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New substrate analogue furin inhibitors derived from 4-amidinobenzylamide

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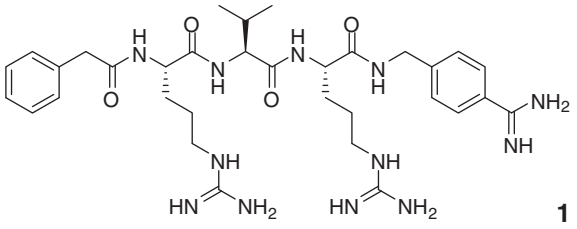
ABSTRACT

A series of new peptidomimetic furin inhibitors was synthesized, which was derived from our previously described lead structure phenylacetyl-Arg-Val-Arg-4-amidinobenzylamide (**1**). Substitution of Val by other amino acid residues revealed several highly potent furin inhibitors with K_i values of less than 2 nM, containing guanidinoalanine, Ile, Phe or Tyr in the P3 position. The replacement of the P2 Arg by Lys was also well accepted, whereas the incorporation of D-amino acids at various positions resulted in poor inhibitors. The use of the 4-amidinobenzylamide group provides convenient synthetic access to stable proprotein convertase inhibitors and derivatives as biochemical tools and for further studies in cell culture.

The proprotein convertase furin is a Ca^{2+}-dependent cellular serine endoprotease with a strong preference for cleavage at multibasic consensus sequences Arg-X-Arg/Lys-Arg↓X.[1] It is involved in the maturation of many proproteins in the secretory pathway, including prohormones, proenzymes and proforms of receptors or extracellular matrix proteins, and therefore has an important function in embryogenesis and homeostasis.[2,3] A comprehensive overview about potential furin substrates provides a recently developed database.[4] In addition to normal physiological processes, furin is most likely involved in diseases, such as cancer,[5] dementia, neurodegenerative disorders, various viral and bacterial infections, and has therefore emerged as a potential target for drug design.[6] Meanwhile, in first proof-of-concept studies it could be demonstrated that synthetic furin inhibitors might have therapeutic benefit in living animals.[7] Various types of synthetic furin inhibitors have been reviewed in the past.[8,9] Among these are pure peptides, such as nona-D-Arg[10]; substrate-analogue peptide mimetics containing non-cleavable ketone-derived peptide bond replacements between the P1 and P1′ residues[11]; and various non-peptidic derivatives based on highly potent guanylated 2,5-dideoxystreptamines,[12] with K_i values <10 nM. Additional non-peptidic lead structures have also been reported; however, most of these compounds exhibit reduced potency.[13,14]

We have recently reported the first furin inhibitors containing a C-terminal 4-amidinobenzylamide as a decarboxylated arginine mimetic. The most potent of these inhibitors, Phac-Arg-Val-Arg-4-Amba **1**, inhibits furin with a K_i value of 0.81 nM and has a similar inhibitory potency against the proprotein convertases PC1/3, PC5/6 and PACE4, whereas PC2 and PC7 or trypsin-like serine proteases were poorly affected.[15]



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Modeling of the binding mode of these inhibitors[15] based on the X-ray structure of mouse furin inhibited by the irreversible inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (1p8j.pdb)[16] revealed that the side chain of the P3 Val is directed away from the enzyme surface towards the solvent and has only loose contact with the carboxyl group of the Glu257 side chain (distance ≈ 3.5–4 Å). Therefore, in this study, we investigated the modification of this P3 residue and prepared additional analogues within this lead structure for comparison.

All inhibitors were prepared by a combination of solid phase and solution synthesis according to the strategy described previously.[15] Briefly, the side-chain protected P5-P2-segment was synthesized by a Fmoc-protocol on 2-chloro-tritylchloride resin using HBTU as a coupling reagent. After weak acidic cleavage from the resin the intermediates were coupled to unprotected 4-amidinobenzylamine × 2HCl using PyBOP/DIPEA in the presence of 6-Cl-HOBT, followed by final side chain deprotection (see Scheme 1 for the synthesis of inhibitors **2** and **7**).

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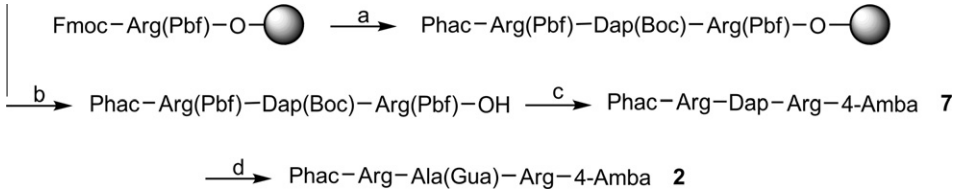

Scheme 1. Synthesis of inhibitors **2** and **7** on Fmoc-Arg(Pbf)-2-chlorotrityl resin. Reagents and conditions: (a) standard Fmoc SPPS; (b) 1% TFA in DCM, 2 × 30 min; (c) (i) 1.5 equiv 4-amidinobenzylamine × 2HCl, 1.1 equiv PyBOP, 3 equiv 6-Cl-HOBt, 3 equiv DIPEA in DMF, 2 h, (ii) TFA/TIS/H_2O (95/2.5/2.5, v/v/v), 3 h, 35 °C; (d) 5 equiv. 1*H*-pyrazole-1-carboxamidine × HCl in 1 $NaCO_3$, 48 h. All final compounds were purified by reversed phase HPLC and obtained as lyophilized powders.

Inhibition constants varied approximately 400-fold (between 1 and 400 nM) among derivatives containing one of the 20 proteinogenic amino acids in the P3 position (Fig. 1). Compared to lead **1**, nearly identical inhibitory activity was found for the Ile inhibitor **3** (K_i of 0.84 nM), whereas the structurally similar Leu derivative **14** was, surprisingly, more than 10-fold less potent. We do not believe that this drop in affinity is related to a simple sterical hindrance of the Leu side chain, because a relatively high potency was also found for the sterically more demanding Phe and Tyr analogues **4** and **5**. In contrast, poor activity (K_i >100 nM) was found for the Glu and Asp inhibitors **21** and **22**, most likely due to electrostatic repulsion from residue Glu257 and the negatively charged active site of furin. Although we expected some benefit from the incorporation of an additional basic amino acid (Arg, Lys) in the P3 position, the resulting inhibitors **6** and **8** showed slightly decreased potency compared to **1**. Based on our model[15] with the Val in P3 position we assume that the side chains of Arg or Lys are also directed towards the solvent and might be too long and flexible to enable any direct interactions with Glu257. To reduce their side chain length we also incorporated the shorter α,β-diaminopropionic acid residue (Dap) in the P3 position and converted its side chain amine into a guanidino group. Whereas the Dap inhibitor **7** exhibited inhibitory activity similar to the Arg compound, its guanylated analogue **2** was more potent, inhibiting furin with a K_i value of 0.83 nM.

Relatively weak potencies, with K_i values of 40 and 36 nM, were found for the P3 Gly and Pro inhibitors, respectively. This might be explained by the lack of any P3 side chain in the case of the P3 Gly inhibitor, and by the elimination of the hydrogen bond between the P3 backbone with the carbonyl oxygen of the furin residue Gly255, which is present in the X-ray structure with a P3 Val,[16] but cannot be formed from a prolyl residue due to the lack of an amide hydrogen.

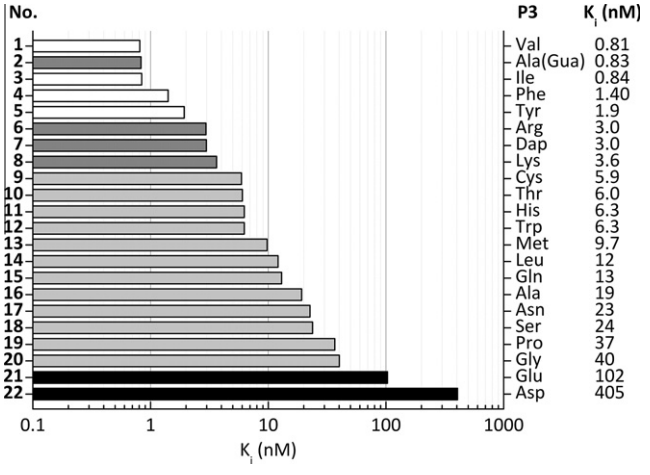

Figure 1. Inhibition of *h*-furin by inhibitors of the type Phac-Arg-P3-Arg-4-amidinobenzylamide. K_i measurements were performed as described previously and are the average of at least two measurements.[13,15] Inhibitors with basic and acidic P3 residues are shown with dark gray and black bars, respectively. Other potent analogues having K_i values <2 nM are presented with white bars and the remaining derivatives in gray.

In a second series (Table 1) we have modified other residues, mainly in the P4 and P2 positions. The vast majority of furin substrates contain an Arg at P4 and P1 positions as a minimal recognition requirement.[1,17] However, a study regarding processing of the wild type pro-insulin-like growth factor 1A demonstrated, that also Lys can be accepted as an alternative P4 residue, although even in that case an Arg residue in a mutated analogue is favoured.[18] Therefore, compound **23** containing a P4 Lys was prepared, but it was found to be a poor furin inhibitor with a 300-fold reduced K_i-value compared to compound **1**, containing an Arg at this position. This drop in the affinity was not surprising, because it is known from the furin crystal structure (1p8j) that the P4 guanidino group is involved in a complex network of salt bridges to the carboxyl groups of Asp264 and Glu236 in a relatively solvent excluded environment and forms also an additional hydrogen bond to the OH of Tyr308. Therefore, it seems to be impossible that all of these interactions can be maintained with a Lys residue in P4 position. Interestingly, the complete elimination of the basic P4 side chain, as present in the neutral urea-containing citrulline derivative **24** and the protected Lys(Cbz) inhibitor **25**, provided compounds with similar potencies as the Lys inhibitor. This also clearly indicates that a Lys cannot be used as an alternative basic P4 residue in these substrate analogue inhibitors.

Peptidic compounds often suffer from limited stability in biological systems due to proteolytic degradation. One strategy to overcome this problem is the incorporation of amino acids in D-configuration, which was applied also for the design of nona-D-Arg. Therefore, some analogues containing D-amino acids were prepared. However, inhibitors **26** and **27**, containing DVal or DAla in the P3 position, were approximately 1000-fold less potent than

Table 1
Inhibition of furin by inhibitors of the type P5-P4-P3-P2-4-Amba (compound **1** is included as reference).

No.	P5	P4	P3	P2	K_i (nM)
1	Phac	Arg	Val	Arg	0.81
23	Phac	Lys	Val	Arg	285
24	Phac	Cit	Val	Arg	238
25	Phac	Lys(Cbz)	Val	Arg	702
26	Phac	Arg	DVal	Arg	1110
27	Phac	Arg	DAla	Arg	1385
28	Phac	DArg	Val	Arg	970
29	Phac	Val	DArg	Arg	7340
30[a]	–	Acetyl	Val	Arg	2390
31[a]	–	Phac	DArg	Arg	3200
32[b]	Phac	Arg	Val	N^{α} (Me)Arg	142
33	Phac	Arg	Val	Lys	1.5
34	Phac	Arg	Dap	Lys	3.7

[a] These analogues are missing any P5 residue.
[b] This inhibitor was synthesized using commercially available Fmoc-N^{α}(methyl)Arg(Mtr)-OH (Bachem) for loading of the 2-chloro-tritylchloride resin.

inhibitor **1**. It should be noted that the inversion of the stereochemistry is not simply an exchange of the hydrogen and side chain attached to the C_α-carbon of an amino acid, but also has a strong impact on the backbone ϕ and ψ dihedral angles. In the Ramachandran plot the torsion angles of D-amino acid residues differ by a 180° rotation from that of L-amino acids.[19] We assume that a change in the backbone conformation completely disrupts important interactions between the Arg side chain and the S4 pocket of furin. Indeed, similar K_i values were found for derivative **30**, which is missing the P4 Arg residue, and for compounds **25** and **28** which contain a Lys(Cbz) or DArg in that position. Even further reduced potency was found for compound **29**, which contains a P4 Val in combination with DArg in the P3 position. This inhibitor might be useful as a negative control in cell culture studies for detection of non-specific effects, because this structure should have a similar overall physicochemical profile as compound **1**. Analogue **30**, which is missing the P4 Arg and contains DArg as the P3 residue, was even slightly more potent than compound **29**. It should be noted that there are relatively small differences in the K_i values between inhibitors **25**–**31** (≈factor of 10); therefore, we assume that they are still able to bind with their P2–P1 segment, which acts as a kind of anchor that provides a basic affinity to furin in the micromolar range.

An additional strategy to stabilize peptides against proteolytic degradation is the modification of peptide bonds, for example, by the incorporation of N^α(methyl)amino acids. As one example Ingles and Knowles demonstrated that the methylation of the P2–P1 amide bond has a dramatic influence on the α-chymotrypsin-catalyzed hydrolysis of acetyl-N^α(methyl)-tyrosine methylester, which was more than 100,000-fold less efficiently cleaved (based on k_{cat}/K_m values) compared to the non-methylated analogue.[20] We have previously used this approach to stabilize hirudin-derived bivalent thrombin inhibitors against thrombin degradation by incorporation of *N*(methyl)arginine as the P1 residue.[21] However, the incorporation of N^α(methyl)Arg as P2 residue resulted in a nearly 175-fold loss in potency of inhibitor **32** as compared to **1**. This was not expected, because based on the X-ray structure of decanoyl-Arg-Val-Lys-Arg-CMK-inhibited mouse furin we did not expect any steric hindrance, and the P2 backbone NH was neither involved in a hydrogen bridge to furin nor to a surrounding water molecule.

In contrast to the P4 position a P2 Lys is well accepted in many furin substrates[1,17] and substrate-analogue inhibitors.[11,22] This was confirmed in this study with inhibitors **33** and **34**, which exhibit only slightly decreased potency compared to the P2 Arg analogues **1** and **7**.

In summary, we have synthesized several new substrate analogue furin inhibitors with a 4-amidinobenzylamide as the P1 residue; various derivatives have inhibition constants in the low nanomolar range. Due to their close structural similarity to our previously described inhibitor **1**, which is also a highly potent inhibitor of PC1/3, PC5/6 and PACE4, we assume that at least some of these analogues should also inhibit other PCs (data not available). The further optimization of the P5 position within this inhibitor type will be described in a following publication.

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