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Supplementary information

New 3-amidinophenylalanine derived inhibitors of matriptase

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Analytical HPLC

Experiments were performed on a Shimadzu LC-10A system with a Nucleodur C_{18} column (5 µm, 100Å , 4.6 x 250 mm, Machery-Nagel, Düren, Germany) with a linear gradient of acetonitrile (10–70 % over 60 minutes, detection at 220 nm) containing 0.1% TFA at a flow rate of 1 ml/min.

Thin Layer Chromatography

Thin layer chromatography (TLC) was applied on precoated "silica gel 60 F254" plates from Merck (Darmstadt, Germany), using n-butanol/acetic acid/water, 4:1:1, v/v/v as mobile phase.

Mass spectrometry

The molecular mass of the synthesized compounds was determined using a QTrap 2000 ESI spectrometer (Applied Biosystems).

Table S1. Analytical data of synthesized inhibitors **4-35**

Compound	Retention time HPLC (min)	TLC R_f	MS calculated as free base	MS found $(M+H)^+$
4	35.3	0.87	607.3	608.2
5	24.0	0.73	551.2	552.3
6	35.4	0.86	607.3	608.2
7	24.3	0.72	551.2	552.3
8	34.6	0.85	607.3	608.2
9	24.6	0.73	551.2	552.2
10	38.6	0.84	657.2	658.2
11	29.4	0.74	601.2	602.2
12	38	0.85	657.2	658.4
13	29.7	0.74	601.2	602.1
14	38.7	0.85	657.2	658.4
15	30.8	0.75	601.2	602.1
16	40.06	0.78	667.3	668.4
17	31.0	0.75	611.2	612.3
18	33.9	0.83	649.3	650.4
19	26.1	0.71	593.3	594.4
20	13.4	0.55	586.3	587.4
21	10.3	0.54	558.2	559.3
22	14.4	0.63	600.3	601.2
23	11.3	0.59	572.3	573.3
24	16.2	0.66	614.3	614.4
25	13.7	0.59	586.3	587.3
26	22.2	0.67	662.3	663.4
27	20.4	0.71	634.3	635.6
28	24.1	0.67	668.3	669.5
29	21.7	0.65	640.3	641.3
30	21.6	0.66	676.3	677.4
31	20.1	0.71	648.3	649.7
32	31.4	0.84	650.3	651.4
33	36.4	0.85	658.2	659.3
34	38.7	0.85	690.3	691.5
35	43.5	0.87	698.2	699.8

Enzyme kinetic measurements

K_i determination for matriptase, thrombin, factor Xa and uPA.

The determination of the K_i values for matriptase, bovine thrombin, human factor Xa and uPA was performed using synthetic chromogenic pNA-substrates. The optical density of the substrate cleavage was measured at 405 nm using a IEMS micoplate reader MF 1401 (Labsystems, Helsinki, Finland).

The used substrates for matriptase, thrombin and FXa were synthesized using standard methods and purified by preparative HPLC. The uPA substrate Pefachrom uPA was purchased from Pentapharm (Basel, Switzerland). All substrates were dissolved in ultrapure water to the appropriate concentrations.

Stock solutions of the inhibitors were prepared with a concentration of 10 mM in DMSO, which were further diluted with Tris HCl buffer pH 8.0 (containing 0.154 M NaCl, 2 % ethanol) to appropriate concentrations. The used concentrations of the inhibitors in the assay were at least 10-fold higher than the enzyme concentration. For the calculation of the molecular weight of the inhibitors, one TFA molecule was added to each basic amidino or amino group, if present. All measurements were performed at room temperature with the following volumina:

- 200 µL 50 mM Tris·HCl buffer pH 8.0 (containing 0.154 M NaCl, 2 % ethanol and inhibitor in appropriate concentrations).
- 25 µL aqueous substrate solution
- start of the reaction by addition of 50 µL enzyme solution

The measurements were stopped by addition of 25 μL 50% acetic acid after appropriate reaction time, when the absorbance at the highest substrate concentration in absence of inhibitor has reached a value between 0.15-0.18. The K_i values were calculated according to the method of Dixon at two different substrate concentrations and five different inhibitor concentrations using an Excel template previously developed by Stürzebecher. The K_i values are the mean of at least two independent measurements.

The following proteases and substrates were used for measurements (Table S2).

Table S2. Used enzymes and substrates.

Enzyme concentration in assay	**Substrate concentration in assay**
matriptase (catalytic domain, expressed and purified as described previously[1]) 2.62×10^{-10} M	$MeSO_2$-D-Cha-Gly-Arg-pNA 182 and 91 µM
human factor Xa (FXa, 200.35 IE/mg, Enzyme Research South Bend, UK) 4.94×10^{-10} M	CH_3OCO-D-Cha-Gly-Arg-pNA 182 and 91 µM
bovine thrombin (1425 IE/mg, purified as described previously[2]) 3.725×10^{-9} M (6.77×10^{-10} M)	$MeSO_2$-D-Cha-Gly-Arg-pNA 182 and 91 µM
human uPA (HS medac, Hamburg, Germany 500,000 I.E.) diluted to 43 IE/mL in assay, molar concentration not exactly known	Bz-ß-Ala-Gly-Arg-pNA (Pefachrome uPA) 182 and 91 µM

Matriptase, FXa and thrombin stock solutions were prepared in 0.9% NaCl solution containing 0.1% HSA or BSA, whereas the uPA preparation already containing HAS was dissolved in a 0.9% NaCl solution.

K_i determination for matriptase-2

Human embryonic kidney (HEK) 293 cells were transfected with the pcDNA4-matriptase-2-Myc-His A plasmid, as reported previously.[3] The activity of matriptase-2 in the conditioned medium of transfected HEK cells (HEK-MT2) was assayed in Tris saline buffer (50 mM Tris, 150 mM NaCl, pH 8.0) at 37 °C by monitoring the release of *para*-nitroaniline from the chromogenic substrate Boc-Gln-Ala-Arg-*para*-nitroanilide (Bachem, Bubendorf, Switzerland) at 405 nm using a Cary 100 UV-vis spectrophotometer (Varian, Darmstadt, Germany). A K_m value of 210 μM was determined with eight different substrate concentrations in duplicate experiments. Inhibition assays were performed in duplicate measurements with five different inhibitor concentrations. IC_{50} values were obtained by nonlinear regression according to equation $v = v_0/(1+[I]/IC_{50})$. The K_i values were calculated using the equation $K_i = IC_{50} / (1 + [S]/K_m)$.[4]

The 10 mM inhibitor stock solutions and a 100 mM stock solution of Boc-Gln-Ala-Arg-*para*-nitroanilide were prepared in DMSO. The final concentration of the substrate was 400 μM and of DMSO was 1.5%. Into a cuvette containing 979 μL prewarmed assay buffer, 11 μL of an inhibitor solution and 4 μL of a substrate solution were added and thoroughly mixed. The reaction was initiated by adding 6 μL of an enzyme solution (5 μg/6 μL total protein of the conditioned medium of HEK-MT2 cells) and was followed over 20 min.

K_i determination for HAT[5]

The inhibition constants were determined with recombinant human airway trypsin-like protease (R&D Systems) at RT according to the method of Dixon using a Safire2 fluorescence plate reader (Tecan) (λ_{ex} = 380 nm; λ_{em} = 460 nm) and D-cyclohexylalanine-Pro-Arg-AMC (synthesized in our lab) as the substrate in 50 mM Tris buffer (pH 9.5) containing 0.05% Brij 58 and 1 mg/mL BSA. The enzyme concentration used in the assay was 23.8 pM, and the substrate concentrations were 50, 100 and 200 lM. Results were obtained from at least two independent experiments.

Crystallization of thrombin and soaking of inhibitor 11

Human α-thrombin (from Enzyme Research Laboratories, South Bend, USA) was dissolved in the crystallization buffer (20 mM NaH_2PO_4, 350 mM NaCl, 2 mM benzamidine, pH 7.5) at 10 mg/ml. The hirudin fragment Acetyl-Hirudin (54-65) achieved from Bachem (Bubendorf, Switzerland) was dissolved in crystallisation buffer at 2.5 mg/mL. In the next step, 40 µL of the solution of the hirudin fragment was mixed with 160 µL of the thrombin solution. After incubation for 1 h at 4 °C, crystallization was carried out at 4 °C by the hanging-drop method. The hirudin/thrombin solution was mixed 1:1 with the reservoir solution (20 mM NaH_2PO_4, 27 % polyethylene glycol 8000, pH 7.5) and 2 µL of this solution were placed in the centre of a cover slip. Immediately after mixing of protein and reservoir buffer microseeding was performed with a horse hair. The wells of the crystallization trays were filled with 500 µL of the reservoir buffer. Subsequently the cover slips were placed over the wells and sealed. Crystals of good diffracting quality could be obtained after 10 to 14 days.
For soaking, a DMSO stock solution of inhibitor **11** (50 mM) was diluted 1:10 with a solution containing crystallization and reservoir buffer 1:1 resulting in the final soaking concentration containing 5 mM of the inhibitor and 10 % DMSO. A medium-size crystal without visible imperfections was selected and transferred into the soaking solution for 1 h.

Data collection and processing

Crystals were prepared for data collection at 110 K using a cryoprotectant solution of 20 % glycerol in reservoir buffer. The data set was collected in-house using radiation from an IµS™ micro focus tube (Incoatec) and an image plate detector (Mar345). Data processing and scaling were performed using the HKL2000 package.[6]

Structure determination and refinement

The coordinates of human thrombin (PDB code 1H8D)[7] were used for molecular replacement with Phaser from the CCP4 program package.[8] For initial rigid body refinement of the protein molecule, followed by repeated cycles of maximum likelihood energy minimization simulated annealing and B-factor refinement the program PHENIX[9] was used. For determining the temperature factors for the structure TLS refinement was applied. The definition of the TLS groups was done with the TLSMD server[10, 11]. A randomly chosen 5 % of all data were used for the calculation of R_{free} and were not used in the refinement. Amino acid side chains were fit into σ-weighted $2F_o - F_c$ and $F_o - F_c$ electron density maps using Coot.[12] After the first

refinement cycle, water molecules and subsequently ions and ligands were located in the electron density and added to the model. Restraints were applied to bond lengths and angles, planarity of aromatic rings and van der Waals contacts. Multiple side chain conformations were built in case an appropriate electron density was observed and maintained during the refinement, and if the minor populated side chain showed at least 20 % occupancy. The final model was validated using PHENIX own validation options or MolProbity.[13] The Ramachandran plot was calculated with PROCHECK.[14] Data collection, unit cell parameters and refinement statistics are given in Table S3. Analysis of temperature factors was done with Moleman.[15] The naming of the protein amino acids was done according to Bode *et al.*.[16]

Protein Data Bank and accession number

Coordinates and structure factors have been deposited in the Protein Data Bank with the following accession code: 4E7R.

Table S3. Data collection and refinement statistics for the thrombin/inhibitor **11** complex.

PDB-Code	**4E7R**
A. Data collection and processing	
Wavelength (Å)	1.54178
Space group	P1
Unit cell parameters	
a, b, c (Å)	50.2, 50.3, 71.8
α, β, γ (°)	97.6, 96.6, 90.6
Matthews coefficient ($Å^3$/Da)	2.4
Solvent content (%)	49.3
Molecules in asymetric unit	2
B. Diffraction data [a]	
Resolution range (Å)	30-2.25 (2.30-2.25)
Unique reflections	32,856
R (I) sym (%)	7.8 (34.8)
Completeness	95.6 (93.1)
Redundancy	2.7 (2.6)
I/σ (I)	13.4 (2.9)
C. Refinement	
Resolution range	29.3 - 2.25
Reflections used in refinement	
(work/free)	29,805 / 1,590
Final R values for all reflections	
(work/free) (%)	17.0 / 23.2
Protein residues (L chain/ H chain)	56 / 500
Sodium ions	4
Inhibitor atoms	80
Water molecules	265
RMSD from ideality	
Bond length (Å)	0.009
Bond angles(°)	1.2
Ramachandran plot (PROCHECK)	
Residues in most favoured regions (%)	85.9
Residues in additionally allowed regions (%)	13.8
Residues in generously allowed regions (%)	0.3
Mean B-factor ($Å^2$)	
Protein (L + H chain)	30.3
Ligand	29.0
Sodium, phosphate, glycerol, NAG	66.5
Water molecules	32.2

[a] Numbers in brackets are for the highest resolution shell.

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