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Identification of potent and compartment-selective small molecule furin inhibitors using cell-based assays

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Abstract

The proprotein convertase furin is implicated in a variety of pathogenic processes such as bacterial toxin activation, viral propagation, and cancer. Several groups have identified non-peptide compounds with high inhibitory potency against furin *in vitro*, although their efficacy in various cellbased assays is largely unknown. In this study we show that certain guanidinyated 2,5-dideoxystreptamine derivatives exhibit interesting *ex vivo* properties. Compound **1b** (1,1'-(4-((2,4-diguanidino-5-(4-guanidinophenoxy)cyclohexyl)oxy)-1,3-phenylene)diguanidine) is a potent and cell-permeable inhibitor of cellular furin, since it was able to retard tumor cell migration, block release of a Golgi reporter, and protect cells against *Bacillus anthracis* (anthrax) and *Pseudomonas aeruginosa* intoxication, with no evident cell toxicity. Other compounds based on the 2,5-dideoxystreptamine scaffold, such as compound **1g** (1,1'-(4,6-bis(4-guanidinophenoxy)cyclohexane-1,3-diyl)diguanidine) also efficiently protected cells against anthrax, but displayed only moderate protection against *Pseudomonas* exotoxin A and did not inhibit cell migration, suggesting poor cell permeability. Certain bisguanidinophenyl ether derivatives such as **2f** (1,3-bis(2,4-diguanidinophenoxy) benzene) exhibited micromolar potency against furin *in vitro*, low cell toxicity, and highly efficient protection against anthrax toxin; this compound only slightly inhibited intracellular furin. Thus, compounds **1g** and **2f** both represent potent furin inhibitors at the cell surface with low intracellular inhibitory action, and these particular compounds might therefore be of preferred therapeutic interest in the treatment of certain bacterial and viral infections.

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No potential conflicts of interest are present.

Graphical Abstract

Keywords

Furin; Proprotein convertases; Furin inhibitors; Cell-based assays; Protease

1. Introduction

The proprotein convertase (PC) furin is a calcium-dependent serine endoprotease with a neutral pH optimum that is widely distributed in mammalian cells and tissues. The furin protein, encoded by the *PCSK3* gene, consists of four different domains: a prodomain, involved in folding and activation; a catalytic domain, which includes the catalytic triad Asp-His-Ser typical of all proprotein convertase family members; the homo B domain (also called the P domain), which is essential for enzyme activity and is known to contribute to enzymatic properties (calcium binding, pH dependence, and substrate specificity); and the C-terminal domain (for reviews see [1, 2]). Furin contains a C-terminal transmembrane domain/cytoplasmic tail within this latter domain, which enables this enzyme to bind cytoplasmic routing proteins and to cleave its substrates within three distinct subcellular compartments, the trans-Golgi network (TGN); the plasma membrane; and the endosomal compartment following its retrieval from the cell surface (reviewed in [3]). Due to this complex cellular itinerary, furin is capable of cleaving a wide range of precursor proteins within the constitutive secretory pathway. Some of the more notable substrates of furin include growth factors and receptors, serum proteins, extracellular matrix components, and protease precursors (*e.g.* matrix metalloproteinases) [4]. Furin typically processes precursor proteins at sites with the consensus sequence Arg-X-X-Arg, where the middle residues are also often basic residues; the most frequent site is Arg-X-Lys/Arg-Arg [1].

The furin knockout mouse dies early during embryonic development; death is thought to derive from an inability to produce correctly processed vital growth factors [5]. Furin is not, however, required for tissue viability after development, as tissue-specific knockout mice exist ([6]; reviewed in [7]), as do cell lines which do not express furin (*e.g.* LoVo; [8]). Furin activity contributes to numerous human pathological conditions such as Alzheimer's disease, arthritis, atherosclerosis, and cancer [9–12]. Overexpression of human furin is correlated with increased carcinogenic potential [4, 13–15]. High furin activity is associated with increased proteolytic processing of the precursor to the membrane type 1-matrix

metalloproteinase (MT1-MMP) [16] and insulin-like growth factor-1 (IGF1), as well as of the IGF1 receptor present on the surface of tumor cells [17]; these actions may explain the correlation of increased furin activity with carcinogenesis. Furthermore, host cell furin participates in the activation of many bacterial toxins, such as anthrax, diphtheria, Shiga and *Pseudomonas aeruginosa* toxins [18, 19] as well as in the activating cleavage of viral envelope glycoproteins necessary for the propagation of many viral pathogens including H5N1 avian influenza, human immunodeficiency, and Ebola viruses [20–22]. Because of its involvement in so many disease-related processes, furin has emerged as a potentially important drug target.

Therapeutic administration of furin inhibitors has been frequently proposed for the treatment of viral and pathogenic infections [23–25]. Additionally, inhibitors of furin and PACE4 (another member of the convertase family) have been used to reduce cancer cell invasiveness [26] and to block tumor growth in mice [17, 27–29]. A number of synthetic inhibitors against furin have been recently generated using the crystal structure of furin [30]. However, most prior studies of non-peptide furin inhibitors have been carried out *in vitro*, providing only limited information on the potency or toxicity of these compounds within cells. Interestingly, a series of non-peptide small molecules based on a 2,5-dideoxystreptamine scaffold was previously shown to potently inhibit furin *in vitro* [31]. In the work presented here, we have performed a systematic structure-activity relationship study of these 2,5-dideoxystreptamine derivatives using multiple cell-based assays, identifying the most potent, non-toxic, and compartment-selective inhibitors of cellular furin activity. In addition, we report novel bis-guanidinophenyl ether compounds with high inhibitory potency for furin *in vitro*; these inhibitors also exhibit low cellular toxicity and efficiently protect cells against anthrax and *Pseudomonas* toxemias.

2. Material and Methods

2.1. Compound synthesis

All of the compounds in the study were synthesized at Hawaii Biotech, Inc. (Aiea, HI). Compounds **1a–c**, **e–k**, **m**, and **p–r** were prepared following the procedures described previously [31]. Compounds **2a–f** (**a**, 1,3-bis(4-guanidinophenoxy)propane; **b**, *cis*-1,3-bis(4-guanidinophenoxy)cyclohexane; **c**, 1,3-bis(4-guanidinophenoxy)benzene; **d**, 1,3-bis(2,4-diguanidinophenoxy)propane; **e**, *cis*-1,3-bis(2,4-diguanidinophenoxy)cyclohexane; **f**, 1,3-bis(2,4-diguanidinophenoxy)benzene) were synthesized via reaction of 1,3-propanediol, *cis*-1,3-cyclohexanediol, or 1,3-benzenediol with 1-fluoro-4-nitrobenzene or 1-fluoro-2,4-dinitrobenzene, followed by hydrogenation, guanidination with *N,N'*-bis(tert-butoxycarbonyl)-*N''*-trifluoromethanesulfonylguanidine, and deprotection with trifluoroacetic acid (Fig. 1).

2.2. Enzyme assays and determination of IC₅₀ values

Soluble human furin was purified from the conditioned medium of methotrexate-amplified, stably transfected CHO DG44 cells as previously described [32]. The furin assay was performed in 96-well polypropylene microtiter plates in a final volume of 100 μ l, containing 100 mM HEPES, pH 7.0, 5 mM CaCl₂, 0.1% Brij 35, 0.1% NaN₃, and 0.1 mg/ml BSA. The

substrate Pyr-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide (pRTKR-MCA; Peptide Institute, Lexington, KY) was used at a final concentration of 100 μ M. Furin was used at a final concentration of 15 nM. Reaction mixtures were incubated at 37°C and fluorescence measurements (380 nm excitation, 460 nm emission) were taken under kinetic conditions every minute for 60 min in a SpectraMax M2 microplate reader. For IC₅₀ assays, serial dilutions of compounds were performed to give final concentrations between 100 nM and 250 μ M in 50 μ l. After a 30-min preincubation at room temperature, pRTKR-MCA was added, and residual enzyme activities were monitored by measuring aminomethylcoumarin fluorescence intensity. Data were analyzed using Prism 5 as described previously [33].

2.3. Enzyme kinetics

Studies of furin inhibition kinetics were carried out at various concentrations of pRTKR-MCA ranging from 0 to 200 μ M in the presence and absence of inhibitors. For all kinetic measurements, the compounds were preincubated with enzyme for 30 min before the addition of substrate. All assays were performed in triplicate in 96-well microplates. Inhibition constants (K_i) were determined using the equation $K_i = IC_{50}/(1+([S]/K_m))$ [32, 34]. The K_m value of furin used for this substrate was 8 μ M, as described previously [35].

2.4. SEAP activity assays

CHO-GRAPfurin cells were obtained as a kind gift of Dr. A. Rehemtulla (University of Michigan). These cells, expressing the hybrid reporter protein GRAPfurin consisting of the secreted alkaline phosphatase (SEAP) protein fused to a Golgi retention signal via a specific furin recognition/cleavage site [36], were plated in 96-well plates and incubated with OptiMem containing 100 μ M of either drug or vehicle for 16 to 20h. The medium was collected, centrifuged, and heated for 30 min at 65°C to inactivate endogenous alkaline phosphatases; SEAP activity remains unaffected after heating. Two and a half μ l of heated medium sample were mixed with 100 μ l of assay buffer (100 mM Tris-HCl, pH 10, 100 mM NaCl, 5 mM MgCl₂) and 100 μ l of 36 μ M 4-methylumbelliferyl phosphate (MUP) in 50 mM Tris, pH 10. Fluorescence was measured by excitation at 365 nm and recording emission at 460 nm continuously during incubation at 37°C for 1 h. SEAP liberated from the furin reporter is secreted; thus, SEAP levels present in the medium are proportional to the activity of Golgi furin [26].

2.5. Cell migration assays

HT1080 fibrosarcoma cells (ATCC# CCL-121) were cultured to 80% confluence in growth medium (MEM with 2 mM L-glutamine, 10% FBS, 1:100 non-essential amino acids, 1 mM sodium pyruvate, 1% penicillin-streptomycin and 1% gentamicin). Cells were plated in Oris Cell Migration Assay (Platypus Technologies) plates (10⁵ cells per well) following the manufacturer's protocol. The next day, the growth medium was removed and the wells were rinsed with Dulbecco's PBS (D-PBS; Ca⁺⁺- and Mg⁺⁺-free), and the cells were incubated in assay medium (MEM with 2 mM L-glutamine, 10% heat shocked FBS, 1:100 non-essential amino acids, 1 mM sodium pyruvate, 1% penicillin-streptomycin and 1% gentamicin) in the presence or absence of inhibitor for 20–24 h at 37°C and 5% CO₂. After incubation the cells were rinsed with D-PBS (containing calcium and 20 mM HEPES), and incubated with the Live / Dead Cell Stain Kit containing 2 μ M calcein AM and 4 μ M ethidium homodimer

(EthD-1) for 30 min at 37°C and 5% CO₂. The fluorescence was measured at 485/528 nm excitation/emission for calcein AM, and 530/645 nm for EthD-1. The experiments were repeated independently 2–3 times for each inhibitor. The results are presented as the percentage of survival $\pm$ SD for the mean of all assays.

2.6. *Pseudomonas* exotoxin A assays

Chinese Hamster Ovary (CHO) DG44 cells were cultured to 80% confluence in growth medium (DMEM:F12 (1:1) with 2 mM L-glutamine, 10% FBS and 1% penicillin-streptomycin). CHO DG44 cells were seeded into microtiter plates (10⁴ per well) and treated 24 h later with 50 ng/ml *Pseudomonas aeruginosa* exotoxin A (List Biologicals) in the presence or absence of 10 or 100 μ M of inhibitor for 20–24 h at 37°C and 5% CO₂. Cell viability was monitored by incubation for 2–4 h with the mitochondrial dye WST-1 (Roche). The experiments were repeated independently 3–5 times for the various sets of inhibitors, using triplicate wells each time. The results are presented as the percentage of survival $\pm$ SD for the mean of all assays.

2.7. Anthrax toxin assays

The effect of all compounds on anthrax toxemia was studied in the murine macrophage cell line RAW267.7. Cells were grown in RPMI medium with 10% fetal bovine serum, seeded into microtiter plates (10⁴ per well), and treated 12 h later with 400 ng/ml protective antigen (PA) and 200 ng/ml lethal factor (LF) (List Biologicals) in the presence of either 10 or 100 μ M of each inhibitor for a 2 h period. Inhibitors were added immediately following treatment of cells with PA + LF. Cell viability was monitored with WST-1. The experiments were repeated independently three times using triplicate wells. The results are presented as the percentage of survival $\pm$ SD from the mean of all assays.

2.8. Cytotoxicity assays

In order to assess the ability of each compound to affect cytotoxicity, a 24 h cytotoxicity assay was performed in CHO DG44, HT1080 and RAW267.7 cells using the mitochondrial dye WST-1. Cells were seeded in 96-well plates to achieve 50% confluence the next day, and then incubated with each compound or vehicle in triplicate. After incubation with inhibitors, cells were further incubated for 2–4 h with 10 μ l of WST-1 reagent per well, and the absorbance was measured at 450 and 600 nm. To distinguish effects on cell proliferation from effects on cell viability, a 2 h cytotoxicity assay was performed in parallel (not shown). No differences were observed at these two periods of incubation. Independent experiments were performed on at least 3 occasions, with the results presented $\pm$ SD for the mean of all assays.

2.9. Molecular modeling

The atomic coordinates of furin were obtained from the X-ray structure of human furin in complex with the competitive inhibitor meta-guanidinomethyl-Phac-RVR-Amba [37] (PDB-entry 4OMC). Enzyme preparation was performed using the program “Molecular Operating Environment” (MOE version 2013). As a validation step, our docking of the co-crystallized ligand was able to closely reproduce the orientation and conformation reported in this X-ray

crystal structure. First, each molecule was aligned to the co-crystallized ligand using the flexible alignment methodology implemented in MOE, and then the best alignment was selected for docking into the catalytic pocket.

3. Results

3.1. Structure-activity relationship studies of guanidinylated 2,5-dideoxystreptamine derivatives using cell-based assays

A previous study identified a series of non-peptide small molecules based on the 2,5-dideoxystreptamine scaffold which exhibited great inhibitory activity against furin *in vitro* [31]. However, in this study only limited data were reported on the cellular potencies of these compounds. Here we have tried to elucidate the structure-activity relationships of these 2,5-dideoxystreptamine derivatives using four different compartment-specific cell-based assays. Table 1 summarizes the cell-based assays and the cell lines used in this work: 1) CHO-GRAPfurin cell/SEAP assay to test furin cleavage within the trans-Golgi network (TGN); 2) HT1080 fibrosarcoma cell migration assay to test the furin-mediated activating cleavage of matrix metalloproteinases in the TGN; 3) CHO-DG44 cell/*Pseudomonas* exotoxin A assay to test furin activity in the endocytic pathway; and 4) RAW264.7 macrophage cell/anthrax assay to examine furin activity at the cell surface. In order to assess the general cytotoxicity of the compounds under study, control cytotoxicity experiments were also conducted in both CHO and HT1080 cells.

For a better structural understanding, the 2,5-dideoxystreptamine compounds were divided into four structurally similar groups. Fig. 2 shows the structures, effects on cellular furin activity, and the cytotoxicity of a set of guanidinylated 2,5-dideoxystreptamine phenyl ether derivatives. The molecules **1a**, **1b** and **1g** exhibited nanomolar potency against furin *in vitro* (Fig. 2A) and showed negligible toxicity either in CHO cells (Fig. 2B) or in HT1080 cells (data not shown) at 100 μ M. However, only **1a** and **1b** were able to inhibit intracellular furin, as judged by the release of SEAP from Golgi stores by cleavage of the reporter at the furin consensus site (Fig. 2C). Both of these compounds were also good inhibitors of cell migration (Fig. 2D). Despite its high potency *in vitro*, compound **1g** was unable to inhibit either TGN-associated furin or cell migration (Fig. 2C, 2D). Interestingly, compound **1b** was capable of blocking both *Pseudomonas* and anthrax toxemia at low doses (Fig. 2E, 2F). Compound **1g** also potently prevented anthrax toxicity (Fig. 2F). Considering its inability to block furin activity in the TGN, and its modest effect on furin activity in the endocytic pathway (as demonstrated by the *Pseudomonas* exotoxin A assays (Fig. 2E)), compound **1g** might represent a good candidate for selective extracellular inhibition. On the other hand, compound **1h**, whose main difference from **1a**, **1b** and **1g** is the absence of guanidino groups at the *para* position, not only showed no or very weak furin inhibition *in vitro* and within cells, but also exhibited poor protection against bacterial toxins.

In an effort to determine whether modification of the ring structure affects assay selectivity, we assessed the effect of a set of guanidinylated 2,5-dideoxystreptamine pyridinyl ether analogs, the heterocyclic analogs of phenyl ether derivatives, on cellular furin activity (Fig. 3). While none of these compounds were toxic (Fig. 3B), in accord with their poor inhibitory

potencies against furin *in vitro* (Fig. 3A), compounds **1m**, **1j** and **1k** showed no or very weak inhibition of cellular furin (Fig. 3C–F). However, compound **1i** exhibited near complete protection against *Pseudomonas* and anthrax toxins at 100 μ M (Fig. 3E, 3F), the former action suggesting its effective uptake by endocytosis. Note that **1i** possesses biological properties similar to those of **1g** (e.g. protection against exotoxin A and anthrax toxin, and no inhibition of TGN-related furin activity), in agreement with its structural homology with this compound (Fig. 2A). These data indicate that the addition of a nitrogen atom to the aromatic ring at the *ortho* position improves the protective action against *Pseudomonas* toxin, but may lessen protection against anthrax toxin.

As shown in Fig. 4, the analogs **1c**, **1e** and **1f**, which possess increasing hydrophobicity via replacement of one or two phenyl groups by naphthyl groups, exhibited high *in vitro* potency against furin (Fig. 4A), although they were also moderately cytotoxic and showed poor inhibitory capacity against intracellular furin (Fig. 4B, 4C). Interestingly, compound **1f** significantly inhibited cell migration, protected against *Pseudomonas* exotoxin A at low concentrations, and effectively blocked anthrax toxemia (Fig. 4D–F). By contrast, **1c** was also able to inhibit migration (more efficiently than **1f**), but did not protect against exotoxin A, and blocked anthrax toxicity less efficiently than **1f** (Fig. 4E, 4F). Compound **1e**, which differs from **1c** by the absence of a guanidino group at the *ortho* position, did not possess any inhibitory effect on migration, but exhibited better protection against bacterial toxins (Fig. 4D–F). In general, however, despite their mild cytotoxicity (**1c**, **1e**), compounds in this third set containing the more hydrophobic groups seem to specifically exhibit increased inhibitory potency on cell migration in comparison to similar, more polar, 2,5-dideoxystreptamine derivatives (Fig. 2D, 3D, and 4D).

The analogous carbamate compounds **1q** and **1p** (Fig. 5A), containing guanidino groups at the *meta*- and *para*-positions of the phenyl ring respectively, displayed micromolar potency against furin *in vitro*, but were moderately cytotoxic (**1q**, **1p**) and, except for **1p**, did not inhibit intracellular furin even at a concentration of 100 μ M (Fig. 5C–D). The analog **1r**, which includes guanidino groups at the *ortho*-position, was totally unable to inhibit furin, either *in vitro* or intracellularly. In comparison to **1q**, the **1p** analog was much less toxic (Fig. 5B), inhibited cell migration by almost 40% (Fig. 5D), and completely protected cells against *Pseudomonas* toxin (Fig. 5E). By contrast, this compound only poorly blocked anthrax toxemia (Fig. 5F). As evidenced by comparing compounds **1p** and **1g** (Fig. 2), the presence of the carbamate groups appears to decrease both the inhibitory capacity on furin *in vitro* and the protective effect against anthrax toxin at the cell surface. However, the carbamate groups likely improve cell permeability and/or uptake, as demonstrated by the superior inhibitory effects of this set of compounds on cell migration and on endosomal activation of exotoxin A at 100 μ M.

3.2. Inhibition of furin activity in vitro and ex vivo by bis-guanidinophenyl ether compounds

Given the limited inhibition of intracellular furin by guanidinylated 2,5-dideoxystreptamine derivatives, we designed and synthesized a series of compounds in which the guanidino phenyl groups were maintained (for maintaining potency against furin) but the middle

linker- the polar 1,3-diguanidinocyclohexane group- was replaced by less polar groups, such as propane (compounds **2a** and **2d**), cyclohexane (compounds **2b** and **2e**), or benzene (compounds **2c** and **2f**), in order to observe whether the cellular activity could be improved. The synthesis of these compounds is described in detail in Fig. 1.

The efficacy of these new molecules in inhibiting furin *in vitro* was initially characterized by determining IC₅₀ values [38] and further by obtaining *K_i*s, as described in “Materials and Methods”. These data revealed that this series of bis-guanidinophenyl ether compounds inhibited furin in the micromolar range *in vitro*, substantially less potent than the guanidinylated 2,5-dideoxystreptamine derivatives (Fig. 6). The mechanism of inhibition was competitive (not shown). Interestingly, the addition of two guanidino groups to the phenyl ring improved the inhibitory potency 3–4 times (i.e. **2a** vs **2d**; **2b** vs **2e**; **2c** vs **2f**). The inhibitory potency of these compounds against furin was also dependent on the middle linker group, in the order of benzene > cyclohexane > propane (Fig. 6). Several dideoxystreptamine furin inhibitors- such as compound **1g**- are known to exhibit substantial cross-inhibition with the proprotein convertases PC5 and PACE4 [31]. While these two enzymes were not available for testing, the bis-guanidinophenyl ether compounds did not inhibit PC2 even at 100 μM, and of compounds **2b** to **2e**, only **2d** and **2e** were found to inhibit PC1/3, and only at high concentrations (51% and 38% inhibition respectively at 100 μM, with no inhibition detected at 10 μM).

Excepting compound **2c**, which was slightly toxic at 10 μM and highly toxic at 100 μM, the bis-guanidinophenyl ether compounds were not toxic at 10 μM, and only certain derivatives such as **2a**, **2b** and **2f** showed slight or modest cell toxicity at 100 μM (Fig. 7A). The high cell toxicity of **2c** may be due to the presence of the middle aromatic group. In fact, the addition of guanidino groups at the *ortho*-positions of the phenyl ring significantly alleviated toxicity (**2c** vs **2f**). Fig. 7B shows that compounds **2a**, **2b** and **2c** were able to inhibit 30–50% of cellular furin activity within the TGN, although these results were directly correlated with cytotoxicity and thus may not reflect actual furin inhibition. Compounds **2d**, **2e** and **2f** inhibited only 10–15% of TGN-associated furin activity, suggesting poor entry into this compartment. All of these compounds were able to inhibit the migration of HT1080 fibrosarcoma cells, the most potent inhibitor being **2c** (Fig. 7C). Again, however, this effect may be associated with cellular toxicity, since these cells were not viable after a 1-day incubation with 100 μM of this compound (data not shown). The remainder of these compounds effectively inhibited cellular migration, from around 40% (**2a**, **2b** and **2d**) to 20–25% (**2e** and **2f**); since only limited cytotoxicity was seen, these compounds represent the second most effective compounds on cell migration, after **1a** and **1c** (60–70% protection).

In the endocytic pathway, compounds **2d**, **2e** and **2f** exhibited good protection against *Pseudomonas* toxemia, increasing the cell viability to 40% in the case of **2f** at 100 μM (Fig. 7D). All of these compounds, except **2c**, also showed moderate protection against *Pseudomonas* exotoxin A at 10 μM, suggesting good uptake into the endosomal system. In agreement with the idea of poor cellular permeability into the TGN of CHO-GRAPfurin cells (Fig. 7B), compounds **2d**, **2e** and **2f** exhibited potent cell activity at the cell surface, with excellent protection against anthrax toxicity at 100 μM inhibitor (Fig. 7E).

Interestingly, inhibitor **2f** showed almost complete protection at only 10 μM ; it was also the most potent of this series *in vitro* ($K_i = 0.4 \mu\text{M}$; Fig. 6). Conversely, compound **2a** was unable to block anthrax toxicity even at 100 μM , which is likely related to its intrinsically low potency *in vitro* towards furin ($K_i = 8.3 \mu\text{M}$, Fig. 6). These data are consistent with the idea that inhibitory potency against anthrax toxemia is most closely correlated with inhibition in *in vitro* assays.

3.3. Docking of 1,3-bis(2,4-diguanidinophenoxy) benzene (compound **2f**) into the catalytic pocket of human furin

In previous studies [39] we emphasized the importance of positively charged groups in furin inhibitors. We also showed a possible binding orientation of 2,5-dideoxystreptamine derivatives into the furin, PC1/3, and PC2 binding sites [33]. Our previously reported binding model of 2,5-dideoxystreptamines into the PC1/3 catalytic site [33] showed a likely preferred orientation of the naphthyl group into the S1 pocket. In this orientation the guanidino group at the *para* position reaches deep into the S1 subsite, making favorable hydrogen bond interactions with the carbonyl oxygen atoms of Pro256 and Ala292.

An example of this binding pose for the novel compound **2f** is shown in Fig. 8. This molecule is the most active amongst the analogous compounds shown in Fig. 6; a comparison with the co-crystallized ligand (guanidinomethyl-phenylacetyl-Arg-Val-Arg-(amidomethyl)-benzamidine (m-guanidinomethyl-Phac-RVR-Amba) [37] shows how compound **2f** is able to nicely orient in a similar pose towards the S2, S3, and S4 sites. These subsites have been proposed to be relevant for achieving specificity in the recently published crystal structure of human furin [37]. In the orientation shown in Fig. 8, however, the S1 subsite is unoccupied. This could potentially represent the reason, at least in part, for the decreased inhibitory potency exhibited by this compound as compared to the submicromolar inhibition exhibited by other 2,5-dideoxystreptamine congeners such as compound **1e** (assuming similar binding poses).

4. Discussion

The proprotein convertase furin is known to act in various subcellular compartments including the TGN, the cell surface, and the endocytic compartment (reviewed in [3]). Despite the fact that the potency of inhibitors directed to furin action on the cell surface (for example the cleavage of the anthrax toxin PA) correlates well with *in vitro* inhibitor potency, inhibitory potency for previously published inhibitors in this cell-based assay is generally several orders of magnitude lower than that observed in the test tube [31, 32, 40–43]. This occurs most likely for reasons of inhibitor stability, very low *in vivo* substrate concentrations, and loss of inhibitor on cell surfaces. Inhibitor access to other subcellular compartments in which furin acts, such as the endosomes and TGN, is likely even more difficult, and the particular biophysical qualities which permit chemical transport across cell membranes or endocytic uptake are still poorly defined. Although reasonably clear structure-activity relationships which generally coincide with prior structural knowledge can be proposed for the 2,5-dideoxystreptamines described in this paper, the challenge of developing structure-activity relationship studies on *in vivo* data is evident. In the work

discussed below, the varying ability of 2,5-dideoxystreptamine derivatives to modulate furin to different extents and to exhibit differing toxicity levels makes these compounds versatile for drug discovery efforts to begin to define compartment-specific furin inhibitors.

The involvement of proprotein convertases, especially furin, in various human pathologies is now well documented (reviewed in [44, 45]). For example, furin expression is elevated in certain kinds of tumors such as those derived from ovary, breast, head and neck, skin, brain and lung compared to normal adjacent tissue [15, 16, 46–49], and furin or furin-like activity contributes to tumor progression, aggressiveness, metastasis, and angiogenesis [27, 50, 51]; reviewed in [12]. Furin processing of substrates such as the metalloproteases stromelysin-3 and pro-membrane type-1 matrix metalloproteinase (proMT1-MMP) facilitates cell invasion and metastasis [14, 52]. In particular, the furin-mediated activation of proMT1-MMP primarily occurs within the TGN, and its mature form, MT1-MMP, is rapidly secreted into the extracellular space [53, 54] where it acts to degrade extracellular macromolecules (*e.g.* fibronectin, fibrin, vitronectin, laminin-1 and -5, and collagens) and adhesion molecules (*e.g.* CD44, integrins, and tissue transglutaminase). Furin-mediated activation of other MMPs such as gelatinase and collagenase 3 also contributes to the metastatic potential of tumor cells [14, 55, 56].

In agreement with the idea that convertase overexpression enhances metastasis, administration of peptide-based convertase inhibitors can limit tumor progression. While the non-selective irreversible convertase inhibitor dec-RVKR-CMK decreases skin tumor progression [57], this highly reactive compound exhibits significant cell toxicity [58]. The most potent peptide-based convertase inhibitors are likely the multi-Leu peptides developed by the Day group, which block viral infection [59] as well as tumor growth [28, 29, 60]; and the peptide analogs recently developed by the Steinmetzer group [25, 61] which potently inhibit bacterial toxin activation. However, because peptide-based drugs suffer from potential stability problems, several groups have instead sought small molecule inhibitors.

Many different kinds of small molecule drugs that act as competitive furin inhibitors and that are relatively specific for this enzyme have now been identified [26, 31, 43]. In a compound library screen, the cell-permeable naphthofluorescein disodium salt B3 prevented cell mobility and invasiveness of human fibrosarcoma HT1080 cells by inhibiting proMT1-MMP processing [26]. We found that the guanidinylated 2,5-dideoxystreptamine derivatives **1a** and **1b** strongly prevented the migration of HT1080 cells (by 60% and 35%, respectively), while showing no significant cell toxicity. Given that these compounds were also able to inhibit TGN furin in GRAPfurin CHO cells, compounds **1a** and **1b** might represent useful inhibitors to reduce cancer cell metastasis.

Other compounds based on the 2,5-dideoxystreptamine scaffold, including **1c**, **1f** and **1p**, also displayed significant inhibition of cell migration (30–40%) with low general cytotoxicity. In contrast, while the bis-guanidinophenyl ether compounds **2a**, **2b** and **2c** also inhibited migration, they were toxic at high doses. For example, **2c** completely inhibited cell mobility, but probably solely as a consequence of a severe decrease in cell viability. In the case of **2d**, **2e** and **2f**, we did not observe cytotoxicity, but these compounds only exhibited a modest 15–35% inhibition of HT1080 cell migration. Taken together, the guanidinylated

2,5-dideoxystreptamine derivatives constitute a promising group of compounds with therapeutic potential in cancer models, and should be subjected to further *in vivo* testing.

Protein convertase cleavages of bacterial and viral proteins are also essential for the pathogenicity of a number of bacteria and viruses [4, 45]. Furin therefore also represents an attractive target for the treatment of certain bacterial and viral infections. In fact, various peptide-based furin inhibitors have been used to block and/or delay anthrax toxemia both in cells and in live animals by inhibiting furin-catalyzed processing of the anthrax PA protein [32, 62, 63]. Additionally, furin has also emerged as a target for the prevention of the cytotoxic activation of *Pseudomonas aeruginosa* exotoxin A and Shiga toxin [25, 40, 59, 64]. Thus, the general convertase inhibitor nona-D-arginine peptide [65, 66] provides efficient topical treatment of severe corneal keratitis caused by *Pseudomonas aeruginosa*, particularly when combined with the antibiotic ciprofloxacin [67]. The present results show that the most promising small molecule compounds for the prevention of anthrax toxemia are likely the 2,5-dideoxystreptamine derivatives **1b** and **1g**, and the bis-guanidinophenyl ether compound **2f**, all of which exhibited complete inhibition of anthrax toxicity at 10 μ M, and none of which displayed significant cell toxicity at this concentration in a variety of cell lines. Compound **1b** was also able to completely prevent *Pseudomonas* toxin cytotoxicity at this same dose. Since compound **1b** also inhibited cell migration, this compound exhibits promise as a potent broad spectrum furin inhibitor for the treatment of furin-related pathologies. Other compounds, including **1i** or **1p**, also completely prevented *Pseudomonas* toxemia, but only at 100 μ M; **1i** was also capable of blocking anthrax toxicity at this same concentration. In general, the compounds based on the bis-guanidinophenyl ether scaffold (except **2a**) potentially inhibited anthrax toxin activation, and showed relatively good protection against *Pseudomonas* exotoxin A at 100 μ M. Taking into account the lack of toxicity and cell permeability of compound **2f** at low doses, its lack of effect on intracellular and endosomal furin, and its potent inhibition of furin at the cell surface, we suggest that this compound might be profitably exploited for selective protection against cell surface furin action (for example as occurs in anthrax infection) with potentially negligible side effects. However, direct evidence for the lack of cell penetrance of this molecule, as well as of other compounds such as **1g**, should be confirmed in *ex vivo* studies using labeled compounds.

In conclusion, we have here elucidated interesting structure-activity relationships for each subgroup of inhibitors; while we could not determine universal molecular characteristics that control the cellular inhibitory activity in each specific compartment, i.e. the TGN, endosome, and cell surface, we have provided “proof of concept” that compartment selectivity of furin inhibitors can be addressed by fine-tuning of molecular structures. We suggest the use of the 2,5-dideoxystreptamine derivative **1b** (1,1'-(4-((2,4-diguanidino-5-(4-guanidinophenoxy)cyclohexyl)oxy)-1,3-phenylene)diguanidine) as a general non-peptide small molecule furin inhibitor to combat cancer and/or infectious agents whose mode of action requires intracellular host furin activity. Additional studies in animals will be necessary to corroborate the efficacy of this promising compound. In addition, we have identified the compartment-selective compounds **1g** and **2f** for the treatment of pathogenic bacterial infections - such as *Pseudomonas aeruginosa* or *Bacillus anthracis* - with poor inhibitory capacity for intracellular furin. We suggest that further development of

compartment-specific inhibitors in cell-based assays will be helpful to avoid potential side effects caused by the deregulation of normal furin activity and to rule out cytotoxic compounds.

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Abbreviations

AMC	7-amino-4-methylcoumarin
CHO	Chinese hamster ovary
ER	endoplasmic reticulum
GRAP	Golgi-retained alkaline phosphatase
LF	lethal factor
MMP	matrix metalloproteinases
PA	protective antigen
PC	proprotein convertase
pRTKR-MCA	Pyr-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide
SEAP	secreted alkaline phosphatase
TGN	trans-Golgi network

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Fig. 1.
Synthesis of bis-guanidinophenyl ether compounds 2a–f.

Fig. 2. Effect of guanidinylated 2,5-dideoxystreptamine phenyl ether derivatives 1a, 1b, 1g and 1h on cellular furin activity

A) The chemical structures and K_i values of compounds 1a, 1b, 1g and 1h. K_i values were taken from the literature [31]. B) CHO cells were incubated with each compound at a final concentration of 100 μM for 24 h at 37°C, and cell viability was monitored by incubation for 2 h with WST-1. C) CHO-GRAPfurin cells, expressing Golgi-retained secreted alkaline phosphatase, was incubated with 100 μM of each inhibitor for 20–24 h at 37°C, and the SEAP activity was measured in the medium. D) Migration of HT1080 cells after incubation with 100 μM of each inhibitor for 24 h at 37°C. E) Protection from *Pseudomonas aeruginosa* exotoxin A cytotoxicity in CHO DG44 cells in the presence of 10 μM and 100 μM of each inhibitor for 20–24 h at 37°C. F) Protection from anthrax toxin cytotoxicity in RAW264.7 cells incubated in the presence of 10 μM and 100 μM of each inhibitor for 2 h at

37°C. Where no error bar is visible, errors were within the range of the data shown.
Compounds showing zero inhibition lack data bars G = guanidino.

Fig. 3. Effect of guanidinylated 2,5-dideoxystreptamine pyridinyl ether derivatives 1i, 1m, 1j and 1k on cellular furin activity

A) Chemical structures and K_i values (taken from [31]). B) CHO cells were incubated with each compound at $100 \mu\text{M}$ for 24 h at 37°C , and cell viability was monitored by incubation for 2 h with WST-1. C) Inhibition of SEAP secretion in the conditioned medium from CHO-GRAPfurin cells incubated with $100 \mu\text{M}$ of each inhibitor for 20–24 h at 37°C . D) Inhibition of migration of HT1080 cells after incubation with each inhibitor ($100 \mu\text{M}$) for 24 h at 37°C . E) Protection from *Pseudomonas aeruginosa* exotoxin A cytotoxicity in CHO DG44 cells in the presence of $10 \mu\text{M}$ and $100 \mu\text{M}$ of each inhibitor for 20–24 h at 37°C . F) Protection from anthrax toxin cytotoxicity in RAW264.7 cells incubated in the presence of $10 \mu\text{M}$ and $100 \mu\text{M}$ of each inhibitor for 2 h at 37°C . Where no error bar is visible, errors were within the range of the data shown. Compounds showing zero inhibition lack data bars G = guanidino.

Fig. 4. Effect of guanidylated 2,5-dideoxystreptamine phenyl/naphthyl ether derivatives **1c**, **1e** and **1f** on intracellular furin activity

A) Chemical structures and K_i values (taken from [31]). B) General cytotoxicity. CHO cells were incubated with each compound at 100 μM for 24 h at 37°C, and cell viability was monitored by incubation for 2 h with WST-1. C) Inhibition of intracellular furin activity. CHO-GRAPfurin cells were incubated with 100 μM of each inhibitor for 20–24 h at 37°C, and SEAP activity was measured in the medium. D) Inhibition of migration of HT1080 cells during incubation of 100 μM of each inhibitor for 24 h at 37°C. E) Protection from cytotoxicity of *Pseudomonas aeruginosa* exotoxin A in CHO DG44 cells in the presence of 10 μM and 100 μM of each inhibitor for 20–24 h at 37°C. F) Protection from cytotoxicity of anthrax toxin in RAW264.7 cells incubated in the presence of 10 μM and 100 μM of each

inhibitor for 2 h at 37°C. Where no error bars are visible, errors were within the range of the data shown. Compounds showing zero inhibition lack data bars G = guanidino.

Fig. 5. Effect of guanidylated 2,5-dideoxystreptamine phenyl carbamate derivatives 1q, 1p and 1r on cellular furin activity

A) Structures of carbamate derivatives and their Kis (taken from [31]). B) CHO cells were incubated with each compound at 100 μ M for 24 h; cell viability was then monitored by incubation for 2 h using WST-1. C) Inhibition of SEAP activity secreted into the conditioned medium by CHO-GRAPfurin cells incubated with 100 μ M of each inhibitor for 20–24 h. D) Inhibition of migration of HT1080 cells during incubation with 100 μ M of each inhibitor for 24 h at 37°C. E) Protection from cytotoxicity of *Pseudomonas aeruginosa* exotoxin A in CHO DG44 cells in the presence of 10 μ M or 100 μ M of each inhibitor for 20–24 h. F) Protection from cytotoxicity of anthrax toxin in RAW264.7 cells incubated in the presence of 10 μ M and 100 μ M of each inhibitor for 2 h. Where no error bars are visible, errors were within the range of the data shown G = guanidino.

Fig. 6. Chemical structures of the bis-guanidinophenyl ether compounds tested as furin inhibitors

2a: 1,3-bis(4-guanidinophenoxy) propane; 2b: 1,3-bis(4-guanidinophenoxy) cyclohexane; 2c: 1,3-bis(4-guanidinophenoxy) benzene; 2d: 1,3-bis(2,4-diguanidinophenoxy) propane; 2e: 1,3-bis(2,4-diguanidinophenoxy) cyclohexane; 2f: 1,3-bis(2,4-diguanidinophenoxy) benzene. Ki values were calculated as described in the "Materials and Methods" G = guanidino.

Fig. 7. Cell-based assays for bis-guanidinophenyl ether compounds

A) CHO cells were incubated with each compound at 10 μ M and 100 μ M for 24 h at 37°C, and cell viability was monitored by incubation for 2 h with WST-1. B) CHO-GRAPfurin cells were incubated with 100 μ M of each inhibitor for 20–24 h at 37°C, and the SEAP activity was measured in the medium. C) Migration of HT1080 cells after incubation of 100 μ M of each inhibitor for 24 h at 37°C. D) Cell toxicity of *Pseudomonas aeruginosa* exotoxin A in CHO DG44 cells in the presence of 10 μ M and 100 μ M of each inhibitor for 20–24 h at

37°C. E) Cytotoxicity of anthrax toxin in RAW264.7 cells incubated in the presence of 10 μ M and 100 μ M of each inhibitor for 2 h at 37°C.

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Fig. 8. Binding orientation of molecule 2f into the furin catalytic pocket

Molecular surface representation of human furin; residues forming the catalytic triad and subsites S1–S4 are colored. Molecule 2f and the co-crystallized ligand [37] are shown as black and yellow stick representations, respectively.

Table 1

Cell-based assays and cell lines used to test furin activity within different cellular compartments. GRAP: Golgi-retained alkaline phosphatase; SEAP: secreted alkaline phosphatase; TGN: trans-Golgi network.

SEAP activity	TGN	CHO-GRAPfurin
Cell migration	TGN	HT1080
<i>Pseudomonas</i> exotoxin A	Endosomes	CHO DG44
Anthrax	Cell surface	RAW264.7