A Bivalent Supramolecular GCP Ligand Enables Blocking of the Taspase1/Importin α Interaction

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General information

All chemicals were purchased either from Fluorochem, Sigma Aldrich or ABCR and used without further purification. The reactions were carried out using dried solvents and under inert gas atmosphere (argon). Reactions were monitored by thin-layer chromatography (TLC), which was performed on 0.2 mm Macherey-Nagel ALUGRAM precoated silica gel aluminum sheets. Spots were visualized using basic KMnO$_4$ solution or by an UV-hand-lamp (254 and 365 nm). Column chromatography was carried out on silica gel 60 (0.063 - 0.2 mm, Merck). Analytical HPLC chromatograms were performed with a system from Dionex and reversed phase column: YMC-ODS-ÅQ (150 mm, Ø = 3 mm, particle size = 5 µm, pore size = 12 nm). Preparative reversed phase chromatography was performed with the MPLC system “SPOT Liquid Chromatography FLASH” of Armen Instruments. The column contained YMC*Gel ODS A RP18 material (50 g, Ø = 25 mm particle size = 50 µm, pore size = 12 nm). Preparative column chromatography was performed on Silica 60 M (0.04 - 0.063 mm). The NMR spectra were recorded on a Bruker Avance HD 600 [$^1$H: 600.13 MHz, $^{13}$C: 150.90 MHz] or Bruker Avance Neo 400 [$^1$H: 400.13 MHz, $^{13}$C: 100.61 MHz] spectrometer. All measurements were performed at room temperature, using d$_1$-chloroform, d$_6$-DMSO or d$_4$-MeOD as solvents. The chemical shifts are referenced relative to the residual proton signals of the solvents in the $^1$H-NMR spectrum (CDCl$_3$: δ = 7.24 ppm, d$_6$-DMSO: δ = 2.50 ppm, d$_4$-MeOD: δ = 3.31 ppm) or relative to the solvent signal in the $^{13}$C-NMR spectrum (CDCl$_3$: δ = 77.16 ppm, d$_6$-DMSO: δ = 39.51 ppm, d$_4$-MeOD: δ = 49.0 ppm). Coupling constants (J) are reported in Hertz (Hz). In the NMR reports: “-” indicates a single bond, “=” a double bond and “>” two single bonds. High resolution mass spectra were measured on a Bruker maXis 4G UHR-TOF.

Scheme 1: Compounds synthesized and investigated in this study.
Figure S1: Synthesis route of 1GC, 2GA, 2G and 2GC. Reagents and conditions: a) 1.) Resin, DMF, RT, 2.) Fmoc(Boc)LysOH, DIPEA, PyBOP, DMF, RT, 3 h, 3.) 1:4 piperidine:DMF, RT, 0.5 h, 4.) Fmoc(Boc/Cbz/Alloc)Lys-OH, DIPEA, PyBOP, DMF, RT, o. n.; 5.) 1:4 piperidine:DMF, RT, 0.5 h, 6.) 1, DIPEA, PyBOP, DMF, RT, 3 h, 7) DCM:TFA:TIS:H₂O 94:1.2:5:2.5, RT, 20 min.; b) 3, PyBOP, DIPEA, DCM, RT 16 h; c) 1.) TFA:DCM 1:1 RT, 1 h, 2.) HCl.
Figure S2: Synthesis route of 2RC. Reagents and conditions: a) 1.) Resin, DMF, RT, 2.) Fmoc(Boc)LysOH, Et₃N, PyBOP, DMF, RT, 4 h, 3.) 1:4 piperidine:DMF, RT, 2 x 20 min, 4.) Fmoc(Cbz)Lys-OH, Et₃N, PyBOP, DMF, RT, o. n.; 5.) 1:4 piperidine:DMF, RT, 2 x 20 min, 6.) BocArg(Pbf)-OH, Et₃N, HATU, DMF, RT, 4 h, 7) DCM:TFA:TIS:H₂O 94:1:2.5:2.5, RT, 2 x 20 min.; b) 3, PyBOP, Et₃N, DMF, RT 19 h; c) 1.) TFA:DCM 1:1 RT, 1 h, 2.) 5 x 0.05 M HCl lyophilisation.
In a 100 ml SPPS vessel, 1.00 eq. 2-chlorotrityl resin (loading 1.55 mmol/g) was suspended in 20 ml DMF and swollen at room RT (room temperature) for 2 h. Then 2.00 - 3.00 eq. of the first amino acid and 6.00 eq. DIPEA were added and at RT shaken overnight. The solvent was suctioned off. The resin was washed with 20 ml MeOH for 10 min and then three times with 20 ml DMF for 5 min. Fmoc deprotection took place with 20 ml of a 1:4 piperidine:DMF mixture. The resin was treated with this solution twice, for 20 minutes. Subsequently the resin was washed five times for 5 min. with 20 ml DMF. 2.00-3.00 eq. of the 2nd amino acid, 2.10 - 3.00 eq. PyBOP and 6.00 eq. DIPEA were added to the resin and dissolved with 20 ml DMF. After the solution was shaken for 3 h, the solution was suctioned off. The Fmoc protective group was then cleaved analogously to the first amino acid and washed five times, for 5 min. with 20 ml DMF. 20 ml DCM and then dried. 10 ml of a 94:1:2.5:2.5 DCM:TFA:TIS:H₂O mixture was added to the resin and shaken for 20 minutes. The resin was then washed three times with 20 ml of DCM. The filtrate was freed from the solvent under reduced pressure and dispersed twice with 20 ml of DCM. The product was then suspended in 20 ml Et₂O and filtered. No further purification of the product was conducted.

**General Procedure B (GP-B)**

1.00 eq. of a primary amine, 2.20 eq. of a carboxylic acid, 2.50 eq. PyBOP and 5.00 eq. DIPEA were solved in DCM. The reaction mixture was stirred at RT for 16 h. The solution became cloudy during the reaction time. The precipitate was filtered and washed with DCM and dried on a rotary evaporator.

**General Procedure C (GP-C)**

10 ml of a 1:1 TFA:DCM mixture was added to the Boc-protected compound and stirred at RT for 1 h. Afterwards the crude product was precipitated with Et₂O and filtered. The crude product was dried on a rotary evaporator and purified via MPLC. After purification, 150 ml of a 3 M hydrochloric acid solution was added to the product. The solvent was distilled off to dryness on a rotary evaporator. This procedure was repeated once. The product was taken up in water and lyophilized.
**Compound 1**

The synthesis of 1 was carried out according to a known literature procedure. All spectra obtained were in accordance with those reported beforehand.  

**Compound 2**

Compound 2 was synthesized according to GP-A. 1.00 g (1.55 mmol/g, 1.00 eq.) 2-chlorotrityl resin, 2.18 g (4.65 mmol, 3.00 eq.) Fmoc-Lys(Boc)-OH, 2.10 g (4.64 mmol, 2.99 eq.) Fmoc-Lys(Alloc)-OH, 1.85 g (4.64 mol, 2.99 eq.) Boc-GCP-OH, 2x 2.42 g (4.65 mmol, 3.00 eq.) PyBOP and 3x 1.60 ml (9.19 mmol, 5.93 eq.) DIPEA were used during the SPPS. 1.05 g (1.43 mmol, 92% based on the resin loading) of 2 as white solid were isolated. **RF** = 0.68 (MeOH:DCM 1:9), **mp:** 127°C (decomposition).

**1H-NMR** (400 MHz, DMSO-d6) δ [ppm] = 11.65 (s, 2H, -NH-), 9.32 (s, 1H, -NH-), 8.57 (s, 1H, -NH-), 3.18 – 2.90 (m, 8H, -C=H), 6.72 (s, 2H, -N=C=), 5.87 (ddt, J = 10.5, 7.6 Hz, 1H, -C=H), 3.04 – 2.91 (m, 2H, -CH2-), 1.80 – 1.65 (m, 2H, -CH2-), 1.65 – 1.52 (m, 2H, -CH2-), 1.46 (s, 9H, -CH3), 1.43 – 1.26 (m, 17H, -CH3 + -CH2-).  

**13C-NMR** (101 MHz, DMSO-d6) δ [ppm] = 173.6 (s, -C(O)OH), 171.9 (s, >C=O), 159.4 (s, >C=O), 155.9 (s, >C=O), 155.5 (s, >C=O), 133.9 (s, -CH3), 116.8 (s, C=CH2), 113.6 (s, -CH3), 112.9 (s, -CH3), 77.4 (s, -C(CH3)3), 64.1 (s, O-CH2-), 52.5 (s, >CH), 51.9 (s, >CH), 31.7 (s, -CH2-), 30.6 (s, -CH2-), 29.2 (s, -CH2-), 29.1 (s, -CH2-), 28.3 (s, -CH3), 27.8 (s, -CH3), 22.9 (s, -CH2-), 22.8 (s, -CH2-).  

**IR (ATR):** [cm⁻¹] = 3315 (NH), 3192 (NH), 3091 (CH arom.), 2978 (CH aliph.), 2933 (CH aliph.), 2866 (CH aliph.), 1687 (C=O), ESI-HRMS m/z (%): 737 [M+H]+ (100), 269 [M-C10H10O2]+ (52), 759 [M+Na]+ (9), 637 [M-C5H7O2]+ (4). Calculated for C33H52O11 [M+H]+: 737.3828; found: 737.3836.

**Compound 4**

0.10 g (0.69 mmol, 1.00 eq.) 3, 1.12 g (1.53 mmol, 2.20 eq.) 2, 0.90 g (1.73 mmol, 2.49 eq.) PyBOP and 0.60 ml (3.44 mmol, 4.97 eq.) DIPEA in 35 ml DCM were implemented according to GP-B. The crude product was purified by column chromatography. Column: V(SiO2) = 300 ml, Ø = 4 cm, eluent = 1:19 MeOH:DCM. 0.76 g (0.48 mmol, 69%) of slightly yellow solid were isolated. **RF** = 0.62 (MeOH/DCM 1:9), **mp:** 125°C (decomposition).

**1H-NMR** (400 MHz, DMSO-d6) δ [ppm] = 11.64 (s, 2H, -NH-), 10.88 (s, 2H, -NH-), 9.33 (s, 2H, -NH-), 8.58 (s, 2H, -NH-), 8.41 (d, J = 7.2 Hz, 2H, -NH-), 7.96 (d, J = 7.8 Hz, 2H, -NH-), 7.76 (s, 2H, -NH-), 7.16 (t, J = 5.0 Hz, 2H, -NH-), 6.83 (s, 4H, =CH-), 6.72 (s, 2H, -NH-), 5.87 (ddt, J = 16.0, 10.5, 5.3 Hz, 2H, =CH3), 5.24 (d, J = 17.2 Hz, 2H, C=CH2), 5.13 (d, J = 10.4 Hz, 2H, C=CH2), 4.54 – 4.29 (m, 6H, -CH2- + >CH2-), 4.25 – 4.06 (m, 2H, >CH3), 3.18 – 2.90 (m, 8H, -CH2-), 2.90 – 2.76 (m, 4H, -CH2-), 1.78 – 1.55 (m, 6H, -CH2- + -CH2-), 1.53 – 1.42 (m, 20H, -CH2- + -CH2-), 1.43 – 1.27 (m, 34H, -CH3 + -CH2-), 1.26 – 1.11 (m, 12H, -CH2-).  

**13C-NMR** (101 MHz, DMSO-d6) δ [ppm] = 171.6 (s,
Compound 2GA

1.00 g (0.63 mmol, 1.00 eq.) 4 were implemented according to GP-C. The product was purified via MPLC (gradient: 40:60 → 70:30 MeOH:H₂O, 100 min.). 0.18 g (0.11 mmol, 17%) of a white solid were isolated. 

**mp.** 92 - 95°C.

1H-NMR (400 MHz, DMSO- d₆) δ [ppm] = 12.54 (s, 2H) a-H, 12.15 (s, 2H) b-H, 8.98 – 8.28 (m, 10H) c-H, 8.14 (d, J = 8.1 Hz, 2H) d-H, 8.04 – 7.72 (m, 8H) e-H, 7.59 (s, 2H) f-H, 7.19 (t, J = 5.6 Hz, 2H) g-H, 6.90 (d, J = 2.7 Hz, 2H) h-H, 5.95 – 5.79 (m, 2H) i-H, 5.24 (dd, J = 17.2, 1.6 Hz, 2H) j-H, 5.14 (dd, J = 10.5, 1.4 Hz, 2H) k-H, 4.51 – 4.33 (m, 6H) l-H, 4.25 – 4.09 (m, 2H) m-H, 3.12 – 2.85 (m, 8H) n-H, 2.81 – 2.63 (m, 4H) o-H, 1.80 – 1.59 (m, 6H) p-H, 1.59 – 1.46 (m, 6H) q-H, 1.46 – 1.25 (m, 16H) r-H, 1.21 (s, 8H) s-H.

13C-NMR (101 MHz, DMSO-d₆) δ [ppm] = 13C-NMR (101 MHz, DMSO)
δ [ppm] = 171.5 (s) a, 171.2 (s) b, 159.8 (s) c, 159.0 (s) d, 155.9 (s) e, 155.6 (s) f, 133.9 (s) g, 132.3 (s) h, 125.6 (s) i, 116.8 (s) j, 115.8 (s) k, 113.6 (s) l, 64.1 (s) m, 53.2 (s) n, 52.4 (s) o, 40.2 (s) p, 38.5 (s) q, 38.4 (s) r, 31.5 (s) s, 31.4 (s) t, 29.2 (s) u, 29.0 (s) v, 28.7 (s) w, 26.5 (s) x, 26.3 (s) y, 22.9 (s) z, 22.3 (s) α.

IR (ATR): [cm⁻¹] = 3280 (NH), 3099 (NH), 2929 (NH), 2860 (NH), 1693 (C=O). 

ESI-HRMS m/z (%): 791 [M+2H]²⁺ (100), 741 [M-C₅H₇O₂]²⁺ (18), 1582 [M+H]⁺ (5). Calculated for C₇₄H₁₂₁N₁₈O₂₀ [M+H]⁺: 1581.8999; found: 1581.8830.

[Diagram of molecule not included in text]
**Compound 5**

Compound 5 was synthesized according to GP-A. 1.00 g (1.55 mmol/g, 1.00 eq.) 2-chlorotrityl resin, 2x 1.45 g (3.09 mmol, 2.00 eq.) Fmoc-Lys(Boc)-OH, 1.24 g (3.11 mol, 2.01 eq.) Boc-GCP-OH, 2x 1.69 g (3.25 mmol, 2.10 eq.) PyBOP and 3x 1.60 ml (9.19 mmol, 5.93 eq.) DIPEA were used during the SPPS. 1.12 g (1.49 mmol, 96% based on the resin loading) of 5 as white solid were isolated. **RF = 0.59** (MeOH/DCM 1:9). **mp:** 187 - 190°C

\(^{1}\text{H-NMR} (400 \text{ MHz, DMSO-d}_6) \delta [ppm] = 12.32 – 11.17 (m, 2H, -NH), 9.37 (s, 1H, -NH), 8.56 (s, 1H, -NH), 8.41 (d, J = 8.0 Hz, 1H, -NH), 8.20 (d, J = 7.6 Hz, 1H, -NH), 6.88 (s, 1H, =CH), 6.86 – 6.81 (m, 1H, =CH), 6.80 – 6.70 (m, 2H, =NH), 4.53 – 4.38 (m, 1H, >CH), 4.20 – 4.06 (m, 1H, >CH), 3.00 – 2.78 (m, 4H, -CH\(_2\)), 1.77 – 1.65 (m, 2H, -CH\(_2\)), 1.64 – 1.52 (m, 2H, -CH\(_2\)), 1.46 (s, 9H, -CH\(_3\)), 1.42 – 1.27 (m, 26H, -CH\(_3\) + -CH\(_2\)). \(^{13}\text{C NMR} (101 \text{ MHz, DMSO-d}_6) \delta [ppm] = 173.5 (s, -(O)OH), 172.0 (s, >C=O), 159.3 (s, >C=O), 156.6 (s, >C=O), 113.4 (s, =CH), 113.0 (s, =CH), 77.3 (s, -(CH\(_3\))\(_2\)), 52.5 (s, >CH), 51.9 (s, >CH), 31.7 (s, -CH\(_2\)), 30.6 (s, -CH\(_2\)), 29.3 (s, -CH\(_2\)), 29.1 (s, -CH\(_2\)), 28.3 (s, -CH\(_3\)), 27.8 (s, -CH\(_3\)), 22.9 (s, -CH\(_3\)), 1.51 – 1.42 (m, 20H, -CH\(_2\) + -CH\(_3\)), 1.41 – 1.09 (m, 64H, -CH\(_3\) + -CH\(_2\)).

IR (ATR): [cm\(^{-1}\)] = 3386 (NH), 3284 (NH), 3082 (CH aliph.), 2978 (CH aliph.), 2931 (CH aliph.), 2860 (CH aliph.), 1687 (C=O). \(^{31}\text{P HRMS} m/z (\%): 753 [M+H\(^{+}\)](100), 227 [M-C\(_{10}H_{22}O_3\)]\(^{2+}\) (26), 653 [M-C\(_{5}H_{2}O_2\)]\(^{+}\) (6), 453 [M-C\(_{10}H_{15}O_4\)]\(^{+}\) (3), 553 [M-C\(_{10}H_{15}O_4\)]\(^{+}\) (1). Calculated for C\(_{55}H_{57}NaO\(_{11}\)) [M+H\(^{+}\)]\(^{+}\): 753.4141; found: 753.4160.

**Compound 6**

0.14 g (1.94 mmol, 0.97 eq.) 3, 1.54 g (2.05 mmol, 2.10 eq.) 5, 1.27 g (2.44 mmol, 2.50 eq.) PyBOP and 0.85 ml (4.88 mmol, 5.01 eq.) DIPEA in 60 ml DCM were implemented according to GP-B. No further purification was performed. 0.84 g (0.52 mmol, 54%) of a white solid was isolated. **RF = 0.49** (MeOH/DCM 1:9). **mp:** 140°C (decomposition).

\(^{1}\text{H-NMR} (400 \text{ MHz, DMSO-d}_6) \delta [ppm] = 11.08 (s, 4H, -NH), 9.33 (s, 2H, -NH), 8.77 – 8.34 (m, 4H, -NH), 8.01 (d, J = 7.9 Hz, 2H, -NH), 7.83 – 7.74 (m, 2H, -NH), 6.84 – 6.78 (m, 4H, -CH), 6.78 – 6.66 (m, 4H, -NH), 4.40 – 4.29 (m, 2H, >CH), 4.22 – 4.06 (m, 2H, >CH), 3.09 – 2.93 (m, 4H, -CH\(_2\)), 2.93 – 2.75 (m, 8H, -CH\(_2\)), 1.75 – 1.55 (m, 6H, -CH\(_2\) + -CH\(_2\)), 1.53 – 1.42 (m, 20H, -CH\(_2\) + -CH\(_3\)), 1.41 – 1.09 (m, 64H, -CH\(_3\) + -CH\(_2\)).

\(^{13}\text{C NMR} (101 \text{ MHz, DMSO-d}_6) \delta [ppm] = 171.7 (s, >C=O), 171.2 (s, >C=O), 165.8 (s, >C=O), 158.5 (s, >C=O), 155.5 (s, >C=O), 113.6 (s, =CH), 113.0 (s, =CH), 77.3 (s, -(CH\(_3\))\(_2\)), 53.2 (s, >CH), 52.6 (s, >CH), 38.5 (s, -CH\(_2\)), 31.8 (s, -CH\(_2\)), 31.5 (s, -CH\(_2\)), 29.3 (s, -CH\(_2\)), 29.2 (s, -CH\(_2\)), 29.0 (s, -CH\(_2\)), 28.7 (s, -CH\(_2\)), 28.3 (s, -CH\(_3\)), 27.8 (s, -CH\(_3\)), 26.3 (s, -CH\(_2\)), 23.0 (s, -CH\(_2\)), 22.7 (s, -CH\(_2\)). IR (ATR): [cm\(^{-1}\)] = 3386 (NH), 3284 (NH), 3082 (CH aliph.), 2978 (CH aliph.), 2931 (CH aliph.), 2860 (CH aliph.), 1687 (C=O). \(^{31}\text{P HRMS} m/z (\%): 807 [M+H\(^{+}\)](100), 372 [M-C\(_{5}H_{2}O_2\)]\(^{2+}\) (13), 757 [M-C\(_{5}H_{2}O_2\)]\(^{2+}\) (12), 707 [M-C\(_{10}H_{15}O_4\)]\(^{2+}\) (1). Calculated for C\(_{76}H_{138}N_{18}O_{20}\) [M+2H\(^{2+}\)]\(^{2+}\): 807.4849; found: 807.4875.
**Compound 2G**

0.12 g (0.07 mmol, 1.00 eq.) 6 were implemented according to GP-C. The product was purified via MPLC (gradient: 20:80 → 50:50 MeOH:H₂O, 100 min.). 0.03 g (0.02 mmol, 24%) of a white solid were isolated. Rf HPLC RP18 (Gradient 10:90 → 100:0 MeOH:H₂O, 30 min.) = 12.5 min. mp.: 92 - 95°C.

**H-NMR** (600 MHz, DMSO-d₆) δ [ppm] = 12.55 (s, 2H) a-H, 12.20 (s, 2H) b-H, 8.81 – 8.49 (m, 10H) c-H, 8.19 (d, J = 8.1 Hz, 2H) d-H, 7.98 (s, 12H) e-H, 7.93 (t, J = 5.4 Hz, 2H) f-H, 7.65 – 7.59 (m, 2H) g-H, 6.93 (dd, J = 3.8, 2.2 Hz, 2H) h-H, 4.47 – 4.39 (m, 2H) i-H, 4.21 – 4.16 (m, 2H) j-H, 3.09 – 2.96 (m, 4H) k-H, 2.79 – 2.70 (m, 8H) l-H, 1.81 – 1.73 (m, 2H) m-H, 1.72 – 1.61 (m, 6H) n-H, 1.61 – 1.50 (m, 10H) o-H, 1.44 – 1.27 (m, 12H) p-H, 1.21 (s, 8H) q-H.

**C-NMR** (151 MHz, DMSO-d₆) δ [ppm] = 171.4 (s) a, 171.2 (s) b, 159.7 (s) c, 159.1 (s) d, 155.6 (s) e, 132.4 (s) f, 125.6 (s) g, 115.9 (s) h, 113.6 (s) i, 53.0 (s) j, 52.5 (s) k, 38.5 (s) l, 38.4 (s) m, 31.3 (s) n, 31.1 (s) o, 29.0 (s) p, 28.7 (s) q, 26.5 (s) r, 26.4 (s) s, 26.3 (s) t, 22.5 (s) u, 22.3 (s) v.

**IR (ATR):** [cm⁻¹] = 3311 (NH), 3235 (NH), 3045 (CH arom.), 2929 (CH aliph.), 1687 (C=O). **ESI-HRMS** m/z (%): 254 [M+4H]^4+ (100), 339 [M+3H]^3+ (98), 507 [M+2H]^2+ (20). Calculated for C₄₆H₆₂N₁₈O₈ [M+2H]^2+: 507.3276; found: 507.3278.

**Compound 7**

Compounds 7 was synthesized according to GP-A. 1.00 g (1.55 mmol/g, 1.00 eq.) 2-chlorotriyl resin, 2.28 g (4.87 mmol, 3.14 eq.) Fmoc-Lys(Boc)-OH, 2.38 g (4.74 mmol, 3.06 eq.) Fmoc-Lys(Cbz)-OH, 1.85 g (4.64 mol, 3.00 eq.) Boc-GCP-OH, 2x 2.42. g (4.65 mmol, 3.00 eq.) PyBOP and 3x 1.60 ml (9.19 mmol, 5.93 eq.) DIPEA were used during the SPPS. 1.11 g (1.41 mmol, 91% based on the resin loading) of 7 as white solid were isolated. RF = 0.64 (MeOH:DCM 1:9). mp.: 90°C (decomposition).

**H-NMR** (400 MHz, DMSO-d₆) δ [ppm] = 11.65 (s, 2H, -NH-), 9.32 (s, 1H, -NH-), 8.57 (s, 1H, -NH-), 8.41 (d, J = 8.0 Hz, 1H, -NH-), 8.19 (d, J = 7.6 Hz, 1H, -NH-), 7.39 –
7.26 (m, 5H, =CH-), 7.23 (t, J = 5.6 Hz, 1H, -NH-), 6.89 – 6.79 (m, 2H, =CH-), 6.76 (t, J = 5.5 Hz, 1H, -NH-), 5.01 (s, 2H, O-CH₂-), 4.46 (td, J = 8.7, 5.1 Hz, 1H, >CH-), 4.17 – 4.06 (m, 1H, >CH-), 3.03 – 2.92 (m, 2H, -CH₂-), 2.92 – 2.82 (m, 2H, -CH₂-), 1.78 – 1.64 (m, 2H, -CH₂-), 1.64 – 1.52 (m, 2H, -CH₂-), 1.46 (s, 9H, -CH₃), 1.43 – 1.24 (m, 17H, -CH₃ + -CH₂-). ¹³C-NMR (101 MHz, DMSO-d₆) δ [ppm] = 173.6 (s, -C(O)OH), 171.9 (s, >C=O), 159.4 (s, >C=O), 158.5 (s, >C=O), 156.0 (s, >C=O), 155.6 (s, >C=O), 137.3 (s, >C=C), 128.3 (s, >CH=CH), 127.7 (s, -CH=), 113.7 (s, =CH-), 112.9 (s, =CH=), 77.4 (s, -C(CH₃)₂), 65.1 (s, O-CH₂-), 52.5 (s, >CH=CH), 51.9 (s, >CH=CH), 31.7 (s, -CH₂-), 30.7 (s, -CH₂-), 29.2 (s, -CH₂-), 29.1 (s, -CH₃), 28.3 (s, -CH₃), 27.8 (s, -CH₂-), 22.9 (s, -CH₂-), 22.8 (s, -CH₂-).

IR (ATR): [cm⁻¹] = 3315 (NH), 3182 (NH), 3086 (CH arom.), 2978 (CH aliph.), 2933 (CH aliph.), 2866 (CH aliph.), 1687 (C=O).

ESI-HRMS m/z (%): 787 [M+H]⁺ (100), 687 [M-C₅H₇O₂]⁺ (17), 453 [M-C₈H₁₂O₄]⁺ (12). Calculated for C₃₇H₅₅N₈O₁₁ [M+H]⁺: 787.3985; found: 787.3978.

**Compound 1GC**

1.00 g (1.27 mmol, 1.00 eq.) 7 were implemented according to GP-C. The product was cleaned via MPLC (gradient: 30:70 → 60:40 MeOH:H₂O (acidified with 1‰ TFA), 80 min.). 0.13 g (0.18 mmol, 15%) of a white solid was isolated. Rₜ HPLC RP18 (Gradient 10:90 → 100 MeOH:H₂O (acidified with 1‰ TFA), 30 min.) = 17.5 min. mp.: 170°C (decomposition).

¹H-NMR (600 MHz, MeOD-d₄) δ [ppm] = 7.34 – 7.30 (m, 4H) a-H, 7.30 – 7.25 (m, 1H) b-H, 7.24 (d, J = 4.1 Hz, 1H) c-H, 7.00 (d, J = 4.1 Hz, 1H) d-H, 5.04 (s, 2H) e-H, 4.52 – 4.42 (m, 2H) f-H, 3.14 (t, J = 6.7 Hz, 2H) g-H, 2.93 (t, J = 7.5 Hz, 2H) h-H, 2.01 – 1.87 (m, 2H) i-H, 1.87 – 1.73 (m, 2H) j-H, 1.73 – 1.62 (m, 2H) k-H, 1.61 – 1.44 (m, 6H) l-H.

¹³C-NMR (151 MHz, MeOD-d₄) δ [ppm] = 175.1 (s) a, 174.8 (s) b, 162.2 (s) c, 161.5 (s) d, 159.2 (s) e, 157.4 (s) f, 138.6 (s) g, 133.5 (s) h, 129.6 (s) i, 129.1 (s) j, 128.8 (s) k, 127.3 (s) l, 116.5 (s) m, 114.3 (s) n, 67.5 (s) o, 55.4 (s) p, 53.1 (s) q, 41.6 (s) r, 40.7 (s) s, 32.6 (s) t, 32.1 (s) u, 30.7 (s) v, 27.9 (s) w, 24.3 (s) x, 23.9 (s) y.

IR (ATR): [cm⁻¹] = 3321 (NH), 3074 (CH arom.), 2943 (CH aliph.), 2873 (CH aliph.), 1662 (C=O). ESI-HRMS m/z (%): 294 [M+2H]²⁺ (100), 453 [M+C₅H₇O₂]⁺ (49), 587 [M+H]⁺ (32). Calculated for C₂₇H₃₉N₉O₁₁ [M+H]⁺: 587.2936; found: 587.2943.
Compound 8

0.28 g (1.94 mmol, 1.00 eq.) 3, 3.21 g (4.08 mmol, 2.10 eq.) 7, 2.52 g (4.84 mmol, 2.50 eq.) PyBOP and 1.00 ml (5.74 mmol, 2.96 eq.) DIPEA in 60 ml DCM were implemented according to GP-B. No further purification was performed. 1.72 g (1.02 mmol, 53%) of a white solid was isolated. RF = 0.59 (MeOH:DCM 1:9). mp.: 149°C (decomposition).

\[ ^1H-NMR \ (400 \text{ MHz, DMSO-d}_6) \delta \ [ppm] = 11.72 (s, 2H, -NH), 10.88 (s, 2H, -NH), 9.34 (s, 2H, -NH), 8.51 (s, 2H, -NH), 8.41 (d, J = 7.4 Hz, 2H, -NH), 7.96 (d, J = 7.9 Hz, 2H, -NH), 7.76 (s, 2H, -NH), 7.40 – 7.25 (m, 10H, =CH), 7.23 (t, J = 5.4 Hz, 2H, -NH), 6.83 (s, 4H, =CH), 6.72 (s, 4H, -CH), 4.47 – 4.31 (m, 2H, >CH), 4.20 – 4.07 (m, 2H, >CH), 3.12 – 2.91 (m, 8H, -CH), 2.91 – 2.78 (m, 4H, -CH), 1.81 – 1.56 (m, 6H, -CH + -CH), 1.56 – 1.44 (m, 20H, -CH + -CH), 1.43 – 1.26 (m, 34H, -CH), 1.21 (s, 12H, -CH).

\[ ^13C-NMR \ (101 \text{ MHz, DMSO-d}_6) \delta \ [ppm] = 171.6 (s, >C=O), 171.2 (s, >C=O), 159.6 (s, >C=O), 158.5 (s, >C=O), 156.1 (s, >C=O), 155.6 (s, >C=O), 137.3 (s, >C=C), 128.3 (s, >CH), 127.7 (s, >CH), 113.0 (s, >CH), 77.3 (s, -CH(C(CH3)3), 65.1 (s, O-CH2), 53.1 (s, >CH), 52.6 (s, >CH), 40.2 (s, -CH2), 38.5 (s, -CH2), 31.8 (s, -CH2), 31.5 (s, -CH2), 29.3 (s, -CH2), 29.2 (s, -CH2), 29.0 (s, -CH2), 28.8 (s, -CH2), 28.3 (s, -CH3), 27.8 (s, -CH3), 26.4 (s, -CH2), 23.0 (s, -CH2), 22.8 (s, -CH2).

IR (ATR): [cm\(^{-1}\)] = 3298 (NH), 3066 (CH arom.), 2974 (CH aliph.), 2931 (CH aliph.), 1693 (C=O)。

ESI-HRMS m/z (%): 841 [M+2H]\(^+\) (100), 1682 [M+H]\(^+\) (3). Calculated for C\(_{82}H_{125}N_{18}O_{20}\) [M+H]\(^+\): 1681.9312; found: 1681.9323.

Compound 2GC

0.20 g (0.12 mmol, 1.00 eq.) 8 were implemented according to GP-C. The product was purified via MPLC (gradient: 40:60 → 70:30 MeOH:H2O, 100 min.). 0.09 g (0.06 mmol, 44%) of a white solid were isolated. Rf HPLC RP18 (Gradient 10:90 → 100:0 MeOH:H2O, 30 min.) = 21.6 min. mp.: 156 - 159°C.

\[ ^1H-NMR \ (600 \text{ MHz, DMSO-d}_6) \delta \ [ppm] = 12.54 (s, 2H) a-H, 12.14 (s, 2H) b-H, 8.78 – 8.43 (m, 10H) c-H, 8.14 (d, J = 8.1 Hz, 2H) d-H, 7.88 (s, 6H) e-H, 7.84 (t, J = 5.4 Hz, 2H) f-H, 7.59 (s, 2H) g-H, 7.38 – 7.27 (m, 10H) h-H, 7.25 (t, J = 5.7 Hz, 2H) i-H, 6.92 – 6.86 (m, 2H) j-H, 5.00 (s, 4H) k-H, 4.46 – 4.36 (m, 2H) l-H, 4.22 – 4.15 (m, 2H) m-H, 3.09 – 2.94 (m, 8H) n-H, 2.76 – 2.68 (m, 4H) o-H, 1.76 – 1.69 (m, 4H) p-H, 1.69 – 1.59 (m, 4H) q-H, 1.56 – 1.47 (m, 6H) r-H, 1.47 – 1.39 (m, 4H) s-H, 1.38 – 1.25 (m, 12H) t-H, 1.23 – 1.13 (m, 8H) u-H.
\[1^3\text{C-NMR} \quad (151 \text{ MHz, DMSO-d}_6) \delta [\text{ppm}] = 171.5 \text{ (s, a), 171.2 (s, b), 159.8 (s, c), 159.0 (s, d), 156.1 (s, e), 155.6 (s, f), 137.3 (s, g), 132.3 (s, h), 128.3 (s, i), 127.7 (s, j), 127.6 (s, k), 125.6 (s, l), 115.8 (s, m), 113.6 (s, n), 65.1 (s, o), 53.2 (s, p), 52.4 (s, q), 40.2 (s, r), 38.5 (s, s), 38.4 (s, t), 31.5 (s, u), 31.4 (s, v), 29.2 (s, w), 29.0 (s, x), 28.7 (s, y), 26.5 (s, z), 22.9 (s, \beta), 22.3 (s, \alpha).\]

\[\text{IR (ATR): \quad [cm}^{-1}] = 3275 (\text{NH}), 3091 (\text{CH arom.}), 3066 (\text{CH arom.}), 3035 (\text{CH arom.}), 2927 (\text{CH aliph.}), 2858 (\text{CH aliph.}), 1687 (\text{C=O}).\]

\[\text{ESI-HRMS} \quad \text{m/z (%): 428 [M+3H]}^+ (100), 383 [(\text{M+3H})-%C_8\text{H}_6\text{O}_2]^+ (53), 641 [\text{M+2H}]^2+ (29), 507 [(\text{M+2H})-%C_{16}\text{H}_{12}\text{O}_4]^2+ (9), 321 [\text{M+4H}]^4+ (5), 1182 [\text{M+H}]^+ (2). \text{Calculated for C}_{62}\text{H}_{94}\text{N}_{18}\text{O}_{12}[\text{M+2H}]^2+: 641.3644; \text{found: 641.3593}.\]

**Compound 9**

Compound 9 was synthesised according to GP-A. 1.00 g (1.55 mmol, 1.00 eq.) 2-chlorotryptil resin was used for the synthesis and 1.00 mL triethylamine (0.7 mmol, 4.62 eq.) were added for every coupling step. For the first step 2.28 g (4.74 mmol, 3.06 eq.) Fmoc-Lys(Boc)-OH, for the second step 2.38 g (4.64 mmol, 3.06 eq.) Fmoc-Lys(Z)-OH with 2.42 g (4.65 mmol, 3.00 eq.) PyBOP and for the last step 2.44 g (4.64 mmol, 3.00 eq.) Boc-Arg(Pbf)-OH and 1.77 g (4.65 mmol, 3.00 eq.) HATU were coupled on the resin. Yield: 0.32 g (0.32 mmol 20% based on the resin loading) The product was isolated without further purification. \(\text{RT} = 0.8 \text{ (MeOH:DCM 1:9). mp.: 114}^\circ \text{C (decomposition).}\)

\[\text{'H-NMR} \quad (400 \text{ MHz, CDCl}_3) \delta [\text{ppm}] = 8.04 - 8.35 (m, 1 H, -NH), 7.45 - 8.01 (m, 3, -NH), 7.21 - 7.37 (m, 6 H, =C=O), 6.09 - 6.74 (m, 1 H, =N), 5.42 - 6.04 (m, 2 H, -NH), 5.05 (br s, 2 H, -C=O, =C=O), 4.04 - 4.57 (m, 3 H, -C=O, =C=O), 2.77 - 3.37 (m, 8 H, -C=O, =C=O), 2.03 - 2.11 (m, 2 H, -C=O, =C=O), 1.65 - 1.94 (m, 6 H, -C=O, =C=O), 1.29 - 1.63 (m, 24 H, -C=O, =C=O).\]

\[\text{IR (ATR): \quad [cm}^{-1}] = 3329 (\text{NH}), 3066 (\text{CH arom.}), 2935 (\text{CH aliph.}), 2868 (\text{CH aliph.}), 1654 (\text{N-C=O}), 1540 (\text{O-C=O}).\]

\[\text{ESI-HRMS} \quad \text{m/z (%): 1017 [M+H]}^+ (100), 1040 [\text{M+Na}]^+ (51). \text{Calculated for C}_{45}\text{H}_{76}\text{N}_{13}\text{O}_{13}\text{S}[\text{M+H}]^+: 1017.5325; \text{found: 1017.5329}.\]
Compound 10

11.4 mg octanediameine (0.10 mmol, 1 eq.) 200.0 mg of Compound 9 (0.20 mmol, 2 eq.), 102.0 mg of PyBOP (0.20 mmol, 2 eq.) and 0.4 mL Et3N (2.89 mmol, 30 eq.) were dissolved in 10.0 mL DMF and stirred for 19 h. The solvent was added to 100.0 mL of ice cold water. The precipitate was filtered off and dried under vacuum. Yield: 83.0 mg (39.0 µmol, yield 39%) of the off-white product was obtained without further purification. RF = 0.68 (MeOH:DCM 1:9).

mp.: 125°C (decomposition).

1H-NMR (400 MHz, DMSO-d6) δ [ppm] = 7.66 - 8.10 (m, 6 H, -NH-), 7.24 - 7.40 (m, 14 H, C-Harom., -NH-), 7.12 - 7.24 (m, 2 H, -NH-), 6.80 - 7.03 (m, 2 H, -NH-), 6.57 - 6.80 (m, 2 H, -NH-), 6.19 - 6.56 (m, 2 H, -NH-), 5.76 (s, 2 H, =NH), 4.92 - 5.07 (m, 8 H, -CH2-), 4.09 - 4.32 (m, 6 H, -CH2-), 3.89 - 3.99 (m, 22 H, -CH2-), 2.89 - 3.09 (m, 22 H, -CH2-), 2.47 (s, 6 H, -CH3), 2.42 (s, 6 H, -CH3), 1.43 - 1.68 (m, 18 H, -CH2-), 1.29 - 1.43 (m, 62 H, -CH3, -CH2-), 1.09 - 1.28 (m, 16 H, -CH2-).

13C-NMR (101 MHz, DMSO-d6) δ [ppm] = 194.2 (s, =C=O), 194.1 (s, >C=O), 194.0 (s, >C=O), 193.9 (s, >C=O), 193.7 (s, >C=O), 133.7 (s, >C=O), 133.6 (s, >C=O), 130.4 (s, =CHarom.), 130.4 (s, =CHarom.), 130.32 (s, =CHarom.), 130.21 (s, =CHarom.), 130.11 (s, =CHarom.), 123.52 (s, C-CH3), 100.08 (s, >CH-), 100.0 (s, >CH-), 99.9 (s, >CH-), 69.9 (s, -CH2-), 69.8 (s, -CH2-), 69.7 (s, -CH2-), 69.6 (s, -CH2-), 67.2 (s, -CH2-), 66.9 (s, -CH2-), 66.5 (s, -CH2-), 66.4 (s, -CH2-), 66.3 (s, -CH2-), 66.3 (s, -CH2-), 10.6 (s, -CH3), 9.4 (s, -CH3), 9.3 (s, -CH3), 7.0 (s, -CH3), 6.1 (s, -CH3), 6.0 (s, -CH3), 5.9 (s, -CH3).

IR (ATR): [cm⁻¹] = 3309 (NH), 3064 (CH arom.), 2972 (CH aliph.), 2933 (CH aliph.), 2868 (CH aliph.), 1685 (C=O).

ESI-HRMS m/z (%): 1072 [M+2H]+' (100). Calculated for C106H168N18O24S2 [M+2H]+: 1072.1048; found: 1072.1053.
Compound 2RC

23 mg (0.01 mmol) of compound 10 was stirred in 10 mL of a 1:1 mixture of TFA and DCM. After 1 h, the solvent was evaporated, and the residue was poured into 12 mL of cold diethylether. The precipitate was filtered off, dried under vacuum, and purified with a preparative HPLC (80/20 H$_2$O/ACN + 0.01% TFA over 26 min $R_t$ = 8.5 min). 15 mg (<0.01 mmol, yield 78 %) of a white solid were obtained. 10 mg of the product was lyophilized 5-times with 0.05 M HCl to obtain the HCl-salt. $R_t$ HPLC RP18 (Gradient 10:90 → 100:0 ACN: H$_2$O + 0.1% TFA, 30 min.) = 13.96 min. mp.: 184°C (decomposition).

^{1}H-NMR (600 MHz, DMSO-d$_6$) δ [ppm] = 8.75 - 8.80 (d, 2H) a-H, 8.26 - 8.30 (s, 2H) b-H, 8.20 (d, 2 H) c-H, 7.95 - 8.15 (m, 12 H) d-H, 7.90 (m, 2 H) e-H, 7.25 - 7.40 (m, 10 H) f-H, 7.18 (s, 2 H) g-H, 5.0 (s, 4 H) h-H, 4.30 (m, 2 H) i-H, 4.15 (m, 2 H) j-H, 3.80 (m, 2 H) k-H, 3.15 (m, 4 H) I-H, 2.95 (m, 8 H) m-H, 2.75 (m, 8 H) n-H, 1.77 (m, 4 H) o-H, 1.60 (m, 16 H) p-H, 1.35 (m, 8 H) q-H, 1.25 (m, 12 H) r-H.

^{13}C-NMR (151 MHz, DMSO-d$_6$) δ [ppm] = 171.44 (s) a, 168.83 (s) b, 157.48 (s) Cc, 156.54 (s) d, 167.72 (s) e, 129.31 (s) f, 129.11 (s) g, 128.82 (s) g, 128.23 (s) h, 128.19 (s) h, 65.59 (s) i, 53.52 (s) j, 52.93 (s) k, 51.89 (s) l, 46.66 (s) m, 31.94 (s) o, 29.64 (s) p, 29.46 (s) p, 29.20 (s) p, 28.71 (s) p, 26.85 (s) q, 26.82 (s) q, 24.34 (s) r, 23.16 (s) s, 22.61 (s) t.

IR (ATR): [cm$^{-1}$] = 3294 (NH), 3079 (CH arom.), 3066 (CH arom.), 3032 (CH arom.), 2972 (CH aliph.), 2887 (CH aliph.), 1684 (C=O).

ESI-HRMS m/z (%): 1238 [M+H]$^+$ (100), 1104 [M+H-1Cbz]$^+$ (87 ), 970 [M+H-2Cbz]$^+$ (16). Calculated for C$_{60}$H$_{104}$N$_{18}$O$_{10}$ [M+H]$^+$: 1237.8256; found: 1237.8280.
Mass spectra of the final compounds

Figure S3: HR-ESI-MS spectrum of compound 1GC. The upper panel represents the whole spectrum, whereas the spectrum shown in the middle is a close-up and the lower panel is the calculated spectrum.

Figure S4: HR-ESI-MS spectrum of compound 2G. The upper panel represents the whole spectrum, whereas the spectrum shown in the middle is a close-up and the lower panel is the calculated spectrum.

Figure S5: HR-ESI-MS spectrum of compound 2GA. The upper panel represents the whole spectrum, whereas the spectrum shown in the middle is a close-up and the lower panel is the calculated spectrum.

Figure S6: HR-ESI-MS spectrum of compound 2GC. The upper panel represents the whole spectrum, whereas the spectrum shown in the middle is a close-up and the lower panel is the calculated spectrum.
Figure S7: HR-ESI-MS spectrum of compound 2RC. The first spectrum represents the deconvoluted spectra. Underneath this spectrum are three pairs of spectra representing the deconvoluted and simulated MS spectra of the fully Cbz-protected 2RC, the mono Cbz-protected 2RC, and the unprotected 2RC respectively. The loss of Cbz groups can be attributed to the ionization process.
NMR spectra

**Figure S8:** $^1$H-NMR of compound 1GC in MeOD-$d_4$ (600 MHz).

**Figure S9:** $^{13}$C-NMR of compound 1GC in MeOD-$d_4$ (151 MHz).
Figure S10: $^1$H-NMR of compound 2GA in DMSO-$d_6$ (400 MHz).

Figure S11: $^{13}$C-NMR of compound 2GA in DMSO-$d_6$ (101 MHz).
Figure S12: $^1$H-NMR of compound 2G in DMSO-$d_6$ (600 MHz).

Figure S13: $^{13}$C-NMR of compound 2G in DMSO-$d_6$ (151 MHz).
Figure S14: $^1$H-NMR of compound 2GC in DMSO-$d_6$ (600 MHz).

Figure S15: $^{13}$C-NMR of compound 2GC in DMSO-$d_6$ (151 MHz).
Figure S16: $^1$H-NMR of compound 2RC in DMSO-$d_6$ (600 MHz).

Figure S17: $^{13}$C-NMR of compound 2RC in DMSO-$d_6$ (151 MHz).
HPLC chromatograms

Figure S18: HPLC chromatogram of compound 1GC.

Figure S19: HPLC chromatogram of compound 2G.

Figure S20: HPLC chromatogram of compound 2GA.
Figure S21: HPLC chromatogram of compound 2GC.

Figure S22: HPLC chromatogram of compound 2RC (upper chromatogram) and the control chromatogram showing the solvents residuals between 2 and 4 minutes (lower chromatogram).
Molecular docking

The program Maestro 11.5 Schroedinger was used for docking. The following 4 ligands were prepared with LigPrep. Tautomers and possible states at pH = 7 +/- 3 were generated. The specified chiralities were retained.

![Compounds used for docking studies.](image)

The crystal structure of Taspase1 was prepared with Protein Preparation. Hydrogens, missing chains, and loops were added. Zero-order bonds to metals and disulfide bonds were created. Water was removed, which was beyond 5 Å away from het groups. Het states were created with the use of Epik (pH = 7 +/- 2). A grid around the amino acids tyr234, arg190, asp228 and ser56 with a size of (36 Å)^3 was generated with glide grid generator (active center).
Figure S24: Top: Grid created around the active centre (36 Å). Amino acids 178-197, 203-216 and 221-233 coloured in turquoise and 197-202 and 217-220 in orange. Bottom: Docking of 2RC (yellow) and 2GC (green) demonstrates the population of the same region on the Taspase loop, although 2GC binds tighter and in a more closed conformation.

The prepared ligands and grid were used for the ligand docking. The method was XP (extra precise). The molecule sampling was flexible. Additionally, the following conditions were chosen: sample nitrogen inversions and ring conformation, bias sampling of torsions for amides and add Epik state penalties to docking score. All protonation degrees were obtained using Ligprep as software.
Table S2: Docking scores calculated with Taspase1 and the corresponding ligand. Docking scores for the best conformations are indicated. The docking scores indicate how strongly a ligand can be stabilized by a certain protein in a defined volume (see pink cube, Fig. S18). The more negative the score, the higher the stabilizing energy. For 2G, two additional free lysines allow for surplus electrostatic interactions. Due to its smaller size, 1GC can access a cavity within the loop, specifically contributing to a more negative score. In contrast, 2GA, 2GC and 2RC are characterized by rather comparable structures and thus docking scores. Indeed, direct comparison reveals that lack of the GCP unit in 2RC might result in decreased binding.

| Compound | Docking Score |
|----------|---------------|
| 2G       | -9.004        |
| 1GC      | -6.832        |
| 2GA      | -6.515        |
| 2GC      | -6.370        |
| 2RC      | -5.634        |

Figure S25: A) In a 3D model, 2GC covers a large portion of the Taspase1 loop necessary for the Importin α interaction and thus indicates steric hindrance. The proenzyme comprises an α-unit (blue) and a β-unit (gray). The β-unit contains a loop region (turquoise) harboring the basic Taspase1/Importin α interface (197KRNKRK202, 217KKRR220 orange), located on two neighboring helices (see Fig. S19). B) 2D ligand-protein interaction diagram reveals a variety of possible interactions of 2GC and Taspase1 via hydrogen bonds (arrowed pink lines). Salt bridges (blue-violet lines) and solvent exposure (blurred grey circles) are also indicated together with polar (cyan), positively (red) and negatively (violet) charged as well as hydrophobic (green) amino acid residues of Taspase1 (aa52-57, 61, 198-209, 224-233). Of note, the hydrophobic cbz protecting group does not interact with the protein. C) In a 3D model of Taspase1 in complex with 1GC, the monomer only covers a small portion of the loop compared to 2GC (A). D) 2D ligand-protein interaction diagram revealing no significant interactions between 1GC and the Importin α/Taspase1 interface. The colour code is the same as in (B), again, relevant Taspase1 amino acid residues are indicated (52-57, 61, 198-209, 224-233). Of note, the cbz group is also not supposed to interact with any hydrophobic groups of Taspase1.
Biological Assays

Cloning

The plasmid for inactive mutant Taspase1_{D233/T234A} was generated as previously described.[3] The plasmid for wildtype Taspase1 was generated as previously described.[4] The plasmid for USF2-GFP was generated as previously described.[5] The gene for Importin α was amplified from a previously described pcDNA3-Importin α-HA plasmid by PCR.[6] During amplification, the ends were modified to introduce ApaI/BamHI restriction sites. Following subcloning into a blunt pJET1.2 vector (Thermo Fisher) according to the CloneJET PCR cloning kit (Thermo Fisher) and plasmid amplification and purification, the Importin α open reading frame was cloned into a modified pET-41b vector containing an N-terminal GST (Glutathione S-transferase) affinity fusion tag and a PreScission protease cleavage site (GeneArt) via ApaI/BamHI. The sequence was verified by sequencing (LCG Genomics).

Purification of recombinant proteins

pET22b-Taspase1_{D233A/T234A}-His and pET22b-WT_Taspase1-His were expressed in E. coli BL21 (DE3). Cells were lysed using ultrasonic sheering and enzymatic lysis with lysozyme. The protein was purified using the His tag for affinity chromatography with a HiTrap FF column (GE Healthcare). Following imidazole elution, Taspase1-His-containing fractions were pooled and loaded onto a Superdex 200 HiLoad16/600 column (GE Healthcare) for size exclusion chromatography. pET41-GST-PreScn-Importin α was expressed in E. coli BL21 (DE3), cells were lysed using sonication and enzymatic lysis with lysozyme, and the soluble fraction obtained with centrifugation and filtration. The protein was purified using the GST tag for affinity chromatography with a glutathione sepharose GSTrap 4B column (GE Healthcare). Following glutathione elution, the GST-Importin α-containing fractions were pooled and loaded onto a Superdex 200 HiLoad16/600 column (GE Healthcare) for size exclusion chromatography. GST-Importin α-containing fractions were pooled, frozen in liquid nitrogen and stored at -20°C.

Pull-down assay

All solutions were prepared with Dulbecco’s Phosphate Buffered saline (Sigma-Aldrich) containing 0.1% (v/v) Triton X-100 (Carl Roth) and 1 mM DTT (Carl Roth) (PBST). All incubation steps were carried out at 4°C to preserve the proteins and all centrifugation steps were carried out at 500 xG. Samples taken for later analysis were mixed with 5x sample buffer and heated to 95°C for 5 min. 50 µM Glutathione Sepharose 4B (Merck) were transferred to a Spin Column (IBA Lifescience), equilibrated with 500 µL PBST followed by centrifugation. As already described,[7] 500 µL 2.5 µM GST-Importin α were added to the column, a sample from the “input” fraction was retained, and the column was incubated for 2 h on a rotator. Samples from each “input” fraction enable to control equal loading of the column and are thus prerequisite to later allow comparative quantification of protein amounts. Unbound protein was removed by three washing steps with PBST followed by centrifugation. 500 µL 2.2 µM inactive Taspase1_{D233/T234A}-His were pre-incubated with the respective concentration of compound on a rotator for 1 h, and again, a sample for the “input” fraction was retained. The free binding sites on the column were blocked with 1% (w/v) BSA (Carl Roth) in PBST for 30 min on a rotator. The blocking solution was removed from the column by centrifugation for 1 min. Subsequently, Taspase1-His pre-incubated with the compound was added to the column and allowed to bind on a rotator for 1 h. Analogously, a sample from the “unbound” fraction was retained, and unbound protein was removed by three washing steps with PBST followed by centrifugation for 1 min. Samples from the “unbound” fraction allow to compare protein amounts with the “input” fraction to avoid unwanted deviation and thus serve as additional assay control. Generally spoken, the “input”, “bound”/“eluted” and “unbound” fractions always have to add up to the same total amount of protein initially used for the assay. Finally, 500 µL 1x sample buffer were added to the column and heated to 95°C for 10 min. Proteins were eluted by centrifugation for 2 min.
SDS-PAGE and Immunoblotting

Here, we used the standard protocols for SDS-PAGE according to Laemmli,[8] and for Immunoblotting according to Towbin.[9] Briefly, for SDS-PAGE, Tris-glycine gels with 7.5% or 10% (v/v) acrylamide in the stacking gel and 4% (v/v) acrylamide in the separating gel were prepared accordingly. For electrophoresis, we used the TetraCell system (BioRad) set to 200 V for 45 min. Proteins were then transferred to a protein-binding membrane using a wet blot tank (Peqlab) set to 360 mA for 90 min at 4°C. To detect the different proteins, the membrane was first reversibly stained with Ponceau S (AppliChem) and then cut between the protein bands according to the Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher). Free binding sites were blocked with 5% (w/v) powdered milk (Carl Roth) or 5% (w/v) Albumin Fraction V (Carl Roth) in Tris buffered saline with Tween-20 (TBST) (Carl Roth) for at least 30 min at room temperature. After that, membranes were incubated with the respective primary antibodies mouse anti-GFP (B-2) 1:1000 (sc-9996, Santa Cruz Biotechnology), mouse anti-α-Tubulin 1:8000 (T6074, Sigma-Aldrich), rabbit anti-Taspase1 1:2000 (sc-85945, Santa Cruz) or mouse anti-Karyopherinα2 1:1000 (sc-55538, Santa Cruz) in 5% (w/v) powdered milk in TBST or mouse anti-Penta-His 1:2000 (34660, Qiagen) in 5% (w/v) Albumin Fraction V in TBST for at least 1 h at room temperature. Unbound antibodies were removed by three washing steps with TBST. Membranes were incubated with the respective secondary antibodies donkey anti-rabbit HPR-coupled 1:10000 (NA934, GE Healthcare) or sheep anti-mouse HPR-coupled 1:10000 (NXA931, GE Healthcare) in 5% (w/v) powdered milk with TBST for 1 h. Unbound antibodies were removed by four washing steps in TBST. For the detection of chemiluminescence, we used the Pierce ECL Plus Western Blotting Substrate (Thermo Fisher) and the Chemidoc Imaging System (BioRad).

Immunoblot quantification

The signals were quantified with Fiji.[10] If necessary, the signal of Taspase1 in the eluted fraction was corrected for Taspase1 bound to the column without Importin α. To correct possible loading differences the signal of Taspase1 in the eluted fraction, values were normalized for the signal of Importin α in the eluted fractions. The data was evaluated using Origin2019 (OriginLab).

Toxicity Assay

1 x 10⁴ cells were cultured in Corning 96 Well microplates (Sigma-Aldrich) in 100 µl Dulbecco’s modified eagle medium (DMEM) (Thermo Fisher Scientific) supplied with 10% (v/v) fetal calf serum (FCS) (Life Technologies GmbH), Antibiotic-Antimycotic (Life Technologies GmbH) and the respective compound concentration. Since the compounds were dissolved in DMSO (Carl Roth), this solvent was included as a reference. Cells were then incubated at 37°C and 5% CO₂ for 24 h. Toxicity was determined via a colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. MTS, in the presence of phenazine methosulfate (PMS), produces a formazan product with an absorbance maximum at 490 nm directly proportional to the number of living, metabolically active cells. Here, dehydrogenase enzymes reduce anabolic cofactors such as nicotinamide adenine dinucleotide and its phosphate (NAD/NADP) to NADH/NADHP required for formazan formation. Briefly, the compound-containing medium was removed, and cells were washed with PBS once to remove excess compound. 100 µL fresh DMEM with 10% FCS and Antibiotic-Antimycotic were added to each well. Following addition of 20 µL Cell Titer AQeous One (Promega), absorption at 490 nm was recorded with the plate reader Promega Glow Max (Promega) after 30 min of incubation. Results were normalized to the DMSO references, compared to the untreated cells and are the mean of at least three replicates ± standard deviation.

Semi-in vitro Taspase1 substrate cleavage assay

5 x 10⁶ eukaryotic 293T cells were cultured in TC dish 100, standard (Sarstedt) with 10 ml DMEM with 10% (w/v)FCS and 1% (w/v) Antibiotic-Antimycotic and transiently transfected with
a plasmid coding for the confirmed Taspase1 substrate USF2 (Upstream stimulatory factor 2) tagged with GFP. 24 h after transfection, cells were detached, washed and resuspended in 500 µl buffer containing 100 mM HEPES (Applichem), 10% Saccharose (Applichem), 10 mM DTT (Applichem), pH 7.9. Cell lysates were prepared by sonicating the cells for at least four times with a Sonopuls mini 20 ultrasonic homogenizer and the ultrasonic probe MS 1.5 (Bandelin) for 20 s and an amplitude of 90%. After centrifugation at 14000 rpm for 20 min at 4°C, the supernatant was transferred into a new reaction tube. For the semi-in vitro Taspase1 substrate cleavage assay, the USF2 cell lysate was incubated with the corresponding compound and 0.4 µg/µl (equals 9 µM) recombinant active Taspase1-His at 37°C and 300 rpm. After 4 and 6 h, samples were collected, mixed with 5x sample buffer and heated to 95°C for 5 min for further analysis by SDS-PAGE and Immunoblotting (see above).

**Pull-down Assay**

![Diagram of pull-down assay]

**Immunoblotting**

- Protein of interest
- Protein-binding membrane
- Primary antibody
- Secondary antibody
- HRP

Proteins are transferred to a protein-binding membrane and identified using a highly specific primary antibody and a detectable secondary antibody.

**SDS-PAGE**

- Purify
- +
- -

Proteins are separated according to their molecular weight by electrophoresis.

**Figure S26:** Schematic workflow of the modified pull-down assay. In a spin column, GST-Importin α was fixed on a sepharose matrix coated with glutathione. After GST bound to glutathione with high affinity, unbound protein was removed by centrifugation. Taspase1-His, with or without ligand pre-incubation (as indicated), was added to the column. Unbound protein was again removed by centrifugation. The column was filled with Laemmi buffer that contains ionic detergents as well as reducing agents and heated to 95°C to denature and thus detach all protein from the matrix. SDS-PAGE was applied to separate the proteins according to their molecular weight followed by immunoblot analysis to identify and quantify the respective proteins.
Figure S27: Immunoblot of the “unbound” fraction after incubation with Taspase1 and ligand. Binding of Importin α to the column was demonstrably not affected by the ligands during the assay. Chemiluminescence images were merged with colorimetric images to allow visualization of the marker (M). Controls included only Taspase1 (C1), GST-Importin α (C2) or only the inhibitor solvent (DMSO).

Figure S28: Only the bivalent GCP-containing but not the arginine-based compound allows to efficiently interfere with the Taspase1/Importin α interaction. A) In our pull-down setup, pre-incubation Taspase1 with 2GC hampers binding to column-bound Importin α in contrast to 2RC. Controls include either only Taspase1 (C1), GST-Importin α (C2) or DMSO treatment (DMSO). Quantification of results comprises the mean of three replicates ± standard deviation.
Semi-in vitro Taspase1 substrate cleavage assay

Figure S29: Schematic workflow of the semi-in vitro Taspase1 substrate cleavage assay. After transient transfection of eukaryotic 293T cells with a plasmid coding for the Taspase1 substrate USF2 (Upstream stimulatory factor 2) tagged with GFP, cell lysates were prepared by sonicating the cells in 500 µl buffer containing 100 mM HEPES (Applichem), 10% (w/v) Saccharose (Applichem), 10 mM DTT (Applichem), pH 7.9.\textsuperscript{[5, 11]} The USF2 cell lysate was incubated with and without the corresponding compound and 0.4 µg/µl recombinant active Taspase1-His at 37°C and continuous rotation at 300 rpm. Taspase1 cleavage activity was assessed by analyzing substrate cleavage in lysate samples collected after 4 h and 6 h via SDS-PAGE and immunoblotting.
Figure S30: Immunoblot of the semi-in vitro Taspase1 substrate cleavage assay indicated an inhibitory effect of 2GC on Taspase1-mediated USF2 cleavage in contrast to 2G, 2GA and 1GC. 293T cells were transiently transfected with USF2-GFP and 24 h after transfection, whole cell lysates were prepared. The cell lysate was incubated with 0.4 µg recombinant Taspase1-His and 500 µM of the different compounds 2GC, 2G, 2GA and 1GC (A) or different concentrations of 2GC (B) at 37°C and continuous rotation at 300 rpm. Controls included cell lysates without active Taspase1 and ligand (w/o) as well cell lysates with active Taspase1 and inhibitor solvent (DMSO). Samples were taken after 4 h and 6 h incubation time and analysed via SDS-PAGE and immunoblotting.
Figure S31: Immunoblot of the semi-in vitro Taspase1 substrate cleavage assay indicated an inhibitory effect of 2GC on Taspase1-mediated USF2 cleavage in contrast to 2RC. 293T cells were transiently transfected with USF2-GFP and 24 h after transfection, whole cell lysates were prepared. The cell lysate was incubated with 0.4 µg recombinant Tapase1-His and 500 µM of the compound 2GC or the arginine-containing control 2RC at 37°C and continuous rotation at 300 rpm. Further controls included cell lysates without active Taspase1 and ligand (w/o) as well cell lysates with active Taspase1 and inhibitor solvent (DMSO). Samples were taken after 4 h and 6 h incubation time and analysed via SDS-PAGE and immunoblotting.
Figure S32: 2GC (orange) affects the cell viability of HeLa and A549 tumor cells in a concentration-dependent manner (A). In contrast, neither the other bivalent compounds 2GA (blue, B) and 2G (light blue, C), nor the monovalent building block 1GC (dark blue, D) decreased tumor cell viability, even when applied at high concentrations of up to 100 µM. This was also evident for the arginine-containing bivalent control compound 2RC (turquoise, E). Of note, we here again included the highest soluble concentration of 2GC (110 µM) to allow a direct comparison between the two compounds (orange).
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