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Original Article

Examination of anthrax lethal factor inhibition by siderophores, small hydroxamates, and protamine

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Background and purpose: Based on their ability to chelate metals, hydroxamate molecules and siderophores have been successfully used as metalloenzyme inhibitors. As the anthrax toxin lethal factor (LF) is a zinc (Zn)-metallopeptidase, an investigation of the ability of some small non-siderophore hydroxamate compounds, 5 hydroxamate-containing siderophores, and 1 catecholate siderophore was undertaken to determine whether these compounds would inhibit LF. In addition, salmon sperm protamine and ethylenediaminetetraacetic acid were investigated.

Methods: A spectrophotometric assay of LF activity, based on its reaction with the substrate (Ac-gly-tyr-βala-arg-arg-arg-arg-arg-arg-arg-arg-val-leu-arg-p-nitroanilide), was used to assess the degree of inhibition of LF by the putative inhibitors. Procedures were implemented to avoid iron contamination of the test solutions and non-ferrated siderophores and hydroxamates were used as potential inhibitors.

Results: The hydroxamate-containing siderophores displayed limited capacities to inhibit LF, as did the low molecular weight hydroxamate compounds. In contrast, the catecholate siderophore enterobactin and the cationic polyamine salmon sperm protamine demonstrated notable inhibition of LF at concentrations ranging from approximately 10 to 200 μM.

Conclusions: The polyamine salmon sperm protamine which mimics the target site of proteins cleaved by LF, was the most effective inhibitor of the molecules examined, while the small molecule hydroxamates and the hydroxamate siderophores were among the poorest. If chelation of the Zn of LF results in LF inhibition by the molecules examined, it is most likely secondary to binding of the putative inhibitors to the active site of LF.

Key words: Anthrax toxin; Protamines; Siderophores

Introduction

Anthrax is a disease most commonly noted among herbivores and people who work with or handle animal products [1,2]. However, the disease can be used as a weapon of bioterrorism and may be inflicted upon an unknowing population with relative ease [3]. The anthrax incidences that occurred in the United States following the terrorism attack in 2001 have raised the need for the medical and civil defense communities to quickly recognize and ameliorate an anthrax challenge that could otherwise be disastrous.

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Anthrax is the result of a 3-component toxin produced by the bacterium *Bacillus anthracis* [1,4-7]. The disease is marked by the production of protective antigen, the protein that allows the penetration of the cell membrane by either edema factor or lethal factor (LF) [4,8]. Edema factor functions within a human cell as a powerful calmodulin and calcium dependent adenylate cyclase, resulting in edema of the tissues [4].

LF functions by a different mechanism; it is a zinc (Zn)-metallopeptidase that can specifically cleave the mitogen-activated protein kinase (MAPK) of the cell [4]. This activity is thought to be responsible for the detrimental [4] and life-threatening effects of the enzyme as immune cells, such as macrophages, are particularly susceptible [6,8]. Effective inhibition of LF could be part of a strategy by which physicians could treat anthrax poisoning.

Siderophores are avid ferric ion–binding molecules secreted by microbes to sequester the metal where it is in short supply [9-14]. In general, the ligands by which siderophores achieve their powerful binding of ferric ion fall into 3 categories: the hydroxamates, the catecholates, and the complexone (polycarboxylate) chemical groups [15-18]. While siderophores are best known and studied for their ferric ion–chelating abilities, they have the potential to bind other metals, such as aluminum, gallium, chromium, copper, and Zn [19-22].

Hydroxamates have been used to bind Zn and have been the basis from which effective matrix metallopeptidase inhibitors were designed [23,24]. Due to metal chelation, hydroxamic acid derivatives inhibited ribonucleotide reductase [25] and a specifically-designed hydroxamate inhibited LF at nM concentrations [26-28]. Siderophores are capable of inhibiting matrix metalloproteinase-2 (MMP-2) [29] and the pyoverdine-like siderophores bind Zn, inhibit MMP-2, and have a direct correlation between their Zn chelation and their MMP-2 inhibitory action [20].

The purpose of this study was to examine whether selected siderophores, particularly hydroxamate siderophores, are effective inhibitors of the Zn-metallopeptidase LF. Although not siderophores, a number of low molecular weight compounds, including hydroxamate compounds such as acetohydroxamic acid, were also examined, as was salmon sperm protamine. The former compounds were examined as small molecules have the ability to inhibit LF [30]. Salmon sperm protamine was examined as a review of the structure of the MAPK consensus sequence sites cleaved by LF, as well as the structure of an artificial substrate developed from the knowledge of these structures [27], resulted in the latter having protein homology to a number of protamines. In addition, cationic polyamines were previously noted as LF inhibitors [8].

Methods

Chemicals and compounds

Anthrax LF was provided by Stephen Leppla (National Institutes of Health, Bethesda, MD, USA, or BEI Resources, Manassas, VA, USA) as a 51 μM solution, which was diluted to aliquots of 1 μM LF containing 200 μg bovine serum albumin/mL. The bovine serum albumin served as a carrier of the LF. Anthrax LF protease substrate II, colorimetric (Ac-gly-tyr-βala-arg-arg-arg-arg-arg-arg-arg-arg-val-leu-arg-p-nitroanilide [pNA]; EMD Biosciences/Calbiochem-EMD Chemicals, Inc., San Diego, CA, USA); aerobactin, schizokinen, and enterobactin (EMC Microcollections, Biophore Research Products Tubingen, Germany); acetohydroxamic acid (Aldrich Chemical Company, Milwaukee, WI, USA); rhodoturilic acid (Frontier Scientific-Porphyrin Products, Logan, UT, USA); ferrichrome-iron-free (deferriferrichrome), L-arginine hydroxymate, L-lysine hydroxymate, and ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, St. Louis, MO, USA); and protamine sulfate (salmon sperm; Fisher Scientific, Hanover Park, IL, USA) were all purchased. Pyoverdine was isolated from *Pseudomonas aeruginosa* American Type Culture Collection 10145 as described by Mishagi et al [31]. Deferrioxamine B was a gift (Novartis, Cambridge, MA, USA).

Compounds and materials were kept iron-free so as to not interfere with any of the chelating effects of the compounds, by employing plastic ware, milliQ water, and passing the assay buffer (see below) through a chelating ion exchange resin (Chelex 100; Biorad, Hercules, CA, USA) prior to its being used to either dissolve the above compounds or to initiate the LF activity assay.

Lethal factor activity assay and analysis

LF activity was monitored via the spectral assay described by Tonello et al [27], which uses a pH 7.4 solution of sodium phosphate (25 mM) and sodium chloride (15 mM) as the buffer for all reactions. LF was present at a concentration of 1 μM and pNA was used at a concentration of 7.26 μM.

Each experiment had duplicate or triplicate samples of either the control or experimental treatments, and 3 controls were consistently used with each compound: pNA and potential inhibitor (no LF); LF with potential inhibitor (no pNA); and LF with pNA (to establish an uninhibited rate in each run). A separate control, consisting of boiled LF with pNA (which showed no activity) versus LF with pNA confirmed that the active enzyme causes the increase in absorption due to LF's cleavage of pNA and that the substrate, pNA, was stable over the time course of each experimental trial, i.e., 2 h. Experimental samples contained pNA, LF, and the potential inhibitor at various concentrations. To begin data collection, the enzyme was added to the cuvette containing buffer, inhibitor, and substrate, and spectral recordings began within 30 sec of LF addition.

All experimental trials were performed using a spectrophotometer (Cary 100; Varian Instruments,

Walnut Creek, CA, USA) and the data was collected using the kinetics software installed with the spectrophotometer. The data was converted into a comma-separated values file, which was then analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) to find an average slope of the curve of change in absorbance/min calculated from the entirety of the 120-min period. These data were then converted into M/min by using the extinction coefficient of pNA (9920/M-cm at 405 nm [EMD Biosciences]). *t*-Tests (1-tail) of the means of experimental trials versus their corresponding controls were performed and statistically different means we are reported at the $p \leq 0.05$ or $p \leq 0.01$ levels.

Results

The hydroxamate siderophores, aerobactin, deferrioxamine B, deferriferrichrome, pyoverdine, and rhodotorulic failed to inhibit the activity of LF at approximately 10 μM concentrations (Table 1). At the higher concentration of about 100 μM, slight inhibition was noted, with the greatest degree of inhibition (30.8%) observed with deferriferrichrome. Although there were insufficient quantities of the hydroxamate siderophore, schizokinen, to perform repeat experiments, it also had little ability to inhibit LF activity at the concentrations tested (12, 120, 238, and 357 μM), with the greatest inhibition (16.6%) occurring with 238 μM concentration.

In contrast to the hydroxamate siderophores, the catecholate siderophore, enterobactin, inhibited LF activity at a concentration of 11 μM (19.1%). Higher concentrations resulted in increasing LF inhibition until a plateau of approximately 50% inhibition occurred at enterobactin concentrations of 83, 105, and 208 μM (Table 1).

Small-molecule hydroxamates did not demonstrate significant LF inhibition (Table 2). While acetohydroxamic acid, L-arginine hydroxamate, and L-lysine hydroxamate did inhibit LF activity at the lower concentrations of 10 to 13 μM (Table 2), the inhibition was slight and higher concentrations of 101 to 266 μM did not demonstrate inhibition of more than 27%. In contrast, EDTA demonstrated significant LF inhibition at both 10 and 100 μM (Table 2). Salmon sperm protamine inhibited LF activity at every concentration examined, with approximately 60% inhibition being noted at a concentration of 10 μM and a peak of about 82% inhibition observed at 75 μM concentration (Fig. 1 and Table 2).

Table 1. Inhibition of lethal factor activity by siderophores.

Proposed inhibitor	Lethal factor inhibition (% ± standard deviation)
Siderophore	
Aerobactin	
11 μM	None
106 μM	None
Deferrioxamine B	
10 μM	None
100 μM	10.4 ± 34
Deferriferrichrome	
10 μM	None
102 μM	30.8 ± 9.4[a]
Enterobactin	
11 μM	19.1 ± 18.0
31 μM	23.5 ± 2.3[a]
52 μM	24.7 ± 16.0
83 μM	50.7 ± 60.0
105 μM	52.6 ± 52.0
208 μM	46.1 ± 94.0
Pyoverdine	
10 μM	None
101 μM	6.2 ± 8.9
Rhodotorulic acid	
12 μM	None
100 μM	14.8 ± 64.0

[a]$p \leq 0.05$.

Discussion

Insignificant LF inhibition by the hydroxamate siderophores examined suggests that the hydroxamate-containing artificial substrate, developed from the knowledge of the target binding by LF [26,27], inhibits LF more by the mimicking of the binding of the substrate to the enzyme's active site than by hydroxamate interaction with the enzyme's Zn atom. That the most effective LF inhibitor was salmon sperm protamine, which contains no hydroxamic acid moieties and was chosen due to its mimicry of the hydroxamate-containing artificial substrate, supports this conclusion. In addition, low molecular weight hydroxamates such as acetohydroxamic acid, L-arginine hydroxamate, and L-lysine hydroxamate were also poor LF inhibitors. The hydroxamate siderophores tested were poor LF inhibitors regardless of their relative molecular weights (Mr), which ranged from a low of Mr 344 for rhodotorulic acid [32] to approximately Mr 1151 for pyoverdine [33].

Interestingly, the best siderophore inhibitor of LF was enterobactin, a catecholate-type siderophore. In this regard, polyphenols from green tea were noted as

Table 2. Inhibition of lethal factor activity by small-molecule hydroxamates and a selected macromolecule.

Variable	Lethal factor inhibition (% ± standard deviation)
Small molecules	
Acetohydroxamic acid	
10 μM	14.2 ± 48.0
101 μM	26.6 ± 8.0[a]
Ethylenediaminetetraacetic acid	
10 μM	65.0 ± 40.0[a]
100 μM	62.9 ± 30.0[a]
L-arginine hydroxamate	
13 μM	6.5 ± 15.0
133 μM	4.8 ± 29.0
266 μM	5.0 ± 7.7
L-lysine hydroxamate	
13 μM	14.1 ± 30.0
126 μM	10.0 ± 9.9
Macromolecule	
Protamine	
10 μM	60.9 ± 9.4[a]
25 μM	68.4 ± 25.0[a]
50 μM	67.4 ± 67.0
75 μM	82.7 ± 10.0[b]
100 μM	77.3 ± 8.1[b]
200 μM	65.3 ± 42.0

[a] $p \leq 0.05$.
[b] $p \leq 0.01$.

highly effective LF inhibitors [34]. It will be of interest to determine whether LF inhibition by enterobactin and polyphenols results from binding to the active site of LF versus the ability of these compounds to chelate the Zn atom of the enzyme. A similar question arises concerning the inhibition noted due to EDTA, that is, whether its binding to the active site or its ability to chelate metal ions (e.g., Zn) is the mechanism of LF inhibition. Investigations employing 1,10-phenanthroline, 1,7-phenanthroline, 4,7-phenanthroline and adding up to 100 μM Zn^{2+}, in addition to the inhibitors of this study, could be used to determine whether metal chelation is the prime mechanism of inhibition. This is due to 1,10-phenanthroline's ability to chelate Zn ions, while its analogues cannot bind to the metal but can bind to the same site on an enzyme. In a similar manner, adding Zn^{2+} to LF inhibited by the compounds examined in this study has the potential to discern whether insufficient concentrations of Zn^{2+} (as would occur if the inhibitors were chelating or removing the metal), or if binding to the active site of LF was the means of inhibition [35].

The original hypothesis of this study, that hydroxamate siderophores, by virtue of metal chelation due to the hydroxamate ligand, would be good LF inhibitors was not substantiated by the LF inhibition data collected. The need for the medical and civil defense communities to be able to quickly and effectively ameliorate the effects of LF remains but, unlike the therapeutic effect that hydroxamate compounds display with enzymes such as matrix metallopeptidase [23,24] and ribonucleotide reductase [25], the low molecular weight hydroxamates and hydroxamate

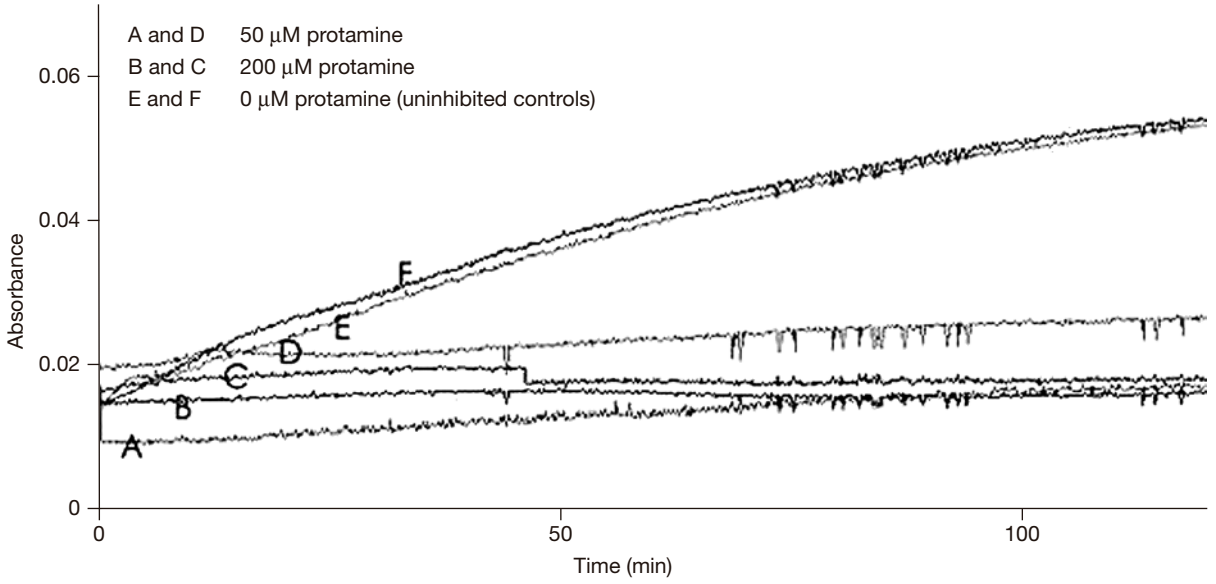

Fig. 1. Protamine inhibition of lethal factor activity observed as the increase of absorbance at 405 nm using the Ac-gly-tyr-βala-arg-arg-arg-arg-arg-arg-arg-arg-val-leu-arg-p-nitroanilide assay.

siderophores examined in this study do not hold promise as therapeutic agents as they failed to significantly inhibit LF.

In contrast, the non-hydroxamate siderophore, enterobactin, and the polyamine-substrate mimicking salmon sperm protamine were the most able inhibitors tested. It is of interest to note that protamine is already used therapeutically to counteract the effect of heparin [36]. That 2 independent inhibitors may engage in a synergistic enhancement, with respect to protection against anthrax lethal toxin, has been noted previously [37]. It will be of interest to determine whether combining the inhibitors, enterobactin and salmon sperm protamine, results in a synergistic increase in the inhibition of LF activity.

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