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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 cell-based assays is largely unknown. In this study we show that certain guanidinylated 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 bis-guanidinophenyl 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.

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. 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

Abbreviations: AMC, 7-amino-4-methylcoumarin; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; G, guanidino group; 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-methyl-coumaryl-7-amide; SEAP, secreted alkaline phosphatase; TGN, trans-Golgi network.

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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. Materials 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*″-tirfluoromethanesulfonylguanidine, and deprotection with trifluoroacetic acid (Fig. 1).

2.2. Enzyme assays and determination of IC_{50} 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 μl, containing 100 mM HEPES, pH 7.0, 5 mM $CaCl_2$, 0.1% Brij 35, 0.1% NaN_3, and 0.1 mg/ml BSA. The substrate Pyr-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide (pRTKR-MCA; Peptides International, Lexington, KY) was used at a final concentration of 100 μM. 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 IC_{50} assays, serial dilutions of compounds were performed to give final concentrations between 100 nM and 250 μM in 50 μl. 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 μM 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 μM, 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 μM of either drug or vehicle for 16–20 h. 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 μl of heated medium sample were mixed with 100 μl of assay buffer (100 mM Tris–HCl, pH 10, 100 mM NaCl, 5 mM $MgCl_2$) and 100 μl of 36 μM 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 (10^5 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%