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4-Amidinobenzylamine-Based Inhibitors of Urokinase

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Abstract—A series of 4-amidinobenzylamine-based peptidomimetic inhibitors of urokinase was synthesized. The most potent one, benzylsulfonyl-D-Ser-Ala-4-amidinobenzylamide **16**, inhibits uPA with a K_i of 7.7 nM but is less selective than **10** with a Gly as P2 residue. Hydroxyamidine and carbonate prodrugs were prepared, which are rapidly converted into the active inhibitors in rats after subcutaneous application.

The plasminogen activator urokinase (uPA) is a trypsin-like serine protease which converts plasminogen to plasmin, which in turn can further activate several matrix metalloproteases (MMP). Plasmin and MMP enable tumor cells to degrade surrounding extracellular matrix proteins, such as laminin and fibronectin, and to intravasate and extravasate lymph and blood vessels. This process is believed to be essential for the dissemination of the tumor and the formation of metastasis. The proteolytic activity of uPA for the cleavage of plasminogen into plasmin is significantly enhanced when it is bound to the surface of tumor cells, which express a specific receptor (uPAR or CD87) for uPA and its zymogen pro-uPA. In addition, the uPA/uPAR-complex initiates several biological events including cell proliferation, migration, chemotaxis and adhesion.[1,2] The strong correlation between increased uPA and uPAR levels found in primary cancer tissues and disease recurrence makes these factors an interesting target for anti-invasive and antimetastatic drugs.[3,4] Among several strategies, the development of selective low molecular weight inhibitors of uPA seems to be a promising way for the development of new cancer therapeutics.[5]

4-Amidinobenzylamine (4-Amba) is a decarboxylated arginine mimetic, which has been widely used for the development of RGD (Arg-Gly-Asp) analogues[6] and protease inhibitors, especially for the thrombin inhibitor melagatran.[7] During a screening among our inhibitor pool using several trypsin-like serine proteases, compound **1** (2-Nas-Gly-4-Amba[8]) was identified to be a moderate uPA inhibitor with a K_i of 9.2 μM (Table 1).[9] In contrast, an analogue with 3-Amba as P1 residue was inactive ($K_i > 1000$ μM). Therefore we assumed that the 4-Amba group significantly contributes to the affinity towards uPA and may be useful for further lead optimization. Initial attempts to improve the affinity of **1** by simple modification of the N-terminal sulfonyl residue or by exchange of the P2 Gly by other L- or D-amino acids were not successful; only for 2-Nas-Ala-4-Amba was a comparable affinity found (K_i 9.6 μM).

Therefore, in the next step P3 amino acids of peptide sequences which were previously shown to be well accepted by uPA in several substrates and inhibitors were attached to Gly-4-Amba. The first sequence was derived from the known irreversible uPA inhibitor Glu-Gly-Arg-chloromethylketone.[10] Secondly, a P3 Ser was incorporated which was shown to be important for the selectivity and the rate of uPA inhibition by variants of the plasminogen activator inhibitor type 1.[11] The third series is based on slow binding arginal inhibitors with a D-Ser in P3 position, which were recently described by Corvas to inhibit uPA after a 30 min incubation period with IC_{50} values in the nanomolar range.[12]

In this report, the design, synthesis, in vitro structure–activity relationships and some pharmacokinetic data of these new uPA inhibitors with a P1 4-Amba group are presented.

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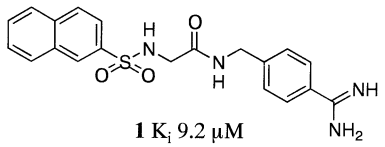
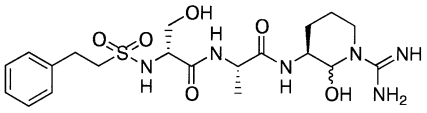


1 K_i 9.2 μM



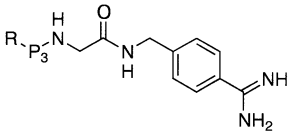
IC_{50} 3.1 nM (described by Corvas)[12]

H-Glu-Gly-4-Amba **2** is slightly less potent than **1** (Table 1). Modification of the N-terminal amino group by a urethane protection further reduced the activity of **3**, therefore no additional analogue with glutamic acid in P3 position were prepared. The incorporation of serine is also accepted; **4** and **5** have similar activity and selectivity as found for **2**. Inhibitors **6** and **7** containing D-serine show a slightly improved anti-uPA activity.

Because only **7** retained the potency towards uPA additional analogues with a D-Ser as P3 residue were prepared (Table 2). A simple acetylation lowers the uPA affinity of **8**; in contrast the Cbz derivative **9** is slightly more active. In the next step the acyl or carbamate protecting groups were replaced by various sulfonyl residues. The benzylsulfonyl analogue **10** has a 330-fold improved activity compared with **6** and was found to be a very selective uPA inhibitor. The 2-phenethylsulfonyl derivative **12** was synthesized by hydrogenation of the *trans*-styrenesulfonyl inhibitor **11**, because it showed the highest affinity in the arginal series described recently.[12] However, **12** was nearly 14 times less potent than **10**. Several other commercially available sulfonyl groups were introduced, but most of the obtained compounds showed reduced uPA inhibition. Therefore we speculated that a methylene spacer is probably essential within the hydrophobic N-terminal sulfonyl group to improve its flexibility and to enable an appropriate binding mode. This could be confirmed by the incorporation of a cyclohexylmethylsulfonyl[13] residue; **15** showed a similar potency and selectivity profile as found for **10**. X-ray[14] and docking[15] investigations for similar tripeptide inhibitors containing an N-terminal benzylsulfonyl group and a D-amino acid in P3 position in complex with thrombin have shown that the main chain of the inhibitor adopts a turn-like structure resulting in a close contact between the phenyl ring of the benzylsulfonamide and the cyclic P1 residue. Therefore, a similar binding mode may explain the high potency found for **10** and **15** in this inhibitor series.

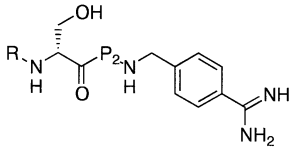
When Ala and Pro were placed in P2 position (**16**, **17**) the uPA affinity was further improved; however, these compounds exhibited a decreased selectivity towards all other proteases investigated, especially for trypsin, plasmin and thrombin. This could be of special interest because some other proteases are also assumed to be involved in tumor spreading.[3,16] However, a strong thrombin inhibition as found for the Pro derivative **17** could potentially influence the blood coagulation system, which may cause side effects such as bleeding complications. Therefore only **10** and **16** were selected for further characterization and modification.

Table 1. Inhibition of uPA and related proteases by inhibitors of the general formula



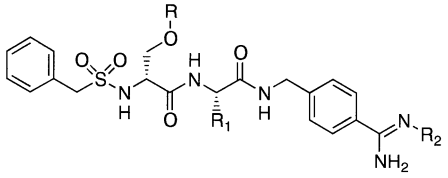
No.	R	P3	K_i (μM)				
			uPA	Plasmin	Trypsin	Thrombin	Factor Xa
1	2-Nas	—	9.2	43	0.65	16	10.6
2	H	Glu	22	>1000	5.0	>1000	>1000
3	Boc	Glu	82	>1000	2.8	>1000	>1000
4	H	Ser	21	>1000	7.0	>1000	>1000
5	Boc	Ser	23	210	3.4	69	110
6	H	D-Ser	12	>1000	6.9	200	>1000
7	Boc	D-Ser	9.0	>1000	4.5	170	140

Table 2. Inhibition of uPA and related proteases by inhibitors of the general formula

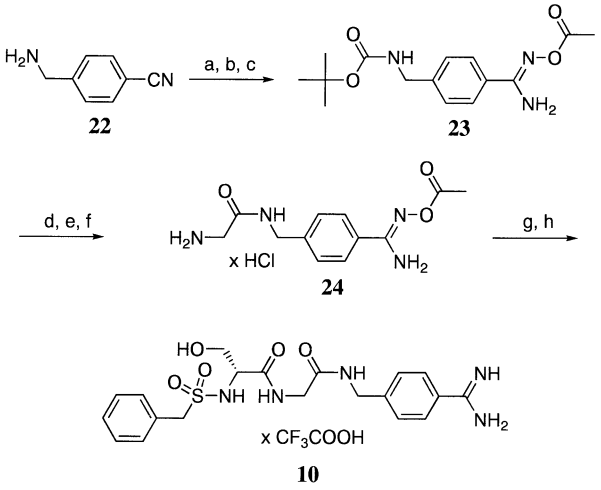


No.	R	P2	K_i (μM)				
			uPA	Plasmin	Trypsin	Thrombin	Factor Xa
8	Acetyl	Gly	41	>1000	14	>1000	>1000
9	Cbz	Gly	4.0	160	4.3	230	140
10	Benzyl-SO_2	Gly	0.036	11	0.15	13	3.0
11	*trans*-Styrene-SO_2	Gly	2.2	140	1.7	15	15
12	2-Phenethyl-SO_2	Gly	0.49	59	0.90	33	28
13	2-Nas	Gly	1.2	32	1.8	11	28
14	Triisopropyl-phenyl-SO_2	Gly	1.5	75	3.4	15	100
15	Cyclohexyl-methyl-SO_2	Gly	0.048	17	0.11	10	2.0
16	Benzyl-SO_2	Ala	0.0077	0.54	0.0033	0.11	2.1
17	Benzyl-SO_2	Pro	0.013	0.15	0.0032	0.012	2.0

Table 3. Inhibition of uPA and related proteases by prodrugs of the formula



No.	R	R_1	R_2	K_i (μM)				
				uPA	Plasmin	Trypsin	Thrombin	Factor Xa
18	H	H	OH	61	>1000	>1000	>1000	>1000
19	CO_2Me	H	H	0.39	10.9	0.064	3.0	0.34
20	CO_2iBut	H	H	0.5	2.4	0.12	4.0	0.5
21	CO_2iBut	CH_3	H	0.043	0.15	0.0048	0.05	0.41



Scheme 1. Reagents and conditions: (a) 1.1 equiv Boc_2O in dioxane/1 N NaOH, 30 min 0 °C, 12 h rt; (b) 1.5 equiv $NH_2OH{\cdot}HCl$ and DIEA in MeOH, reflux 4 h and 12 h rt; (c) 2 equiv acetic anhydride in AcOH, 20 min rt; (d) 1 N HCl/AcOH, 45 min rt; (e) 1 equiv Boc-Gly-OH, PyBop and 3 equiv DIEA in DMF, 15 min 0 °C, 2 h rt; (f) 1 N HCl/AcOH, 45 min rt; (g) 1 equiv benzylsulfonyl-D-Ser(Bzl)-OH, PyBop and 3 equiv DIEA in DMF, 15 min 0 °C, 2 h rt.; (h) H_2 and 10% Pd/C in acetic acid overnight, preparative HPLC.

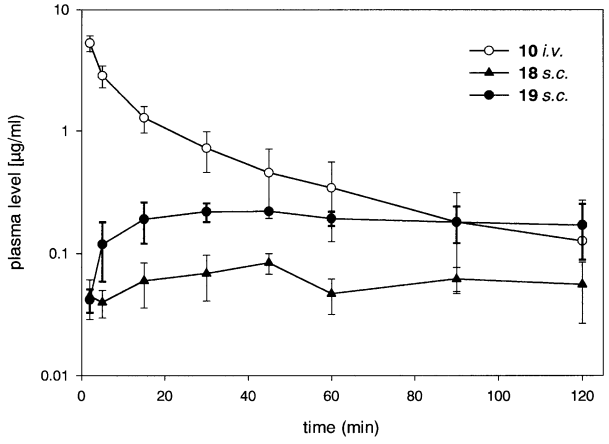


Figure 1. Time course of plasma levels in rats after iv or sc injection of a dose of 1 mg/kg of selected inhibitors ($n=3$).

Different prodrugs have been prepared (Table 3), because the analysis of the plasma levels of **10** after iv application (1 mg/kg) to rats revealed an unsatisfactory fast elimination from the circulation with a half life shorter than 10 min.[17] In **18** the basic amidino group is masked as hydroxyamidine resulting in a P1 4-hydroxyamidinobenzylamide, a modification known from the development of an orally active prodrug of the thrombin inhibitor melagatran.[18] In **19**–**21** the D-serine hydroxyl group was reversibly blocked as carbonate.[12] While prodrug **18** was inactive in all K_i determinations, the carbonates **19**–**21** surprisingly retained some uPA affinity and possess an enhanced potency towards related enzymes assayed in vitro.

The inhibitors **18** and **19** were subcutaneously administered to rats at a dose of 1 mg/kg, and as expected, only **10** could be detected in plasma (Fig. 1). This indicates that these prodrugs are easily converted in free **10**. In contrast to an iv application of **10**, upon sc application of **19** a sustained plasma concentration of circa 0.2 μg/mL was achieved; the plasma level of **18** was likewise lower but also nearly constant.

The described 4-amidinobenzylamine-containing peptidomimetics represent a new class of potent, fast-binding inhibitors of uPA including **10**, which was found to be exceptionally selective, and **16**, which had a higher uPA-affinity but lower selectivity. They are easily prepared from inexpensive 4-cyanobenzylamine and lack the potentially labile stereogenic center present in the arginine-based transition-state analogues.

Synthesis

The synthesis of inhibitors **2**–**17** is exemplarily described by the preparation of **10** (Scheme 1). 4-Cyanobenzylamine **22** was initially Boc protected and converted into the acetyloxamidine **23**.[19] The Boc group was removed, and the intermediate was coupled with Boc-Gly-OH using PyBOP/DIEA. In the next step the N-terminal protecting group was cleaved to give **24**, followed by the coupling of benzylsulfonyl-D-Ser(Bzl)-OH. The final hydrogenation resulted in **10**. **18** was obtained from benzylsulfonyl-D-Ser(tBut)-Gly-4-cyanobenzylamine by the treatment with 1.5 equiv $NH_2OH{\cdot}HCl$ and diisopropylethylamine in refluxing methanol for 4 h; the

serine protecting group was cleaved with 90% trifluoroacetic acid (45 min at rt). The prodrugs **19** und **20** were prepared from **10** by reaction with 2 equiv of the appropriate chloroformate in pyridine; despite several side products approximately 25% of the desired compounds could be isolated by preparative HPLC.

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