Chemical tools to monitor and control human proteasome activities

Bruin, G. de

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Author: Bruin, G. de
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CHAPTER 8

Development of an inhibitor and activity-based probe selective for β1c

Introduction

Proteasome inhibitors in clinical use and evaluated as clinical candidates for the treatment of haematological cancers do not distinguish between constitutive (cCPs) and immunoproteasomes (iCPs) (see chapter 3). The reason for this may be historical: initially cCPs, and in particular the β5c subunits, were thought to be the essential targets in treating haematological cancers with proteasome inhibitors. From later studies it became apparent that the two clinical drugs that target proteasomes and that were developed as β5c inhibitors (bortezomib and carfilzomib) inhibit next to β5c also β5i as well as a number of other cCP and iCP subunits. In order to establish which cCP/iCP subunits are ideally targeted for the treatment of various tumours, but also to discriminate their individual role in antigen processing and presentation, it would be advantageous to have available a set of inhibitors and activity-based probes uniquely modulating one of each of the six cCP/iCP subunits. In the previous chapters a number of such inhibitors and probes is described. For instance, chapter 7 reports on the development of selective β5c selective inhibitors and a β5c-selective activity based probe (ABP). This chapter describes the development of an inhibitor and ABP with high selectivity for β1c.

The nature of the S1 pocket of β1c promotes hydrolysis of peptide bonds C-terminally of acidic amino acids and as such, the catalytic activity of β1c is designated as peptidylglutamyl-peptide hydrolyzing (PGPH)[1] or cysteine-aspartic-acid-protease (caspase)-like.[2] To assess the activity of β1c, Z-Leu-Leu-Glu-AMC is often used as fluorogenic substrate, even though the hydrolysis rate of this substrate is rather low.[2] To overcome this limitation, Ac-Nle-Pro-Nle-Asp-AMC was developed which showed a much higher cleavage rate, indicating that β1c prefers Asp over Glu at P1. Indeed, direct comparison of Ac-Gly-Pro-Leu-Asp-AMC with Ac-Gly-Pro-Leu-Glu-AMC showed a two-fold slower conversion of the latter substrate.[2] This finding was confirmed by a systematic study in which substrate preferences of all proteasome subunits were analysed using tripeptide epoxyketones (EK) with different P1 amino acids.[3] It was found that Ac-PAD-EK and Ac-LAD-EK showed ten-fold lower IC_{50} values than Ac-PAE-EK and Ac-LAE-EK (see chapter 4). The preference of β1c for substrates and inhibitors with acidic residues on P1 can be explained by the increased hydrophobicity of the S1 pocket of β1i caused by mutations (Val20 and Leu45 in β1i versus Thr20 and Arg45 in β1c, see chapter 4).

Although Ac-PAD-EK shows complete selectivity for β1c over all subunits, the IC_{50} for β1c is rather high (1.4 µM, Figure 1). In addition, Ac-PAD-EK lacks an azide or alkyne functionality for direct attachment of a fluorophore in order to obtain a β1c selective ABP. In this chapter a more potent β1c inhibitor based on Ac-PAD-EK and β1c/β1i specific inhibitor NC-001[4] is described (Figure 1). This inhibitor is equipped with an azide group, which allowed direct functionalization with a fluorophore and yielded the first ABP selective for β1c.

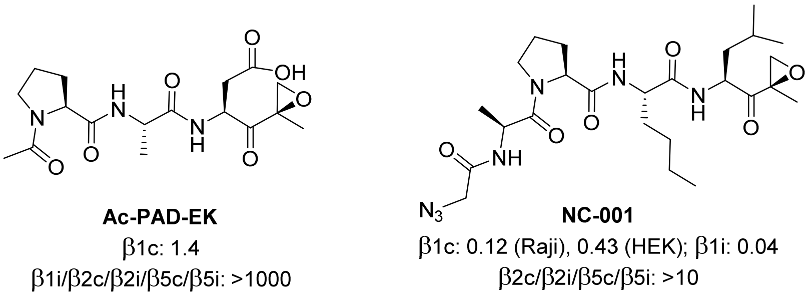
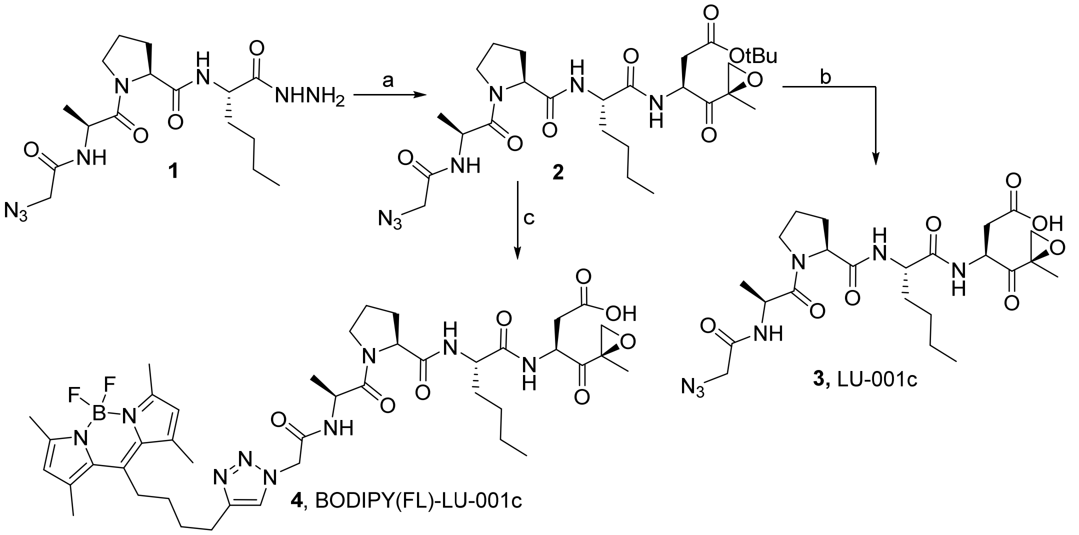


Figure 1. Structures and IC_{50} values (µM) of NC-001 (β1c/β1i selective) and Ac-PAD-EK (β1c selective). IC_{50} values have been determined by ABPP in Raji or HEK cell lysate.

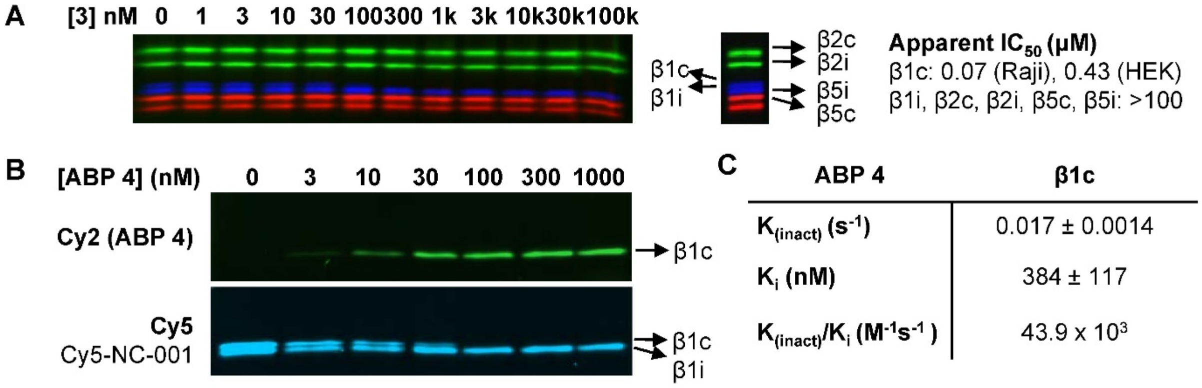
Results and discussion

NC-001 is 10-fold more potent for β1c than Ac-PAD-EK, but also targets β1i (Figure 1). In addition, elongation of tripeptide Ac-PLL-EK with a P4 Ala residue resulted in a four-fold lower IC_{50} value for β1c (see chapter 4). Therefore it was hypothesized that substitution of the P1 Leu residue of NC-001 for Asp (compound **3**, LU-001c) would result in more a potent β1c inhibitor. For the synthesis of compound **3**, first an azide coupling between hydrazide **1**[5] and TFA·H-Asp(OtBu)-EK (synthesized as described in chapter 4) was performed providing compound **2** (Scheme 1). Treatment of **2** with dry TFA yielded compound **3**. Significant amounts of side products were formed due to partial reduction of the epoxide during the hydrogenation of Cbz-Asp(OtBu)-EK and significant hydrolysis of the epoxyketone moiety during the final deprotection (see also chapter 4). Therefore, HPLC purification was necessary and compound **3** could be isolated in excellent purity but only in a low yield. Evaluation of LU-001c **3** in Raji lysate showed a 20-fold improved potency for β1c compared to Ac-PAD-EK (Figure 2A). Both in Raji and HEK lysate, the IC_{50} values of LU-001c **3** for β1c are comparable to NC-001 (Figure 1 and 2). In addition, up to concentrations of 100 µM no inhibition of other proteasome subunits was observed. On the contrary, in intact RPMI-8226 cells, no inhibition of β1c could be observed at concentration up to 100 µM, most likely due to poor cell permeability caused by the negatively charged carboxylic acid moiety.



Scheme 1. Synthesis of LU-001c (2). Reagents and conditions: a) 1. i. tBuONO, HCl, DMF, -35°C. ii DiPEA, TFA·H-Asp(OtBu)-EK, -35°C-RT; b) TFA, 1.9 % (total yield over a and b); c) 1. TFA. 2. BODIPY(FL)-alkyne, $CuSO_4$, NaAsc, DMF/H_2O, 31%.

Since LU-001c **3** has been equipped with an azide functionality, a fluorophore can be attached straightforwardly by copper(I)-catalysed azide-alkyne cycloaddition (CuAAC). For further studies aimed at establishing the composition of 20S proteasome core particles (see chapter 10) a β1c probe equipped with a BODIPY(FL) fluorophore was desired. Therefore, compound **2** was treated with TFA and directly used (without purification) in the CuAAC reaction with BODIPY(FL)-alkyne[6]. ABP **4** (BODIPY(FL)-LU-001c) was isolated in a reasonable yield and excellent purity after purification by HPLC. Assessment of ABP **4** in Raji lysate showed good selectivity and potency for β1c (Figure 2B). Labelling of the other proteasome subunits was not observed and therefore the kinetic constants could only be determined for β1c (Figure 2C).



ABP 4	β1c
$K_{(inact)}$ (s^{-1})	0.017 ± 0.0014
K_i (nM)	384 ± 117
$K_{(inact)}/K_i$ ($M^{-1}s^{-1}$)	43.9 x 10^3

Figure 2. Evaluation of LU-001c (3) and ABP 4 in Raji lysate. A) Inhibition profile (Raji lysate) and apparent IC_{50} values of LU-001c (**3**) as determined in Raji and HEK lysates. B) Evaluation of ABP **4**. Raji lysates were treated with indicated concentrations of ABP **4** for 1 h, followed by the addition of Cy5-NC-001 (100 nM end concentration, labels β1c/β1i, see chapter 3) for 1 h. C) Kinetic constants of ABP **4** 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 the β1c selective inhibitor Ac-PAD-EK and β1c/β1i selective inhibitor NC-001, LU-001c (**3**) was synthesized, which showed increased inhibitory potency for β1c. LU-001c (**3**) can therefore be considered as the most selective and potent β1c selective inhibitor known to date. Due to poor cell permeability, this inhibitor can only be used to block β1c in cell lysates. In addition, LU-001c (**3**) was used to synthesize the first ABP able to selectively label β1c (BODIPY(FL)-LU-001c, ABP **4**) in lysates. Although this probe has not been evaluated in intact cells, it is envisioned that this probe also suffers from poor cell permeability. It might be possible to overcome the poor cell permeability of LU-001c (**3**) and ABP **4** by converting the carboxylic acid moiety to an ester (for instance a methyl- or ethyl ester), which can be hydrolysed by esterases once the compound has entered the cell.

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). 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-Asp(OtBu)-EK was synthesized as described in chapter 4.

N_3Ac-Ala-Pro-Nle-Asp-EK (LU-001c,3)

Compound **4** was synthesized as reported before.[5] The title compound was prepared via azide coupling of hydrazide **4** and TFA·H-Asp(OtBu) epoxyketone. Hydrazide **4** (57 mg, 144 µmol, 1 equiv.) was dissolved in DMF and cooled to -30 °C. tBuONO (23 µL, 1.1 equiv.) and HCl (100 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. The epoxyketone as free amine was added to the reaction mixture as a solution in DMF. DiPEA (144 µL, 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 or DCM and extracted with H_2O (3×). The organic layer was dried over $MgSO_4$ concentrated and purified by flash column chromatography (0-3% MeOH in DCM), provided tert-butyl protected product **2**, which was deprotected by treatment with dry TFA (1 mL) for 10 min, followed by the addition of toluene and concentration. Purification by HPLC (C_{18}, 10-30% MeCN, 0.1% TFA, 15 min gradient), followed by lyophilization provided the product (1.46 mg, 1.9%). Complex NMR due to presence of rotamers, peaks of major rotamer are reported. ^1H NMR (600 MHz, MeOD) δ 4.80 – 4.74 (m, 1H), 4.63 (q, *J* = 7.0 Hz, 1H), 4.46 (dd, *J* = 8.4, 4.5 Hz, 1H), 4.29 – 4.23 (m, 1H), 3.92 – 3.85 (m, 2H), 3.80 (dt, *J* = 9.8, 6.8 Hz, 1H), 3.65 (dt, *J* = 10.1, 6.7 Hz, 1H), 3.23 (d, *J* = 4.7 Hz, 1H), 2.92 (dd, *J* = 12.3, 4.9 Hz, 1H), 2.79 (dd, *J* = 16.5, 4.2 Hz, 1H), 2.70 (dd, *J* = 16.3, 7.6 Hz, 1H), 2.21 (tdd, *J* = 15.0, 9.7, 5.6 Hz, 1H), 2.15 – 2.07 (m, 1H), 2.07 – 1.97 (m, 2H), 1.80 (ddt, *J* = 15.2, 10.8, 5.2 Hz, 1H), 1.71 – 1.57 (m, 1H), 1.40 – 1.30 (m, 7H), 1.22 (s, 3H), 0.97 – 0.88 (m, 3H). ^{13}C NMR (151 MHz, MeOD) δ 207.14, 174.28, 174.11, 173.74, 173.24, 169.87, 61.51, 54.67, 53.25, 52.47, 50.51, 49.57, 48.63, 36.13, 32.77, 30.48, 28.93, 26.04, 23.45, 16.82, 14.27. LC-MS (linear gradient 0 → 50% MeCN, 0.1% TFA, 13.5 min): R_t (min): 7.70 (ESI-MS (m/z): 538.07 $(M+H)^+$). HRMS: calculated for $C_{23}H_{35}N_7O_8$ 538.26199 $[M+H]^+$; found 538.26202

BODIPY(FL)-LU-001c (4)

Tert-butyl protected LU-001c **2** (10 mg, 17 µmol) was treated with dry TFA (1 mL) for 15 min, followed by co-evaporation with dry toluene to afford crude LU-001c, which was directly used in the next step. To a degassed solution of LU-001c (crude, 17 µmol) and BODIPY(FL)-alkyne (8.3 mg, 25 µmol, 1.5 equiv.) in DMF (1 mL) under an argon atmosphere was added $CuSO_4 \cdot 5H_2O$ (0.5 equiv, 8.5 µmol (100 µL from degassed stock solution of 85 µmol/mL)) and NaAsc (0.75 equiv, 12.8 µmol (100 µL from degassed stock solution of 128 µmol/mL)). After stirring overnight, the reaction mixture was immediately purified by HPLC (C_{18}, 40-70% MeCN, 0.1% TFA, 10 min gradient), providing the product as a yellow powder after lyophilisation (4.62 mg, 5.3 µmol, 31%). ^1H NMR (600 MHz, MeOD) δ 7.77 (s, 1H), 6.14 (s, 2H), 5.20 – 5.12 (m, 2H), 4.80 (dd, *J* = 7.5, 4.9 Hz, 1H), 4.63 (q, *J* = 7.0 Hz, 1H), 4.45 (dd, *J* = 8.3, 4.6 Hz, 1H), 4.28 (dd, *J* = 8.7, 5.3 Hz, 1H), 3.77 (dt, *J* = 9.4, 6.8 Hz, 1H), 3.65 (dt, *J* = 9.6, 6.3 Hz, 1H), 3.24 (d, *J* = 5.0 Hz, 1H), 3.06 (dd, *J* = 10.3, 6.8 Hz, 2H), 2.94 (d, *J* = 5.0 Hz, 1H), 2.84 – 2.79 (m, 3H), 2.73 (dd, *J* = 16.5, 7.6 Hz, 1H), 2.46 (s, 6H), 2.43 (d, *J* = 6.1 Hz, 6H), 2.26 – 2.17 (m, 1H), 2.16 – 1.90 (m, 5H), 1.88 – 1.77 (m, 1H), 1.77 – 1.68 (m, 2H), 1.68 – 1.59 (m, 1H), 1.51 (s, 3H), 1.46 – 1.33 (m, 7H), 1.00 – 0.90 (m, 3H). ^{13}C NMR (151 MHz, MeOD) δ 206.71, 173.80, 173.66, 172.81, 167.13, 154.52, 148.16, 147.52, 141.87, 132.21, 124.54, 122.19, 67.75, 61.11, 59.93, 54.24, 52.87, 52.32, 35.74, 32.39, 31.97, 30.36, 30.03, 28.70, 28.50, 25.62, 25.57, 23.03, 16.77, 16.38, 16.13, 14.02, 13.87. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 13.0 min):R_t (min): 7.35, (ESI-MS (m/z): 846.07 $(M-F)^+$). HRMS: calculated for $C_{42}H_{58}BF_2N_9O_8$ 866.45422 $[M+H]^+$; found 866.45426.

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 MgCl2, 10% glycerol, 2 mM ATP. Cell lysate labelling and competition experiments were performed at 37°C. Probe cocktail consist of: 100 nM Cy5-NC-001, 30 nM BODIPY(FL)-LU-112, 100 nM BODIPY(TMR)-NC-005-VS, used as premixed 10x concentrated cocktail in DMSO which is incubated with cell lysate for 60 min. 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 inhibitor (10x stock in DMSO) at indicated concentrations for 1 h at 37 °C, followed by addition of probe cocktail (10x stock, 1.1 µL) and SDS-PAGE as described above. For IC_{50} determinations, intensities of bands were measured by fluorescent densitometry and divided by the intensity of bands in mock-treated extracts. Average values of three independent experiments were plotted against inhibitor concentrations. IC_{50} values were calculated using GraphPad Prism software.

Evaluation of ABP 4

Cell lysates (diluted to 10-15 µg total protein in 9 µL buffer) were exposed to ABP **4** (10x stock in DMSO) at indicated concentrations for 1 h at 37 °C. Residual β1c/β1i activity is labelled by Cy5-NC-001 (100 nM end concentration). SDS-PAGE analysis is performed as described above.

Determination of kinetic constants

Raji cell lysates (10 µg/ 9 µL) were incubated with increasing concentrations of ABP **3** (160, 320, 640, 1280, 2560, 5120 nM) for different lengths of time (0, 1, 2, 5 min). 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 2, supporting figure S2 for example.

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