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Development of substrate analogue inhibitors for the human airway trypsin-like protease HAT

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ABSTRACT

A series of substrate analogue inhibitors of the serine protease HAT, containing a 4-amidinobenzylamide moiety as the P1 residue, was prepared. The most potent compounds possess a basic amino acid in the D-configuration as P3 residue. Whereas inhibitor **4** (K_i 13 nM) containing proline as the P2 residue completely lacks selectivity, incorporation of norvaline leads to a potent inhibitor (**15**, K_i 15 nM) with improved selectivity for HAT in comparison to the coagulation proteases thrombin and factor Xa or the fibrinolytic plasmin. Selected inhibitors were able to suppress influenza virus replication in a HAT-expressing MDCK cell model.

Human influenza viruses cause acute infection of the respiratory tract that affects millions of people during seasonal outbreaks and occasional pandemics worldwide.[1] Currently, two drugs targeting the viral neuraminidase (NA) and the M2 channel blockers amantadine and rimantadine are approved for the treatment of influenza. Resistance to these drugs have been observed and exacerbate the situation.[2,3]

The replication cycle of influenza viruses is initiated by its surface glycoprotein hemagglutinin (HA). HA mediates the binding of the virus to sialic acid containing receptors of the host cells and, after endocytosis, the fusion of the viral envelope with the endosome membrane. This process is termed uncoating and enables the release of the viral genomic RNA into the cytosol of the host cell.[4] HA is synthesized as HA0 precursor and has to be cleaved by host endoproteases into disulfide-linked HA1 and HA2 subunits to become fusogenic and is thus a crucial step for infectivity and spread of influenza viruses. The HAs of most influenza strains, including the H1, H2 and H3 subtypes, which typically infects humans, contain a single arginine as the P1 residue at its cleavage site.[5] Recently it was shown that the trypsin-like serine proteases HAT (human airway trypsin-like protease or TMPRSS11D), TMPRSS2 (epitheliasin or transmembrane protease serine 2) and TMPRSS4 (CAP 2 or transmembrane protease serine 4), which are expressed in the human respiratory tract, efficiently cleave the HA0 of various influenza strains with a monobasic cleavage site.[6,7] In addition, HAT and TMPRSS2 also support the multicycle influenza virus replication in HAT- or TMPRSS2-expressing MDCK cells.[6] Additionally, we could demonstrate that HAT is proteolytically active on the cell surface, whereas it seems that TMPRSS2 cleaves HA0 in the secretory pathway within the cell.[8] Due to its location on the cell surface and its better accessibility HAT might be a potential target for the treatment of influenza infections. Recently, a first peptidic arginal-derived HAT inhibitor with a K_i value of 54 nM has been developed.[9]

HAT belongs to the type II transmembrane serine proteases and has a mosaic-like structure with its protease domain in the C-terminal part.[10] So far no X-ray structure of HAT is available. However, the sequence of its protease domain has significant similarity to other trypsin-like serine proteases, such as the clotting proteases thrombin[11] and factor Xa[12] or the fibrinolytic urokinase (uPA).[13] These proteases are all inhibited by substrate analogue structures containing a 4-amidinobenzylamide as a decarboxylated P1 arginine mimetic in combination with a P3 amino acid in the D-configuration.[14,15] Starting from a homology model[16–18] of HAT based on the X-ray structure of DESC1[19] (2oq5.pdb) we assumed that such 4-amidinobenzylamide derivatives should be suitable HAT inhibitors.

A first screen with available compounds, like the previously described factor Xa inhibitor 1[12] and the uPA inhibitors 2 and 3[13] revealed also some inhibition of HAT with K_i-values >50 nM. These analogues have been previously used for preliminary experiments to demonstrate the inhibition of proteolytic activation and propagation of influenza viruses in HAT-expressing MDCK cells.[20]

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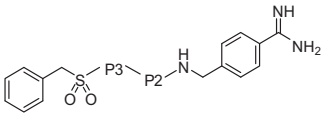


	P3-P2	K_i (nM)[21]
1	D-Arg-Gly	430
2	D-Ser-Ser	78
3	D-Ser-Lys(Cbz)	51

Therefore, we screened[22] and synthesized additional analogues of this inhibitor type. Based on previous experience with related thrombin,[23] factor Xa[12] and urokinase[13] inhibitors it was known that proline is a suitable P2 residue in substrate analogue inhibitors of various trypsin-like serine proteases. Consequently, we prepared a series of P2 proline analogues with several P3 amino acids in the D-configuration and maintained the important P4 benzylsulfonyl[15] and the P1 4-amidinobenzylamide[24] (Table 1).

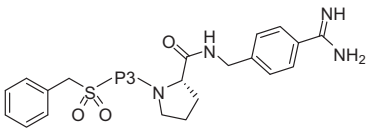
Only the replacement of glycine in inhibitor **1** by proline resulted in an approximately 20-fold improved inhibition constant of compound **5**, which could marginally be enhanced by incorporation of a P3 *homo*-D-arginine (**4**, K_i 13 nM). Obviously, the conformationally constrained proline is well accepted by the S2 site of HAT. However, proline is also a suitable P2 residue for many other trypsin-like serine proteases and its incorporation often leads to lack of specificity. Indeed, compound **5** is also a potent inhibitor of thrombin (3.5 nM), factor Xa (2.4 nM), plasma kallikrein (8.3 nM), matriptase (55 nM) and matriptase 2 (170 nM), thus completely lacking any selectivity.[25,26] The other P2 proline analogues have reduced potency, however, some of them still inhibit HAT with K_i-values <100 nM, including several inhibitors with hydrophobic P3 residues. Based on the K_i-values it seems that HAT has some preference for positively charged P3 residues, whereas the acidic D-aspartyl residue (**17**) is poorly accepted.

Interestingly, the *tert*-butyl protected D-aspartic- or D-glutamic acid inhibitors **6** and **7** show relatively high potency with K_i-values <50 nM. Therefore, we used analogues with a free D-aspartic- and D-glutamic acid side chain in the P3 position as suitable starting point for further modifications and incorporated various piperazine derivatives and other cyclic amines in an additional series (Table 2).

The strongest inhibitory potency within this series was obtained with a 1-(2-pyrimidyl)-piperazine coupled to the side chain of D-glutamic acid (**18**). However, all other analogues had reduced activity; so we decided to keep D-arginine in the P3 position to study the influence of further P2 substitutions.

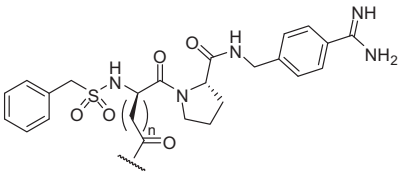
Results from Table 3 indicate that valine, isoleucine and alanine as well as its close analogues α-aminobutyric acid and norvaline are suitable P2 residues and provide inhibitors with K_i-values ⩽ 30 nM. We assumed that some of these analogues could have an improved selectivity compared to the unspecific proline derivative **5**. Therefore, we selected some inhibitors and determined their K_i-values against the coagulation proteases thrombin and factor Xa and the fibrinolysis enzyme plasmin (Table 4). Inhibitor **31** is the only compound that has stronger potency against HAT compared to the other proteases. Especially compounds **4** and **5** have a significantly higher affinity to thrombin and factor Xa. The inhibitory potency against thrombin could be strongly reduced by incorporation of a P2 serine residue (**36**). A similar effect was previously observed in a series of analogous urokinase inhibitors[13]; however, inhibitor **36** is still a relatively potent factor Xa inhibitor. In contrast, all selected compounds from the piperazine series (**18**, **19**, **21**) showed a reduced affinity for factor Xa but were still relatively potent thrombin inhibitors (K_i <15 nM). It should be noted that various compounds also inhibit the protease domain of TMPRSS2 with K_i-values <100 nM (e.g., 20, 53 and 68 nM for derivatives **5**, **30** and **31**, respectively). Detailed results regarding the

Table 1
Inhibition of HAT by P2 proline inhibitors of the general formula



Compound	P3	K_i (nM)[21]
4	D-*homo*-Arg	13
5	D-Arg	19
6	D-Glu(OtBu)	36
7	D-Asp(OtBu)	38
8	D-Lys	40
9	D-Lys(Cbz)	53
10	D-*homo*-Phe	63
11	D-Val	76
12	D-Phe	98
13	D-Cha	108
14	D-Leu	120
15	D-Phe(4-Amidino)	168
16	D-Phe(4-CN)	311
17	D-Asp	1425

Table 2
Inhibition of HAT by inhibitors of the general structure



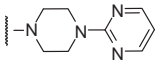
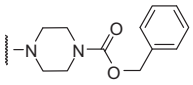
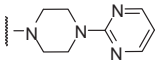
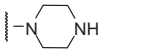
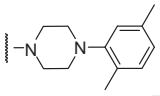
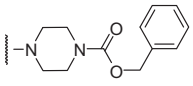
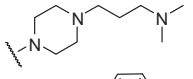
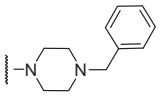
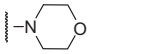
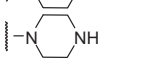
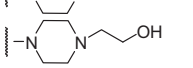
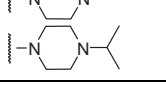
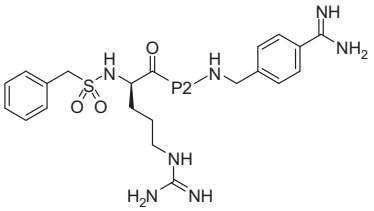
Compound	n	P3	K_i (nM)[21]
18	2		17
19	2		38
20	1		43
21	2		68
22	1		73
23	1		135
24	1		149
25	1		158
26	1		183
27	1		202
28	1		210
29	1		221

Table 3
Modifications in the P2 position



Compound	P2	K_i (nM)[21]
30	Abu[a]	14
31	Nva[b]	15
32	Val	22
33	Ile	23
34	Ala	30
35	Lys(Cbz)	34
36	Ser	34
37	Leu	47
38	Phe	57
39	Asp	422
40	Arg	457
41	Asp(OtBu)	512

[a] α-Aminobutyric acid.
[b] Norvaline.

Table 4
Specificity of selected compounds

Compound	K_i (nM)			
	HAT[21]	Thrombin[28]	FXa[28]	Plasmin[28]
4	13	0.39	2.7	17.5
5	19	3.5	2.4	39
6	36	6.7	31	1520
8	40	1.0	52	63
18	17	5.2	129	23,130
19	38	9.8	397	1160
21	68	13.5	565	114
30	14	29	2.1	3030
31	15	68	37	2110
32	22	66	21	2810
34	30	74	1.4	590
36	34	1610	14.5	161

expression of the proteolytic domain of TMPRSS2 in *Escherichia coli* and inhibition studies with these substrate analogue inhibitors will be published in due course.[27]

The relatively selective HAT inhibitor **31**, its close analogue **30** and the most potent compound from the piperazine series (**18**) were selected to inhibit influenza virus propagation in cell culture. For this assay MDCK cells were used which express HAT under doxycycline-induced transcriptional activation.[8] These MDCK cells were infected with various human influenza strains and incubated with the respective inhibitors for 24 h to allow multiple cycles of viral replication. Subsequently infected cells and comet-like spread of infection were immunostained using antibodies against the viral nucleoprotein (NP) as described previously.[20] As expected, multicycle replication of viruses is observed in doxycycline-treated cells in the absence of inhibitors, whereas no virus spread is visible in cells lacking doxycycline-induced expression of HAT (Fig. 1).

At a concentration of 1 μM (not shown) selected inhibitors and compound **5**, which serves as control,[8] show only a negligible effect against all three tested virus strains, whereas at 10 μM all compounds, especially inhibitors **18** and **30**, strongly suppress multicycle virus replication.

The synthesis of the inhibitors was performed according to previously described methods[12,13,26] and is exemplarily described only for the most potent analogue **4** and its precursors **8** and **9** and for inhibitor **18** (Schemes 1 and 2). Briefly, benzylsulfonylchloride (**42**) was introduced to H-D-Lys(Cbz)-OH followed by coupling of H-Pro-4-amidinobenzylamide × 2HCl[29] (**44**) to give inhibitor **9**. Cleavage of the Cbz-group provided inhibitor **8**, which was converted into the D-*homo*-Arg analogue **4** by reaction with 1*H*-pyrazole-1-carboxamidine.[30]

Inhibitor **18** was synthesized according to Scheme 2 starting with inhibitor **6**. Cleavage of the *tert*-butyl group provided compound **45**, which was used for PyBOP mediated coupling of 1-(2-pyrimidyl)-piperazine to give analogue **18**.

In summary, the replacement of glycine by proline in the initial inhibitor **1** improved the K_i-value by a factor of 20, but leads to poor selectivity. In fact, thrombin and factor Xa are even more inhibited than HAT by various compounds. Interestingly, the incorporation of P2 norvaline provided an inhibitor (**31**) with similar potency against HAT and improved selectivity against the other tested three trypsin-like serine proteases. Furthermore, this

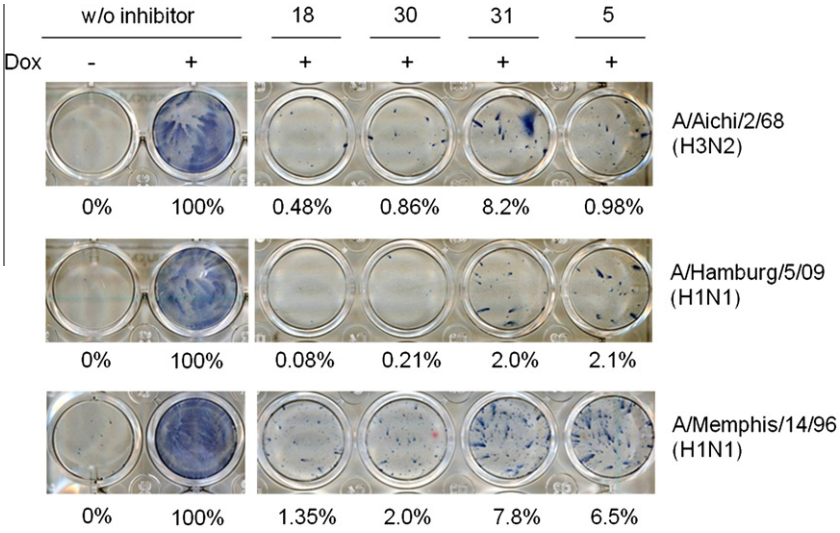
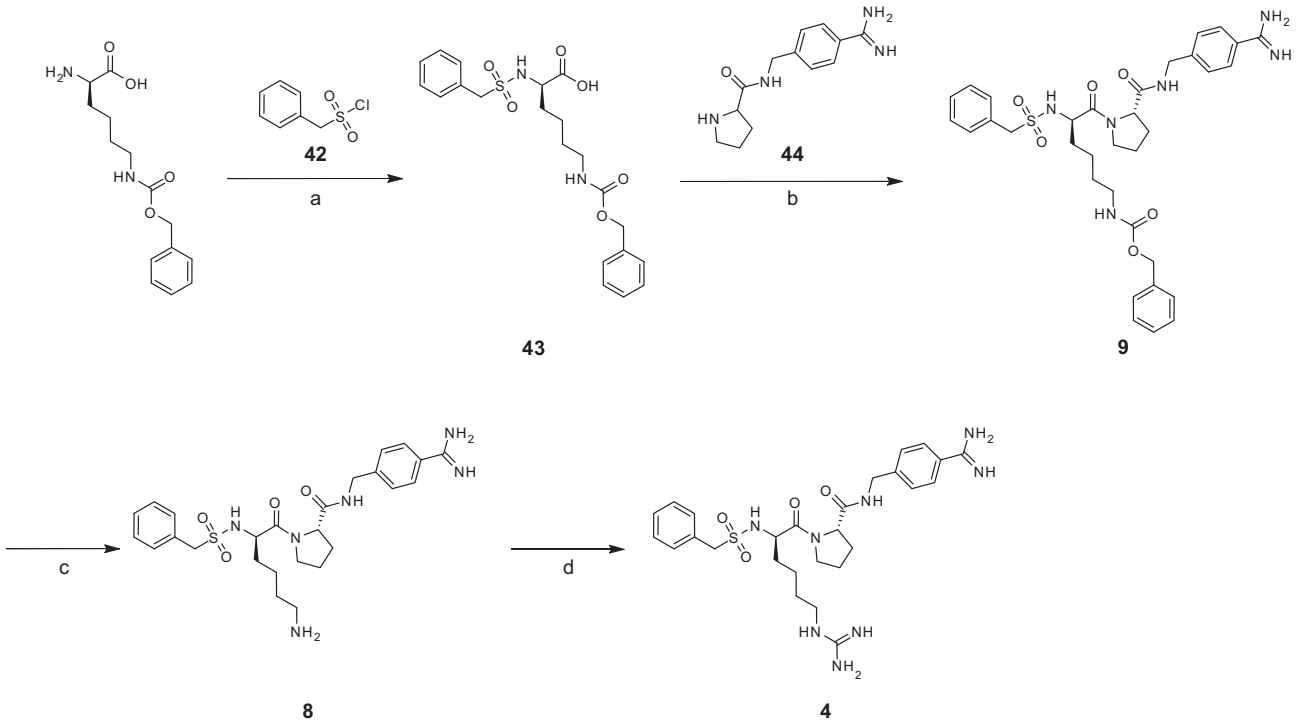
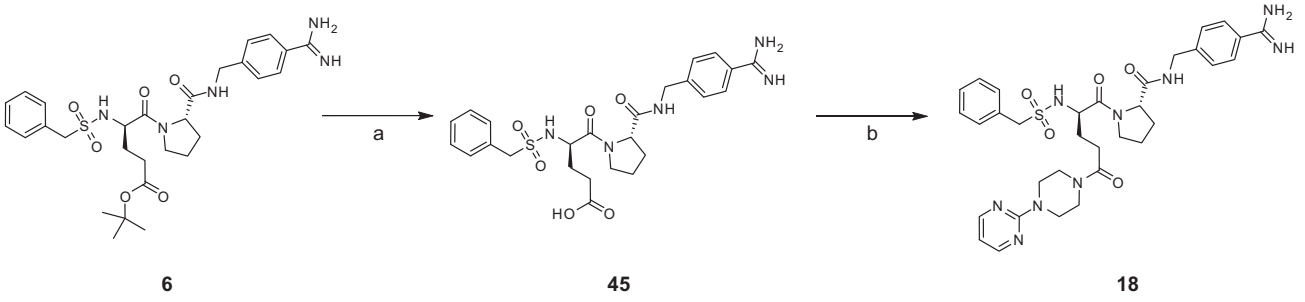


Figure 1. Inhibition of influenza virus propagation by inhibitor treatment in MDCK–HAT cells. Cells were infected with different influenza A viruses and incubated with or without doxycycline (Dox) in the presence of 10 μM of inhibitors **5**, **31**, **30** and **18** at 37 °C. After 24 h the cells were immunostained with influenza virus NP-specific antibodies. Infection of MDCK–HAT cells treated or not with doxycycline in the absence of inhibitors are used as control. Immunostained cells were counted using a microscope, and the percentage of infected cells compared to control (100%) is given below each well.



Scheme 1. Synthesis of inhibitors **4**, **8** and **9**. Reagents and conditions: (a) 2.2 equiv trimethylsilylchloride, 2.2 equiv diisopropylethylamine reflux in dry dichloromethane for 1 h followed by addition of 1.1 equiv **42** and 1.1 equiv diisopropylethylamine at 0 °C for 15 min, then room temperature for 3 h, (b) 1 equiv **44**, 1.1 equiv HBTU, 1.1 equiv HOBt, 3 equiv diisopropyethylamine in DMF at room temperature, 12 h, (c) 10% Pd/C, H_2 atmosphere, in 90% acetic acid, room temperature for 24 h, (d) 3 equiv 1*H*-pyrazole-1-carboxamidine hydrochloride, 4.5 equiv diisopropylethylamine in DMF, 16 h. All final compounds were purified by reversed phase HPLC to a purity of >95% according to HPLC analysis and UV detection at 220 nm and obtained as lyophilized powders.



Scheme 2. Reagents and conditions: (a) trifluoroacetic acid for 1 h, (b) 1.1 equiv 1-(2-pyrimidyl)-piperazine, 1 equiv PyBOP, 2 equiv diisopropylethylamine, room temperature, 6 h.

norvaline inhibitor **31**, its close analogue **30** and compound **18** from the piperazine series were effective in suppressing replication of H1 and H3 influenza viruses in a HAT-expressing MDCK cell model.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.06.033.

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21. 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 Safire^2 fluorescence plate reader (Tecan) (λ_{ex} = 380 nm; λ_{em} = 460 nm) and D-cyclohexylalanine-Pro-Arg-AMC 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 μM. Results were obtained from at least two independent experiments.
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