $[\text{M}]^+$; found 1161.66479 LC-MS (linear gradient 10 $\rightarrow$ 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_2CCH)-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 $\rightarrow$ 20% EtOAc/pent) provided the title compound (309 mg, 0.93 mmol, 72%). ^1H 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_2CCH)-OMe (9)

Boc-Tyr(OCH_2CCH)-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_2CCH)-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. ^1H 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_2CCH)-OMe (10)

Boc-D-Ala-Tyr(OCH_2CCH)-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_2CCH)-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 $\rightarrow$ 75% EtOAc/pent) provided the title compound (396 mg, 0.86 mmol, 93%). ^1H 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(OCH₂CCH)-NHNH₂ (**11**)

To a solution of 3MeIndAc-D-Ala-Tyr(OCH₂CCH)-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). ^1H NMR (400 MHz, $\text{CDCl}_3/\text{MeOD}$) (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.

RT: 0.00 - 13.20

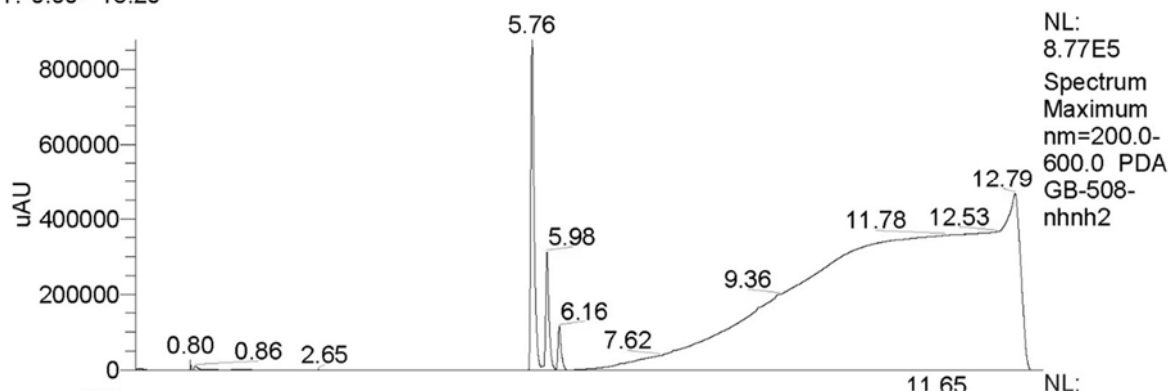


Figure 6. HPLC trace of compound **11**. Retention time (min)/(m/z): 5.76/461.07 (alkyne); 5.98/463.07 (alkene); 6.16/465.00 (alkane).

3MeIndAc-D-Ala-Tyr(OCH₂CCH)-Cha-EK (**12**)

Hydrazide **12** (47 mg, 0.1 mmol, 1 equiv.) was dissolved in DMF (2 mL) and cooled to -30 °C. $t\text{BuONO}$ (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 H₂O (3x). 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%). ^1H 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 μ mol) and Cy5-alkyne (13 mg, 24 μ mol, 1.5 equiv.) in DMF (1 mL) under an argon atmosphere was added $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.5 equiv, 8 μ mol (100 μ L from degassed stock solution of 8 μ mol/mL)) and NaAsc (0.75 equiv, 12 μ mol (100 μ L from degassed stock solution of 120 μ mol/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 μ mol, 28%). ^1H 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 $\text{C}_{73}\text{H}_{90}\text{N}_9\text{O}_7$ 1204.69577 $[\text{M}]^+$; found 1204.69580 LC-MS (linear gradient 10 $\rightarrow$ 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 μ mol) and Cy5-alkyne (5.0 mg) in DMF (0.8 mL) under an argon atmosphere was added $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.5 equiv, 4.9 μ mol (100 μ L from degassed stock solution of 49 μ mol/mL)) and NaAsc (0.75 equiv, 7.4 μ mol (100 μ L from degassed stock solution of 74 μ mol/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 μ mol, 75%). ^1H 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 $\text{C}_{63}\text{H}_{85}\text{F}_2\text{N}_{10}\text{O}_7$ 1131.65653 $[\text{M}]^+$; found 1131.65649. LC-MS (linear gradient 10 $\rightarrow$ 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 MgCl_2 , 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 μ g total protein in 9 μ L 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 μ g/ 9 μ L) were incubated with appropriate inhibitor(s) (1 μ L 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

β 5c: Raji cell lysates (10 μ g/ 9 μ L) were incubated LU-035i (1 μ M, 1 μ L from 10x stock, to inhibit β 5i) and NC-001 2.5 μ M, 1 μ L from 10x stock, to inhibit β 1c/ β 1i) 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 μ M; Full labelling of β 5c is achieved by incubation with 30 μ M of probe for 1 h.

β 5i: Raji cell lysates (10 μ g/ 9 μ L) were incubated LU-005c (1 μ M, 1 μ L from 10x stock, to inhibit β 5c) and NC-001 2.5 μ M, 1 μ L from 10x stock, to inhibit β 1c/ β 1i) 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 β 5i is achieved by incubation with 100 nM of probe for 1 h.

ABP 14

β 1c: Raji cell lysates (10 μ g/ 9 μ L) were incubated LU-001i (1 μ M, 1 μ L from 10x stock, to inhibit β 1i) and NC-005 5.0 μ M, 1 μ L from 10x stock, to inhibit β 5c/ β 5i) 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 μ M; Full labelling of β 1c is not achieved by incubation with 30 μ M of probe for 1 h, however, higher concentrations did show too much background. Therefore the labelling with 30 μ M of probe for 1 h was used as a reference and was determined to be 94%.

β 1i: Raji cell lysates (10 μ g/ 9 μ L) were incubated LU-001c (10 μ M, 1 μ L from 10x stock, to inhibit β 1c) and NC-005 5.0 μ M, 1 μ L from 10x stock, to inhibit β 5c/ β 5i) 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 β 1i 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.

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