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Incorporation of neutral C-terminal residues in 3-amidinophenylalanine-derived matriptase inhibitors

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ABSTRACT

A novel series of matriptase inhibitors based on previously identified tribasic 3-amidinophenylalanine derivatives was prepared. The C-terminal basic group was replaced by neutral residues to reduce the hydrophilicity of the inhibitors. The most potent compound **22** inhibits matriptase with a K_i value of 0.43 nM, but lacks selectivity towards factor Xa. By combination with neutral N-terminal sulfonyl residues several potent thrombin inhibitors were identified, which had reduced matriptase affinity.

Matriptase, also named MT-SP1, TADG-15, ST14, or epithin in mice, is a type II transmembrane serine protease that contains a short N-terminal intracellular domain and a larger extracellular region.[1] Matriptase has a complex multidomain structure, with its trypsin-like protease domain located at the C-terminus of the protein on the cell surface. Many insights regarding the physiological role of matriptase have been obtained with matriptase-null mutant mice. Matriptase has a critical role in the proper development of a number of epithelial tissues, including epidermis, oral epithelium, thymic epithelium, and hair follicles. Matriptase knockout mice die shortly after birth due to severe dehydration and impaired epidermal barrier function.[2] These studies with knockout mice revealed a defect in the proteolytic processing of the epidermal polyprotein profilaggrin into filaggrin monomer units, which are required for proper epidermis development.[3] In addition, matriptase may also act as an activator of prostasin, a glycophosphatidylinositol-anchored membrane serine protease. This was supported by the observation that matriptase and prostasin knockout mice have nearly identical phenotypes.

Under normal conditions the activity of matriptase is tightly regulated by the endogenous hepatocyte growth factor activator inhibitors HAI-1 and/or HAI-2, which are type I transmembrane proteins that contain two Kunitz-type serine protease inhibitor domains.[4] Recently matriptase has also been identified as a potential drug target in oncology based on many reports that have causally linked the overexpression of matriptase or an increased matriptase/HAI-1 ratio to a variety of epithelial tumors.[5] However, it must be noted that in some tumor studies also a downregulation of matriptase and HAI-1 expression was detected.[6]

In addition to both profilaggrin and prostasin, which have to be processed for normal embryogenesis and epithelial development, other potential matriptase substrates have been identified, which are probably involved in tumorigenicity. Among them are the proform of hepatocyte growth factor, also named scatter factor (pro-HGF/SF),[7] pro-uPA, the protease activated receptor PAR-2,[8] the insulin-like growth factor binding protein-related protein-1 (IGFBP-rP1),[9] and stromelysin (MMP-3).[10]

In a first publication on matriptase inhibitors we described the development of tribasic secondary amides of sulfonylated 3-amidinophenylalanine, such as **1** (Table 1).[11] Despite excellent potency for matriptase and high selectivity towards other trypsin-like serine proteases, such compounds have no potential for oral bioavailability due to their high hydrophilicity and consequently poor penetration. Therefore, with the assumption that it should be possible to convert the 3-amidinophenylalanine into a less basic, more hydrophobic hydroxyamidino prodrug similar to that used for the anti-metastatic uPA inhibitor mesupron and the thrombin inhibitor prodrug ximelagatran,[12] we focused our efforts on the elimination of the positively charged groups in the N- and C-terminal

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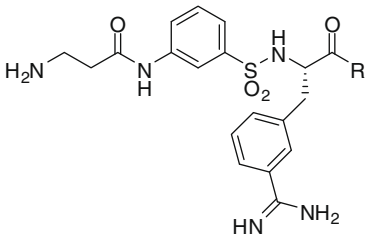
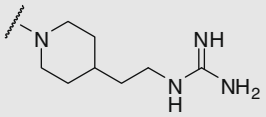
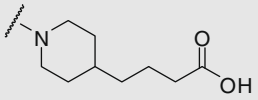
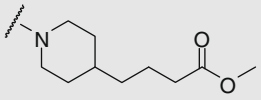
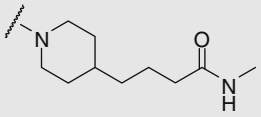
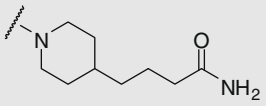
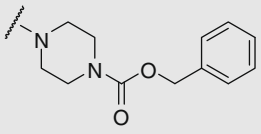
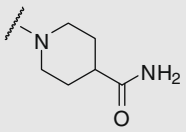
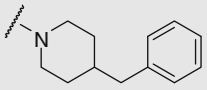
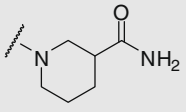
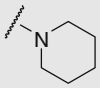
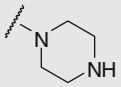
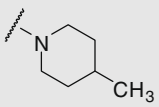
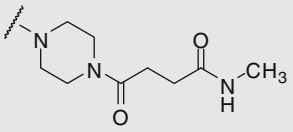
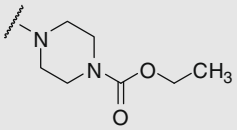
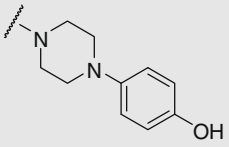
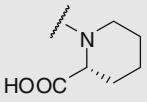
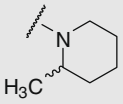
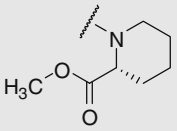
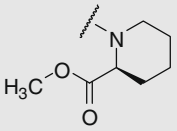
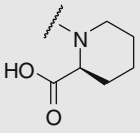
Table 1
Inhibition of matriptase and other trypsin-like serine proteases by inhibitors of the general formula



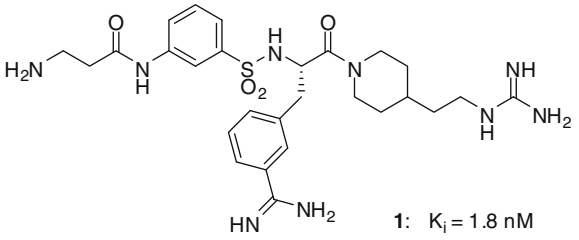
No.	R	K_i (μM)				
		Matriptase	Plasmin	uPA	Thrombin	fXa
1		0.0018	5.0	1.7	6.0	9.5
2		0.255	3.3	17	14	18
3		0.0061	2.8	2.5	0.60	6.1
4		0.0063	1.6	3.3	0.85	10
5		0.012	2.9	2.1	0.67	8.2
6		0.0075	1.3	3.3	1.4	19
7		0.013	1.2	3.3	1.25	2.5
8		0.017	2.8	2.2	0.35	3.4
9		0.031	1.3	7.4	2.1	15
10		0.033	3.3	2.6	0.27	10
11		0.037	2.56	0.88	71.7	2.1
12		0.053	6.2	3.0	0.11	4.4

(*continued on next page*)

Table 1 (*continued*)

No.	R	K_i (μM)				
		Matriptase	Plasmin	uPA	Thrombin	fXa
13		0.053	3.6	16.9	29	44
14		0.065	13.4	9.2	5.1	26
15		0.15	2.5	8.37	14.5	2.5
16		0.18	9.4	7.3	2.3	11
17		0.47	4.5	34.5	0.395	17
18		0.1	4.9	76	24.2	0.10
19		2.4	69.4	126	10.6	127
20		11	79	193	159	30

inhibitor segments. The results of the N-terminal modification were previously described,[13] in the present publication we report the results of the C-terminal replacements.

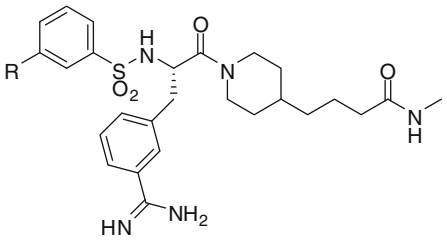


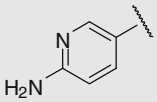
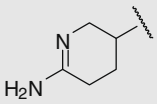
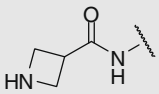
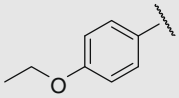
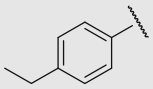
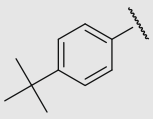
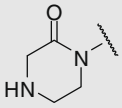
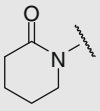
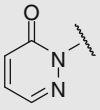
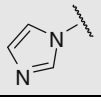
1: $K_i = 1.8$ nM

The X-ray structure of a similar inhibitor (number 8 in our previous publication)[11] complexed with matriptase revealed that the guanidinoethyl group is directed into a negatively charged cleft of matriptase lined by the carboxylate groups of Asp96 and Asp60b and the carbonyl groups of His57 and Ile60. However, this guanidyl group was not directly involved in contacts to the protease and formed only some water-mediated hydrogen bonds. To probe the importance of the guanidyl group for matriptase inhibition we introduced an oppositely charged 4-piperidylbutanoic acid. As expected, compound **2** has only poor activity (Table 1); however, a high potency was retained for the analogous methyl ester **3**, which was prepared as a precursor of **2**. Replacement of the somewhat labile methyl ester by the isosteric methyl amide resulted in similar potency (**4**), whereas the activity of the non-substituted amide was slightly reduced (**5**). A relatively high potency was observed also for the Cbz-protected piperazide **6**, the isonepecotic acid amide **7,** and the benzylpiperidide **8**. Most of these analogues retained sufficient selectivity for matriptase compared to the other examined trypsin-like serine proteases. Additional modifications resulted in significantly reduced matriptase affinity (**9**–**20**).

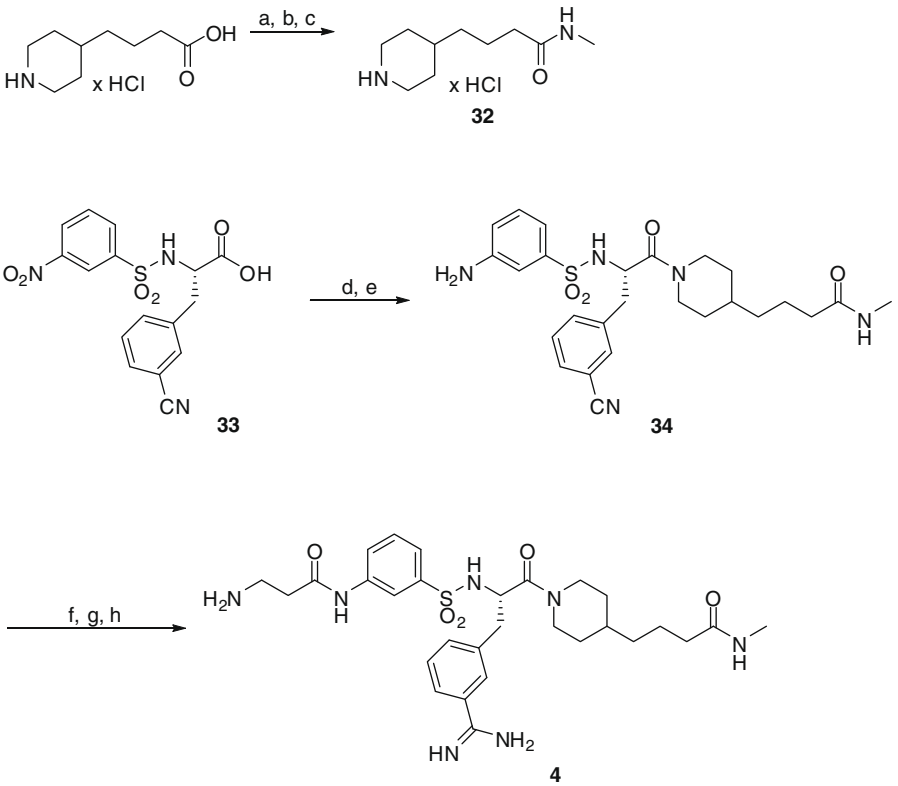
In the further course of this work, the N-terminal β-alanyl amide was replaced with selected groups as described in the preceding paper.[13] We maintained the C-terminal 4-piperidylbutanoyl methylamide, which should have an improved stability over the analogous methyl ester and which has also a slightly reduced molecular weight compared to the similar potent Cbz-protected piperazide (Table 2). As found previously with similar analogues,[13] also in this series inhibitor **22** containing an N-terminal 3-(6-ami-

Table 2
Inhibition of matriptase and other trypsin-like serine proteases by inhibitors of the general formula

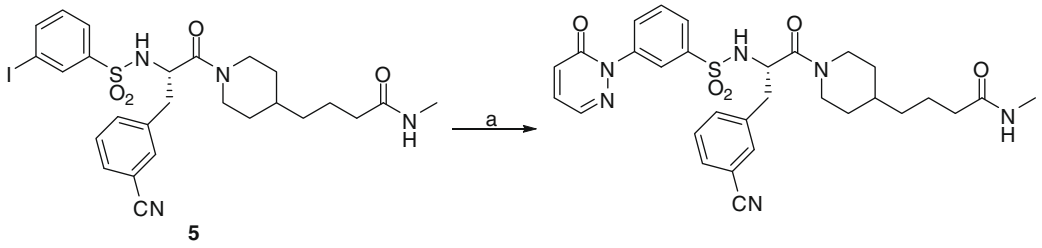


No.	R	K_i (μM)				
		Matriptase	Plasmin	uPA	Thrombin	fXa
21		0.010	1.17	9.5	0.01	0.163
22		0.00043	0.078	7.1	0.02	0.0057
23		0.016	0.354	4.7	0.66	6.93
24		0.028	0.211	11.9	0.0055	0.641
25		0.024	0.606	13.5	0.0036	0.371
26		0.087	0.41	17	2.05	0.834
27		0.024	1.0	45	0.92	0.012
28		0.030	1.22	26	0.0011	0.704
29		0.098	0.66	24	0.0084	0.85
30		0.177	0.95	11	0.0013	1.8
31		0.238	2.7	31	0.053	6.7

no-2,3,4,5-tetrahydropyridin-3-yl)benzenesulfonyl group is the most potent analogue, but additionally efficiently inhibits factor Xa with a K_i value of 5.7 nM. In contrast, the 4-ethoxy- and 4-ethylphenyl derivatives (**24**, **25**) are stronger thrombin than matriptase inhibitors, whereas the more bulky *tert*-butyl analogue (**26**) is obviously not able to occupy the hydrophobic aryl binding site of thrombin, but still maintains some affinity to matriptase, which possesses a more open S3/S4-binding region.



Scheme 1. Reagents and conditions: (a) 1.1 equiv $(Boc)_2O$, NaOH (pH 9) in dioxane/water 2:1, 15 min 0 °C, 3 h at room temp, (b) 10 equiv CH_3–NH_2·HCl, 1.5 equiv HBTU, 4.5 equiv DIPEA in ethyl acetate, 5 h stirring at 40 °C, (c) 1 N HCl in acetic acid, 1 h room temp., (d) 1.1 equiv of **32**, PyBop, 3.0 equiv DIEA in DMF, 15 min 0 °C and 3 h room temp, (e) zinc powder in acetic acid, 2 h, room temp, (f) Cbz-β-Ala-OH, *N*-methyl-morpholine, isobutyl chloroformate, −15 °C, 10 min in DMF, followed by addition of **34**, 1 h, −15 °C, overnight at room temp, (g) (i) 2 equiv hydroxylamine·HCl and DIPEA, reflux in ethanol, 4 h, stirring overnight at room temp and evaporation of the ethanol; (ii) 2.5 equiv Ac_2O in acetic acid, 30 min room temp and evaporation; (iii) H_2 and Pd/C as catalyst in 90% acetic acid, stirring over night at 30 °C; (h) purification by preparative reversed phase HPLC.[1]



Scheme 2. Reagents and conditions: (a) (i) 5 equiv pyridazinone, 2 equiv K_3PO_4, 10 mol % (1*R*,2*R*)-*N*,*N*′-dimethyl-1,2-cyclohexanediamine, 4 mol % CuI in DMF under N_2, stirring 1.5 h at 110 °C, ii: flash chromatography on silica gel using a DCM/MeOH gradient.

A third series of analogues containing a C–N-bond between both rings within the sulfonyl residue was prepared (**27**–**31**). The piperazinone and piperidinone inhibitors still inhibit matriptase with K_i values $\leqslant$30 nM, whereas the other compounds were less efficient. Some of the more hydrophobic analogues possess a remarkable activity as thrombin inhibitors (e.g., **28**, **30**), whereas again in this case the basic piperazin-2-one derivative **27** preferentially inhibits factor Xa.

Two hydroxyamidino prodrugs[12] of analogues **24** and **26** were prepared and orally applied to rats via a gastric feeding tube at a dose of 10 mg/kg (n = 3). These prodrugs were selected due to their high *c*log *P* values of 3.75 and 5.02, respectively. However, the HPLC or LC/MS-analysis of the plasma samples at different times over a period of 180 min never revealed any concentration of the prodrugs or their active metabolites above the detection limit (100 nmol/L or 0.065 μg/mL for the parent compounds, and 200 nmol/L or 0.13 μg/mL for both prodrugs). In addition, neither inhibitor nor prodrug could be detected in kidney, liver, or in the urine. These results clearly indicate that both prodrugs are not orally available. In contrast, after oral application of the reference compound ximelagatran[12] (20 mg/kg, n = 3), a double prodrug of the thrombin inhibitor melagatran that also contains a hydroxyamidino prodrug moiety, significant levels could be detected for the drug melagatran (C_{max} = 1.0 μmol/L) as well as for the mono prodrug *N*-hydroxy-melagatran (C_{max} = 1.1 μmol/L) over a period of 120 min.

However, although we could not demonstrate oral bioavailability for these types of matriptase inhibitors, we used several analogues of this and our preceding series in tumor cell-based assays to elucidate further the role of matriptase in invasion, pro-uPA , and pro-HGF cleavage with subsequent c-Met activation and phosphorylation. The results of these studies will be published elsewhere.[14]

All inhibitors summarized in Table 1 were synthesized using standard procedures, an example of which is described for the preparation of **4** (Scheme 1). Briefly, commercially available 4-(piperidin-4-yl)butanoic acid was converted into its methylamide

32. This intermediate was coupled to the 3-cyanophenylalanine derivative **33**, followed by reduction of the nitro group and coupling of Cbz-β-Ala-OH using the mixed anhydride method. The conversion of the nitrile into the amidine **4** was performed according to the method of Judkins.[11,15] Appropriate commercially available secondary amines were used as starting material for the synthesis of other compounds given in Table 1. All inhibitors with free carboxyl group were obtained from their analogous methyl esters by saponification in the final step.

The biphenyl-3-sulfonyl derivatives (**21**, **24**–**26**) were obtained by Suzuki coupling as previously described.[13] Inhibitor **22** was obtained by hydrogenation of purified **21** using Pd/C as catalyst in 90% acetic acid over 2 days. The coupling of the nitrogen heterocycles to the aryl iodide for inhibitors **27** to **31** was performed by a copper-catalyzed reaction (Scheme 2).[16] The final conversion of the nitrile into the amidine was performed as described above.

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