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Short communication

Proteolytic assay-based screening identifies a potent inhibitor of anthrax lethal factor

Hae-Chul Park [a], So-Ra Sung [a], Su Min Lim [a], Jong-Sun Lee [b], Sung-Kun Kim [b], Moon-Young Yoon [a,*]

[a] Department of Chemistry and Research Institute of Natural Sciences, Hanyang University, Seoul 133-761, Republic of Korea
[b] Department of Chemistry and Biochemistry, Baylor University, Waco, TX 76798, USA

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ABSTRACT

Anthrax lethal factor (LF), a Zn^{2+}-dependent metalloprotease, is a key virulence component of anthrax toxin. Here, we used proteolytic assay-based screening to identify novel LF inhibitors from a naturally extracted chemical library. The screening identified four compounds that inhibited *in vitro* proteolytic activity of LF with an IC_{50} of low micromolar range (11–20 μM). Three of these compounds were toxic to the mouse macrophage-like cell line, RAW 264.7. Compound 200 was non-toxic, however, and successfully protected Raw 264.7 cells from a lethal toxin challenge with an IC_{50} of 39.2 μM. We also identified possible binding modes of compound 200 by molecular docking.

1. Introduction

Bacillus anthracis, the etiological agent of anthrax, is a gram-positive bacterial pathogen of humans and animals [1]. *B. anthracis* poses a significant threat as an agent of biological warfare and terrorism, with a significant capacity to cause mortality. Virulence of this bacterium relies on an antiphagocytic capsular antigen, unique among bacterial capsules, that consists of poly-D-glutamic acid and the anthrax toxin. Anthrax toxin is made up of three proteins (protective antigen, PA; lethal factor, LF; and edema factor, EF), which combine to form edema and lethal toxins. PA is an 83 kDa protein that binds to PA cell-surface receptors and is activated by proteolytic removal of the N-terminal 20 kDa fragment, allowing the remaining receptor-bound portion (PA63) to oligomerize into a heptameric ring. The PA heptamer binds LF and EF and translocates them into the cytosol, where they produce major, often lethal, damage [2]. Current treatment, which includes administration of appropriate antibiotics against *B. anthracis,* has limitations since the high levels of secreted toxin may remain in circulation for several days and continue to damage the host even after the bacteria may have been killed. Comprehensive research on anthrax toxin has been carried out, given its major role in the pathogenesis of *B. anthracis*.

Among the toxin components, LF is the dominant virulence factor in anthrax pathogenesis, suggesting a key role for the toxin. LF is a Zn^{2+}-dependent metalloprotease, which specifically cleaves mitogen-activated protein kinase kinase (MAPKK) family members, leading to macrophage lysis of toxin-challenged cells [3,4]. Several classes of chemically distinct classes of LF inhibitors have been identified from a wide variety of approaches, including library screening and optimization [5,6], MS-based screening [7], scaffold-based NMR screening [8], and use of synthetic peptides as LF substrates [9]. These LF inhibitors act in low micromolar to nanomolar concentrations with various advantages. In particular, GM6001 was widely known as a metalloprotease inhibitor [14] and used for control inhibitor of assays [8].

Here we used proteolytic assay-based screening to identify potent LF inhibitors from a naturally extracted chemical library, and then evaluated the ability of potential compounds to protect Raw 264.7 macrophages from anthrax lethal toxin. Finally, we identified possible binding sites of one potent compound in LF by molecular docking.

2. Results and discussion

2.1. Proteolytic assay-based screening

In this study, we used a previously reported proteolytic assay-based method to identify LF inhibitors [10]. Briefly, full-length

* Corresponding author. Department of Chemistry, Hanyang University, 17-Haengdong-dong, Seoul 133-791, Republic of Korea. Tel.: +82 2 2220 0946; fax: +82 2 2299 0762.
E-mail address: myyoon@hanyang.ac.kr (M.-Y. Yoon).

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MEK1 (Mitogen-Activated Protein Kinase Kinase 1) with a GST moiety at the N-terminus (GST-MEK) was prepared as a substrate, and N-terminal cleavage of the peptide bond between Pro8 and Ile9 of MEK1 by LF was analyzed. The assay was carried out in a standard buffer containing 25 mM HEPES, pH 7.4, 1 mM DTT, 10% glycerol, 50 μM $CaCl_2$, 5 mM $MgCl_2$ and 380 nM GST-MEK1 and the LF activity was determined by the decrease of GST-MEK (~72 kDa) band intensity, which resulted in two bands corresponding to a GST moiety containing peptides 1–8 of MEK1 (28 kDa) and a remaining MEK1 (Ile9-end, 44 kDa) moiety. For inhibition tests, naturally extracted compounds were pre-incubated for 10 min with LF in the standard assay buffer, and the assay was initiated by the addition of GST-MEK followed by 30 min incubation at 37 °C. The reaction was terminated and the inhibition potency was analyzed based on the band intensity of cleaved products on SDS-PAGE.

To identify LF inhibitors, a naturally extracted chemical library (representing 480 compounds) was screened against LF proteolytic activity (Fig. 1A). The screening identified 52 initial hits that appeared to show inhibition of GST-MEK cleavage by LF. Of the 52 hits identified in the primary screening, the initial effect could not be reproduced in 17, and 31 showed <50% inhibition at a concentration of 500 μM. The remaining four compounds displayed >95% inhibition at 500 μM. The structures of the four compounds are shown in Fig. 1B.

2.2. Determination of IC_{50} values

Proteolytic activity of LF in the presence of varied concentrations of compounds 106, 109, 112, and 200 was measured to determine the *in vitro* IC_{50} value of each. All four compounds showed dose-dependent inhibition of LF proteolytic activity (Fig. 2). An SDS-PAGE image of compound 200 inhibition is shown in Fig. 2A (data not shown for the other three compounds). Band intensities were analyzed using ImageJ software [11]. The relative activities were

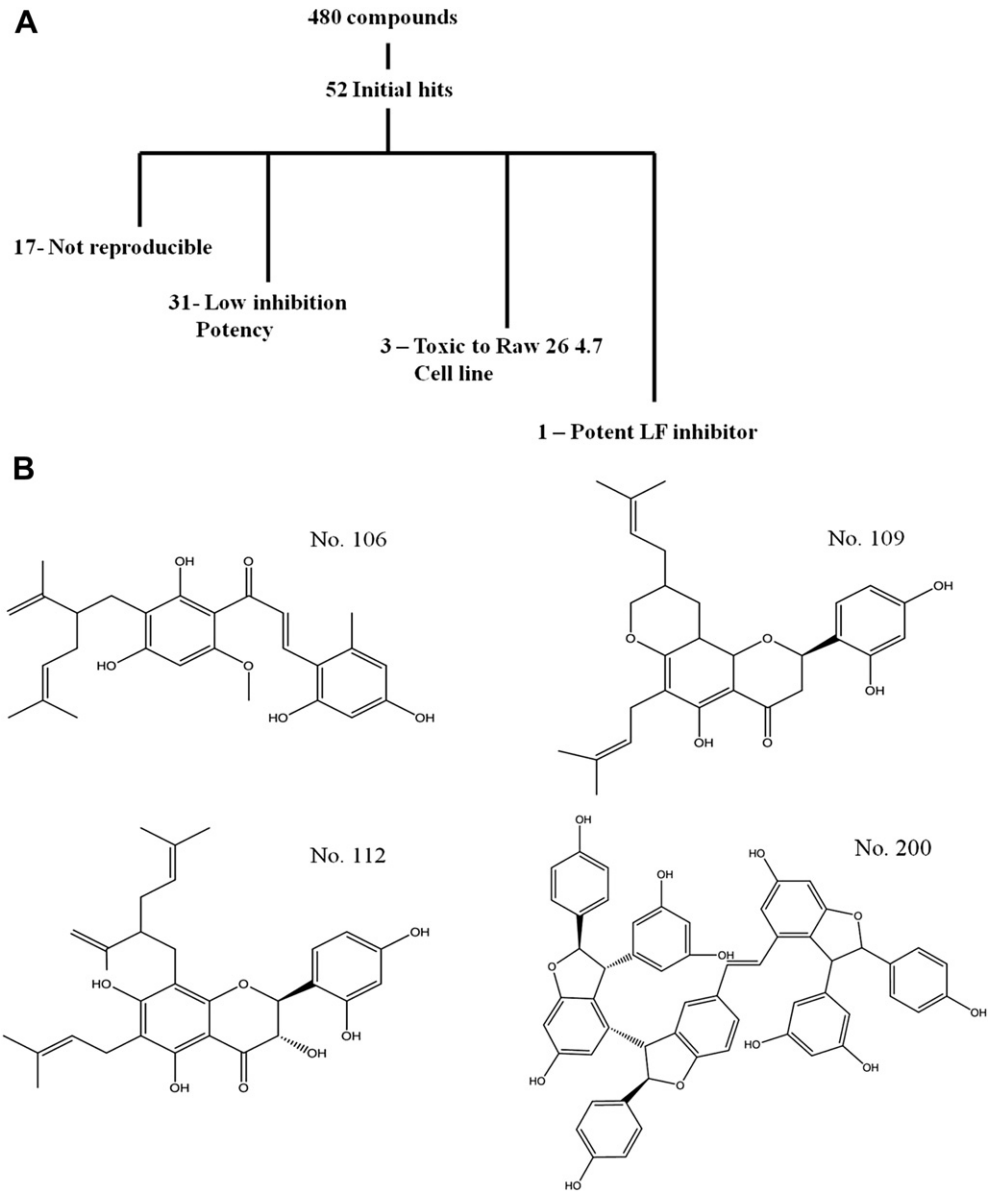

Fig. 1. Summary of proteolytic assay-based screening. (A) Schematic summary of 480 naturally extracted compounds as LF inhibitors. (B) Structure of identified hit compounds 106, 109, 112 and 200 (IUPAC chemical name of 200: 5-(4-(2-((2R,2′R,3R,3′R)-3′-(3,5-dihydroxyphenyl)-6′-hydroxy-2,2′-bis(4-hydroxyphenyl)-2,2′,3,3′-tetrahydro-3,4′ bibenzofuran-5-yl)ethyl)-6-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydrobenzofuran-3-yl)benzene-1,3-diol).