

Supporting Information

Entry into a New Class of Potent Proteasome Inhibitors having High Antiproliferative Activity by Structure-Based Design

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Experimental procedures and analytical data for all intermediate and final compounds. Description of biochemical and cellular assays.

Experimental Section

General Methods. All starting materials were obtained from commercial sources unless otherwise specified. Melting points were determined with a Büchi B-545 apparatus and are uncorrected. ^1H NMR spectra (δ , ppm) were recorded using Varian 300 and 400 (300 MHz and 400 MHz, respectively) spectrometers with tetramethylsilane as the internal standard. Mass spectra were obtained with a Micromass Platform II spectrometer, and high resolution mass spectra were obtained with a Finnigan MAT 900 spectrometer. Elemental analyses were performed by Solvias AG, Basel, Switzerland. Column chromatography was performed with Merck silica gel 60 (0.04 – 0.063 mm). Preparative MPLC was carried out using a C18 column (Merck LICHROPREP RP-16, 15-25 μM ; detection at 215 nm) using a water/acetonitrile gradient containing 0.1% of trifluoroacetic acid. The purity of the compounds was assessed by analytical HPLC: i) system 1: linear gradient over 10 min of $\text{CH}_3\text{CN}/0.09\%$ TFA and $\text{H}_2\text{O}/0.1\%$ TFA from 1:4 to 1:0 and 3 min at 1:0; flow rate 1 mL/min, detection at 215 nm (Nucleosil 100-3 C18HD, 125 x 4.6 mm column); ii) system 2: linear gradient over 2.5 min of $\text{CH}_3\text{CN}/0.09\%$ TFA and $\text{H}_2\text{O}/0.1\%$ TFA from 1:49 to 1:0 at 4 mL/min, detection at 215 nm (Chromolith SpeedROD 50 x 4.6 mm column); single peak at the indicated retention time (t_{R}). Yields refer to chromatographically and spectroscopically pure compounds.

3,4,5-Trimethoxy-L-phenylalanine. N-Acetyl-3,4,5-trimethoxy-*L,D*-phenylalanine was prepared from commercially available 3,4,5-trimethoxybenzaldehyde and *N*-acetylglycine according to a literature procedure (E.M. Oltz, R. C. Bruening, M. J. Smith, K. Kustin and K. Nakanishi in *J. Am. Chem. Soc.* **1988**, *110* (18), 6162-6172). Alternatively, the N-acetyl-3,4,5-trimethoxy-*L,D*-phenylalanine methyl ester was obtained from 3,4,5-trimethoxybenzaldehyde and malonic acid dimethyl ester following also known protocols (M. Tanaka, T. Wakamatsu and H. Mitsuhashi, *EP 511666*; L. Tedeschi, D. Enders, *Organic Lett.* **2001**, *3* (22), 3515-3517; K.T. Okamoto, J. Clardy, *Tetrahedron Lett.* **1987**, *28* (42), 4969-4972; M. Skof, J. Svete, M. Kmetec, S. Golic-Grdadolnik, B. Stanovnik, *Eur. J. Org. Chem.* **1999**, 1581-1584). The resolution of the racemic N-acetyl-3,4,5-trimethoxy-*L,D*-phenylalanine methyl ester was performed by enzyme-catalyzed hydrolysis of the L-ester using Alcalase® (Novo Nordisk) as described in literature (J.J. Nestor, Jr., T.L. Ho, R.A. Simpson, B.L. Horner, G.H. Jones, G.I. McRae and B.H. Vickery, *J. Med. Chem.* **1982**, *25* (7), 795-801; O. D. Tyagi and P. M. Boll, *Indian J. Chem.* **1992**, 851-854).

$[\alpha]_{\text{D}}^{20} = -18.9^{\circ}$ ($c = 1.02$, H_2O). Analytical HPLC, $t_{\text{R}} = 1.88$ min (system 1). ^1H NMR (400 MHz, D_2O) δ 6.54 (s, 2H), 3.86 (m, 1H), 3.74 (s, 6H), 3.64 (s, 3H), 3.11 (dd, $J = 14.5$ Hz, $J = 5.1$ Hz, 1H), 2.95 (dd, $J = 14.4$ Hz, $J = 8.3$ Hz, 1H). HRMS (ESI) calcd for $\text{C}_{12}\text{H}_{17}\text{NO}_5$: 278.0999; found: 278.0997. To confirm its enantiomeric purity, 3,4,5-trimethoxy-D-phenylalanine ($[\alpha]_{\text{D}}^{20} = +19.7^{\circ}$ ($c = 1.02$, H_2O)) was also prepared and the two compounds were analysed using a chiral HPLC system (Chirobiotic T-Teicoplanin, water/EtOH (1:1, v/v), 1 mL/min): $t_{\text{R}} = 6.21$ min (3,4,5-trimethoxy-L-phenylalanine, ee > 99%) and $t_{\text{R}} = 7.92$ min (3,4,5-trimethoxy-D-phenylalanine, ee > 99%).

N^α-Boc-3,4,5-Trimethoxy-L-phenylalanine. To a stirred suspension of 3,4,5-trimethoxy-L-phenylalanine (20 g, 56.3 mmol) in a mixture of dioxane (180 mL) and 0.5 N NaOH (160 mL) was added Boc_2O (17 g, 78.3 mmol). The mixture was stirred for 4 h at room temperature and, after removal of the dioxane under reduced pressure, it was extracted with petroleum ether. The aqueous layer was acidified to pH 3 with 1N HCl at 0 °C. The precipitate was collected by filtration, washed with water and dried under vacuum at 50 °C to give the title compound as white crystals (22.3 g, 80%). mp 76-80 °C. Analytical HPLC, $t_{\text{R}} = 4.83$ min (system 1). $[\alpha]_{\text{D}}^{20} = +13.4^{\circ}$ ($c = 1.01$; CH_3OH). ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.61 (s, 1 H), 7.08 (d, $J = 8.4$ Hz, 1 H), 6.54 (s, 2 H), 4.08 (m, 1 H), 3.73 (s, 6 H), 3.59 (s, 3 H), 2.94 (dd, $J = 14.5$ Hz, 4.7 Hz, 1 H), 2.72 (m, 1 H), 1.30 (s, 9 H). HRMS (ESI) calcd for $\text{C}_{17}\text{H}_{25}\text{NO}_7$: 354.1558; found: 354.1559.

(S)-2-Amino-N-(2-hydroxy-4-methoxy-benzyl)-3-methyl-butylamide·HCl (compound a in scheme 1). This compound was obtained according to a literature procedure (Billich, A.; Scholz, D.; Charpiot, B.; Gstach, H.; Lehr, P.; Peichl, P.; Rosenwirth, B. *Antiviral Chemistry & Chemotherapy* **1995**, 6 (5), 327-336) MS (ES, m/z): 252.3 $[\text{MH}^+]$. ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 9.80 (s, 1 H), 8.63 (m, 1H), 8.16 (s, 3H), 7.06 (d, $J = 8$ Hz, 1H), 6.42 (s, 1H), 6.34 (d, $J = 8$ Hz, 1H), 4.08.4.32 (m, 2H), 3.66 (s, 3H), 3.57 (d, $J = 8$ Hz, 1H), 2.05 (m, 1H), 0.9 (s, 3H), 0.88 (s, 3H). Anal. ($\text{C}_{13}\text{H}_{20}\text{N}_2\text{O}_3 \times \text{HCl}$): C,H,Cl; N: *calcd*, 9.79; *found* 9.10.

[(S)-1-[(S)-1-(2-Hydroxy-4-methoxy-benzylcarbamoyl)-2-methyl-propylcarbamoyl]-2-(3,4,5-trimethoxy-phenyl)-ethyl]-carbamic acid tert-butyl ester. To a solution of N^α-Boc-3,4,5-trimethoxy-L-phenylalanine (5.6 g, 15.8 mmol) and (S)-2-amino-N-(2-hydroxy-4-methoxy-benzyl)-3-methyl-butylamide·HCl (5.46 g, 18.9 mmol) in 25 mL of DMF was added DIEA (10.72 mL, 63 mmol). After stirring for 30 min at room temperature, TPTU (5.15 g, 17.3 mmol) was added at 0 °C and the reaction was continued for 3 h at room temperature. The reaction was quenched with water (150 mL), and the mixture was extracted with AcOEt. The organic layer was washed with water, a saturated aqueous NaHCO₃ solution, brine, dried over Na₂SO₄ and evaporated to give the title compound, which was crystallized from AcOEt (6.0 g, 65%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.56 (s, 1 H), 8.33 (m, 1 H), 7.67 (d, *J* = 9.0 Hz, 1 H), 7.04 (d, *J* = 8.6 Hz, 1 H), 6.98 (d, *J* = 8.2 Hz, 1 H), 6.55 (s, 2 H), 6.35 (d, *J* = 2.3 Hz, 1 H), 6.32 (dd, *J* = 8.2 resp. 2.3 Hz, 1 H), 4.1-4.25 (m, 4 H), 3.74 (s, 6 H), 3.65 (s, 3 H), 3.60 (s, 3 H), 2.92 (dd, *J* = 13.5 resp. 4.0 Hz, 1 H), 2.65 (m, 1 H), 1.96 (m, 1 H), 1.31 (s, 9 H), 0.84 (d, *J* = 6.6 Hz, 2 H). Analytical HPLC *t*_R = 6.02 min (system 1); HRMS (ESI) calcd for C₃₀H₄₃N₃O₉: 590.3072; found: 590.3073.

(S)-2-[(S)-2-Amino-3-(3,4,5-trimethoxy-phenyl)-propionylamino]-N-(2-hydroxy-4-methoxy-benzyl)-3-methyl-butylamide·HCl. To a solution of [(S)-1-[(S)-1-(2-hydroxy-4-methoxy-benzylcarbamoyl)-2-methyl-propylcarbamoyl]-2-(3,4,5-trimethoxy-phenyl)-ethyl]-carbamic acid tert-butyl ester (5.98 g, 10.14 mmol) in dioxane (10 mL) was added 4 N HCl in dioxane (30 mL) and the mixture was stirred for 90 min at room temperature. After removal of the solvent under reduced pressure, the residue was resuspended in Et₂O and filtered to obtain compound the title compound (5.33 g, 100%). mp 187-189 °C. Analytical HPLC *t*_R = 3.94 min (system 2). MS (ES, *m/z*) = 490.3. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.61 (s, 1 H), 8.59 (d, *J* = 8.6 Hz, 1 H), 8.41 (m, 1 H), 8.10 (s, 3 H), 6.99 (d, *J* = 8.6 Hz, 1 H), 6.56 (s, 2 H), 6.38 (s, 1 H), 6.32 (d, *J* = 8.6 Hz, 1 H), 4.29 (m, 1 H), 4.22 (dd, *J* = 14.8, *J* = 5.8 Hz, 1 H), 4.08 (dd, *J* = 14.9, *J* = 5.5 Hz, 1 H), 3.73 (s, 6 H), 3.66 (s, 3 H), 3.63 (s, 3 H), 3.06 (dd, *J* = 14.1, *J* = 4.7 Hz, 1 H), 2.90 (m, 1 H), 1.98 (m, 1 H), 0.87 (m, 6 H). Anal. (C₂₅H₃₅N₃O₇ x HCl x 0.52 H₂O) C, H, Cl, H₂O; N: *calcd*, 7.85; *found*, 7.40.

(S)-N-(2-Hydroxy-4-methoxy-benzyl)-3-methyl-2-[(S)-2-[2-(3-phenoxy-phenyl)-acetylamino]-3-(3,4,5-trimethoxy-phenyl)-propionylamino]-butyramide (3). To a solution of 3-phenoxy-phenylacetic acid (1.54 g, 6.61 mmol) in DMF abs. (20 mL) were added DIEA (2.33 mL, 13.32 mmol) and TPTU (2.02g; 6.73 mmol). The mixture was stirred at room temperature for 30 min, and then (S)-2-[(S)-2-amino-3-(3,4,5-trimethoxy-phenyl)-propionylamino]-N-(2-hydroxy-4-methoxy-benzyl)-3-methyl-butylamide-HCl (3.15 g, 6 mmol) was added. After 16 h at room temperature, the reaction was quenched with water (200 mL) and the precipitate was filtered. The residue was dissolved in hot CH₂Cl₂/CH₃OH (9/1), filtered and the solvent evaporated under reduced pressure to afford **3**, which was crystallised from CH₃OH/Et₂O (3.22 g, 77%). mp 209-210 °C. MS (ES, *m/z*) 699.8 (MH⁺). Analytical HPLC *t_R* = 6.63 min (system 1). ¹H NMR (400 MHz, DMSO-*d*₆), δ ppm. 9.56 (s, 1 H), 8.32 (d, *J* = 8.6 Hz, 1 H), 8.27 (m, 1 H), 7.88 (d, *J* = 9.0 Hz, 1 H), 7.36 (t, *J* = 8.2 Hz, 2 H), 7.18 (t, *J* = 8.2 Hz, 1 H), 7.11 (t, *J* = 7.4 Hz, 1 H), 6.96 (m, 3 H), 6.85 (m, 2 H), 6.79 (m, 1 H), 6.53 (s, 2 H), 6.36 (d, *J* = 2.4 Hz, 1 H), 6.31 (dd, *J* = 8.6 Hz, *J* = 2.7 Hz, 1 H), 4.61 (m, 1 H), 4.21-4.06 (m, 3 H), 3.69 (s, 3 H), 3.65 (s, 3 H), 3.60 (s, 3 H), 3.41 (m, 2 H), 2.93 (dd, *J* = 14.1 Hz, *J* = 4.3 Hz, 1 H), 2.72 (m, 1 H), 1.93 (m, 1 H), 0.79 (m, 6 H). Anal. (C₃₉H₄₅N₃O₉ x 0.2 H₂O) C, H, N, H₂O.

2-(S)-[3-(3,4-Dimethoxy-phenyl)-2-phenylacetylamino-propionylamino]-N-(2-hydroxy-4-methoxy-benzyl)-3-methyl-butylamide (4). This compound was prepared as described for compound **3** using phenylacetic acid and (S)-2-[(S)-2-amino-3-(3,4-dimethoxy-phenyl)-propionylamino]-N-(2-hydroxy-4-methoxy-benzyl)-3-methyl-butylamide-HCl. Yield: 36 mg (55%). MS (ES, *m/z*) 578.5 (MH⁺). Analytical HPLC *t_R* = 2.01 min (system 2). ¹H NMR (300 MHz, DMSO-*d*₆), δ ppm. 9.60 (s, 1 H), 8.32 (m, 2 H), 7.88 (d, *J* = 8.0 Hz, 1 H), 7.15 (m, 3 H), 7.04 (m, 2 H), 6.97 (dd, *J* = 7.9 Hz, 2 H), 6.81 (s, 1 H), 6.70 (dd, *J* = 7.9 Hz, 2 H), 6.50-6.30 (m, 3 H), 4.55 (m, 1 H), 4.20-4.10 (m, 3 H), 3.69 (s, 3 H), 3.65 (s, 3 H), 3.60 (s, 3 H), 2.90 (dd, *J* = 15.0 Hz, *J* = 3.9 Hz, 1 H), 2.65 (dd, *J* = 15.0 Hz, *J* = 11.0 Hz, 1 H), 1.93 (m, 1 H), 0.80 (m, 6 H). Anal. (C₃₂H₃₉N₃O₇) C: *calcd*, 66.53; *found* 66.12; H: *calcd*, 6.80; *found* 7.24; N: *calcd*, 7.27; *found* 6.67; O: *calcd*, 19.39; *found* 19.24.

**2-(S)-{3-(3,4-Dimethoxy-phenyl)-2-[2-(3-phenoxy-phenyl)-acetylamino]-propionylamino}-
(S)-N-(2-hydroxy-4-methoxy-benzyl)-3-methyl-butylamide (5).** This compound was prepared as described for compound **3** using 3-phenoxy-phenylacetic acid and (S)-2-[(S)-2-amino-3-(3,4-dimethoxy-phenyl)-propionylamino]-N-(2-hydroxy-4-methoxy-benzyl)-3-methyl-butylamide-HCl. Yield: 1.34 g (75 %). MS (ES, m/z) 670.0 (MH⁺). Analytical HPLC t_R = 6.60 min (system 1). ¹H NMR (400 MHz, DMSO-*d*₆), δ 9.60 (s, 1 H), 8.26 (m, 2 H), 7.89 (d, J = 8.6 Hz, 1 H), 7.36 (d, J = 7.4 Hz, 1 H), 7.34 (d, J = 8 Hz, 1 H), 7.18 (t, J = 8.0 Hz, 1 H), 7.10 (t, J = 7.3 Hz, 1 H), 6.96 (m, 3 H), 6.8 (m, 4 H), 6.7 (m, 2 H), 6.34 (d, J = 2.4 Hz, 1 H), 6.30 (dd, J = 8.2 Hz resp. 2.5 Hz, 1 H), 4.57 (m, 1 H), 4.18-4.1 (m, 3 H), 3.66 (s, 3 H), 3.65 (s, 3 H), 3.64 (s, 3 H), 3.38 (m, 2 H), 2.91 (m, 1 H), 2.68 (m, 1 H), 1.91 (m, 1 H), 0.77 (m, 6 H). Anal. (C₃₈H₄₃N₃O₈ x 0.21 H₂O) C,H,N, H₂O.

Biochemical assays. Peptidase activity was monitored using the following substrates (Bachem AG, Switzerland): chymotrypsin-like activity with Suc-Leu-Leu-Val-Tyr-AMC; trypsin-like activity with Boc-Leu-Arg-Arg-AMC; and post-glutamyl-peptide hydrolytic-like activity with Z-Leu-Leu-Glu-AMC. Briefly, 100 ng of purified human 20S proteasome was incubated for 1 h at 37 °C with or without the inhibitor in 50 μ l of assay buffer [20 mM Tris-HCl, pH 8.0; 5 mM CaCl₂]; after this time, the corresponding fluorogenic peptide substrate (c= 80 μ M or 100 μ M, final concentration in the well) was added and the reaction mixture was incubated for 3 h at 37 °C. The rates of hydrolysis were monitored in a FLUOROSKAN ASCENT™ microtiter plate reader by recording the fluorescence of hydrolyzed fluorescence AMC group with an excitation filter of 355 nm and an emission filter of 460 nm. The initial linear portions of the curves were used to calculate the IC₅₀ value (the concentration of inhibitor at which the rate of the activity of the 20S proteasome catalyzed hydrolysis of the corresponding fluorogenic peptide substrate is reduced at 50 %).

Inhibition of the proteasome activity in culture cells. MDA-MB435 cells (0.5 x 10⁶ cells/well) were pre-cultured overnight at 37 °C in the recommended media and under 5 % CO₂ in 12-well culture plates (1 ml total volume). The 20S proteasome inhibitor was added at different concentrations to the culture in a total volume of 1 ml of fresh medium. Incubation continued for 16 h at 37 °C and under 5 % CO₂. After this time, the supernatant was aspirated to collect the detached cells. Cells were collected by centrifugation and the cell pellet was washed x2 with 1.0

ml of cold PBC. The pellet was lysed in 50 ml of M-OER/Pierce by vortexing and left for 10 min at room temperature to yield lysate A. The adherend cells were washed twice with 1 ml of cold PBS and lysed in 0.2 ml of M-PER/Pierce by carefully pipetting up and down, and shaking the plate for 10 min at room temperature. The lysate was collected together with 50 μ l of lysate A, spun down for 5 min at 10,000 g at 4 °C and stored at -20 °C.

a) *Chymotrypsin-like activity of the proteasome.* 10 μ l of lysate was added to 96-well black Cliniplates (Labsystems/Bioconcept – CH nr. 9502870) followed by the addition of the specific substrate Suc-Leu-Leu-Val-Tyr-AMC (Bachem/CH, nr. I-1395) to a final concentration of 80 μ M. The working buffer was 20 mM Tris HCl pH 8.0, 5 mM CaCl₂. The total volume per well was 100 μ l and the incubation time was 60 to 180 min at 37 °C. Relative fluorescence was measured in a fluorescent reader (Ascent, Labsystems/Bioconcept, CH) at 355 / 460 nm. Limiting quantities of extract were used to assure a proportional relationship between fluorescence signal intensity and amount of enzymatic activity added to the assay. Inhibition of the fluorescence signal upon *ex vivo* incubation of the extract with a standard proteasome inhibitor (e.g. 1 μ M of MG132, Sigma/Saint Louis, USA, nr. C-2211) was performed on all extracts. For this purpose, 10 μ l of lysate was incubated with MG132 in a total volume of 50 μ l for 60 min at room temperature before the substrate was added.

b) *Post-glutamyl-peptide hydrolytic-like activity of the proteasome.* 10 μ l of lysate was added to 96-well black Cliniplates (Labsystems/Bioconcept – CH nr. 9502870) followed by the addition of the specific substrate Z-Leu-Leu-Glu-AMC (Calbiochem/CH nr, 539141) to a final concentration of 80 μ M. The working buffer was 20 mM Tris HCL pH 8.0, 5 mM CaCl₂. The total volume per well was 100 μ l and the incubation time was 60 to 180 min at 37 °C. Relative fluorescence was measured in a fluorescent reader (Ascent, Labsystems/Bioconcept, CH) at 355 / 460 nm.

c) *Trypsin like activity of the proteasome.* The experimental set-up was as described above except that the substrate Z-Ala-Arg-Arg-AMC (Bachem/CH nr. I-1125) was used at a final concentration of 50 μ M in 20 mM Tris HCl pH 8.0 without calcium chloride.

Cell proliferation assay. Tumour cells were cultured at 37 °C in an incubator with a 5 % v/v CO₂ and 80 % relative humidity atmosphere. Cells were seed on day 0 at 1.5 x 10³ cells/well into 96-well micrititer plates and incubated overnight. Two-fold serial compound dilutions performed in cell culture medium were added on day 1. Highest drug concentration tested was 1 μ M.

Following incubation of the cell plates for an additional 4 or 6 days, a time period that allowed for at least two populations doublings in the vehicle-treated controls, cells were fixed with 3.3 % v/v glutaraldehyde, washed with water and stained with 0.05 % w/v methylene blue. After washing, the dye was eluted with 3 % HCl and the optical density measured at 665 nm with a SpectraMax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA). IC₅₀ values were determined by mathematical curve-fitting using the SoftPro3.0 software (Molecular Devices, Sunnyvale, CA), applying the formula $(OD \text{ treated} - OD \text{ start}) / (OD \text{ control} - OD \text{ start}) \times 100$. The IC₅₀ was defined as the drug concentration that at the end of the incubation period led to 50 % inhibition of net growth (i.e. increased in cell mass) compared to untreated control cultures.