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Cross-inhibition between furin and lethal factor inhibitors ☆

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Abstract

Bacillus anthracis synthesizes two toxins composed of the three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF). The cleavage of PA on the cell surface by the convertase furin leads to the translocation of LF and EF into the cytosol. We have investigated the cross-inhibitory activities of the furin inhibitors hexa-D-arginine amide (D6R) and nona-D-arginine amide (D9R), which block the proteolytic activation of PA; and of the LF inhibitor In-2-LF, a peptide hydroxamate. D6R and D9R inhibit LF with IC_{50s} of 300 and 10 μM, respectively; conversely, In-2-LF also inhibits furin (IC_{50} 2 μM). In-2-LF was efficiently cleaved by furin with the concomitant loss of inhibitory activity on both LF and furin. Incubation of In-2-LF with LF however generated a product that retained partial inhibitory activity against LF. Combined treatment of cells with D6R and In-2-LF enhanced protection against anthrax lethal toxin, indicating that combined administration of inhibitors could represent an effective therapeutic approach.

Anthrax is a severe, often fatal bacterial disease that occurs when *Bacillus anthracis* spores infect alveolar macrophages; the ensuing infection results in septicemia and toxemia. The major virulence factors of *Bacillus anthracis* are the cytotoxic proteins lethal factor (LF) and edema factor (EF) [1]. The third polypeptide secreted by the anthrax bacterium is protective antigen (PA), which is cleaved by furin at the cell surface to result in the production of an N-terminal fragment, PA_{20}, and a C-terminal fragment, PA_{63} (reviewed in [2]). Seven molecules of PA_{63} then coassemble and become inserted in the cell membrane; this PA complex is essential for the translocation of LF and EF into the cytosol of the cell [3,4].

Various therapeutic strategies against anthrax have been proposed to date (e.g., antibiotic treatments [5]) and strong effort has been focused on the development of an efficacious and safe anthrax vaccine [6,7]. In any case prompt treatment of exposed individuals is required in order to avoid the deleterious effects of toxin build-up, for which there are currently no effective therapies. It has been shown that a specific peptide inhibitor of the metalloprotease LF, termed In-2-LF, can block anthrax toxicity in RAW264.7 and J774.A1 cells [8]. Our group has shown that the furin inhibitor hexa-D-arginine (D6R), through its blockade of PA cleavage, is also effective in reducing anthrax toxin pathogenesis in vitro and in vivo [9,10]. We have recently observed that nona-D-arginine (D9R) inhibits furin activity in vitro with higher efficacy (Kacprzak et al., in press). Because D6R, D9R, and In-2-LF all contain a similar polyarginine-rich core sequence, we have examined the possibility of cross-inhibition of these compounds on furin and LF.

☆ *Abbreviations:* D6R, hexa-D-arginine amide; D9R, nona-D-arginine amide; EF, edema factor; LF, lethal factor; PA, protective antigen; PEA, *pseudomonas* exotoxin A, In-2-LF, lethal factor inhibitor 2.

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Materials and methods

Peptides. The peptide hydroxamate In-2-LF was purchased from Calbiochem (La Jolla, CA). D6R and D9R were synthesized by Pepceuticals, (New Orleans, LA) and purified by reverse-phase HPLC to greater than 99% purity. Because of the high content of basic amino acids, both peptides contained approximately 40% by weight of associated salt (trifluoroacetate); the results have not been corrected for this percentage.

Determination of inhibitory activity against furin. Mouse furin was purified from the medium of stably transfected CHO cells as previously described [10]. The assays were carried out in 96-well round-bottomed polypropylene plates and the fluorescence was measured in a 96-well fluorometer (Labsystems, Hampshire, UK) at 380 nm excitation and 460 nm emission. The assay for furin was performed at 37 °C using pERTKR-MCA as described previously [11] at pH 7.0 in 100 mM Hepes, 5 mM $CaCl_2$, and 0.1% Brij 35. The total volume was 50 μl. Unless otherwise stated, the final substrate concentration for all furin assays was 200 μM.

Determination of inhibitory activity against LF. LF was obtained either as previously described [12] or acquired from Calbiochem. The LF enzyme assay was carried out as previously described [13]. Briefly, 25 μl of a 6 μM solution of the peptide substrate 7-hydroxy-4-methyl-3-acetylcoumarinyl-K(QSY-35)GG)-NH_2 (10 μM final concentration; Calbiochem) was added in assay buffer (20 mM Hepes, pH 7.0/1 mM $CaCl_2$/0.1 mg/ml BSA/0.01% Tween 20). Test compounds were added in DMSO (1.5 μl; this amount of DMSO applied alone did not modify activity). The enzymatic reaction was monitored after the addition of 18.5 μl LF in assay buffer (10 nM LF in the 50 μl final volume) for a 15 min period (380 nm excitation and 460 nm emission). All assays were performed in triplicate.

Cleavage of In-2-LF by furin and LF. In-2-LF (40 μM) was incubated at 37 °C with furin or LF in their respective assay buffers. Aliquots of 20 μl were removed at the indicated times, placed into 480 μl of ice-cold 0.1% trifluoroacetic acid, and immediately frozen. Peptides in each aliquot were separated using a 5 μm Beckman ODS column (0.46 × 25 cm) with a linear gradient of 0–40% acetonitrile containing 0.1% trifluoroacetic acid over 60 min at a flow rate of 1 ml/min. Absorbance was monitored at 218 nm. Parallel reactions containing buffer instead of furin or LF were also analyzed. In order to examine the inhibitory potency of cleavage fragments, a similar experiment was performed and aliquots were removed at the same time points, boiled, and used (at a final concentration of 0.7 μM In-2-LF) to test inhibitory activity on LF and furin.

Cell culture and cytotoxicity assay. The inhibitory effect of D6R and In-2-LF on anthrax toxemia was studied in RAW264.7 cells. RAW264.7 cells (10^4 per well) were seeded into microtiter plates and treated 12 h later with 400 ng/ml PA and 200 ng/ml LF in the presence of 5 μM D6R and In-2-LF or 5 μM of each inhibitor for a 2 h period. Inhibitors were added immediately after treatment of cells with PA + LF. Cell viability was monitored with the compound WST-1 (Roche Diagnostics) using the manufacturer's protocol; this assay reflects the activity of mitochondrial dehydrogenase present in living cells. The difference in the absorbance at 450 and 630 nm was measured 1 h after addition of WST-1 to cells. The results are presented as the percentage of survival ± SD.

Results

In-2-LF inhibits furin

As the sequences of the peptidyl inhibitors D6R, D9R, and In-2-LF all share a polyarginine core (Fig. 1A), we determined the extent of cross-inhibition between these inhibitors and their respective enzymes (furin and LF) in a 15 min enzymatic assay. The results of the furin assay show a strong inhibitory effect of In-2-LF on furin at submicromolar concentrations (Fig. 1B). Kinetic analysis showed that the inhibition of

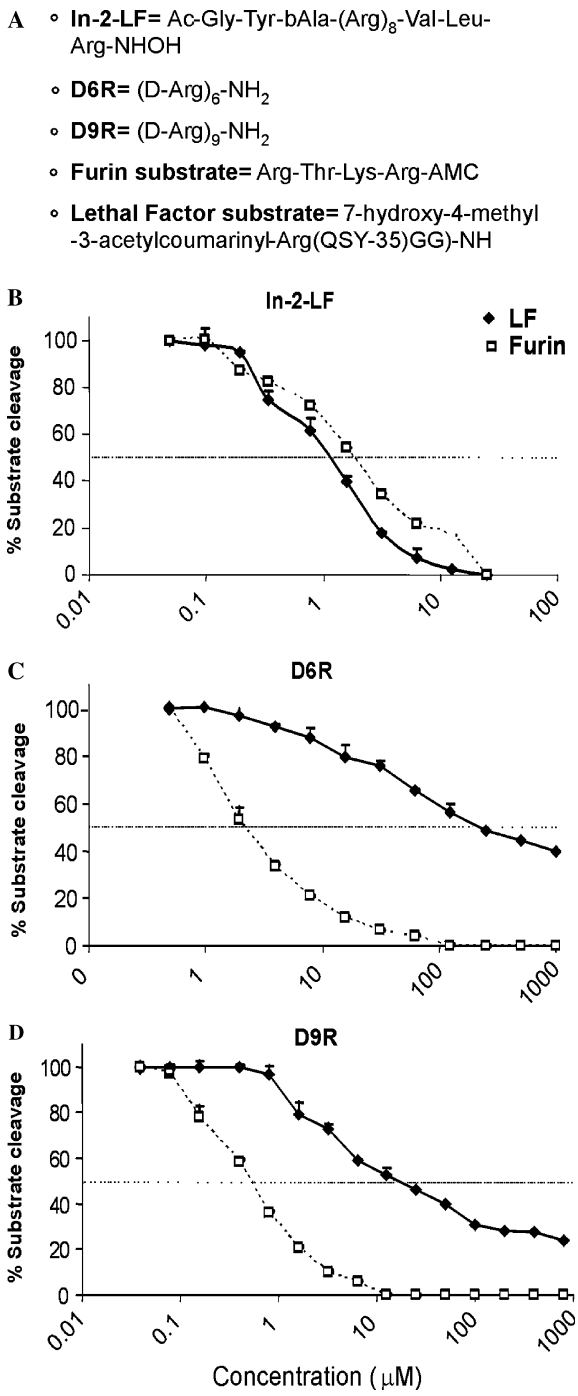

Fig. 1. Effect of In-2-LF, D6R, and D9R on furin and LF activity. (A) Structure of the furin and LF inhibitors and their fluorogenic substrates. (B) In-2-LF inhibits both LF and furin (doses from 0 to 100 μM were tested). (C) D6R and (D) D9R inhibit furin and LF (0 to 1 mM). Data are presented as means ± SD ($n = 3$). Fifty percent inhibition is indicated with a line. These data are derived from initial linear rates of substrate hydrolysis (maximum substrate cleavage of 13%) where the maximum cleavage rate is 4.3 pmol/min.

In-2-LF on furin was strictly competitive, with a K_i of 49 nM (data not shown). Given the differences between our study with regard to substrate type and concentration, these results showing submicromolar inhibition of LF by In-2-LF are in fairly good agreement with those of Tonello et al. [8] (K_i of 1 nM).

D6R and D9R inhibit LF activity

D6R and D9R also inhibited LF, although D9R was much more potent. Thus, while D6R reached 50% inhibition at 300 μM (Fig. 1C), the IC_{50} of D9R was 10 μM (Fig. 1D). In contrast to In-2-LF, D6R never inhibited more than 60% of LF activity, and D9R never attained more than 80% inhibition of this enzyme. These non-standard kinetics do not allow us to calculate the K_is of D6R or D9R, although the results support the idea that poly-D-arginines exhibit mixed competitive and non-competitive inhibition of LF.

Furin and LF cleave In-2-LF

We first tested whether In-2-LF can be cleaved by furin by using reverse-phase HPLC (Fig. 2). In-2-LF was effectively cleaved by furin after 60 min of incubation, generating five different peptide products (Fig. 2, left). Incubation of In-2-LF with LF also led to cleavage of this inhibitor, although in this case there were only two peptide products (Fig. 2, right).

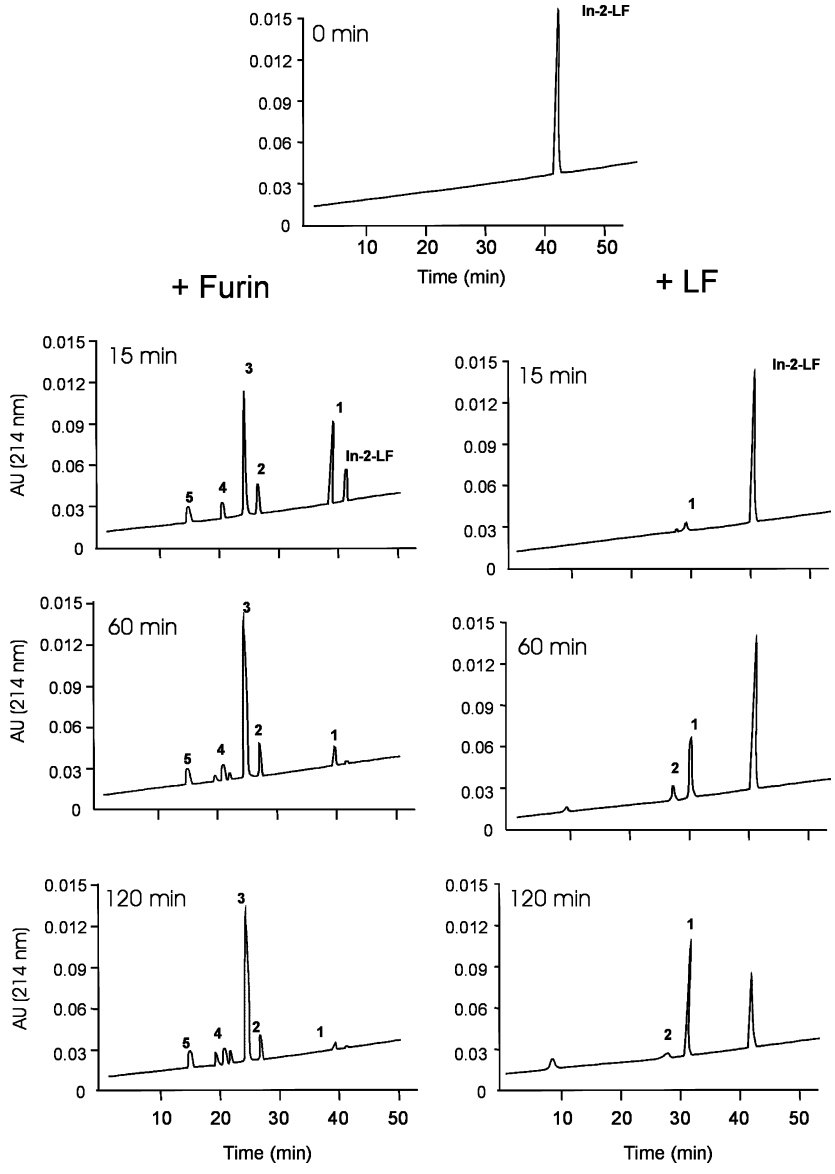

Fig. 2. Cleavage of In-2-LF by furin and LF. In-2-LF was incubated with LF (right) and furin (left) at 37 °C at different time points (15, 30, 60, and 120 min) prior to separation by HPLC as described under "Materials and methods." The In-2-LF fragments generated are labeled with numbers and the peak corresponding to unprocessed In-2-LF is also indicated in the panel. AU, absorbance units.

Incubation of In-2-LF with furin reduces its inhibitory activity against furin and LF

Enzymatic assay of the cleavage products of In-2-LF after furin incubation showed a reduction of inhibitory activity on furin and LF in a time-dependent manner (Fig. 3A). Preincubation of In-2-LF with furin for 120 min reduced its ability to inhibit furin and LF. On the other hand, preincubation of In-2-LF with LF still resulted in products with good inhibition of LF activity (Fig. 3B). We tested the possibility that D6R or D9R could be cleaved by furin and LF, but no cleavage was observed (data not shown).

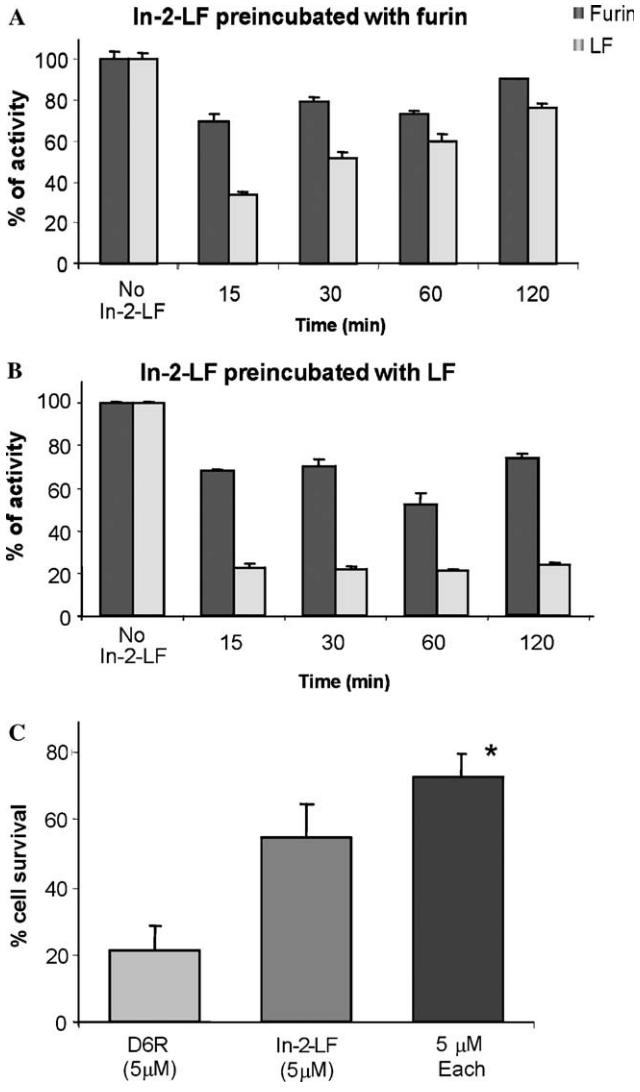

Fig. 3. (A,B) Inhibitory activity of In-2-LF cleaved by furin and LF. In-2-LF was incubated with furin (A) or LF (B) at 37 °C. The inhibitory activity of the processed In-2-LF was measured after 15, 30, 60, and 120 min of incubation. Data are presented as percentage control activity (without In-2-LF). (C) Increased protection against anthrax toxemia using combined treatment with In-2-LF and D6R. RAW264.7 cells were treated with 400 ng/ml PA and 200 ng/ml LF 12 h after subculturing in the presence of 5 μM D6R, 5 μM In-2-LF, or 5 μM of each inhibitor combined. Inhibitors were added immediately after treatment of cells with PA + LF, and cell growth was monitored after a 2 h treatment. Results are presented as the percentage of survival. D6R combined with In-2-LF enhances the inhibition when compared to D6R alone ($p < 0.01$). All cells not treated with the inhibitors exhibited almost zero percentage survival after 2 h and the inhibitors alone did not have any toxic effect on the cells (data not shown).

Combined treatment with D6R and LF increases the resistance of RAW cells to anthrax toxemia

RAW264.7 cells were treated with anthrax toxin and either 5 μM D6R or 5 μM In-2-LF, or with a combination of these two inhibitors (Fig. 3C). Cell growth was monitored with the compound WST-1 (Roche Diagnostics). The results are presented as the percentage of survival and represent data assessed at 2 h after intoxication, when the control cells given toxins alone exhibited 0% survival. Applied separately, each inhibitor provided partial protection at 5 μM (22% of cell survival for D6R and 55% in the case of In-2-LF). The combination of the two inhibitors reproducibly exhibited an enhanced protective effect when compared to D6R alone (73% of cell survival, $p < 0.01$). The results are presented as the average of four independent experiments.

Discussion

The mechanism of anthrax toxemia begins with the cleavage of PA by furin, which is required for entry of LF and EF into cells [14]. Once in the cytosol, LF cleaves signaling proteins such as MAPK-kinase, whereas EF, an adenylate cyclase, catalyzes the synthesis of cyclic AMP (cAMP) (reviewed in [15]). We have previously found that polyarginine furin inhibitors suppress anthrax toxemia as well as other furin-mediated toxemias [9,16]. Tonello et al. [8] have shown that the peptide hydroxamate In-2-LF, an LF inhibitor, blocks anthrax toxemia in RAW264.7 cells. Interestingly, D6R and In-2-LF share a degree of homology, as both contain a long polyarginine core. Library screenings by Cantley and co-workers [17] have also recently identified LF inhibitors with arginine-rich sequences which are essential for inhibitory activity. The common requirement for arginine-rich sequences between furin and LF inhibitors prompted us to study the potential inhibitory cross-reaction of LF and furin inhibitors.

Our results demonstrate that In-2-LF, in addition to inhibiting LF, also represents a potent inhibitor of furin. Thus, the effect of In-2-LF in preventing anthrax toxemia in RAW264.7 cells [8] is most likely not only due to its inhibitory effect on LF but also to its inhibition of furin. On the other hand, our data indicate that the inhibitory capacity of In-2-LF may be reduced in a time-dependent manner following furin exposure, as furin-mediated cleavage of In-2-LF results in products that have no inhibitory effect on furin.

Our studies also show that the polyarginines D6R and D9R represent LF inhibitors, though both are much less potent than In-2-LF. Since LF acts intracellularly, these peptides, like In-2-LF, must cross the cell membrane in order to effect inhibition. Recent findings indicate that polyarginines of a certain length have efficient access to the intracellular space [18,19], supporting this notion. We have observed that D6R is able to readily cross the plasma membrane of CHO cells, showing a partial localization within a perinuclear compartment (data not shown). Although both D6R and D9R are not as potent against LF as is In-2-LF, their stability to degradation implies that they are more likely to reach LF-containing compartments. In addition, since the cleavage of In-2-LF by furin generates peptides that have no inhibitory effect on LF, inhibition of cell surface-associated furin by polyarginines should enhance the ability of intact In-2-LF to reach intracellular LF.

D6R and In-2-LF exhibited competitive inhibitory kinetics on furin; In-2-LF also inhibits LF in a competitive manner. The inhibition of D6R on LF was complex, and never reached more than 60% in the case of D6R and 80% in the case of D9R; thus, no kinetic constants could be calculated. This phenomenon could be due to differential binding of these two types of inhibitors to the active pockets of each enzyme; for example, polyarginines may not have complete access to the active site of LF. A comparison of the three-dimensional structures of furin [20] and LF [21] reveals that the extended acidic substrate-binding pocket of furin is considerably wider and more accessible than that of LF, indicating that the binding of D6R and D9R to LF is likely to differ significantly from its interaction with furin. However, the cross-inhibition of each enzyme by the non-cognate inhibitors implies some degree of structural similarity.

Our previous studies with D6R demonstrate that this inhibitor is efficient in preventing anthrax toxemia, both in vitro and in vivo [10], results supported by the present study. Combined treatment with D6R and In-2-LF resulted in enhanced cellular survival against anthrax intoxication, most likely due to a dual mechanism. First, inhibition of furin by D6R will inhibit both PA and In-2-LF cleavage. Second, the greater amounts of intact In-2-LF produced by blockade of furin activity will result in enhanced inhibition of LF. Combined treatment with both furin and LF inhibitors may therefore represent a more effective therapeutic approach to the blockade of anthrax toxemia than the use of either inhibitor alone.

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