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Identification of Novel Host-Targeted Compounds That Protect from Anthrax Lethal Toxin-Induced Cell Death

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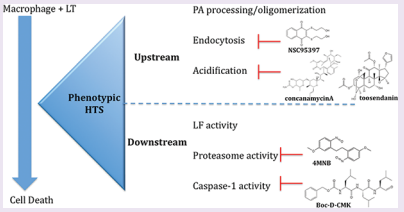
ABSTRACT: Studying how pathogens subvert the host to cause disease has contributed to the understanding of fundamental cell biology. *Bacillus anthracis*, the causative agent of anthrax, produces the virulence factor lethal toxin to disarm host immunity and cause pathology. We conducted a phenotypic small molecule screen to identify inhibitors of lethal toxin-induced macrophage cell death and used an ordered series of secondary assays to characterize the hits and determine their effects on cellular function. We identified a structurally diverse set of small molecules that act at various points along the lethal toxin pathway, including inhibitors of endocytosis, natural product inhibitors of organelle acidification (e.g., the botulinum neurotoxin inhibitor, toosendanin), and a novel proteasome inhibitor, 4MNB (4-methoxy-2-[2-(5-methoxy-2-nitrosophenyl)ethyl]-1-nitrosobenzene). Many of the compounds, including three drugs approved for use in humans, also protected against the related *Clostridium difficile* toxin TcdB, further demonstrating their value as novel tools for perturbation and study of toxin biology and host cellular processes and highlighting potential new strategies for intervening on toxin-mediated diseases.



Chemical perturbation of biological systems is a powerful method for studying cellular pathways and processes. Bioactive small molecules identified from forward chemical genetic screens can serve as tools to dissect these processes when linked to a target or mechanism of action. Bacterial toxins can similarly mediate such perturbations when they induce phenotypes such as cell death by co-opting or disrupting host cellular processes. Thus, the identification of small molecules that suppress toxin-induced phenotypes in chemical suppressor screens can help not only to elucidate the mechanism of intoxication and identify new therapeutic antitoxin targets but also to provide novel tools for studying fundamental host cell biology.

Anthrax toxin, which is elaborated by *Bacillus anthracis*, the causative agent of anthrax, is an example of one such toxin that co-opts a large number of host factors to enter cells and induce death. It is composed of three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF).[1] LF is a zinc metalloprotease that cleaves MAP kinase kinases[2−4] and NLRP1b,[5] while EF is an adenylate cyclase.[6] Individually the toxin components have no effect, but when combined in a binary fashion to form their respective AB toxins, PA and LF (forming lethal toxin, LT) cause the death of experimental animals and cultured macrophages,[7−10] and PA and EF (forming edema toxin, ET) cause tissue edema[11] and death in a rabbit model of inhalational anthrax.[12,13] PA, the toxin B subunit, shuttles both LF and EF into the cytosol.

After PA binds to one of two anthrax toxin receptors, it is processed by a host furin protease and oligomerizes into heptamers or octamers that relocalize to lipid rafts[14] (Figure 1). Up to three or four molecules of LF and/or EF bind to the oligomer,[15,16] and the toxin−receptor complexes undergo endocytosis. Following endocytosis, the internalized vesicles are trafficked to early endosomes, where toxin−receptor complexes are sorted into intraluminal vesicles.[17] A drop in pH in the endosome causes a conformational change in PA, converting the heptamer from the prepore to the pore state, which inserts into the membrane[18−20] and forms a functional pore through which LF and EF translocate into the lumen of the intraluminal vesicles.[1] LF and EF are finally delivered to the cytosol when the vesicles undergo back-fusion with the late endosomal membrane.[17]

After entering the cytosol in J774A.1 cells and other susceptible murine macrophages, LT induces rapid caspase-1-dependent cell death.[8] Activation of caspase-1 involves the NLRP1b inflammasome and can be prevented by proteasome

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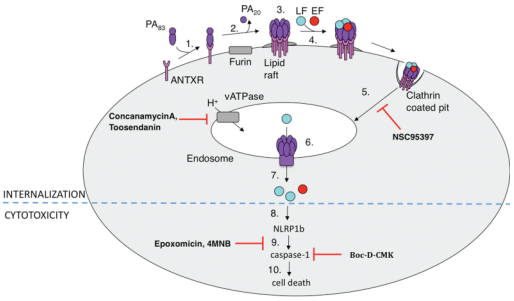

Figure 1. Cytoplasmic delivery of anthrax toxin. 1. PA binds to one of two anthrax toxin receptors on the cell surface; 2. PA is processed by a host furin protease; 3. PA_{63} remains bound to the receptor and oligomerizes into a heptamer or octamer; 4. binding of EF and/or LF; 5. clathrin-mediated endocytosis of the toxin–receptor complexes and trafficking to the endosome; 6. upon endosome acidification, PA undergoes a conformational change and forms a pore in the endosomal membrane; 7. translocation of EF and LF through the PA pore into the cytosol; 8. LF cleaves NLRP1b; 9. caspase-1 activation is dependent on proteasome activity; 10. caspase-1-dependent cell death.

inhibitors. LF has recently been shown to cleave NLRP1b from susceptible rat macrophages, although it is not clear that this signal alone is sufficient to induce caspase-1 activation and cell death.[5] MAP kinase kinases are also substrates of the LF protease; however, it is currently unclear whether down-regulation of the MAP kinase pathway contributes to caspase-1-dependent cell death in susceptible murine macrophages.

Previous small molecule screens for inhibitors of LT have predominantly yielded novel inhibitors of endosomal acidification, including the antiarrhythmic drugs amiodarone and bepridil,[21] and known bioactive compounds diphyllin and niclosamide.[22] A screen also identified the napthoquinone NSC 95397, which has previously been described as an inhibitor of Cdc25 protein phosphatase. The exact mechanism of action of NSC 95397 was unclear, but it was proposed to protect via activation of MAP kinases to trigger an antiapoptotic effect upon LF treatment.[23]

Here, we describe an approach combining a high-throughput small molecule screen to identify novel inhibitors of host proteins involved in the LT pathway and a series of secondary assays that allow systematic elucidation of their mechanisms of action. We identified several small molecules that provide complete protection of J774A.1 murine macrophages from LT, including compounds that prevent LT internalization and a compound that prevents LT cytotoxicity by inhibiting the proteasome. Many of the identified internalization inhibitors prevented endocytosis of the toxin. Interestingly, while two natural products, the botulinum neurotoxin inhibitor toosendanin and the glycosylated sterol **5**, appear to inhibit endosome acidification, they were able to inhibit LT-induced cell death at a lower concentration than that needed to affect endosome acidification, suggesting that they may have an additional mechanism of action. However, unlike its action on botulinum neurotoxin,[24] toosendanin did not appear to inhibit translocation of LF through the PA pore in *in vitro* and cell-based assays. Because many toxins that need to gain access to the host cell cytosol share conserved mechanisms of entry, compounds that inhibit events involved in toxin internalization may act as broad toxin inhibitors. We show that inhibitors of endocytosis and endosomal acidification identified in this screen, including toosendanin and three drugs approved for use in humans, also inhibit cell death induced by the bacterial toxin TcdB from *Clostridium difficile*, a pathogen of significant current interest due to the rising incidence and mortality in hospital-acquired infection.

RESULTS AND DISCUSSION

Small Molecule Screen for Inhibitors of Anthrax LT.

We performed a suppressor screen of LT by screening for compounds that rescue J774A.1 murine macrophages from LT challenge. Cells were preincubated with a compound library in 384-well format for 2 h and then treated with PA and LF for 6 h, which kills approximately 98% of cells. Cell survival was measured using CellTiter-Glo, a luminescence-based assay that quantitates ATP. The average Z' factor for the assay was 0.67.

A short assay time was chosen to allow us to screen inhibitors of essential cell functions that would otherwise be toxic over longer periods of time. We screened 31,350 small molecules at an average concentration of approximately 33 μM. The libraries screened were part of the Broad Institute collection and included bioactive compounds with annotated mechanisms of action, commercially available compounds, diversity-oriented synthetic (DOS) compounds,[25] natural products, and natural product extracts (Table 1). We cherrypicked the top 0.5% of compounds based on the strength of protection from LT and reproducibility between the two replicates (Table 1; Figure 2)

Table 1. Compounds Cherrypicked from Small Molecule Screen

library	screened	cherrypicked
DOS	9570	23
commerical	8580	31
bioactives	6930	63
natural product extracts	3630	19
purified natural products	2640	24
total	**31350**	**160**

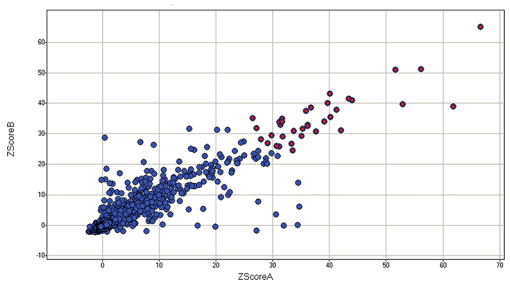

Figure 2. Small molecule screen: primary data from screening day 2. The plot shows the z score for replicates A and B for each compound screened. Compounds that were cherrypicked and retested are shown in red.

and retested them in an 8-point dose–response assay. Of these 160 compounds, 126 (79%) reproducibly rescued J774A.1 cells from death. We proceeded to follow up with the 49 compounds that protected 100% of cells from LT and exhibited low toxicity toward J774A.1 cells in this short time-period. Active compounds identified also included the proteasome inhibitor epoxomicin, the general caspase inhibitor Boc-D-CMK, and the vacuolar ATPase inhibitor concanamycin A,[26] which served as positive controls for the screen and secondary assays. Interestingly, the screen also identified three drugs approved

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Table 2. Characterization of Internalization Inhibitors and Selected Control Compounds from the Small Molecule Screen[a]

ID	name	function	LeTx MIC (μM)	(μM)LeTx IC_{50}	PA/LFn-DTA	EdTx	transferrin/dextran uptake	TcdB
1	concanamycin A	vacuolar ATPase inhibitor	0.001	0.0005	+	+	−	+
2	epoxomicin	proteasome inhibitor	0.125	0.04	*b*	−	−	−
3	destruxin B[61]	vacuolar ATPase inhibitor	0.625	0.156	+	+	nd	+
4	NSC 95397	Cdc25 protein phosphatase inhibitor	1.25	0.625	toxic	+	+	+
5	ACon1_001348	unknown	2.5	1.25	+	+	−	+
6	helenalin[62]	NF-κB inhibitor	2.5	1.75	+	+	+	+
7	(−)-terreic acid[63]	BTK inhibitor (PH-domain)	10	4	toxic	+	+	−
8	WP1066[64]	Stat3 inhibitor	5	2.5	nd	+	+	+
9	CBChromo1_000185	unknown	5	1.25	toxic	+	+	+
10	CBChromo1_000186	unknown	5	3.75	toxic	+	+	+
11	ACon1_000439	unknown	5	3.5	toxic	+	+	+
12	pizotifen malate[65]	serotonin receptor antagonist	10	5	*b*	+	+	+
13	TimTec1_005928	unknown	6.25	3.75	+	+	+	+
14	bisindolylmaleimide I[66]	protein kinase C inhibitor	7.5	1.875	+	+	+	+
15	SD-1029[67]	JAK2 inhibitor	10	3.75	nd	+	+	+
16	tilorone[68]	induces interferon production	10	7.5	*b*	+	+	+
17	ACon1_001234	unknown	10	5	+	+	+	+
18	MDL-12330A hydrochloride[69]	adenylate cyclase inhibitor and calcium channel blocker	20	10	+	+	+	+
19	cucurbitacin[70]	JAK/Stat3 inhibitor	20	10	toxic	+	+	+
20	stattic[71]	Stat3 inhibitor	12.5	7.5	nd	+	nd	*b*
21	disulfiram[72]	aldehyde dehydrogenase inhibitor	20	7.5	*b*	+	+	+
22	radicicol[73,74]	Hsp90 and Src kinase inhibitor	20	15	+	+	+	+
23	ChemDiv3_007213	unknown	20	7.5	toxic	+	+	−
24	ACon1_000166	unknown	20	15	*b*	+	+	+
25	ChemDiv 0892-0507	unknown	20	15	+	+	inhibits dextran but not transferrin uptake	+
26	aeroplysinin-1[75]	EGFR inhibitor	25	10	+	+	+	+
27	TimTec1_001156	unknown	25	15	+	+	+	+
28	Neuro1_000172	unknown	40	30	toxic	+	nd	+
29	cantharidic acid[76]	protein phosphatase 2A inhibitor	>40	20	*b*	+	+	−
30	bisindolyl maleimide V[77]	negative control compound for PKC inhibition studies	>40	20	+	+	nd	+
31	natural product extract	unknown	234[c]	100[c]	+	nd	−	−
32	natural product extract	unknown	29[c]	10[c]	+	nd	−	−
33	toosendanin	inhibitor of BoNT	0.183	0.06	+	nd	−	+
34	4MNB	proteasome inhibitor	40	20	−	−	nd	+

[a]The minimum inhibitory concentration (MIC) required for 100% protection from LT and the concentration required for 50% protection (IC_{50}) are shown. All compounds designated as ET inhibitors inhibited at least 50% of cAMP induced by ET, while 4MNB and epoxomicin did not inhibit ET at all. Compounds that inhibited at least 50% of transferrin and dextran uptake were annotated as inhibitors of endocytosis. Protection of cells from TcdB-induced cell death ranged from 20% to 56%. Structures are shown in Figure 3. (nd = not done). [b]Compound did not protect LF_N-DTA or LT over 24 h assay. [c]Concentration in ng mL^{-1}.

for use in humans (pizotifen malate, disulfiram, and tilorone), which could be potential therapeutics for anthrax disease (Table 2).

Secondary Assays To Categorize Hits. We next proceeded to characterize the active compounds by performing a number of secondary assays. First, we determined whether the compounds inhibited toxin internalization (binding, uptake, and delivery into the cytosol) or cytotoxicity (LF protease activity and induction of cell death). We took advantage of a toxin chimera LF_N-DTA, which is a fusion protein consisting of the N-terminal domain of LF and the enzymatic domain of diphtheria toxin. LF_N-DTA enters the cytosol in a PA-dependent manner identical to that of LF but kills cells by a completely different mechanism involving inhibition of elongation factor 2 and activation of apoptosis. We tested the ability of compounds to protect J774A.1 cells from PA/LF_N-DTA challenge over 24 h, as compounds that protect against both LF and LF_N-DTA likely act during internalization. In addition, the longer time course of this experiment allowed us to distinguish compounds that might exhibit toxicity to the host. Of the 46 compounds tested, we identified 15 internalization inhibitors that protected cells from both LT and PA/LF_N-DTA over 24 h, and one cytotoxicity inhibitor (Table 2). We were unable to annotate the remaining 30 compounds due to toxicity toward J774A.1 cells over 24 h, or because they did not protect cells from LT over this time scale. Compounds that protect cells from LT in the 6 h assay but not the 24 h assay likely affect the kinetics of internalization or cytotoxicity, but do not completely block toxin action.

Next, to further confirm that compounds were internalization or cytotoxicity inhibitors, as well as to characterize the inhibitors that we were unable to assign with the 24 h LF_N-DTA assay, we tested the ability of the compounds to protect cells against the effects of EF, which also enters in a PA-dependent manner but results in elevated cAMP levels. Using a competitive ELISA-based assay, we measured the concentration

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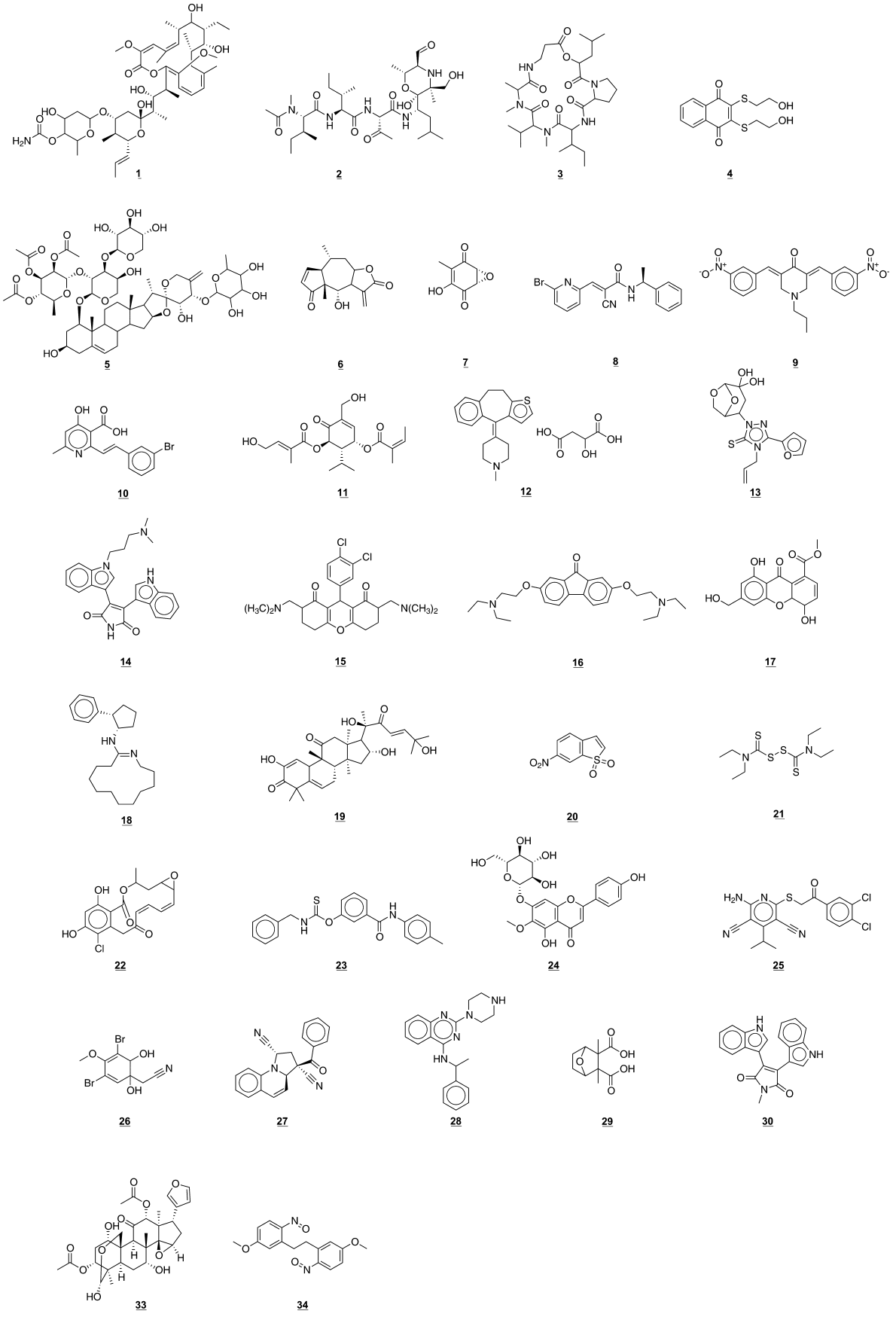

Figure 3. Structures of selected hits from the small molecule screen. Structures of compounds from Table 2.

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of cAMP in lysates from cells treated with ET for 5 h. By these two assays, we identified a total of 31 LT inhibitors that prevent internalization and 18 inhibitors of cytotoxicity (Figure 3 and Table 2).

Multiple Inhibitors of Internalization Prevent Endocytosis. Next, to determine whether the internalization inhibitors prevent endocytosis of the toxin−receptor complex, we tested their ability to inhibit the uptake of transferrin and dextran. Iron-loaded transferrin enters cells by binding to transferrin receptors on the cell surface and undergoing clathrin-mediated endocytosis.[27] Following release of Fe^{3+} in the endosome, transferrin enters the recycling endosome and is returned to the cell surface. Dextrans are hydrophilic polysaccharides that can be used as a fluid-phase marker for uptake via endocytosis, phagocytosis, or macropinocytosis.[28,29] J774A.1 cells were pretreated with compound and then incubated with fluorescently labeled dextran and transferrin for 2 h or 15 min, respectively, and washed, and then fluorescence was measured using a plate reader. Twenty-three out of 27 of the internalization inhibitors tested in this assay prevented uptake of transferrin and dextran by more than 50%, suggesting that they protect against LT by inhibiting endocytosis of the toxin (Table 2). Of note, we found that the napthoquinone NSC 95397 (compound **4**) is an internalization inhibitor that prevents transferrin and dextran uptake. NSC 95397 has previously been identified in a small molecule screen of LT inhibitors and was suggested to protect against LT-induced cell death by inhibiting MEK cleavage, as well as to upregulate MAP kinase signaling.[23] Here, we show that, in fact, NSC 95397 protects against LT-induced cell death by inhibiting toxin uptake.

The enrichment for endocytosis inhibitors could be due to the complexity of endocytosis and its requirement for numerous host proteins, such as actin, clathrin, and the unconventional adaptor AP-1.[30,31] Furthermore, endocytosis of toxin−receptor complexes is regulated by post-translational modifications of anthrax toxin receptors. In the absence of PA binding, toxin receptors are palmitoylated,[32] which serves to prevent endocytosis and receptor turnover in the absence of ligand. PA binding triggers redistribution of the PA−receptor complexes to cholesterol-rich lipid rafts,[30] likely due to a conformational change in the receptor that causes depalmitoylation or association with other membrane proteins. Src-kinase mediated tyrosine phosphorylation of the cytoplasmic tail of the receptors recruits the E3 ligase Cbl in a β-arrestin-dependent manner,[31,33] leading to ubiquitination of the cytoplasmic tail and thus promoting interaction with components of the endocytic machinery that contain Ub-interacting domains.

Toosendanin Inhibits Entry of LT into the Cytoplasm. Three of the internalization inhibitors are natural products, or natural product extracts, that did not inhibit uptake of dextran and transferrin (Table 2). Two of the natural products (extracts **31** and **32**) were fractions of an extract from the plant *Melia azedarach*, while the structure of the third natural product, the glycosylated sterol **5** (IC_{50} 1.25 μM), was known (Figure 3). Extract **32** was particularly potent, with an upper IC_{50} limit of 10 ng mL^{-1}, assuming that the extract consisted only of the active molecule.

Because **31** and **32** are both from *Melia azedarach*, we searched for known compounds produced by this plant. Toosendanin is a triterpenoid from *Melia azedarach*[34] and has been described to have *in vivo* activity against botulinum neurotoxin (BoNT)[24,35−37] (Figure 3). We found that toosendanin phenocopied the effects of **31** and **32**, by preventing LT and PA/LF_N-DTA-induced cell death without inhibiting transferrin and dextran uptake. Toosendanin had an IC_{50} of 56 nM (32 ng mL^{-1}) against LT, indicating that it is likely the active compound in these extracts (Figure 4).

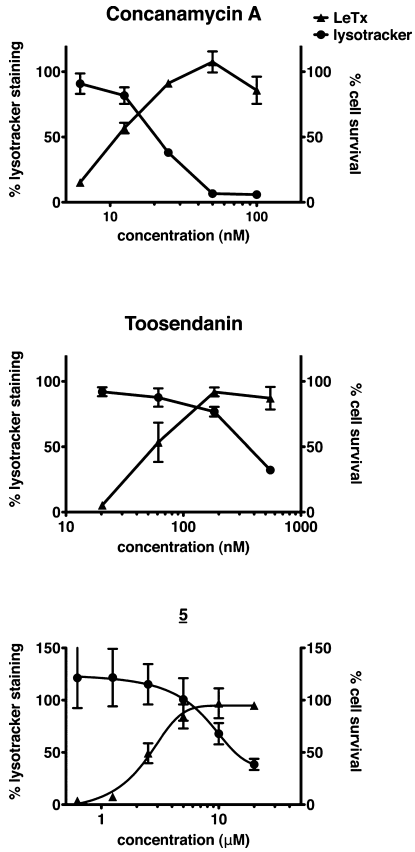

Figure 4. Internalization inhibitors of LT that inhibit endosome acidification. Inhibition of lysotracker staining requires a higher concentration of compound than protection from LT.

Since pore formation and translocation of LF require an acidic endosome, we tested whether toosendanin and sterol **5** inhibit organelle acidification using the cell-permeable pH-sensitive dye lysotracker red, which accumulates in acidic organelles. Cells were preincubated with compound and then incubated with lysotracker red, before fluorescence was measured using a plate reader. Since lysotracker staining responds to changes in pH in both endosomes (pH 5−6.5) and lysosomes (pH 4−5), full inhibition of lysotracker staining suggests that both endosomes and lysosomes are neutralized, while partial inhibition of lysotracker staining likely corresponds to neutralization of endosomes but not lysosomes. Therefore, as noted for the vacuolar ATPase inhibitor concanamycin A (Figure 4, 25 nM) full inhibition from LT-induced cell death corresponds to around 60% inhibition of lysotracker staining.

We found that both toosendanin and **5**, along with concanamycin A, inhibited lysotracker staining at sufficiently high concentrations (Figure 4). However, we noted that in contrast to the positive control concanamycin A, toosendanin and **5** protect close to 100% of cells from LT at concentrations where they exhibit minimal or no inhibition of lysotracker staining (Figure 4). For example, 5 μM **5** protected 84% of cells

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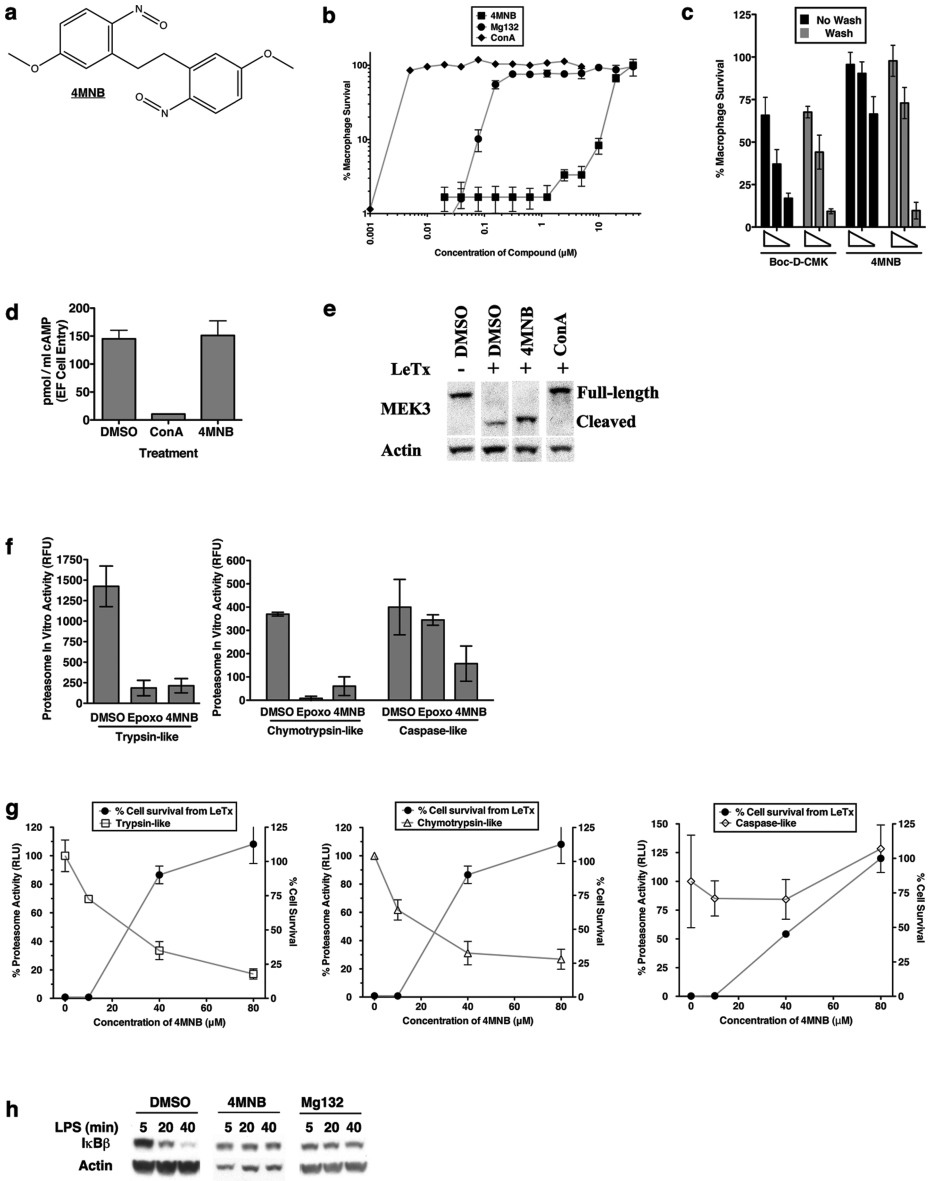

Figure 5. 4MNB protects against LT-induced cytotoxicity by inhibiting the proteasome. (a) Structure of 4MNB. (b) J774 cells were treated with compound, followed by LT, and cell survival was measured. ConA is the internalization inhibitor concanamycin A, and Mg132 is a proteasome inhibitor that prevents LF-induced cytotoxicity. (c) J774 cells were treated with compound (Boc-D-CMK 10, 5, 2.5 μM and 4MNB 80, 40, 20 μM), washed, and exposed to LT, and cell survival was measured. (d) J774 cells were treated with compound (0.5 μM ConA and 40 μM 4MNB) and exposed to ET, and cAMP levels were measured by ELISA. (e) J774 cells were treated with compound (40 μM 4MNB and 0.5 μM ConA) and exposed to LT, and lysates were probed for MEK3 cleavage. (f) Lysates from J774 cells were treated with compound (10 μM Epoxo and 40 μM 4MNB) and incubated with substrates of the three proteasome proteases, and activity was determined by measuring fluorescence of cleaved substrates. (g) J774 cells were treated with 4MNB and incubated with substrates of the proteasome or LT. Proteasome activity was determined by measuring luminescence of cleaved substrate, and cell survival was measured. (h) J774 cells were treated with compound (80 μM 4MNB and 20 μM Mg132) and exposed to LPS (100 ng mL^{-1}), and lysate was collected and probed for evidence of IκBβ degradation.

from LT but had no effect on lysotracker staining, while 183 nM toosendanin protected 92% of cells from LT but inhibited only 23% of lysotracker staining (Figure 4). These results raise the possibility that while these compounds do inhibit endosomal acidification at higher concentrations, at lower concentrations where protection against LT is still observed they may inhibit internalization by a different mechanism.

Toosendanin has been reported to protect cells against botulinum toxin (BoNT), produced by the pathogen *Clostridium botulinum*. BoNT is an AB toxin that enters cells via the endocytic route in a similar manner to LT. Previously, toosendanin has been shown to inhibit BoNT oligomerization and channel formation by the heavy chain[38,39] and to directly inhibit light chain translocation by stabilizing the interaction between the light and heavy chains.[24] Given the similarities between anthrax toxin and BoNT translocation, we hypothesized that toosendanin could inhibit LT by a similar mechanism. However, toosendanin appeared to have no effect on LF_N translocation through PA pores formed in planar lipid bilayers or on LT-induced cell death when PA pore formation in the plasma membrane and LF translocation into the cytoplasm was initiated by a low pH pulse (data not shown). This suggests that toosendanin may protect cells from LT and BoNT by different mechanisms.

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Toosendanin appears to be a potent inhibitor of LT-induced cell death, and our data suggest that it may protect cells via more than one mechanism. Toosendanin is known to have inhibitory and excitatory effects on a broad range of membrane ion channels, including potassium channels, calcium channels, and the botulinum neurotoxin (BoNT) channel, and therefore disruption of the vacuolar ATPase could explain its inhibition of endosomal acidification.[24,40−45] Interestingly, toosendanin has inhibitory activity against BoNT in animal models[24,35−37] and has also been shown to inhibit hepatitis C virus replication in a cell culture model,[46] suggesting that it might be a useful therapeutic agent in a variety of infectious diseases, including anthrax.

Inhibitors of Toxin Entry Also Protect against the AB Toxin TcdB. We hypothesized that compounds that inhibit LT, ET, and PA/LF_N-DTA might also inhibit related AB toxins, given the conservation of some mechanisms in the uptake of several different toxins, such as endocytosis and endosomal acidification. Thus, we sought to test the identified internalization inhibitors against the toxin TcdB from *Clostridium difficile*. TcdB is an AB toxin that enters the cytosol through a self-made pore in the endosomal membrane in a similar manner to LT. Once in the cytosol, TcdB glycosylates the small GTPases Rho, Rac, and Cdc42, causing actin depolymerization and ultimately cell death.[47,48] Almost all of the internalization inhibitors that prevented transferrin and dextran, as well as toosendanin and the endosomal acidification inhibitor **5**, also protected CHO cells from TcdB (Table 2). Notably, the approved drugs piztofen malate, tilorone, and disulfiram were among the inhibitors of TcdB, further demonstrating the value of these novel compounds as potential therapeutics and as tools to study toxin and host cell biology. Of note, toosendanin protected only 30% of cells from TcdB, while the endosomal acidification inhibitor concanamycin A afforded 100% protection. This suggests that toosendanin does not strongly inhibit endosome acidification, supporting the hypothesis that it likely provides 100% protection from LT by an additional mechanism.

4MNB Is a Novel Cytotoxicity Inhibitor. One small molecule of interest that was identified as a cytotoxicity inhibitor is the compound 4-methoxy-2-[2-(5-methoxy-2-nitrosophenyl)ethyl]-1-nitrosobenzene (4MNB, Figure 5a), which completely protects J774A.1 macrophages from LT-induced death at 40 μM and has an IC_{50} of ∼18 μM (Figure 5b). Washing cells after treatment with 4MNB (or Boc-D-CMK[49]) does not dramatically reduce the protection of macrophages from LT at several concentrations of 4MNB (Figure 5c), suggesting it is an irreversible inhibitor. 4MNB did not inhibit the increase in cAMP concentration induced by ET, indicating that it acts after toxin internalization (Figure 5d).

4MNB Does Not Protect from LT by Inhibition of Bcl-2/Bcl-XL. 4MNB was previously reported to bind and inhibit Bcl-2 and Bcl-XL,[50,51] which are homologous antiapoptotic factors that protect cells from death by binding to Apaf-1 and preventing activation of caspase-9.[52,53] To test if inhibition of Bcl-2/Bcl-XL could account for 4MNB's observed inhibition of LT cytotoxicity, we tested several well-known inhibitors of Bcl-2/Bcl-XL including HA14-1,2-methoxy-antimycin A3, Gossypol, and Antimycin A.[54] None of these inhibitors protected cells from LT-induced cell death at any of the concentrations tested (data not shown), indicating that 4MNB protects from LT-induced cytotoxicity via a different target.

4MNB Does Not Affect MEK Cleavage. Because LF is a metalloprotease that cleaves the cytosolic MEK signaling molecules,[2] we tested whether 4MNB inhibits the protease activity of LF. We treated macrophages with 4MNB prior to LT exposure, then harvested lysate, and probed by Western blot analysis for evidence of MEK3 cleavage. While the internalization inhibitor concanamycin A prevents MEK cleavage by preventing translocation of LF into the cytosol, 4MNB did not prevent MEK3 cleavage at concentrations that protect cells from LT-induced death (Figure 5e). These data confirm that 4MNB does not affect translocation of LF into the cytosol and demonstrate that it does not inhibit the protease activity of LF in whole cells.

4MNB Is a Novel Proteasome Inhibitor. We next examined whether 4MNB protects cells from LT by inhibiting the protease activity of the proteasome. If 4MNB directly inhibits the proteasome, it should affect one of three protease activities of the proteasome: chymotrypsin-like, trypsin-like, or caspase-like activity. To test this possibility, we treated macrophage lysate with 4MNB, or a known proteasome inhibitor control, epoxomicin (Epoxo),[55] and then added one of three synthetic proteasome substrates that result in fluorescence when cleaved by the corresponding specific protease activity. Using this assay, we found that 4MNB directly inhibits all three proteasome protease activities *in vitro* (Figure 5f).

To further confirm these results, we assayed the activity of three proteasomal protease activities in intact cells, in the presence and absence of 4MNB. We used three different cell permeable substrates specific for each protease activity that result in luminescence upon cleavage and tested for protection from LT-induced death in a parallel assay. 4MNB inhibits the proteasome trypsin and chymotrypsin-like protease activities at concentrations close to that required for protection of macrophages from LT, but not the caspase-like activity (Figure 5g). These data demonstrate that in addition to the previously reported inhibition of Bcl-2/Bcl-XL, 4MNB also directly inhibits two of the protease activities of the proteasome *in vivo*.

To demonstrate that 4MNB can prevent the degradation of true cellular proteasome substrates, we tested its ability to inhibit the canonical LPS (lipopolysaccharide)-induced IκBβ degradation that results in NFκB activation. Cells were treated with 4MNB followed by LPS for different time-periods to stimulate the degradation of IκBβ. Lysates were then harvested and assayed by Western blot analysis for the presence of IκBβ. In untreated (DMSO) cells, a dramatic reduction in IκBβ occurs by 40 min; however, 4MNB prevents the degradation of IκBβ similar to the control proteasome inhibitor Mg132 (Figure 5h). These data demonstrate that 4MNB protects macrophages from LT-induced death through inhibition of the protease activity of the proteasome.

In summary, 4MNB did not inhibit the increase in cAMP concentration induced by ET or cleavage of MEK3 by LF in intact cells but did inhibit proteasome protease activity *in vitro* and *in vivo*. Despite its ability to inhibit all three protease activities *in vitro*, 4MNB appears to only inhibit the trypsin and chymotrypsin-like activities to a significant degree *in vivo*, leaving the caspase-like activity intact in treated cells. 4MNB also inhibited IκBβ degradation, indicating that its activity extends beyond substrates in the LT pathway.

Conclusion. Through a forward chemical genetic screen, we have identified several novel small molecule inhibitors of LT-induced cell death, including three drugs approved for use in

humans that could potentially be used as antitoxin therapeutics. We screened a diverse set of small molecules and note that the top hits were enriched for bioactive small molecules and natural products (Table 1). We characterized hits from the screen by performing an ordered series of secondary assays based on current knowledge of the LT pathway to elucidate their function, assessing whether the compounds were internalization inhibitors (compounds that prevent cytosolic delivery of toxin) or cytotoxicity inhibitors (compounds that inhibit cytotoxicity induced by LF protease activity in the cytosol). We found that a large proportion of the hits were inhibitors of internalization, which is not surprising, given the large number of host proteins and numerous steps involved in toxin trafficking (e.g., delivery of toxin from the endosome to the cytosol, sorting of the toxin−receptor complexes into intraluminal vesicles, PA pore formation, translocation of LF and EF into the lumen of the intraluminal vesicle, and back-fusion of the vesicles with the late endosomal membrane to deliver toxin into the cysotol [17]). We also identified a number of compounds that inhibited cytotoxicity. Of particular interest, we found that 4MNB is a novel proteasome inhibitor that does not affect toxin entry.

Since the steps involved in internalization are required for many essential cellular processes, it is possible that inhibitors of these processes could exhibit toxicity to the host cell or organism. Indeed, we found that 8 compounds were toxic to J774A.1 macrophages over 24 h (Table 2), which could preclude their use in *in vivo* assays. However, these compounds remain useful tools for studying the LT pathway and host cell biology.

The identification of novel host-targeted inhibitors of LT-induced cell death provides a structurally diverse set of tool compounds that can be used to perturb and study both the LT pathway and fundamental cellular processes. By systematically applying a series of secondary assays, we were able to assign mechanisms of action to inhibitors. In some cases, such as toosendanin and NSC 95397, the inhibitors appear to have multiple possible mechanisms by which they protect against LT. However, with further investigation, these mechanisms can be distinguished to clarify the relevant mode of action to LT intoxication.

METHODS

Cells, Antibodies, and Reagents. J774A.1 cells were obtained from American Type Cell Culture Collection (ATCC), cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing L-glutamine, 10% (v/v) fetal bovine serum (Hyclone), and penicillin-streptomycin (Mediatech Inc.), and maintained at 37 °C with a humidified atmosphere containing 5% CO_2. Secondary HRP goat anti-rabbit antibody and antibodies to MEK3 (sc-959), I$\kappa B\beta$ (sc-9130), and actin were from Santz Cruz Biotechnology. LF_N-DTA[56] was a kind gift from the Collier lab (Harvard Medical School). EF was obtained from List Biological Laboratories. Compounds were obtained from Spectrum Chemicals, Gaia Chemical Corporation, Ryan Life Sciences, Biomol International, Alexis Biochemicals Calbiochem, and EMD. LF substrate 3 was from Calbiochem, and proteasome substrates from Promega.

Protein Expression and Purification. PA and LF were expressed in BL21STAR(DE3) *Escherichia coli* grown in ECPM1 medium.[57] Cultures were induced at 30 °C with isopropyl-1-thio-β-D-galactopyranoside (IPTG).

Recombinant PA was overexpressed in the periplasm from pET22b (Invitrogen). Periplasmic proteins were purified by osmotic shock by first resuspending pelleted cells in 4 mL of 20% (v/v) sucrose, 1 mM EDTA, and 20 mM Tris-HCl (pH 8.5) per gram of cells. Cells were then resuspended in 5 mM magnesium sulfate with 20 mM Tris-HCl pH 8.5 and 1 mM PMSF, and the periplasmic fraction was clarified by centrifugation. PA was purified by anion-exchange chromatography followed by size-exclusion chromatography using an S-200 Superdex gel filtration column (Amersham Biosciences).[58]

His_6-tagged LF cloned into pET15b was purified as previously described.[58,59] Cells were resuspended in 50 mM Tris-HCl pH 7.5, 150 mM sodium chloride with 1x Complete Protease Inhibitor without EDTA, lysozyme and DNase I, then sonicated and centrifuged to clarify the lysate. His_6-tagged protein was purified by nickel column, and the N-terminal H_6-tag was removed by thrombin cleavage overnight at 25 °C in Buffer C with 2.5 mM calcium chloride and 2.1 U thrombin mg^{-1} protein (GE Healthcare). Proteins were further purified by anion exchange and size exclusion chromatography as described above.

TcdB was purified from *C. difficile* strain VPI 10463 according to published methods. Protein purity was assessed by SDS-PAGE.[60]

High-Throughput Small Molecule Screen. J774A.1 cells (4×10^3) in 20 μL were plated in white 384-well plates and incubated overnight. Then 100 nL of compound in DMSO was transferred to plates, and cells were incubated for 2 h at 37 °C. Next 10 μL of LT (16 nM PA, 8 nM LF) was added, and cells were incubated at 37 °C for 6 h. Cell viability was assessed by adding an equal volume of Cell Titer Glo diluted 1:6 in PBS and measuring luminescence using an Envison Multilabel Plate reader. Compounds were screened in duplicate, and raw data was submitted to the Broad Chemical Biology screening platform for data analysis and calculation of z scores. ($z = (x - \mu)/\sigma$ where x is the raw luminescence score, μ is the mean of the mock (DMSO)-treated well, and σ is the standard deviation of the mock-treated wells.)

Small Molecule Retesting and PA/LF_N-DTA Assay. Compounds were retested in duplicate with 8-point dose response curves, with typical concentrations between 33 μM and 0.015 μM in 3-fold dilutions. LT (16 nM PA, 8 nM LF) was added, and cells were incubated at 37 °C for 6 h. Cells were also screened with LT (16 nM PA, 660 pM LF) for 24 h, with PA/LF_N-DTA (16 nM PA, 100 pM LF_N-DTA) for 24 h, and with compound alone for 24 h to assess toxicity. Cell Titer Glo was used to measure cell viability as described previously.

ET Assay. J774A.1 cells (5×10^4) in 100 μL were plated in 96-well plates and incubated overnight. Cells were preincubated with compound for 2 h, before the addition of ET (11 nM PA, 1.2 nM EF) for 5 h at 37 °C. The concentration of cAMP in cell lysates was measured by cAMP enzyme immunoassay according to the manufacturers instructions (Enzo Life Sciences).

Transferrin/Dextran. J774A.1 cells (3.5×10^4) were plated in black, optical 96-well plates and incubated overnight. Cells were pretreated with compound for 2 h, followed by addition of dextran-Alexa Fluor 488 10,000 MW (100 $\mu g\ mL^{-1}$) (Invitrogen) and incubation at 37 °C for 75 min, and then addition of transferrin-Alexa Fluor 546 (10 $\mu g\ mL^{-1}$) (Invitrogen) and incubation at 37 °C for 15 min. Cells were washed with 3 × 100 μL DMEM (without phenol red), and 50 μL PBS was added to plates. Fluorescence was measured using a SpectraMax M5 plate reader (Molecular Devices) to assess transferrin and dextran uptake. Cell Titer Glo was added to the plate to measure the number of cells in each well.

TcdB Assay. Chinese Hamster Ovary (CHO) cells (9×10^4) in 30 μL were plated in white 384 well plates containing compound and incubated at 37 °C for 2 h. TcdB (35 nM) was added, and the cells were incubated at 37 °C for 3 h. CellTiterGlo was used to measure cell viability as previously described.

Lysotracker Assay. J774A.1 cells (3.5×10^4) were plated in black, optical 96-well plates and incubated overnight. Cells were pretreated with compound for 2 h, followed by addition of lysotracker red DND-99 (100 nM) (Invitrogen) and incubation for 1 h at 37 °C. Cells were washed with 3 × 100 μL DMEM (without phenol red) and fixed with 4% (w/v) paraformaldehyde. Fluorescence was measured using a SpectraMax M5 plate reader.

MEK Cleavage Assay. Macrophages (1×10^6) were plated per well in a 6-well dish overnight. Cells were incubated with compound for 2 h, then LT (LF 1−2 nM and PA 11 nM) was added, and cells

were incubated for 2 h. Total cell lysates were made, protein concentrations were measured and normalized, and proteins were separated on an SDS-PAGE, transferred to PVDF, and probed with anti-MEK3 antibodies.

Whole Cell Proteasome Assay. Macrophages (5×10^5 per well) were plated in a 96-well plate and incubated overnight. Cells were then preincubated with compound for 2 h and then mixed with cell-permeable substrates in the provided buffer (Promega). Kinetic reads of luminescence were taken every 5 min for 30 min.

***In Vitro* Proteasome Assay.** Cells (7×10^6) were plated in 10 cm dishes, incubated overnight, washed with PBS, and harvested in assay buffer (50 mM Tris [pH 7.5], 140 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, 0.5 mM DTT, 2 mM ATP, and protease inhibitor cocktail (Sigma)). Cells were lysed using sonication, and lysate was diluted in assay buffer and distributed into wells of a 384- or 96-well plate. Compound was added, then substrate (ProteasomeGlo, Promega) was added quickly, and kinetic readings were taken for fluorescence or luminescence (depending on substrate used).

IκBβ Blot. Cells were treated in 6-well plates with compounds for 2 h and then treated with 100 ng mL^{-1} LPS for the indicated period of time. Lysates were probed by Western blot for IκBβ and actin for loading control. In a parallel LT assay, all compounds protected ∼100% from LT-induced cell death.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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