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Chemical Genetic Screening Identifies Critical Pathways in Anthrax Lethal Toxin-Induced Pathogenesis

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SUMMARY

Anthrax lethal toxin (LT)-induced cell death via mitogen-activated protein kinase kinase (MAPKK) cleavage remains questionable. Here, a chemical genetics approach was used to investigate what pathways mediate LT-induced cell death. Several small molecules were found to protect macrophages from anthrax LT cytotoxicity and MAPKK from cleavage by lethal factor (LF), without inhibiting LF enzymatic activity or cellular proteasome activity. Interestingly, the compounds activated MAPK-signaling molecules, induced proinflammatory cytokine production, and inhibited LT-induced macrophage apoptosis in a concentration-dependent manner. We propose that induction of antiapoptotic responses by MAPK-dependent or -independent pathways and activation of host innate responses may protect macrophages from anthrax LT-induced cell death. Altering host responses through a chemical genetics approach can help identify critical cellular pathways involved in the pathogenesis of anthrax and can be exploited to further explore host-pathogen interactions.

INTRODUCTION

Host-pathogen interactions are regulated both by virulence factors and by host components that either protect the host or promote pathogenesis. Historically, significant efforts have focused on identifying microbial targets for the development of both therapeutic and preventive measures, while the host factors involved in microbial pathogenesis have not been fully explored. Identifying host proteins that are involved in the pathogenesis of a microbial disease and developing host-oriented therapeutics will help circumvent the problems of acquired or engineered resistance to existing antimicrobials.

Secreted by *Bacillus anthracis*, anthrax lethal toxin (LT), a pore-forming heterocomplex of protective antigen (PA) and lethal factor (LF), cleaves several members of the mitogen-activated protein kinase kinase (MAPKK/MEK) family [1–3]. Recent studies suggest that LT severely impairs the host immune response by disrupting the MAP kinase-signaling network in macrophages [1–4] and in dendritic cells [5, 6], thereby allowing the growth of bacteria and establishment of infection. However, cytotoxicity studies revealed that cultured macrophages from inbred mouse strains vary in their sensitivity to LT-induced cell death [3, 7]. Certain mouse strains whose macrophages are resistant to LT-induced cytotoxicity exhibit MAPKK cleavage [3]. In a recent study, we showed a relationship between LT-induced MAPKK cleavage and impaired bactericidal activity of nonhuman primate alveolar macrophages in the absence of cell death [8]. Thus, these studies suggest that there is no direct relationship between MAPKK cleavage and susceptibility to LT-induced cytotoxicity and that other cellular proteins besides MAPKK may be involved in anthrax pathogenesis.

An emerging technology, chemical genetics utilizes small organic molecules as probes in high-throughput cell-based screening assays to identify critical signaling pathways that regulate biological function [9, 10]. To better understand the mechanism of anthrax LT-induced cell death, we used a diverse library of small molecules to perturb macrophage function during anthrax LT treatment. Cell viability assays identified several compounds that protected macrophages from LT-induced cell death. These compounds did not inhibit in vitro LF enzymatic activity or cellular proteasome activity. However, the compounds did protect MAPKK from cleavage by LF in macrophages, thus suggesting that the activation or inhibition of cellular factor(s) was responsible for the observed macrophage viability following LT treatment. The compounds activated ERK1/2, p38, and SAPK/JNK MAPK-signaling molecules and inhibited LT-induced apoptosis of macrophages. Possible induction of antiapoptotic

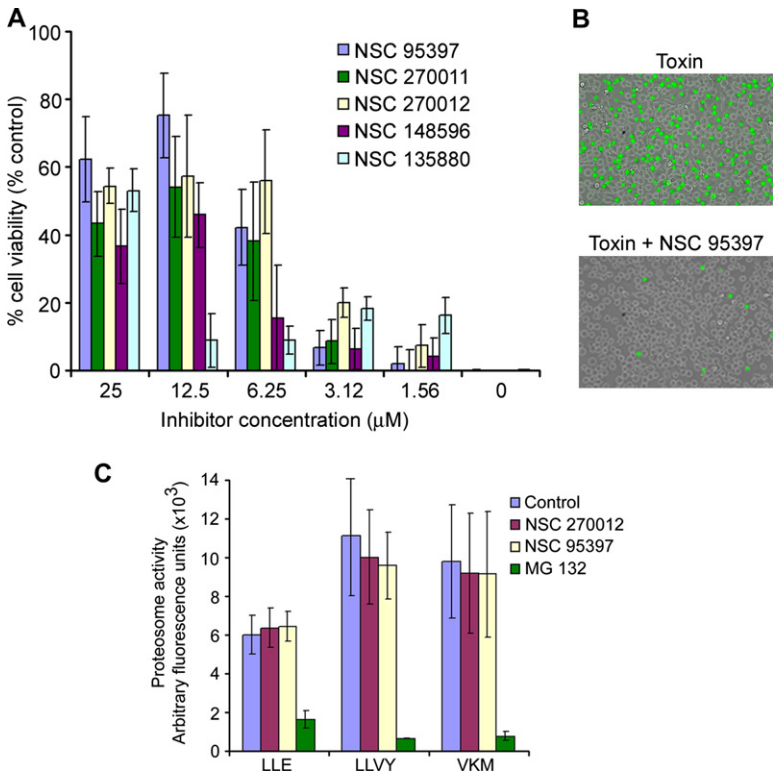


Figure 1. Bioactive Small Molecules Protect Macrophages from the Lethal Action of Anthrax Toxin and Do Not Inhibit Proteasome Activity

(A) J774A.1 macrophages were pretreated with medium containing DMSO (control) or with the small molecules for 30 min and then further incubated with the anthrax LT. After 4 hr, cell viability was measured using a MTT assay. The data represent the averages ± SD for three independent experiments.

(B) Live imaging of J774A.1 macrophages treated with anthrax LT alone (top panel) or with the anthrax toxin and compound NSC 95397 (bottom panel) in the presence of the membrane-impermeable SYTOX green dye. The 4 hr incubation time is shown in the figure.

(C) Compounds do not inhibit proteasome activity. Cell lysates prepared from untreated J774A.1 cells were incubated with 100 μM of the different fluorogenic peptide substrates (SLLVY-AMC, Z-LLE-AMC, and Z-VKM-AMC) in the presence of either medium containing DMSO (control), compound NSC 270012 (20 μM), NSC 95397 (20 μM), or MG132 (10 μM). Increased fluorescence was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm using a fluorescence plate reader. Data shown are from the 30 min incubation time point. The data represent the averages ± SD for three independent experiments.

genes through MAPK-dependent or -independent pathways may be responsible for the observed phenotype.

RESULTS

Small Molecules Protect Macrophages from Anthrax Lethal Toxin-Induced Cytotoxicity but Do Not Inhibit LF or Proteasome Activity

To identify compounds that inhibit anthrax lethal toxin cytotoxicity, we screened a chemically diverse set of small molecules from the National Cancer Institute (NCI) Open Chemical Repository (~1000 compounds) in a toxin-induced cell death assay. J774A.1 macrophages in multiwell plates were preincubated with either medium containing DMSO (control) or compounds, and then anthrax toxin was added. Cell viability was determined using a MTT dye assay. Five compounds, NSC 148596, NSC 135880, NSC 95397, NSC 270011, and NSC 270012, inhibited anthrax LT cytotoxicity with ED_{50} values ranging from 5 to 25 μM (Figure 1A). At concentrations above 25 μM, the compounds themselves were toxic to the cells, but below 25 μM their efficacy to enhance survival increased in a dose-dependent manner. We confirmed the protective effect of the most potent compound, NSC 95397, by visualizing with time-lapse imaging the uptake of the membrane-impermeable SYTOX green dye by dead cells (for complete movies, see Movies S1 and S2). When treated with both NSC 95397 and toxin, relatively few macrophages took up the dye (Figure 1B, bottom panel) compared to those treated with toxin alone (Figure 1B, top panel), thus showing that the compound could indeed protect J774A.1 macrophages from the lethal action of anthrax toxin.

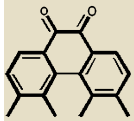
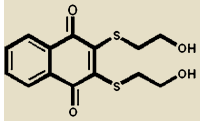
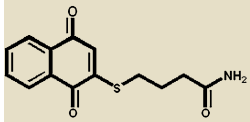
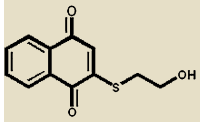
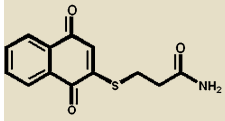
To determine if the identified compounds protected J774A.1 cells by inhibiting LF enzymatic activity, we tested all five compounds in vitro using an HPLC-based LF assay [11]. None of the compounds inhibited the ability of LF to cleave peptide substrate (data not shown), suggesting that the compounds protected the cells by acting on a cellular component within the macrophages.

The proteasome, a multicatalytic protease, causes extralysosomal degradation of cellular proteins and plays a role in apoptosis [12]. Functional proteasome activity is reported to be indispensable for anthrax LT to kill macrophage-like cell lines such as RAW264.7 [13]. To determine if the identified compounds inhibited proteasome activity in macrophages, protein lysates from macrophages were incubated with three different fluorogenic proteasome substrates (LLE, LLVY, and VKM) in the presence of either NSC 95397, NSC 270012, or a known proteasome inhibitor, MG132. As shown in Figure 1C, neither NSC 95397 nor NSC 270012 inhibited proteasome activity compared to MG132, suggesting that they protected anthrax LT-treated cells by a mechanism other than inactivating the proteasome machinery.

Identified Compounds Inhibit Cdc25B In Vitro, but Not in Toxin-Treated Macrophages

Because the identified compounds do not directly inhibit anthrax LF enzymatic activity in vitro, this strongly

Table 1. Chemical Structures of Small-Molecule Inhibitors with Percent Cdc25B Inhibition at a Compound Concentration of 10 μM, and ED_{50} Values for Macrophage Protection

Structure	NSC Number	% Inhibition (Cdc25B)	ED_{50} (μM)
	135880	99	25.0
	95397	83	6–12
	270012	72	6–12
	148596	67	—
	270011	25	6–12

suggests that they targeted cellular proteins. The two compounds, NSC 95397 and NSC 135880, were previously reported by Lazo and coworkers [14] as Cdc25 dual-specificity phosphatase inhibitors. To determine if the identified compounds inhibited Cdc25B activity, we tested them in an in vitro phosphatase assay. The identified compounds exhibited a broad range of Cdc25B inhibition. Table 1 shows the chemical structures of the five inhibitors, their percentage phosphatase inhibition at concentrations of 10 μM, and their ED_{50} values (obtained from the cell-based MTT assay).

Cdc25B is a dual-specific phosphatase that regulates the entry of all eukaryotic cells into mitosis by activating the cdc2/cyclin B mitotic kinase complex [15]. Although activation of cdc2 during progression into mitosis is controlled at several different steps, the critical regulatory step is dephosphorylation of its Thr14 and Tyr15 residues [16, 17]. To investigate if Cdc25B was a cellular target involved in anthrax LT-induced cell death, we determined the phosphorylation state of cdc2 and if G2/M arrest occurred in J774A.1 macrophages following compound treatment. Cell lysates from compound-treated macrophages did not show increased phosphorylation of cdc2 either by western blotting or after immunoprecipitation of the cdc2/cyclin B complex. In addition, the macrophages did not exhibit G2/M arrest following compound treatment, although G2/M arrest could be achieved using nocodozole (data not shown). Thus our data suggest that Cdc25B was not involved in anthrax LT pathogenesis.

The Compounds Prevent Cleavage of MAPKK and Activate Downstream MAPK-Signaling Molecules

Anthrax LF, a Zn^{2+}-dependent metalloprotease, cleaves several isoforms of MAPKK (MEK), including MEK1, MEK2, MEK3, MEK4, MEK6, and MEK7, but not MEK5 [3, 18, 19]. To determine the MEK cleavage pattern in the macrophages, cell lysates prepared from J774A.1 cells pretreated with NSC 95397 or NSC 270012 (20 μM), in the presence or absence of anthrax LT, were analyzed by western blotting using antibodies to the MEK isoforms MEK1, MEK2, MKK3, and MKK4. As seen in Figure 2A, the compounds partially to completely protected each of these four isoforms of MEK, which correlates with the observed protection against cytotoxicity after toxin and compound treatment (Figure 1).

Cleavage of the different isoforms of MEK by anthrax LT disrupts downstream signaling to molecules including the extracellular regulated kinases (ERK1 and ERK2, also called p44/42), p38 kinase, and SAPK/JNK. Because we observed partial to complete protection from enzymatic cleavage of the different isoforms of MEK (Figure 2A), we next examined the phosphorylation states of downstream MAPK-signaling molecules. We prepared cell lysates from J774A.1 cells treated with or without toxin and in medium containing DMSO (control), NSC 95397, or NSC 270012 (20 μM). The lysates were analyzed by western blotting using antibodies that recognize either the phosphorylated or total levels of endogenous ERK1/2, p38, and SAPK/JNK. Our results show that, compared to control-treated J774A.1 cells, macrophages treated with the compounds showed increased phosphorylation of ERK1/2, p38, and SAPK/JNK, both in the absence and presence of LT (Figure 2B). These results suggest that, regardless of the toxin treatment, the compounds were able to activate MAPK-signaling molecules. A time-dependent study of the phosphorylation state of MAPK revealed an increase in the phosphorylation states of ERK1/2, p38, and SAPK/JNK within 60 min in NSC 95397-treated macrophages, compared to controls (Figure 2C). Similar results were observed with NSC 270012 (data not shown). Although control (medium containing DMSO) treatment alone seemed to show high levels of phosphorylated ERK1/2, densitometric scans from three independent experiments showed a 1- to 3-fold fold increase in ERK1/2 phosphorylation in compound-treated macrophages (data not shown). The observed increased phosphorylation was more pronounced for ERK2 than ERK1.

MAPK Nuclear Translocation and Activation of Transcription Factors

Once activated, MAPKs such as ERK1/2, p38, and SAPK/JNK can either phosphorylate other kinases in the cytoplasm or they may translocate to the nucleus, where they phosphorylate and activate nuclear proteins such as transcription factors. To determine if activated p38 translocated to the nucleus following compound

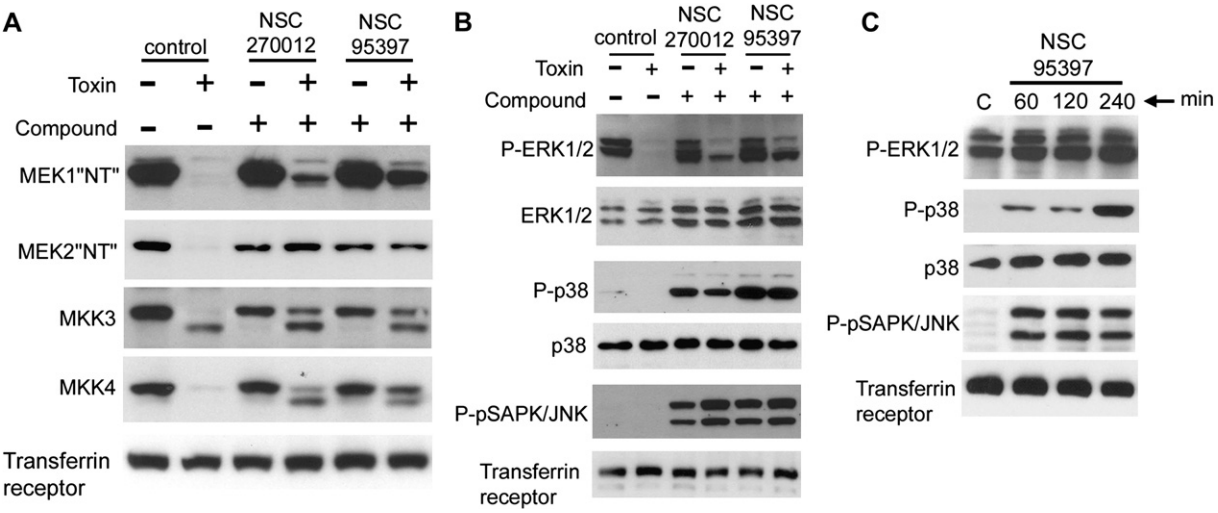


Figure 2. Partial to Complete Protection of MEK Cleavage and Activation of MAPK-Signaling Molecules by Bioactive Small Molecules

J774A.1 cell lysates were treated with either medium containing DMSO (control) or anthrax toxin and in the presence or absence of compounds NSC 2700012 or NSC 95397 (20 μM). After 4 hr of incubation, cell lysates were prepared and analyzed by western blotting using antibodies to (A) MEK1 NT, MEK2 NT, MKK3, and MKK4 and (B) phospho- (P)-ERK1/2, P-p38, P-pSAPK/JNK, ERK1/2, p38, and transferrin receptor. (C) Macrophages were treated with compound NSC95397 and at time points of 60, 120, and 240 min, and cell lysates were prepared and western blotted with antibodies that recognize either the total p38 or phosphorylated ERK1/2, p38, SAPK/JNK. The control ("C") lane is lysate prepared from macrophages incubated for 240 min in medium containing DMSO.

treatment, we treated cells with medium containing DMSO (control) or NSC 95397 (20 μM) for 4 hr. The cells were then fixed, stained with p38 antibody, and visualized by confocal microscopy (Figure 3A). Phosphorylated p38 localized to the nucleus after compound treatment, while nuclear staining of p38 was not observed in control cells.

To determine if compound-activated MAPKs were functionally active and could phosphorylate specific transcription factors, we measured kinase activity in protein lysates from compound and/or toxin-treated cells using Elk-1, ATF-2, and c-*jun* as substrates of the ERK1/2, p38, and SAPK/JNK kinases, respectively. As shown in Figure 3B, compound-activated MAPKs, in either the absence or presence of the toxin, can phosphorylate their respective substrates in vitro. Taken together, these studies suggest that the compounds, through an unknown mechanism, activated ERK1/2, p38, and SAPK/JNK MAPK-signaling molecules, which then translocated to the nucleus and phosphorylated transcription factors.

Induction of Proinflammatory Cytokines

Prior studies report that anthrax LT severely impairs the ability of the macrophages to induce proinflammatory cytokines [3, 21]. Because the compounds activated MAPK signaling both in the absence and presence of toxin, we next investigated if this induced production of proinflammatory cytokines. We treated macrophages with medium containing DMSO (control), NSC 95397, or NSC 270012 for 4 hr in either the absence or presence of toxin. Culture supernatants were analyzed for the presence of cytokines, including IL-12p70, MCP-1, TNF-α, IL-10, and IL-6. The compounds, either in the absence or presence of LT, induced high levels of TNF-α, while LT alone impaired the ability of macrophages to secrete TNF-α (Figure 4). There was no increase in secretion of IL-12p70, IL-10, and IL-6 by macrophages after compound treatment (data not shown). The macrophages constitutively produced high levels of MCP-1 (>5000 pg/ml), and these levels were not altered after toxin and/or compound treatment (data not shown). Our results suggest that MAPK phosphorylation (Figure 3B) and the induction of signals via TNF-α receptors most likely provide survival signals to macrophages.

The Compounds Inhibit LT-Induced Apoptosis in Macrophages

Anthrax LT induces apoptosis of susceptible [22] or activated macrophages [2]. To investigate if the compounds inhibited LT-induced apoptosis, J774A1 macrophages were incubated for 3 hr (Figure 5A, left panel) or 4 hr (Figure 5A, right panel) with anthrax LT in the absence or presence of different concentrations of NSC 95397 and then stained with a mixture of YO-PRO-1, a green fluorescent dye that detects early apoptosis-specific changes in membrane permeability, and PI, a late apoptosis/necrosis-specific red fluorescent dye. Hoechst dye (blue) was used to stain the cell nuclei. Cells treated with the toxin and compound showed a concentration-dependent inhibition of apoptosis (Figure 5A, bottom panels) and increased cell viability (Figure 5A, top panels) compared to toxin control. Representative images of macrophages

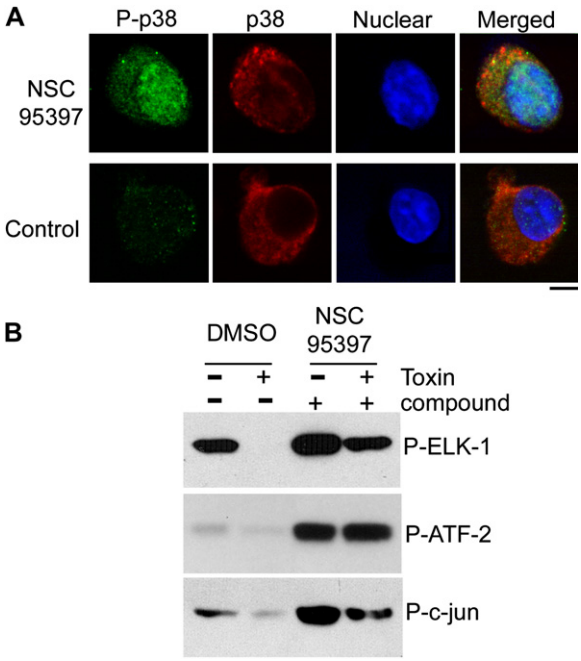


Figure 3. Activated p38 Is Translocated to the Nucleus and Phosphorylates Transcription Factors In Vitro

(A) J774A.1 cells were treated with medium containing DMSO (control) or NSC 95397 (20 μM) for 4 hr and then fixed, permeabilized, and stained with p38 or phospho-p38 (P-p38) primary antibodies, which were then detected using Alexa 568- or Alexa 488-conjugated secondary antibodies, respectively. The nucleus was stained with Hoechst dye. Scale bar = 5 μm.

(B) The kinase activity of compound-activated ERK1/2, p38, and SAPK/JNK signaling molecules was examined by in vitro kinase assays using Elk-1, ATF-2, and c-*jun* as substrates respectively.

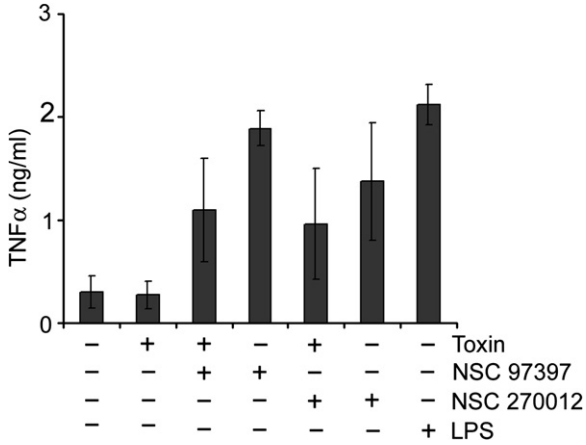


Figure 4. Compounds Induce TNF-α Secretion

J774A.1 macrophages were untreated or treated with either LPS (100 ng/ml), medium containing DMSO, anthrax toxin (PA 80 ng/ml and LF 16 ng/ml), NSC 95397 (20 μM), NSC 270012 (20 μM), or a combination of compound and toxin. After 4 hr, culture supernatants were harvested and analyzed by cytometric bead array. The data represent average ± SD macrophage responses from at least three individual experiments.

treated with anthrax LT and NSC 95397 (Figure 5B, bottom panel) show that fewer cells take up the apoptosis indicator green fluorescent YO-PRO-1 dye (Figure 5B, left panel) and the late apoptosis/necrosis red fluorescent PI dye (Figure 5B, middle panel) compared to the toxin control (Figure 5B, top panel).

DISCUSSION

To date, the cellular and molecular interactions between *B. anthracis* virulence factors and host cells have been poorly understood. Anthrax LT, a virulence factor produced by *B. anthracis*, turns off host immune responses by disrupting MAPK-signaling pathways in immune cells such as macrophages [1–4] and dendritic cells [5, 6]. This inhibits the clearance of the bacteria by phagocytosis and ultimately results in massive bacteremia and toxemia. Anthrax LT at high concentration causes cytolysis of susceptible J774A.1 macrophages. However, macrophages from different inbred strains of mice vary in their sensitivity to the lethal effects of anthrax LT [7, 23]. Furthermore, macrophages resistant to LT-induced cell death still exhibit MEK cleavage [7]. This indicates that there is no direct relation between MEK cleavage and cell death, and hence additional cellular factors may play a role in LT-induced cytotoxicity. A number of other cellular processes or factors that have been reported to participate in anthrax LT-induced cytotoxicity include proteasome activity [13], intracellular calcium stores [24, 25], protein synthesis [26], and reactive oxygen intermediates [27]. Recent studies by Wei et al. [28] show that low-density lipoprotein receptor-related protein 6 (LRP6), a cellular protein, is involved in the internalization of the anthrax LT complex. Intracellular signaling molecules like phosphatases have also been implicated in anthrax LT-induced cytotoxicity [20]. These studies suggest that anthrax LT modulates a plethora of effects, and that the concerted action of multiple cellular players is responsible for anthrax pathogenesis.

Identifying critical pathways and host cell proteins that play a key role in either the early stages of infection, or during late stages when high levels of LT are produced, would help researchers better understand the disease process and help them develop novel therapeutic approaches. In recent years, small organic molecules have been extensively exploited as tools to perturb biological systems. By activating or inactivating protein functions, compounds identified in chemical genetic screens can help uncover pathways and cellular targets involved in normal or disease processes [9, 29]. However, formidable problems with using the chemical genetics approach include identifying the cellular target proteins and possible indirect effects of the small molecules. Nonetheless, mechanistic studies in combination with bioinformatics and biological simulation models can provide clues to the compounds' mechanisms of action, the interplay between different cellular players, and the possible roles of associated targets in pathogenesis.

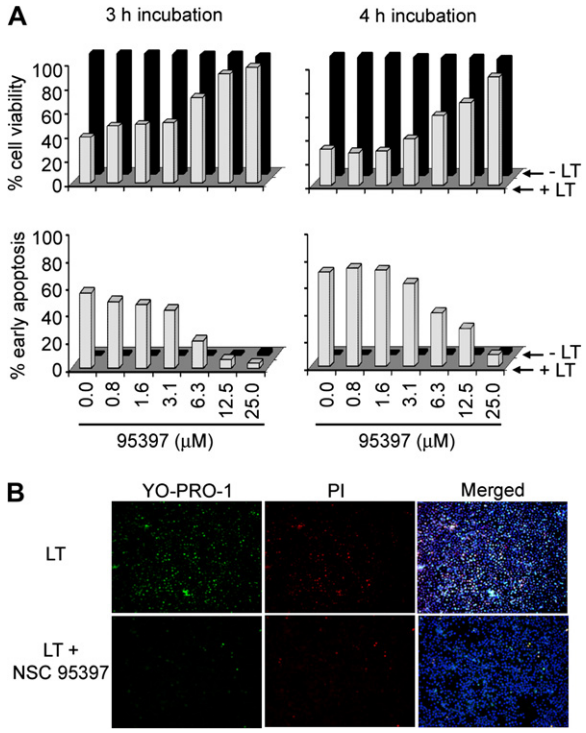


Figure 5. Compounds Inhibit Anthrax LT-Induced Apoptosis

(A) Representative data of J774A.1 macrophages incubated for 3 hr (left panel) or 4 hr (right panel) with anthrax LT in the absence or presence of different concentrations of the compound NSC 95397. The compound showed dose-dependent inhibition of LT-induced apoptosis (bottom panel) and increased cell viability (top panel).

(B) Representative images of macrophages treated with either anthrax LT (top panels) or LT and NSC 95397 (25 μM; 4 hr incubation) (bottom panels) and showing apoptosis as indicated by uptake of YO-PRO-1 green fluorescent dye (left panel) or late apoptosis/necrosis as indicated by uptake of PI, a red fluorescent dye (middle panel). The right panel shows green and red fluorescence merged with Hoechst staining of all nuclei (blue).

In the present study, we used a chemical genetics approach combined with a phenotypic screen to reveal pathways and cellular targets that play a role in anthrax LT-induced cell death. Small molecules from the NCI Open Chemical Repository (>1000) were screened for their ability to protect macrophages from the lethal action of anthrax toxin. Subsequently, several compounds were found to protect macrophages from LT-induced cytotoxicity, most likely by a mechanism that activates MAPKs and induces the proinflammatory cytokine TNF-α, both of which may provide survival signals to macrophages. The compounds show a concentration-dependent inhibition of macrophage apoptosis, and their protective action is not mediated by inhibition of LF, the proteosome, or the Cdc25B phosphatase activity in anthrax LT-treated macrophages.

One characteristic feature of the identified compounds (NSC 95397, NSC 135880, NSC 270011, and NSC 270012; Table 1) is that they are all good electrophiles and, thus, have the potential to modify amino and thiol groups of the proteins indiscriminately. Despite these seemingly ubiquitous structural motifs, studies by Lazo et al. [14] have shown that compounds NSC 95397 and NSC 135880 are potent and selective inhibitors of Cdc25B compared with either the dual-specificity phosphatase VHR or the protein tyrosine phosphatase PTP1B. One implication of these findings is the prospect that specific modifications to the electrophilic phenanthrene and naphthalene dione scaffolds of NSC 135880 and NSC 95397 (respectively) may generate greater specificity for their intended target. This is particularly interesting given that these compounds demonstrated irreversible binding, thus signifying the likelihood of covalent modification of Cdc25B.

Although Cdc25 dual-specificity phosphatases are important regulators of cell cycle control and mitogenic signal transduction, our cell cycle experiments showed that Cdc25B was not the cellular target of the compounds in macrophages. An interesting observation during the cell cycle study was that cells arrested in the G2/M phase using nocodozole were less susceptible to anthrax LT-induced death compared to cells that were released from G2/M arrest and then treated with LT (Figure S1). The functional significance of this observation remains to be determined.

In our study, the compounds NSC 95397 and NSC 270012 induced the phosphorylation of MAPK-signaling molecules (ERK1/2, p38, and SAPK/JNK) in J774A.1 macrophages. Induced phosphorylation of ERK1/2 by NSC 95397 and its hydroxyl derivatives, as well as Cpd 5, a naphthoquinone derivative, and PM-20, a phenyl maleimide, has recently been reported in Hep3B human hepatoma cell line [30, 31]. A fluorinated derivative of Cpd5, also induced the phosphorylation of ERK1/2, p38, and JNK1/2 signaling in Hep3B cells [32]. The induced phosphorylation of ERK1/2 in Hep3B cells is likely due to inhibition of an (as yet) undefined ERK phosphatase(s) activity but not of the dual-specificity ERK phosphatase MKP-1 [30–32].

The exact mechanism of how the identified compounds protect macrophages remains unclear. One possibility is that the compounds may be preventing the entry of LF into the cells. However, our in vitro ELISA studies (data not shown) revealed that the LF can bind to $(PA_{63})_7$ in the presence of NSC 95397. Since this compound does not interfere with the interaction of LF with $(PA_{63})_7$, this suggests that LF can enter the cells in the presence of the compound. In a recent study, Kau et al. [20] hypothesized that hyperphosphorylated MEK1 protein may not be a suitable target for anthrax LT. However, we did not observe hyperphosphorylation of MAPKK after treating macrophages with our compounds (data not shown). In addition we found that anthrax LF in vitro cleaved both the phosphorylated and nonphosphorylated forms of MEK (Figure S2). Thus the observed protection in our cell-based assay was not due to the inability of LT to cleave hyperphosphorylated MAPKK. To address the question regarding why active LF cannot efficiently cleave endogenous MAPKK, microarray and protein array

studies are ongoing to examine the effects of the compounds on the macrophages in presence of anthrax toxin.

An interesting observation in our study was the concentration-dependent inhibition of LT-induced apoptosis by the compounds. This effect could be mediated through the activated MAPK-signaling pathway and, perhaps, specifically by activated p38. Prior studies by Park et al. [2] showed that survival of activated macrophages depends upon the activity of p38 and NF-κB signaling molecules. After macrophage activation, the p38-dependent, antiapoptotic signal is relayed to the cAMP-responsive transcription factor (CREB), which in turn synergizes with NF-κB to induce the transcription of two antiapoptotic genes, serpin plasminogen-activator inhibitor 2 (PAI-2) and Bfl-1/A1 [33]. It has also been shown that in activated human monocytes, phosphorylated p38 stabilizes TNF-α and antiapoptotic Bcl2 mRNA transcripts [34] and protects against TNF-α-mediated apoptosis [35].

Alternatively, crosstalk between TNF receptors, MAPKs, and NF-κB may account for the compound-induced protection against LT-mediated cytotoxicity. Ligation of TNF-α to its receptors can induce phosphorylation of p38, SAPK/JNK, and ERK signaling molecules [36–38]. Activated ERK phosphorylates TNF receptors in macrophages [39, 40], which protects these cells from apoptosis in a silicosis model [41]. Moreover, TNF receptor-activated NF-κB signals also protect cells from apoptosis by inducing expression of TRAF1/2 and cIAP1/2 genes [42].

Based on the observations in this study and data from the published literature (discussed above), we propose a model for how the compounds may be protecting macrophages from anthrax LT-induced cell death (Figure 6). In particular it appears that the nature of the stimulus and the triggered signaling pathways determine either apoptotic or antiapoptotic responses (Figure 6A). For example, activation of Toll-like receptor 4 (TLR4) induces macrophage apoptosis through signaling pathways that are not yet well characterized [43]. On the other hand, TLR4 activation can also initiate antiapoptotic responses through activation of p38 MAPK-signaling pathways. The pathogen *B. anthracis* produces LF, a virulence factor that inhibits MAPK activation. This contributes to the apoptosis of activated macrophages [2]. In our study, the compound-induced activation of MAPKs triggered an antiapoptotic response (Figure 6B). This response may be mediated via activation or inactivation of unidentified cellular factor(s) or inhibition of phosphatase(s) downstream of the MAPKK-signaling molecules. Activated MAPKs may synergize with NF-κB to induce antiapoptotic gene expression resulting in survival of the LT-treated macrophages. Alternatively, induced secretion of cytokines like TNF-α by the compounds may be responsible for activating MAPK-signaling molecules. Our data did not distinguish whether the compounds or the induced TNF-α that activated the MAPK-signaling molecules. The contribution of activated SAPK/JNK signaling molecules in compound-induced cell survival still remains to be determined.

Although our study did not identify the direct target(s) of our protective compounds in macrophages, we did rule out several leading hypotheses and highlighted the importance of activated p38 and ERK1/2 to macrophage survival. This demonstrates the power of applying a chemical genetic approach to the study of host-pathogen interactions for identifying the host proteins and pathways involved in infection and disease progression. This approach can be exploited to identify the key host players in other pathogenic systems, both to better understand the disease process and to identify host-based therapeutic targets, which may be more effective against resistant bacterial strains or engineered biothreat organisms.

SIGNIFICANCE

Chemical genetics involves the use of small molecules as probes to perturb dynamic cellular processes, thereby helping define critical pathways and factors involved in health and disease. In this study, we employed molecular scaffolds with pharmacophoric diversity to identify compounds that protected macrophages from the lethal action of the anthrax toxin. Next, we selected the subset of active compounds that lacked direct inhibitory activity on LF or the cellular proteasome in order to explore downstream targets related to the anthrax toxin mechanism of action. Interestingly, compounds fulfilling these criteria were previously identified as inhibitors of a downstream target to Cdc25 dual-specificity phosphatases, thus exposing a common link between targets of interest for cancer and bioterror therapeutics. Subsequent investigations demonstrated that these compounds induce the phosphorylation of MAPK-signaling molecules and the production of the proinflammatory cytokines such as TNF-α. We propose that induction of antiapoptotic responses via MAPK-dependent or -independent pathways and the activation of host innate responses may protect macrophages from anthrax LT-induced cell death. More importantly, this type of chemical genetic approach can provide new insights into the function of several signaling pathways during the pathogenesis of multiple diseases that share common downstream pathways.

EXPERIMENTAL PROCEDURES

The Compounds

Small molecules examined in this study were obtained from the NCI Open Chemical Repository. The chosen compounds comprise a range of molecular scaffolds covering a diversity of pharmacophoric space. Based on the structure of NSC 95397, related napthoquinone- and dione-containing derivatives were subsequently examined.

Cell-Based Cytotoxicity Assay

J774A.1 macrophages were preincubated with medium containing 2% DMSO (control) or different concentrations of the NCI compounds ranging from 0 to 25 μM. After 30 min, cells were treated with PA (80 ng/ml) and LF (16 ng/ml). After a 4 hr incubation with the toxin, 25 μl of MTT (1 mg/ml) dye was added and the cells were further incubated for 2 hr. The reaction was stopped by adding an equal volume of lysis buffer (50% DMF and 20% SDS [pH 4.7]). Plates were incubated overnight at 37°C and absorbance read at 570 nm in a multiwell plate reader.

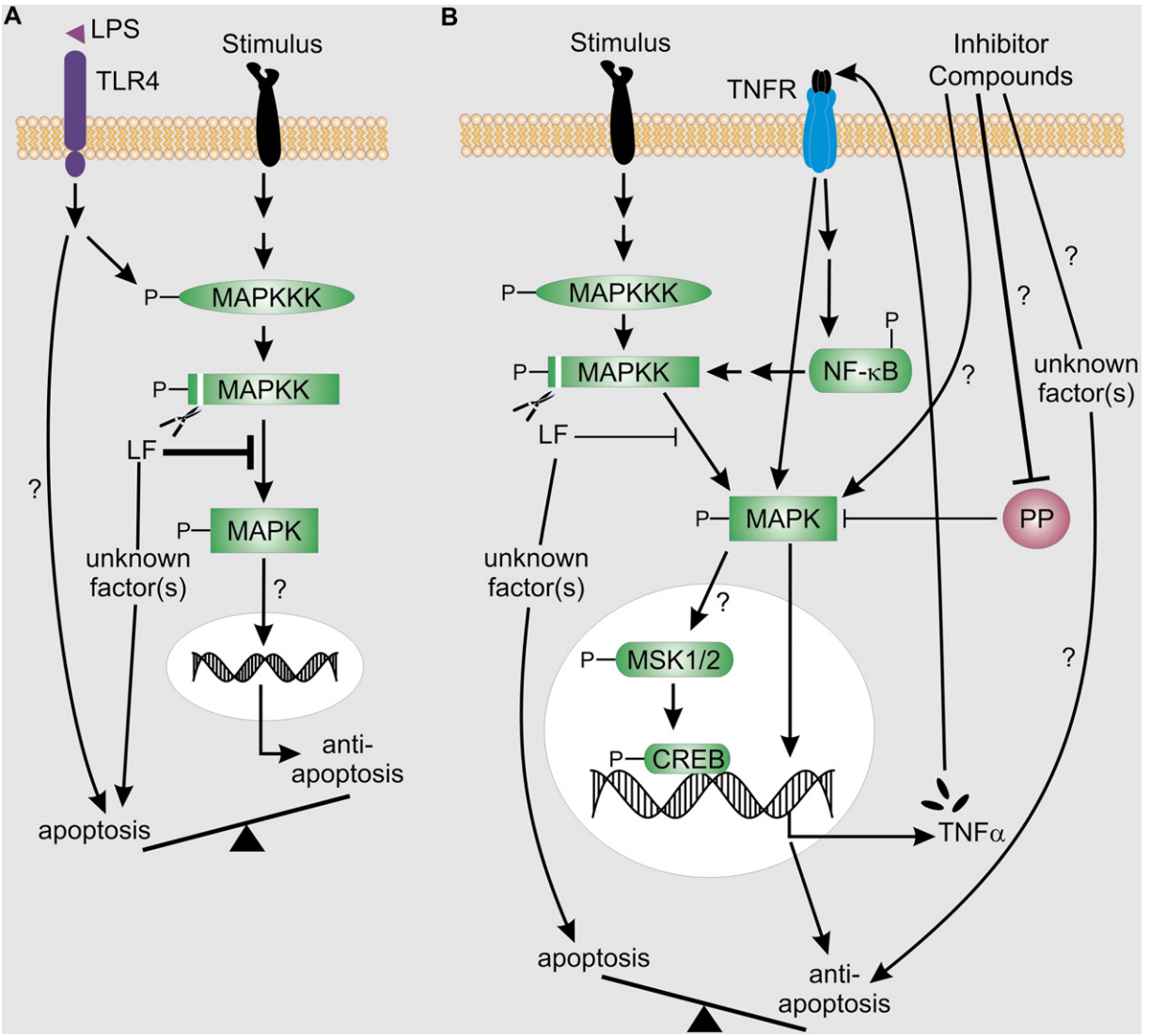


Figure 6. Proposed Model of LT-Treated Macrophage Survival Based on our Mechanistic Studies and Published Literature

(A) Different stimuli can trigger apoptotic and antiapoptotic pathways [2, 33, 35–40, 42]. TLR4 activation can induce either an apoptotic response or, via MAPK activation, can trigger antiapoptotic response. Anthrax LF cleaves MAPKK and disrupts downstream signaling events, thereby inducing apoptosis of susceptible macrophages.

(B) Compounds, via inhibition of protein phosphatases (PP) or activation/inactivation of unknown cellular factor(s), activate MAPK-signaling molecules and thereby trigger antiapoptotic response. Alternatively, induction of proinflammatory cytokines like TNF-α by the compound may activate MAPK-signaling molecules.

In Vitro Cdc25b Phosphatase Assay

The in vitro activity of the Cdc25B phosphatase was measured as described previously [14, 44]. Briefly, reaction mix (25 μl) containing assay buffer (30 mM Tris [pH 8.0], 75 mM NaCl, 1 mM EDTA, 0.033% BSA, and 1 mM DTT), 40 μM of 3-O-methyl fluorescein phosphate (OMFP) substrate, 0.7 μg/ml of His-Cdc25B catalytic domain, and compound or DMSO (control) was incubated for 1 hr at room temperature. Removal of the phosphate group from the substrate by Cdc25B caused an increase in fluorescence that was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm.

HPLC-Based LF Assay

The HPLC-based LF assay was carried out as described previously [11]. Briefly, the reaction was set up in a 30 μl volume. Master mix containing 40 mM HEPES buffer (pH 7.2), 0.05% Tween 20, 100 μM $CaCl_2$, and 1 μg/ml of LF was added to each tube containing 20 μM peptide and 10 μM of select compounds. The reaction mixture was incubated for 30 min at 30°C and then stopped by adding solution containing 8 M guanidine hydrochloride and 0.3% TFA. The cleaved products were separated on a Hi-Pore C18 column (Bio-rad laboratories, Richmond, CA) using 0.1% TFA (solvent A) and 0.1% TFA/70% acetonitrile (solvent B). The column effluent was monitored at 365 nm, where the substrate and C-terminal cleavage products showed stronger absorbance.

Measurement of Proteasome Activity

J774A.1 macrophages were lysed by sonication (3 × 5 s pulses) in proteasome buffer (50 mM Tris [pH 7.5], 140 mM NaCl, 5 mM $MgCl_2$, 1 mM

EGTA, 10% glycerol, 0.5 mM DTT, 2 mM ATP, and protease inhibitor cocktail). After centrifugation, cell extracts containing 40 μg of total protein were incubated with 100 μM of the different fluorogenic peptide substrates (SLLVY-AMC, Z-LLE-AMC, and Z-VKM-AMC) and in the presence of the compounds NSC 270012 or NSC 95397 (20 μM), or proteasome inhibitor MG132 (10 μM). Kinetic measurements were made every minute for 30 min at an excitation and emission maxima of 380 and 460 nm, respectively, using a fluorescence plate reader (Tecan, Safire).

Immunoblot Analysis

J774A.1 macrophages (~1 × 10^6) seeded in a six-well plate were preincubated for 30 min at 37°C with either medium containing DMSO (control) or with 20 μM of the compounds NSC 95397 or NSC 270012. Anthrax LT (80 ng/ml PA and 16 ng/ml LF) was added to the appropriate wells and further incubated for various time points as indicated in the figure legends. Macrophages were harvested and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1% Triton X-100, 10 mM NaF, 1 mM sodium orthovanadate, protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktail I and II (Sigma). The cell lysates were incubated for 30 min on ice, and insoluble material was removed by centrifugation. Cell extracts (30 μg) were electrophoresed on SDS-PAGE and then subjected to western blotting. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL).

Antibodies

Antibodies used in this study included antibodies that recognize p38 and phospho-p38 MAP kinase, ERK1/2 and phospho-ERK1/2 MAP kinase, and phospho-SAPK/JNK and pan-SAPK/JNK. These were all purchased from Cell Signaling Technology, Beverly, MA. Anti-MEK1 NT was from Upstate Biotechnology, while antibodies to MKK2 NT, MKK3 C-19, and MKK4 C-20 were from Santa Cruz Biotechnology.

Live Imaging and Confocal Microscopy

For live imaging studies, J774A.1 macrophages were treated either with toxin (80 ng/ml PA and 16 ng/ml LF) diluted in medium containing DMSO (2%) or with toxin and NSC 95397 (20 μM). Live imaging of the cells was carried out in the presence of the membrane-impermeable nucleic acid dye SYTOX green (Molecular Probes, Eugene OR) over a period of 4 hr using a Nikon TE300 inverted microscope with a heated enclosure to maintain the cells at 37°C. Bright-field and fluorescence image pairs were collected at 10 min intervals with a SPOT RT CCD camera (Diagnostic Instruments, Sterling Heights, MI) and two Uniblitz shutters (Vincent Associates, Rochester, NY) controlled by MetaMorph imaging software (Molecular Devices Corporation, Downingtown, PA).

To determine the subcellular localization of activated p38, J774A.1 grown on chambered coverslips were incubated with medium containing DMSO (control) or NSC 95397 (20 μM) for 4 hr and then fixed with 3.7% formaldehyde and permeabilized in 0.2% Triton X-100 for 15 min at room temperature. The cells were blocked overnight at 4°C in buffer consisting of 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), incubated with primary antibody overnight at 4°C, washed three times with PBS, and then treated with the appropriate Alexa 488- or Alexa 568-conjugated secondary antibodies for 1 hr at 37°C. The cells were washed three times with PBS and then treated with Hoechst dye to stain the cell nuclei. Fluorescence was visualized using a Bio-Rad 2000MP confocal/multiphoton system attached to a Nikon TE300 inverted microscope. Images of cells with and without compound were collected using identical settings, and any subsequent contrast enhancement was likewise performed identically.

Kinase Assays

J774A.1 macrophages were preincubated with either medium containing DMSO (control) or NSC 95397 alone for 30 min and then further incubated in the absence or presence of anthrax LT (80 ng/ml PA and 16 ng/ml LF). After 4 hr incubation, cell lysates were prepared in lysis buffer (detailed in western blot analysis) containing 1% Triton X-100 and protease and phosphatase inhibitor cocktails. The insoluble cellular debris was removed by centrifugation. The protein concentration of the resulting supernatant was determined. To measure MAPK activity in the lysates, a nonradioactive kinase assay kit was used as per manufacturer's instructions (Cell Signaling Technologies). Briefly, phosphorylated ERK1/2, p38, or SAPK/JNK proteins were immunoprecipitated from 200 μg of cell lysate with appropriate antibodies, and an in vitro kinase assay was performed using Elk-1, ATF-2, and c-*jun* proteins as substrates, respectively. The phosphorylated substrates were detected by immunoblotting with phospho-substrate-specific antibodies.

Cytokine Measurement

Culture supernatants collected from J774A.1 macrophages treated with either LPS (100 ng/ml), medium containing DMSO, anthrax LT (80 ng/ml PA and 16 ng/ml LF), NSC 95397 (20 μM), NSC 270012 (20 μM), or a combination of compound and toxin were analyzed for secreted proteins using the mouse inflammation cytometric bead array kit (BD Biosciences, San Jose, CA) according to the manufacturer's directions. All analyses were performed on a FACSCalibur (BD Biosciences).

Apoptosis/Necrosis Assay

J774A.1 macrophages (1 × 10^4 cells/well) were seeded in a 96-well plate. The next day, cells were treated with either medium containing DMSO (control) or with anthrax lethal toxin (80 ng/ml PA and 16 ng/ml LF) in the absence or presence of different concentrations of the compound NSC 95397. After incubation for 3 or 4 hr, medium was removed and cells were incubated for 20 min with a mix of YO-PRO-1 (0.5 μM) and propidium iodide (PI; 10 μg/ml) indicator dyes diluted in PBS. Cells were washed and fixed in 1.8% paraformaldehyde. Cells were then stained with Hoechst dye, and images from nine sites per well collected and analyzed using the Discovery-1 high-content screening system (Molecular Devices, Downingtown, PA). Images were analyzed with the Cell Health module of MetaXpress imaging analysis software. Total cell counts were based on number of Hoechst-stained cell nuclei, while colocalization with YO-PRO-1 staining was scored as early apoptotic, colocalization with PI staining was scored as necrotic, and colocalization with both YO-PRO-1 and PI together was scored as late apoptotic.

Supplemental Data

Supplemental Data include two figures and two movies and can be found at http://www.chembiol.com/cgi/content/full/14/3/245/DC1/.

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