Highly potent inhibitors of the proprotein convertase furin as potential drugs for the treatment of infectious diseases

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Background: Furin and furin-like proprotein convertases are involved in disease related processes and emerged as potential drug targets.

Results: The incorporation of basic acyl residues in P5 position provides highly potent inhibitors of furin, PC1/3, PC4, PACE4, and PC5/6.

Conclusion: These inhibitors could be potential drugs for the treatment of infectious diseases.

Significance: Development of the most potent synthetic inhibitors of furin-like proprotein convertases.

SUMMARY

Optimization of our previously described peptidomimetic furin inhibitors was performed and yielded several analogues with a significantly improved activity. The most potent compounds containing an N-terminal 4- or 3-(guanidinomethyl)phenylacetyl residue inhibit furin with $K_i$ values of 16 and 8 pM, respectively. These analogues inhibit other proprotein convertases, such as PC1/3, PC4, PAEC4 and PC5/6 with similar potency, whereas PC2, PC7 and trypsin-like serine proteases are poorly affected. Incubation of selected compounds with MDCK cells over a period of 96 hours revealed that they exhibit great stability, making them suitable candidates for further studies in cell culture. Two of the most potent derivatives were used to inhibit the hemagglutinin cleavage and viral propagation of a highly pathogenic avian H7N1 influenza virus strain. The treatment with inhibitor 24 (4-(guanidinomethyl)phenylacetyl-Arg-Val-Arg-4-amidinobenzylamide) resulted in significantly delayed virus propagation compared to an inhibitor-free control. The same analogue was also effective in inhibiting Shiga toxin activation in HEp-2 cells. This antiviral effect, as well as the protective effect against a bacterial toxin, both suggest that inhibitors of furin or furin-like proprotein convertases could represent promising lead structures for future drug development, in particular for the treatment of infectious diseases.
Furin, the translational product of the fur gene, was discovered in 1986 (1). Due to its homology with the Kex2 prohormone processing serine protease from Saccharomyces cerevisiae, furin was identified as the first member of the family of secretory proprotein convertases (PC) in humans, all of which contain a subtilisin-like protease domain (2,3). Nine human PCs have now been identified. Two of these, SKI-1 and PCSK9, cleave their substrates after paired hydrophobic amino acid residues, such as leucine or isoleucine, whereas the other seven classical PCs (furin, PC2, PC1/3, PACE4, PC4, PC5/6, and PC7) strictly require a basic amino acid as a P1 residue (4,5).

Furin is ubiquitously expressed as type I-transmembrane protein and is present both in vertebrates and invertebrates. The protein is mainly located in the Golgi and TGN, but may also circulate through the endosomal system to the cell surface and back to the TGN and it is partially shed as a soluble, truncated enzymatically active enzyme (6-8). Furin catalyses the physiological activation of proforms of receptors, hormones,zymogens and cell surface proteins (9). It has a more rigid preference for a basic amino acid in the P2 position than other furin-like PCs, leading to its preferred consensus sequence Arg-X-Lys/Arg-Arg↓-X, where bulky hydrophobic residues in P1′ position are unfavourable (10,11). A study of furin-deficient mice demonstrated the importance of this enzyme during embryogenesis: the knockout of the fur gene led to an early death at embryonic day 11 due to the failure of ventral closure and embryonic turning (12). However, studies with furin-deficient cell lines (13) and a liver-specific interferon-inducible knock-out mouse showed no obvious adverse effects implying that other PCs may be able to compensate for furin deficiency due to partly overlapping expression patterns and subcellular localizations (14,15). In addition to its normal physiological role, furin contributes to the maturation of many disease-related proteins and is involved viral and bacterial infections, tumorigenesis, neurodegenerative disorders, diabetes or atherosclerosis (7,16,17). For example, many viruses contain fusogenic surface glycoproteins which must be cleaved by furin or a furin-like PC as a prerequisite for virus propagation. Among these are the hemagglutinin (HA) of H5 and H7 subtypes of the highly pathogenic avian influenza viruses which cause bird flu, and the surface glycoproteins of the HIV, Ebola, Marburg and measles viruses (7,18,19). In addition, furin is involved in various bacterial infections. It processes the protective antigen precursor of Bacillus anthracis, a component of anthrax toxin; it also cleaves Pseudomonas exotoxin, Shiga toxin, Shiga-like toxins, and diphtheria toxin, thereby contributing to their pathogenicity (7). Also among its substrates are growth factors, matrix metalloproteases and adhesion molecules, all of which are important for tumor progression and malignancy (20,21). Due to its involvement in many disease-related processes, furin has emerged as a potential drug target.

The first furin inhibitors were based on irreversibly inhibiting chloromethyl ketones (CMKs) (18,22). Several other inhibitor types were later designed (23). In addition to macromolecular compounds, such as α1-antitrypsin Portland (24), mutated forms of eglin c (25), and the synthetic complete 83-mer prodomain of furin (26), various types of oligopeptidic and small molecule inhibitors have also been prepared. Certain peptide inhibitors were derived from either substrate sequences (27) or from the furin prodomain (28), or were identified by screening of peptide libraries (29). A strong inhibitory activity was found for polyarginines, such as nona-D-arginine, which inhibits furin with a K<sub>i</sub> value of 1.3 nM (30). By contrast, most nonpeptide small molecules possess only moderate activity in the micromolar range (23,31,32). An exception is the series of potent tetrasubasic 2,5-dideoxystreptamine derivatives, one of which inhibits furin with a K<sub>i</sub> value of 6 nM (33).

Recently, we have developed a series of reversible competitive substrate analogue furin inhibitors containing decarboxylated arginine mimetics in the P1-position (34). Phenylacetyl-Arg-Val-Arg-4-amidinobenzylamide (1) possesses a K<sub>i</sub>-value of 0.81 nM and has a similar potency against PC1/3, PACE4 and PC5/6, whereas PC2, PC7 and several trypsin-like serine proteases were poorly inhibited. Modification of the P3 position provided several compounds with similar potency, whereas the replacement of the P4 Arg residue or the incorporation of amino acids in D-configuration was not accepted (35). Despite its excellent activity in vitro, compound 1...
showed reduced potency in a cellular assay as an inhibitor of the cleavage of the fowl plague hemagglutinin of the subtype H7, possessing a multibasic furin cleavage site. We assumed that this might be related to limited accessibility of these inhibitors to their intracellularly-located target, furin.

To improve the cellular performance of these inhibitors we have synthesized new analogues derived from our lead structure 1. Examination of the X-ray structure of mouse furin covalently bound to the irreversible substrate analogue inhibitor decanoyl-Arg-Val-Lys-Arg-CMK revealed that its P5 residue is directed into the solvent and might be a suitable residue for further optimization (36). In a first approach, based on results obtained during the development of the initial decanoylated chloromethyl ketone inhibitor (22,37) and recent reports regarding the improved membrane permeability of octaarginine after acylation with a hexanoyl residue (38), we have incorporated saturated and unsaturated fatty acid residues in the P5 position. In a second approach, the linear acyl groups were replaced by hydrophobic cyclic residues; and in a third series, we modified the inhibitors with various basic P5 groups. In this paper we describe the synthesis and characterization of these new furin inhibitors. The most potent analogues were examined for their ability to block both, fowl plague virus (FPV) propagation and activation of Shiga toxin. Our successful use of these new furin inhibitors suggests broad therapeutic potential.

EXPERIMENTAL PROCEDURES

Information regarding the reagents used for synthesis, and analytical methods for inhibitor characterization is provided as supporting information.

Synthesis of inhibitors

The inhibitors were synthesized by a combination of solid phase peptide synthesis and solution synthesis, as described previously (34,35). Briefly, the P2-P5 segments were synthesized by Fmoc-strategy on acid-labile 2-chlorotrityl chloride resin, which allowed the mild cleavage of the intermediates from the resin under side chain protection. After coupling of the unprotected P1 4-aminobenzylamine in solution, all protecting groups were removed and the inhibitors purified by preparative reverse-phase HPLC and obtained as lyophilized trifluoroacetic acid (TFA) salts. In case of inhibitors 20, 22, 24, 26 and 28 a final guanylation was performed by treatment of the amine precursors with 1H-pyrazole-1-carboxamide (39). As an example, the synthesis strategy of the most potent inhibitor 26 and its amine precursor 25 is shown in scheme 1. Enzyme kinetics with furin

The inhibition constants of inhibitors 1-9 and 12-18 (Table 1) were determined with recombinant soluble human furin (30) at room temperature according to the method of Dixon (40) using the fluorescence plate reader Safire 2 (Tecan, Switzerland) at λex = 380 nm, λem = 460 nm and Pyr-Arg-Thr-Lys-Arg-AMC as substrate (Bachem, Switzerland) in 100 mM HEPES buffer pH 7.0, containing 0.2 % Triton X-100, 2 mM CaCl2, 0.02 % sodium azide and 1 mg/ml BSA, as described previously (34). The lowest inhibitor concentration used was at least 10 times higher than the enzyme concentration in the assay to avoid tight-binding conditions.

The kinetic analysis of inhibitors 10 and 11 revealed nonlinear curves in the Dixon plot (Figure 1); therefore, only IC50 values could be obtained for both compounds. Only for the purpose of comparison, additional IC50 values were also determined for certain other inhibitors (7, 12-14), although for these analogues KI values were obtainable. The v, I data pairs of the IC50-curves (Figure 2) were fitted to the three-parameter equation 1, where v is the steady-state velocity at different inhibitor concentrations, v0 is the constant velocity in absence of inhibitor, I is the inhibitor concentration, and s a slope factor.

\[ v = \frac{v_0}{1 + \left(\frac{I}{IC_{50}}\right)^s} \]

(1)

The v, I data pairs of the tight binding inhibitors 19-28 were fitted to equation 2 (41), where v0 is the constant velocity in absence of inhibitor, I and E are the inhibitor and enzyme
concentrations, and $K_i^*$ is an apparent inhibition constant at the substrate concentration used.

$$v = v_0 \cdot \frac{[(K_{i'} + I - E)^2 + 4K_{i'} \cdot E]^{1/2} - (K_{i'} + I - E)}{2 \cdot E}$$

(2)

The true $K_i$ values were calculated from these apparent inhibition constants using equation 3.

$$K_i = \frac{K_{i'}}{1 + \left( \frac{S}{K_m} \right)}$$

(3)

Information on enzyme kinetic studies with the other PCs is given in the supporting information.

Size measurements of the inhibitor assemblies

To detect micelle formation of analogues with long acyl residues in the P5 position light scattering measurements were performed by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a 10 mW HeNe laser at a wavelength of 633 nm at 25°C, as described previously (42). Scattered light was detected at an angle of 173° with laser attenuation, and the measurement position adjusted automatically by the Malvern software. Values given are the means ± standard deviation of three independent experiments with each experiment comprising three measurements of the same sample with at least 10 runs each, as determined by the instrument (Zetasizer Software 6.01).

Stability measurements of inhibitors with HPLC

Stability measurements of inhibitors 20 and 24 were performed by incubating the compounds at 30 or 10 µM final concentrations in Dulbecco’s modified Eagle medium (DMEM) in the presence or absence of MDCK cells under cell culture conditions (37°C, 5% CO₂, and 80% humidity). At different time points (0, 24, 48, 72, and 96 h) 200 µL of the supernatant were removed, centrifuged (30,000 × g, 15 min) and 100 µL analyzed by analytical reverse-phase HPLC; quantitative determination of the inhibitor peak area yielded the amount of inhibitor remaining at each time point.

Inhibition of hemagglutinin cleavage and inhibition of multiple-cycle replication of FPV

All information regarding the MDCK cells used, the cell culture conditions, the antisera for HA cleavage detection, the SDS-PAGE including Western blot analysis, and the avian influenza viruses A/chicken/Rostock/34 (H7N1) has been provided previously (34,43) and is additionally contained in the supporting information. Quantitative determination of the infectious virus particles released from cells after multiple-cycle replication was performed by a microplaque assay (44).

Inhibition of Shiga toxin activation

The [³H]Leucine was purchased from Hartman Analytic (Braunschweig, Germany) and the Shiga toxin was a generous gift from Dr. J.E. Brown (U.S. Army Medical Research Inst. of Infectious Diseases, Frederick, MD). The HEp-2 cells used were maintained in DMEM (45). For cytotoxicity assays, cells were seeded into 24-well plates at a density of 5×10⁴ cells/well one day before the experiment. The Shiga toxin cytotoxicity assay was performed as previously described (46).

RESULTS

Design and activity of inhibitors

All structures, analytical data and $K_i$ values of the newly synthesized furin inhibitors are summarized in Table 1; data for the previously described inhibitors 1 and 3 are provided for reference.

A comparison of the $K_i$ values determined for inhibitors 2-9 revealed that the P5 residue clearly contributes to binding affinity, because analogue 2 - lacking an acyl group - has an approximately 10-fold reduced activity compared to 1 or 3. In contrast, there were only marginal differences, with inhibition constants close to 1 nM, between compounds 2-7. A gradually loss in inhibitory potency was observed with longer acyl chains possessing...
more than 10 carbon atoms, e.g., for the myristoylated derivative 9 a $K_i$ value of 50 nM was determined. Interestingly, we could not obtain inhibition constants for the palmitoyl and stearoyl inhibitors 10 and 11, due to nonlinear curves in the Dixon plot (see Figure 1 for inhibitor 11).

Because of these non-linear curves in the Dixon plot, only IC$_{50}$ values were determined for both analogues. For comparison we also used other inhibitors with known $K_i$ values for these IC$_{50}$ measurements (Figure 2). We assumed that the non-linear curves in the Dixon plots and reduced potency in IC$_{50}$ measurements of compounds 10 and 11 might be related to a kind of inhibitor assembly or micelle formation. To overcome this problem, related non-saturated analogues 12-14 also containing 16 or 18 carbon atoms were synthesized, because it is known that acyl residues containing cis-double bonds are more flexible and exhibit a reduced tendency for micelle formation. For these three inhibitors, linear curves in the Dixon plots were observed, allowing $K_i$ determinations according to a normal competitive reversible mechanism (Table 1). Interestingly, the linoyl derivative 14 ($K_i$-value 5.4 nM) was significantly more potent than the two analogues containing only one double bond.

To verify the different tendencies of saturated and unsaturated analogues to form micelles, light scattering experiments by photon correlation spectroscopy were performed; we surmised that this could yield better insight into the substructure of liquid formulations. The formation of self-organising structures such as micelles results in a change in the intensity of scattered light; in addition, the hydrodynamic diameter of the micellar structures can be determined. The tendency of the selected inhibitors to form micelles was investigated in a concentration range from 0.02 µM to 200 µM. The fluid acetyl-, octanoyl- and decanoyl (Dec) derivatives 3, 6 and 7 showed no concentration-dependent change in scattered light intensity (Figure 3). A significant increase in signal intensity was, however, observed at concentrations above 200 µM for the decanoyl compound (data not shown). The stearyl-, oleoyl- and linoyl-derivatives 11, 13 and 14, all possessing 18 carbon atom-long P5 chains, showed a concentration-dependent (stearyl 0.4-0.5 µM; oleoyl 1-1.5 µM; linoyl 40-50 µM) increase in scattered light intensity, indicating micelle formation. The sizes of the micelles were determined as 468 ± 28.5 nm (stearyl; at 0.5 µM); 324 ± 22.5 nm (oleoyl; at 2 µM) and 330 ± 32.4 nm (linoyl; at 40.0 µM). The mean diameters of the micellar systems also increased with increasing inhibitor concentrations. At the highest concentration investigated (i.e., 200 µM) the structures reached diameters of 2,928 ± 431 nm (stearyl, see Figure S1 in supporting information), 2,183 ± 377 nm (oleoyl), and 1,478 ± 285 nm (linoyl).

In addition to inhibitors 3-14 with linear acyl residues we also prepared compounds 15-18 containing cyclic and relatively hydrophobic P5 groups. These analogues were less potent than our lead 1 and were therefore not further studied. Because various furin substrates contain an additional arginine or lysine residue in the P5 or P6 position (11), a third inhibitor series made by incorporation of basic P5 residues was prepared (19-28). Significantly improved inhibition was observed with all of these compounds (Table 1). The inhibition constants for these derivatives had to be determined under tight-binding conditions, i.e., at similar concentrations of enzyme and inhibitor. In contrast to classical inhibitors (used $[I] >> [E]$) the kinetic analysis of tight binding inhibitors according to equation 2 requires a knowledge of the active enzyme concentration. Active site titration of serine proteases is normally done with appropriate ester substrates, leading to a rapid formation of an acyl enzyme (47,48). For furin, active site titration has been described with the amide substrate Boc-Arg-Val-Arg-Arg-AMC, which also shows burst kinetics (49); however, this substrate was not available to us. Other groups have performed active site titration of PCs by incubating enzymes with different concentrations of an irreversible CMK inhibitor around the expected enzyme concentration for a defined time (approximately 30 min) and measuring the residual enzyme activity (24).

The resulting plots (Figure 4) provide the active enzyme concentration as the intersection with the X-axis. Our attempts to perform this kind of active site titration with the commercially available Dec-Arg-Val-Lys-Arg-CMK in the same buffer as used for enzyme kinetics revealed that a preincubation period of 30 min
was not sufficient. In our hands, a stable value of approximately 1.2 nM for the enzyme concentration was not obtained until after approximately 2 h of preincubation (Figure 4A and 4B). Despite correcting for the peptide content of the CMK inhibitor provided by the supplier, this value of 1.2 nM was slightly higher than the furin concentration of 0.95 nM, calculated based on protein content determination. We assumed that the difference between the titrated furin value and the protein concentration might be arisen from an uncertainty of the true inhibitor concentration, perhaps due to instability of the CMK derivative in the buffer at pH 7.0. Therefore, the stability of the CMK inhibitor in the buffer used for enzyme kinetics was also tested by analytical HPLC. The data could be fitted to a first-order reaction, providing a half-life of approximately 11.4 h (see Figure S2 in supporting information). Therefore, we believe that the active site titration using a CMK inhibitor is influenced by a certain amount of inaccuracy in the concentration of the inhibitor.

In order to reconcile these different furin concentration values, we used a different approach to determine the concentration of active furin; this approach has been described for the K_i determination of the highly potent thrombin inhibitor hirudin. In this case the true concentration of the hirudin isolated from leech was unknown, and therefore its concentration was simply modified by an additional parameter during fitting using equation 2 (50). By analogy, in addition to K_i we used the unknown furin concentration as a second parameter for fitting the v, I data pairs obtained from measurements with the highly potent tight-binding inhibitors to equation 2. The value of approximately 0.81 nM obtained (Figure 5 for inhibitor 26) was slightly lower than the calculated furin concentration of 0.95 nM determined from the protein content. To confirm the validity of this approach, we attempted to fit the measured data using a slightly higher (1.0 nM) or lower (0.6 nM) fixed enzyme concentration. In both cases it was impossible to fit the measured data to equation 2 (Figure 5, dotted and dashed lines). To avoid errors in the concentrations of the tight-binding inhibitors 19 - 28 used, their peptide contents were calculated based on the nitrogen content obtained from elemental analysis. These experimentally-determined peptide contents were between 90 - 97% of the calculated values in all cases, assuming that one molecule of trifluoroacetic acid is attached as a counter-ion to each basic group in the inhibitor.

Linear progress curves were observed during all kinetic measurements, indicating a reversible inhibition mechanism for all inhibitors. Several compounds containing a basic P5 residue were found to be highly potent tight-binding inhibitors of furin. The most potent analogues 24 and 26, modified by an N-terminal p- or m-guanidinomethyl-phenylacetyl group, possess K_i values of 16 and 8 pM, respectively. Selected inhibitors of this series were also tested against the other furin-like PCs and the trypsin-like serine protease thrombin, in order to examine their selectivity (table 2).

Compounds 21-26 are also picomolar inhibitors of PC1/3, PC4, PC5/6 and PACE4, whereas they possess reduced activity against PC7, and are weak PC2 or thrombin inhibitors. It should be noted that all new derivatives shown in table 2 also have poor potency against the clotting factor Xa and the fibrinolytic enzyme plasmin (data not shown). Nearly all inhibition constants against these proteases were found to be > 10 µM, except for inhibitors 24 and 26, which inhibit plasmin with K_i values of 7.3 and 2.5 µM, respectively. This selectivity profile is similar to that previously reported for inhibitor 1 (34).

Inhibition of HA cleavage and influenza virus propagation

The infectivity of the influenza virus depends on the correct cleavage of its surface glycoprotein precursor HA0 into the subunits HA1 and HA2. In the case of the highly pathogenic avian influenza subtypes H5 and H7, this processing is performed at a multibasic cleavage site by furin or PC5/6 during transport of the HA from the endoplasmic reticulum to the plasma membrane. It has been previously suggested that the inhibition of this step might offer a new possibility for antiviral therapy (51,52). Therefore, we used the furin inhibitors 22 and 24 to investigate their effect on HA cleavage and FPV replication in cell culture. Prior to this, however, we examined the cytotoxicity of
these analogues on the viability of the MDCK cells using the MTT assay (53), because normal virus propagation is possible only in living cells. At concentrations of 25 and 50 µM, inhibitors 22, and 24 were relatively well tolerated by MDCK cells (reduction in cell viability < 20 % after 24 h and < 30 % after 48 h. It should be noted that certain other compounds, especially inhibitors 9, 10, 11 and 13 had a significant cytotoxic effect (see figure S3 in supporting information). Therefore, all analogues with pronounced cytotoxicity were not further investigated in the cell culture experiments.

In a first experiment we investigated the cleavage of HA (PSKKRKKR↓GLFG) in FPV in the presence of different concentrations of inhibitors 22 and 24. Both derivatives caused a concentration-dependent inhibition of HA processing (Figure 6). It should be noted that the efficacy of inhibitor 22 is similar to that of the previously described irreversible inhibitor decanoyl-Arg-Val-Lys-Arg-CMK (= 80% inhibition at 50 µM inhibitor concentration), as shown before (34). An improved potency was found for inhibitor 24, even at the lowest concentration of 1 µM, it still reduced the HA processing by approximately 40%. In contrast, we could not detect any beneficial effect with inhibitors modified by various fatty acid residues in a screening experiment (see figure S5 in the supporting information). Therefore, these analogues were not further investigated.

Although HA processing is a prerequisite for virus infectivity, the percentage of HA cleavage inhibition provides no clear correlation regarding the amount of remaining infectious virus. Therefore, in an additional experiment the multiple-cycle replication of FPV in the presence of inhibitors 22 and 24 was investigated, followed by a microplaque test, which clearly indicates the amount of infectious viruses. Figure 7 shows the number of released infectious virus particles from MDCK cell cultures at different times over a period of 48 h. Compared to the control lacking inhibitor, both compounds significantly reduced and delayed virus propagation.

The inhibition experiments of multiple-cycle replication of FPV were performed over a period of 48 h. Therefore, we also determined the stability of both inhibitors in cell culture medium in the presence and absence of MDCK cells by analytical reverse-phase HPLC over a period of 96 h. These measurements revealed that both compounds were stable in pure medium, whereas a time-dependent decrease of their concentration was observed in medium containing MDCK cells. After 96 h of incubation, the concentration of inhibitor 24 was reduced by approximately 30 and 50% when using 30 or 10 µM inhibitor concentrations, respectively (Figure 8 for inhibitor 24; data for inhibitor 22 are provided in Figure S3 in the supporting information). At present we cannot distinguish whether the concentration decrease in the cell supernatant that we observed is caused by degradation and/or modification of the inhibitors; or by cellular uptake, which would presumably be required for the effective inhibition of intracellularly-localized furin.

Inhibition of Shiga toxin activation

Various toxin-producing bacteria, such as Shigella dysenteriae or pathogenic Escherichia coli strains, can cause serious infections in humans that may lead to diarrhea, hemolytic uremic syndrome (HUS) and kidney failure (54). Previous experiments in cell culture revealed that intracellular furin efficiently cleaves the A-subunit of the toxin from Shigella dysenteriae into two disulfide-bonded fragments. The thereby activated Shiga toxin has N-glycosidase activity which removes a single adenine from the cellular 28 S rRNA of the 60 S ribosomal subunit. This eventually leads to inhibition of protein synthesis in the intoxicated cells (46). Therefore, we tested the ability of inhibitors 1 and 24 to protect HEp-2 cells from intoxication by Shiga toxin. As a control, we used cells lacking inhibitor treatment and cells treated with phenylacetyl-Val-DArg-Arg-4-Amba, a structurally closely related analogue of inhibitor 1, which possesses poor furin affinity (§29 in reference(35)). In vitro, activation of Shiga toxin is also possible using trypsin; therefore, the nonspecific trypsin-like serine protease inhibitor benzylsulfonyl-dSer-Lys(Cbz)-4-Amba (#33 in reference (55) which inhibits trypsin with a K<sub>i</sub> value of 5 nM but has negligible affinity against furin, was also included as an additional control. The
effect of Shiga toxin on protein synthesis in HEp-2 cells in the absence and presence of these inhibitors is shown in figure 9a.

In the control lacking added inhibitor, a Shiga toxin concentration of approximately 4.4 ng/ml was required to reduce cellular protein synthesis by 50% (ID$_{50}$), whereas approximately 6-fold higher toxin concentrations were required to achieve this reduction in the presence of inhibitor 24. A slight protection (1.8-fold) was observed in the presence of compound 1, whereas negligible efficacy was observed with the poor furin inhibitor Phac-Val- dArg-Arg-4-Amba and the trypsin-like serine protease inhibitor benzylsulfonyl-DSer-Lys(Cbz)-4-Amba. It should be noted that additional inhibitors were screened in this assay (data not shown). The irreversible inhibitor dec-Arg-Val-Lys-Arg-CMK had a slightly reduced protective effect compared to inhibitor 24, whereas only a marginal effect was found for compounds 4, 6 and 8, which were well tolerated by the used HEp-2 cells. In contrast, we observed a very strong cytotoxic effect of inhibitors 9, 10, 12, and 13 modified by longer fatty acid chains in P5 position on HEp-2 cells in absence of Shiga toxin (see figure S4 in supporting information). Therefore these inhibitors were not further investigated.

Based on these results a second experiment with varying concentrations of inhibitor 24 was performed. No protection was observed at 0.1 µM, whereas a concentration dependent protection was seen at all higher inhibitor concentrations, e.g., 5.4-fold ± 0.9 at 10 µM (Figure 9b). These results suggest that inhibitor 24 has a protective effect against intoxication of HEp-2 cells by Shiga toxin, most likely due to inhibition of furin.

DISCUSSION

The implication of furin or furin-like PCs in various diseases is now well established; therefore, efficacious inhibition of these proteases might potentially offer a novel treatment strategy. Although our previously described inhibitors containing a C-terminal 4-amidinobenzylationamide (4-Amba) residue had high in vitro potency, their efficacy in cell culture as inhibitors of the H7N1 influenza virus propagation was only in the micromolar range, with IC$_{50}$ values around 10 µM. The X-ray structure of furin inhibited by Dec-Arg-Val-Lys-Arg-CMK (36) suggested that the P5 acyl residue might be a suitable position for further optimization of these inhibitors. Due to reports regarding the use of stearylated arginine-rich peptides as efficient transfection systems (56,57) and experience with the enhanced activity of decanoylated CMK-derived furin inhibitors in cell culture assays, we performed a stepwise prolongation of the P5 acyl chain. Most of the acylated analogues possess a stronger affinity for furin as compared to inhibitor 2 which lacks a P5 residue. This might be explained by interactions found for the P5 carbonyl oxygen in the X-ray complex with the CMK inhibitor (36), which should have a similar backbone conformation compared to our peptidomimetic inhibitors. In the X-ray structure, the decanoyl oxygen binds intramolecularly to one $\omega$-nitrogen of the P4-Arg, which leads to a stabilization of the bound inhibitor conformation and, via a bridging water molecule, to the amide nitrogen of the furin residue Glu257. It seems reasonable to assume that the elimination of both interactions could reduce the potency of analogue 2 by an order of magnitude. Only for longer saturated acyl residues with more than 12 carbon atoms we did observe a gradual decrease in inhibitory activity.

Using light scattering experiments we could demonstrate that these decreased inhibitory activities might be related to the formation of micelles at concentrations above 0.2 µM. Similar critical micelle formation concentrations (CMCs) in the range of 0.8-0.9 µM were determined for stearylated pentadecapeptides that block sialylgalactose structures on the surface of host cells, which act as receptors for the influenza virus hemagglutinin (58). In this case the micelles enabled multiple binding and therefore a stronger blockade of the host cell receptors, which resulted in significantly improved inhibition of the virus propagation compared with the non-acylated peptides. In contrast, the saturated C14-C18-furin inhibitors 9-11 exhibit a relatively low inhibitory potency. We surmise that micelles formed by these inhibitors present the inhibitory structures directly on their surfaces. If this is the case, these inhibitors might be less accessible to furin due to steric hindrance. This assumption is supported by results obtained during the
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design of biotinylated furin inhibitors bound to a streptavidin matrix, which were used for affinity purification. In that case we observed that specific furin binding required sufficient linker length between the biotin and the inhibitory moieties (59). A linker length of 9 atoms was clearly not long enough, whereas an 18 atom-long linker enabled efficient furin binding. An additional hint for micelle formation as the reason for the reduced potency of the saturated C14-C18 inhibitors was deduced from the incorporation of the linoyl group in inhibitor 14. This acyl chain contains two cis-double bonds and therefore reduces the tendency for micelle formation (Figure 3). In consequence, inhibitor 14 with an inhibition constant of 5.3 nM possesses an inhibitory potency close to the analogues with shorter acyl groups. However, compounds 14, 13 and 11 also reduced the viability of MDCK cells. Therefore, such compounds are not suited for investigations of furin-related processes in cell culture.

Interestingly, despite structural differences all inhibitors containing a basic P5 residue exhibit improved activity in enzyme kinetic studies. This is quite different to the influence of the basic P4 residue in substrate-analogue inhibitors, where only an arginine is accepted, as found previously (35). Replacement by lysine at this site caused an approximately 350-fold decrease in affinity, which was comparable to that of inhibitors containing a non-basic citrulline or side chain protected Lys(Cbz) as the P4 residue. Therefore, the improved inhibition constants found for all inhibitors with a basic P5 residue might be partially dependent on faster association of the inhibitors due to a stronger electrostatic binding contribution to the unusually acidic active site of furin. This effect might be comparable to the binding of the highly acidic C-terminal hirudin tail to the basic fibrinogen recognition exosite of thrombin, which contains many Lys and Arg residues. Although it is known from various X-ray structures that several Glu side chains in the C-terminal tail region of hirudin are directed into the solvent and therefore are not involved in direct salt or hydrogen bridges to basic thrombin residues (60), their substitution by glutamine results in weaker thrombin inhibition. Kinetic studies indicated that in all cases where an increase in the dissociation constant (weaker inhibition) was observed, it was predominantly due to a decrease in the association rate constant, whereas the dissociation rate constant was only marginally influenced (61). In contrast, significant differences exist in the inhibitory potency of the furin inhibitors described here, which suggests that the basic P5 groups of the most potent analogues do form specific bonds with furin. Preliminary modeling of inhibitor 26 in complex with furin suggests binding of its P5-guanidino group to the side chain of Glu230 (Figure S7 in supporting information). However, this Glu230 is relatively solvent-exposed at the furin surface and is not part of a well-defined binding pocket.

The most potent compounds 24 or 26 might also be useful tools for convenient active site titration of furin, PC1/3, PC4, PACE4 or PC5/6. Their strong potency leads to significant inhibition at concentrations below that of the enzyme in the assay. A similar approach was previously described for hirudin titration by thrombin (50) or for thrombin titration by hirudin (62). In general, all analogues tested in this series exhibited low selectivity against other furin-like PCs, such as PC1/3, PC4, PACE4 or PC5/6. Although models of other PCs (63) which are based on the known furin and kexin coordinates have been built (36,64,65), without actual experimental structures it is presently impossible to explain why other PCs, for example PC2 and PC7, are less affected by our inhibitors. The consequences of reduced selectivity of such compounds in practical use are also unknown. A high specificity for the target enzyme would be generally preferred, because it should lead to fewer side effects. However, due to overlapping expression pattern of the PCs, it could perhaps represent an advantage to inhibit more than one PC, to avoid compensation by other PCs in a pathophysiological situation. In contrast, the weak affinity of these analogues for trypsin-like serine proteases is beneficial for the maintenance of blood homeostasis.

The inhibition of host cell proteases such as furin could be a promising approach for the development of new anti-infective drugs. Because a host enzyme is targeted, this strategy would avoid the often-observed emergence of resistance due to mutations in the commonly addressed viral targets. Both inhibitors 22 and 24 caused a significant
inhibition in the HA cleavage of a highly pathogenic H7N1 avian influenza strain in virus-infected cell culture assays. Compared to previous results with the reference inhibitor 1 (34) the efficacy of analogue 2 was clearly improved; at the lowest concentration of 1 µM, approximately 35% of the uncleaved HA0 was still maintained. As already discussed, it is impossible to deduce an exact correlation of the extent of HA-cleavage inhibition with an effect on virus propagation. More meaningful is the determination of infectious virus particles in a multiple-cycle replication assay using a microplaque test. Compared to a control without any inhibitor, only 5% of the amount of infectious virus particles were detected 48 h after treatment with inhibitor 24. However, animal experiments will be necessary to demonstrate that such delayed virus propagation will result in a beneficial therapeutic effect. Due to their different modes of action, these host cell protease inhibitors could also be useful candidates for combination therapy with already established anti-influenza drugs, for example neuraminidase inhibitors.

In addition to their antiviral effects, furin inhibitors could also represent potential drugs for the treatment of bacterial infections. Various types of furin inhibitors have been used to delay the anthrax toxemia both in cells and in live animals by inhibiting furin-catalyzed processing of the anthrax protective antigen (30,66,67). An additional example is the inhibition of the furin-dependent activation of the cytotoxic Pseudomonas aeruginosa exotoxin (24,68). Stimulated by the recent outbreak of enterohemorrhagic E. coli (EHEC) infections in Germany, which produce Shiga-like toxins, we tested selected furin inhibitors on their ability to reduce the Shiga toxin activation in cell culture. Inhibitor 24 provided a significant dose-dependent protection against Shiga toxin-induced cytotoxicity in cell culture. However, treatment with inhibitor 24 was unable to afford complete protection against cytotoxicity. This finding is in agreement with earlier studies of cells lacking furin (46) and with studies of mutated Shiga toxins lacking the furin cleavage site (69), suggesting that alternative ways exist, whereby cells can cleave and activate Shiga toxin. It should be noted that furin inhibitor 24 provided no protection against the plant toxin ricin, which has the same effect on ribosomes as Shiga toxin (54), i.e., both toxins remove a single adenine from the 28S RNA of the 60S ribosome. This demonstrates that inhibitor 24 does not interfere with the similar enzymatic action of these toxins. Therefore, furin seems to be the relevant target for the inhibition of Shiga toxin activation in vivo.

In conclusion, we have developed a series of stable and highly potent synthetic furin inhibitors with a relatively broad specificity towards similar PCs, with the exception of PC2 and PC7. Such compounds might be interesting tools for further studies on the physiological role of furin-like PCs and can serve as a starting point for drug development useful for the treatment of furin-related diseases.

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**Supporting information** - This material contains additional information regarding the chemicals used for synthesis, the analytical methods for inhibitor characterization, the enzyme kinetic measurements with hPC1/3, hPC2, mPC4, hPACE4, hPC5/6, hPC7 or thrombin, the sample preparation for light scattering measurements, measurements of the stability of the irreversible inhibitor decanoyl-Arg-Val-Lys-Arg-CMK in buffer, additional information regarding the used viral assays in cell culture, results of cytotoxicity measurements with selected inhibitors, a screening of selected inhibitors regarding the HA-cleavage inhibition, stability measurements with inhibitor 22 in pure cell culture medium and in medium containing MDCK cells, and stereo figures of the modeled complex of inhibitor 26 bound to mouse furin.
SCHEME AND FIGURE LEGENDS

**SCHEME 1.** Synthesis of inhibitors 25 and 26. The N-terminal Boc-protected P5-P2 segment was prepared on 2-chlorotriyl chloride resin using 3-(Boc-aminomethyl)-phenylacetic acid for the coupling of the P5 group and otherwise using a standard Fmoc protocol. (a) cleavage from resin with 1% TFA in DCM, 2 x 30 min, drying *in vacuo*; (b) 1.5 equiv 4-aminomethylbenzamidine · 2 HCl, 1.7 equiv PyBOP, 4.5 equiv 6-Cl-HOBt, 10 equiv DIPEA in DMF, 2 h; (c) TFA/TIS/H$_2$O (95/2.5/2.5, v/v/v), 3 h at 35 °C, precipitation in cold diethyl ether, preparative reversed phase HPLC; (d) 5 equiv 1H-pyrazole-1-carboxamidine · HCl in 1 M Na$_2$CO$_3$, 24 h, purification by preparative HPLC.

**FIGURE 1.** Dixon plot for the stearoyl inhibitor 11, the shown data are the mean and standard error of three independent measurements. The non-linear curves (substrate concentrations 32.5 (●) and 13 (○) µM) do not permit the calculation of a true K$_i$ value according to a classical competitive mechanism. Only for purpose of better visualization, the data points have here been connected by fitting to an exponential growth curve. The dashed line parallel to the X-axis represents 1/V$_{max}$.

**FIGURE 2.** IC$_{50}$-dermination for inhibitors 6 ●, 10 ▲, 11 ○, 13 ●, and 14 ○. The shown data are the mean and standard error of three independent measurements and were performed at a fixed substrate concentration of 8.13 µM. The data were fitted to equation 1.

**FIGURE 3.** Concentration dependency of light scattering (in %) determined for inhibitors 3 (●), 7 (●), 11 (○), 13 (▲) and 14 (△) in the buffer used for enzyme kinetics. Dotted lines indicated no micelle formation in the concentration range used.

**FIGURE 4.** Active site titration attempts of furin using Dec-Arg-Val-Lys-Arg-CMK. (A) Determination of the remaining enzyme activity after preincubation of furin with different concentrations of the irreversible inhibitor Dec-Arg-Val-Lys-Arg-CMK (times for preincubation indicated in the graphs). (B) The concentration of active furin obtained was dependent on the preincubation time, providing a relatively constant value of approximately 1.2 nM only after two hours of preincubation.

**FIGURE 5.** Enzyme kinetic analysis of inhibitor 26 in presence of 8.13 µM substrate. The measured data were fitted to equation 2, providing a furin concentration of 0.81 nM as a parameter (solid line). In contrast, fits performed using different constant enzyme concentrations were unsatisfactory (e.g., furin fixed to a concentration of 1 nM (dotted line) and fixed to 0.6 nM (dashed line)).

**FIGURE 6.** Inhibition of HA cleavage by different concentrations of compounds 22 and 24. (A) MDCK cell cultures were infected with FPV at a multiplicity of infection (MOI) of 100. Inhibition of HA cleavage at different inhibitor concentrations was analyzed 24 h post infection. Proteins from virus particles (HA0, 82 kDa; nucleoprotein NP, 56 kDa; HA1, 50 kDa; neuraminidase NA, 50 kDa) were immunochemically detected after SDS-PAGE followed by Western blot analysis. (B) Quantification of HA cleavage inhibition by Western blot analysis (n=2, including standard errors). The maximum amount of HA0, obtained by inhibition with 50 µM of inhibitor 24, was normalized to 100% cleavage inhibition. Other HA0 band intensities at different concentrations were measured and normalized by standardization of each HA0 value correlating with the corresponding NP band. Experimental details are provided in the supporting information.

**FIGURE 7.** Inhibition of multiple cycle replication of FPV in cell culture in the absence (○) and in the presence of 50 µM of inhibitor 22 (▼) and 24 (▵). Cultures of MDCK cells were inoculated with FPV at an MOI of 0.0001. The inhibitor was added to the medium at a final concentration of 50 µM. At different times (0, 18, 24, 48 h p.i.) the amount of infectious virus (as plaque-forming units (PFU) per ml) released into the medium was determined by a microplaque assay (44).

**FIGURE 8.** Stability measurements with inhibitor 24 at two different concentrations in pure cell culture medium (30 µM (▼), 10 µM (●)) and in medium containing MDCK cells (30 µM (▼), 10 µM (○)). In all cases 100 µL of the inhibitor containing medium or supernatant from cell culture was analyzed by analytical HPLC, which corresponds to approximately 3.7 µg in case of the 30 µM
solution and to 1.2 µg in case of the 10 µM solution. The shown data are the mean of two measurements including standard errors.

FIGURE 9. Intoxication of HEp-2 cells by Shiga-toxin (n≥3 for all experiments). (A) The intoxication was performed in presence of selected inhibitors (all used at 25 µM; control lacking inhibitor in black, ID₅₀ 4.4 ng/ml; inhibitor 24 in red, ID₅₀ 26.1 ng/ml; inhibitor 1 in blue, ID₅₀ 7.83 ng/ml; Phac-Val-dArg-Arg-4-Amba in magenta, ID₅₀ 6.1 ng/ml; benzylsulfonyl-dSer-Lys(Cbz)-4-Amba in green, ID₅₀ 3.69 ng/ml). (B) The intoxication was performed in presence of various concentrations of inhibitor 24. No protection was observed at an inhibitor concentration of 0.1 µM.
Table 1: Inhibition of furin by inhibitors of the general formula

| No. | P5             | HPLC (min) | MS calc. | MS found<sup>a</sup><sup>b</sup> | K<sub>i</sub> (nM) | IC<sub>50</sub> (nM) |
|-----|----------------|------------|----------|---------------------------------|-----------------|-------------------|
| 1   |                | 24.2       | 678.41   | 679.4<sup>a</sup>               | 0.81            | nd<sup>e</sup>    |
| 2   |                | 15.12      | 560.37   | 281.21<sup>b</sup>              | 7.6             | 33                |
| 3   |                | 16.83      | 602.38   | 302.25<sup>b</sup>              | 1.0             | nd<sup>e</sup>    |
| 4   |                | 20.40      | 630.41   | 316.26<sup>b</sup>              | 0.67            | nd<sup>e</sup>    |
| 5   |                | 26.29      | 658.44   | 330.30<sup>b</sup>              | 0.78            | nd<sup>e</sup>    |
| 6   |                | 32.73      | 686.47   | 344.29<sup>b</sup>              | 0.67            | 2.3               |
| 7   |                | 38.78      | 714.5    | 358.32<sup>b</sup>              | 1.6             | 8.3               |
| 8   |                | 15.16<sup>c</sup> | 742.53   | 372.32<sup>b</sup>              | 5.6             | nd<sup>e</sup>    |
| 9   |                | 20.40<sup>c</sup> | 770.56   | 386.37<sup>b</sup>              | 50              | 396               |
| 10  |                | 25.38<sup>c</sup> | 798.6    | 400.37<sup>b</sup>              | nd<sup>e</sup>  | 80                |
| 11  |                | 30.66<sup>c</sup> | 826.63   | 414.49<sup>b</sup>              | nd<sup>e</sup>  | 14010             |
|   | Structure | MW  | pIC50  | EC50 $^{b}$ | nd$^{e}$ | Ref. |
|---|-----------|-----|--------|-------------|---------|------|
| 12 | ![Structure 12](attachment:structure12.png) | 54.50 | 796.58 | 399.80$^{b}$ | nd$^{e}$ | 289  |
| 13 | ![Structure 13](attachment:structure13.png) | 58.20 | 824.61 | 413.63$^{b}$ | nd$^{e}$ | 272  |
| 14 | ![Structure 14](attachment:structure14.png) | 55.10 | 822.6 | 412.56$^{b}$ | 5.3 | 22   |
| 15 | ![Structure 15](attachment:structure15.png) | 31.72 | 746.33 | 374.24$^{b}$ | 1.2 | nd$^{e}$ |
| 16 | ![Structure 16](attachment:structure16.png) | 27.02 | 714.37 | 358.25$^{b}$ | 5.3 | nd$^{e}$ |
| 17 | ![Structure 17](attachment:structure17.png) | 27.78 | 694.4 | 348.27$^{b}$ | 2.4 | nd$^{e}$ |
| 18 | ![Structure 18](attachment:structure18.png) | 36.80 | 782.43 | 329.31$^{b}$ | 8.5 | nd$^{e}$ |
| 19 | ![Structure 19](attachment:structure19.png) | 16.50 | 673.45 | 337.88$^{b}$ | 0.096$^{d}$ | nd$^{e}$ |
| 20 | ![Structure 20](attachment:structure20.png) | 19.30 | 715.47 | 358.88$^{b}$ | 0.085$^{d}$ | nd$^{e}$ |
| 21 | ![Structure 21](attachment:structure21.png) | 15.70 | 659.43 | 330.91$^{b}$ | 0.070$^{d}$ | nd$^{e}$ |
| 22 | ![Structure 22](attachment:structure22.png) | 17.30 | 701.46 | 351.88$^{b}$ | 0.062$^{d}$ | nd$^{e}$ |
| 23 | ![Structure 23](attachment:structure23.png) | 18.10 | 707.43 | 354.89$^{b}$ | 0.033$^{d}$ | nd$^{e}$ |
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![Chemical structures and data]

19.70 749.46 375.91\(^b\) 0.016\(^d\) nd\(^e\)

19.48 707.43 354.73\(^b\) 0.037\(^d\) nd\(^e\)

20.41 749.46 750.38\(^a\) 0.008\(^d\) nd\(^e\)

19.50 707.43 708.5\(^a\) 0.127\(^d\) nd\(^e\)

21.50 749.46 750.5\(^a\) 0.291\(^d\) nd\(^e\)

\(^a\)(M+H); \(^b\)(M+2H)^+/2; \(^c\)HPLC starting condition: 30% acetonitrile containing 0.1% TFA, all other HPLC measurements started at 1% acetonitrile (see supporting information), \(^d\)K\(_i\) values determined under tight-binding conditions; \(^e\)nd, not determined.
Table 2: Inhibition of furin-like PCs and thrombin by selected inhibitors.

| No. | Kᵢ (pM) | hFurin | hPC1/3 | mPC4 | hPC5/6 | hPACE4 | hPC7 | hPC2 | thrombin |
|-----|---------|--------|--------|------|--------|--------|------|------|----------|
| 1   |         | 810    | 750    | n.d. | 1.6×10³ | 600    | 6.2×10⁶ | 3.1×10⁵ | 2.3×10⁷ |
| 21  |         | 70     | 52     | 136  | 69     | 75     | 1.2×10⁵ | >10⁶  | 2.5×10⁷ |
| 22  |         | 62     | 11     | 68   | 13     | 24     | 9.1×10⁴ | >10⁶  | 1.8×10⁶ |
| 23  |         | 33     | n.d.   | 24   | 6.3    | 30     | 2.7×10⁴ | >10⁶  | 3.6×10⁷ |
| 24  |         | 16     | n.d.   | 11.2 | 1.5    | 17     | 2.3×10⁴ | >10⁶  | 1.3×10⁷ |
| 25  |         | 37     | 44     | 140  | 22     | 74     | 6.4×10⁴ | >10⁶  | 1.1×10⁷ |
| 26  |         | 8      | 1.7    | 23   | 2.9    | 5.1    | 3.2×10⁴ | >10⁶  | 5.9×10⁶ |

*The data for inhibitor 1 were included as reference and taken from a previous publication (34). bIt should be noted that Kᵢ values < 5 pM were determined for inhibitors 23 and 24 against hPC1/3 based on a single measurement only, due to the limited amount of the enzyme. Therefore, these data are not included in the table.
Scheme 1:

\[
\begin{align*}
\text{Boc} & \quad \text{NH} & \quad \text{Boc} & \quad \text{NH} & \quad \text{Boc} & \quad \text{NH} \\
& \quad \text{Arg(Pbf)} & \quad \text{Val} & \quad \text{NH} & \quad \text{NH} \\
\text{CONH} & \quad \text{CONH} & \quad \text{CONH} & \quad \text{CONH} \\
\text{Boc} & \quad \text{NH} & \quad \text{Boc} & \quad \text{NH} & \quad \text{Boc} & \quad \text{NH} \\
& \quad \text{Arg(Pbf)} & \quad \text{Val} & \quad \text{NH} & \quad \text{NH} \\
\text{CONH} & \quad \text{CONH} & \quad \text{CONH} & \quad \text{CONH} \\
\text{Boc} & \quad \text{NH} & \quad \text{Boc} & \quad \text{NH} & \quad \text{Boc} & \quad \text{NH} \\
& \quad \text{Arg(Pbf)} & \quad \text{Val} & \quad \text{NH} & \quad \text{NH} \\
\text{CONH} & \quad \text{CONH} & \quad \text{CONH} & \quad \text{CONH} \\
\end{align*}
\]
Figure 1

![Graph showing the relationship between inhibitor concentration and activity.](http://www.jbc.org/Downloaded from)
Figure 2
Figure 4

A

B
Figure 6

A

\[
\begin{array}{c|cccc|cccc}
\text{[I] (µM)} & 0 & 1 & 10 & 25 & 50 & 1 & 10 & 25 & 50 \\
\hline
HA0 & & & & & & & & & \\
NP & & & & & & & & & \\
NA/HA1 & & & & & & & & & \\
\end{array}
\]

B

\[
\begin{array}{c|cccc|cccc}
\text{inhibitor 22} & 0 & 1 & 10 & 25 & 50 & 1 & 10 & 25 & 50 \\
\hline
\text{cleavage inhibition (%)} & & & & & & & & & \\
\end{array}
\]
Figure 7

[Graph showing infectious virus amount (PFU/mL) over time (h) with different data points and error bars.]

Potent furin inhibitors for the treatment of infectious diseases
Figure 8
Potent furin inhibitors for the treatment of infectious diseases

Figure 9A

[Graph showing the effect of Shiga toxin on [3H]leucine incorporation.]

Figure 9B

[Bar graph showing the x-fold protection from cytotoxicity with inhibitor 24.]
Highly potent inhibitors of the proprotein convertase furin as potential drugs for the treatment of infectious diseases

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