Published in final edited form as:

ACS Infect Dis. 2018 December 14; 4(12): 1746–1754. doi:10.1021/acsinfecdis.8b00231.

The role of a small molecule in the modulation of cell death signal transduction pathways

Stella Hartmann^{1,‡}, David J. Nusbaum^{2,‡}, Kevin Kim¹, Saleem Alameh¹, Chi-Lee C. Ho², Renae L. Cruz², Anastasia Levitin¹, Kenneth A. Bradley^{2,*}, and Mikhail Martchenko^{1,*}

¹School of Applied Life Sciences, Keck Graduate Institute, 535 Watson Drive, Claremont, CA 91711

²Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, 609 Charles E Young Drive E, Los Angeles, CA, 90095

Abstract

Inflammasomes activate caspase-1 in response to molecular signals from pathogens and other danger stimuli as a part of the innate immune response. A previous study discovered a small-molecule, 4-fluoro-N'-[1-(2-pyridinyl)ethylidene]benzohydrazide, which we named DN1, that reduces the cytotoxicity of anthrax lethal toxin (LT). We determined that DN1 protected cells irrespectively of LT concentration and reduced the pathogenicity of an additional bacterial exotoxin and several viruses. Using the LT cytotoxicity pathway, we show that DN1 does not prevent LT internalization and catalytic activity, or caspase-1 activation. Moreover, DN1 does not affect the proteolytic activity of host cathepsin B, which facilitates the cytoplasmic entry of toxins. PubChem Bioactivities lists two G protein-coupled receptors (GPCR), type-1 angiotensin II receptor and apelin receptor, as targets of DN1. The inhibition of phosphatidylinositol 3-kinase, phospholipase C, and protein kinase B, which are downstream of GPCR signaling, synergized with DN1 in protecting cells from LT. We hypothesize that DN1-mediated antagonism of GPCRs modulates signal transduction pathways to induce a cellular state that reduces LT-induced pyroptosis downstream of caspase-1 activation. DN1 also reduced the susceptibility of *Drosophila melanogaster* to toxin-associated bacterial infections. Future experiments will aim to further characterize how DN1 modulates signal transduction pathways to inhibit pyroptotic cell death in LT-sensitive macrophages. DN1 represents a novel chemical probe to investigate host cellular mechanisms that mediate cell death in response to pathogenic agents.

Graphical Abstract

*To whom correspondence should be addressed: Mikhail Martchenko mikhail_martchenko@kgi.edu, Kenneth A. Bradley kenneth.bradley@roche.com.

David J. Nusbaum: Keck School of Medicine of the University of Southern California

Kevin Kim: University of California at Santa Barbara

Kenneth A. Bradley: F. Hoffmann-La Roche

[‡]These authors contributed equally to this work

Author Contributions

K.A.B., M.M., and A.L. designed research; S.H., D.J.N., K.K., S.A., C-L.C.H., and R.L.C. performed research; all analyzed data; and S.H., D.J.N., K.A.B., A.L., and M.M. wrote the paper. S.H. and D.J.N. contributed equally.

Keywords

Anthrax toxin; broad-spectrum; host-oriented; pyroptosis; drug discovery; G protein-coupled receptor

Bacillus anthracis is the bacterium that causes anthrax. Vegetative bacilli secrete toxins and proliferate in the host, ultimately killing the organism, which emphasizes the need for new inhibitors of bacteria and toxins. *B. anthracis* contains a virulence plasmid, pX01, which encodes three toxin subunits that can form two distinct AB toxins: edema toxin (ET) and lethal toxin (LT)¹. Both toxins consist of two protein subunits: protective antigen (PA), a subunit that binds to host cell receptors (B), and a catalytic (A) subunit responsible for toxicity. ET is comprised of PA and edema factor (EF), and LT is comprised of PA and lethal factor (LF).

PA binds host cell-surface receptors: tumor endothelial marker 8 (TEM8) or capillary morphogenesis gene 2 (CMG2)^{2–3}. Following receptor binding, a host membrane protease called furin cleaves a 20-kD subunit from an 83-kD PA (PA₈₃) monomer, yielding 63-kD PA (PA₆₃). Oligomerization sites on PA₆₃ subunits allow the formation of a heptamer or octamer known as the PA pre-pore⁴. EF and LF bind to the pre-pore, which is internalized via clathrin-mediated endocytosis⁴. During acidification of the endosome, the PA pre-pore converts to the PA pore, distinguished by the formation of a β -barrel structure. PA pore establishes itself on the endosomal membrane and allows the acid-denatured catalytic subunits to translocate to the cytosol and renature⁵.

LF is a zinc-dependent protease with multiple targets in the host cell. LF proteolytically cleaves the N-terminus of mitogen-activated protein kinase kinases (MAPKKs) and Nlrp1b¹. Disruption of the MAPKK pathway hinders the immune response, which allows the bacteria to replicate within the host⁶. Nlrp1b is a cytosolic sensor protein that together with caspase-1 forms inflammasome complex⁷. Once stimulated, the inflammasome triggers caspase-1-mediated maturation of cytokines and a rapid pro-inflammatory cell death, called pyroptosis, which is characterized by cell lysis and release of cellular contents. In macrophages that possess an LT-sensitive allele of *Nlrp1b*, LF directly cleaves and activates the Nlrp1b inflammasome and induces caspase-1-mediated processing of the cytokines interleukin

(IL)-1 β and IL-18 and pyroptotic cell death⁸. Mice with an LT-sensitive allele of *Nlrp1b* are resistant to *B. anthracis*, suggesting that inflammasome-mediated pyroptosis in macrophages is an innate immune response to anthrax infection⁹.

The LT intoxication pathway and mechanisms of inflammasome-mediated pyroptosis are not fully characterized. Chemical genetic screens are an effective approach for studying the host cellular response to LT. One such screen identified various small molecules that protect RAW264.7 murine macrophages from LT-induced pyroptosis¹⁰. Here we describe the characterization of 4-fluoro-N'-[1-(2-pyridinyl)ethylidene]benzohydrazide (DN1) (Fig. 1a), identified in this screen as an inhibitor of LT-induced pyroptosis.

RESULTS AND DISCUSSION

DN1 Protects Macrophages from Pyroptosis Induced by LT.

DN1 was identified as an inhibitor of LT-induced pyroptosis in murine macrophages¹⁰. To characterize DN1's protective activity, we performed viability assays to test the dose-response of DN1 to varying concentrations of LT during a 4-hour intoxication in RAW264.7 macrophages. The data showed that the range of efficacious and cytotoxic concentrations of DN1 are overlapping. DN1 protected macrophages from LT-induced death at concentrations higher than 1.7 μ M; DN1 was cytotoxic in macrophages at concentrations above 1.7 μ M, as indicated by decreased viability of cells treated with only DN1 (Fig. 1b). This may suggest that DN1 targets pathways important to the cell. DN1 protected cells independently of LT concentration, suggesting that DN1 may affect a host target.

A time-course LT intoxication test showed that 3.1 μ M DN1 is protective when applied to cells either before or at the time of toxin exposure, but not after toxin addition (Fig. 1c). This demonstrates that its molecular target may be involved in LF entry into the cytosol, or in the process of pyroptosis.

To determine the mechanism of action of DN1, we tested whether DN1-mediated protection from LT requires translational changes. DN1 protected RAW264.7 macrophages pre-treated with the translation inhibitor cycloheximide from LT-induced pyroptosis (Fig. 1d), suggesting that the protective activity of DN1 is not due to synthesis of new proteins.

Evaluation of DN1 ability to inhibit various bacterial toxins.

In order to investigate whether the protection of DN1 is specific to anthrax toxin, the chemical was studied for its ability to reduce LFnDTA cytotoxicity. LFnDTA is a chimeric protein that consists of the N-terminal PA binding site of LF (LFn) and a catalytic portion of the diphtheria toxin A-chain of (DTA). LFnDTA has previously been utilized as an LF substitute, and it kills cells by a mechanism that is different from LF¹¹. While the cellular uptake of LF and LFnDTA both rely on PA, the cytotoxicity pathways of the two toxins are distinct: unlike LT, LFnDTA is known to induce apoptosis by inhibiting protein synthesis¹². We demonstrated that low μ M range of DN1 protects cells from LFnDTA-PA killing (Fig. 2a), suggesting that DN1 targets a step required in the cell death pathways induced by both LFnDTA + PA and LT.

Other pathogenic agents that induce apoptosis include cholera toxin (CT)¹³ and *Pseudomonas aeruginosa* exotoxin A (PE)¹⁴, which are delivered to the cytoplasm from the endoplasmic reticulum (ER)¹⁵. We demonstrated that DN1 does not reduce cellular lethality caused by these toxins (Fig. 2b). These data indicate that DN1 may inhibit a pathway that is shared by some but not all mechanisms of cell death. The cytotoxicity of DN1 in this experiment was lower compared to that observed in Figure 1. One of the reasons for such difference in the cytotoxicity between Figures 1b and 2b could be the differences in the cell viability assays: ATPLite and MTT were used in respective experiments. Alternatively, the drug could show different cytotoxicity levels because of the difference in the length of the assays: cells were treated with DN1 for 24 hours, rather than for 4 hours as in Figure 1b.

DN1 Does Not Inhibit PA Pore Formation.

Because DN1 blocked cell cytotoxicity of LT and LFnDTA-PA but not CT or PE, we next sought to determine whether DN1 inhibits PA dependent toxin entry. In acidified endosomes PA pre-pores undergo a conformational transition to PA pores⁴, which resist being dissociated by SDS and appear as an oligomer on SDS-PAGE⁴. We used immunoblot to monitor PA heptamerization in the presence and in the absence of DN1. Treatment of cells with 1.56, 6.25, and 12.5 μ M of DN1 did not inhibit PA pore formation in these macrophages (Fig. 3a). This result suggests that DN1 acts downstream of PA internalization and pore formation.

DN1 Does Not Inhibit the Intracellular Proteolytic Activity of Cathepsin B.

The translocation of LF from acidified host endosomes to the cytosol is performed by the cathepsin B (CTSB)^{16–17}. To further investigate how DN1 mediates the protection of host cells from LT cytotoxicity, we used CTSB-specific substrate to test whether DN1 reduces CTSB proteolytic activity in RAW264.7 cells via fluorescence resonance energy transfer (FRET). We observed that pretreatment of cells with 3.1 μ M DN1 did not affect CTSB enzymatic activity (Fig. 3b). We included 25 μ M amodiaquine, a known inhibitor of CTSB, as a control. We confirmed that amodiaquine, which was previously shown to inhibit LT in cells¹⁷, reduced 41% of CTSB activity. This result suggests that the mechanism of action of DN1 is downstream of toxin endocytosis.

DN1 Does Not Inhibit Catalytic Activity of LF.

In order to test whether DN1 targets cleavage of MAPKKs by LF, we determined the proteolysis of MAPKK2 by western blotting. While in LF-PA exposed RAW264.7 macrophages MAPKK-2 was cleaved, treatment of cells with DN1 at 6.25, 12.5, and 25.0 μ M did not prevent this effect (Fig. 3c). In this experiment DN1 was tested at concentrations higher than in previous experiments because the cells were exposed to the drug for only 1 hour prior to being lysed. This result suggests that the mechanism of action of DN1 is downstream of cytoplasmic entry and subsequent catalytic activity of LF.

DN1 Does Not Inhibit Caspase-1 Activation.

Because DN1 does not inhibit toxin entry or catalytic activity of LF in the cytosol, we tested whether DN1 blocks LT-mediated caspase-1 activation, an event downstream of delivery of

LF to the cytoplasm. Macrophages were challenged with LT in the presence of DN1 or EGA, a small molecule [4-bromobenzaldehyde N-(2,6-dimethylphenyl)semicarbazone] identified in a previous screen known to reduce the entry of LT into the cytosol¹⁰. The FLICA (Fluorescent Labeled Inhibitor of Caspases) reagent is a fluorescent-labeled peptide that binds to activated caspase-1. While LT activated caspase-1 and EGA prevented this activation, fluorescent signal detected in cells treated with both DN1 and LT was not significantly different than the signal from control cells treated with only LT (Fig. 3d). This result shows that DN1 does not prevent the LT-induced caspase-1 activation and suggests that the mechanism of action of DN1 is downstream of caspase-1 activity.

DN1 Synergizes with PLC and PI3K Inhibitors to Protect Macrophages from LT.

PubChem Bioactivities datamining revealed that DN1 acts as an antagonist of two GPCR receptors (Fig. 4a): type-1 angiotensin II receptor (AGTR1) and apelin receptor (APLNR). The efficacies (IC₅₀) of DN1 against AGTR1 and APLNR are 1.958 and 2.280 μ M, respectively.

To determine whether DN1 protects cells specifically through antagonistic binding of DN1 to GPCRs, we tested inhibitors of phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K), enzymes activated by signaling cascades induced by APLNR and AGTR1, for their capability to reduce LT-induced cellular killing. PLC inhibitor U-73122 and PI3K inhibitor LY-294002 each synergized with a sub-protective dose of DN1 (1 μ M) to protect macrophages from LT-induced pyroptosis (Fig. 4b). Individually, these inhibitors were insufficient for protection (Fig. 4c). This suggests a role for these enzymes and downstream effectors in mediating pyroptosis in response to LT. PLC and PI3K inhibitors in combination did not protect cells from LT (Fig. 4d), indicating that other host factors are involved. An inhibitor of AKT, also called protein kinase B, which is downstream of PI3K in the signaling pathway, did not protect cells from LT, but displayed a modest synergistic effect with 1 μ M DN1 (Fig. 4e).

These results suggest that PLC and PI3K activity play a role in Nlrp1b-mediated pyroptosis. PI3K activates AKT that promotes cell proliferation and apoptosis resistance¹⁸. An AKT inhibitor alone did not protect cells from LT but exhibited a modest synergistic effect with a sub-protective dose of DN1 (Fig. 4e), suggesting a possible role for effectors downstream of AKT in regulating pyroptosis. PLC converts phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃), which regulates the release of Ca²⁺ from the ER¹⁹. Because inhibition of PLC and PI3K—alone or in combination—failed to protect RAW264.7 cells from LT without DN1 treatment (Fig. 4d), it is likely that additional factors are involved in the pyroptotic response.

Evaluation of Anti-Bacterial Activity of DN1.

During anthrax, the host experiences both bacteremia and toxemia. We studied if DN1 possesses anti-bacterial property by utilizing *B. cereus* as a surrogate for *B. anthracis*. *B. cereus* was used in this study because it known to genetically resemble *B. anthracis*, although this bacterium lacks anthrax toxins²⁰. We applied 1 μ L of 10 mM DN1 on Gram-positive *B. cereus* and Gram-negative *Serratia liquefaciens* agar-media grown lawns. We

tested *S. liquefaciens* as a representative of Gram-negative bacteria as well as a surrogate for the more pathogenic *S. marcescens*²¹. We observed that this amount of DN1 decreased the growth of *B. cereus* but not *S. liquefaciens* (Fig. 5a). In order to determine the effective range of DN1, two-fold dilutions of this drug were applied on the lawn of *B. cereus*. An approved antibiotic for the treatment of *B. anthracis* infection, Levofloxacin, was used as a control (Fig. 5b). We observed that application of 1 μ L of 5 and 10 mM of DN1 reduced the growth of *B. cereus* (Fig. 5b). However, the anti-bacterial effect produced by DN1 was smaller than the one observed with Levofloxacin. In these experiments, the application of DN1 onto a solid bacterial media produces a concentration-gradient. To find the precise effective anti-bacterial concentrations of DN1, we evaluated it on bacteria grown in liquid media. One hundred μ M DN1 and two-fold dilutions were tested, and only 100 μ M, 50 μ M, and 25 μ M of DN1 were effective in reducing the grow-rate of *B. cereus* (Fig. 5c). At these concentrations, DN1 is cytotoxic to macrophages in other experiments (Fig. 1). These data suggest that DN1 does not have properties of an effective anti-bacterial agent.

Evaluation of Anti-Bacterial Activity of DN1 in a Fly Model of Infection.

In order to test whether DN1 elicits an anti-bacterial effect *in vivo*, we tested whether this drug affects the ability of *Drosophila melanogaster* to withstand bacterial challenges. Fruit flies were chosen for our study because they are an accepted model-system to study innate immunity. We performed all of the following experiments with 5–6-day old (post-eclosion) female flies in order to reduce any potential variability in the sensitivity of flies to bacterial infection due to age and gender differences. In our experiments flies were pricked according to the needle-pricking method²². Both, *B. cereus* and *S. liquefaciens* are known to secrete cytotoxic pore-forming toxins^{23–25}. We infected flies with *B. cereus* and *S. liquefaciens*, since these bacteria secrete pore-forming toxins, which were shown to enter into cells by endocytosis and are cytotoxic and insecticidal^{24, 26}. Initially, we pricked flies with *B. cereus* as previously described²⁷ and confirmed that flies are sensitive to *B. cereus* challenge. The addition of rifampicin, a known antibiotic active against *B. cereus*²⁸, to the fly media effectively protected flies from bacterial infection at 120 μ M (Fig. 6a). The exposure of uninfected flies to rifampicin did not kill any flies (Fig. 6a). None of the flies pricked with a PBS-dipped needle and a non-pricked group of flies (CO₂ only) died in this experiment (Fig. 6a). This *in vivo* infection model presented the opportunity to test the efficacy of DN1 against bacterial infections.

In addition to being sensitive to *B. cereus*, *Drosophila* has previously been reported to be sensitive to *S. marcescens*²⁹. We infected 5–6 days old female flies with either *B. cereus* (Fig. 6b) or with *S. liquefaciens* (Fig. 6c), and we treated them with DN1 by adding this drug to fly-media at different concentrations during the bacterial infection experiment. While none of the conditions achieved a minimally acceptable statistical significance, we observed that 40 μ M of DN1 in the media decreased the sensitivity of *Drosophila* to *B. cereus* and *S. liquefaciens* challenges ($P=0.07$ for both bacteria), whereas 120 μ M of DN1 partially protected against *B. cereus* only (Fig. 6b–c). DN1 at 120 μ M did not affect the survival rates of *S. liquefaciens* infected flies (Fig. 6c). Although no statistical significance was achieved, these results collectively suggest the trend that DN1 has a potential in decreasing the sensitivity of flies to bacterial infections.

In light of the fact that DN1 did not kill *S. liquefaciens* directly (Fig. 5), and that the anti-*B. cereus* effect of DN1 is only seen at very high and cytotoxic doses, this data suggests that the anti-bacterial efficacy of DN1 may also be host-oriented.

Evaluation of Anti-Viral Property of DN1.

Since DN1 acts as an inhibitor of certain toxins, we investigated whether its efficacy extends to viruses. Other viruses are also known to induce pyroptosis in host cells. These viruses include herpes simplex virus 1³⁰, human cytomegalovirus³¹, and Rift Valley fever virus³². We showed that DN1 decreased the pathogenicity of all three viruses (Table 1) with an EC50 of 0.48–2.9 μM. These efficacious concentrations of DN1 are comparable to doses effective against anthrax toxins (Fig. 1b), and these data support the conclusion that DN1 reduces cell death pathway caused by certain pathogenic agents by inhibiting a host target.

DN1 inhibited herpes simplex virus 1 and rift valley fever virus that enter into the cytoplasm through ER^{33–34}. Additionally, the cellular entry of these two viruses are clathrin-independent^{35–36}. Moreover, DN1 inhibited human cytomegalovirus, which enters into the cytoplasm by pH-independent fashion³⁷. Taken together, these observations argue that DN1 acts as an anti-viral compound by inhibiting cell-death pathways, rather than cell-entry pathways.

Future experiments will aim to further characterize how DN1 modulates signal transduction pathways to inhibit pyroptotic cell death in LT-sensitive macrophages. Ultimately, the use of DN1 as a probe may lead to the discovery of a novel role for one or more host factors in the regulation of Nlrp1b-induced pyroptosis in response to LT. Moreover, DN1 may be used as probe to discover new proteins involved in bacterial pathogenesis.

METHODS

Compound Preparation

4-fluoro-N'-[1-(2-pyridinyl)ethylidene]benzohydrazide (DN1) (Chembridge (Compound ID: 5325699)), U-73122 (a PLC inhibitor; Cayman Chemical), LY-294002 (a PI3K inhibitor; Sigma-Aldrich), and AKT Inhibitor VIII (Cayman Chemical) stocks were solubilized in DMSO.

Toxins Tests

RAW264.7 macrophages were plated in 384-well plate at 2×10^3 cells in a volume of 40 μL per well in DMEM with 25 mM HEPES. Cells were incubated with drugs for periods of time indicated in figure legends at 37°C and 5% CO₂. Cells were intoxicated with LT or LFnDTA-PA at 37°C for various times and at various concentrations as listed in figure legends. Ten μL of ATPLite reagent (PerkinElmer) was added to wells and the Wallac Victor 3V plate reader (Perkin Elmer) was used to record emitted photon intensity in relative luminescence units.

In other experiments, 2×10^4 of RAW264.7 macrophages per well were seeded in 96-well plate one day prior to the experiment in 100 μL of media. Prior to the addition of toxins, cells were treated for 1 hour with various concentrations of DN1. Cholera toxin (CT) or *P.*

aeruginosa exotoxin A (PE) (List Biological Laboratories) were also added to cells for 24 hours. Cells were pre-treated with identical DN1 concentrations for 1 hour. The resulting PE and CT concentrations were 2.0 and 4.0 µg/ml, respectively. Cell survival was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, also known as MTT assay¹⁷. RAW264.7 % survival is described as the % of live cells compared to cells exposed to DMSO alone (100%).

Western Blot Assays

Two million of RAW264.7 cells were seeded in 2 mL per well onto a 6-well plate. On the next day, various concentrations of DN1 were added, and the macrophages were incubated for 1 hour. Cells were treated with either PA or LT for the time listed in the figure legends. Macrophages were then lysed using lysis buffer (NP-40) that contained 1× HALT protease/phosphatase inhibitor. Cell lysates were analyzed for protein concentration via the Bradford assay. Proteins were analyzed using SDS-PAGE on a 7.5% polyacrylamide gel, followed by a transfer to a PVDF membrane. Membranes were probed with anti-MAPKK-2 (Santa Cruz Biotechnologies), rabbit anti-PA (Covance), or anti-β-tubulin (Sigma-Aldrich) antibody and developed using goat anti-rabbit secondary antibody, which is conjugated with horseradish peroxidase (Invitrogen).

FLICA Assay

Macrophages were plated on a 384-well plate at 2×10^4 cells/well in 20 µL complete media and incubated overnight. The next day, cells received 10 µL DN1, EGA, or media for 1 hour before intoxication with 500 ng/mL LT for 1.5 hours. FLICA reagent (Immunochemistry) was added to cells for 1 hour, followed by the addition of Hoechst 33342 that stained nuclei. Cells were analyzed using an ImageXpressmicro automated high content image screening system. Pictures were analyzed using MetaXpress software (Molecular Devices).

Cathepsin B Enzymatic Activity Assays

CTSB activity was evaluated using an InnoZyme™ CTSB activity assay kit (EMD Millipore). CTSB proteolytic activity was measured by treating macrophages for 1 hour with 3.1 µM of DN1. RAW264.7 cells were then lysed and the CTSB proteolytic activity was tested with a labeled substrate. A cleaved substrate generates fluorescence that was measured at an excitation and emission wavelengths of 370 nm 450 nm, respectively (Molecular Devices, Spectra Max 384 PLUS). The rate of substrate cleavage in the presence of DN1 was analyzed by the Microsoft Excel LINEST analysis and compared to the CTSB rate in the absence of the drug.

Bacterial tests

B. cereus (ATCC 14579) was propagated in LB media at 37°C, and *S. liquefaciens* (ATCC 27592) was grown in tryptic soy broth at 30°C. In agar media assays, the optical density at 600 nm (OD₆₀₀) of bacterial overnights was measured. The OD₆₀₀ values were converted to cells/mL according to McFarland's scale³⁸. 600×10^6 bacterial cells were added to solid media on 25 cm petri dishes. DN1 was applied on the plate surface as 1 µL of 10 mM stock.

In experiments using liquid assays, the bacterial overnight culture was resuspended in new liquid bacterial medium to OD₆₀₀ of 0.1 and plated in 100 µL into wells of a 96-well plate. Various concentrations of DN1 were then added to wells. The bacteria were incubated in plates at 37°C with constant shaking. The OD₆₀₀ was determined every 610 seconds for 12 hours by a microplate reader.

***D. melanogaster* needle-prick microbial infection**

Wild type *D. melanogaster* Oregon-R-C strain was received from Bloomington Drosophila Stock Center (stock #5). Flies were propagated on a standard Bloomington fly culture medium (cornmeal and molasses) at 23°C and 80% humidity. Drugs were added to fly media by placing 60 µL of either 3.3 mM or 10 mM of drug stocks to 5 mL of fly media, which resulted in 40 µM and 120 µM final drug concentrations, respectively.

The bacterial cells from overnight cultures (5 ml) were centrifuged, and the supernatants were discarded and resuspended in PBS (100 µl). The stainless-steel surgical pins (0.012 mm tip width) (Roboz Surgical Instruments) used for pricking were sterilized by autoclaving. Flies were anesthetized with CO₂ delivered via a Flystuff Flypad and infected by pricking in the thoracic cavity with the prepared surgical needle dipped in the microbial suspension. As a pricking control, female flies were pricked with needles dipped in PBS. To ensure that CO₂ exposure has no effects on survivability of flies, a control was devised where flies were not pricked, but exposed to CO₂ for the duration of the entire experiment. During drug-testing experiments, pricked female flies were returned to fly vials with food that did or did not contain different concentrations of DN1 or Rifampicin and were incubated at 25°C. Flies were infected in groups of ten flies. Following infection, the expired flies were recorded in intervals of 30 minutes for 16 hours. Each experiment was reproduced at least three times.

Viral tests

Viral cytopathic effect (CPE) inhibition was used to perform the viral tests. Confluent or near-confluent monolayers of cultured cells were prepared in 96-well plates. Human foreskin fibroblasts (HFF) and African green monkey kidney epithelial cells (Vero 76) were cultured in DMEM with 10% FBS and 1% PSG. For antiviral assays DMEM was supplemented with 50 µg/mL gentamicin 2% FBS. DN1 was tested at 0.1, 1.0, 10, and 100 µM. Each concentration of DN1 was tested in 3 wells with infected cells and 2 wells with uninfected cells. Each plate included 6 wells with untreated cells and 6 wells with infected cells. The growth media was removed, and DN1 was applied in 100 µL to wells at twice the concentration. An additional 100 µL of virus solution at <100 50% tissue culture infectious doses (TCID₅₀) was applied to virus infection wells. Medium alone was added to DN1 control and cell growth control wells. Other wells received virus as a control, but in the absence of DN1. Cells were grown until virus control wells reached maximum CPE. Cells were stained with 0.011% neutral red for 2 hours. The staining solution was removed, and the cells were rinsed with PBS. The dye is delivered into live cells, and the dye content was measured at 540 nm. Cell survival was described as the percentage of live cells compared to untreated cells treated.

ACKNOWLEDGEMENTS

We thank Kseniya Polukhina for technical assistance with FRET experiments. A.L. acknowledges support from The Kenneth T. and Eileen L. Norris Foundation. M.M. acknowledges support from City of Hope Comprehensive Cancer Center through the KL2 Mentored Career Development Award Program of the Inland California Translational Consortium (GR720001).

Abbreviations Used

PA	protective antigen
LF	lethal factor
LT	anthrax lethal toxin comprising PA and LF
PA83	83 kDa PA subunit
PA63	63 kDa PA subunit
LFnDTA	diphtheria toxin fused with the PA binding domain of LF
DTA	A-chain of diphtheria toxin
CT	cholera toxin
PE	<i>Pseudomonas aeruginosa</i> exotoxin A
LF_N	N-terminus region of LF comprising the PA binding domain of LF
CTSB	cathepsin B
MAPKK	mitogen-activated protein kinase kinases
FRET	Fluorescence Resonance Energy Transfer

REFERENCES

1. Mock M; Fouet A, Anthrax. Annu Rev Microbiol 2001, 55, 647–71. DOI: 10.1146/annurev.micro.55.1.647. [PubMed: 11544370]
2. Scobie HM; Rainey GJ; Bradley KA; Young JA, Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc Natl Acad Sci U S A 2003, 100 (9), 5170–4. DOI: 10.1073/pnas.0431098100. [PubMed: 12700348]
3. Bradley KA; Mogridge J; Mourez M; Collier RJ; Young JA, Identification of the cellular receptor for anthrax toxin. Nature 2001, 414 (6860), 225–9. DOI: 10.1038/n35101999. [PubMed: 11700562]
4. Miller CJ; Elliott JL; Collier RJ, Anthrax protective antigen: prepore-to-pore conversion. Biochemistry 1999, 38 (32), 10432–41. DOI: 10.1021/bi990792d. [PubMed: 10441138]
5. Naik S; Brock S; Akkaladevi N; Tally J; McGinn-Straub W; Zhang N; Gao P; Gogol EP; Pentelute BL; Collier RJ; Fisher MT, Monitoring the kinetics of the pH-driven transition of the anthrax toxin prepore to the pore by biolayer interferometry and surface plasmon resonance. Biochemistry 2013, 52 (37), 6335–47. DOI: 10.1021/bi400705n. [PubMed: 23964683]
6. Erwin JL; DaSilva LM; Bavari S; Little SF; Friedlander AM; Chanh TC, Macrophage-derived cell lines do not express proinflammatory cytokines after exposure to Bacillus anthracis lethal toxin. Infect Immun 2001, 69 (2), 1175–7. DOI: 10.1128/IAI.69.2.1175-1177.2001. [PubMed: 11160016]
7. Moayeri M; Sastalla I; Leppla SH, Anthrax and the inflammasome. Microbes Infect 2012, 14 (5), 392–400. DOI: 10.1016/j.micinf.2011.12.005. [PubMed: 22207185]

8. Hellmich KA; Levinsohn JL; Fattah R; Newman ZL; Maier N; Sastalla I; Liu S; Leppla SH; Moayeri M, Anthrax lethal factor cleaves mouse nlrp1b in both toxin-sensitive and toxin-resistant macrophages. *PLoS One* 2012, 7 (11), e49741 DOI: 10.1371/journal.pone.0049741. [PubMed: 23152930]
9. Terra JK; Cote CK; France B; Jenkins AL; Bozue JA; Welkos SL; LeVine SM; Bradley KA, Cutting edge: resistance to *Bacillus anthracis* infection mediated by a lethal toxin sensitive allele of Nalp1b/Nlrp1b. *J Immunol* 2010, 184 (1), 17–20. DOI: 10.4049/jimmunol.0903114. [PubMed: 19949100]
10. Gillespie EJ; Ho CL; Balaji K; Clemens DL; Deng G; Wang YE; Elsaesser HJ; Tamilselvam B; Gargi A; Dixon SD; France B; Chamberlain BT; Blanke SR; Cheng G; de la Torre JC; Brooks DG; Jung ME; Colicelli J; Damoiseaux R; Bradley KA, Selective inhibitor of endosomal trafficking pathways exploited by multiple toxins and viruses. *Proc Natl Acad Sci U S A* 2013, 110 (50), E4904–12. DOI: 10.1073/pnas.1302334110. [PubMed: 24191014]
11. Blanke SR; Milne JC; Benson EL; Collier RJ, Fused polycationic peptide mediates delivery of diphtheria toxin A chain to the cytosol in the presence of anthrax protective antigen. *Proceedings of the National Academy of Sciences* 1996, 93 (16), 8437–8442.
12. Milne JC; Blanke SR; Hanna PC; Collier RJ, Protective antigen-binding domain of anthrax lethal factor mediates translocation of a heterologous protein fused to its amino- or carboxy-terminus. *Mol Microbiol* 1995, 15 (4), 661–6. [PubMed: 7783638]
13. Allam M; Bertrand R; Zhang-Sun G; Pappas J; Viallet J, Cholera toxin triggers apoptosis in human lung cancer cell lines. *Cancer Res* 1997, 57 (13), 2615–8. [PubMed: 9205066]
14. Morimoto H; Bonavida B, Diphtheria toxin- and *Pseudomonas* A toxin-mediated apoptosis. ADP ribosylation of elongation factor-2 is required for DNA fragmentation and cell lysis and synergy with tumor necrosis factor- α . *J Immunol* 1992, 149 (6), 2089–94. [PubMed: 1517572]
15. Johannes L; Decaudin D, Protein toxins: intracellular trafficking for targeted therapy. *Gene Ther* 2005, 12 (18), 1360–8. DOI: 10.1038/sj.gt.3302557. [PubMed: 15902276]
16. Ha SD; Ham B; Mogridge J; Saftig P; Lin S; Kim SO, Cathepsin B-mediated autophagy flux facilitates the anthrax toxin receptor 2-mediated delivery of anthrax lethal factor into the cytoplasm. *J Biol Chem* 2010, 285 (3), 2120–9. DOI: 10.1074/jbc.M109.065813. [PubMed: 19858192]
17. Zilbermintz L; Leonardi W; Jeong SY; Sjodt M; McComb R; Ho CL; Retterer C; Gharaibeh D; Zamani R; Soloveva V; Bavari S; Levitin A; West J; Bradley KA; Clubb RT; Cohen SN; Gupta V; Martchenko M, Identification of agents effective against multiple toxins and viruses by host-oriented cell targeting. *Sci Rep* 2015, 5, 13476 DOI: 10.1038/srep13476. [PubMed: 26310922]
18. Shanware NP; Bray K; Abraham RT, The PI3K, metabolic, and autophagy networks: interactive partners in cellular health and disease. *Annu Rev Pharmacol Toxicol* 2013, 53, 89–106. DOI: 10.1146/annurev-pharmtox-010611-134717. [PubMed: 23294306]
19. Kadamur G; Ross EM, Mammalian phospholipase C. *Annu Rev Physiol* 2013, 75, 127–54. DOI: 10.1146/annurev-physiol-030212-183750. [PubMed: 23140367]
20. Hartmann S; Lopez Cruz R; Alameh S; Ho CC; Rabideau A; Pentelute BL; Bradley KA; Martchenko M, Characterization of Novel Piperidine-Based Inhibitor of Cathepsin B-Dependent Bacterial Toxins and Viruses. *ACS Infect Dis* 2018, 4 (8), 1235–1245. DOI: 10.1021/acsinfecdis.8b00053. [PubMed: 29749721]
21. Traub WH, Antibiotic susceptibility of *Serratia marcescens* and *Serratia liquefaciens*. *Chemotherapy* 2000, 46 (5), 315–21. DOI: 10.1159/000007304. [PubMed: 10965096]
22. Apidianakis Y; Rahme LG, *Drosophila melanogaster* as a model host for studying *Pseudomonas aeruginosa* infection. *Nat Protoc* 2009, 4 (9), 1285–94. DOI: 10.1038/nprot.2009.124. [PubMed: 19680242]
23. Remuzgo-Martinez S; Aranzamendi-Zaldunbide M; Pilares-Ortega L; Icardo JM; Acosta F; Martinez-Martinez L; Ramos-Vivas J, Interaction of macrophages with a cytotoxic *Serratia liquefaciens* human isolate. *Microbes Infect* 2013, 15 (6–7), 480–90. DOI: 10.1016/j.micinf.2013.03.004. [PubMed: 23524146]
24. Chakroun M; Banyuls N; Bel Y; Escrache B; Ferre J, Bacterial Vegetative Insecticidal Proteins (Vip) from Entomopathogenic Bacteria. *Microbiol Mol Biol Rev* 2016, 80 (2), 329–50. DOI: 10.1128/MMBR.00060-15. [PubMed: 26935135]

25. Hertle R, Serratia type pore forming toxins. *Curr Protein Pept Sci* 2000, 1 (1), 75–89. [PubMed: 12369921]
26. Dodd SJ; Hurst MR; Glare TR; O’Callaghan M; Ronson CW, Occurrence of sep insecticidal toxin complex genes in *Serratia* spp. and *Yersinia frederiksenii*. *Appl Environ Microbiol* 2006, 72 (10), 6584–92. DOI: 10.1128/AEM.00954-06. [PubMed: 17021209]
27. Ma J; Benson AK; Kachman SD; Hu Z; Harshman LG, *Drosophila melanogaster* Selection for Survival of *Bacillus cereus* Infection: Life History Trait Indirect Responses. *Int J Evol Biol* 2012, 2012, 935970 DOI: 10.1155/2012/935970. [PubMed: 23094195]
28. Vogler AJ; Busch JD; Percy-Fine S; Tipton-Hunton C; Smith KL; Keim P, Molecular analysis of rifampin resistance in *Bacillus anthracis* and *Bacillus cereus*. *Antimicrob Agents Chemother* 2002, 46 (2), 511–3. [PubMed: 11796364]
29. Nehme NT; Liegeois S; Kele B; Giammarinaro P; Pradel E; Hoffmann JA; Ewbank JJ; Ferrandon D, A model of bacterial intestinal infections in *Drosophila melanogaster*. *PLoS Pathog* 2007, 3 (11), e173 DOI: 10.1371/journal.ppat.0030173. [PubMed: 18039029]
30. Takasu A; Masui A; Hamada M; Imai T; Iwai S; Yura Y, Immunogenic cell death by oncolytic herpes simplex virus type 1 in squamous cell carcinoma cells. *Cancer Gene Ther* 2016, 23 (4), 107–13. DOI: 10.1038/cgt.2016.8. [PubMed: 26987291]
31. Huang Y; Liu L; Ma D; Liao Y; Lu Y; Huang H; Qin W; Liu X; Fang F, Human cytomegalovirus triggers the assembly of AIM2 inflammasome in THP-1-derived macrophages. *J Med Virol* 2017, 89 (12), 2188–2195. DOI: 10.1002/jmv.24846. [PubMed: 28480966]
32. Ermler ME; Traylor Z; Patel K; Schattgen SA; Vanaja SK; Fitzgerald KA; Hise AG, Rift Valley fever virus infection induces activation of the NLRP3 inflammasome. *Virology* 2014, 449, 174–80. DOI: 10.1016/j.virol.2013.11.015. [PubMed: 24418550]
33. Spiegel M; Plegge T; Pohlmann S, The Role of Phlebovirus Glycoproteins in Viral Entry, Assembly and Release. *Viruses* 2016, 8(7). DOI: 10.3390/v8070202.
34. Turcotte S; Letellier J; Lippe R, Herpes simplex virus type 1 capsids transit by the trans-Golgi network, where viral glycoproteins accumulate independently of capsid egress. *J Virol* 2005, 79 (14), 8847–60. DOI: 10.1128/JVI.79.14.8847-8860.2005. [PubMed: 15994778]
35. Devadas D; Koithan T; Diestel R; Prank U; Sodeik B; Dohner K, Herpes simplex virus internalization into epithelial cells requires Na⁺/H⁺ exchangers and p21-activated kinases but neither clathrin- nor caveolin-mediated endocytosis. *J Virol* 2014, 88 (22), 13378–95. DOI: 10.1128/JVI.03631-13. [PubMed: 25210183]
36. Harmon B; Schudel BR; Maar D; Kozina C; Ikegami T; Tseng CT; Negrete OA, Rift Valley fever virus strain MP-12 enters mammalian host cells via caveola-mediated endocytosis. *J Virol* 2012, 86 (23), 12954–70. DOI: 10.1128/JVI.02242-12. [PubMed: 22993156]
37. Haspot F; Lavault A; Sinzger C; Laib Sampaio K; Stierhof YD; Pilet P; Bressolette-Bodin C; Halary F, Human cytomegalovirus entry into dendritic cells occurs via a macropinocytosis-like pathway in a pH-independent and cholesterol-dependent manner. *PLoS One* 2012, 7 (4), e34795 DOI: 10.1371/journal.pone.0034795. [PubMed: 22496863]
38. Mc FJ, The nephelometer: aN instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *Journal of the American Medical Association* 1907, XLIX (14), 1176–1178. DOI: 10.1001/jama.1907.25320140022001f.

Figure 1: Evaluation of the efficacy of DN1 against anthrax toxins.

(a) The structure of DN1. DN1 is 4-fluoro-N'-[1-(2-pyridinyl)ethylidene] (Chembridge ID# 5325699, PubChem CID 5720743).

(b) DN1 protects RAW264.7 cells from LT-induced cell death independent of LT concentration. RAW264.7 cells were incubated with various concentrations of DN1 ranging from 0.1 to 12.5 μ M for 1 hour, then intoxicated with 250, 500 or 1,000 ng/mL LT for 4 hours. Cell viability was measured using the ATPLite viability assay (b-d). Mean Relative Luminescence Units from each of three independent experiments were normalized to cells only control values to determine percent cell viability.

(c) The determination of effective timing of DN1. RAW264.7 cells were treated with 3.1 μ M DN1 prior to, at the time of, or after the intoxication with 500 ng/mL LF for 4 hours. DN1 was added at times ranging from 1 hour prior to 2 hours post-intoxication in 30-minute intervals. Cell viability is defined as the percentage of surviving cells relative to untreated cells. Data were analyzed using one-way ANOVA, followed by the Dunnett's post-test using luminescence data from cells treated with a DN1 control. Data represent percent survival

with standard deviation obtained in triplicate assays done in a representative experiment.

**** indicates $p < 0.0001$.

(d) DN1 protects cells from LT in the presence of the translation inhibitor cycloheximide.

Cells were treated with or without 100 $\mu\text{g/mL}$ cycloheximide and DN1 for two hours prior to intoxication with 500 ng/mL LT for 4 hours. Data represent mean values with standard deviation obtained in triplicate assays done in a representative experiment.

Figure 2: The effect of DN1 on various bacterial toxins.

(a) DN1 protects RAW264.7 cells from LFnDTA, a diphtheria toxin chimera. RAW264.7 cells were incubated with various concentrations of DN1 for 1 hour then incubated with 1 ng/mL LFnDTA + 500 ng/mL PA in a 24-hour intoxication assay. Cell viability was measured using the ATPLite viability assay. Mean Relative Luminescence Units from each of three independent experiments were normalized to cells only control values to determine percent cell viability.

(b) The effect of DN1 on the sensitivity of cells treated with bacterial toxins. RAW264.7 cells were pretreated with indicated DN1 concentrations for 1 hour, and then treated with 2 μ g/mL of cholera toxin (CT) or 4 μ g/mL of *Pseudomonas aeruginosa* Exotoxin A (PE) for 24 hours. RAW264.7 cell survival was measured by the MTT assay. Each data point shown for cell survival assays indicates the mean $\pm$ SD value obtained in triplicate assays done in a representative experiment. At least three such experiments were routinely carried out.

Figure 3: DN1 does not inhibit cellular entry and activity of LF.

(a) DN1 does not inhibit PA pore formation. RAW264.7 cells received 1.56 μ M, 6.25 μ M, or 12.5 μ M DN1, 25 μ M EGA, or DMSO solvent control 1 hour prior to treatment with 4.15 μ g/mL PA for 1 hour. Cells were lysed in NP-40 and analyzed by SDS-PAGE, and western blots were performed with cell lysates using anti-PA antibody. Data are representative of three independent experiments.

(b) DN1 does not inhibit the proteolytic activity of host cathepsin B (CTSB). FRET assay showing the activity of CTSB without drugs, or with addition of 3.1 μ M DN1 or 25 μ M amodiaquine (AQ). RAW264.7 cells were treated with DN1 or AQ for 1 hour prior to lysis and determination of cellular CTSB activity.

(c) DN1 does not inhibit MAPKK-2 cleavage. RAW264.7 cells received 6.25 μ M, 12.5 μ M, or 25.0 μ M DN1, or a DMSO vehicle control and were incubated with 500 ng/mL LT for 2.5

hours. Cells were lysed and analyzed by SDS-PAGE and western blot using anti-MAPKK-2 and anti-tubulin antibodies. Data are representative of three independent experiments.

(d) DN1 does not inhibit caspase-1 activation. RAW264.7 cells received 6.25 μ M DN1 or 12.5 μ M EGA for 1 hour prior to intoxication with 500 ng/mL LT for 2 hours. 5 μ L FLICA reagent was then added to each well for 1 hour, followed by 10 μ L Hoechst reagent for 10 minutes, and analyzed by automated fluorescence microscopy. Data represent mean values with standard deviation obtained in triplicate assays done in a representative experiment.

a

Figure 4. A search for DN1 targets suggests a model for DN1-mediated reduction of LT-induced pyroptosis.

(a) PubChem Bioactivities of DN1 lists two G protein-coupled receptor (GPCR) receptors, type-1 angiotensin II receptor and apelin receptor, as targets of DN1. This schematic illustrates the phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K) pathways activated by these GPCRs. The PLC and PI3K pathways may affect LT-induced pyroptosis through inositol triphosphate (IP3)-Ca²⁺ and protein kinase B (AKT), respectively. Small molecules U-73122 and LY-294002 inhibit PLC and PI3K, respectively.

(b-e) Compounds were applied 1 hour before treating cells with 500 ng/mL LT for 4 hours. In all experiments, cell viability was measured using the ATPLite viability assay. Mean Relative Luminescence Units from each of three independent experiments were normalized to cells only control values to determine percent cell viability.

(b) PLC and PI3K inhibitors synergize with DN1 in protecting cells from LT. Cells were pre-treated with a range of concentrations of U-73122 (a PLC inhibitor) or LY-294002 (a

PI3K inhibitor) with a sub-protective dose of DN1 (1 μ M). Sub-protective amount of DN1 was tested with and without LT as controls.

(c) PLC and PI3K inhibitors do not protect cells from LT individually. Cells were pre-treated with a range of concentrations of U-73122 or LY-294002.

(d) PLC and PI3K inhibitors alone do not synergize to protect cells from LT-induced pyroptosis. Cells were pre-treated with serial dilutions of a cocktail of PLC inhibitor U-73122 (top dose: 3.33 μ M) and PI3K inhibitor LY-294002 (top dose: 100 μ M).

(e) DN1 and protein kinase B (Akt) inhibitor synergize in protecting cells from LT in RAW264.7 cells. Cells were treated with a range of concentrations of Akt Inhibitor VIII with or without 1 μ M DN1.

Figure 5: The effect of DN1 on the bacterial growth.

(a) Agar DN1 diffusion susceptibility assay of *Bacillus cereus* and *Serratia liquefaciens*.

Plates were treated with 1 μ L of 10 mM of DN1 right after spreading of 200 μ L of liquid microbial cultures and left to incubate overnight.

(b) Agar DN1 diffusion susceptibility assay of *B. cereus*. Plates were treated with 1 μ L of

two-fold serial dilutions of 10 mM DN1 or with 1 μ L of 10mM levofloxacin (positive control) right after spreading microbial culture and left to incubate overnight. (a-b) An equal volume of drug solvent, DMSO, was also included as a negative control.

(c) The ability of DN1 to affect the growth of *B. cereus* in liquid LB was measured. The growth kinetics of *B. cereus* was measured in liquid medium in the 96-well plate, with and without various concentrations of DN1. The plates were grown with constant shaking at 37°C and the 600 nm absorbance was measured over time. Each data point shown indicates

the mean $\pm$ SD value obtained in triplicate assays done in a representative experiment. At least three such experiments were carried out.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6: The effect of DN1 on the sensitivity of flies to *Bacillus cereus* and *Serratia liquefaciens* infections.

(a) Ten female *Drosophila melanogaster* per group were pricked with *B. cereus* in the absence and in the presence of 120 μM rifampicin (Rif) in the fly food as a positive control. The *B. cereus* with and without Rif survival curves were statistically analyzed based on the Log-rank (Mantel-Cox) test.

(b-c) The effect of 40 and 120 μM DN1 on infected and uninfected *D. melanogaster* were measured over time. Flies were pricked with either *B. cereus* (b) or *S. liquefaciens* (c). Flies pricked with PBS-dipped needle and a non-pricked group of flies (CO₂ only) were included as control groups (a-c). Bacteria with and without 40 μM DN1 survival curves were statistically analyzed based on the Log-rank (Mantel-Cox) test.

Table 1.

The effect of DN1 on the pathogenicity of Human Cytomegalovirus (HCV) and Herpes Simplex Virus-1 (HCV-1) in human foreskin fibroblast (HFF) cells, as well as and Rift Valley fever virus in Vero 76 cells. The 50% effective (EC50, virus-inhibitory) concentrations and 50% cytotoxic (CC50, cell-inhibitory) concentrations were determined. CC50 divided by EC50 indicate the selectivity index (SI) value.

Virus	Strain	Cell line	EC50 μ M	CC50 μ M	SI50
Human cytomegalovirus	AD169	HFF	0.48	1.92	4
Herpes simplex virus 1	E-377	HFF	0.48	2.4	5
Rift Valley fever virus	MP12	Vero 76	2.9	5.5	1.9