Identification of Small Molecules That Suppress Ricin-Induced Stress-Activated Signaling Pathways

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Abstract

Ricin is a member of the ribosome-inactivating protein (RIP) family of plant and bacterial toxins. In this study we used a high-throughput, cell-based assay to screen more than 118,000 compounds from diverse chemical libraries for molecules that reduced ricin-induced cell death. We describe three compounds, PW66, PW69, and PW72 that at micromolar concentrations significantly delayed ricin-induced cell death. None of the compounds had any demonstrable effect on ricin’s ability to arrest protein synthesis in cells or on ricin’s enzymatic activity as assessed in vitro. Instead, all three compounds appear to function by blocking downstream stress-induced signaling pathways associated with the toxin-mediated apoptosis. PW66 virtually eliminated ricin-induced TNF-α secretion by J774A.1 macrophages and concomitantly blocked activation of the p38 MAPK and JNK signaling pathways. PW72 suppressed ricin-induced TNF-α secretion, but not p38 MAPK and JNK signaling. PW69 suppressed activity of the executioner caspases 3/7 in ricin toxin- and Shiga toxin 2-treated cells. While the actual molecular targets of the three compounds have yet to be identified, these data nevertheless underscore the potential of small molecules to down-regulate inflammatory signaling pathways associated with exposure to the RIP family of toxins.

Introduction

Ricin, a heterodimeric glycoprotein found in the seeds of the castor bean plant (Ricinus communis), is an extraordinarily potent toxin. Ricin’s enzymatic subunit (RTA) is an RNA N-glycosidase that irreversibly inactivates eukaryotic ribosomes through hydrolytic cleavage of a conserved adenosine residue within the sarcin-ricin loop (SRL) of 28S rRNA [1,2]. Ricin’s binding subunit (RTB) is a galactose- and N-acetylgalactosamine (Gal/GalNac)-specific lectin that mediates attachment, endocytosis, and trafficking of RTA from the plasma membrane to the endoplasmic reticulum (ER) [3]. Then through a process known as retro-translocation (or dislocation), RTA is threaded across the ER membrane and into the cytoplasm, [4,5,6,7]. Once in the cytoplasm, RTA refolds into its enzymatically active conformation and initiates ribosome depurination at a rate estimated to exceed 1500/min [8].

As a direct consequence of rRNA depurination, RTA activates the so-called ribotoxic stress response (RSR) [9]. The RSR is associated with damage to 28S rRNA by a variety of toxic agents [10]. Through a mechanism that has yet to be fully elucidated, 28S rRNA damage stimulates cellular stress-activated protein kinases (SAPK), including p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) pathways. Activation of these and possibly other SAPKs by RTA leads to increased production of pro-inflammatory cytokines and apoptosis-mediated cell death [11,12,13]. The MAP3K, ZAK, has been identified as the being responsible for activating the p38 MAPK and JNK pathways in response to ricin [9,11,14].

Because ricin is a Category B biothreat agent, there is considerable interest in the identification of small molecules that block its cytotoxic effects [15]. In a recent report, we performed a cell-based, high-throughput screen (HTS) of >80,000 compounds from 17 commercially available chemical libraries [16]. In that initial screen, we identified a number of compounds that potentially interact with RTA’s active site. In this study, we have screened an additional 118,000 compounds and have identified three new compounds that partially protect cells from the effects of ricin. Characterization of these compounds suggests they function not by interacting with ricin per se, but rather, by blocking stress-activated pathways associated with ricin-induced cell killing. This study is significant in that it contributes to an emerging body of evidence that suggests small molecule inhibitors of cell death and inflammation may have utility, alone or in combination with immunotherapeutics, as countermeasures against ricin and other related biothreat agents.

Experimental Procedures

Cell culture, reagents, and materials

Vero (CCL-81), J774A.1 (TIB-67), and THP-1 (TIB-202) cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Vero and J774A.1 cells were routinely propagated in antibiotic-free Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in 95% air and 5% CO2, as described [16]. THP-1 cells were propagated in Roswell Park Memorial Institute (RPMI) medium...
with 10% FBS. Cell culture methods have been described previously [16].

Ricin (*Ricinus communis* agglutinin II), ricin-FTTC, and RTA were obtained from Vector Laboratories (Burlingame, CA). Ricin was dialyzed against PBS to remove sodium azide prior to use. Shiga toxin 2 (Stx2) was a gift from Dr. Cheleste Thorpe (Tufts Medical Center, Boston MA). CellTitre-Glo™, control RNA (luc mRNA), and Bright-Glo™ Luciferase Assay System were purchased from Promega (Madison, WI). Luminescence was measured using an EnVision® (Perkin Elmer, Waltham, MA) or a SpectraMax® L Molecular Devices (Sunnyvale, CA) microplate luminometer. Goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) was purchased from Sandoz-Hoffmann-La Roche (Basel, Switzerland). Nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH). Electrochemical luminescence (ECL) reagent was purchased from Pierce Scientific (Rockford, IL). Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences).

**HTS of small-molecule libraries**

Primary screening of ∼118,700 pure compounds from the commercially available chemical libraries (Actimol TimTec, Bionet, ChemDiv, CEREP, Enamine, I.F. Lab, Maybridge, and Peakdale) was performed at the National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases (NSRB) at Harvard Medical School (Boston, MA) as previously described [16]. Briefly, Vero cells suspended in DMEM + 10% FBS (25 µl; 1.0 × 10⁵ cells) were seeded in 384-well opaque plates and incubated overnight at 37°C to allow the cells to adhere. Test compounds (100 nM; ∼30–90 µM final concentration) were then added to the assay wells. The cells were then incubated at 37°C for 1 h before the addition of 5 µl of ricin (∼0.08 nM final concentration). The cells were then incubated at 37°C for 48 h and cell viability was measured using CellTitre-Glo™. HTS data were analyzed essentially as described previously [16]. The Z-prime factor (*Z'*) of robustness of an assay, for each test plate was determined as described [17]. Compounds were “cherry picked” if they met the following criteria: (i) were present on test plates with *Z'* ≥ 0.5; (ii) gave a Z-score ≥ 2.0; and (iii) inhibited ricin-induced cytotoxicity by ≥ 50%. Compounds that conferred 50–80% cell viability were considered moderate hits, whereas compounds that conferred ≥ 80% cell viability were classified as strong.

**Secondary and tertiary analysis of small molecule inhibitors**

Secondary screens were performed as described for the primary screen but with one major modification: Chery picked compounds (1.2 µl; 5 mg/ml in DMSO) from the primary screen were either diluted 10-fold in DMSO and then transferred (30–90 µM final concentration) to Vero cell assay plates using pin arrays, or transferred directly to Vero cells plates without dilution using PocketTips™. Tertiary analyses with graded concentrations of test compounds were performed essentially as described [16]. Briefly, Vero cells suspended in DMEM + 10% FBS (120 µl; 1.0×10⁶ cells) were seeded in a 96-well plate and incubated overnight at 37°C. 1 µl of 2-fold serially diluted (10 to 0.078 mM in DMSO) test compound was added in triplicate to the assay wells. An equal volume of DMSO was added in triplicate to the positive and negative control wells. The cells were incubated at 37°C for 30 min before 6.4 µl of ricin (∼0.2 nM final concentration) was added to the assay wells. Cells were then incubated at 37°C for 24 h before viability of the cells was measured. Purity of the test compounds (>90%) was confirmed by liquid chromatography mass spectrometry (LCMS) analysis and the final concentration of DMSO in each assay well was ∼0.8% v/v.

**Protein synthesis inhibition assay**

Vero cells (5.0×10⁴ cells/ml), grown overnight in 24-well plates, were incubated with test compounds (25 µM) for 30 min before ricin (∼0.2 nM final concentration) treatment. Eight hours later, the growth medium was replaced with Met/Cys-free DMEM (Invitrogen, Carlsbad, CA) supplemented with 10 µCi/ml Met-Cys (PerkinElmer, Boston, MA). Two hours later, the cells were washed with PBS and then treated with 3% of ice-cold trichloroacetic acid (TCA). Cell debris was scrapped from the plate and transferred to a scintillation vial with 5 ml of EcoScint (National Diagnostics, Atlanta, GA). Radioactivity was measured using Beckman LS 6300 Scintillation Counter (Ramsey, MN).

**In vitro translation (IVT) assay**

The rabbit reticulocyte protein translation assays contained test compounds (3–94 µM), RTA (1.0 nM), luc mRNA (10 ng/ml), and DMSO (0.33% final) and were done as described previously [16,18,19,20]. Retic Lysate IVT™ kit was purchased from Applied Biosystems/Ambion (Austin, TX).

**Inhibition of ricin binding to cell surfaces**

FITC-labeled ricin (0.5 µg/ml; ∼8.0 nM) was incubated with varying concentrations (1 to 100 µM) of test compounds, DMSO (negative control) or galactose (30 mg/ml; ∼167 µM) for 30 min at 4°C before being applied to THP-1 cells (3×10⁵/ml). Following 30 min incubation at 4°C, the cells were washed to remove unbound ricin and then subjected to flow cytometry [21].

**Cytometric bead array (CBA)**

The mouse inflammatory CBA kit (BD Biosciences, San Diego, CA) was used to measure Interleukin (IL)-6, IL-10, monocyte chemotactic protein-1 (MCP-1), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-12p70 in cell supernatants [22].

**Activation of p38 MAPK and SAPK/JNK**

J774A.1 cells were treated with test compounds (20 µM final concentration) or DMSO for 30 min before addition of ricin (0.2 nM final concentration). Cells were collected 6 h later for analysis of activated p38 MAPK using the nanoradioactive kit (Cell Signaling Technology, Beverly, MA). For analysis of activated SAPK/JNK, cells were detached from the culture plates using a cell scraper and then collected by centrifugation. The resulting cells were suspended in 1 vol of Laemmli sample buffer containing 5% β-mercaptoethanol, boiled, and subjected to SDS-PAGE and Western blotting with phospho-p38 MAPK and phospho-JNK-specific antibodies purchased from Cell Signaling Technology.

**In vitro inhibition of p38-α MAPK activity**. p38-α MAPK was immunoprecipitated from ricin-treated cell lysate (as described above) and then incubated for 5 min at RT with varying concentrations (5–40 µM) of the test compounds before the addition of ATP and ATF-2. The mixture was incubated for 30 min at 30°C, and then subjected to dot blot or Western blot analysis. ImageJ software was used for quantification of signal densities on exposed X-ray films.

**Inhibition of caspases 3/7 activities.** Vero cells (10,000 cells/well) were treated with test compounds (0.6–78.5 µM) for 30 min before treatment with ricin (0.2 nM) or Stx2 (1.5 nM) for

**Inhibitors of Ricin-Induced Stress Signaling**

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concentrations and PW72 were themselves not toxic to Vero cells, even at early endosomes and the TGN [23,24]. Compounds PW66, PW69 blocks retrograde transport of ricin (and Shiga toxin) between 24 h (Fig. S2). Retro-2 is a recently identified small molecule that blocks retrograde transport of ricin (and Shiga toxin) between early endosomes and the TGN [23,24]. Compounds PW66, PW69 and PW72 were each more effective respectively in both Vero and J774A.1 (Table 1; Figures 1, 2; Fig. S1). In fact, PW66, PW69 and PW72 were each more effective than Retro-2 at reducing toxin-induced death when assessed at 24 h (Fig. S2); Retro-2 is a recently identified small molecule that blocks retrograde transport of ricin (and Shiga toxin) between early endosomes and the TGN [23,24]. Compounds PW66, PW69 and PW72 were themselves not toxic to Vero cells, even at concentrations ≥100 µM (Table S1). For these reasons, we chose to investigate them in greater detail. The remaining 29 commercially available compounds in Group A are being pursued in a separate study (P. Wahome and N. Mantis, manuscript in preparation).

We next examined the inhibitory activity of select analogs of compounds PW66, PW69 and PW72 (Table 1; Figure 2). Three analogs of PW66 (B, D and E) had EC$_{50}$s similar to PW66. Analogue C, on the other hand, was 4-fold less effective than PW66, possibly due to the proximity of two nitrogen atoms in the pendant groups (pyridines) of compound. Compounds PW69B and PW72B had ricin inhibitory activities similar to PW69 and PW72, respectively (Table 1).

Finally, using the Vero cell cytotoxicity assay, we tested PW66, PW69 and PW72 in various combinations and concentrations in order to determine whether a mixture of the compounds would be more effective than individual compounds at reducing ricin-induced cell death. Surprisingly, preliminary checkerboard analysis did not reveal any evidence of synergy or even additivity between the compounds (data not shown). While our initial supposition based on these studies is that the compounds are not more effective when combined, a further more detailed analysis using models such as Bliss independence or Loewe additivity is required before such a conclusion can be fully substantiated.

**Table 1. Compounds described in this study.**

| Compound | Systematic Name | ID | Vendor | EC$_{50}$ (µM) |
|----------|----------------|----|--------|---------------|
| PW66     | 4-(5-pyridin-3-yl| 4056795$^b$ | Peakdale | 8 |
| PW66B    | 4-(5-Phenyl-1H-pyrazol-4-y| 4378208$^b$ | Peakdale | 10 |
| PW66C    | 3-(5-Phenyl-1H-pyrazol-4-yl| 2568949$^b$ | Peakdale | 36 |
| PW66D    | 4-(3-Pyridin-2-yl| 3000655$^b$ | Peakdale | 9 |
| PW66E    | 2-(5-(3-Pyridinyl)-1H-pyrazol-4-yl| 4285370$^b$ | Peakdale | 9 |
| PW69     | 6-oxo-N-(1-phenylbutan-2-yl| 6623219$^b$ | ChemDiv | 23 |
| PW69B    | 6-oxo-N-(4-phenylbutan-2-yl| 20885127$^b$ | ChemDiv | 22 |
| PW72     | 1-(3-(furan-2-yl)-7-(furan-2-y| 4342949$^b$ | Enamine | 31 |
| PW72B    | 1-(3-(furan-2-yl)-5-(4| 16358890$^b$ | Enamine | 26 |

$^a$Source of the compound; $^b$PubChem ID; $^c$Chemsper ID; $^d$Vendor ID.

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that none of the compounds (3–94 μM) had any demonstrable impact on RTA’s ability to arrest protein synthesis or on translation itself (Fig. S4; data not shown). From these studies we conclude that PW66, PW69 or PW72 must interfere with ricin’s cytotoxic effects at step(s) downstream of ribosome arrest.

PW66 and PW72 (but not PW69) reduce ricin-induced TNF-α secretion by macrophages

The fact that PW66, PW69 and/or PW72 did not interfere with protein synthesis inhibition per se, led us to hypothesize that they suppress toxin-induced SAPK activation and/or apoptosis. Induction of the RSR by RTA, for example, triggers the p38 MAPK and JNK pathways, resulting in the secretion of the pro-inflammatory cytokine TNF-α [10,12,25,26]. To examine the influence of each of the compounds on TNF-α production, J774A.1 cells were treated with the PW66, PW69 or PW72 for 30 min prior to ricin treatment. TNF-α levels were then measured in culture supernatants 24 h later. We found that ricin treatment alone resulted in ~10-fold increase in TNF-α in cell supernatants (Fig. 3). PW66 completely blocked ricin-induced TNF-α production, whereas PW72 reduced TNF-α levels by >50%. PW69, in contrast, did not impact ricin-induced TNF-α production. These data suggest that PW66 and PW72 likely block activation of the p38 MAPK and/or JNK signaling pathways in response to ricin treatment.
PW66 inhibits activation of the p38 MAPK and JNK pathways

We used a coupled immunoprecipitation/ATF-2-phosphorylation assay to determine whether PW66 and/or PW72 interfere with ricin-induced activation of p38 MAPK. As expected, treatment of J774A.1 cells with ricin alone resulted in a significant increase in the endogenous levels of phospho-p38 MAPK, as evidenced by the high signal intensity of phosphorylated ATF-2 (Fig. 4A). No such activation of ATF-2 was evident when cells were treated with PW66. Indeed, PW66 demonstrated a dose-dependent capacity to inhibit ricin-induced phospho-p38 MAPK (Fig. 4B). In contrast, PW69 and PW72 did not reduce (but, in fact, marginally enhanced) activation of p38 MAPK (Fig. 4A). None of the compounds had any effect on the endogenous levels of unphosphorylated p38 MAPK in Vero cells (Fig. 4C).

To examine whether PW66 influences p38 MAPK phosphotransferase activity, we immunoprecipitated p38 MAPK from ricin-treated cells and then performed an in vitro ATP-2 phosphorylation reaction in the presence or absence of PW66. Indeed, PW66 demonstrated a dose-dependent capacity to inhibit ricin-induced phospho-p38 MAPK (Fig. 4B). In contrast, PW69 and PW72 did not reduce (but, in fact, marginally enhanced) activation of p38 MAPK (Fig. 4A). None of the compounds had any effect on the endogenous levels of unphosphorylated p38 MAPK in Vero cells (Fig. 4C).

To test whether PW66 had a similar inhibitory effect on activation of the JNK pathway, total cell lysates were probed by Western blot with antibodies specific for phospho-JNK. As expected, ricin alone caused an increase in phospho-JNK levels (Fig. 5). Treatment of cells with compound PW66 completely blocked ricin-induced activation of JNK, whereas neither PW69 nor PW72 had any effect on phospho-JNKs levels (Fig. 5, upper panel). None of the compounds had notable effect on the endogenous levels of unphosphorylated JNK in Vero cells (Fig. 5, lower panel).

Compounds PW69 inhibit caspases 3/7 activities

Since neither PW69 nor PW72 blocked ricin-induced SAPK pathways, we postulated that they might act on another aspect of toxin-mediated cell death, such as activation of executioner caspases 3 and 7 [27]. To this end, Vero cells were pretreated with PW69, PW72 or PW66 for 30 min prior to ricin exposure. Activities of executioner caspases 3 and 7 were determined using a luciferase-based substrate. We found that ricin treatment alone resulted in a ~10 fold increase in caspase 3/7 activities, as compared to mock-treated cells (Fig. 6A). Neither PW66 nor PW72 significantly influenced caspase activation. PW69, on the other hand, demonstrated a dose-dependent reduction in caspase 3/7 activities (Fig. 6A; data not shown). Moreover, PW69 interfered with toxin-induced DNA fragmentation in J774A.1 cells, while PW66 and PW72 did not (data not shown). To examine whether PW69 could also interfere with the activity of caspases 3 and 7 in Vero cells treated with a different inducer of apoptosis, cells were treated with Stx2. PW69 exhibited a dose-dependent inhibition of Stx2-induced caspase (Figure 6B). These data suggest that PW69 interferes with ricin- and Stx2-induced apoptosis.

Discussion

Two very different experimental screening strategies have been employed over the past two decades in an effort to identify small molecule inhibitors of ricin (and Shiga toxins), with very different outcomes. On the one hand, virtual library screening has led to the identification of three broad classes of active site (or near active site) inhibitors [15]. For example, virtual screening identified pteroic acid (PTA), a small molecule that was subsequently shown by X-ray crystallography to bind RTA’s active site with nearly perfect complimentarity [28]. Subsequent rational design and medicinal chemistry strategies have been employed to develop a diversified collection of pterin-based compounds, like 7-carboxy...
pterin (7CP), that make additional contacts with RTA and that improve relative IC50s [29].

Cell-based screening strategies, on the other hand, have led to the identification of small molecules that (partially) protect cells from ricin-induced death. The compounds identified to date by this strategy do not act on the toxin per se, but rather interfere with cellular processes required for toxin intracellular transport or trafficking [15]. Using a cell-based HTS, Haslam and colleagues identified two compounds, 75 and 134, from the known bioactive and ChemDiv3 chemical libraries [30]. Compound 75 inhibited intracellular transport of Shiga toxin 1 (Stx1) to perinuclear recycling endosomes, while compound 134 inhibited transport of Stx1 at a post-recycling endosome stage. Stechmann and colleagues identified Retro 2 from a screen of 16,500 compounds in the ChemBridge library [24].

We have now completed a high-throughput, cell-based screen of more than 118,000 compounds from diverse chemical libraries and identified three compounds, PW66, PW69 and PW72 with varying capacities to reduce ricin-induced cell death. Like the other cell-based screens, none of the compounds we identified appear to act on ricin directly. Rather, PW66, PW69 and PW72 apparently act by blocking toxin-induced activation of one or

Figure 4. Compound PW66 interferes with activation of p38 MAPK in ricin-treated cells. (A) J774A.1 cells were treated with ricin (0.2 nM) with or without indicated compounds for 6 h before cells were lysed. Phospho-p38 MAPK was immunoprecipitated from cell lysates and used in an in vitro ATF-2 phosphorylation assay, as described in the Experimental Procedures. Shown are the results of a dot-blot analysis. (B) J774A.1 cells were treated with ricin (0.2 nM) with or without PW66 at indicated concentrations as shown in Panel A, except that ATF-2 was subjected to SDS-PAGE and Western blotting. (C) Western blot analysis of total p38 MAPK from ricin- or ricin + compound-treated cells, as indicated. Shown are results of representative experiments from three or more independent experiments.

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Figure 5. Compound PW66 interferes with activation of SAPK/JNK in ricin-treated cells. J774A.1 cells were treated with ricin (0.2) in the presence or absence of indicated compounds for 6 h before cells were lysed and subjected to SDS-PAGE and Western blot analysis with (A) phospho-JNK or (B) JNK specific antibodies. Shown are results of representative experiments from three or more independent experiments.

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Figure 6. Compound PW69 interferes with the activity of caspases 3/7. Vero cells were treated with (A) ricin (0.2 nM) or (B) Stx2 (1.5 nM) in the absence or presence of indicated concentrations of PW69. Cells were then lysed and incubated with caspases 3/7 substrates, as described in the Experimental Procedures. The results are from a representative experiment that was performed in triplicate and showed <10% variation. One-way ANOVA with Dunnett’s multiple comparison test was performed and p values less than 0.05 were considered statistically significant (**).
more SAPK or pro-apoptotic pathways. Collectively, PW66, PW69 and PW72 constitute a class of “downstream” ricin inhibitors, in contrast to Retro 2 and Compounds 75/134, which interfere with trafficking of ricin from the plasma membrane to the ER.

Our data are consistent with PW66 functioning as an inhibitor of ZAK (also known as MRK and MLKT-2), the upstream MAP3K responsible for activating the p38 MAPK and JNK pathways in response to ricin and other ribotoxic stressors [9,11,14]. Jandhyala and colleagues demonstrated that treatment of HCT-8 or Vero cells with the ZAK inhibitor DHP-2 (200 nM) blocked ricin-induced IL-6 production and suppressed activation of both p38 MAPK and JNK pathways [11]. In this study we demonstrated that PW66 is similar to DHP-2 in that it suppressed ricin-induced TNF-α in J774 cells and p38 MAPK and JNK pathways in Vero cells. Although we have not demonstrated that PW66’s target is in actually ZAK, it is interesting to note that compound PW66 is structurally related to DHP-2, an aryl-substituted dihydro-pyrrrolopyrazole quinoline [31]. The fact that PW66 was identified from among the more than 118,000 compounds screened in this study attests a possible central role of ZAK (or a ZAK-like MAP3K) in orchestrating ricin-induced stress-activated signaling pathways.

It is not immediately obvious how ZAK inhibitors like DHP-2 (and possibly PW66) interfere with ricin-induced cell death. On the one hand, there is considerable evidence for “cross talk” between pathways involved in inflammation and apoptosis, particularly via the p38 MAPK pathway [12,26,32,33,34]. Higuchi et al, for example, reported a decrease in ricin-induced apoptosis of murine macrophage (e.g., RAW 264.7) when the cells were treated with a specific p38 MAPK inhibitor [12]. Magun and colleagues, on the other hand, recently reported that ricin-mediated release of the pro-inflammatory cytokine IL-1β via the NALP3 inflammasome in bone marrow-derived macrophages is enhanced, rather than suppressed, by inhibition of SAPK phosphorylation [35]. Because ricin triggers the SAPKs, as well as other proinflammatory pathways, sorting out the relevance of specific inhibitors in dampening ricin’s pathophysiology at the cellular and tissue levels awaits comprehensive animal studies.

The mechanism(s) by which PW69 and PW72 limit ricin-induced cell killing are yet to be determined. To our knowledge, neither compound is structurally related to any previously described inhibitors of ricin or apoptosis [15,16,20,24,30,36]. PW72 was effective at blocking ricin-induced TNF-α release by J774 cells, but did not suppress signaling via p38 MAPK or JNK. Based on this result, we hypothesize that PW72 must interfere with TNF-α synthesis, intracellular trafficking, and/or proteolytic release from the cell surface [37]. PW69, on the other hand, blocked ricin-induced up-regulation of executioner caspases 3 and 7, strongly suggesting that this compound works by interfering with cellular progression to apoptosis. The fact that PW66, PW69 and PW72 all appear to partially protect cells from ricin-induced killing by targeting host proteins or host pathways, provides further support to the idea that inhibitors of toxin-induced SAPK pathways could be utilized, alone or in conjunction with immunotherapies, to mitigate inflammatory responses initiated by ricin or related RIPs in systemic and mucosal compartments [9].

Supporting Information

Figure S1 Compounds PW66, PW69 and PW72 inhibit ricin cytotoxicity. Vero cells were treated with ricin (0.2 nM; dashed lines) or pretreated with PW66 (open circles), PW69 (open squares), or PW72 (open triangles) at the indicated concentrations for 30 min before ricin was added. Cell viability was measured at (A) 40 hr or (B) 72 hr as described in the Experimental Procedures. Each panel shows results of a representative experiment from three independent experiments that were done in triplicate and showed <10% correlation of variation (% CV) for individual experiment. (TIF)

Figure S2 Inhibition of ricin cytotoxicity by Retro 2. Vero cells were treated with ricin (0.2 nM; dashed lines) or pretreated with Retro 2 (filled triangles) at the indicated concentrations for 30 min before ricin was added. Cell viability was measured at 24 hr, as described in the Experimental Procedures. Shown are results of a representative experiment from three independent experiments that were done in triplicate and showed <10% correlation of variation (% CV) for individual experiment. (TIF)

Figure S3 Compounds PW66, PW69, and PW72 do not inhibit the effect of ricin on protein biosynthesis. Vero cells were treated with ricin (0.2 nM) or pretreated with 25 μM of PW66, PW72, PW69 or Retro 2 for 30 min before an aliquot of the growth medium (DMEM + 10% FBS) with or without ricin was added. The cells were incubated for 8 hr at 37°C, pulsed with 10 μCi/ml 35Met,35Cys for 2 hr, washed, treated with 5% TCA, and the activity of incorporated radioisotopes was measured as described in the Experimental Procedures. Shown are results of a representative experiment that was done in quadruplicate and showed <10% correlation of variation (% CV) for individual experiment. (TIF)

Figure S4 Compounds PW66, PW69, and PW72 do not inhibit the enzymatic activity of RTA or significantly impact protein synthesis in vitro. Individual test compound (94 μM) or DMSO (carrier solvent for compounds) was mixed with RTA (1.6 nM) or PBS (pH 7.4) and then added to an in vitro translation reaction in which luciferase mRNA was present as template. Translation of the luciferase mRNA was determined by addition of Bright-GloTM substrate and measurement of light emission with a luminometer, as described in the Experimental Procedures. Shown are results of a representative experiment that was done in duplicate and showed <10% correlation of variation (%CV) for individual experiment. (TIF)

Table S1 Viability of Vero cells treated with varying concentrations of PW66, PW69, PW72 or Retro 2. (DOC)

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Author Contributions

Conceived and designed the experiments: PGW NJM. Performed the experiments: PGW SA. Analyzed the data: PGW SA NJM. Contributed
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References

1. Endo Y, Minui K, Motizuki M, Tsurugi K (1987) The mechanism of action of ricin and related toxins on eukaryotic ribosomes. J Biol Chem 262: 5908–5912.  
2. Endo Y, Tsurugi K (1987) RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J Biol Chem 262: 8125–8130.  
3. Rutener E, Ready M, Robertus JD (1987) Structure and evolution of ricin B chain. Nature 326: 624–626.  
4. Redmann V, Oresic K, Tortorella LL, Cook JP, Lord M, et al. (2011) Dislocation of ricin toxin a chains in human cells utilizes selective cellular factors. J Biol Chem 286: 21231–21238.  
5. Slominska-Wojewodka M, Gregers TF, Wchalchi S, Sandvig K (2006) EDEM is involved in retrotranslocation of ricin from the endoplasmic reticulum to the cytosol. Mol Biol Cell 17: 1664–1675.  
6. Sokolowska I, Wchalchi S, Wegrzyn G, Sandvig K, Slominska-Wojewodka M (2011) A single point mutation in ricin A-chain increases toxin degradation and inhibits EDEM-dependent ER retrotranslocation. Biochem J 436: 371–385.  
7. Simpson JC, Roberts LM, Romisch K, Davey J, Wolf DH, et al. (1999) Ricin A chain utilises the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast. FEBS Lett 459: 80–84.  
8. Endo Y, Tsurugi K (1988) The RNA N-glycosidase activity of ricin A-chain. The characteristics of the enzymatic activity of ricin A-chain with ribosomes and with rRNA. J Biol Chem 263: 8735–8739.  
9. Jandhyala DM, Thorpe CM, Magun B (2012) Ricin and Shiga toxins: effects on host cell signal transduction. Curr Top Microbiol Immunol 357: 41–65.  
10. Iordanov MS, Pribnow D, Magun JL, Dinh TH, Pearson JA, et al. (1997) Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. Mol Cell Biol 17: 3373–3381.  
11. Jandhyala DM, Ahioualnia A, Obrig T, Thorpe CM (2008) ZAK: a MAP3K kinase that translocates Shiga toxin- and ricin-induced proinflammatory cytokine expression. Cell Microbes 10: 1468–1477.  
12. Higuchi S, Tamura T, Oda T (2003) Cross-talk between the pathways leading to the induction of apoptosis and the secretion of tumor necrosis factor-alpha in ricin-treated RAW 264.7 cells. J Biochem (Tokyo) 134: 927–933.  
13. Smith WE, Kane AV, Campbell ST, Acheson DW, Cochran BH, et al. (1997) Shiga toxin I triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. Infection & Immunity 71: 1497–1504.  
14. Sauter KA, Magun EA, Iordanov MS, Magun B (2010) ZAK is required for doxorubicin, a novel ribotoxic stressor, to induce SAPK activation and apoptosis in HaCaT cells. Cancer Biol Ther 10: 251–266.  
15. Wahome PG, Robertus JD, Mantis NJ (2012) Small-molecule inhibitors of ricin and Shiga toxins. Curr Top Microbiol Immunol 357: 179–207.  
16. Wahome PG, Bai Y, Neal LM, Robertus JD, Mantis NJ (2010) Identification of small-molecule inhibitors of ricin and shiga toxin using a cell-based high throughput screen. Toxicol In Vitro 26: 313–323.  
17. Zhang JH, Chung TD, Oldenburg KR (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 4: 67–73.  
18. Neal LM, O’Hara J, Brey RN II, Mantis NJ (2010) A monoclonal immunoglobulin G antibody directed against an immunodominant linear epitope on the ricin A chain confers systemic and mucosal immunity to ricin. Infect Immun 78: 552–561.  
19. Bai Y, Monzongo AF, Robertus JD (2009) The X-ray structure of ricin A chain with a novel inhibitor. Arch Biochem Biophys 483: 23–28.  
20. Bai Y, Wilt B, Wahome PG, Mantis NJ, Robertus JD (2010) Identification of new classes of ricin toxin inhibitors by virtual screening. Toxicol 56: 526–534.  
21. Yermakova A, Mantis NJ (2011) Protective immunity to ricin toxin conferred by antibodies against the toxin’s binding subunit (RTB). Vaccine 29: 7825–7835.  
22. Yoder JM, Aslam RU, Mantis NJ (2007) Evidence for widespread epithelial damage and coincident production of monocytectoxic protein 1 in a murine model of intestinal ricin intoxication. Infect Immun 75: 1745–1750.  
23. Park JK, Kahn JN, Tumer NE, Pang YP (2012) Chemical Structure of Retro-2, a Compound That Protects Cells against Ribosome-Inactivating Proteins. Sci Rep 2: 631.  
24. Stechmann B, Bai SK, Gobbo E, Lopez R, Merer G, et al. (2010) Inhibition of retrograde transport protects mice from lethal ricin challenge. Cell 141: 231–242.  
25. Korcheva V, Wong J, Corless C, Iordanov M, Magun B (2005) Administration of ricin induces a severe inflammatory response via nonredundant stimulation of ERK, JNK, and P38 MAPK and provides a mouse model of hemolytic uremic syndrome. Am J Pathol 166: 233–339.  
26. Korcheva V, Wong J, Lindauer M, Jacoby DB, Iordanov MS, et al. (2007) Role of apoptotic signaling pathways in regulation of inflammatory responses to ricin in primary murine macrophages. Mol Immunol 44: 2761–2771.  
27. Bergbauer T, Fink ML, Cookson ET (2009) Pyroptosis: host cell death and inflammation. Nat Rev Microbiol 7: 99–109.  
28. Yan X, Hollis T, Smith T, Day P, Monzongo AF, et al. (1997) Structure-based identification of a ricin inhibitor. J Mol Biol 266: 1043–1049.  
29. Pruett JM, Jasbeway KR, Manzano LA, Bai Y, Andyn EV, et al. (2011) Substituted pterins provide a new direction for ricin A-chain inhibitors. Eur J Med Chem 46: 3608–3615.  
30. Saenz JB, Doggett TA, Hashlam DB (2007) Identification and characterization of small molecules that inhibit intracellular toxin transport. Infect Immun 75: 4552–4561.  
31. Wang X, Mader MM, Toth J, Xu Y, Jin N, et al. (2005) Complete inhibition of anisomycin and UV radiation but not cytokine induced JNK and p38 activation by an aryl-substituted dihydropyropolyrazole quinoline and mixed lineage kinase 7 small interfering RNA. J Biol Chem 280: 19298–19305.  
32. Cargnello M, Roux PP (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 75: 50–83.  
33. Leyva-Illescas D, Chen RL, Lee MS, Tosh VI (2012) Regulation of cytokine and chemokine expression by the ribotoxic stress response elicited by Shiga toxin type 1 in human macrophage-like THP-1 cells. Infect Immun. 34. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270: 1326–1331.  
35. Lindauer M, Wong J, Magun B (2010) Ricin Toxin Activates the NALP3 Inflammasome. Toxins (Basel) 2: 1500–1514.  
36. Saenz JB, Sun WJ, Chang JW, Li J, Bursulaya B, et al. (2009) Golgicide A reveals essential roles for GRB1 in Golgi assembly and function. Nat Chem Biol 5: 157–165.  
37. Adrain C, Zelik M, Christova Y, Taylor N, Freeman M (2012) Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. Science 333: 225–228.