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Inhibition of the Proteolytic Activity of Anthrax Lethal Factor by Aminoglycosides

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The pathogenesis of anthrax is determined by three plasmid-encoded proteins of the Gram positive *Bacillus anthracis*: protective antigen (PA), edema factor (EF), and lethal factor (LF).[1] These proteins are individually nontoxic. Their toxic effects during anthrax infection require cooperation: self-assembly of PA into a heptamer mediates entry of EF and LF into mammalian cells (Figure 1A). Once inside the cell, EF causes edema via Ca^{2+}/calmodulin-dependent adenylate cyclase activity. LF, a Zn-dependent endopeptidase, specifically cleaves the N-terminus of the D-domain of mitogen-activated protein kinase kinases (MAPKK), thereby abrogating an essential signal transduction pathway of the host macrophage.[2−4] Strains of *B. anthracis* deficient in EF remain pathogenic, while those lacking LF become attenuated. LF is therefore considered the dominant virulence factor of anthrax,[5,6] and some synthetic aromatic and peptide-like cationic small-molecule inhibitors have been reported.[7]

We have begun a search for novel LF inhibitors using an in vitro fluorescent assay (Figure 1B).[8] A library of approximately 3000 compounds was screened in a 96-well assay format.[9] Neomycin B, a commonly utilized antibiotic of the aminoglycoside family, emerged as the most potent inhibitor of LF (Table 1, Figure 2). Other common aminglycosides,[10] as well as novel synthetic aminoglycosides, were tested in a secondary round of screening. Table 1 lists the K_i values of the aminoglycosides that show the highest inhibitory activities against LF. These aminoglycosides were observed to be competitive inhibitors of LF. Preliminary investigation of the binding mechanism of these aminoglycosides showed that their inhibitory activity is pH dependent and most potent in the physiological pH range. The binding affinity of the aminoglycosides is also dependent on the ionic strength of the assay environment: the higher the salt concentration (e.g., KCl), the lower the affinity.

Examination of the recently determined X-ray crystal structure of LF shows that the active site of the protease consists of a broad, deep, 40 Å groove with a highly negative electrostatic potential, containing clusters of Glu/Asp as well as Gln/Asn residues.[7,11] Acidic residues Asp328, Glu333, Glu370, Glu 372, Glu374, Asp387, Asp394, Asp647, Glu651, Glu662, Glu687, Asp706, Glu733, and Glu735 are located at or within the vicinity of this active site. Computational docking experiments with neomycin B and OP71 support the experimental results that the active site of LF has the propensity to bind to these molecules.[12] The lowest-energy docked conformations of the neomycin B/LF and OP71/LF complexes show that neomycin B and OP71 reside within the vicinity of the catalytic Zn(II) (Figures 3 and 4). Negatively charged residues (Asp328, Glu374, Asp394, Glu651, Glu662, Glu687, Asp706, Glu733, and Glu735) surround the aminoglycosides, with many making direct contacts via electrostatic and H-bonding interactions. Interestingly, the neomycin B/LF complex indicates that the hydroxyl group on C-4 of ring I is within coordination distance (2.0 Å) from the catalytic Zn(II).

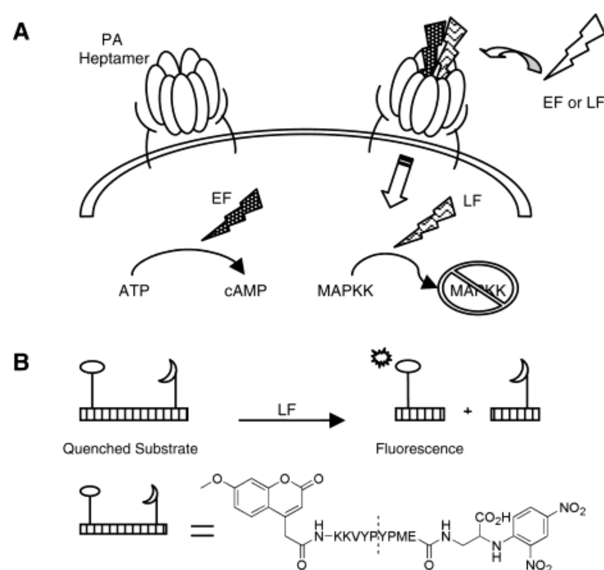

Figure 1. (A) PA assisted entry of EF and LF. (B) Fluorescent substrate for the screening of LF inhibitors.

Table 1. Inhibition Constants of Aminoglycosides for LF and Their Corresponding Dissociation Constants for RNA

aminoglycosides	lethal factor K_i (nM)[a]	16S rRNA A-Site K_d (nM)[14]
neomycin B	7.0 ± 2.1	200 ± 36
OP71	14.1 ± 4.4	41.6 ± 2.2
OP76	14.4 ± 4.5	141 ± 9
OP463	28.5 ± 8.8	166 ± 20

[a] 10 mM KHEPES pH7.4, 0.1 mg/mL BSA, 33 nM LF, fluorescent substrate, and inhibitors.

The inhibition of LF by aminoglycosides is an unexpected finding as they are more commonly known as RNA binders and not as enzyme inhibitors. LF appears to be the first example of a protease that is inhibited by aminoglycosides. Aminoglycoside antibiotics such as neomycin B exert their antibacterial activities by binding selectively to the major groove of the bacterial A-site decoding-region of 16S ribosomal RNA, causing deleterious misreading of the genetic code.[13] Table 1 also lists the corresponding affinities

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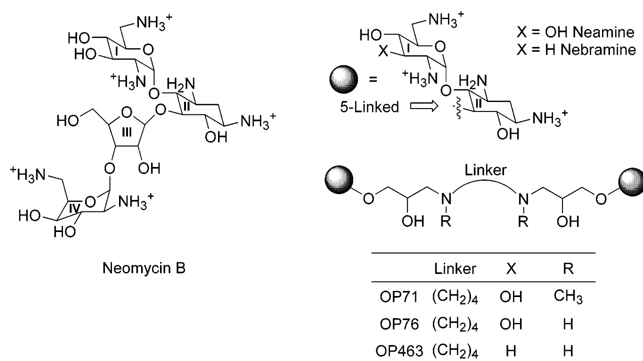

	Linker	X	R
OP71	$(CH_2)_4$	OH	CH_3
OP76	$(CH_2)_4$	OH	H
OP463	$(CH_2)_4$	H	H

Figure 2. Structures of neomycin B and synthetic aminoglycoside dimers.

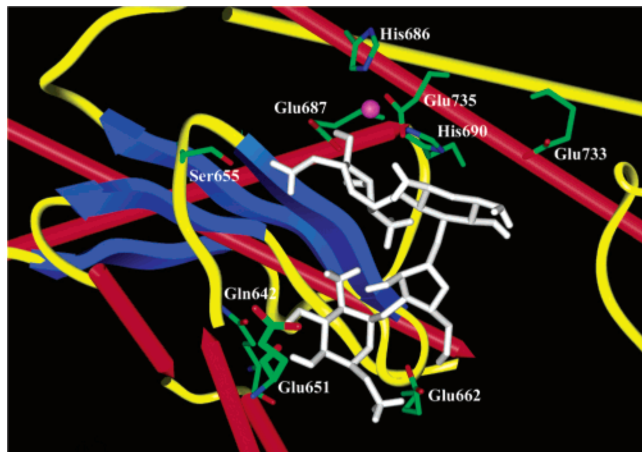

Figure 3. Lowest-energy docked conformation of neomycin B/LF complex. Neomycin B resides near the catalytic Zn(II) in the active site.

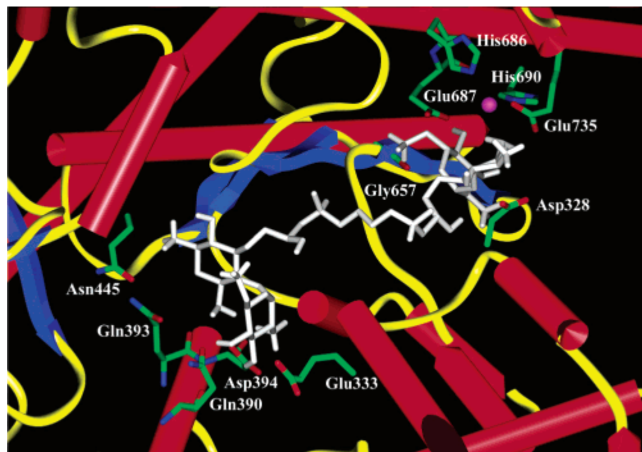

Figure 4. Lowest-enery docked conformation of OP71/LF complex. OP71 resides near the catalytic Zn(II) in the active site.

of neomycin B, as well as OP71, OP76, and OP463, for the bacterial 16S rRNA A-Site.[14] In addition, these aminoglycosides have also been shown to be effective antibiotics, with MIC (minimum inhibitor constant) values in the single digit micromolar range.[14]

The shape and directionality of the hydroxyl and ammonium groups on the aminoglycosides may contribute to the observed specificity of these molecules as good inhibitors of LF. Further study of these molecules against furin,[15] a serine protease with a negative electrostatic potential active site on the macrophage cell surface that activates PA to mediate LF entry into the cell, showed no inhibition activity.

In summary, this study showed that the antibiotic neomycin B also targets the LF of *B. anthracis*. The dual function of this drug may provide a new strategy for the protection against anthrax. The other synthetic aminoglycosides with better antibiotic activity could be further improved to increase their potency against LF. Work is in progress to evaluate the efficacy of neomycin B and synthetic aminoglycosides in a macrophage assay.

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Supporting Information Available: Measurements of distances between H-bonds and electrostatic interactions from ligand docking experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Assay condition: 20 mM HEPES pH 7.4, 0.1 mg/mL BSA, 3.3 nM LF, and 1 μM LF-10 quenched fluorescent substrate. Additional Zn(II) was not added as the enzyme has been pre-loaded with Zn from Calbiochem.
(10) Approximately 60 synthetic aminoglycosides of tobramycin derivatives and neamine/nebramin dimers, and commercial aminoglycosides (apramycin, butirosin, Geneticin, hygromycin, kanamycin, lividomycin, paromomycin, ribostamycin, sisomicin, spectinomycin, streptinomycin, and tobramycin) were tested during the secondary round of screening.
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