Acta Biochim Biophys Sin (2008): 848-854 | © 2008 Institute of Biochemistry and Cell Biology, SIBS, CAS | All Rights Reserved 1672-9145
http://www.abbs.info; www.blackwellpublishing.com/abbs | DOI: 10.1111/j.1745-7270.2008.00470.x

Design of peptide inhibitors for furin based on the C-terminal fragment of histone H1.2

Suming Wang[1,3#], Jinbo Han[2,3#], Yanfang Wang[2#], Wuyuan Lu[4], and Chengwu Chi[2,3*]

[1] School of Life Sciences, University of Science and Technology of China, Anhui 230027, China
[2] Institute of Protein Research, College of Life Sciences and Technology, Tongji University, Shanghai 200092, China
[3] Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[4] Institute of Human Virology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21201, USA

The mammalian proprotein convertase furin has been found to play an important role in diverse physiological and pathological events, such as the activation of viral glycoproteins and bacterial exotoxins. Small, non-toxic and highly active, furin inhibitors are considered to be attractive drug candidates for diseases caused by virus and bacteria. In this study, a series of peptide inhibitors were designed and synthesized based on the C-terminal fragment of histone H1.2, which has an inhibitory effect on furin. Replacing the reactive site of inhibitors with the consensus substrate recognition sequence of furin has been found to increase inhibitory activity greatly. The most potent inhibitor , I_4, with 14 amino acid residues has a K_i value of 17 nM for furin. Although most of the synthesized peptides were temporary inhibitors, the inhibitor I_5, with nine amino acids, retained its full potency, even after a 3 h incubation period with furin at 37 ºC. These inhibitors may potentially lead to the development of anti-viral and anti-bacterial drug compounds.

Keywords furin; inhibitor; histone H1.2; peptide synthesis

In the secretory pathway, proproteins are limited and cleaved by a family of proteolytic enzymes called proprotein convertases (PCs). PCs are calcium-dependent serine proteases whose catalytic domain shares some sequence similarities with that of the bacterial subtilisin [1]. This cleavage is an important process widely used to regulate the activation of peptides and proteins that play significant roles in various biological events that are implicated in both homeostasis and various diseases [2]. Furin, a mammalian PC, was the first to be identified, and it has been extensively studied. Furin has been shown to have effects on different substrates, such as blood-clotting factors, growth factors, hormone receptors, matrix metalloproteinases and ion channels [3–5]. Bacterial exotoxins, such as diphtheria toxin, anthrax toxin, and viral envelope glycoproteins of HIV and the SARS virus, are also processed by furin [2,6–9]. Furthermore, many studies have indicated that increased furin activity is closely related to the malignancy of various tumors [10]. Thus, furin is an attractive target for therapeutic drugs.

Many furin inhibitors have been studied, including small molecular PC inhibitors and protein-based inhibitors [11]. Each small molecular PC inhibitor is categorized as a peptide inhibitor, peptidomimetic inhibitor or non-peptide inhibitor [12–14], while protein-based inhibitors include polypeptides derived from the prodomain of PC [15–17], bioengineered proteins [18–21], and some endogenous proteins [22–26]. Among them, α_1-antitrypsin Portland and polyarginine have been used to prevent the activation of bacterial toxin, the processing of envelope glycoprotein in viral replication and the metastasis of cancer [10,27,28].

By comparison, small peptide inhibitors are more attractive furin inhibitors, since they are more potent but have low toxicity. Many peptide inhibitors have been investigated; for example, some of them were designed based on the sequence of PC prodomain or PC partner proteins and the lysine active domain of the mung bean trypsin inhibitor (MBTI) [29,30]. Other peptide inhibitors

Received: June 2, 2008 Accepted: August 10, 2008
This work was supported by grants from the National Basic Research Program of China (No. 2004CB719904) and the National Natural Science Foundation of China (No. U0632001)
#These authors contributed equally to this work
*Corresponding author: Tel, 86-21-54921165; Fax, 86-21-54921011; E-mail, zwqi@sibs.ac.cn

include the consensus substrate recognition sequence of furin, the C-terminals of which are modified by an active group (–CMK, –CHO, =NOH or –CH=NNHCONH$_2$) [17, 31]. Meanwhile, the stability of small peptide inhibitors has been improved by cyclic peptide inhibitors, such as chymotrypsin inhibitor 2 from the barley serine proteinase inhibitor-2 [32], sunflower trypsin inhibitor-1 [33], and the Lys fragment of mung bean trypsin inhibitor [34].

In our previous study, three highly active inhibitors against furin were purified from porcine liver and identified as C-terminal truncated fragments with different sizes of histone H1.2. The inhibitory activities of these fragments were greater than that of the full-length histone H1.2, and it has been suggested that inhibitory activity against furin relies upon the C-terminal domain [35]. In the same study, a synthesized 36 amino acid peptide of the C-terminal fragment retained inhibitory activity against furin; however, this 36 amino acid peptide with a K_i value of 5.1×10^{-7} M is too long for wide application and lacks the ability to inhibit the activity of furin efficiently. In this study, we used this small amino acid peptide as a template to design a shorter but more potent and stable furin inhibitor. Seven peptide inhibitors derived from the 36 amino acid peptide were synthesized, and their potency and stability against furin were characterized. Of them, we found a nonapeptide with high stability and a K_i value of 2.7×10^{-8} M, which may serve as a leading compound for the development of therapeutic drugs for furin-mediated diseases, such as HIV.

Materials and Methods

Materials

The fluorogenic substrate pyrArg-Thr-Lys-Arg-7-amino-4-methylcoumarin (MCA) was purchased from Bachem Bioscience (San Diego, USA). All Fmoc amino acids and Fmoc resins were obtained from Applied Biosystems (Foster City, USA).

Peptide synthesis

All the linear peptides were synthesized using the standard Fmoc chemistry. The protected peptide was independently grown on a Wang-resin, using the HBTU (O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate)/HOBT (N-Hydroxybenzotriazole) amino acid activation method. Solid phase peptide synthesis was performed on a 433A peptide synthesizer (Applied Biosystems). The protected amino acids were Fmoc-Ser (tBu), Fmoc-Lys (tBoc), Fmoc-Thr (tBu), Fmoc-Arg (Pbf) and Fmoc-Asp (otBu). The resin was incubated in TFA containing 5% p-cresol and a few drops of triethylsilane and thioanisole for 1.5 h at room temperature for cleavage. The crude peptides were precipitated by cool anhydrous diethyl ether and purified by reverse phase HPLC.

All the cyclic peptides were synthesized through their corresponding linear peptide thioester precursors by intramolecular native chemical ligation [36]. The linear peptide thioester precursors were prepared using the Boc solid phase method with *in situ* neutralization [37]. Typically, S-trityl-β-mercaptopropionic acid was preactivated with HBTU/DIEA (N,N-diisopropylethylamine) and introduced to Leu-Pam resin. After deprotection with neat TFA, the first amino acid from the C-terminal was coupled to the resin with a free thiol group using HBTU/DIEA as coupling reagent. After the chain elongation was finished, all the protection groups were removed and peptides were cleaved from resin by HF (Hydrogen Fluoride)/p-cresol (90:10) at 0 ºC. The peptides were precipitated and washed with cold diethyl ether and purified by reverse phase HPLC. The cyclization of the linear peptides was performed on 0.25 M phosphate buffer containing 6 M guanidine hydrochloride, pH 7.4, overnight. The reaction was monitored by RP-HPLC and the cyclic product was purified by HPLC and identified by electrospray ionization-mass spectrometry.

Peptide purification

The synthetic peptides were desalted on a Sephadex G15 column (Amersham Biosciences, Piscataway, USA), washed with buffer A (0.1% TFA in water), lyophilized, dissolved in buffer A and then purified on a Zorbax C18 column (9.4×250 mm) (Agilent, Palo Alto, USA) by HPLC. The peptides were equilibrated with buffer A at a flow rate of 2 ml/min and eluted in a gradient of 0% buffer B (0.1% TFA in acetonitrile) for 5 min and 0%–30% buffer B for 25 min. The molecular masses of all synthetic peptides were determined with an ABI API2000 Q-trap mass spectroscope (Applied Biosystems).

K_i measurement and stability assay

The fluorogenic MCA substrate (pyrArg-Thr-Lys-Arg-MCA) was used for the furin activity assay. To determine the inhibitory activity, different amounts of the inhibitors were first incubated with a fixed amount of enzyme (1.7 μM) at 37 ºC for 3 min in a final volume of 1 ml HEPES buffer (100 mM HEPES, pH7.5, 1 mM $CaCl_2$, 0.5% Triton X-100, and 1 mM β-mercaptoethanol), and the residual enzyme activity was then measured with an F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). For stability assay, the inhibitors were incubated with furin for different periods (0, 30, 60, 90, 120, 150 and 180

min), and then the inhibitory activity was measured. Enzymes incubated without inhibitors were measured as control. The excitation and emission wavelengths were 370 nm (slit width, 10 nm) and 460 nm (slit width, 10 nm), respectively. The K_i values of inhibitors against furin were determined by Dixon's plot (1/V against I) using two different concentrations of substrate (1.0 μM, 1.5 μM). The substrate concentration for stability assay was 1.0 μM. Data from three measurements were averaged and graphically analyzed with an equation to obtain the equilibrium inhibition constant K_i.

HPLC assay of stability of peptide inhibitors

HPLC was used to study the stability of peptide inhibitors; 20 μg peptide inhibitors with or without incubation with furin (1.7 μM) in HEPES buffer at 37 ºC for 3 h were placed into 300 μl buffer A and centrifugated. The supernate was then loaded to a PepMap C18 column (4.6×250 mm) (Applied Biosystems). I_5 was equilibrated with buffer A at a flow rate of 0.8 ml/min and eluted in a gradient of 0% buffer B for 5 min and 0%–50% buffer B for 25 min. I_4 was equilibrated with 10% buffer B at a flow rate of 0.8 ml/min and eluted in a gradient of 10% buffer B for 5 min and 10%–50% buffer B for 20 min. The molecular masses of all peaks were determined with an ABI API2000 Q-trap mass spectroscope.

Table 1 Molecular weights (MW) of the synthetic peptides

Inhibitors	Observed MW	Calculated MW
I_1	3570.0	3569.4
I_2	1455.6	1455.8
I_3	1580.7	1580.9
I_4	1666.8	1667.0
I_5	1039.5	1039.3
I_6	1223.8	1224.4
I_7	1394.9	1394.7
I_8	1651.2	1651.0

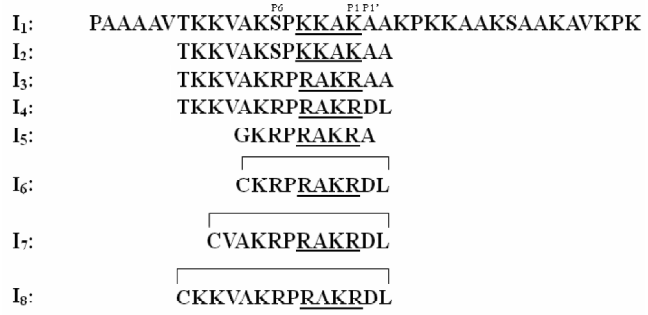

Fig. 1 Amino acid sequences of synthetic peptide inhibitors I_1 with 36 amino acids was previously reported [35]; I_2–I_8 are different mutants of I_1. I_6, I_7 and I_8 are cyclic peptides linked by the N-terminal cysteine and the C-terminal leucine.

Results

Optimization of inhibitor

As reported in our previous work, a peptide, bearing a K_i value of 5.1×10^{-7} M, with 36 amino acid residues (PAAATVTKKVAKSP***KKAK***AAKPKKAAKSAAKAVKPK) derived from the C-terminal fragment of histone H1.2 possesses a potent inhibitory activity against furin [35]. Based on this 36 amino acid peptide template (termed I_1), a series of shorter peptides was designed and synthesized (**Table 1**) (**Fig, 1**). The first step in optimization was to shorten the 36 amino acid peptide from both the N- and C-terminals. The resulting peptide, termed I_2, with 14 amino acid residues exhibited a 10-fold lower inhibitory activity than I_1. To improve the potency of I_2, the second step introduced the consensus substrate recognition sequence of furin into the reactive site. Furin recognizes a specific RXRAKR↓ site. The peptide I_3 was then designed by replacing the P_2 residue with Lys and P_1, P_4 and P_6 residues with Arg. These replacements led to a decrease in the K_i value of I_3 by approximately 5.8×10^{-8} M, suggesting that the consensus substrate sequence of furin is essential for the inhibitor. When two alanine residues at the P_1' and P_2' positions of I_3 were replaced with Asp (P_1') and Leu (P_2'), respectively, to achieve I_4, the K_i value for furin further decreased three-fold, indicating that a negatively charged residue at the P_1' site is favorable. Though the 14 amino acid peptides I_3 and I_4 have appropriate inhibitory activities, their relatively large sizes restrain their application. The third step was to remove the N-terminal Thr-Lys-Lys-Val and C-terminal Ala residues flanking the reactive site of the inhibitor I_3 to obtain I_5. The truncation at both termini had no apparent impact on the inhibitory activity of I_5, resulting in a nonapeptide inhibitor with a K_i value of 2.7×10^{-8} M. To protect the peptides from possible *in vivo* degradation by exopeptidase, three cyclic peptide inhibitors with 10, 12 and 14 amino acid residues were also synthesized in the thioester formation, between the N-terminal cysteine and the C-terminal Leu. Unexpectedly, the inhibitory potencies of the peptides I_6, I_7 and I_8 decreased by 160, 35 and 5 folds, respectively, compared to I_4 (**Table 2**).

Stability analysis

Table 2 Inhibitory constants of the various synthetic peptide inhibitors against furin

Peptide inhibitors	Inhibition constants K_i (M)
I_1	$5.1\pm0.15\times10^{-7}$
I_2	$4.4\pm0.19\times10^{-6}$
I_3	$5.8\pm0.30\times10^{-8}$
I_4	$1.7\pm0.22\times10^{-8}$
I_5	$2.7\pm0.07\times10^{-8}$
I_6	$2.8\pm0.51\times10^{-6}$
I_7	$5.9\pm 0.35\times10^{-7}$
I_8	$8.3\pm0.10\times10^{-8}$

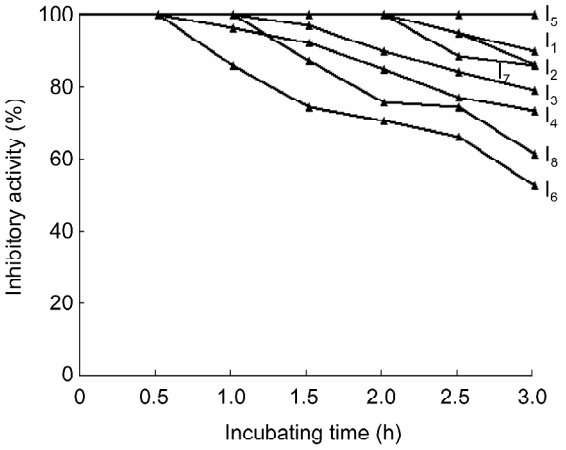

Fig. 2 Stability of the inhibitors incubated with furin for different lengths of time Incubation was carried out at 37 ºC in HEPES buffer (100 mM HEPES, pH7.5; 1 mM $CaCl_2$, 0.5% Triton X-100, and 1 mM β-mercaptoethanol).

Stability assays were carried out to measure the stability of the inhibitors over several hours. To measure the stability of these inhibitors, the IC_{50} concentrations of the inhibitors were used based on their K_i values. The substrate concentration for the stability assay was 1.0 μM. Enzymes incubated without inhibitors were used as a control to confirm that furin activity would not change during incubation in buffer at 37 ºC. The initial inhibitory activity of each inhibitor was marked as 100%; their inhibitory activities at indicated time points were then compared with initial activity and normalized as percentage values. Inhibitory activities were measured three times.

When the synthesized peptides (I_1–I_8) were incubated with furin for an indicated time, their inhibitory activities gradually decreased in a time-dependent manner, with the exception of I_5. The most stable inhibitor, I_5, retained 100% potency against furin, even after a 3 h incubation period. In contrast, the inhibitor I_6 was the least stable with a 50% activity loss during the same time period. Among inhibitors I_2, I_3 and I_4, the activity of the one with the highest inhibitory activity (K_i-I_4<K_i-I_3<K_i-I_2) decayed the fastest. I_4 is five amino acids longer than I_5; however, I_5 is more stable than I_4. Compared with I_4, the cyclic peptides I_6 and I_8 lost their potencies much more quickly, suggesting that the cyclization of peptide is not helpful in the optimization of a furin inhibitor (**Fig. 2**).

To confirm the stability analysis results further, HPLC was also used to measure stability. Since I_4 and I_5 are the most active peptide inhibitors, they were selected to be incubated with furin for 0 h or 3 h, and then separated by HPLC. As shown in **Fig. 3(A)**, after 3 h incubation with furin at 37 ºC (right panel), the HPLC profile of I_5 was the same as that of I_5 incubated with furin for 0 h (left panel). The figure inserted on the right shows the molecular weight of the peak marked I_5, as measured by mass spectrum. Consistent with our stability assay, the HPLC profile of I_4 showed that it decreased and a new peptide was generated, with a retention time of 9.5 min on HPLC, after incubating with furin at 37 ºC for 3 h [**Fig. 3(B)**]. The molecular weight of this newly generated peptide was 1,438.6 kDa [**Fig. 3(C)**], which is a good match with the calculated molecular weight of peptide cleaved from I_4 between P_1 and P_1'. This indicates that the instability of these inhibitors was caused by the cleavage of furin at the C-terminal of P_1.

Discussion

Since furin has been found to be related to bacterial and viral infections, the development of atherosclerosis [38], Alzheimer's disease [39], and the metastasis of cancer [10], it has become an important therapeutic target for those types of diseases. Furin inhibitors are capable of neutralizing bacterial exotoxins and preventing viral infections. Until now, proteinase inhibitor 8 and histone H1.2 have been reported as naturally synthesized possible inhibitors of furin in mammals [23,35]. The C-terminal fragment of histone H1.2 was found to be more potent than full-length histone H1.2, with a K_i value of 310 nM against furin. This C-terminal fragment of histone H1.2 has many advantages over other furin inhibitors; for example, its small size and lack of a disulfide bond means it is easily synthesized and purified. However, modifications are needed to promote its potency and stability before it can be applied therapeutically. In this study, we successfully optimized this C-terminal fragment to be a

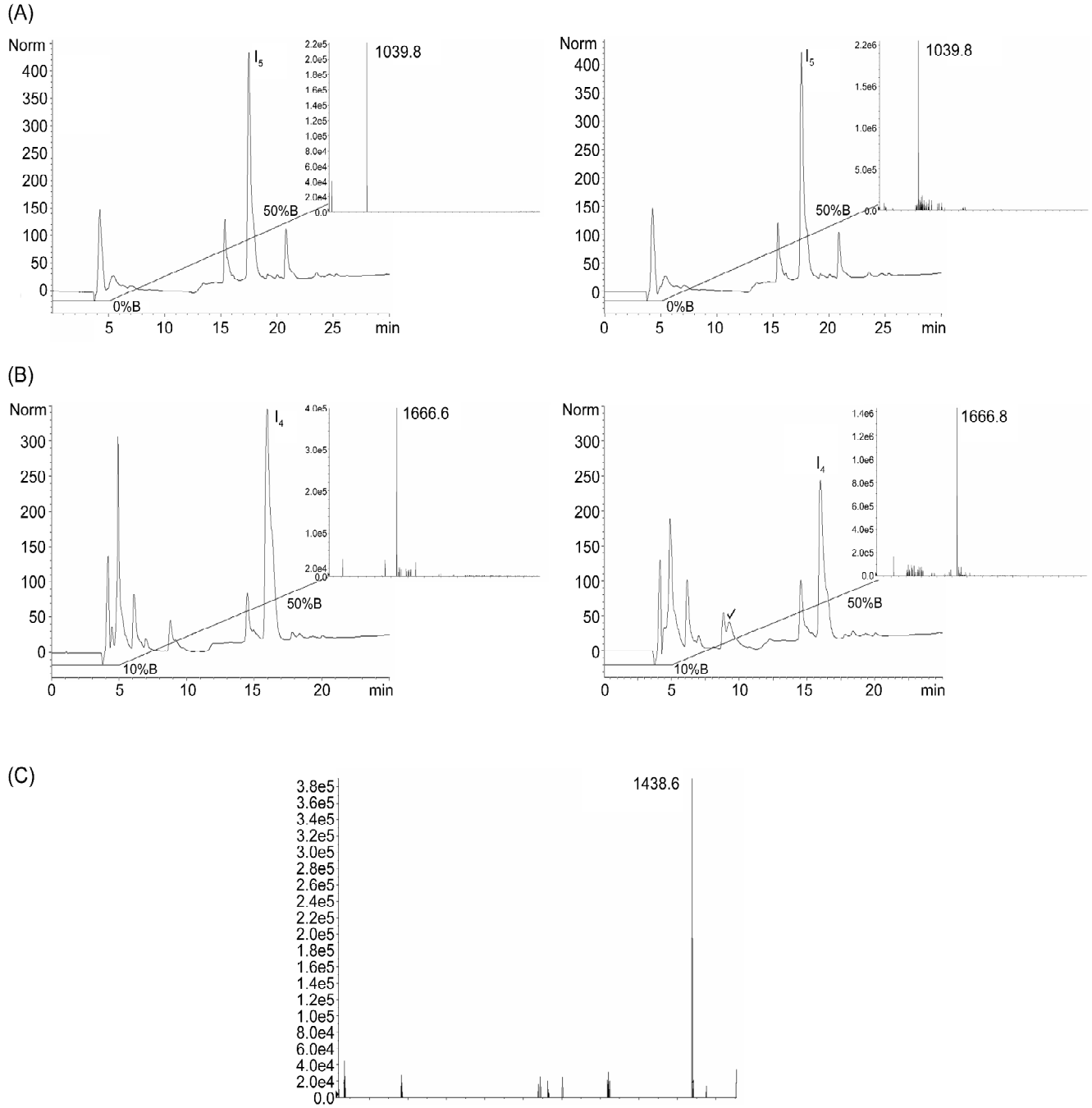

Fig. 3 HPLC profile of inhibitors incubated with furin (A) I_5 incubated with furin for 0 h (left panel) and 3 h (right panel) at 37 ºC. Inset images: molecular weight of I_5 measured by mass spectrum. (B) I_4 incubated with furin for 0 h (left panel) and 3 h (right panel) at 37 ºC. Inset images: molecular weight of I_4 measured by mass spectrum. (C) Molecular weight of the newly generated peptide marked with a tick mark.

more potent and stable furin inhibitor, and thus made it an attractive and potential candidate for use as a therapeutic drug.

The crystal structure of the catalytic domain of mouse furin indicates that the active site of furin forms an extended substrate-binding groove that is lined with many negatively charged residues [40]. Studies of furin inhibitors have shown that peptides comprised of positively charged residues are better furin inhibitors [13,41]. There are three pockets in the substrate binding sites of furin [4,42]: S_1,

S_2 and S_4. In general, the S_1 pocket of furin needs Arg in the P_1 site of the substrate/inhibitor, the S_2 pocket interacts with Lys in the P_2 site, and the S_4 pocket favorably interacts with Arg in the P_4 site. As furin does not have an S_3 pocket, the P_3 site of the substrate/inhibitor is optional; thus, a favorable substrate of furin would have the conserved RAKR↓ sequence. Furin also has another secondary pocket in the substrate binding sites, the S_6 pocket. It can interact with Arg in the P6 site of the substrate/inhibitor. Our previous study showed that only one site cleaved by furin exists in the C-terminal of histone H1.2 (K175–K178) [35]. Based on this cleavage site, I_1 with 36 amino acids was designed and found to be a potent furin inhibitor, with a K_i value of 5.1×10^{-7} M. To shorten the original I_1 peptide, the 14 amino acid peptide inhibitor I_2 was then designed by removing the N- and C-terminal residues flanking the reactive site of I_1; the inhibitory activity of I_2 was thus reduced almost 10-fold. In order to increase inhibitory potency, we further designed I_3 and I_4 based on the optimal cleavage site (RXRAKR↓DL). The mutations at the reactive site markedly increased the inhibitory activities of I_3 and I_4, indicating that the consensus substrate sequence of furin is preferable to achieve high inhibitory activity. At the same time, substitution with Asp and Leu at the P_1' and P_2' positions may also increase inhibitory activity three-fold (**Table 2**). By docking the nonapeptide (RERRRKKRG) with furin [43], the S_1, S_2, S_4 and S_6 pockets are at one side. The cyclic peptides (I_6–I_8) in our study form rigid structures, and not all the amino acids in the reactive site are able to bind to the S_1, S_2, S_4 and S_6 pockets of furin. The cyclic peptides achieved greater structural flexibility when the length of the circle increased, and accordingly, the inhibitory activity increases with the elongation of peptide from I_6 to I_8.

Like other peptide inhibitors of furin, most of the synthesized peptides (I_1–I_8) were temporary inhibitors, as their inhibitory activities gradually decreased in a time-dependent manner. Notably, the lower the K_i value, the more quickly activity decayed (**Fig. 2**). One exception was nonapeptide I_5, with a K_i value of 2.7×10^{-8} M, in which no apparent change in inhibitory activity was found, even after a 3 h incubation period with furin at 37 ºC. Three cyclic peptides were also designed to improve inhibitor stability. Unexpectedly, cyclization increased neither the potency nor the stability of the inhibitor.

In summary, based on the C-terminal fragment of Histone H1.2, a series of furin inhibitors were designed. Among them, I_4 exhibited the highest inhibitory activity, and I_5 was the most stable. These inhibitors may serve as ideal lead compounds for the development of therapeutic drugs used in the fight against furin-mediated diseases, such as HIV.

Acknowledgement

We would like to thank Dr. Iris. Lindberg (Louisiana State University, New Orleans, USA) for the purified recombinant mouse furin.

References

1 Han KK, Martinage A. Post-translational chemical modification(s) of proteins. Int J Biochem 1992, 24: 19–28
2 Thomas G. Furin at the cutting edge: From protein traffic to embryogenesis and disease. Nat Rev Mol Cell Biol 2002, 3: 753–766
3 Hughey RP, Bruns JB, Kinlough CL, Harkleroad KL, Tong Q, Carattino MD, Johnson JP *et al*. Epithelial sodium channels are activated by furin-dependent proteolysis. J Biol Chem 2004, 279: 18111–18114
4 Rockwell NC, Krysan DJ, Komiyama T, Fuller RS. Precursor processing by kex2/furin proteases. Chem Rev 2002, 102: 4525–4548
5 Wachter A, Schwappach B. The yeast CLC chloride channel is proteolytically processed by the furin-like protease Kex2p in the first extracellular loop. FEBS Lett 2005, 579: 1149–1153
6 Bergeron E, Vincent MJ, Wickham L, Hamelin J, Basak A, Nichol ST, Chrétien M *et al*. Implication of proprotein convertases in the processing and spread of severe acute respiratory syndrome coronavirus. Biochem Biophys Res Commun 2005, 326: 554–563
7 Hallenberger S, Bosch V, Angliker H, Shaw E, Klenk HD, Garten W. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature 1992, 360: 358–361
8 Klimpel KR, Molloy SS, Thomas G, Leppla SH. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. Proc Natl Acad Sci USA 1992, 89: 10277–10281
9 Tsuneoka M, Nakayama K, Hatsuzawa K, Komada M, Kitamura N, Mekada E. Evidence for involvement of furin in cleavage and activation of diphtheria toxin. J Biol Chem 1993, 268: 26461–26465
10 Bassi DE, Lopez De Cicco R, Mahloogi H, Zucker S, Thomas G, Klein-Szanto AJ. Furin inhibition results in absent or decreased invasiveness and tumorigenicity of human cancer cells. Proc Natl Acad Sci USA 2001, 98: 10326–10331
11 Basak A. Inhibitors of proprotein convertases. J Mol Med 2005, 83: 844–855
12 Brinkerhoff CJ, Podsiadlo P, Komiyama T, Fuller RS, Blum O. Protease inhibitors formed *in situ* from copper and tridentate chelates: a generalized approach towards metal-based pharmaceuticals. Chembiochem 2002, 3: 1141–1143
13 Kacprzak MM, Peinado JR, Than ME, Appel J, Henrich S, Lipkind G, Houghten RA *et al*. Inhibition of furin by polyarginine-containing peptides: nanomolar inhibition by nona-D-arginine. J Biol Chem 2004, 279: 36788–36794
14 Podsiadlo P, Komiyama T, Fuller RS, Blum O. Furin inhibition by compounds of copper and zinc. J Biol Chem 2004, 279: 36219–36227
15 Bissonnette L, Charest G, Longpre JM, Lavigne P, Leduc R. Iden-

tification of furin pro-region determinants involved in folding and activation. Biochem J 2004, 379: 757–763
16 Boudreault A, Gauthier D, Lazure C. Proprotein convertase PC1/3-related peptides are potent slow tight-binding inhibitors of murine PC1/3 and Hfurin. J Biol Chem 1998, 273: 31574–31580
17 Komiyama T, VanderLugt B, Fugere M, Day R, Kaufman RJ, Fuller RS. Optimization of protease-inhibitor interactions by randomizing adventitious contacts. Proc Natl Acad Sci USA 2003, 100: 8205–8210
18 Heinz DW, Hyberts SG, Peng JW, Priestle JP, Wagner G, Grutter MG. Changing the inhibitory specificity and function of the proteinase inhibitor eglin c by site-directed mutagenesis: functional and structural investigation. Biochemistry 1992, 31: 8755–8766
19 Jean F, Stella K, Thomas L, Liu G, Xiang Y, Reason AJ, Thomas G. Alpha1-Antitrypsin Portland, a bioengineered serpin highly selective for furin: application as an antipathogenic agent. Proc Natl Acad Sci USA 1998, 95: 7293–7298
20 Komiyama T, Fuller RS. Engineered eglin c variants inhibit yeast and human proprotein processing proteases, Kex2 and furin. Biochemistry 2000, 39: 15156–15165
21 Lu W, Zhang W, Molloy SS, Thomas G, Ryan K, Chiang Y, Anderson S *et al*. Arg15-Lys17-Arg18 turkey ovomucoid third domain inhibits human furin. J Biol Chem 1993, 268: 14583–14585
22 Braks JA, Martens GJ. 7B2 is a neuroendocrine chaperone that transiently interacts with prohormone convertase PC2 in the secretory pathway. Cell 1994, 78: 263–273
23 Dahlen JR, Jean F, Thomas G, Foster DC, Kisiel W. Inhibition of soluble recombinant furin by human proteinase inhibitor 8. J Biol Chem 1998, 273: 1851–1854
24 Fei H, Li Y, Wang LX, Luo MJ, Ling MH, Chi CW. Nonhistone protein purified from porcine kidney acts as a suicide substrate inhibitor on furin-like enzyme. Acta Pharmacol Sin 2000, 21: 265–270
25 Fricker LD, McKinzie AA, Sun J, Curran E, Qian Y, Yan L, Patterson SD *et al*. Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. J Neurosci 2000, 20: 639–648
26 Mbikay M, Seidah NG, Chretien M. Neuroendocrine secretory protein 7B2: structure, expression and functions. Biochem J 2001, 357: 329–342
27 Kibler KV, Miyazato A, Yedavalli VS, Dayton AI, Jacobs BL, Dapolito G, Kim SJ *et al*. Polyarginine inhibits gp160 processing by furin and suppresses productive human immunodeficiency virus type 1 infection. J Biol Chem 2004, 279: 49055–49063
28 Sarac MS, Cameron A, Lindberg I. The furin inhibitor hexa-D-arginine blocks the activation of Pseudomonas aeruginosa exotoxin A *in vivo*. Infect Immun 2002, 70: 7136–7139
29 Cameron A, Appel J, Houghten RA, Lindberg I. Polyarginines are potent furin inhibitors. J Biol Chem 2000, 275: 36741–36749
30 Li Y, Huang Q, Zhang S, Liu S, Chi C, Tang Y. Studies on an artificial trypsin inhibitor peptide derived from the mung bean trypsin inhibitor: chemical synthesis, refolding, and crystallographic analysis of its complex with trypsin. J Biochem 1994, 116: 18–25
31 Basak A, Schmidt C, Ismail AA, Seidah NG, Chretien M, Lazure C. Peptidyl substrates containing unnatural amino acid at the P'1 position are potent inhibitors of prohormone convertases. Int J Pept Protein Res 1995, 46: 228–237
32 Villemure M, Fournier A, Gauthier D, Rabah N, Wilkes BC, Lazure C. Barley serine proteinase inhibitor 2-derived cyclic peptides as potent and selective inhibitors of convertases PC1/3 and furin. Biochemistry 2003, 42: 9659–9668
33 Korsinczky ML, Schirra HJ, Rosengren KJ, West J, Condie BA, Otvos L, Anderson MA *et al*. Solution structures by 1H NMR of the novel cyclic trypsin inhibitor SFTI-1 from sunflower seeds and an acyclic permutant. J Mol Biol 2001, 311: 579–591
34 Tao H, Zhang Z, Shi JH, Shao XX, Cui DF, Chi CW. Template-assisted rational design of peptide inhibitors of furin using the lysine fragment of the mung bean trypsin inhibitor. FEBS J 2006, 273: 3907–3914
35 Han J, Zhang L, Shao X, Shi J, Chi C. The potent inhibitory activity of histone H1.2 C-terminal fragments on furin. FEBS J 2006, 273: 4459–4469
36 Dawson PE, Muir TW, Clark-Lewis I, Kent SB. Synthesis of proteins by native chemical ligation. Science 1994, 266: 776–779
37 Schnölzer M, Alewood P, Jones A, Alewood D, Kent SB. *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. Int J Pept Protein Res 1992, 40: 180–193
38 Stawowy P, Fleck E. Proprotein convertases furin and PC5: targeting atherosclerosis and restenosis at multiple levels. J Mol Med 2005, 83: 865–875
39 Pinnix I, Council JE, Roseberry B, Onstead L, Mallender W, Sucic J, Sambamurti K. Convertases other than furin cleave beta-secretase to its mature form. FASEB J 2001, 15: 1810–1812
40 Henrich S, Cameron A, Bourenkov GP, Kiefersauer R, Huber R, Lindberg I, Bode W *et al*. The crystal structure of the proprotein processing proteinase furin explains its stringent specificity. Nat Struct Biol 2003, 10: 520–526
41 Apletalina E, Appel J, Lamango NS, Houghten RA, Lindberg I. Identification of inhibitors of prohormone convertases 1 and 2 using a peptide combinatorial library. J Biol Chem 1998, 273: 26589–26595
42 Nakayama K. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. Biochem J 1997, 327: 625–635
43 Guo XL, Li L, Wei DQ, Zhu YS, Chou KC. Cleavage mechanism of the H5N1 hemagglutinin by trypsin and furin. Amino Acids 2008, 35: 375–382